eman ta zabal zazu

Universidad del País Vasco   Euskal Herriko Unibertsitatea

Glial localization of the cannabinoid CB$_1$ and CB$_2$ receptors in a mouse model of Alzheimer's disease

DOCTORAL THESIS

Itziar Terradillos Irastorza

Doctoral Thesis presented by

ITZIAR TERRADILLOS IRASTORZA

Supervised by:

Prof. Dr. PEDRO ROLANDO GRANDES MORENO

Prof. Dra. IZASKUN ELEZGARAI GABANTXO

Department of Neurociences

Faculty of Medicine and Nursing

University of the Basque Country (UPV/EHU) Leioa, 2021

1.SUMMARY

Alzheimer's disease (AD) is a devastating neurodegenerative disorder highly prevalent in the elderly and the most common cause of dementia. However, the pathogenesis of this disease is still unclearNeuronal death is the most significant cellular change that occurs in the central nervous system (CNS) before clinical symptoms appear in AD, but there are also changes in glial cells.

The identification of human cannabinoid receptors and their roles in health and disease has meant an important advance in recent decades. Several laboratories have tried to clarify the usefulness of targeting cannabinoid receptors in AD due to their neuroprotective effect and the role of cannabinoids in preventing microglial activation and Aβ-induced neurotoxicity. It is therefore of special interest to study in detail the endocannabinoid system (ES) during the progression of AD. Alteration of the endocannabinoid tone usually associates with changes in the expression and/or function of CB_1R and CB_2R, the main cannabinoid receptors. However, conflicting results have been obtained about the CB_1R in AD, and the study of the CB_2R is even more complex given the lack of specific antibodies against this receptor. Astrocytes interact closely with microglia, and both cells are implicated in reactive gliosis in AD. Thus, Aβ-containing aggregates induce a chronic inflammatory response leading to activation of both microglia and astrocyte that surround and invade amyloid plaques. This reactive gliosis results in abnormal morphology and proliferation of astrocytes and microglia. Whether these histopathological changes reflect beneficial, detrimental or inconsequential activity of glial cells in neurodegenerative events remains unclear. On top of that, very little is known about the localization and expression of cannabinoid receptors in glial cells in AD.

We hypothesise in this doctoral thesis that the glial expression of CB_1R and CB_2R is altered in the subiculum of a mouse model of AD, a brain region particularly affected by large accumulation of plaques, as a result of concomitant subcellular changes in microglial cells and astrocytes.

Based on this hypothesis, I have studied the microglia and astrocytes in the subiculum of 6.5-7.5 month-old $CB2^{EGFP/f/f}$/5xFAD mice with plaques and neuronal damage already formed at that age and in control $CB2^{EGFP/f/f}$ mice (López et al., 2018). They also express the enhanced green fluorescent protein (EGFP) under the same promoter as the CB_2R gene. The $CB2^{EGFP/f/f}$/5xFAD and $CB2^{EGFP/f/f}$ mice were kindly provided by Dr. Julián Romero (Universidad Francisco de Vitoria, Madrid), in the context of the active collaboration established with his laboratory. I used a double pre-embedding immunogold and

immunoperoxidase method for electron microscopy, to quantify the CB_1R expression in microglia and astrocytes and CB_2R in microglia.

As to CB_1R in $CB2^{EGFP/f/f}$/5xFAD mice, an increase in both the number and percentage of CB_1R-positive microglial processes with respect to the total number of microglial processes was observed in the subiculum, as well as in the number of microglial gold particles per normalised area. The density of CB_1R particles decreased, but this seemed to be due to the increase in the perimeter of the microglial processes. In the case of astrocytes, no changes were seen in: number or percentage of CB_1R-positive processes; number of CB_1R immunoparticles per normalised area; density of CB_1R astrocytic processes. However, an increase in the number of CB_1R particles per astrocytic process was observed. Thus, CB_1R expression seems to increase in reactive microglia in the subiculum of $CB2^{EGFP/f/f}$/5xFAD mice (though CB_1R density decreases), while it remains constant in astrocytes with the number of CB_1R particles increasing proportionally to the increase in the perimeter of the reactive astrocytic processes.

As to CB_2R in $CB2^{EGFP/f/f}$/5xFAD mice, there was an increase in: number and percentage of GFP-positive processes; number of GFP particles per microglial area; number of microglial GFP particles per normalised area. Therefore, CB_2R was overexpressed in the subiculum of the $CB2^{EGFP/f/f}$/5xFAD mice.

The findings of my thesis on the correlation between glial reactivity and the CB_1R and CB_2R expression in microglial cells and astrocytes, aim to contribute to the understanding of the role of the endocannabinoid system in the pathophysiology of Alzheimer´s disease.

-**Aβ:** Beta-amyloid

-**ABC:** Avidin-biotin-peroxidase complex

-**AC:** Adenylate cyclase

-**AD:** Alzheimer's disease

-**AEA:** Arachidonoylethanolamine

-**AICD:** APP intracellular domain

-**APP**: Amyloid precursor protein

-**BACE1:** β-site amyloid precursor protein 1

-**BBB:** Blood-brain barrier

-**BDNF:** Brain-derived neurotrophic factor

-**BSA:** Bovine serum albumin

-**CA:** Cornu ammonis

-**CA1:** Cornu ammonis 1

-**CB$_1^{-/-}$:** Cannabinoid receptor type 1-knock out

-**CB$_2^{-/-}$:** Cannabinoid receptor type 2-knock out

-**CB$_1$R:** Cannabinoid receptor type 1

-**CB$_2$R:** Cannabinoid receptor type 2

-**CNS:** Central nervous system

-**DAB:** Diaminobenzidine

-**DAG:** Diacylglycerol

-**DAGL:** Diacylglycerol lipase

-**DAMPs:** Danger-associated molecular patterns

-**DG:** Dentate gyrus

-**EC:** Entorhinal cortex

-**ECBs:** Endocannabinoids

-**EGFP:** Enhanced green fluorescent protein

-**ERK:** Extracellular signal-regulated kinase

-**ES**: Endocannabinoid system

-**EtOH:** Ethanol

-**FAAH:** Fatty acid amide hydrolase

-**FAD:** Familial Alzheimer Disease

-**GFAP:** Glial fibrillary acidic protein

-**GFP:** Green fluorescent protein

-**GLAST:** Glutamate aspartate transporter 1

-**GPCRs:** G protein-coupled receptors

-**GPR18:** G protein-coupled receptor 18

-**GPR55:** G protein-coupled receptor 55

-**hAPP:** Human amyloid precursor protein

-**HIV:** Human immunodeficiency virus

-**H_2O_2:** Hydrogen peroxide

-**Iba1:** Ionized calcium-binding adapter molecule 1

-**IL-1β:** Interleukin-1β

-**IL-4:** Interleukin-4

-**IL-10**: Interleukin-10

-**iNOS:** Inducible nitric oxide synthase

-**IP3:** Inositol triphosphate

-**IRES:** Internal ribosomal entry site sequence

-**LPS:** Lipopolysaccharide

-**LRP1:** Lipoprotein receptor-related protein1

-**LS:** Limbic system

-**MAPK:** Mitogen-activated protein kinase

-**MHCII:** Major histocompatibility complex type II

-**NAPE:** N-acyl-phosphatidylethanolamines

-**NAPE-PLD:** N-acyl-phosphatidylethanolamine-selective phospholipase D enzyme

-**NArPE:** N-arachadonoyl-phosphatidylethanolamine

-**NFTs**: Neurofibrillary tangles

-**NGF:** Nerve growth factor

-**NO:** Nitric oxide

-**NT:** Neurotransmitter

-**NT-3:** Neurotrophin 3

-**NT-4/5:** Neurotrophin 4/5

-**ObR:** Leptin receptor

--**OH:** Hydroxyl

-**O^{2-}:** Superoxide

-**PAMPs:** Pathogen-associated molecular patterns

-**PB:** Phosphate buffer

-**PBS:** Phosphate buffered saline

-**PE:** Phosphatidylethanolamine

-**PI:** Phosphatidylinositol

-**PI3K:** Phosphatidylinositol 3-kinase

-**PKA:** Protein kinase A

-**PKB:** Protein kinase B

-**PLC:** Phospholipase C

-**PLD:** Phospholipase D

-**PPAR-α:** Peroxisome proliferator-activated receptor α

-**PPAR- γ:** Peroxisome proliferator-activated receptor γ

-**PreS:** Presubiculum

-**ROS:** Reactive oxygen species

-**RT:** Room temperature

-**RT-PCR:** Reverse transcription polymerase chain reaction

-**SPs:** Senile plaques

-**SUB:** Subiculum

-**TBS 1X:** 1X Tris-buffered saline

-**TGF-β:** Transforming growth factor beta

-**THC:** Δ9-tetrahydrocannabinol

-**TNF-α:** Tumour necrosis factor-α

-**TRPV1:** Transient receptor potential channel V1

-**UTR**: Untranslated region

-**VSCCs:** Voltage-sensitive Ca_2^+ channels

-**WT:** Wild-type

-**5xFAD:** Five familial Alzheimer's disease mutations

-**2-AG:** 2-arachidonoylglycerol

-**τ:** Tau

2. INTRODUCTION

2.1. ALZHEIMER'S DISEASE:

Alzheimer's disease (AD) is the most frequent neurodegenerative disorder in older people and is the most common cause of dementia (Glenner and Wong, 1984; Verkhratsky et al., 2019). Auguste Deter was the first patient diagnosed with AD by Dr. Alois Alzheimer in 1906. He suffered from a form of progressive pre-senile dementia and over time developed memory loss as well as other behavioural sequelae, dying at the age of 55. Brain autopsy revealed the presence of senile plaques (SPs) and neurofibrillary tangles (NFTs) visible by silver staining as well as significant brain shrinkage (for review Mufson et al., 2015).

AD is characterised by impaired memory and cognitive abilities, dementia, dysfunction in activities of daily living and behavioural disturbances. The pathogenesis of this disease is still unclear, as abundant pathological changes have been observed in the post-mortem brains of AD patients (Medeiros and LaFerla, 2013). Aggregation and deposition of beta-amyloid (Aβ) peptides associated with the formation of SPs, and hyper phosphorylation of tau (τ) protein, associated with the generation of NFTs, have been postulated to play an important role in their development. However, other mechanisms are known to be involved in the pathogenesis of AD. These include synapse dysfunction, inflammation, oxidative stress, alterations in mitochondrial metabolism and loss of cholinergic neurons (for review Moreira et al., 2006; for review Querfurth and LaFerla, 2010; for review Bloom, 2014; Bobkova and Vorobyov, 2015). Therefore, AD most commonly occurs as a sporadic multifactorial disease (for review Frost and Li, 2017).

SPs are extracellular deposits composed of dystrophic and degenerated neurites, microglia and reactive astrocytes, but mainly of Aβ-peptide, a catabolic product of amyloid precursor protein (APP) catabolism, which forms fibrils and aggregates. Three main types of SPs are distinguished. On the one hand, diffuse plaques, which are non-fibrillar amyloid deposits that do not alter the neuropil or induce a glial response and therefore do not usually lead to cognitive impairment. On the other hand, amyloid plaques, with a more or less dense centre (De la Vega and Zambrano, 2013). Finally, there are compact or neuritic plaques, also called SPs, with a dense core and the presence of APP immunopositive (APP[+]) dystrophic neurites, which are toxic in nature and specific to AD, containing astrocytes and activated microglia (De la Vega and Zambrano, 2013; for review Selkoe and Hardy, 2016).

Two processes of APP proteolysis have been described: non-amyloidogenic and amyloidogenic (Fig. 1). In the non-amyloidogenic pathway, the enzyme α-secretase cleaves the APP releasing a soluble fragment (sAPPα) and another fragment C83 on which γ-

secretase also acts, giving rise to p3 and APP intracellular domain (AICD). AICD is released into the cytoplasm, and is targeted to the nucleus, mediating the transcription of certain genes (for review Querfurth and LaFerla, 2010; for review Coronel et al., 2019). sAPPα is a soluble peptide, which is then easily eliminated by the body. In the amyloidogenic pathway, the enzyme β-secretase or β-site amyloid precursor protein 1 (BACE1) releases a soluble fragment (sAPPβ) and the carboxy-terminal fragment C99. This C99 fragment is cleaved by γ-secretase within the cell membrane, forming the AICD and Aβ-peptides (Aβ 1-40 and Aβ 1-42) (De la Vega and Zambrano, 2013; for review Coronel et al., 2019).

Moreover, Aβ species acts as a negative feedback on γ-secretase activity. In AD, however, consecutive cleavage of APP by first β-secretase or BACE1 and then γ-secretase predominates, forming insoluble Aβ peptide (Aβ 1-42) which is excreted by neurons (for review Querfurth and LaFerla, 2010; De la Vega and Zambrano, 2013; for review Frost and Li, 2017). Glial cells (astrocytes and microglia) then unsuccessfully attempt to eliminate the Aβ 1-42, generating an inflammatory process that, together with the toxic effect of Aβ 1-42 itself, contributes to neuronal damage.
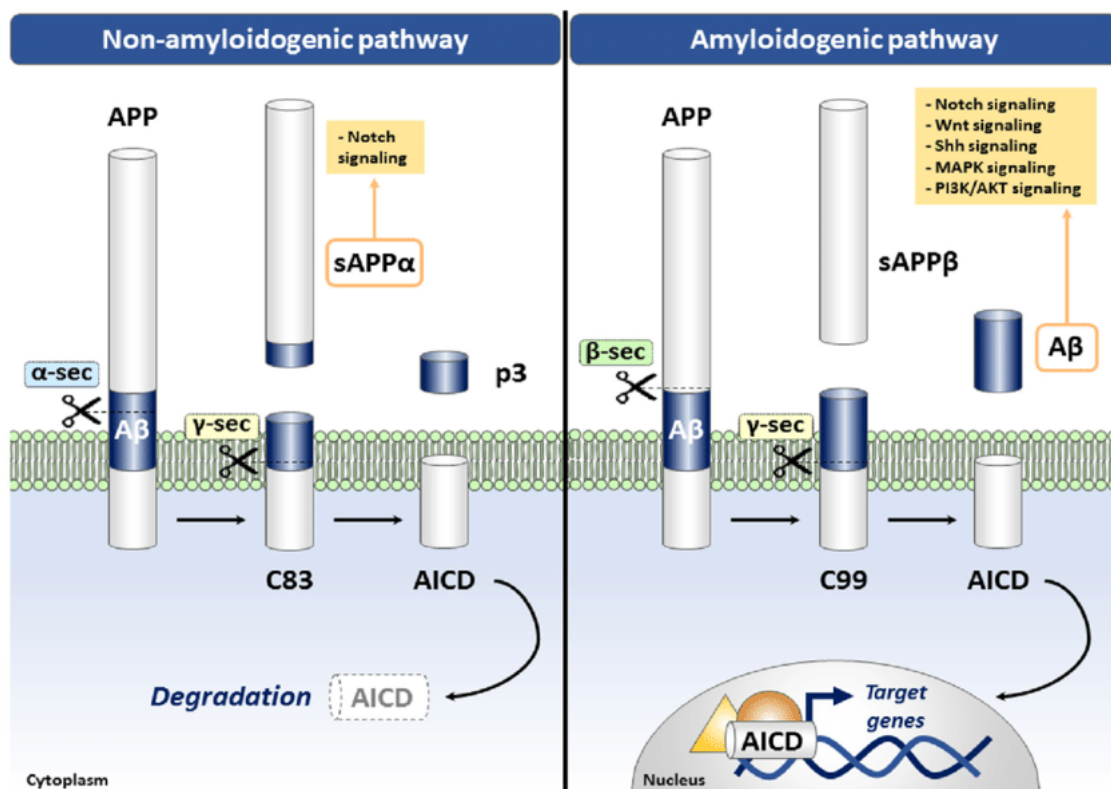

Figure 1. APP proteolysis processes. Two processes of APP proteolysis have been described, one amyloidogenic and one non-amyloidogenic. (From Coronel et al., 2019).

As for NFTs, they constitute neurofibrillary degeneration and are generated by intracellular deposits of τ protein. The normal τ protein forms the "bridges" that correctly hold together the microtubules that make up the neuronal cytoskeleton, but in AD (partly due to the toxic action of Aβ 1-42) there is an abnormal hyper phosphorylation of the τ protein. The hyper phosphorylation of τ proteins forms paired helical filaments associated with ubiquitin, which causes the destruction of microtubules and neurofilaments, disassembling the cytoskeleton and giving rise to neurofibrillary degeneration (De la Vega and Zambrano, 2013; for review Selkoe and Hardy, 2016).

Both types of lesions (SPs and NFTs) can also be found in the brains of healthy elderly people. What really marks the histopathological diagnosis is their number and location, being their number and density correlated with the intensity of dementia in these patients (De la Vega and Zambrano, 2013).

In early-onset AD, abnormal proteolytic processing of human amyloid precursor protein (hAPP) occurs due to mutations in genes encoding hAPP or presenilins 1 or 2 (transmembrane proteins that constitute the catalytic subunit of the γ-secretase enzyme), increasing the production and extracellular deposition of hAPP-derived Aβ peptides (for review Wyss-Coray and Mucke, 2002; for review Selkoe, 2001).

In vivo studies in mouse models of AD reveal that Aβ deposits have a direct toxic effect on neurons, causing dendritic simplification, with loss of dendritic spines and neuritic dystrophies, as well as inflammation and cell death (Spires et al., 2005; Meyer-Luehmann et al., 2008; for review Cline et al., 2018). Thus, exposure to oligomeric Aβ results in synaptic dysfunction in early AD, with reduction in the density of dendritic spines (Shankar et al., 2007) as well as changes in their morphology (Shankar et al., 2007; Ortiz-Sanz et al., 2020). These phenomena correlate with alterations in synaptic networks (Shankar et al., 2007), and this in turn has an impact on the degree of cognitive impairment in AD patients (Terry et al., 1991).

In an attempt to maintain brain function and cognition during the onset of dementia in early AD pathology, neuronal organisation allows for remodelling as a compensatory mechanism. That is, the hippocampus, a critical structure in the medial temporal lobe memory circuitry, is affected in the early stages of AD and in response to pathological extracellular deposition of Aβ and the formation of intracellular NFTs sets in motion an adaptive response of synaptic and intraneuronal remodelling (for review Mufson et al., 2015). These changes occur before neuronal death and the formation of SPs in the early stages of AD (Terry et al.,

1991). Therefore, Aβ may promote dual effects by acutely enhancing dendritic complexity and spine density (Ortiz-Sanz et al., 2020). Thus, changes in dendritic morphology and density are early pathological features that characterise AD (for review Bakota and Brandt, 2016), and recent findings suggest that dendritic spine plasticity may provide cognitive resilience against dementia at these stages of the disease (Boros et al., 2017). In this way, a "latent" period in disease progression is described, which seems to be associated with the involvement of compensatory mechanisms in the brain, capable of temporarily or permanently protecting the brain from neurodegenerative processes. These compensatory/adaptive brain mechanisms are thought to be activated in response to the malfunctioning of various brain systems: antioxidant, neurotrophic, neurotransmitter (NT), immune and others (Bobkova and Vorobyov, 2015). However, prolonged exposure to Aβ inevitably causes microstructural changes at the synapse that result in increased NT release, failure of synaptic plasticity and memory loss (Koppensteiner et al., 2016). These changes in dendritic spines and neuronal loss occur in a region-specific manner (Golovyashkina et al., 2015).

Nevertheless, the pathophysiology of AD is far from being understood and, despite extensive biomedical scientific efforts, currently available treatments offer only a partial and transient improvement of symptoms and a slight delay in disease progression (for review Massoud and Leger, 2011; Beauquis et al., 2013). This chronic age-related neurodegenerative pathology is expanding intensively in modern human society and has been observed to begin long before its clinical manifestations (Bobkova and Vorobyov, 2015). Increasing evidence suggests that SPs and NFTs are not the first pathological changes in AD (Beauquis et al., 2013).

On the one hand, growing proofs suggest that oxidative stress plays an important role in the pathophysiology of the disease. Intracellular oxidative balance is tightly regulated, so one would expect multiple signalling pathways to be activated in neurons of AD patients for the regulation of compensatory mechanisms. Oxidative stress occurs early in disease progression, long before pathological features (SPs and NFTs) develop. Thus, in the early stages of AD, there is an increase in oxidative stress levels, mainly generated by the interaction between reactive oxygen species (ROS) formed by mitochondria and transition metals. This induces adaptive neuronal responses, such as MAP kinase activation, Aβ deposition and τ protein hyper phosphorylation, to prevent neuronal damage and/or death. However, as AD progresses and ROS levels steadily increase, efficient clearance of metal-Aβ complexes and hyper phosphorylated τ protein would be overwhelmed by their disproportionately high generation.

This would lead to uncontrolled growth of SPs and NFTs, together with increased ROS generation, resulting in neuronal damage and subsequent neuronal death (for review Moreira et al., 2006). Therefore, it is also hypothesised that Aβ deposition and hyper phosphorylated τ protein are compensatory responses and subsequent adaptations to ensure that neuronal cells do not succumb to oxidative damage in the early stage of disease development (for review Moreira et al., 2006).

In addition, toxic forms of the Aβ peptide can induce Ca^{2+} entry into neurons by oxidative stress or by forming an oligomeric pore in the membrane, making neurons vulnerable to excitotoxicity and apoptosis. Hence, AD compromises the ability of neurons to regulate Ca^{2+} entry, efflux and subcellular compartmentalisation (for review Bezprozvanny and Mattson, 2008).

On the other hand, increasing evidence suggests that the glia-mediated inflammatory immune response is an important component of AD neurophysiology. The close association of astrocytes and activated microglia with SPs and cells undergoing neurofibrillary degeneration and the release of soluble cytokines strongly suggests that inflammatory processes may play an important role in the pathogenesis of AD (for review Unger, 1998; Mrak and Griffin, 2005; Dzamba et al., 2016).

This doctoral thesis analyses the pathological changes that appear in microglia and astrocytes in five familial Alzheimer's disease mutations (5xFAD) transgenic mice (Table 1). The 5xFAD transgenic mouse was developed in 2006, and overexpresses APP and PSEN1 by 5xFAD mutations: Human APP by three mutations in the APP transgene (695) [the Swedish (K670N, M671L), Florida (I716V) and London (V7171) mutations], and human PSEN1 by two mutations in the PSEN1 transgene (M146L and L286V) (Oakley et al., 2006). The 5xFAD line became congenic in the C57BL/6J background, and exhibit amyloid deposition, gliosis and progressive neuronal loss (Oakley et al., 2006; Oblak et al., 2021; Zhang et al., 2021) accompanied by cognitive and motor deficits, recapitulating many of the features of human AD. However, in the 5xFAD model, NFTs do not usually appear (Oakley et al., 2006).

2.2. ASTROCYTES:

Among the cellular changes in the central nervous system (CNS) that occur before clinical symptoms appear in AD, the most significant at the cellular level is neuronal death, but since astroglial cells are responsible for maintaining homeostasis in the brain, they are also closely

related (Verkhratsky et al., 2019). The pathological modification of astrocytes in the brains of patients with dementia was first described by Alois Alzheimer (Simchowicz, 1911). He observed abundant glial cells located in SPs (Simchowicz, 1911). Subsequently, post-mortem analyses of human tissues in the late stages of the disease have shown widespread reactive astrogliosis, with the inclusion of astrocytes in SPs (Nagele et al., 2003; for review Nagele et al., 2004; for review Rodriguez et al., 2009; Olabarria et al., 2010; Verkhratsky et al., 2019).
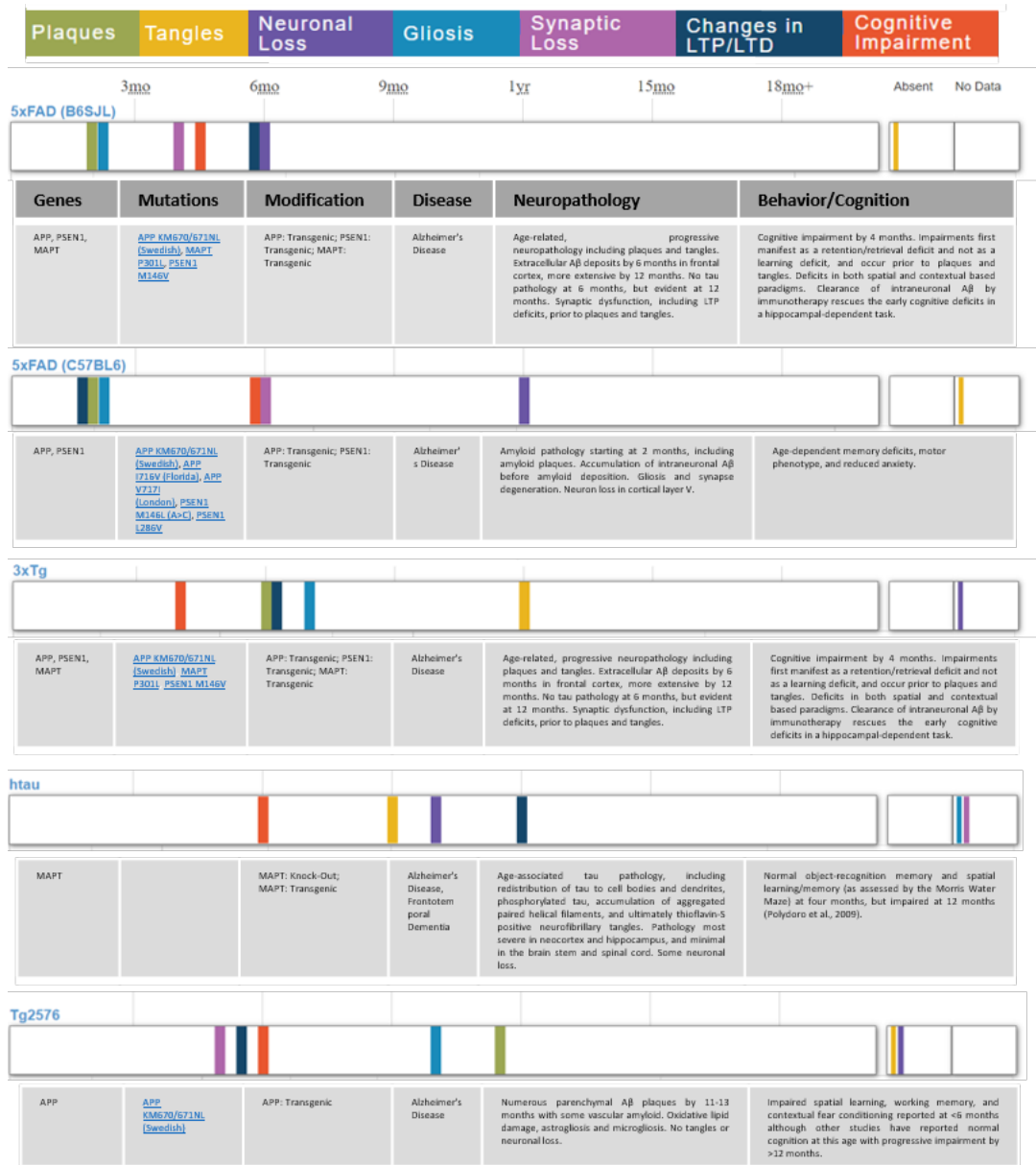
Reactive astrogliosis is therefore an archetypal morphological feature of AD, which has been observed both in human tissue and in isolated brains from animal models of the disease (for review Nagele et al., 2004; for review Rodriguez et al., 2009; for review Heneka et al., 2010; Beauquis et al., 2013; for review Verkhratsky et al., 2010).

Astrocytes, the most abundant cell type in the brain, form part of the basis of the grey matter and are the main element of the brain's homeostatic system. They shape the brain microarchitecture, form neuronal-glial-vascular units, regulate the blood-brain barrier (BBB), control the CNS microenvironment and act as a defence against a multitude of insults. In transgenic models of AD, hypertrophic reactive astrocytes surround SPs, and astroglial atrophy can be observed throughout the brain parenchyma, which could explain the early changes in synaptic plasticity and cognitive impairments that develop before severe neurodegenerative alterations (for review Rodriguez et al., 2009). Furthermore, recent evidence suggests that early stages of AD are linked to an accumulation of astrocytes at sites of Aβ deposition, suggesting that these lesions generate chemotactic molecules that mediate their recruitment (Wyss-Coray et al., 2003). This phenomenon leads to alterations in synaptic connectivity, imbalance in NT homeostasis and neuronal death through increased excitotoxicity (Wyss-Coray et al., 2003; for review Verkhratsky et al., 2010). Reactive astrocytes surrounding SPs also appear to be involved in the inflammatory process of the disease (Medeiros and LaFerla, 2013). Complementary *in vitro* studies have shown that in fact astrocytes surrounding SPs are involved in the formation of these plaques and in the local inflammatory response. These glial cells are activated by Aβ 1-42 oligomers and this makes them secrete cytokines such as IL-1β and other toxic products (for review Nagele et al., 2004; for review Heneka et al., 2010; for review Rodriguez et al., 2009; Hou et al., 2011; Beauquis et al., 2013). This evidence therefore suggests a key role for astrocytes in modulating Aβ-induced cellular toxicity in the brain (Beauquis et al., 2013).

In a transgenic model of Alzheimer's disease (3XTg-AD) (Table 1), hypertrophic reactive astrocytes have been described surrounding SPs, but atrophy of astroglial cells has been observed in the rest of the brain parenchyma. Early atrophy of astrocytes may be

pathologically relevant in the progression of familial Alzheimer Disease (FAD) (also in animal models of AD that reproduce FAD-like pathology). Atrophic changes are associated with medial and distal thin secondary processes, resulting in a reduction in overall astroglial arborisation and thus a possible decrease in glial synaptic coverage (for review Rodriguez et al., 2009). Another study using the same mouse model (3xTg-AD) (Table 1) shows that the overall astroglial cell density is not affected by either age or AD conditions, that is, neither ageing nor AD pathology is associated with cell loss. Despite this, widespread atrophy of glial fibrillary acidic protein (GFAP)-positive astrocytes in both the dentate gyrus (DG) and Cornu Ammonis 1 (CA1) has been described in the AD brain. This atrophy is manifested by a decrease in the surface area and volume of GFAP-positive profiles appearing in DG in the early stages of the disease as soon as 6 months of age. A similar hypotrophy of GFAP-positive astrocytes in the CA1 hippocampal region has also been described at 18 months of age. Thus, signs of atrophy have been described in astrocytes located at more than 50 μm from the plaque borders (all cells with soma within 50 μm of the plaque border are considered plaque-associated), with decreased soma volumes and reduced number of main processes, their arborisation and overall surface area and volume. However, astrocytes associated with SPs show clear signs of reactivity: hypertrophy, thickened processes, enlarged cell bodies and overall increased volume and surface area of GFAP-positive profiles. Therefore, differential changes in astrocyte morphology can be observed according to their relationship with SPs (Olabarria et al., 2010).

Table 1. Different transgenic mouse models of Alzheimer's disease. Each model shows distinct signs of the disease such as plaques (green), tangles (yellow), neuronal loss (dark purple), gliosis (light blue), synaptic loss (light purple), changes in LTP/LTD (dark blue) and cognitive impairment (orange) at various ages (modified from http://www.alzforum.org/research-models/alzheimers-disease).

Plaques	Tangles	Neuronal Loss	Gliosis	Synaptic Loss	Changes in LTP/LTD	Cognitive Impairment

3mo | 6mo | 9mo | 1yr | 15mo | 18mo+ | Absent | No Data

5xFAD (B6SJL)

Genes	Mutations	Modification	Disease	Neuropathology	Behavior/Cognition
APP, PSEN1, MAPT	APP KM670/671NL (Swedish), MAPT P301L, PSEN1 M146V	APP: Transgenic; PSEN1: Transgenic; MAPT: Transgenic	Alzheimer's Disease	Age-related, progressive neuropathology including plaques and tangles. Extracellular Aβ deposits by 6 months in frontal cortex, more extensive by 12 months. No tau pathology at 6 months, but evident at 12 months. Synaptic dysfunction, including LTP deficits, prior to plaques and tangles.	Cognitive impairment by 4 months. Impairments first manifest as a retention/retrieval deficit and not as a learning deficit, and occur prior to plaques and tangles. Deficits in both spatial and contextual based paradigms. Clearance of intraneuronal Aβ by immunotherapy rescues the early cognitive deficits in a hippocampal-dependent task.

5xFAD (C57BL6)

APP, PSEN1	APP KM670/671NL (Swedish), APP I716V (Florida), APP V717I (London), PSEN1 M146L (A>C), PSEN1 L286V	APP: Transgenic; PSEN1: Transgenic	Alzheimer's Disease	Amyloid pathology starting at 2 months, including amyloid plaques. Accumulation of intraneuronal Aβ before amyloid deposition. Gliosis and synapse degeneration. Neuron loss in cortical layer V.	Age-dependent memory deficits, motor phenotype, and reduced anxiety.

3xTg

APP, PSEN1, MAPT	APP KM670/671NL (Swedish), MAPT P301L, PSEN1 M146V	APP: Transgenic; PSEN1: Transgenic; MAPT: Transgenic	Alzheimer's Disease	Age-related, progressive neuropathology including plaques and tangles. Extracellular Aβ deposits by 6 months in frontal cortex, more extensive by 12 months. No tau pathology at 6 months, but evident at 12 months. Synaptic dysfunction, including LTP deficits, prior to plaques and tangles.	Cognitive impairment by 4 months. Impairments first manifest as a retention/retrieval deficit and not as a learning deficit, and occur prior to plaques and tangles. Deficits in both spatial and contextual based paradigms. Clearance of intraneuronal Aβ by immunotherapy rescues the early cognitive deficits in a hippocampal-dependent task.

htau

MAPT		MAPT: Knock-Out; MAPT: Transgenic	Alzheimer's Disease, Frontotemporal Dementia	Age-associated tau pathology, including redistribution of tau to cell bodies and dendrites, phosphorylated tau, accumulation of aggregated paired helical filaments, and ultimately thioflavin-S positive neurofibrillary tangles. Pathology most severe in neocortex and hippocampus, and minimal in the brain stem and spinal cord. Some neuronal loss.	Normal object-recognition memory and spatial learning/memory (as assessed by the Morris Water Maze) at four months, but impaired at 12 months (Polydoro et al., 2009).

Tg2576

APP	APP KM670/671NL (Swedish)	APP: Transgenic	Alzheimer's Disease	Numerous parenchymal Aβ plaques by 11-13 months with some vascular amyloid. Oxidative lipid damage, astrogliosis and microgliosis. No tangles or neuronal loss.	Impaired spatial learning, working memory, and contextual fear conditioning reported at <6 months although other studies have reported normal cognition at this age with progressive impairment by >12 months.

In addition, several studies have shown that astrocytes can accumulate substantial amounts of Aβ intracellularly (Nagele et al., 2003; Thal et al., 2000). In some astrocytes closely associated with SPs, an accumulation of Aβ has been observed with specific antibodies (Nagele et al., 2003; for review Rodriguez et al., 2009). The contribution of astroglia to the clearance and degradation of Aβ was suggested about 15 years ago, but this aspect is still unclear (for review Guénette, 2003; Nicoll and Weller, 2003; Verkhratsky et

al., 2019). Thus, in a transgenic Tg2576 mouse model (Table 1) of AD expressing mutant APP, reactive astrocytes that were associated with SPs were found to express zinc-dependent neprilysin metalloendopeptidase, an enzyme capable of degrading Aβ (Apelt et al., 2003). Further studies are needed to determine whether astrocytes degrade Aβ intracellularly or extracellularly, and whether removal of Aβ from the brain is among the normal homeostatic functions of astrocytes (Wyss-Coray et al., 2003). Some studies implicate astrocytes in Aβ clearance (Shaffer et al., 1995; Wyss-Coray et al., 2003), but others show that they inhibit microglia-mediated phagocytosis of SPs. Today it remains unclear whether the reactive astrocytes that accumulate around Aβ deposits have neuroprotective or destructive functions (DeWitt et al. 1998; Wyss-Coray et al. 2003).

Although Aβ production is mainly associated with neurons, there are several reports involving astrocytes in this process (for review Frost and Li, 2017). The contribution of astrocytes in Aβ production is also not fully characterised. Neurons were long considered to be the main source of Aβ as they express its producing enzymes (Laird et al., 2005; Verkhratsky et al., 2019). Healthy astrocytes do not seem to express these enzymes, however, their expression can be induced in astrocytes under chronic stress or neuroinflammatory conditions (Blasko et al., 2000; Leuba et al., 2005; Zhao et al., 2011; Orre et al., 2014; for review Frost and Li, 2017; Verkhratsky et al., 2019). Thus, expression of β-secretase or BACE1 has been found in reactive astrocytes in AD mouse models expressing mutant hAPP (Hartlage-Rübsamen et al., 2003; for review Rossner et al., 2005; Verkhratsky et al., 2019).

In addition, there is evidence that β-secretase or BACE1 is expressed in human and rodent brain neurons and also by reactive astrocytes near SPs in aged Tg2576 transgenic mice (Table 1) brains (Hartlage-Rübsamen et al., 2003; for review Rossner et al., 2005). Furthermore, BACE1 expression has been observed in reactive astrocytes in animal models of chronic gliosis and in the brains of AD patients (Brugg et al., 1995; for review Frost and Li, 2017).

The expression of APP and its mRNA in rat astrocytes and the presence of multiple proinflammatory cytokines that appear to regulate APP levels in mouse brain astrocytes have also been detected. This implies that in the neuroinflammatory context of AD, reactive astrocytes express higher levels of APP than at resting, therefore, they may produce more Aβ (Brugg et al., 1995; for review Frost and Li, 2017). Altogether, reactive astrocytes have higher levels of all three components required for Aβ production: APP, BACE1 and γ-secretase (for review Frost and Li, 2017). This suggests that reactive astrocytes might be playing an important role in the development of AD (Hartlage-Rübsamen et al., 2003). Modulating the state of astrocytes, reversing or halting their degeneration or regulating astroglial reactivity

could alter the course of AD delaying the disease or modifying the cognitive impairment (Verkhratsky et al., 2019).

2.3. MICROGLIA:

The brain maintains microglial levels in a finely tuned balance between local proliferation and apoptosis (Askew et al., 2017). It constitutes between 5% and 12% of all glial cells in the rodent brain and between 0.5% and 16% in humans (for review Gomez-Nicola and Perry, 2015; for review Sarlus and Heneka, 2017). Microglia forms the main inflammatory cells in the CNS and play key roles in responses to CNS damage. This cell type is rapidly activated by most CNS insults and accumulates in large numbers at sites of injury. This phenomenon termed reactive microgliosis is a hallmark of nearly all CNS pathologies, including trauma, stroke, inflammation, autoimmunity and neurodegenerative diseases such as AD (Ajami et al., 2007).

Although microglia is a specialised population of tissue macrophages resident in the CNS parenchyma known as "the macrophage of the brain" over the years, microglial cells have also many important roles in CNS development, function and repair, synaptic organisation, neuronal excitability, debris removal or trophic support (for review Gomez-Nicola and Perry, 2015; Grabert et al., 2016).

Their surveillance, constantly scanning the brain's microenvironment for minor perturbations of CNS homeostasis, is able to detect the presence of neurotoxic substances or inflammatory mediators from the systemic circulation, as they are in close communication with the BBB. Phagocytic microglia can rapidly detect and eliminate damaged or apoptotic neurons, preventing lesion of neighbouring cells and helping to maintain the turnover of specific cell populations. In addition, the phagocytic capacity of microglia is particularly important in development (pruning microglia), when they can contribute to the elimination of supernumerary synapses in specific neuronal pathways. Microglia also modulates the synapses then, influencing neuronal activity (neuromodulatory microglia) (for review Gomez-Nicola and Perry, 2015).

Microglial cells express a wide range of receptors that act as molecular sensors, allowing them to recognise an exogenous or endogenous insult and to initiate an immune response (for review Sarlus and Heneka, 2017). They therefore act as active sensors of alterations in their microenvironment and are capable of elaborating a broad spectrum of responses to restore tissue homeostasis (for review Gomez-Nicola and Perry, 2015). Loss of homeostasis or tissue

changes result in associated conditions that induce the emergence of various dynamic microglial processes, including changes in surface phenotype, cellular mediators secretion, cell morphology and proliferative responses (this is referred to as the 'activated state') (for review Sarlus and Heneka, 2017). Thus, the local environment will be a key influence in shaping the microglial phenotype (Grabert et al., 2016) and, consequently, the microglial transcriptome is regionally heterogeneous in the CNS. Considering that the microenvironment is not uniform across brain regions, variations in neuronal subtypes, NT profiles, hemodynamics and metabolism could influence the local microglial phenotype, again highlighting its functional diversity (Grabert et al., 2016; for review Sarlus and Heneka, 2017). They are hence cells of great plasticity showing different morphological and functional phenotypes. Two categories of activation have been described (Fig. 2): classical (M1) and alternative (M2). Specifically, stimulation with interferon-γ or lipopolysaccharide (LPS) promotes the activation of the microglia towards the M1 phenotype, which releases inflammatory mediators: cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), chemokines, redox molecules, co-stimulatory proteins and major histocompatibility complex type II (MHCII). These mediators increase inflammation leading to cell and tissue injury. In contrast, cytokines such as interleukin-4 (IL-4) and interleukin-10 (IL-10) promote the M2 polarised state. This type of microglia synthesises cytokines with anti-inflammatory activity, such as transforming growth factor beta (TGF-β) and IL-10, which have neuroprotective properties. These anti-inflammatory mediators will promote brain repair by removing cellular debris, reducing local inflammation and participating in tissue recovery. Under neuroinflammatory conditions, microglial cells get an amoeboid morphology and adopt a classical M1 or alternative M2 phenotype depending on the nature of the local environment (for review Nakagawa and Chiba, 2014; for review Salvi et al., 2017).
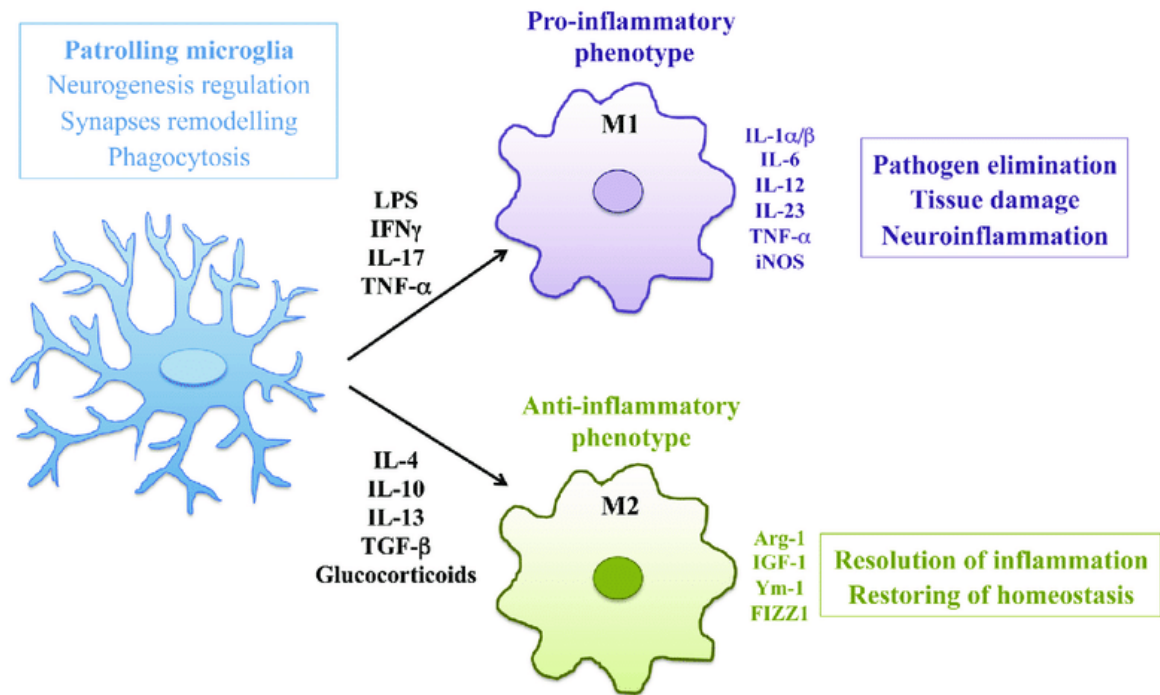
Figure 2. Activation and polarization of microglia under resting conditions and during neuroinflammation. The different functional states of microglia are represented with their associated morphology and phenotype (From Salvi et al., 2017).

Microglial activation and its M1/M2 polarisation have been observed in various forms of CNS damage (Lin et al., 2017). However, numerous studies have shown that microglia can express M1 and M2 markers at the same time (Almolda et al., 2015), suggesting the existence of mixed phenotypes (for review Sarlus and Heneka, 2017).

Imaging techniques have reinforced the traditional view that microglial morphology oscillates between "branched quiescent" and "activated amoeboid". However, microglia is highly dynamic with considerable functions and variable morphologies as they cycle, migrate, undulate, phagocytose, and extend or retract thin and thick processes. Thus, the three-dimensional space occupied by microglia is constantly changing as they migrate and move their cellular processes, reversibly transition from a simple rounded shape to a complex branched form with secondary and tertiary branches endowed with diffuse or even more branched tips (for review Karperien et al., 2013). This transition can be very rapid or, alternatively, microglia can remain in one form for years (Colton et al., 2000). Based on such flexible morphology in the mature CNS, unbranched and intermediate forms of microglia are considered as "activated", "reactive" or "intermediate", with a few or many robust processes protruding. This type of microglia is activated to perform an immuno-inflammatory function that includes gathering at injury sites, other cells recruitment and activation, proliferation,

phagocytosis, debris clearance and contribution to healing. In their fully branched forms, microglia is actively involved in essential physiological functions acting as guardian cells ensuring the proper functioning of neurons, providing neurotrophic substances, regulating NTs and hormones, mediating pain, protecting neurons from damage and responding to changes in the microenvironment. These branching microglial cells change and move in many ways as they perform their multiple tasks (for review Karperien et al., 2013). In AD, a variety of microglial reactions occurs at different stages of the disease (for review Sarlus and Heneka, 2017). Thus, unlike in the healthy brain, microglia proliferate and accumulate around SPs in order to promote their elimination (for review Gomez-Nicola and Perry, 2015). In response to neurodegeneration and Aβ aggregation, microglia adopts an activated state called priming (for review Perry and Holmes, 2014). Activated microglia in AD increases the expression of inflammatory markers such as CD14, IL-1β, p40, CCL-3, CCL-4, CXCL-1 and iNOS (Martin et al., 2017). Also, increased microglial proliferative activity has been described in mouse models of AD (Table 1) (Kamphuis et al., 2012; Olmos-Alonso et al., 2016) and in post-mortem brain samples from patients with the disease (Gomez-Nicola et al., 2013). Therefore, activated microglia together with immunoglobulins and complement components are closely associated with Aβ deposits in the brains of AD patients and mouse models (Table 1) (Eikelenboom and Stam, 1982; Frautschy et al., 1998; for review Sarlus and Heneka, 2017).

Upon contact between microglia and Aβ, Aβ binds to the microglia's pattern recognition receptors (PRRs) that are innate immune cell receptors designed to respond to danger- or pathogen-associated molecular patterns (DAMPs or PAMPs), resulting in the activation of resting microglia. Cytokines released by the activated microglia increase phagocytosis as well as Aβ uptake and clearance. However, long-term microglia activation drives proliferation and chronic inflammation that cause neurotoxicity, neurodegeneration and disrupting Aβ phagocytosis. Therefore, in the pathogenesis of AD, microglia activation may play a dual role: 1), acute microglia activation may lead to decrease Aβ accumulation through an increase in phagocytosis and clearance; 2), chronic microglia activation contributes to neurotoxicity and synaptic degeneration by triggering several pro-inflammatory cascades (for review Sarlus and Heneka, 2017).

2.4. ENDOCANNABINOID SYSTEM:

The endocannabinoid system (ES) is an important neuromodulatory system consisting of lipid molecules, specific receptors and several enzymes responsible for their synthesis and

degradation (for review Lu and Mackie, 2016; for review Talarico et al., 2019). The main endocannabinoids (ECBs) are the eicosanoids arachidonoylethanolamine (AEA), also known as anandamide, and 2-arachidonoylglycerol (2-AG). They are synthesised in the postsynaptic terminal from membrane phospholipids, and function as retrograde messengers at presynaptic cannabinoid receptors (for review Aso and Ferrer, 2014; for review Pertwee, 2015; Monory et al., 2015; for review Lu and Mackie, 2016). The ES negatively regulates the release of several NTs in an activity-dependent manner, thereby influencing the excitability of neuronal circuits (for review Katona and Freund, 2012; Monory et al., 2015). The ECBs are not stored in cells and released from vesicles, rather they are generated "on demand" (Min et al., 2010). In general, pharmacological and electrophysiological data have shown that Ca^{2+} influx can induce endocannabinoid synthesis as well as the activation of some metabotropic receptors (for review Petrocellis et al., 2004). After being synthesised, these endocannabinoids are released from the depolarised postsynaptic neuron and activate presynaptic cannabinoid receptor type 1 (CB_1R), suppressing the release of NTs from inhibitory or excitatory presynaptic terminals (for review Hashimotodani et al., 2007). Thus, by this mechanism, the ES causes a transient and long-lasting suppression of NT release from presynaptic cells (for review Ueda et al., 2011). Therefore, retrograde signalling is the primary mode by which endocannabinoids mediate short- and long-term forms of plasticity at excitatory and inhibitory synapses. However, endocannabinoid signalling is mechanistically more complex and diverse than originally thought (for review Castillo et al., 2012).

The synthesis and degradation pathways of endocannabinoids have attracted much attention since their discovery (for review Ueda et al., 2011). 2-AG and AEA are formed and degraded in completely different pathways, despite structural and functional similarities (for review Ueda et al., 2011).

In the case of 2-AG, which is much more abundant than AEA in the CNS (for review Hashimotodani et al., 2007), the phosphatidylinositol (PI)-phospholipase C (PLC)/diacylglycerol lipase (DAGL) synthesis pathway is probably the most important (for review Ueda et al., 2011). After simultaneous activation of voltage-dependent Ca^{2+} channels and $G_{q/11}$-coupled metabotropic receptors, PLC is activated and produces diacylglycerol (DAG) from PI. This DAG is then converted to 2-AG by DAGL which is stimulated by high intracellular Ca^{2+} levels (Min et al., 2010). Therefore, strong neuronal depolarisation activates voltage-dependent Ca^{2+} channels that induces Ca^{2+} entry into the postsynaptic neuron and the rise in intracellular Ca^{2+} concentration (for review Hashimotodani et al., 2007). As with the CB_1R, the localization and expression level of the DAGL appears to be unique in each brain

region, which would regulate the threshold for 2-AG-mediated retrograde suppression of excitatory or inhibitory transmission. Moreover, it is known that the ES modulates synaptic plasticity in the hippocampus, in particular CB_1R and 2-AG play a key role, pointing to its functional participation in memory processing within the adult brain (for review Kano et al., 2009; Peñasco et al., 2019).

AEA is a lipid of the N-acylethanolamine group. It is biosynthesised via a phospholipid-dependent pathway, namely the enzymatic hydrolysis of the corresponding N-acyl-phosphatidylethanolamines (NAPE) (for review Piomelli, 2003). Specifically, AEA is synthesised via N-arachadonoyl-phosphatidylethanolamine (NArPE), which originates from the union of arachidonic acid and a membrane-bound phosphatidylethanolamine (PE). The synthesis of NAPEs, including NArPE, is accomplished by the transfer of a fatty acyl group from the phospholipids to the N-position of the primary amine of the PE by one of a series of N-acyltransferases that are either calcium-dependent or calcium-independent. Next, the release of AEA from NArPE is carried out by hydrolysis by phospholipases (for review Biringer, 2021), in particular by a NAPE-selective phospholipase D (PLD) enzyme (NAPE-PLD) with low affinity for other membrane phospholipids. The presence of NAPE has been confirmed in murine brain, testis and leukocytes. The precursor/product relationship between NArPE and AEA in the CNS was further confirmed by the findings of a similar distribution of both compounds in different areas of the brain (Bisogno et al., 1999; for review Petrocellis et al., 2004). Following cannabinoid receptor activation, endocannabinoids are degraded by enzymatic hydrolysis (for review Lu and Anderson, 2017). AEA is hydrolysed by fatty acid amide hydrolase (FAAH) in postsynaptic neurons, or by a more complex process involving the eicosanoid biosynthesis machinery (for review Biringer, 2021). On the other hand, 2-AG is predominantly hydrolysed by monoacylglycerol lipase (MAGL) in presynaptic neurons (for review Lu and Anderson, 2017; for review Talarico et al., 2019).

Among the main receptors of the ES, the best known are CB_1R and the cannabinoid receptor type 2 (CB_2R). Other cannabinoid receptors such as the transient receptor potential channel V1 (TRPV1), peroxisome proliferator-activated receptors α and γ (PPAR-α and PPAR-γ, respectively), the G protein-coupled receptor 55 (GPR55) and the G protein-coupled receptor 18 (GPR18) have also been found (for review Stella, 2009; for review Izzo et al., 2009; for review Stella, 2010; for review Fezza et al., 2014; for review Rapino et al., 2018; for review Guerrero-Alba et al., 2019).

The first cannabinoid receptor to be cloned and characterised was CB_1R in 1991, and the second, CB_2R, was first identified in 1993. Their distribution is very different, with CB_1R

being abundantly expressed in the CNS, whereas CB_2R is expressed at a much lower level. This implies that CB_1R is primarily responsible for the psychoactivity of exogenous cannabinoids and the physiological actions of endocannabinoids in the CNS. Studies with CB_1R-knock out ($CB_1^{-/-}$) mice and CB_1R-specific antagonists have endorsed the role of the CB_1R in brain function and dysfunction (Munro et al., 1993; for review Di Marzo et al., 2004; for review Stella, 2009; for review Zou and Kumar, 2018).

Both receptors (CB_1R and CB_2R) belong to the seven transmembrane domain receptor superfamily and are metabotropic G protein-coupled receptors (GPCRs) (for review McAllister and Glass, 2002; Sánchez et al., 2003). CB_1Rs are coupled to $G_{i/o}$ proteins and modulate the activity of numerous ion channels and second messengers (for review Straiker and Mackie 2006; for review Stella, 2010). Furthermore, different effects on cellular functions have been observed depending on the type of receptor stimulation. For example, acute activation of neuronal CB_1R for milliseconds to seconds reduces neurotransmission and controls intrinsic excitability (Mackie and Hille, 1992; for review Stella, 2010). However, their sustained activation for minutes or hours stimulates intracellular signals, which modify enzyme activity and the expression of specific genes (Marsicano et al., 2003; for review Stella, 2010).

CB_2R also bind to G_i proteins, but probably not to G_o proteins (Glass and Northup 1999; Munro et al., 1993; for review Stella, 2010). It shares 44% protein structure with CB_1Rs, but shows a different pharmacological profile and expression pattern (Felder et al., 1995; for review Stella, 2010).

Cannabinoid receptor activation by ECBs is known to result in the modulation of multiple intracellular signal transduction pathways (for review McAllister and Glass, 2002; Sánchez et al., 2003). Thus, when CB_1R and CB_2R are stimulated by 2-AG or AEA, intracellular signalling events are triggered through the activation of $G_{i/o}$ proteins (for review Di Marzo et al., 2004; for review Jean-Gilles et al., 2010). This has several consequences, such as inhibition of adenylate cyclase (AC) with subsequent inactivation of the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) phosphorylation pathway, stimulation of mitogen-activated protein kinase (MAPK) and activation of extracellular signal-regulated kinase (ERK). These intracellular events can modulate synaptic communication, modify enzymatic activity and regulate gene expression, among other effects (for review Guzmán et al., 2002; for review Piomelli, 2003; for review Di Marzo et al., 2004; for review Stella, 2010). CB_1R also inhibits N- and P/Q-type voltage-sensitive Ca_2^+ channels (VSCCs) and activates K^+ channels (for review Guzmán et al., 2002). In addition, through the activation of

PLC-γ via the βγ subunits of $G_{i/o}$ proteins, there is also stimulation of phosphatidylinositol 3-kinase (PI3K) with subsequent synthesis of inositol triphosphate (IP3) which induces intracellular Ca_2^+ mobilisation (for review Guzmán et al., 2002; for review Petrocellis et al., 2004). CB_1R has also been proposed to trigger more complex protein phosphorylation cascades via PI3K and protein kinase B (PKB) (Gómez del Pulgar et al., 2000; for review Di Marzo et al., 2004).

Cannabinoids therefore exert most of their effects in the CNS via CB_1R (Gómez del Pulgar et al., 2000), as it is the most abundant cannabinoid receptor expressed at high levels in the hippocampus, cortex, cerebellum and basal ganglia, regulating important brain functions such as cognition and memory, emotions, motor control, feeding or pain perception. CB_1R is considered the most abundant G protein-coupled receptor in the mammalian brain (for review Piomelli, 2003). Its expression occurs mainly in neurons and glial cells (for review Wilson and Nicoll, 2002). Specifically, electron microscopy studies have shown that CB_1R is abundant in presynaptic terminals, but it is also localized in postsynaptic structures and glia. Among CB_1R-expressing synapses under physiological conditions, inhibitory GABAergic interneurons tend to have a much higher content of this receptor than excitatory glutamatergic synapses (Monory et al., 2006; Katona et al., 2006; for review Katona and Freund, 2012; for review Lu and Mackie, 2016). In astrocytes, the expression of CB_1R is even smaller (Gutiérrez-Rodríguez et al., 2018). In our studies performed using electron microscopy, about 56% of the observed CB_1R labelling was found in GABAergic terminals, 12% in glutamatergic terminals and 6% in astrocytes. The rest of the immunoparticles were located in mitochondria (15%) or other compartments, which could be microglial cells, lysosomes, endosomes, etc. (Bonilla-Del Río et al., 2019, 2020). Moreover, this enrichment of CB_1R expression at inhibitory synapses varies greatly between brain regions (for review Howlett, 2002; for review Stella, 2009). Different types of interneurons also differ in their CB_1R content, for example, perisomatic basket cells and inhibitory dendritic cells associated with Schaffer's collaterals. This is consistent with the different efficacy of endocannabinoid-mediated synaptic plasticity at these synapses (Lee et al. 2010; for review Katona and Freund, 2012). Transgenic animal models with cell type-specific CB_1R deletion are useful tools to elucidate the contribution to behaviour and network activity of the endocannabinoid signalling at specific sites (Marsicano et al., 2003; Monory et al., 2006; for review Katona and Freund, 2012).

In situ hybridisation, immunocytochemistry and autoradiography demonstrated the presence of CB_2R in spleen, thymus, tonsils, bone marrow, pancreas, splenic

macrophage/monocyte preparations, mast cells, peripheral blood leukocytes as well as in a variety of cultured immune cell models (for review Howlett, 2002). CB$_2$R was initially identified in macrophages as a peripheral receptor. Subsequently, CB$_2$R expression in the brain was described by reverse transcription-polymerase chain reaction (RT-PCR), in situ hybridisation and immunohistochemistry. Still, there is much controversy about the presence of this receptor in the CNS. Some immunohistochemical studies indicate the presence of CB$_2$R in healthy brain neurons (Van Sickle et al., 2005; Gong et al., 2006), but they did not include the negative controls needed to assess immunostaining specificity, particularly CB$_2$R knock out mice (Gong et al., 2006; Onaivi et al., 2006, 2008; for review Stella, 2010). However, other studies limit the presence of CB$_2$R to glia, specifically to microglia. Furthermore, it has been proposed that microglial expression is dependent on the level of cellular activation (Carlisle et al., 2002; Maresz et al., 2005; López et al., 2018). Thereby, neuronal damage is associated with microglial activation and its switch to pro-inflammatory phenotypes that leads to the release of free radicals and toxins and the up-regulation of CB$_2$R expression. Furthermore, CB$_2$R expressed in activated microglia regulates microglial migration and promotes microglial polarisation to the anti-inflammatory phenotype (Lin et al., 2017). Thus, endocannabinoids produced by damaged neurons and lesion-stimulated astrocytes act on CB$_2$R expressed in microglia switching the cells to an anti-inflammatory phenotype (for review Stella, 2009). CB$_2$R expression increases sharply in pathological conditions with increase neuroinflammation, such as AD, multiple sclerosis, Down syndrome or human immunodeficiency virus (HIV) encephalitis (for review Benito et al., 2008; López et al., 2018). However, the lack of specific antibodies to label CB$_2$R casts doubts on these results (Gong et al., 2006; for review Atwood and Mackie, 2010; Marchalant et al., 2014; López et al., 2018). Finally, microglial cells might also express CB$_1$R whose activation in cell cultures regulates specific functions related to microglia-mediated immunity (for review Stella, 2010).

Western blotting and immunocytochemical studies have shown CB$_2$R in human astrocytes (Sheng et al., 2005). In addition, CB$_2$R has been described in astrocytes associated with SPs in post-mortem brains of AD patients (Benito et al., 2003; for review Kano et al., 2009). However, several studies restricted CB$_2$R expression to microglial cells (Benito et al., 2003, 2008; López et al., 2018). The CB$_1$R in astrocytes plays a role in their metabolic functions. For example, CB$_1$R activation in cultured rat astrocytes increases the rate of glucose oxidation and ketogenesis involved in brain energy supply (Blazquez et al., 1999; for review Stella, 2010). This may be due to the existence of CB$_1$R in astrocytic mitochondria,

as the presence of CB_1R in astrocytes is not only described in the cytoplasmic membrane. Therefore, CB_1R has also been detected in astrocytic mitochondrial membrane of mouse hippocampus, prefrontal cortex, piriform cortex and nucleus accumbens, demonstrating that these glial cells also contain functional mitochondrial CB_1R (Jimenez-Blasco et al., 2020). Activation of these receptors reduces mitochondrial soluble AC activity and PKA-dependent phosphorylation. Consequently, mitochondrial CB_1Rs in astrocytes interfere with glucose metabolism and lactate production, which affects neuronal functions and behaviour (Jimenez-Blasco et al., 2020). The astrocytic CB_1R has also been shown to play an indispensable metabolic role in maintaining optimal leptin receptor (ObR) expression and function (Bosier et al., 2013). Furthermore, astrocytic CB_1Rs moderate the ability of these cells to produce inflammatory mediators (Sheng et al., 2005; for review Stella, 2010). In addition, activation of hippocampal CB_1R-expressing astrocytes leads to Ca^{2+} mobilisation from internal stores, which stimulates glutamate release ultimately activating NMDA receptors on pyramidal neurons (Navarrete and Araque, 2008). Therefore, given the ability of astrocytic CB_1Rs to regulate energy metabolism and to mediate neuron-glia interactions, they seem to play an important role in a variety of key regulatory functions (for review Stella, 2010).

Altogether, both astrocytes and microglia express cannabinoid receptors in an activity-dependent manner (for review Bilkei-Gorzo, 2012), and their activation may regulate the differentiation, functions and viability of these cells (for review Stella, 2010).

2.4.1. ENDOCANNABINOID SYSTEM AND ALZHEIMER'S DISEASE:

The efficacy of current therapies against AD is limited, highlighting the need to develop new agents to prevent or delay the disease process. The ES has been shown to modulate the main pathological processes occurring during the neurodegeneration pathway, and has therefore become a novel strategy to tackle this illness (for review Aso and Ferrer, 2014). The ES appears to have a CB_1R-dependent neuroprotective function. Indeed, an accelerated decline in learning performance at maturity has been observed in $CB_1^{-/-}$ mice (Bilkei-Gorzo et al., 2005), which may be related to the lack of these documented neuroprotective effects (Sinor et al., 2000; Bilkei-Gorzo et al., 2005). In AD, changes in cannabinoid receptor expression may depend on the stage of the disease. In this regard, CB_1R and CB_2R have different expression patterns: the highest level of activity is shown by hippocampal and frontal CB_1R in the early stages of AD, but appears to decrease as the disease develops. However, CB_2R is more expressed at advanced stages when

neuroinflammation is more evident and microglia and astrocytes are activated (for review Di Marzo et al., 2015; for review Talarico et al., 2019).

The potential of cannabinoids to target several processes involved in AD pathogenesis, such as oxidative stress, Aβ and τ protein metabolism, inflammation, mitochondrial dysfunction, and excitotoxicity, is regarded as a therapeutic strategy (Fig. 3) (Casarejos et al., 2013; for review Talarico et al., 2019). Thus, by inhibiting presynaptic glutamate release (Hampson et al., 1998; Marsicano et al., 2003; Monory et al., 2006) and blocking voltage-dependent Ca^{2+} channels (Mackie and Hille, 1992; Twitchell et al., 1997), ECBs have a neuroprotective effect against excitotoxicity (Sinor et al., 2000; Marsicano et al., 2002). In addition, *in vitro* and *in vivo* studies have shown that certain cannabinoid compounds confer neuroprotective effects against Aβ. Direct stimulation of cannabinoid receptors (via 2-AG) or inhibition of endocannabinoid degrading enzymes (MAGL) facilitate Aβ clearance *in vitro* and *in vivo*. These actions appear to be mediated through cannabinoid receptors, as the modulation or inhibition of CB_1R or CB_2R mitigates the effect of this cannabinoid treatment. Further, following cannabinoid treatment, the levels of lipoprotein receptor-related protein 1 (LRP1), the Aβ transporter protein, increase in brain and plasma, explaining the increased Aβ transit from the brain to the periphery via BBB (Bachmeier et al., 2013; for review Talarico et al., 2019).

A large number of studies have also shown that mitochondrial function is impaired in the early stages of AD and worsens with disease progression (for review Maruszak and Żekanowski, 2011; for review Talarico et al., 2019). In addition, brain metabolism is reduced in AD decreasing energy production. Thus, the limited glycolytic capacity of the cells leads to increase mitochondrial aerobic oxidative phosphorylation in order to meet their energy needs. However, oxidative phosphorylation is a major source of endogenous and toxic free radicals, such as hydrogen peroxide (H_2O_2), hydroxyl (-OH) and superoxide (O^{2-}) (for review Moreira et al., 2006; for review Talarico et al., 2019). In this regard, ECBs can prevent the production of ROS and reduce nitric oxide (NO) by inhibiting the expression of inducible nitric oxide synthase (iNOS). This effect leads, in turn, to a significant inhibition of NO-dependent τ protein hyper phosphorylation in a concentration-dependent manner. Thus, cannabinoids also play an important role in τ hyper phosphorylation. Actually, *in vitro* studies in Aβ-stimulated C6 rat glioma cells have shown that this phenomenon occurs through a selective activation of CB_1R (Esposito et al., 2006; for review Talarico et al., 2019). There are also studies reporting a Δ9-tetrahydrocannabinol (THC)-mediated decrease in mitochondrial NADH oxidase activity involved in mitochondrial complex I activity.

However, these mitochondrial effects of cannabinoids are concentration-dependent (Athanasiou et al., 2007).

Cannabinoids have also been shown to prevent Aβ-induced neurodegeneration by reducing microglial activation. Both CB$_1$R and CB$_2$R expressed in microglia are involved in this action. Therefore, the role of these receptors in inhibiting neuroinflammation by preventing ROS formation and microglial release of cytokines has been documented (Ramírez et al., 2005; Martín-Moreno et al., 2011; Casarejos et al., 2013; for review Talarico et al., 2019).
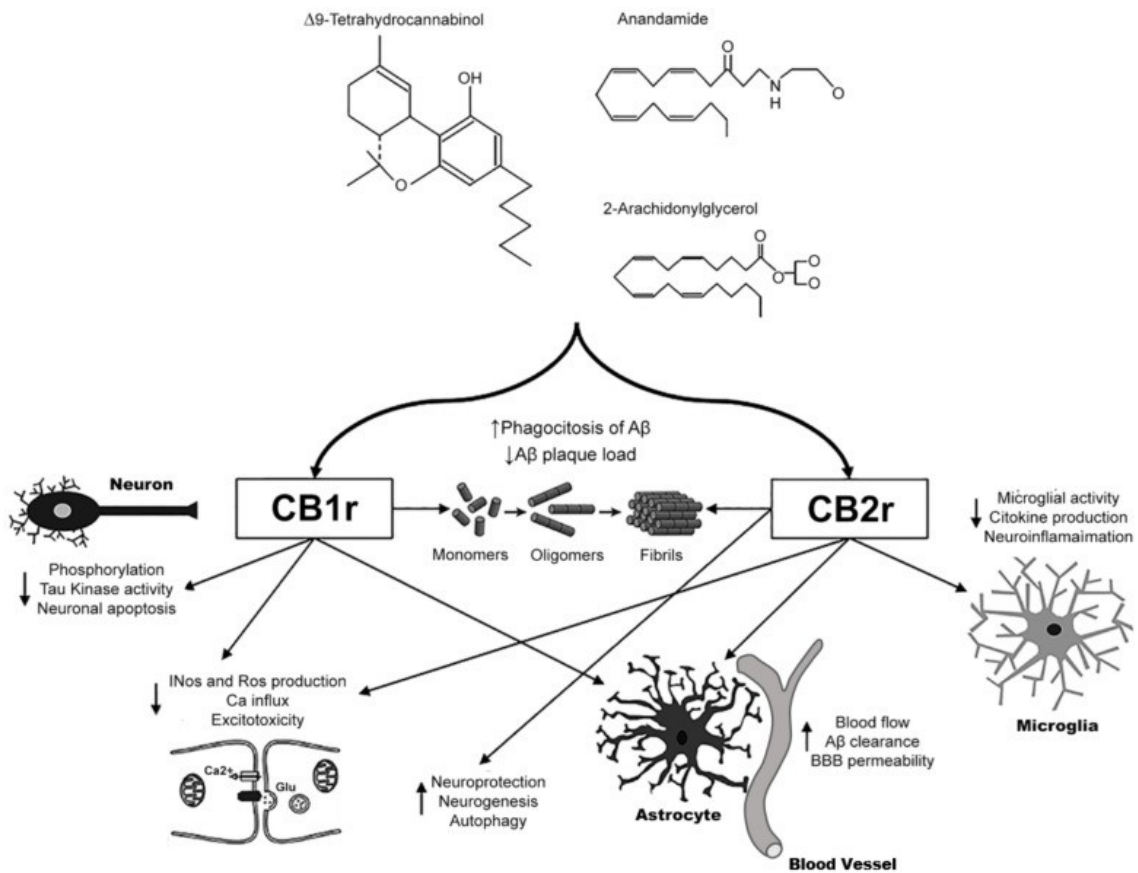
Figure 3. Diagram of the beneficial effects of the cannabinoid system in the pathology of AD. Cannabinoid treatment can modulate multiple AD processes, such as Aβ and τ processing, neuroinflammation, microglial activation, mitochondrial dysfunction and excitotoxicity (From Talarico et al., 2019).

2.5. LIMBIC SYSTEM:

The limbic system (LS) of the brain regulates a range of behaviours essential for the subsistence of all vertebrate species, including humans. It connects external cues or stimuli of emotional, social or motivational relevance with a specific set of appropriate, contextual

and species-specific behavioural outputs. Many of these behaviours are enhanced by learning and experience reinforcement, but most of them are innate meaning that they manifest themselves without prior learning. These innate behaviours focused on the survival of the individual or progeny and the propagation of the species include courtship, maternal care, defence and the establishment of social hierarchy. Activation of the circuits that regulate these behaviours begins in the periphery with sensory stimulation such as touch, sound and, especially in rodents, smell. These stimuli are then processed in the brain by a set of delineated structures that primarily include the amygdala and hypothalamus. An animal's inability to detect or correctly process social or environmental cues results in abnormal behaviour and increases the risk of attack and/or predation. Although the basic neuroanatomy of these connections is well established, much remains unknown about how information is processed within innate circuits (for review Sokolowski and Corbin, 2012).

Degenerative changes in the LS play an important role in the genesis of AD (Van Hoesen and Hyman, 1990). The first neurodegenerative events in AD appear in the trans-entorhinal cortex and subsequently spread to the entorhinal cortex (EC) and hippocampus. Later, this neurodegenerative process spreads through the temporal, frontal and parietal lobes (Thompson et al., 2003; for review Thompson et al., 2007; Verkhratsky et al., 2019). In the later stages of the disease, there is widespread atrophy of the brain parenchyma with significant loss of neurons and synaptic contacts. This atrophy includes both white and grey matter (Simchowicz et al., 1911; Verkhratsky et al., 2019). In particular, the hippocampal formation is severely damaged in AD being a focal point of the pathology (Van Hoesen and Hyman, 1990).

2.5.1. HIPPOCAMPAL FORMATION:

The hippocampal formation is located in the temporal lobe of the human brain. It consists of a group of distinct cytoarchitectonic structures: DG; hippocampus proper or cornu ammonis (CA); EC, which especially in rodents is divided into medial and lateral portions; subiculum (Fig. 4) (for review O'Mara et al., 2001; for review Rajmohan and Mohandas, 2007). The main reason for grouping these structures together is that they are linked by prominent and largely unidirectional connections that form a functional entity. However, their components vary between authors (Insausti and Amaral, 2004).

- *HIPPOCAMPUS:*

The CA or hippocampus is a layered structure (archicortex) consisting of the outer stratum oriens, stratum pyramidale, stratum radiatum and stratum lacunosum-moleculare layer, plus the stratum lucidum in the CA3 region. In terms of differences in cytoarchitecture and connectivity, the hippocampus is divided into four fields (named by Lorente de Nó in 1934): CA1, CA2, CA3 and CA4. There is disagreement as to whether or not CA4 is part of the CA, or whether it is a separate structure. The hippocampus adjoins the alveus, a thin fibre layer that joins to form the fimbria and, subsequently, the crura of the fornix, which is the main efferent pathway of the hippocampal formation. The fornix crura converge to form the fornix body and then the fornix columns that reach the mammillary bodies of the hypothalamus (for review Rajmohan and Mohandas, 2007; El Falougy et al., 2008).

- *SUBICULUM:*

While the physiology and functions of the DG and CA have been extensively studied, the subiculum has received little attention (for review O'Mara et al., 2001), despite being an important input and output region of the hippocampal formation (Fig. 4). This structure occupies a key position in the neural circuitry of learning and memory formation (Ishihara and Fukuda, 2016). A wide variety of information (space, time, etc.) is processed in the CA1 area and the medial/lateral EC, and then both regions synapse with the subiculum, which actively modifies and integrates these inputs. In addition, subicular pyramidal cells exhibit various forms of synaptic plasticity and form recurrent circuits (for review Matsumoto et al., 2019).
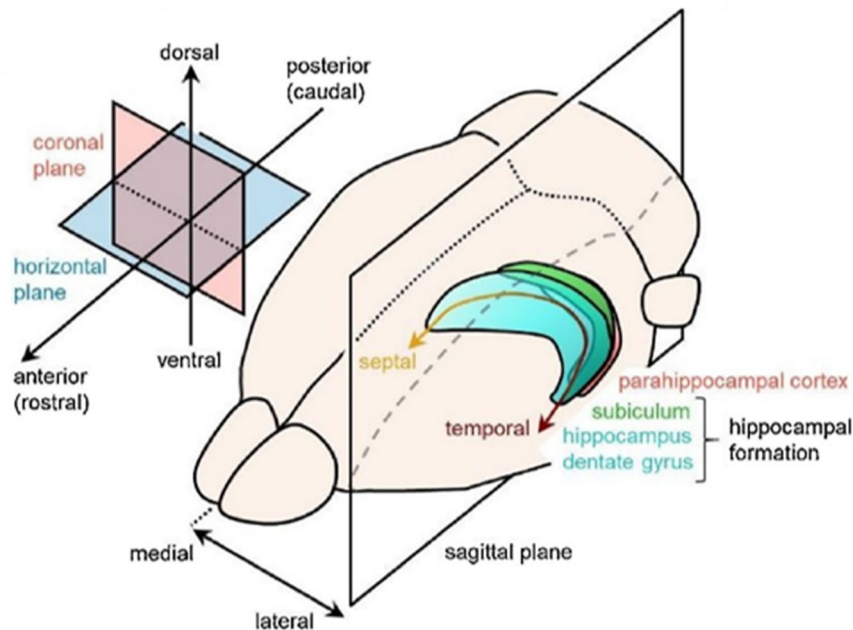
Figure 4. Anatomy of the rat subiculum (Modified from Matsumoto et al., 2019). The hippocampal formation is shown in green and blue, and the parahippocampal cortex in red. The hippocampal formation includes the hippocampus and DG, shown in blue, and the subiculum in green. Three representative reference axes and their corresponding cardinal planes are shown: the anteroposterior (rostrocaudal), dorsoventral and mediolateral axes, which are perpendicular to the coronal, horizontal and sagittal planes, respectively. In the C-shaped hippocampal formation in rodents, the end closest to the septum is named the septal pole, while the end adjoining the temporal lobe is the temporal pole (for review Matsumoto et al., 2019).

The subicular complex is usually subdivided into three zones: subiculum proper, presubiculum (PreS) and parasubiculum. There are examples of different subdivisions. For example, Lorente de Nó (1934) described a region between the CA1 and the subiculum called prosubiculum which today many researchers suggest is a transitional region and should not be defined separately. Brodmann (1909) also recognised a separate region in several species: the post- or retrosubicularis region; more recent authors suggest that this region may be considered part of the presubiculum. Later, van Groen and Wyss supported again the idea of the post-subiculum as a fourth region of the subicular complex (van Groen and Wyss, 1990; for review O'Mara et al., 2001). Thus, the subdivisions of the subicular complex have been a controversial issue over the last century.

Most of the subiculum is located between the PreS and the CA1 region of the hippocampus with, an area adjacent to the retrosplenial cortex at the septal end (Ishihara and Fukuda, 2016). Little is known about the internal structure of the subiculum that remains controversial. The subiculum is a characteristic three-layered structure of the allocortex, in contrast to the six-layered EC typical of neocortex. The three main layers of the subiculum

are: molecular layer, which is continuous with the CA1 strata lacunosum-moleculare and radiatum; extended pyramidal cell layer, containing the somata of the principal neurons; and polymorphic layer (for review O'Mara et al., 2001). The molecular layer is close to the hippocampal fissure; the pyramidal cell layer lies in the middle between the molecular and the polymorphic layer; the polymorphic layer is closer to the alveus and is continuous with the CA1 stratum oriens (for review Matsumoto et al., 2019). The molecular and polymorphic layers are also referred to as superficial and deep layers based on their proximity to the hippocampal fissure and alveus, respectively. On the other hand, the terms proximal and distal are used to refer to the region of the subiculum closest to the CA1 region and the region of the subiculum closest to the PreS, respectively. Up to five layers have been distinguished in the proximal subiculum that contains larger neurons with lower cell density, especially in the middle and deep layers. The distal subiculum, however, consists of smaller neurons with relatively homogeneous soma size and higher cell density (Ishihara and Fukuda, 2016). In addition to this classification, immunohistochemical investigations have further characterised the internal structure of the subiculum based on differential cytoarchitecture and immunoreactivity for calbindin (Fujise et al., 1995; for review Matsumoto et al., 2019).

The main cell type of the subiculum is the pyramidal cell. They exhibit a lower degree of axonal collateralisation than CA1 pyramidal cells and project to only a few brain areas. Nevertheless, these neurons have axon collaterals that reach the alveus, and in turn have multiple synaptic terminals both in the pyramidal cell layer and the apical dendrites (Harris et al., 2001; for review Matsumoto et al., 2019). Cell packing in the subicular pyramidal layer is more diffuse than in CA1 (for review O'Mara et al., 2001). In addition, many smaller neurons intermingled with the pyramidal cells are considered to be subicular interneurons, however, they are poorly characterised (for review O'Mara et al., 2001; for review Matsumoto et al., 2019).

2.5.2. CONNECTIVITY OF THE HIPPOCAMPAL FORMATION:

The EC projection to the hippocampal formation is segregated: layer III entorhinal neurons project to the CA1 and the subiculum, while layer II stellate cells project to the DG, CA2 and CA3. The layer II pyramidal cells (island cells) of the medial EC also project to the CA1 and to a lesser extent to the subiculum (for review Matsumoto et al., 2019).

The perforant pathway is considered the major pathway to the hippocampus, where fibres from glutamatergic neurons in layers II and III of the EC pass through the subiculum

and reach the granule cell layer of the DG. Glutamatergic mossy fibres then extend from the DG to the CA3 pyramidal cells. Next, although some CA3 efferent fibres project to the fimbria, many CA3 axons emit Schaffer collaterals that reach ipsilateral CA1 dendrites (stratum radiatum and stratum oriens). Finally, from CA1, the fibres extend to the alveus, the fimbria and then to the fornix. In addition, the subiculum also receives a massive input of important projections from the CA1 region. Therefore, the subiculum is innervated mainly by CA1 and the EC (for review O'Mara et al., 2001; Ishihara and Fukuda, 2016; for review Matsumoto et al., 2019).

Three hippocampal efferent systems are formed: the precommissural fornix, the nonfornical fibres and the postcommissural fornix. The precommissural fibres of the fornix originate in the CA and the subiculum and travel within the fimbria, crura and body of the fornix. Non-fornical fibres project directly from the hippocampus to entorhinal area, posterior cingulate cortex, retrosplenial cortex and amygdala. Postcommissural fibres terminate mainly in the mammillary bodies, but some fibres also project to the anterior nuclei of the thalamus, the bed nucleus of the stria terminalis and the ventromedial nucleus of the hypothalamus. The CA fibres terminate exclusively in the septal nucleus, while the subicular fibres are distributed to the nucleus accumbens, anterior olfactory nucleus, septal nuclei, precommissural hippocampus, medial frontal cortex and rectus gyrus. This broad projection pattern implies that both the subiculum and the CA1 region play a fundamental role in the organisation of hippocampal output (for review Mark et al., 1995; for review Rajmohan and Mohandas, 2007; Ishihara and Fukuda, 2016; for review Matsumoto et al., 2019).

In AD, subicular projection neurons are particularly affected. Damage to these hippocampal outflow pathways leads to disconnection and isolation of the hippocampal formation (Hyman et al., 1984). In the brain of 5xFAD mice, Aβ deposition (and gliosis) begins at 2 months and reach a very large burden, especially in the subiculum and deep cortical layers (Oakley et al., 2006). Thus, pathology first appears in the subiculum and gradually spreads to interconnected limbic brain regions in a specific pattern, over 3-15 months (Rönnbäck et al., 2012; George et al., 2014). Thus, Aβ accumulation sequentially affects areas that receive neural projections from previously affected brain regions. The spatio-temporal progression of AD neuropathology along neuronal pathways has been suggested by several studies, but the underlying mechanisms are still unclear. The disease then affects brain regions that receive inputs from the subiculum, functionally connected sequentially in an order that is in accordance with known anatomical connections, such as the retrosplenial cortex, mammillary body and thalamus (Fig. 5) (Rönnbäck et al., 2012). In

studies of AD patients with different levels of cognitive impairment, a prevalent atrophy of the presubicular-subicular complex was observed from the early stages of the disease. This finding is consistent with neuropathological observations in AD patients and probably reflects the severe degeneration of the perforant pathway as it penetrates the hippocampus via the subicular field in its course from the entorhinal cortex to the DG. These studies therefore pointed to a non-homogeneous pattern atrophy in the hippocampus of AD patients, with prevalent involvement of the presubicular-subicular complex from the earliest stages of the disease that remains evident in the later stages (Carlesimo et al., 2015; Lindberg et al., 2017). Therefore, the study of neuronal damage and glial changes in the subiculum is fundamental to understanding AD pathology, given the major involvement of this system in the regulation of multiple brain functions (for review Matsumoto et al., 2019).
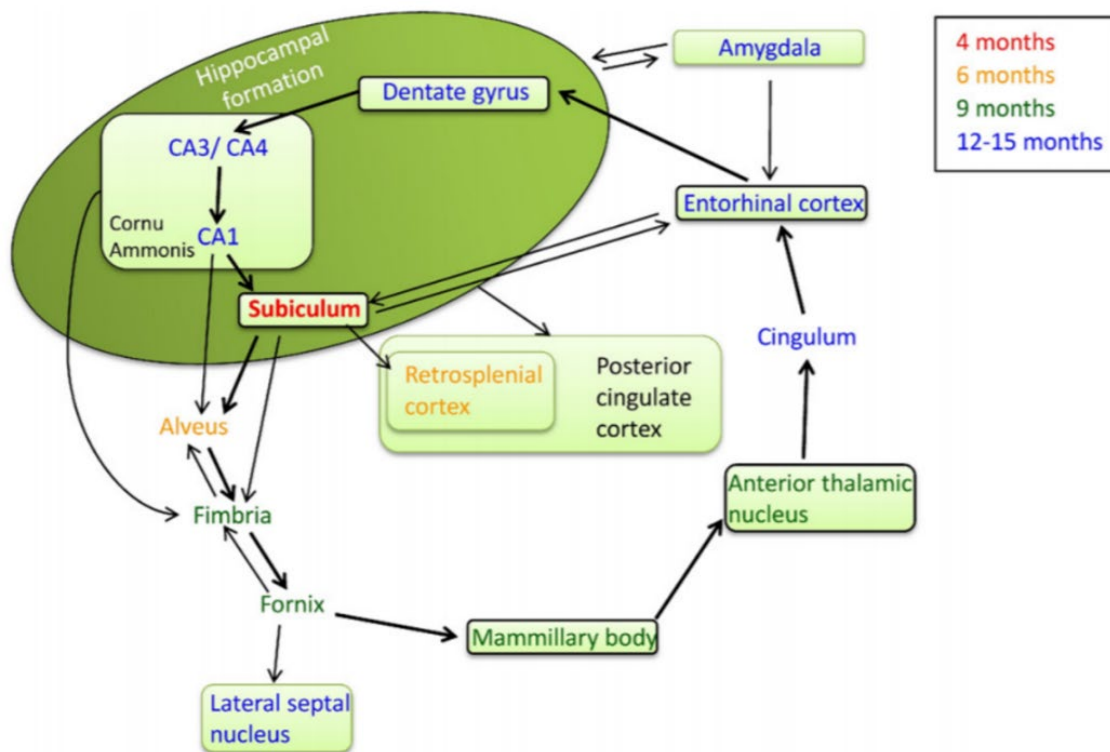
Figure 5. Schematic of the progression of diffuse amyloid deposition in a mouse model of Alzheimer's disease (TgAPParc). Relevant anatomical connections between brain regions are marked with arrows and the colour of the text marks the age at which the first diffuse amyloid deposits are detected (Modified from Rönnbäck et al., 2012).

3. WORKING HYPOTHESIS

Abundant pathological changes have been observed in post-mortem brains of AD patients and in animal models of the disease (Medeiros and LaFerla, 2013). These alterations include aggregation and deposition of Aβ peptides associated with the formation of SPs, hyper phosphorylation of τ protein associated with the development of NFTs, neuronal death, synapse dysfunction, inflammation, oxidative stress and alterations in mitochondrial metabolism (for review Moreira et al., 2006; for review Querfurth and LaFerla, 2010; for review Bloom, 2014; Bobkova and Vorobyov, 2015). However, the pathogenesis of AD is still unclear. In the last years, the role that inflammatory cells may play in AD is being investigated as it seems that they may affect multiple pathological mechanisms. Thus, a new field of research focused on glial cells is opening up.

Microglia is the main inflammatory cell type in the CNS and plays a key role in responses to CNS damage (Ajami et al., 2007). When activated in the course of disease, microglial cells decrease Aβ accumulation by increasing phagocytosis and clearance, and contribute to neurotoxicity and synaptic degeneration by triggering several pro-inflammatory cascades (for review Sarlus and Heneka, 2017). Astrocytes, on the other hand, seem to become more reactive in areas of increased injury surrounding SPs (for review Rodriguez et al., 2009) and appear to be involved in the inflammatory process of the disease (Medeiros and LaFerla, 2013).

The efficacy of current therapies to treat AD is limited, emphasizing the need to develop new agents to prevent or delay the neurodegenerative process (for review Massoud and Leger, 2011; Beauquis et al., 2013). The ES is an important neuromodulatory system (for review Talarico et al., 2019; for review Lu and Mackie, 2016) and the cannabinoids effects on several processes involved in the pathogenesis of AD suggest their potential therapeutic benefits in the disease (for review Talarico et al., 2019). However, little is known about the expression and localization of cannabinoid receptors in glial cells in AD. We hypothesise in my doctoral thesis that glial expression of CB_1R and CB_2R is altered in the subiculum of a mouse model of AD, a brain region particularly affected by large accumulation of SPs, as a result of subcellular changes happening in microglial cells and astrocytes. Therefore, knowing the correlation between glial reactivity in AD and the microglial and astroglial expression of CB_1R and CB_2R would help to better understand the role of the ES in the pathophysiology of the disease.

4. OBJECTIVES

The overall aim of my doctoral thesis was to investigate the glial expression of the cannabinoid CB_1R and CB_2R in a mouse model of AD, in particular, the astrocytic and microglial reactivity in the subiculum as well as the localization of CB_1R in astroglia and microglia and CB_2R in microglia.

I focused on the subiculum because: 1) it accumulates the highest amount of SPs; 2) it shows a large glial reaction; 3) it is one of the regions that most incipiently shows signs of degeneration; and 4) it is involved in memory formation.

The specific objectives were to:

1. Study microglial morphology.

Does AD affect the area, perimeter and number of microglial processes?

2. Investigate astrocyte morphology.

Does AD alter the area, perimeter and number of astrocytic processes?

3. Explore CB_1R localization in microglia.

Does AD impact on CB_1R expression in microglia?

4. Analyse CB_1R localization in astrocytes.

Does AD modify CB_1R expression in astrocytes?

5. Decipher CB_2R expression in microglia.

Does AD influence CB_2R expression in the microglial cells?

To achieve these objectives, I used immunoelectron microscopy in $CB2^{EGFP/f/f}$/5xFAD and control $CB2^{EGFP/f/f}$ mice.

5. MATERIAL AND METHODS

5.1. ETHICS STATEMENT:

The protocols for animal care and use were approved by the Committee of Ethics for Animal Welfare of the University of the Basque Country (M20/2015/093) and were in accordance to the European Communities Council Directive of September 22, 2010 (2010/63/EU) and Spanish regulations (Real Decreto 53/2013, BOE 08-02-2013). Efforts were made to minimize the number and suffering of animals.

5.2. EXPERIMENTAL ANIMALS:

This work was carried out with CB2$^{EGFP/f/f}$ transgenic mice that co-express five AD mutations (5xFAD). They were provided by Dr. Julián Romero Paredes (Universidad Francisco de Vitoria, Madrid), as part of the collaboration with his laboratory. The CB2$^{EGFP/f/f}$ mice were generated at the Genoway facilities (Lyon, France) by designing a targeting strategy consisting of the insertion of an enhanced green fluorescent protein (EGFP) reporter gene, preceded by an internal ribosomal entry site sequence (IRES) into the 3' untranslated region (UTR) of the mouse cnr2 gene. This results in expression of the reporter gene (EGFP) under the control of the mouse endogenous cnr2 promoter, and transcription of the same bicistronic mRNA as the CB$_2$R protein. In addition, these mice co-express 5xFAD mutations at the same time. The 5xFAD mice with a C57BL/6J background were purchased from Jackson Laboratory (Bar Harbor, Main, USA). To obtain the co-expression, 5xFAD mice were mated with CB2$^{EGFP/f/f}$ mice for at least five generations to generate CB2$^{EGFP/f/f}$/5xFAD mice (López et al., 2018). This 5xFAD model does not seem to express the mutation that causes this neurofibrillary degeneration (Oblak et al., 2021), but it could perhaps occur indirectly through neuronal degeneration and Aβ 1-42 deposits.

To test the specificity of the GFP antibody, cannabinoid receptor type 2-knock out (CB$_2^{-/-}$) mice which do not express CB$_2$R (therefore nor GFP) were used (Fig. 8; Fig. 9 a,b). CB2$^{EGFP/f/f}$ mice and constitutive knock out (CB$_2^{-/-}$) mice are homozygous. These homozygous mice were identified by PCR and further verified by Southern blot analysis (López et al., 2018).

The subicular region of male mice aged between 6.5 and 7.5 months with amyloid deposits and dystrophic neurites already visible was used in my thesis. Area, perimeter, number of processes and CB$_1$R expression in astrocytes, were studied in 5,596.477 µm^2 of 5 CB2$^{EGFP/f/f}$, and in 7,681.429 µm^2 of 7 CB2$^{EGFP/f/f}$/5xFAD mice. In addition, the area, perimeter and number of microglial processes were measured in 7,900.338 µm^2 of 7 CB2$^{EGFP/f/f}$, and in 10,792.572 µm^2 of 10 CB2$^{EGFP/f/f}$/5xFAD mice. For the study of CB$_1$R in

microglia, 6,344.998 μm^2 in 5 CB2$^{EGFP/f/f}$, and 7,268.343 μm^2 in 7 CB2$^{EGFP/f/f}$/5xFAD mice were analysed. Finally, 3,524 μm^2 in 3 CB$_2^{EGFP/f/f}$, and 4,078 μm^2 in 3 CB2$^{EGFP/f/f}$/5xFAD mice were examined for CB$_2$R in microglia.

5.3. PRESERVATION OF BRAIN TISSUE:

Mice were anaesthetised with ketamine/xylazine (100mg/10mg/kg body weight, intraperitoneal injection) and subsequently perfused transcardially at room temperature (RT) through the left ventricle. First with phosphate buffered saline (PBS) 0.1 M (pH 7.4) for 20 sec, and then with the fixative solution composed of 4% formaldehyde (freshly depolymerised from paraformaldehyde), 0.2% picric acid and 0.1% glutaraldehyde in PBS 0.1 M (pH 7.4) for 10-15 minutes, with a fixative solution volume of 80 mL per mouse. The brains were then removed from the skull and post-fixed in the fixative solution for approximately one week at 4ºC. Subsequently, they were stored in 1:10 diluted fixative solution at 4°C with 0.025% sodium azide, for proper preservation. Brains were cut into coronal sections at 50 μm on a vibratome and stored in 12- or 24-well cell culture plates with 1 mL of phosphate buffer (PB) 0.1 M (pH 7.4) with 0.025% sodium azide at 4°C.

5.4. AVIDIN-BIOTIN PEROXIDASE METHOD FOR LIGHT MICROSCOPY

I used the following protocol:

1. 50 μm coronal brain sections cut with the vibratome containing the subicular region were selected. They were collected in 12- or 24-well culture plates and kept floating in PB 0.1M (pH 7.4) at RT.

2. The sections were preincubated with a blocking solution of 10% bovine serum albumin (BSA), 0.1% sodium azide and 0.5% Triton X-100 in 1X Tris-buffered saline (TBS 1X) (pH 7.4) for 30 min at RT.

3. They were then incubated with the primary anti-CB$_1$R (1:100), anti-GFP (1:500), anti-ionized calcium-binding adapter molecule 1 (Iba1) (1:500) or anti-glutamate aspartate transporter 1 (GLAST) (0.3 μg/mL) antibody (Table 2) prepared in the blocking solution, gently shaken for 2 days at 4°C or 1 day at RT.

4. Sections were washed with 1% BSA and 0.5% triton X-100 in TBS 1X for 30 min to remove excess of antibody (3 x 1 min and 2 x 10 min).

5. They were followed by incubation with a biotinylated anti-guinea pig (1:200, Biotin-SP-AffiniPure Goat Anti-Guinea Pig IgG), biotinylated anti-rat (1:200, Biotin-SP-AffiniPure Goat Anti-Rat IgG) or a biotinylated anti-rabbit secondary antibody (1:200, Biotin-SP-

AffiniPure Donkey Anti-Rabbit IgG) diluted in the washing solution for 1 h on a shaker at RT.

6. They were washed with 1% BSA and 0.5% triton X-100 (3 x 1 min and 2 x 10 min) in TBS 1X.

7. Afterwards, tissue was incubated with the avidin-biotin peroxidase complex (ABC) (1:50 avidin-biotin peroxidase complex, Elite, Vector Laboratories, Burlingame, CA, USA) prepared in the washing solution, for 1 h at RT.

8. Samples were washed with 1% BSA and 0.5% Triton X-100 in TBS 1X (3 x 1 min) and lastly with PB 0.1M (pH 7.4) and 0.5% Triton X-100 (2 x 10 min).

9. Labelling was revealed with the chromogen 0.05% diaminobenzidine (DAB) in PB 0.1 M (pH 7.4) containing 0.5% triton X-100 and 0.01% hydrogen peroxide for 3.5 min at RT.

10. It was followed by washes in PB 0.1 M (pH 7.4) with 0.5% triton X-100 (3 x 1 min and 2 x 10 min).

11. Tissue sections were mounted on gelatinised slides.

12. Once they were dried, were dehydrated in graded ethanol (EtOH) (50°, 70°, 96° and 100°) for 5 min each.

13. Sections were rinsed with xylene (3 x 5 min).

14. The slides wee cover slipped with DPX.

15. Subicular tissue was examined and photographed with a Zeiss AxioCam light microscope coupled to a Zeiss AxioCam HRc camera.

5.5. DOUBLE PRE-EMBEDDING IMMUNOGOLD AND IMMUNOPEROXIDASE METHOD FOR ELECTRON MICROSCOPY:

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

1. Four to five sections per brain containing the caudal hippocampus and subiculum were selected and placed on a new plate.

2. They were pre-incubated in a blocking solution (1mL/well) consisting of 10% BSA, 0.02% saponin and 0.1% sodium azide in TBS 1X (pH 7.4), for 30 min on the shaker (300 rpm) at RT.

3. Tissue was then incubated with two primary antibodies (Table 2): a guinea pig polyclonal anti-CB$_1$R antibody diluted 1:100 (Frontier Institute Co., ltd) or a rat monoclonal anti-GFP antibody diluted 1:500 (Nacalai) in combination with a rabbit polyclonal anti-GLAST antibody (0.3 µg/mL; Gifted by Prof. Niels Christian Danbolt), or a rabbit polyclonal anti-Iba1 antibody diluted 1:500 (FUJIFILM Wako Pure Chemical Corporation). The solution

contained 10% BSA in TBS 1X, 0.1% sodium azide and 0.004% saponin. Incubation was performed on a shaker for 2 days at 4°C.

4. Subsequently, five washes (3 x 1 min and 2 x 10 min) in 1% BSA/TBS 1X were made to remove excess of antibody.

5. For incubation with secondary antibodies (Table 2): sections were incubated with the 1.4 nm gold-conjugated goat anti-guinea pig IgG antibody (Fab' fragment, 1:100, Nanoprobes Inc, Yaphank, NY, USA) or with the 1.4 nm gold-conjugated goat anti-rat IgG antibody (Fab' fragment, 1:100, Nanoprobes Inc, Yaphank, NY, USA). They were also incubated with biotinylated anti-rabbit IgG antibody (1:200, Biotin-SP-AffiniPure Donkey Anti-Rabbit IgG) diluted in 1% BSA/TBS 1X with 0.004% saponin on a shaker for 4 h at RT.

6. Tissue was washed were (3 x 1 min and 2 x 10 min) in 1% BSA/TBS 1X on a shaker at RT to remove excess of antibody thoroughly.

7. Sections were then incubated with ABC (1:50) prepared in washing solution (1 mL/well) for 1.5 h at RT.

8. They were washed in 1% BSA/TBS 1X (3 x 1 min, 1 x 10 min) and kept in the same washing solution overnight at 4°C under agitation.

9. The following day, tissue was postfixed with 1% glutaraldehyde in TBS 1X (1 mL/well) for 12 min at RT.

10. Afterwards, sections were washed in double distilled water (3 x 10 min each).

11. They were transferred to test tubes.

12. Gold particles were silver-intensified with the HQ Silver kit (Nanoprobes Inc., Yaphank, NY, USA; 1 mL/tube) in the dark for 12 min at RT.

13. After intensification, the sections were transferred to glass vials (15 mL, 3 x 5 cm) and washed three times in double distilled water (1 min each) in the dark.

14. They were washed with PB 0.1 M (pH 7.4) several times in the dark for 30 min.

15. The biotinylated antibody was revealed with 0.05% DAB prepared in PB 0.1 M (pH 7.4) containing 0.5% triton X-100 and 0.01% hydrogen peroxide (1 mL/vial) for 3.5 min at RT, followed by several washes in PB 0.1 M (pH 7.4) (3 x 1 min and 2 x 10 min).

16. The sections were osmified (1% osmium tetroxide in PB 0.1 M (pH 7.4) (1 mL/vial) in the dark for 20 min.

17. They were washed in PB 0.1 M (pH 7.4) (3 x 1 min and 2 x 10 min).

18. Subsequently, sections were dehydrated in EtOH (50%, 70%, 96%; 5 min/each) followed by 100% EtOH (3 x 5 min) (1 mL/vial).

19. They were cleared in propylene oxide (3 x 5 min, 1mL/vial).

20. Sections were pre-embedded in 1:1 propylene oxide/Epon 812 resin (1 mL/vial) on a shaker overnight at RT.

21. Then, they were embedded in pure Epon 812 resin (1 mL/vial) for at least of 2 h at RT.

22. Tissue was then placed between two glass slides wrapped in aluminium foil and left in the oven at 60°C for 2 days.

23. The resin-embedded sections were separated from the aluminium foil and the subiculum was trimmed out.

24. Then, tissue was glued onto a resin mould to cut 1 μm-thick section with a histo-diamond knife (Diatome USA) on an ultramicrotome, and was stained with 1 % toluidine blue.

25. When the area of interest was reached, 50 nm-thick sections were cut with an ultra-diamond knife (Diatome USA) and collected on nickel mesh grids.

26. After drying the grids for at least two h in the hood, sections were counterstained with 2.5% lead citrate (1 drop/grid) for 20 min at RT.

27. Finally, they were washed in double distilled water 1 drop/grid (5 x 10 min each).

28. The counterstained grids were examined with a transmission electron microscope (JEOL JEM 1400 Plus, Canada) and tissue was photographed using a Hamamatsu FLASH digital camera inserted in the electron microscope. Anatomical landmarks were taken to locate the subiculum region.
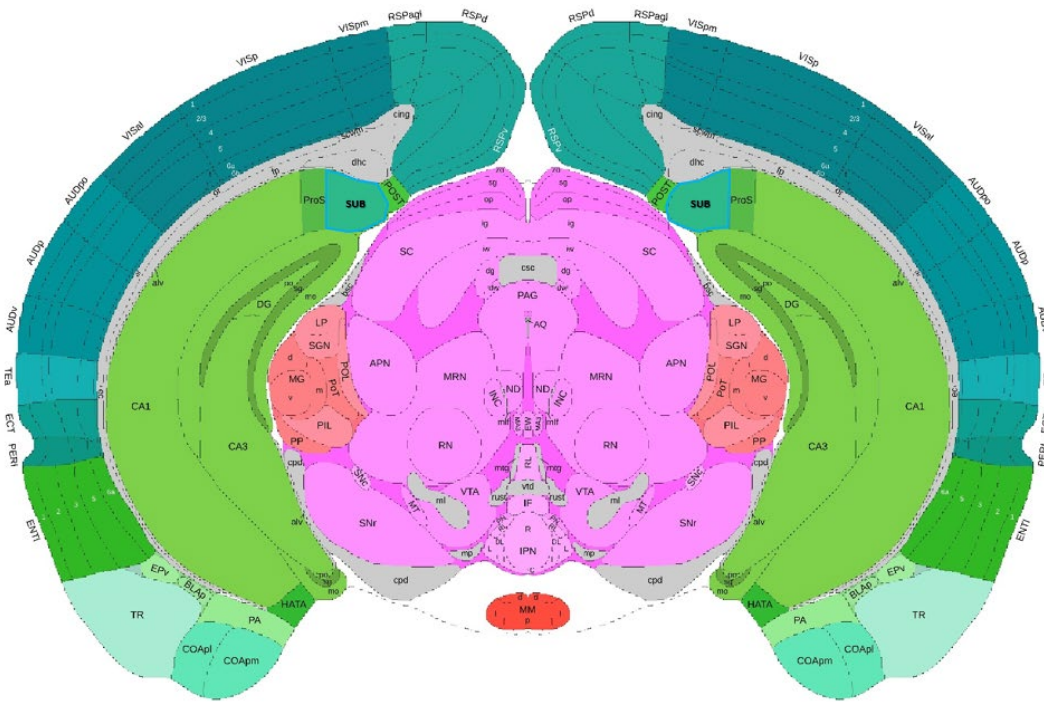
Figure 6. Coronal section of the mouse brain (modified from Allen Mouse Brain Atlas; © 2004 Allen Institute for Brain Science). Allen Mouse Brain Atlas. Available at: atlas.brain-map.org. The subiculum (SUB) is outlined in blue.
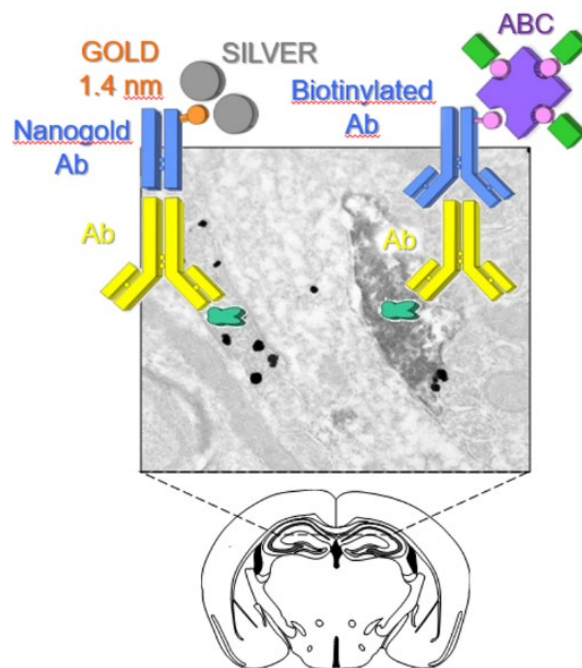
Figure 7. Double immunolabelling method for transmission electron microscopy (Adapted with permission from Puente et al., 2019).

Experiments were performed under the same conditions. In addition, negative controls omitting the primary antibodies were also used. Furthermore, the CB$_1$R antibody was tested in CB$_1^{-/-}$ brain (Fig. 9 c, d, e) and the GFP antibody in CB$_2^{-/-}$ mice (Fig. 8; Fig. 9 a, b).

Finally, the specificity of the other antibodies applied was thoroughly confirmed in previous studies (Bjørnsen et al., 2007; Delcambre et al., 2016).
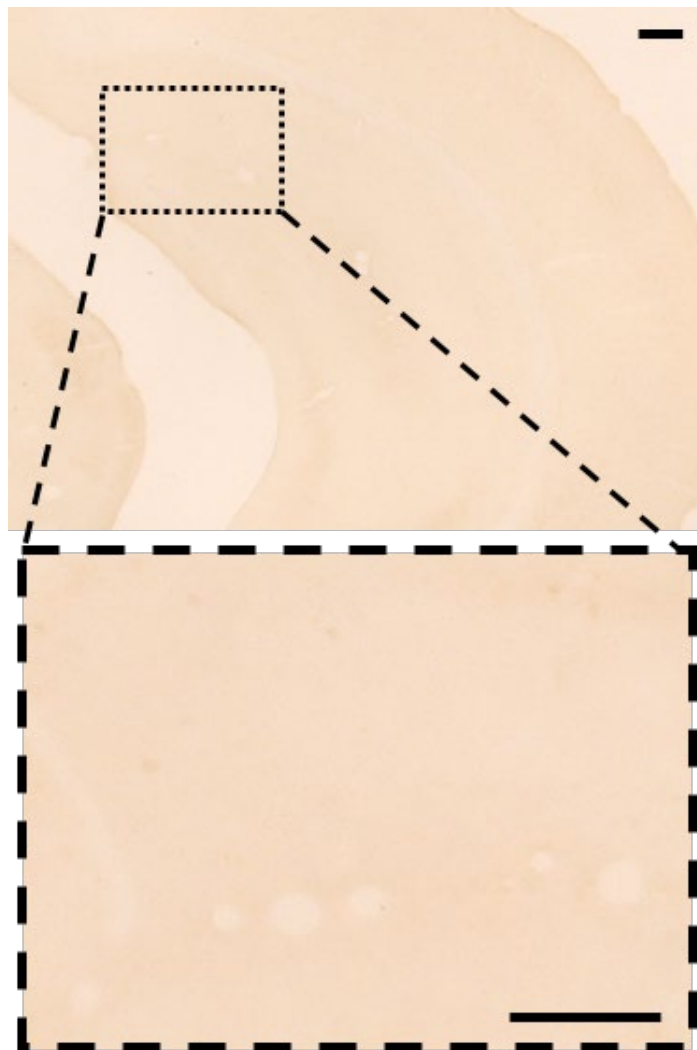
Figure 8. CB$_2^{-/-}$ mouse brain using anti-GFP antibody. Avidin-biotin peroxidase method for light microscopy. No sign of specific staining is visible in the subiculum (enlarged framed area). Scale bars: 200 μm.
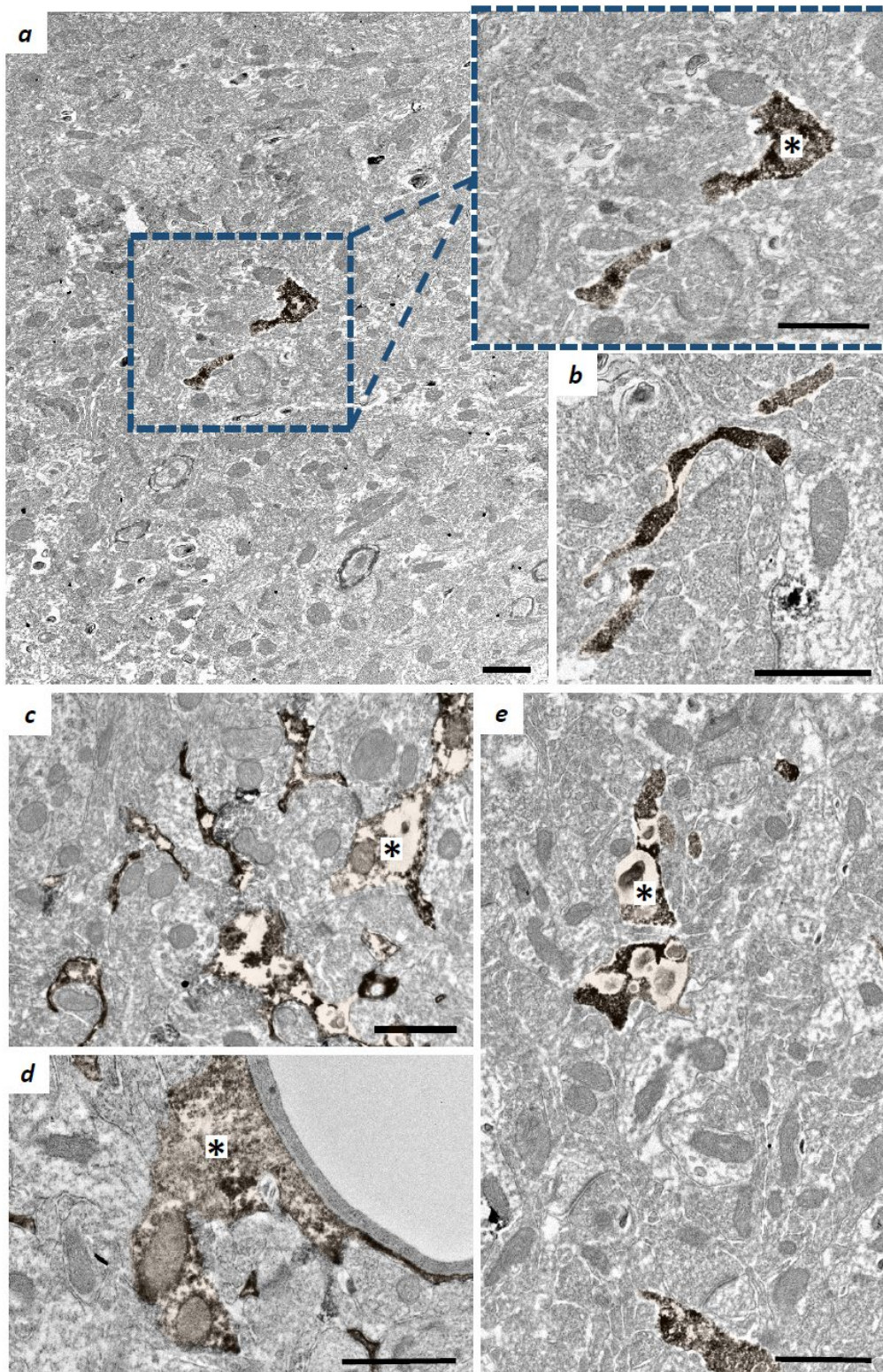
Figure 9. Subiculum of knock out mice. Double immunogold and immunoperoxidase method for electron microscopy. Antibodies were tested in $CB_2^{-/-}$ (a, b) and $CB_1^{-/-}$ mice (c, d, e). (a, b) Only residual GFP/CB_2 background is seen in the images showing simultaneous labelling for Iba1 (DAB, brown, *) and GFP/CB_2 (gold). Also, non-specific residual background is observed when combined GLAST (DAB, brown, *) with CB_1R (gold) (c, d), or Iba1 (DAB, brown, *) with CB_1R (gold) (e). Scale bars: 1 μm.

Table 2. **Details of the antibodies used.**

ANTIBODY	MANUFACTURER	HOST	CONCENTRATION
Anti-CB$_1$R polyclonal; CB1-GP-Af530; AB_2571593	Frontier Institute Co., ltd	Guinea pig	(1:100)
Anti-A522 (EAAT1 [GLAST]) polyclonal; Ab#314; AB_2314561	Gifted by Prof. Niels Christian Danbolt University of Oslo	Rabbit	(0.3 µg/mL)
Anti-Iba1 polyclonal; 019-19741	FUJIFILM Wako Pure Chemical Corporation	Rabbit	(1:500)
Anti-GFP monoclonal; GF090R	Nacalai	Rat	(1:500)
Biotin-SP-AffiniPure Goat Anti-Guinea Pig IgG (H+L); polyclonal; AB_2337394	Jackson Immuno Research	Goat	(1:200)
Biotin-SP-AffiniPure Donkey Anti-Rabbit IgG (H+L); polyclonal; AB_2340593	Jackson Immuno Research	Donkey	(1:200)
Biotin-SP-AffiniPure Goat Anti-Rat IgG (H+L); polyclonal; AB_2338179	Jackson Immuno Research	Goat	(1:200)
1.4 nm gold-conjugated Nanogold®-Fab´Goat anti-Guinea Pig IgG (H+L) #2055	Nanoprobes	Goat	(1:100)
1.4 nm gold-conjugated Nanogold®-Fab´Goat anti-Rat IgG (H+L) #2008	Nanoprobes	Goat	(1:100)

5.7. QUANTITATIVE SCORING AND STATISTICAL ASSESSMENT OF CB_1 RECEPTORS AND GFP LABELLING:

To ensure homogeneous labelling between all samples, only the first 1.5 μm from the section surface of each specimen was collected (antibody penetration is not reliable beyond this depth). Areas were photographed randomly within the subiculum. Electron micrographs containing microglial or astrocytic cell bodies were discarded as their large size could give inaccurate values. CB_1Rs in astrocytes were assessed in GLAST-immunoreactive astrocytic processes. CB_1Rs in microglia were investigated in Iba1-immunopositive microglial elements. The proportion of cell compartments labelled for CB_1R was then tabulated. Positive labelling was considered when at least one CB_1R immunoparticle was within 30 nm of the given membrane compartment studied. CB_1R density (particles/μm membrane) was also determined by counting CB_1R immunoparticles in the positive compartments. Membrane length (perimeter) was measured with the Image-J software (NIH; RRID: SCR_003070).

CB_2R was assessed in Iba1-positive microglial processes. GFP gold particles were counted and differentiated between their localization in membrane (at a maximum distance of 30 nm from the membrane) or cytosol (at a greater distance than 30 nm). This distinction was made to determine where GFP localizes, as it does not necessarily have to be in the same place of CB_2R within the cell.

All values were 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 statistical tests were performed. Data were analysed by means of non-parametric or parametric tests: Mann–Whitney U test or Student's Unpaired t-test (* $P < 0.05$).

Minor contrast and brightness adjustments were made to the figures using Image-J software (NIH; RRID: SCR_003070), Adobe Photoshop and Gimp.

6. RESULTS

6.1. GLIAL MORPHOLOGY IN THE SUBICULUM OF CB2$^{EGFP/F/F}$/5xFAD MICE.

To have an overview of the general changes occurring in glial cells in the subiculum of CB2$^{EGFP/f/f}$/5xFAD compared to CB2$^{EGFP/f/f}$ mice, Iba1 and GLAST labelling of microglia and astrocytes, respectively, were visualized using the avidin-biotin peroxidase method for light microscopy. Changes in staining density of both glial cells were detected (Fig. 10). Microglia identified in CB2$^{EGFP/f/f}$ (Fig. 10a, a´) occupied a much larger area in CB2$^{EGFP/f/f}$/5xFAD (Fig. 10b, b´). This increase appeared to be related to both the number of microglial cells and the thickness of their processes, suggesting the existence of microglial activation. In the case of astrocytes, GLAST staining seen in CB2$^{EGFP/f/f}$ (Fig. 10c, c´) was also more intense in CB2$^{EGFP/f/f}$/5xFAD, compatible with astrocytic hypertrophy (Fig. 10d, d´). These findings were further analysed by electron microscopy.
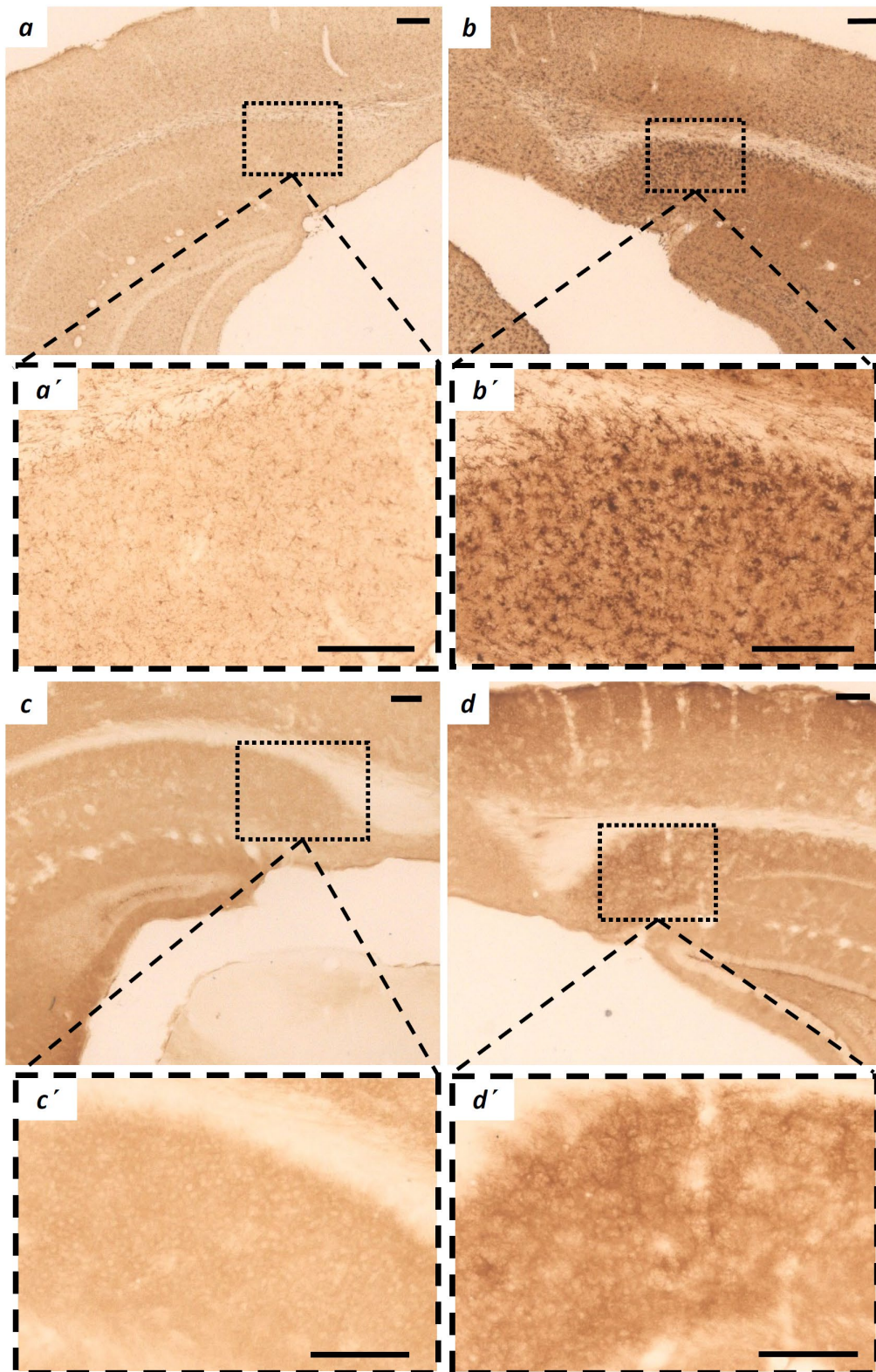
Figure 10. Iba1 and GLAST in the subiculum of CB2$^{EGFP/f/f}$ and CB2$^{EGFP/f/f}$/5xFAD mice. Avidin-biotin peroxidase method for light microscopy. Subicular Iba1 staining observed in CB2$^{EGFP/f/f}$ microglia (a, a′) is drastically increased in enlarged CB2$^{EGFP/f/f}$/5xFAD microglia (b, b′). GLAST staining seen in CB2$^{EGFP/f/f}$ (c, c′) is also stronger in CB2$^{EGFP/f/f}$/5xFAD (d, d′). Scale bars: 200 μm.

6.2. CB$_1$ RECEPTOR EXPRESSION IN THE SUBICULUM OF CB2$^{EGFP/F/F}$/5xFAD MICE.

To get an overview of the changes occurring in CB$_1$R expression in the subiculum region of CB2$^{EGFP/f/f}$/5xFAD compared to CB2$^{EGFP/f/f}$ mice, tissue was stained with antibody against CB$_1$R (Table 2) in combination with the avidin-biotin peroxidase method for light microscopy. The labelling observed in CB2$^{EGFP/f/f}$ (Fig. 11a, a') got more patchy appearance in CB2$^{EGFP/f/f}$/5xFAD (Fig. 11b, b').Thus, there appeared to be areas of lower staining which could correspond to SPs surrounded by more intense CB$_1$R immunoreactivity (Fig. 11b, b'). These changes in CB$_1$R expression were further analysed in both microglia and astrocytes by electron microscopy.
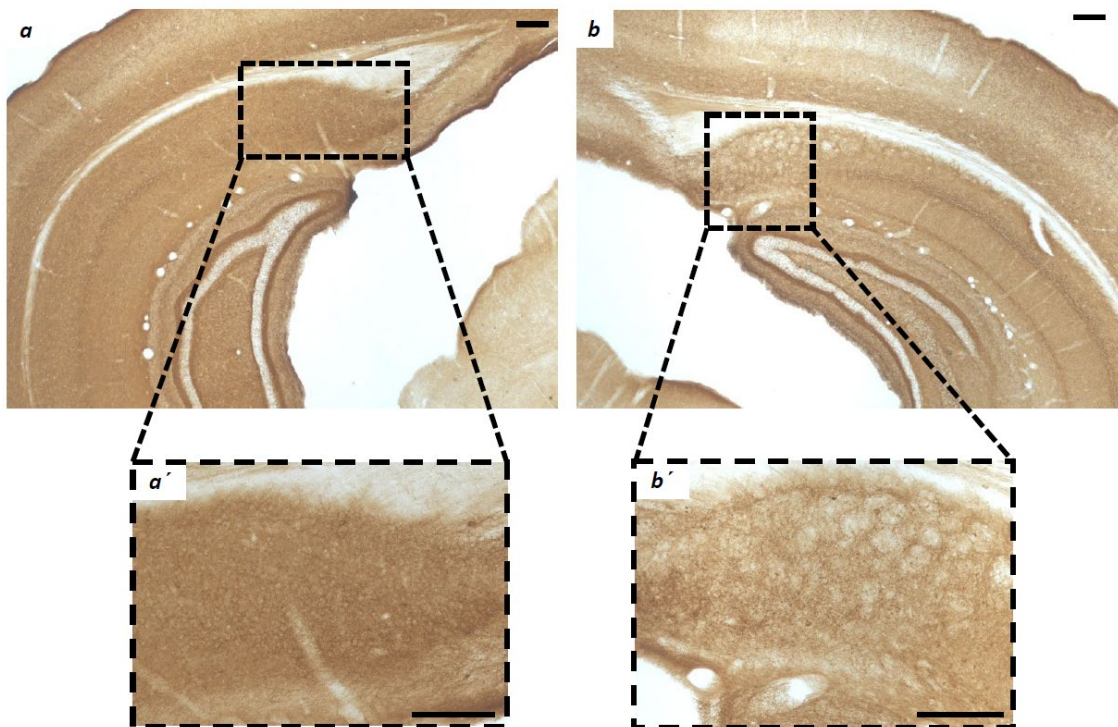
Figure 11. CB$_1$R immunostaining in the subiculum of CB2$^{EGFP/f/f}$ and CB2$^{EGFP/f/f}$/5xFAD mice. The CB$_1$R staining observed in the subiculum of CB2$^{EGFP/f/f}$ (a, a′) changes to a patchy appearance in CB2$^{EGFP/f/f}$/5xFAD, which seems to correspond to lesion sites (b, b′). Scale bars: 200 μm.

First, I assessed the microglial morphology. DAB immunostaining was used to identify Iba1-containing microglial processes (Fig. 12). While in controls only scattered microglial processes were observed (Fig. 12 a-c), numerous Iba1-positive processes surrounding plaques (Fig. 12 d and g) and dystrophic neurites (Fig. 12 d-h) were seen in the CB2$^{EGFP/f/f}$/5xFAD mice. Furthermore, a significant increase in the area of the microglial processes was observed in CB2$^{EGFP/f/f}$/5xFAD (0.3229 ± 0.05282 µm^2; ***$p < 0.0001$; Fig. 13a) relative to CB2$^{EGFP/f/f}$ mice (0.1000 ± 0.01754 µm^2). This change was also visible in the total area per sample normalised to 100 µm^2 (CB2$^{EGFP/f/f}$/5xFAD: 2.074 ± 0.5156 µm^2; ***$p < 0.0001$; CB2$^{EGFP/f/f}$: 0.3485 ± 0.06955 µm^2; Fig. 13b). In addition, a significant increase in the perimeter of the microglial prolongations was observed in CB2$^{EGFP/f/f}$/5xFAD (2.200 ± 0.1248 µm; ***$p < 0.0001$) versus CB2$^{EGFP/f/f}$ (1.260 ± 0.1036 µm; Fig. 13c). This increase could also be noticed in the perimeter of the total microglial processes per sample normalized to 100 µm^2 (15.73 ± 1.929 µm in CB2$^{EGFP/f/f}$/5xFAD; 4.334 ± 0.5345 µm in CB2$^{EGFP/f/f}$; ***$p < 0.0001$; Fig. 13d). Finally, significant changes were also detected in the number of microglial processes (7.260 ± 0.6304 in CB2$^{EGFP/f/f}$/5xFAD; 3.413 ± 0.4092 in CB2$^{EGFP/f/f}$; ***$p < 0.0001$; Fig. 13e).

Figure 12. Microglia in the subiculum of CB2$^{EGFP/f/f}$ and CB2$^{EGFP/f/f}$/5xFAD mice. Double pre-embedding immunogold (CB$_1$R) and immunoperoxidase (Iba1) method for electron microscopy. Microglial processes show Iba1 labelling (DAB immunodeposits, brown, *). In control CB2$^{EGFP/f/f}$, a few slender microglial elements are observed (a, b, c). However, thick processes of reactive microglia appear in CB2$^{EGFP/f/f}$/5xFAD (d, e, f, g, h). Plaques (pink; d, g) and numerous dystrophic neurites (blue; d, e, f, g, h) are also identified. Notice membrane and cytosolic CB$_1$R particles in microglial processes of both mutants, with particular abundance in supposedly degenerating inhibitory terminals in CB2$^{EGFP/f/f}$/5xFAD (yellow; f, h). Scale bars: 1 μm.
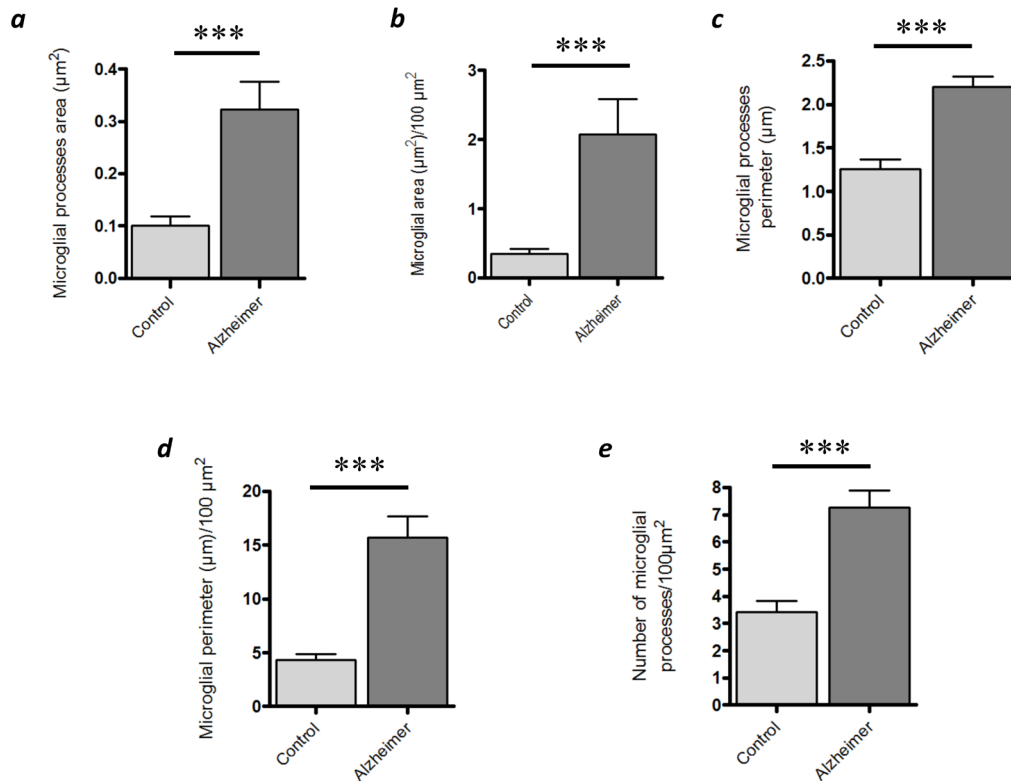
Figure 13. Quantification and statistics of microglial ultrastructure in the subiculum of CB2$^{EGFP/f/f}$ (control) and CB2$^{EGFP/f/f}$/5xFAD (Alzheimer) mice. (a) Microglial processes area. (b) Microglial area normalized to 100 μm^2. (c) Microglial processes perimeter. (d) Microglial perimeter normalized to 100 μm^2. (e) Number of microglial processes in 100 μm^2. Data were analysed by non-parametric or parametric tests (Mann–Whitney U-test or Student's t-test). Mann–Whitney U-test or Student's t-test. $p < 0.05$*; $p < 0.01$**; $p < 0.001$***; $p < 0.0001$****. All data are represented as mean ± S.E.M.

By using GLAST-DAB, the area, perimeter and number of astrocytic elements were analysed in the subiculum of CB2$^{EGFP/f/f}$ and CB2$^{EGFP/f/f}$/5xFAD mice (Fig. 14).

There was a significant increase in the area of the astrocytic processes (CB2$^{EGFP/f/f}$/5xFAD: 0.2598 ± 0.01853 μm^2; CB2$^{EGFP/f/f}$: 0.1565 ± 0.006515 μm^2; ***$p <$ 0.0001; Fig. 15a). However, no significant differences were observed in the total area per sample occupied by astrocytic processes normalized to 100μm^2 (CB2$^{EGFP/f/f}$/5xFAD: 8.993 ± 0.8664μm^2; CB2$^{EGFP/f/f}$: 7.415 ± 0.6552 μm^2; p: 0.1711; Fig. 15b). There was also a significant increase in the perimeter of the astrocytic processes (CB2$^{EGFP/f/f}$/5xFAD: 2.833 ± 0.08486 μm; CB2$^{EGFP/f/f}$: 2.116 ± 0.04741 μm; ***$p <$ 0.0001; Fig. 15c). Nevertheless, no differences in the total perimeter of astrocytic processes per sample were detected either (CB2$^{EGFP/f/f}$/5xFAD: 99.89 ± 8.087 μm; CB2$^{EGFP/f/f}$: 100.1 ± 6.811 μm; p: 0.9820; Fig 15d). Consistent with these results, a significant decrease in the number of astrocytic processes in the CB2$^{EGFP/f/f}$/5xFAD (35.52 ± 2.661 per 100 μm^2) relative to CB2$^{EGFP/f/f}$ (47.33 ± 2.709 per 100 μm^2) was observed (**p: 0.0036; Fig. 15e).
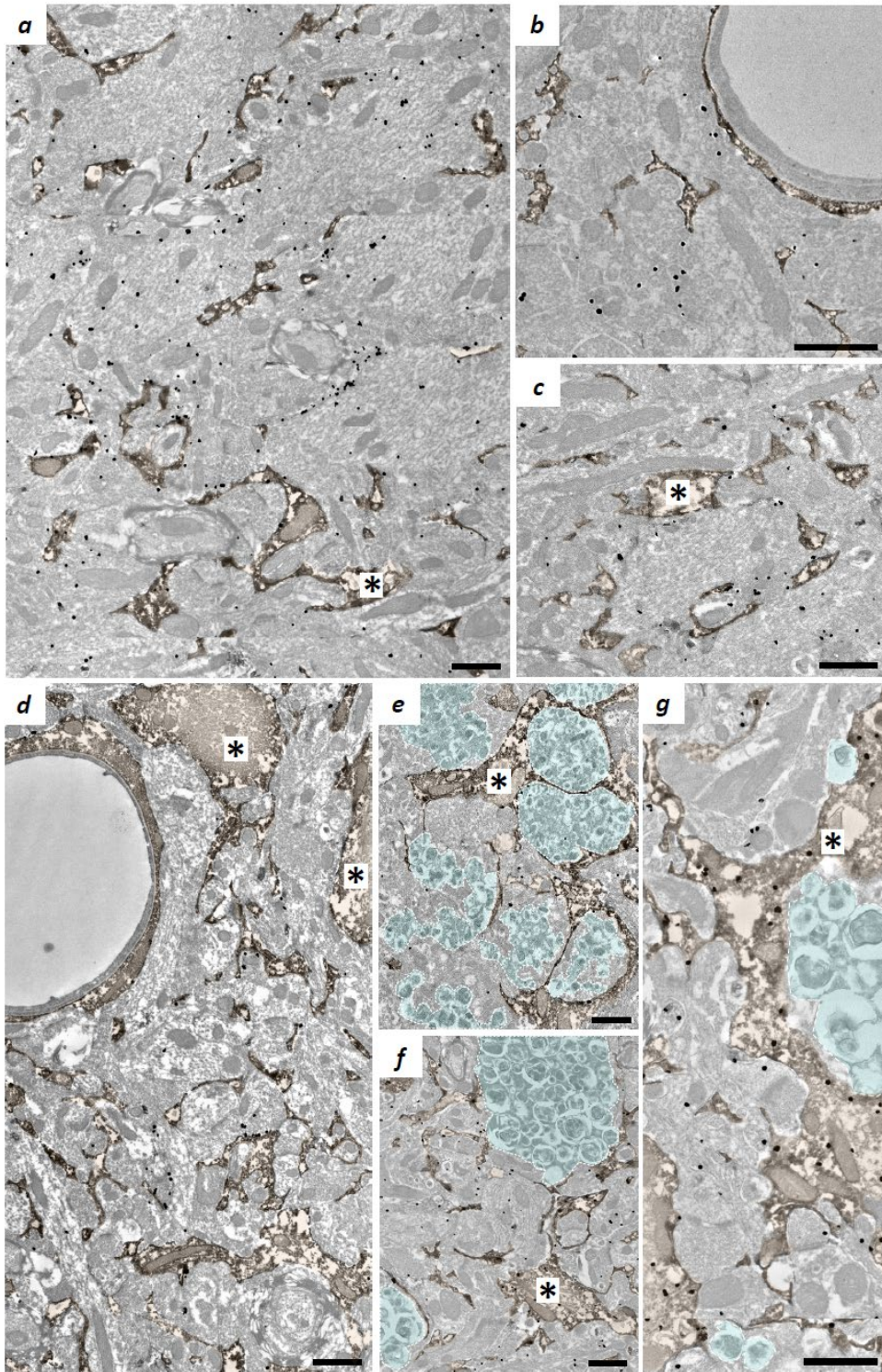
Figure 14. Astroglia in the subiculum of CB2$^{EGFP/f/f}$ and CB2$^{EGFP/f/f}$/5xFAD mice. Double pre-embedding immunogold (CB$_1$R) and immunoperoxidase (GLAST) method for electron microscopy. GLAST-positive astrocytic processes (DAB immunodeposits, brown, *) seen in CB2$^{EGFP/f/f}$ (a, b, c) are thicker in CB2$^{EGFP/f/f}$/5xFAD (d, e, f, g). Observe astrocytic elements closely surrounding dystrophic neurites in the AD mouse (light blue; e, f, g). Scale bars: 1 μm.
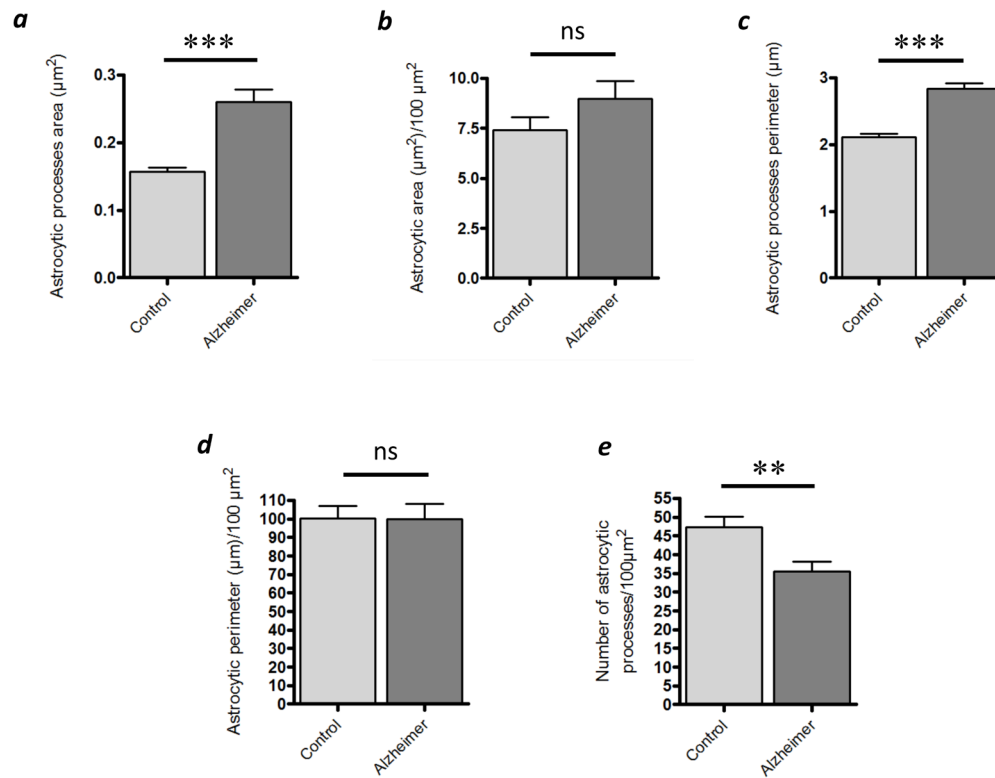
Figure 15. Quantification and statistical assessment of morphological parameters of astrocytes in the subiculum of CB2$^{EGFP/f/f}$ (control) and CB2$^{EGFP/f/f}$/5xFAD (Alzheimer) mice. (a) Astrocytic processes area. (b) Astrocytic area normalized to 100 μm^2. (c) Astrocytic processes perimeter. (d) Astrocytic perimeter normalized to 100 μm^2. (e) Number of astrocytic processes in 100μm^2. Data were analysed by non-parametric or parametric tests (Mann–Whitney U-test or Student's t-test). Mann–Whitney U-test or Student's t-test. $p < 0.05$*; $p < 0.01$**; $p < 0.001$***; $p < 0.0001$****. All data are represented as mean ± S.E.M.

6.5. Microglial localization of the CB_1 receptor in the subiculum of $CB2^{EGFP/F/F}$/5xFAD mice.

CB_1R immunoparticles were localized in the membranes of Iba1-positive microglial processes in both $CB2^{EGFP/f/f}$ and $CB2^{EGFP/f/f}$/5xFAD mice (Fig. 12, 16). The analysis revealed a significant increase in CB_1-positive microglial processes was seen in $CB2^{EGFP/f/f}$/5xFAD (0.9942 ± 0.1259) compared to $CB2^{EGFP/f/f}$ mice (0.3254 ± 0.07758; ***p <0.0001; Fig. 17a). In addition, a significant increase in the proportion of CB_1-positive elements was detected in $CB2^{EGFP/f/f}$/5xFAD mice (6.27 ± 1.15 %; $CB2^{EGFP/f/f}$: 3.79 ± 2.10 %; **p: 0.0033; Fig. 17b). However, CB_1R density in the positive microglial processes was significantly reduced in $CB2^{EGFP/f/f}$/5xFAD ($69.44 \pm 7,577$ particles/100 μm of membrane; $CB2^{EGFP/f/f}$: 135.5 ± 24.78/100 μm of membrane; **p: 0.0023; Fig. 17c). Furthermore, significant differences in the total number of microglial CB_1R particles per 100 μm^2 were observed between $CB2^{EGFP/f/f}$/5xFAD (1.31 ± 0.18) and the $CB2^{EGFP/f/f}$ (0.48 ± 0.13 particles; ***p: 0.0009; Fig. 17d). Finally, there were no differences in the number of CB_1R immunoparticles per microglial process between both mutants ($CB2^{EGFP/f/f}$/5xFAD: 1.333 ± 0.08347 particles; $CB2^{EGFP/f/f}$: 1.450 ± 0.2112 particles; p: 0.7736; Fig. 17e).
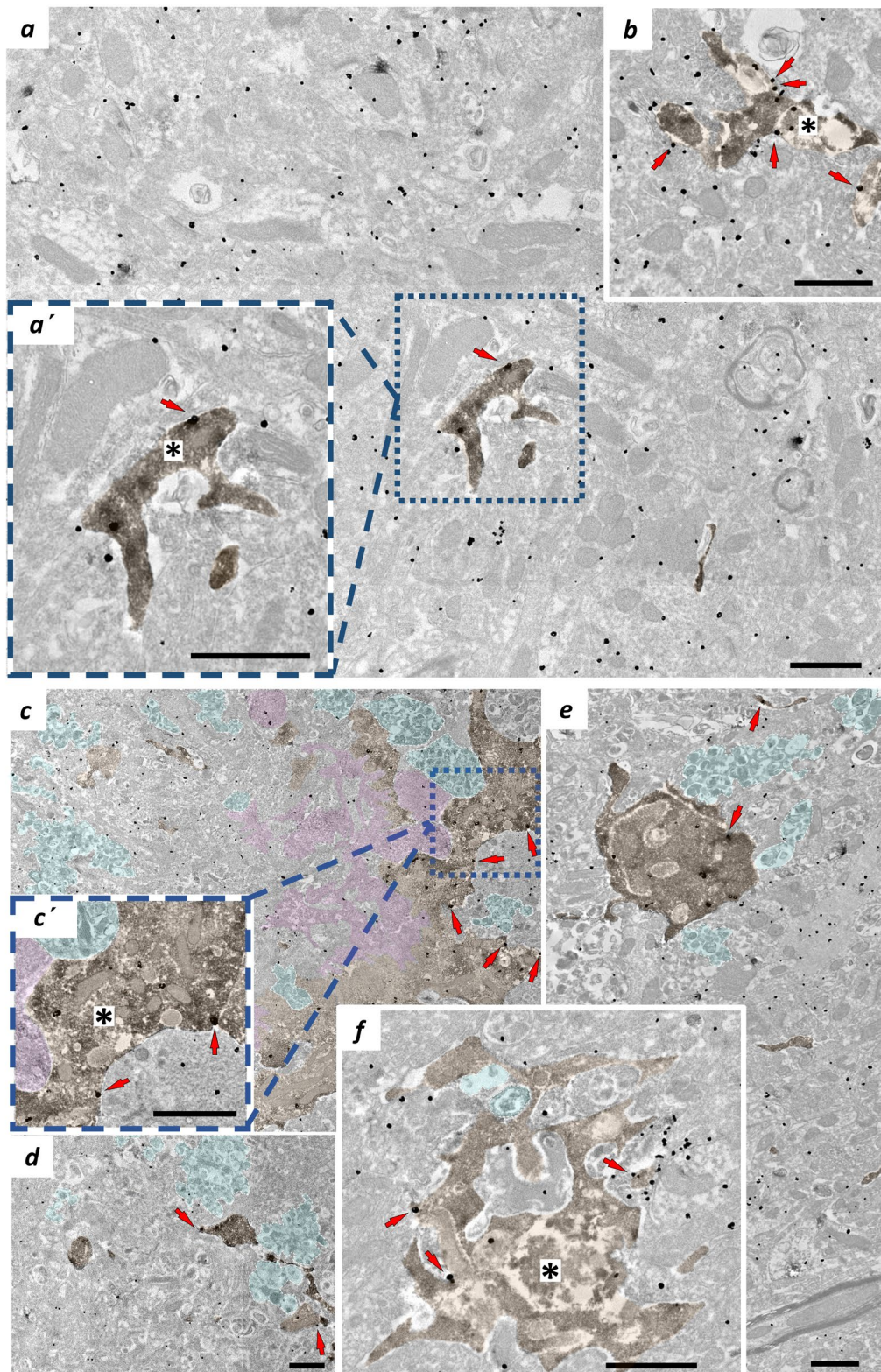
Figure 16. CB₁R localization in microglial compartments in the subiculum of CB2^EGFP/f/f and CB2^EGFP/f/f/5xFAD mice. Double pre-embedding immunogold (CB₁R) and immunoperoxidase (Iba1) method for electron microscopy. Some CB₁R gold particles (red arrows) are observed on microglial membranes (DAB immunodeposits, brown, *) in both CB2^EGFP/f/f (a, a´, b) and CB2^EGFP/f/f/5xFAD (c, c´, d, e, f). Plaques (pink) (c) and dystrophic neurites (blue) are present in CB2^EGFP/f/f/5xFAD. Scale bars: 1 μm.
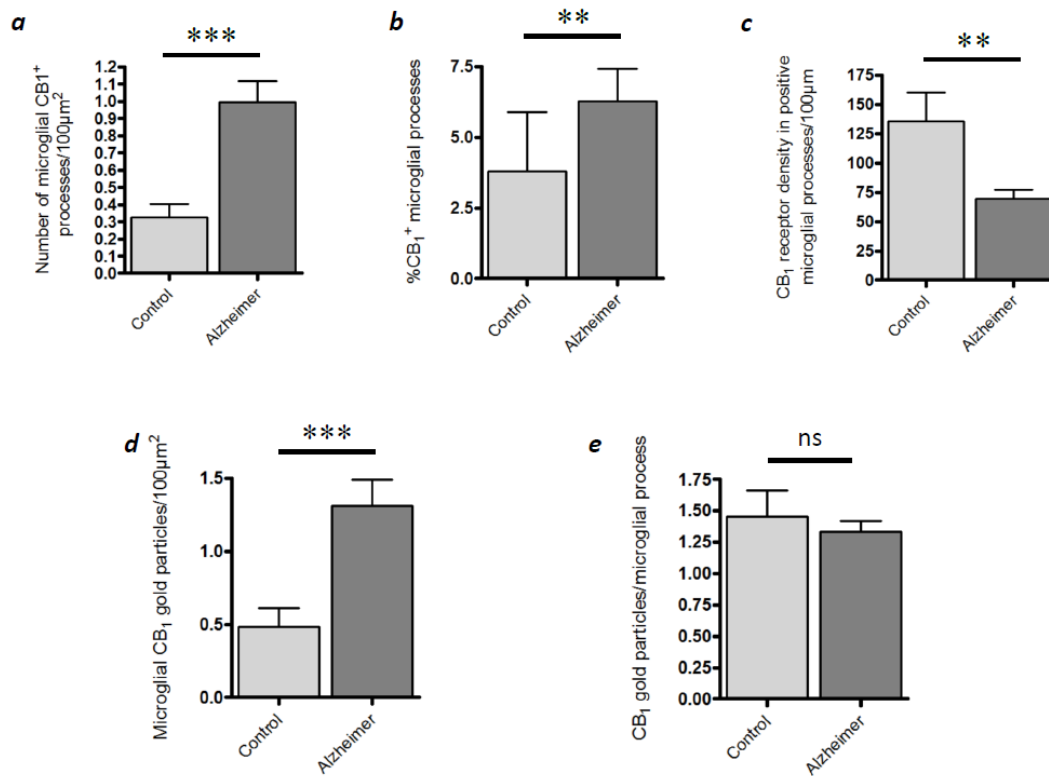
Figure 17. Statistical analysis of the microglial CB$_1$R localization in the subiculum of CB2$^{\text{EGFP/f/f}}$ and CB2$^{\text{EGFP/f/f}}$/5xFAD mice. (a) Number of microglial CB$_1$R positive processes per 100μm^2. (b) Percentage of CB$_1$R positive microglial processes. (c) CB$_1$R density in positive microglial elements per 100μm. (d) Microglial CB$_1$R particles per 100 μm^2. (e) CB$_1$R labelling per microglial element. Data were analysed by non-parametric or parametric tests (Mann–Whitney U-test or Student's t-test). Mann–Whitney U-test or Student's t-test. $p < 0.05$*; $p < 0.01$**; $p < 0.001$***; $p < 0.0001$****. All data are represented as mean ± S.E.M.

I used the CB2$^{EGFP/f/f}$ and CB2$^{EGFP/f/f}$/5xFAD mice expressing GFP under the same promoter as the CB$_2$R to investigate the CB$_2$R localization in microglia (Fig. 18, 19). However, although both proteins are synthesised under the same promoter, are not bound together, thus they are not necessarily located at the same sites within the cell. Said this, GFP immunoreactivity (CB$_2$R) was not observed in CB2$^{EGFP/f/f}$ in the light microscope (Fig. 18a, 18a′), while punctate immunostaining was manifest in the CB2$^{EGFP/f/f}$/5xFAD subiculum (Fig. 18b, 18b′). Then, GFP distribution in membrane and cytosolic compartments was studied in the electron microscope. Iba-1 immunopositive microglial processes localized GFP (CB$_2$R) in both CB2$^{EGFP/f/f}$ and CB2$^{EGFP/f/f}$/5xFAD mice (Fig. 19). The statistical analysis revealed that the GFP-positive microglial processes counted in CB2$^{EGFP/f/f}$/5xFAD (0.7126 ± 0.2311) increased significantly with respect to CB2$^{EGFP/f/f}$ (0.1648 ± 0.07686) (*p: 0.0176; Fig. 20a). In addition, a significant increase in the percentage of GFP-positive microglial ramifications was noticed in CB2$^{EGFP/f/f}$/5xFAD (16.71 ± 3.664 %) relative to CB2$^{EGFP/f/f}$ (5.430 ± 2.631 %; *p: 0.0191; Fig. 20b). The total number of GFP particles per area of microglial ramifications was significantly greater in CB2$^{EGFP/f/f}$/5xFAD (1.238 ± 0.2534) than in CB2$^{EGFP/f/f}$ mice (0.6962 ± 0.4138; *p: 0.0467; Fig. 20c). Furthermore, the number of GFP particles in microglial branches per 100 μm^2 was statistically higher in CB2$^{EGFP/f/f}$/5xFAD (0.8343± 0.2962) than in CB2$^{EGFP/f/f}$ (0.1648± 0.07686; *p: 0.0176; Fig. 20d). Noticeably in CB2$^{EGFP/f/f}$/5xFAD, the percentage of GFP immunoparticles localized in microglial membranes (77.22 ± 11.40 %) was significantly higher than the proportion distributed in the cytosol (22.78 ± 11.40 %; *p: 0.0106; Fig. 20e). In the case of CB2$^{EGFP/f/f}$, 100% of the GFP particles were found in microglial membranes.
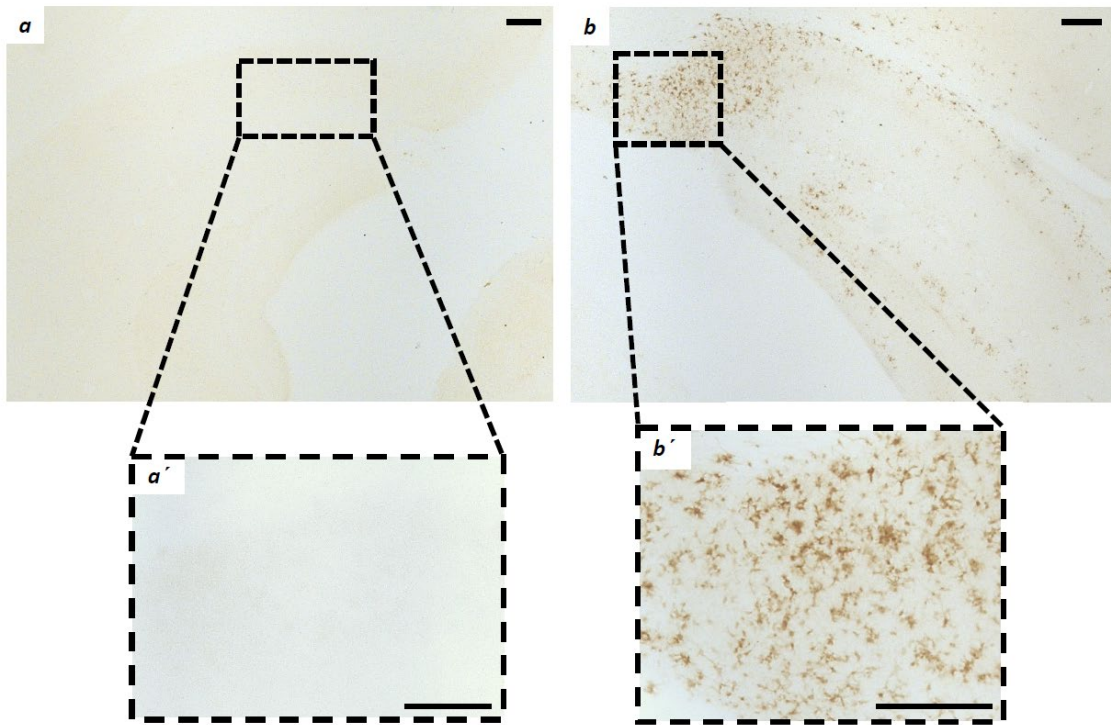
Figure 18. GFP immunostaining in the subiculum of CB2$^{EGFP/f/f}$ and CB2$^{EGFP/f/f}$/5xFAD mice. Avidin-biotin peroxidase method for light microscopy. No staining is seen in CB2$^{EGFP/f/f}$ (a, a′); however, GFP immunoreactivity appears dispersed in the hippocampus but accumulates in the subiculum of CB2$^{EGFP/f/f}$/5xFAD (b, b′). Scale bars: 200 μm.
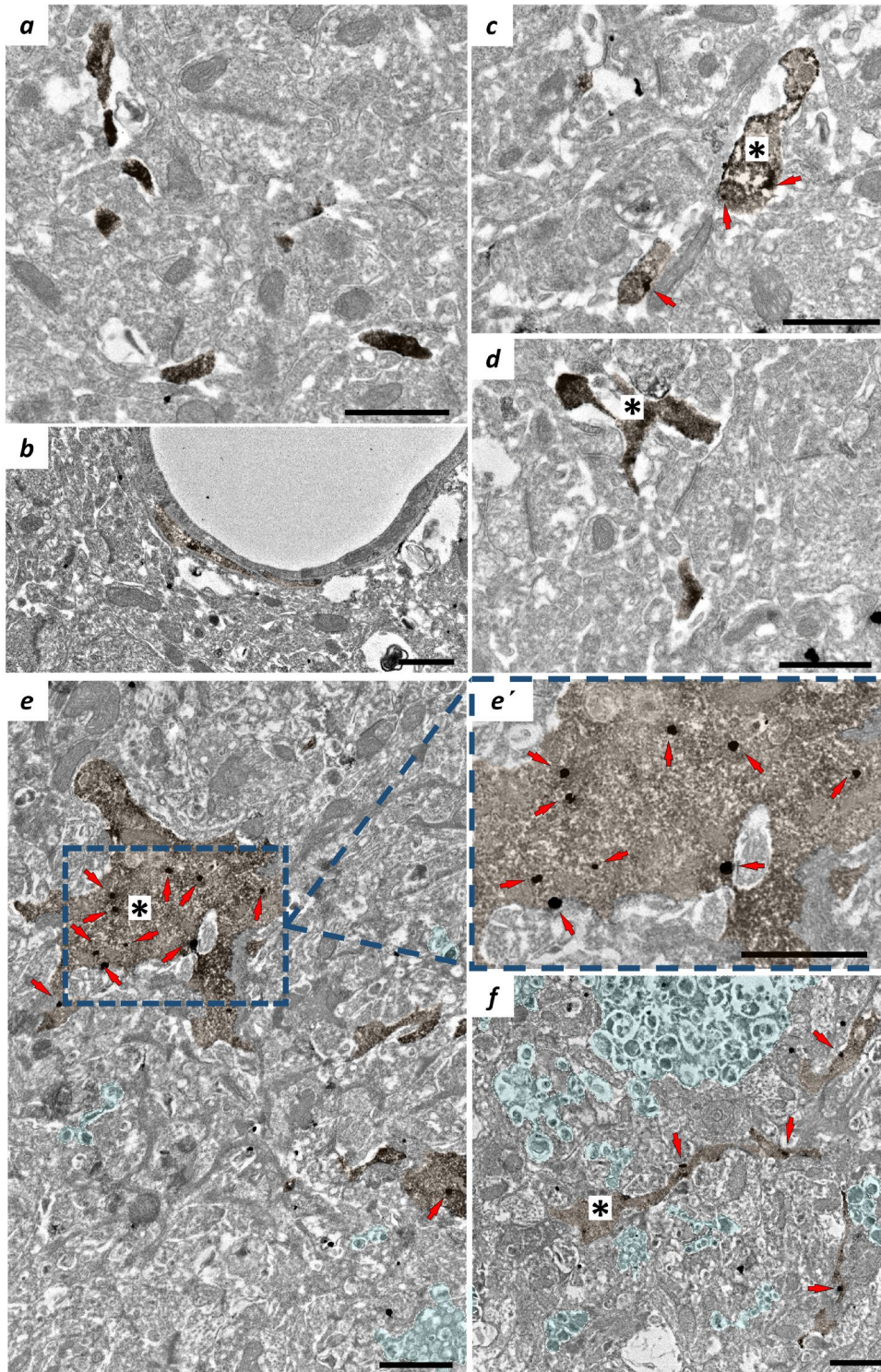
Figure 19. Microglial GFP localization in the subiculum of CB2^EGFP/f/f and CB2^EGFP/f/f/5xFAD mice. Double pre-embedding immunogold (GFP) and immunoperoxidase (Iba1) method for electron microscopy. GFP particles (red arrows) are localized in Iba1-positive microglial elements (DAB immunodeposits, brown, *). In CB2^EGFP/f/f (a, b, c, d), only membrane localization of GFP is observed (arrows, c). In CB2^EGFP/f/f/5xFAD, GFP particles are found in both membranes and cytosol (e, e´, f). Notice dystrophic neurites (blue) in CB2^EGFP/f/f/5xFAD (e, f). Scale bars: 1 μm.
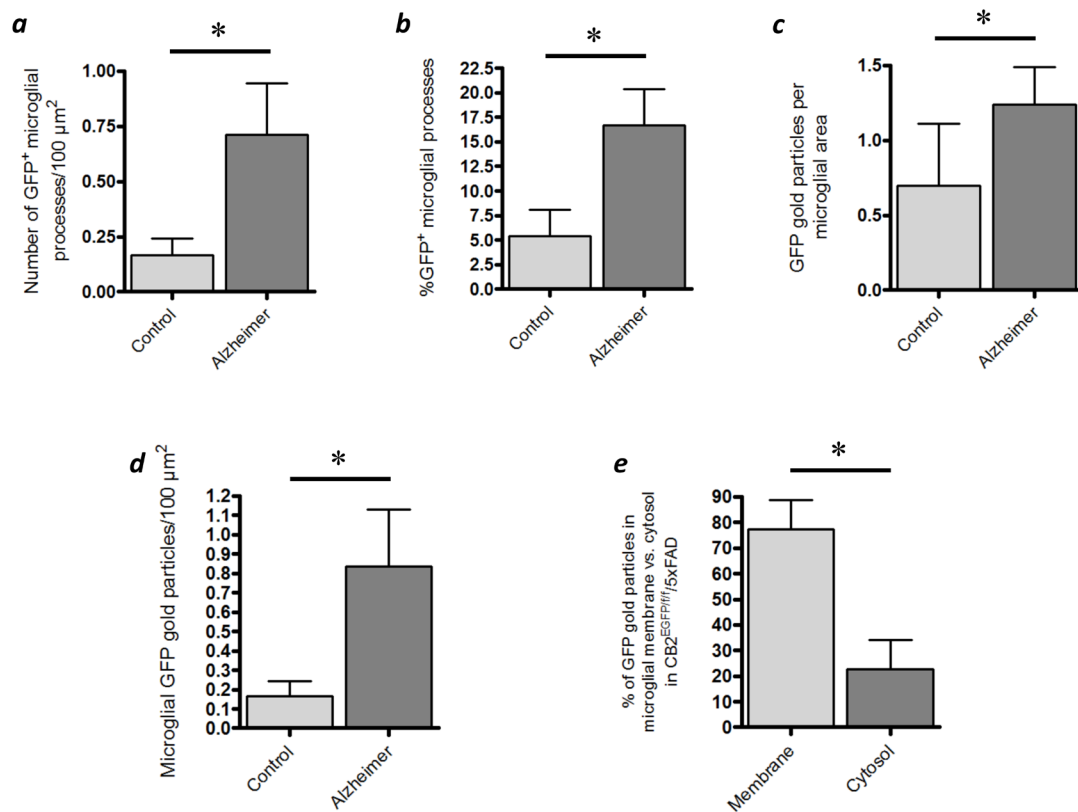
Figure 20. Statistics of the microglial GFP (CB₂R) localization in the subiculum of CB2^EGFP/f/f and CB2^EGFP/f/f/5xFAD mice. (a) Number of microglial GFP positive processes per 100μm². (b) Percentage of GFP positive microglial processes. (c) GFP gold particles per microglial area. (d) Microglial GFP immunoparticles per 100 μm². (e) Percentage of GFP particles in microglial membrane vs. cytosol in CB2^EGFP/f/f/5xFAD. Data were analysed by non-parametric or parametric tests (Mann–Whitney U-test or Student's t-test). Mann–Whitney U-test or Student's t-test. $p < 0.05$*; $p < 0.01$**; $p < 0.001$***; $p < 0.0001$****. All data are represented as mean ± S.E.M.

6.7. Astroglial localization of the CB$_1$ receptor in the subiculum of CB2$^{\text{EGFP/F/F}}$/5xFAD mice.

To determine how CB$_1$R expression is affected in reactive astrocytes surrounding dystrophic neurites and senile plaques in Alzheimer's pathology, the subiculum of CB2$^{\text{EGFP/f/f}}$ and CB2$^{\text{EGFP/f/f}}$/5xFAD mice was studied. In the electron microscope, CB$_1$Rs were localized to membranes of astrocytic processes (GLAST positive) in both mutants (Fig. 21), as previously reported by our laboratory (Han et al., 2012; Bosier et al., 2013; Gutiérrez-Rodríguez et al., 2018; Bonilla-Del Río et al., 2019, 2021). No significant differences were detected in the number of CB$_1$R-positive astrocytic prolongations between both mice (within per 100 μm^2, CB2$^{\text{EGFP/f/f}}$: 8.661 $\pm$ 0.8977 processes; CB2$^{\text{EGFP/f/f}}$/5xFAD: 7.967 $\pm$ 1.224 processes; p: 0.3094; Fig. 22a). Furthermore, the percentage of CB$_1$R positive astrocytic branches was statistically similar between CB2$^{\text{EGFP/f/f}}$/5xFAD (21.24 $\pm$ 2.37%) and CB2$^{\text{EGFP/f/f}}$ mice (17.75 $\pm$ 1.21%; p: 0.2303; Fig. 22b). There was also no significant difference in CB$_1$R density in astrocytic positive processes (CB2$^{\text{EGFP/f/f}}$/5xFAD: 57.64 $\pm$ 2.105 particles; CB2$^{\text{EGFP/f/f}}$: 59.83 $\pm$ 2.324; p: 0.2209; Fig. 22c), nor in the number of astrocytic CB$_1$ particles per sample (CB2$^{\text{EGFP/f/f}}$/5xFAD: 12.78 $\pm$ 2.174 particles; CB2$^{\text{EGFP/f/f}}$: 11.63 $\pm$ 1.265; p: 0.6716; Fig. 22d). However, the number of CB$_1$R particles in positive astrocytic processes was significantly higher in CB2$^{\text{EGFP/f/f}}$/5xFAD (1.603 $\pm$ 0.05081 particles/branch) than in CB2$^{\text{EGFP/f/f}}$ (1.343 $\pm$ 0.03909; ***p: 0.0005; Fig. 22e).
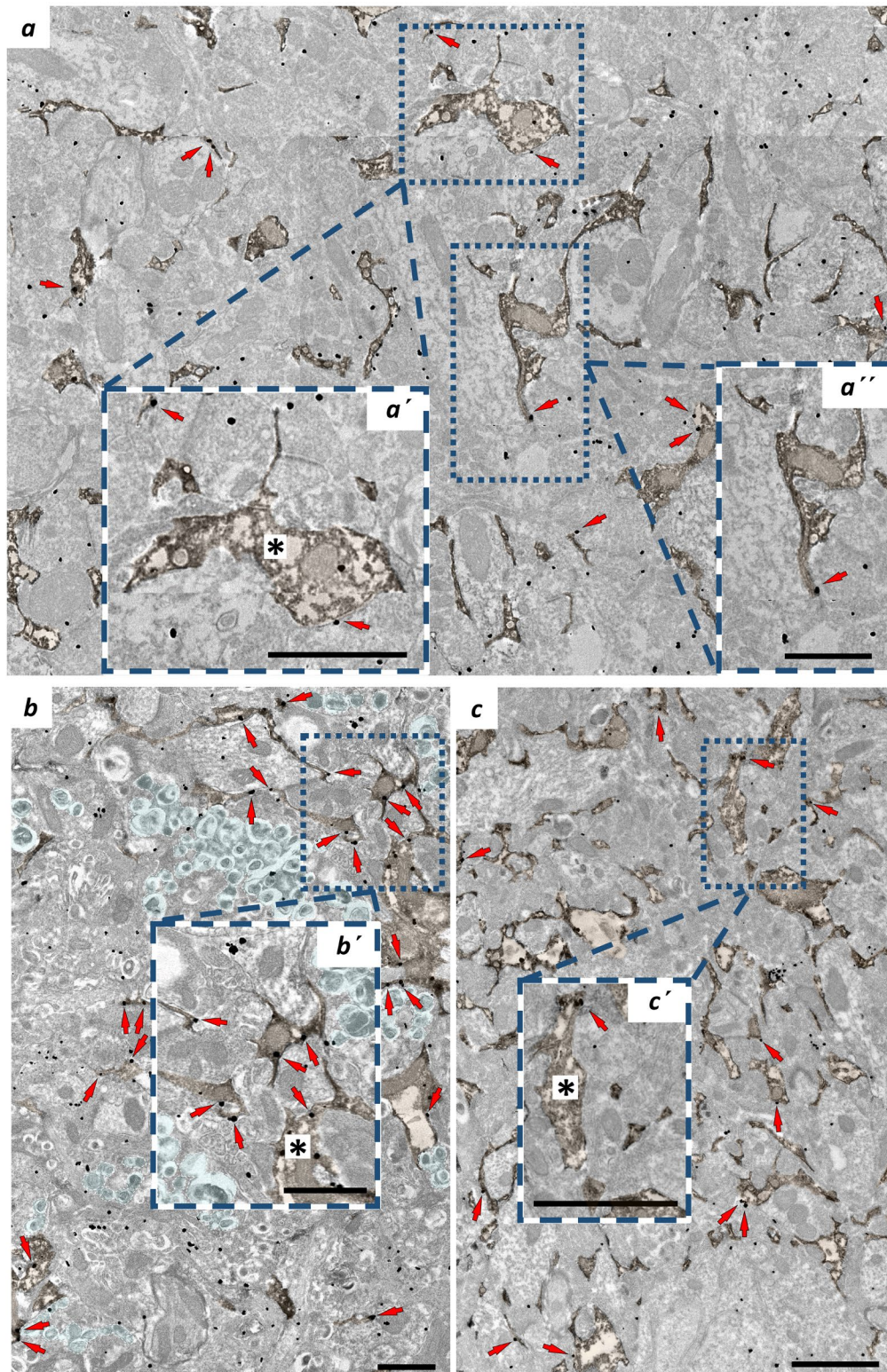
Figure 21. Localization of astroglial CB$_1$R in the subiculum of CB2$^{EGFP/f/f}$ and CB2$^{EGFP/f/f}$/5xFAD mice. Double pre-embedding immunogold (CB$_1$R) and immunoperoxidase (GLAST) method for electron microscopy. CB$_1$R particles (red arrows) localize to plasma membranes of GLAST-positive astrocytic processes (DAB, brown, * immunodeposits) in CB2$^{EGFP/f/f}$ (a, a′, a′′) and CB2$^{EGFP/f/f}$/5xFAD (b, b′, c, c′). Observe dystrophic neurites (blue) in CB2$^{EGFP/f/f}$/5xFAD (b). Scale bars: 1 μm.
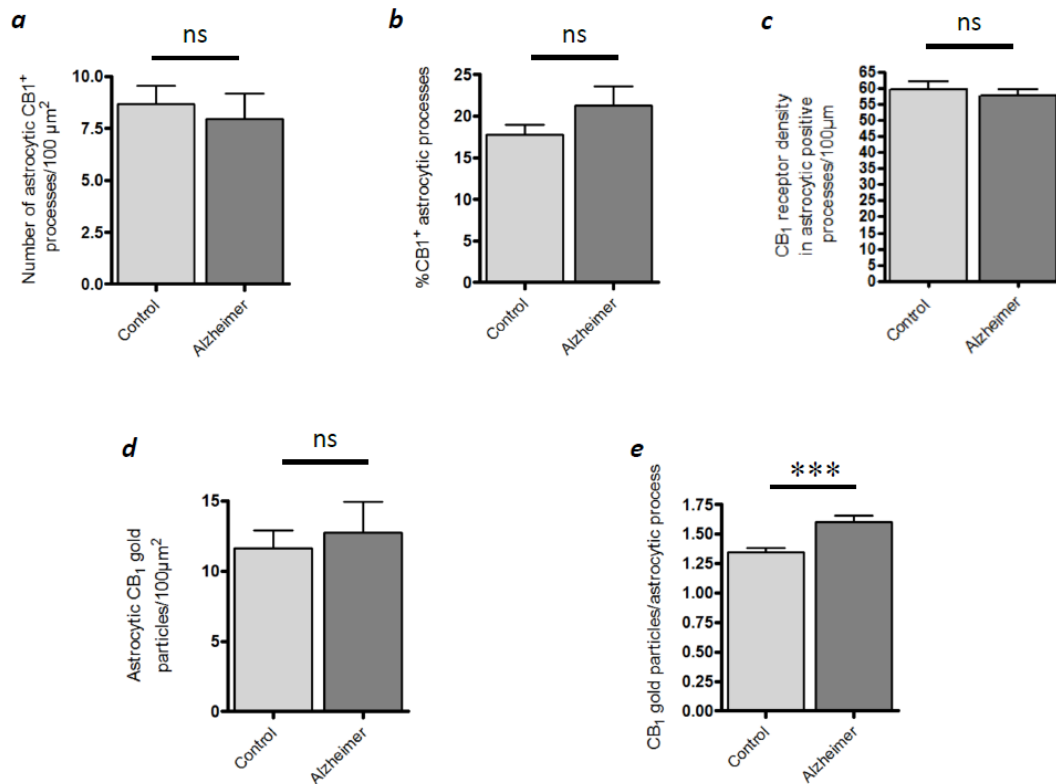
Figure 22. Statistical assessment of the CB₁R localization in subicular astrocytes of CB2$^{EGFP/f/f}$ and CB2$^{EGFP/f/f}$/5xFAD mice. (a) Number of astrocytic CB₁R$^+$ processes in 100μm^2. (b) Percentage of CB₁R$^+$ astrocytic processes. (c) CB₁R density in positive astrocytic processes per 100μm. (d) Astrocytic CB₁R gold particles per 100 µm^2. (e) CB₁R particles per astrocytic process. Data were analysed by non-parametric or parametric tests (Mann–Whitney U-test or Student's t-test). Mann–Whitney U-test or Student's t-test. $p < 0.05^*$; $p < 0.01^{**}$; $p < 0.001^{***}$; $p < 0.0001^{****}$. All data are represented as mean ± S.E.M.

7. DISCUSSION

In my doctoral thesis, I have used a transgenic mice expressing EGFP under the control of the gen cnr2 promoter that were crossed with mice expressing 5 familial Alzheimer's disease mutations (5xFAD; CB2$^{EGFP/f/f}$/5xFAD) and their controls CB2$^{EGFP/f/f}$ (López et al., 2018). All these mice were kindly provided by Dr. Julián Romero and his laboratory (Faculty of Experimental Sciences, Universidad Francisco de Vitoria, Pozuelo de Alarcón) within the frame of our exciting collaborative work.

Numerous murine models have been generated to study AD and its underlying pathogenesis (Table 1). These models have been essential to understand the development and progression of the disease and to gain insight into its mechanisms and risk factors (for review Jankowsky and Zheng, 2017; Oblak et al., 2021). However, the degree of pathological features in the animal models is limited compared to the events happening in human AD. Therefore, adequate characterisation of the AD mouse models has been necessary to improve their translational value in clinical drug design and treatment development (Oblak et al., 2021). In this sense, a deep phenotyping study was conducted comparing 5xFAD mice with wild-type (WT) mice, analysing changes in transcriptomic, electroencephalogram, *in vivo* imaging, biochemical characterisation and behavioural assessments. The study revealed that 5xFAD mice recreate human AD in the context of (1) body frailty and abnormal motor function, (2) sex-based alterations in gene status and expression, (3) immune system involvement in disease development, and (4) amyloidosis. Therefore, the use of this animal model in AD studies was recommended (Oblak et al., 2021).

I focused on the subiculum because it is strategically located in the control of hippocampal outflow (for review Mark et al., 1995; for review Rajmohan and Mohandas, 2007; Ishihara and Fukuda, 2016; for review Matsumoto et al., 2019). At the same time, the subiculum is one of the first and most damaged brain regions in AD causing cognitive deficits as a consequence of the hippocampal disconnection (Hyman et al., 1984; Oakley et al., 2006; López et al., 2018).

I have studied microglia and astrocytes in the subiculum of CB2$^{EGFP/f/f}$/5xFAD mice (6.5-7.5 month-old) with plaques and neuronal damage already formed at that age. Aβ-containing aggregates induce a chronic inflammatory response leading to activation of both microglia and astrocytes, among other events (Benito et al., 2003). Thus, plaques are surrounded and invaded by activated microglia (Yin et al., 2017; for review Smit et al., 2021) and reactive astrocytes (Kamphuis et al., 2014; Kato et al., 1998; for review Smit et al., 2021). Astrocytes

are specifically known to interact closely with microglia, and both cells are implicated in reactive gliosis in AD (McAlpine et al., 2021; for review Smit et al., 2021). This reactive gliosis results in abnormal morphology and proliferation of astrocytes and microglia. Whether these histopathological changes reflect beneficial, detrimental or inconsequential activity of glial cells in neurodegenerative events remains unclear (for review Hansen et al., 2018). Much of the research agrees that neuroinflammation begins at the earliest stage of AD with both positive and negative consequences. The brain's immune system recognises abnormal protein accumulation as a harmful stimulus and, as a defence, initiates a physiological reaction by activating glial cells and releasing pro-inflammatory molecules. However, when these processes are prolonged over time, they can contribute to neuronal dysfunction and cell death, favouring disease´s progression (for review Sarlus and Heneka, 2017).

Furthermore, there seems to be a communication between microglia and astroglia that is beneficial in AD. For example, upon detection of Aβ deposits, microglia increases IL-3Rα expression, the specific IL-3 receptor (also known as CD123). Astrocytes, in turn, constitutively secrete IL-3, which upon binding to IL-3Rα receptors generates a response that triggers transcriptional, morphological and functional programming of microglia to endow these cells with an acute immune response programme, increased motility and ability to aggregate and remove Aβ and τ aggregates. These changes mediated by both glial cells in collaboration would therefore limit AD pathology and cognitive impairment (McAlpine et al., 2021).

I observed glial reactivity in both microglia and astrocytes, with an increase in the area and perimeter of their processes. I also detected in the electron microscope the presence of plaques and a multitude of dystrophic neurites in the CB2$^{EGFP/f/f}$/5xFAD mice, further confirming the usefulness of this animal model for studying the pathophysiology of AD. The results obtained in my study have contributed to determine the changes that occur in the area, perimeter and number of these glial cell processes in AD, demonstrating the presence of significant alterations in the subicular cytoarchitecture, with overt microglial and astrocytic reactivity and abundant neuronal death (Scuderi et al., 2020).

7.1.1. MICROGLIAL CHANGES IN THE SUBICULUM IN ALZHEIMER'S PATHOLOGY

I have shown in my thesis a very significant increase in the number, area and perimeter of microglial processes in CB2$^{EGFP/f/f}$/5xFAD mice. Thus, there was an obvious microglial activation in the subiculum of these AD mutant mice by the age of 6.5-7.5 months. There is

ample evidence documenting microglia-mediated inflammatory response in the AD brain. Microglial cells are normally responsible for tissue maintenance and immune surveillance of the brain. Amyloid deposition in AD triggers microglial diversity due to microglial activation that reflects both cell plasticity and their ability to transition into activated states (Cameron and Landreth, 2010; for review Hansen et al., 2018). Thus, a variety of microglial reactions can be observed at different stages of the disease (for review Sarlus and Heneka, 2017). Human studies using PET techniques have shown a correlation between decreased cognitive abilities and microglial activation in patients with mild cognitive impairment (for review Blasko et al., 2004; Cagnin et al., 2007; Okello et al., 2009). In post-mortem samples from AD patients (Gomez-Nicola et al., 2013) and in murine models of the disease, microglial proliferation strongly correlates with microglial activation and plaque deposition (for review Blasko et al., 2004; Maeda et al., 2007; Kamphuis et al., 2012; Olmos-Alonso et al., 2016). Therefore, the dynamic nature of microglial cells, such as their great phenotypic diversity, highlights the need to characterise this cell type in AD (for review Mandrekar-Colucci and Landreth, 2010). Recent studies have indicated that mouse models of AD show a change in microglial activation status in response to disease progression. Furthermore, the number of microglial cells and their size increase directly in proportion to the size of the plaque they surround. Microglia in the immediate vicinity of amyloid plaques also proliferates, allowing the accumulation of these cells at the periphery of amyloid deposits. Plaque-associated microglial cells extend their processes and encompass Aβ deposits and, through this association, may regulate plaque dynamics in transgenic mouse models of AD (for review Mandrekar-Colucci and Landreth, 2010; for review Kettenmann et al., 2011). Thus, with the accumulation of Aβ oligomers, microglia is activated rapidly to eliminate these harmful stimuli. However, *in vivo* studies suggest that the beneficial functions of microglia gradually diminish over the course of the disease and a "toxic" microglia phenotype turns out to pop up. Furthermore, there are experimental studies in mouse describing microglial dysfunction leading to neuronal loss through many mechanisms, namely, Aβ accumulation after reduced amyloid clearance, excessive secretion of inflammatory cytokines, microglia-mediated synaptic engulfment, or τ-containing exosomes release (for review Shen et al., 2017). Therefore, the proliferation and activation of microglia around amyloid plaques in an attempt to achieve their clearance is a hallmark of AD (for review Gomez-Nicola and Perry, 2015). Conversely, impaired microglia and altered microglial responses to Aβ are associated with an increased risk of AD (for review Hansen et al., 2018).

Astrocytes are the most abundant cells in the brain (for review Blasko et al., 2004) and are essential for proper neuronal and, ultimately, brain function (for review Verkhratsky and Nedergaard, 2018; for review Smit et al., 2021). Given the importance of this cell population in the CNS, we wanted to analyse the occurrence of changes in astrocytes in the mouse models studied in my doctoral thesis. I observed that there was a significant increase in the area and perimeter of GLAST-positive astrocytic processes offset by a significant decrease in their number in CB2$^{EGFP/f/f}$/5xFAD mice. That is, there were fewer but larger astrocytic processes in the subiculum of CB2$^{EGFP/f/f}$/5xFAD compared to CB2$^{EGFP/f/f}$ mice.

To clearly identify astrocytic compartments at the ultrastructural level, an anti-A522 (EAAT1 [GLAST]) antibody (Ab#314) targeting the C-terminal residues 522-541 of rat EAAT1 (Hu et al., 2020) kindly provided by Prof. Niels Christian Danbolt (University of Oslo), was used. GLAST is selectively expressed in astrocytes, and my assays have shown that GLAST is restricted to astrocytes and localizes intracellularly, with no detectable labelling in nerve terminals as previously described (Lehre et al., 1995; Schmitt et al., 1997). There may be differences in GLAST expression between rodents and humans, but meta-analysis studies showed that the distribution of the transporter is very similar in both species (Li et al., 2012). Therefore, this antibody has proven to be a good strategy for labelling astrocytes to study astrocytic changes in the AD model.

The view of astrocytes has changed dramatically in the last few years, from the concept of mainly supportive cells to multifunctional cells that allow neurons to progressively specialise in information processing tasks. Thus, astrocytes have been described as organising not only the structural architecture of the brain, but also its communication pathways and plasticity (for review Blasko et al., 2004). In addition to modulating synaptic strength and activity, they also positively and negatively regulate neurogenesis and glycogenesis in neurogenic regions of the adult human brain from resident precursor cells (Song et al., 2002; for review Goldman, 2003), through reciprocal paracrine interactions between astrocytes, endothelial cells and ependymal cells (for review Goldman, 2003; Cambier et al., 2005). In addition, astrocytes play an important role in inflammatory processes (Wyss-Coray et al., 2003; for review Blasko et al., 2004). Other functions include maintenance of ionic homeostasis, regulation of extracellular levels of excitatory amino acids such as glutamate, vascular coupling, synaptic plasticity, circuit building, synapse turnover, waste disposal or higher functions such as the sleep-wake cycle, food intake or memory (for review Blasko et al., 2004; for review Verkhratsky and Nedergaard, 2018; for review Escartin et al., 2019).

The astrocyte appearance is different in the diseased brain, as this cell reacts to almost all alterations in brain homeostasis, through major morphological and molecular changes known as astrogliosis (for review Escartin et al., 2019). In a mouse model of binge drinking, for example, astrocytes increase the surface area and reduce the number of processes in the adult hippocampus following exposure to EtOH during adolescence (Bonilla-Del Río et al., 2019). In AD, astroglial reactivity in close association with Aβ aggregates shows a rise in intermediate filament proteins and hypertrophy of cell bodies (for review Escartin et al., 2019; for review Smit et al., 2021). Unlike microglia, astrocytes are able to clear and degrade Aβ aggregates without mediators or stimuli such as opsonins or cytokines (Bard et al., 2000; Wyss-Coray et al., 2003; for review Blasko et al., 2004). In addition, the reactive astrocytes can release cytokines and growth factors similar to those produced by microglia (McGeer and McGeer, 1995), and secrete trophic substances for neurons, such as nerve growth factor (NGF) (Aguado et al., 1998), the neurotrophic signalling molecule S100β (Mrak and Griffin, 2001, 2005), brain-derived neurotrophic factor (BDNF) (Hock et al., 2000), neurotrophin 3 (NT-3) (Blondel et al., 2000), or neurotrophin 4/5 (NT-4/5). The release of these substances, together with transporter molecules and enzymes involved in excitatory amino acid metabolism or antioxidant pathways, may contribute to the protection of neurons and other brain cells by controlling the production of potentially toxic substances (for review Blasko et al., 2004).

In summary, functional changes associated with astrocytic reactivity may have a direct impact on synaptic communication and neuronal network function, which could contribute to cognitive impairment (for review Escartin et al., 2019; for review Smit et al., 2021).

7.2. Cannabinoid receptors in subicular glial cells affected by Alzheimer's pathology

To investigate the expression of the major cannabinoid CB_1R and CB_2R in microglial and astroglial cells in AD, the subiculum of $CB2^{EGFP/f/f}$/5xFAD and $CB2^{EGFP/f/f}$ mice was studied in my doctoral thesis. The main findings were that the CB_1R and CB_2R expression suffers remarkable changes in glial cells of the AD mouse. Not least, the localization of CB_1Rs in microglial processes in the subiculum of controls and closely surrounding amyloid plaques and dystrophic neurites in the subiculum of the AD model, supports previous suggestions of the presence of the receptor in this glial cell type (see below).

Low expression of CB_1R and CB_2R in glial cells could be accurately detected by immunoelectron microscopy. Thus, a combined preembedding immunogold and

immunoperoxidase method has previously been shown to be an optimal approach for the localization of CB1Rs in astrocytes (Gutiérrez-Rodríguez et al., 2018; Puente et al., 2019). Further, the experiments I performed in this work showed that this combined immunoelectron microscopy protocol was also optimal for CB$_1$R and GFP localization in microglia. I detected an increase in the microglial expression of CB$_1$R and CB$_2$R, and an unalterable CB$_1$R density in astrocytes despite the reactivity of their processes. The expression of CB$_1$R and CB$_2$R in microglia changes depending on the microglial phenotype and activation profile (for review Stella, 2010). CB$_2$Rs are selectively and abundantly overexpressed in microglia in the vicinity of NPs (for review Bedse et al., 2015) and cannabinoids increase CB$_1$R gene expression through CB$_2$Rs (for review Haspula and Crark, 2020), so high CB$_2$R expression could affect CB$_1$R expression near the lesions. Therefore, it appears that microglia overexpresses both cannabinoid receptors in a proinflammatory state as an autoregulatory mechanism to decrease glial reactivity (for review Benito et al., 2008). The increased expression of CB$_1$R and CB$_2$R could have a protective effect in AD.

7.2.1. CANNABINOID CB$_1$ RECEPTORS IN SUBICULAR MICROGLIA OF CB2$^{EGFP/F/F}$ AND CB2$^{EGFP/F/F}$/5xFAD MICE

There are pieces of evidence indicating that microglial cells constitutively express CB$_1$Rs (for review Stella, 2009; Ribeiro et al., 2013) which mediate some of the effects of cannabinoids on these cells (for review Kaplan, 2013). Thus, endocannabinoids would primarily target this receptor in resting microglia (Navarro et al., 2018). However, microglia hardly expressed (if any) CB$_1$R (and CB$_2$R) at resting conditions. Actually, specific CB$_1$R antibodies were unable to detect CB$_1$R in microglial cells of the healthy brain. However, CB$_1$R was observed in cultured microglia of several species but not of humans (Stefano et al., 1996; Sinha et al., 1998; Waksman et al., 1999; Carlisle et al., 2002; Molina-Holgado et al., 2002; Facchinetti et al., 2003; Klegeris et al., 2003; Walter et al., 2003).

As I have mentioned in the Introduction section, CB$_1$R expression under normal conditions is very high in inhibitory terminals (Gutiérrez-Rodríguez et al., 2017; for review Katona and Freund, 2012; for review Lu and Mackie, 2016), low in excitatory terminals (Gutiérrez-Rodríguez et al., 2017; Katona et al., 2006; Monory et al., 2006) and even lower in astrocytes (Gutiérrez-Rodríguez et al., 2018). Our laboratory assessed in previous studies that ~ 56% of the CB$_1$R labelling is in GABAergic terminals, ~12% in glutamatergic terminals, ~6% in astrocytes and ~15% in mitochondria (Gutiérrez-Rodríguez et al., 2018;

Bonilla-Del Río et al., 2019). Noticeably, ~11% of the CB1R immunoparticles were localized elsewhere (Gutiérrez-Rodríguez et al., 2018; Bonilla-Del Río et al., 2019) that might correspond to CB_1Rs located at lysosomes/endosomes (Rozenfeld and Devi, 2008) or another cell compartments like microglia.

In this work, I have sought to clarify what changes occur in CB_1R expression under AD conditions with obvious microglial activation. I could observe in the subiculum using the microglial marker Iba1 that labels all types of microglial subpopulations (Ito et al., 1998; Okere and Kaba, 2000; Hirayama et al., 2001; Shapiro et al., 2008), the presence of CB_1Rs in microglial membranes of control $CB2^{EGFP/f/f}$ mice. These findings are in line with the recent preliminary evidences of our laboratory also using high resolution immunocytochemical techniques for electron microscopy, indicating the CB_1R localization in ~ 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 (unpublished). It is a low percentage that might be changed under pathological conditions.

I also revealed a significant increase in the number and percentage of CB_1R-positive microglial processes in $CB2^{EGFP/f/f}$/5xFAD relative to $CB2^{EGFP/f/f}$ mice. However, there were not significant changes in the number of CB_1R particles per microglial branch, but there was a significant decrease in CB_1R density due to the perimeter increase of microglial processes. Furthermore, there were more CB_1R-positive microglial ramifications, suggesting that more microglial processes are under the influence of CB_1Rs in $CB2^{EGFP/f/f}$/5xFAD. Preliminary results in our laboratory indicate that CB_1Rs 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. This percentage is higher than what my results showed in 6.5-7.5 month-old mice, as the proportion of CB_1R-positive processes in $CB2^{EGFP/f/f}$/5xFAD was ~7%. This discrepancy should be studied in detail because it suggests that CB_1R expression increases in this mouse model as the degeneration makes progress. Accordingly, CB_2R is more expressed at advanced stages of AD when neuroinflammation is more evident (see below; for review Di Marzo et al., 2015; for review Talarico et al., 2019).

CB_1R expression increases in many inflammatory and neurodegenerative diseases like AD (for review Bisogno and Di Marzo, 2010; Ribeiro et al., 2013). Microglial activation also elicits a significant increase in 2-AG and AEA production with respect to resting microglia. These ECBs activate, in turn, more CB_1R and CB_2R and their signalling cascades, amplifying the anti-inflammatory and protective microglial phenotype (for review Mecha et al., 2016; for review Duffy et al., 2021). How CB_1Rs regulate microglial cell function is controversial

(for review Stella, 2010). In this sense, microglial CB$_1$R activation inhibits liposaccharide-induced NO release (Stefano et al., 1996; Waksman et al., 1999) and MPTP-induced oxidant production (Chung et al., 2011), among other effects (for review Kaplan, 2013). Studies have also reported that CB$_1$R interacts with CB$_2$R in heteromers, which form different quaternary structures depending on whether microglia is resting or activated (Navarro et al., 2018). This would explain the different effects on the role of CB$_1$R and CB$_2$R in immune modulation, microglial activation and the potential to combat neuroinflammation (for review Kaplan, 2013; for review Stella, 2010; Navarro et al., 2018).

7.2.2. CANNABINOID CB$_2$ RECEPTORS IN SUBICULAR MICROGLIA OF CB2$^{EGFP/F/F}$ AND CB2$^{EGFP/F/F}$/5xFAD MICE

As to CB$_2$Rs, only some mRNA has been detected in healthy brain suggesting that this receptor is not much expressed in quiescent microglia (Munro et al., 1993; Derocq et al., 1995; Galiègue et al., 1995; Schatz et al., 1997; Griffin et al., 1999; McCoy et al., 1999; Sugiura et al., 2000; Carlisle et al., 2002). In fact, CB$_2$R was considered a peripheral receptor for its very high expression in spleen and human leukocytes (Munro et al., 1993; Galiègue et al., 1995). Some years ago, CB$_2$R was localized in perivascular microglial cells of the human cerebellar white matter (Núñez et al., 2004). Cultured microglia also expresses CB$_2$Rs (Carlisle et al., 2002; Facchinetti et al., 2003; Klegeris et al., 2003; Walter et al., 2003) regulated by some pathogens and cytokines (Carayon et al., 1998; Waksman et al., 1999, Derocq et al., 2000; Lee et al., 2001; Gardner et al., 2002). In addition, microglial cell lines with high proliferation rate express CB$_2$Rs (Walter et al., 2003; Carrier et al., 2004). In brain, CB$_2$R is expressed in activated microglia in certain conditions. For instance, in neuropathic pain models increases CB$_2$R in microglia of the spinal cord, but not in a peripheral chronic inflammatory pain model (Zhang et al., 2003). There is also an increase in microglial CB$_2$R in inflammation (Maresz et al., 2005) and in activated microglia in brain tissue of patients with AD or multiple sclerosis mostly at the lesion sites (Benito et al., 2003; Yiangou et al., 2006), in the vicinity of tumors (for review Guzmán et al., 2001) and in activated microglia of a simian model of acquired immunodeficiency syndrome dementia (Benito et al., 2005). The first attempts to localize CB$_2$Rs in the CNS in basal conditions failed because CB$_2$R could only be seen in pathological conditions, as described before. Nevertheless, the CB$_2$R receptor was not only detected in microglia (for review Cabral et al., 2008; for review Atwood and Mackie, 2010) but also in neurons (Van Sickle et al., 2005; Zhang et al., 2014). However, there are serious concerns about the CB$_2$R localization in the CNS due to the lack of specific

CB$_2$R antibodies (for review Atwood and Mackie, 2010; for review Lu and Mackie, 2016) that precludes the demonstration of the CB$_2$R distribution in the brain. To circumvent this limitation, new strategies have been developed over the recent past based on new genetic strategies using mouse lines in order to clarify the CB$_2$R localization in the brain, as the one used in my doctoral thesis. In this mouse model, GFP expression was below detection levels (López et al., 2018). However, the authors observed that in CB2$^{EGFP/f/f}$/5xFAD mice was coincident with the presence of neuritic plaques in several brain regions. Furthermore, the GFP (CB$_2$R) expression was restricted to microglial cells in the vicinity of the neuritic plaques. Importantly, this pattern of GFP (CB$_2$R) expression was not seen in CB$_2$$^{-/-}$/5xFAD mice (López et al., 2018).

In agreement with these previous observations, I have demonstrated in my doctoral thesis a significant increase in the number and proportion of GFP-positive microglial processes as well as in microglial GFP particles in the subiculum of CB2$^{EGFP/f/f}$/5xFAD mice. Interestingly, CB$_2$R particles were also detected in the subicular microglia of mice without pathology (CB2$^{EGFP/f/f}$). This suggests the existence of a basal CB$_2$R expression in microglia in the healthy brain. Alternatively, this expression might be representing early signs of ageing associated with the age of the control CB2$^{EGFP/f/f}$ mice (6.5-7.5 months).

The CB$_2$R expression increases in activated microglia as a response to certain neuropathological and neuroinflammatory conditions. ECBs produced by damaged neurons and injury-stimulated astrocytes activate CB$_2$R expressed in microglia (for review Stella, 2009). In this way, they regulate immune functions in these cells, stimulating microglial proliferation and migration and reducing at the same time neurotoxic factors such as TNFα or free radicals (Walter et al., 2003; Carrier et al., 2004; Ramirez et al., 2005; Eljaschewitsch et al., 2006; Dirikoc et al., 2007; Lin et al., 2017), then having microglia lower harmful effects at lesion sites (for review Stella, 2010). Altogether, the CB$_2$R increase in microglial cells observed in my doctoral thesis would represent a compensatory mechanism to mitigate the negative consequences of the inflammatory and neurodegenerative mechanisms associated to AD. The increased expression of CB$_2$Rs in microglial cells opens new perspectives on both the functional role of this receptor in the CNS and its therapeutic implications for the development of new anti-inflammatory treatments (for review Benito et al., 2008).

7.2.3. CANNABINOID CB$_1$ RECEPTORS IN SUBICULUR ASTROGLIA OF CB2$^{EGFP/F/F}$ AND CB2$^{EGFP/F/F}$/5xFAD MICE

Taking into account the ongoing morphological changes in astrocytes around NPs, it was timely to investigate the astrocytic CB$_1$R expression as this receptor plays important roles in astrocytes. For example, CB$_1$R increases the rate of glucose oxidation and ketogenesis (Blazquez et al., 1999; for review Stella, 2010), modulates their ability to produce inflammatory mediators (Sheng et al., 2005; for review Stella, 2010) or participates in endocannabinoid-mediated neuron-astrocyte communication (Navarrete and Araque, 2008). In addition, high levels of FAAH have been found around NPs in astrocytes (Benito et al., 2003; for review Bedse et al., 2015; for review Abate et al., 2021). These evidences altogether suggest that changes in the ES do occur in astrocytes closer to AD lesions.

To determine the contribution of reactive astrocytes to AD pathogenesis, it is necessary to understand the effects of their functional changes ultimately leading to dementia (for review Smit et al., 2021). Thus, CB$_1$R expression in subicular astrocytes were assessed in CB2$^{EGFP/f/f}$/5xFAD mice. I observed a significant increase in the number of CB$_1$R particles per astrocytic process in CB2$^{EGFP/f/f}$/5xFAD, but there were not significant differences with CB2$^{EGFP/f/f}$ in the number of CB$_1$R particles nor in the number or percentage of CB$_1$R-positive processes. I also observed no significant differences in CB$_1$R density between the two transgenic mice. Therefore, it seems that as the perimeter of the reactive astrocytic processes augments, CB$_1$Rs also increase keeping unchangeable density and receptor expression. Thus, there were adaptive changes in CB$_1$R expression, as there were fewer but larger astrocytic processes with more CB$_1$R in their membranes. Hence, although there was a clear astrocytic reactivity around the plaques, there were not changes in CB$_1$Rs. Yet, it remains to be elucidated whether the CB$_1$R expression in astrocytes varies depending on the receptor distance to dystrophic neurites and NPs. Finally, it would be interesting to investigate other components of the ES in CB2$^{EGFP/f/f}$/5xFAD astrocytes.

Astrocytic swelling leads to astroglial dysfunction (for review Adermark and Bowers, 2016) and disruption of GFAP found in the astrocyte intermediate filaments (Renau-Piqueras et al., 1989). Astrocytes participate in the inflammatory response through their capacity to release pro-inflammatory molecules (for review Farina et al., 2007) that can be diminished by anti-inflammatory reactions mediated by ECBs acting on astroglial CB$_1$Rs (for review Metna-Laurent and Marsicano, 2015). Furthermore, swollen astrocytic processes 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). Hence, because of the astrocytic swelling, it is reasonable to expect an impairment of the astroglial anti-inflammatory reaction around the plaques and dystrophic neurites seen in the subiculum of the $CB2^{EGFP/f/f}$/5xFAD mice. In addition, the supposedly resulting disturbance of NT clearance and gliotransmission may lead to deficits in synaptic plasticity (for review Dzyubenko et al., 2016) and, consequently, cause brain dysfunction. It remains to be determined whether the astrocytic alterations correlate with changes in GLAST expression in the $CB2^{EGFP/f/f}$/5xFAD mice.

The astrocytic CB_1Rs together with the basal endocannabinoid tone play important roles in brain functions such as synaptic plasticity, memory and behaviour (Han et al., 2012; Navarrete and Araque, 2008; for review Perea et al., 2009; Navarrete and Araque, 2010). For instance, CB_1Rs in astrocytes mediate the spatial working memory deficit and *in vivo* LTD reported at the hippocampal CA3-CA1 synapses after acute cannabinoids exposure (Han et al., 2012). It appears that astrocytic CB_1R expression is regulated by multiple factors, as it can vary under different conditions as well as in transgenic animals lacking other cannabinoid receptors. For example, TRPV1 knock out mice show a significant decrease in CB_1R density in astrocytes (Egaña-Huguet et al., 2021). In addition, following acute THC injection, a significant increase in astroglial CB_1Rs has been described by our laboratory (Bonilla-Del Río et al., 2021). In addition, this increase could somehow affect the morphology of these cells, since a decrease in the area of astrocytic processes was also reported after acute THC exposure (Bonilla-Del Río et al., 2021) which might affect on brain function and behavior (Tahir et al., 1992; Suárez et al., 2000).

Altogether, changes in astroglial morphology and their inflammatory consequences may contribute to cognitive impairment. The consequences that astroglial dysfunction may have in AD are poorly understood (for review Verkhratsky and Nedergaard, 2018; for review Smit et al., 2021), though studies focusing on astrocyte function and astrocyte-neuron interactions are being reported (for review Escartin et al., 2019; for review Smit et al., 2021). This will undoubtedly lead to a better understanding of the role of astrocytes in health and disease, which is essential for understanding the full spectrum of AD pathogenesis. This information will enable the development of innovative astrocyte-targeted therapies orientated to counteract the astrocyte-induced damage, or facilitate its repair and/or recovery when the damage is not yet too severe and irreversible (for review Smit et al., 2021).

7.2.4. Therapeutic potential of cannabinoid-based treatments in Alzheimer's disease

Cannabinoids exhibit pleiotropic activity and target in parallel several processes involved in AD pathogenesis (for review Aso and Ferrer, 2014). Thus, cannabinoids may have beneficial effects by reducing Aβ aggregation, inhibiting τ protein hyperphosphorylation and ROS generation, among others (Casarejos et al., 2013; for review Aso and Ferrer, 2014; for review Talarico et al., 2019; Soto-Mercado et al., 2020). The most significant changes observed in this doctoral thesis are the increase in both CB_1R and CB_2R in microglia, which could be regarded as potential targets for cannabinoid-based therapies to treat AD. As for the effect of cannabinoids on microglia, it remains to be deciphered but seems very promising (Esposito et al., 2006; for review Talarico et al., 2019). Therefore, broad-spectrum compounds capable of controlling glial activation with a combination of neuroprotective and anti-inflammatory effects could be taken into account as a novel therapeutic approach for AD. However, the effect of therapeutic interventions targeting glial cells depends on the right balance between attenuating harmful effects and, at the same time, maintaining the brain's beneficial defence mechanisms (for review Hansen et al., 2018; Scuderi et al., 2020).

This doctoral work has therefore served to characterize the morphological changes and expression of CB_1R in astrocytic and microglial cells in a 5xFAD mouse model aged between 6.5 and 7.5 months. In addition, thanks to the $CB2^{EGFP/f/f}$ and $CB2^{EGFP/f/f}/5xFAD$ models, we have been able to characterize the microglial expression of the CB_2R. Both receptors, which seem to be so important at the glial level in the course of this pathology, may be taken into account as therapeutic targets in AD, a disease with no effective treatment to date.

8. CONCLUSIONS

The conclusions of my doctoral thesis are:

1. Microglia in the subiculum of the $CB2^{EGFP/f/f}$/5xFAD mouse shows an increase in the area, perimeter and number of processes.

2. Astrocytes in the subiculum of the $CB2^{EGFP/f/f}$/5xFAD mouse display a decrease in the number but an increase in the area and perimeter of the ramifications. Thus, the area and perimeter per sample area remain constant.

3. The number and proportion of CB_1R immunopositive microglial processes increase significantly in the subiculum of $CB2^{EGFP/f/f}$/5xFAD mouse, but the number of CB_1R particles per microglial process remains steady. This, together with the increased branch perimeter in $CB2^{EGFP/f/f}$/5xFAD mouse, results in a reduction in microglial CB_1R density.

4. The number and percentage of GFP-positive microglial processes increase significantly in the subiculum of $CB2^{EGFP/f/f}$/5xFAD mouse. There is also a significant increase in the number of microglial GFP particles per microglial process area, and total area per sample. Thus, there is an increase in microglial CB_2Rs in the $CB2^{EGFP/f/f}$/5xFAD mouse.

5. The number of astrocytic CB_1R particles per process increases, but the CB_1R density remains constant in the subiculum of $CB2^{EGFP/f/f}$/5xFAD mice. Therefore, CB_1R expression in astrocytes does not change in this mouse model of AD.

6. Overall, this doctoral thesis has demonstrated the existence of glial alterations and changes in CB_1R and CB_2R expression in the subiculum of $CB2^{EGFP/f/f}$/5xFAD mice that may be contributing to the cognitive impairment caused by AD.

9. BIBLIOGRAPHY

-Abate G, Uberti D, Tambaro S. Potential and Limits of Cannabinoids in Alzheimer's Disease Therapy. Biology 2021;10(6):542.

-Adermark L, Bowers MS. Disentangling the role of astrocytes in alcohol use disorder. Alcoholism: Clinical and Experimental Research 2016; 40(9):1802-1816.

-Allen Mouse Brain Atlas [Internet]. Allen Institute for Brain Science; 2004 [Retrieved Jun 9, 2021]. Available from: atlas.brain-map.org/.

-Aguado F, Ballabriga J, Pozas E, Ferrer I. TrkA immunoreactivity in reactive astrocytes in human neurodegenerative diseases and colchicine-treated rats. Acta neuropathologica. 1998; 96(5):495-501.

-Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. Nat. Neurosci. 2007;10(12):1538-1543.

-Almolda B, de Labra C, Barrera I, Gruart A, Delgado-Garcia JM, Villacampa N, et al. Alterations in microglial phenotype and hippocampal neuronal function in tablw mice with astrocyte-targeted production of interleukin-10. Brain Behav. Immun. 2015; 45:80-97.

-Apelt J, Ach K, Schliebs R. Aging-related down-regulation of neprilysin, a putative β-amyloid-degrading enzyme, in transgenic Tg2576 Alzheimer-like mouse brain is accompanied by an astroglial upregulation in the vicinity of β-amyloid plaques. Neurosci. Lett. 2003; 339(3):183-186.

-Araque A, Carmignoto G, Haydon PG, Oliet SH, Robitaille R, Volterra A. Gliotransmitters travel in time and space. Neuron 2014; 81(4):728-739.

-Askew K, Li K, Olmos-Alonso A, Garcia-Moreno F, Liang Y, Richardson P, et al. Coupled proliferation and apoptosis maintain the rapid turnover of microglia in the adult brain. Cell reports 2017; 18(2):391-405.

-Aso E., Ferrer I. Cannabinoids for treatment of Alzheimer's disease: moving toward the clinic. Frontiers in pharmacology 2014; 5:37.

-Aso E, Ferrer I. CB2 cannabinoid receptor as potential target against Alzheimer's disease. Frontiers in neuroscience 2016; 10:243.

-Athanasiou A, Clarke AB, Turner AE, Kumaran NM, Vakilpour S, Smith PA, et al. Cannabinoid receptor agonists are mitochondrial inhibitors: a unified hypothesis of how cannabinoids modulate mitochondrial function and induce cell death. Biochem. Biophys. Res. Commun. 2007; 364(1):131-137.

-Atwood BK, Mackie K. CB2: a cannabinoid receptor with an identity crisis. Br. J. Pharmacol. 2010; 160(3):467-479.

-Bachmeier C, Beaulieu-Abdelahad D, Mullan M, Paris D. Role of the cannabinoid system in the transit of beta-amyloid across the blood–brain barrier. Molecular and Cellular Neuroscience 2013; 56:255-262.

-Bakota, L., and Brandt, R. Tau biology and Tau-directed therapies for Alzheimer's disease. Drugs 2016; 76, 301–313.

-Bard F, Cannon C, Barbour R, Burke RL, Games D, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K. Peripherally administered antibodies against amyloid β-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nature medicine. 2000; 6(8):916-9.

-Beauquis J, Pavía P, Pomilio C, Vinuesa A, Podlutskaya N, Galvan V, et al. Environmental enrichment prevents astroglial pathological changes in the hippocampus of APP transgenic mice, model of Alzheimer's disease. Exp. Neurol. 2013;239:28-37.

-Bedse G, Romano A, Cianci S, Lavecchia AM, Lorenzo P, Elphick MR, et al. Altered expression of the CB1 cannabinoid receptor in the triple transgenic mouse model of Alzheimer's disease. J. Alzheimer's Dis. 2014; 40(3):701-712.

-Bedse G, Romano A, Lavecchia AM, Cassano T, Gaetani S. The role of endocannabinoid signaling in the molecular mechanisms of neurodegeneration in Alzheimer's disease. J. Alzheimer's Dis. 2015;43(4):1115-1136.

-Benito C, Nunez E, Tolon RM, Carrier EJ, Rabano A, Hillard CJ, et al. Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. J. Neurosci. 2003; 23(35):11136-11141.

-Benito C, Kim WK, Chavarria I, Hillard CJ, Mackie K, Tolon RM, et al. A glial endogenous cannabinoid system is upregulated in the brains of macaques with simian immunodeficiency virus-induced encephalitis. J. Neurosci. 2005; 25(10):2530-2536.

-Benito C, Tolon R, Pazos M, Nunez E, Castillo A, Romero J. Cannabinoid CB2 receptors in human brain inflammation. Br. J. Pharmacol. 2008; 153(2):277-285.

-Bezprozvanny I, Mattson MP. Neuronal calcium mishandling and the pathogenesis of Alzheimer's disease. Trends Neurosci. 2008; 31(9):454-463.

-Bilkei-Gorzo A, Racz I, Valverde O, Otto M, Michel K, Sastre M, et al. Early age-related cognitive impairment in mice lacking cannabinoid CB1 receptors. Proc. Natl. Acad. Sci. U.S.A. 2005; 102(43):15670-15675.

-Bilkei-Gorzo A. The endocannabinoid system in normal and pathological brain ageing. Philosophical Transactions of the Royal Society B: Biological Sciences 2012; 367(1607):3326-3341.

-Biringer RG. The rise and fall of anandamide: processes that control synthesis, degradation, and storage. Mol. Cell Biochem. 2021; 476(7):2753-2775.

-Bisogno T, Berrendero F, Ambrosino G, Cebeira M, Ramos J, Fernandez-Ruiz J, et al. Brain regional distribution of endocannabinoids: implications for their biosynthesis and biological function. Biochem. Biophys. Res. Commun. 1999; 256(2):377-380.

-Bisogno T, Di Marzo V. Cannabinoid receptors and endocannabinoids: role in neuroinflammatory and neurodegenerative disorders. CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders) 2010; 9(5):564-573.

-Bjørnsen L, Eid T, Holmseth S, Danbolt N, Spencer D, De Lanerolle N. Changes in glial glutamate transporters in human epileptogenic hippocampus: inadequate explanation for high extracellular glutamate during seizures. Neurobiol. Dis. 2007; 25(2):319-330.

-Blasko I, Veerhuis R, Stampfer-Kountchev M, Saurwein-Teissl M, Eikelenboom P, Grubeck-Loebenstein B. Costimulatory effects of interferon-γ and interleukin-1β or tumor necrosis factor α on the synthesis of Aβ1-40 and Aβ1-42 by human astrocytes. Neurobiol. Dis. 2000; 7(6):682-689.

-Blasko I, Stampfer-Kountchev M, Robatscher P, Veerhuis R, Eikelenboom P, Grubeck-Loebenstein B. How chronic inflammation can affect the brain and support the development of Alzheimer's disease in old age: the role of microglia and astrocytes. Aging cell 2004; 3(4):169-176.

-Blázquez C, Sánchez C, Daza A, Galve-Roperh I, Guzmán M. The stimulation of ketogenesis by cannabinoids in cultured astrocytes defines carnitine palmitoyltransferase I as a new ceramide-activated enzyme. J. Neurochem. 1999; 72(4):1759-1768.

-Blondel O, Collin C, McCarran WJ, Zhu S, Zamostiano R, Gozes I, Brenneman DE, McKay RD. A glia-derived signal regulating neuronal differentiation. Journal of Neuroscience. 2000; 20(21):8012-20.

-Bloom GS. Amyloid-β and tau: the trigger and bullet in Alzheimer disease pathogenesis. JAMA neurology 2014; 71(4):505-508.

-Bobkova N, Vorobyov D - Institute of Cell Biophysics, Russian Academy of Sciences, 142290 Pushchino, Moscow Region,,Russia. The brain compensatory mechanisms and Alzheimer's disease progression: a new protective strategy. Neural Regen. Res. 2015; 10(5):696-697.

-Bonilla-Del Río I, Puente N, Peñasco S, Rico I, Gutiérrez-Rodríguez A, Elezgarai I, et al. Adolescent ethanol intake alters cannabinoid type-1 receptor localization in astrocytes of the adult mouse hippocampus. Addict. Biol. 2019; 24(2):182-192.

-Bonilla-Del Río I, Puente N, Mimenza A, Ramos A, Serrano M, Lekunberri L, et al. Acute Δ9-tetrahydrocannabinol prompts rapid changes in cannabinoid CB1 receptor immunolabelling and subcellular structure in CA1 hippocampus of young adult male mice. J. Comp. Neurol. 2021; 529(9):2332-2346.

-Boros, B. D., Greathouse, K. M., Gentry, E. G., Curtis, K. A., Birchall, E. L., Gearing, M., et al. Dendritic spines provide cognitive resilience against Alzheimer's disease. Ann. Neurol. 2017; 82, 602–614.

-Bosier B, Bellocchio L, Metna-Laurent M, Soria-Gomez E, Matias I, Hebert-Chatelain E, et al. Astroglial CB1 cannabinoid receptors regulate leptin signaling in mouse brain astrocytes. Molecular metabolism 2013; 2(4):393-404.

-Brodmann K. Vergleichende Lokalisationslehre der Grosshirnrinde in ihren Prinzipien dargestellt auf Grund des Zellenbaues. : Barth; 1909.

-Brugg B, Dubreuil YL, Huber G, Wollman EE, Delhaye-Bouchaud N, Mariani J. Inflammatory processes induce beta-amyloid precursor protein changes in mouse brain. Proc. Natl. Acad. Sci. U.S.A. 1995; 92(7):3032-3035.

-Cabral G, Raborn E, Griffin L, Dennis J, Marciano-Cabral F. CB2 receptors in the brain: role in central immune function. Br. J. Pharmacol. 2008; 153(2):240-251.

-Cagnin A, Kassiou M, Meikle SR, Banati RB. Positron emission tomography imaging of neuroinflammation. Neurotherapeutics 2007; 4(3):443-452.

-Cambier S, Gline S, Mu D, Collins R, Araya J, Dolganov G, et al. Integrin αvβ8-mediated activation of transforming growth factor-β by perivascular astrocytes: an angiogenic control switch. The American journal of pathology 2005; 166(6):1883-1894.

-Cameron B, Landreth GE. Inflammation, microglia, and Alzheimer's disease. Neurobiol. Dis. 2010; 37(3):503-509.

-Carayon P, Marchand J, Dussossoy D, Derocq J, Jbilo O, Bord A, et al. Modulation and functional involvement of CB2 peripheral cannabinoid receptors during B-cell differentiation. Blood, The Journal of the American Society of Hematology 1998; 92(10):3605-3615.

-Carlesimo GA, Piras F, Orfei MD, Iorio M, Caltagirone C, Spalletta G. Atrophy of presubiculum and subiculum is the earliest hippocampal anatomical marker of Alzheimer's

disease. Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring 2015; 1(1):24-32.

-Carlisle S, Marciano-Cabral F, Staab A, Ludwick C, Cabral G. Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. Int. Immunopharmacol 2002; 2(1):69-82.

-Carrier EJ, Kearn CS, Barkmeier AJ, Breese NM, Yang W, Nithipatikom K, et al. Cultured rat microglial cells synthesize the endocannabinoid 2-arachidonylglycerol, which increases proliferation via a CB2 receptor-dependent mechanism. Mol. Pharmacol. 2004; 65(4):999-1007.

-Casarejos MJ, Perucho J, Gomez A, Munoz MP, Fernandez-Estevez M, Sagredo O, et al. Natural cannabinoids improve dopamine neurotransmission and tau and amyloid pathology in a mouse model of tauopathy. J. Alzheimer's Dis. 2013; 35(3):525-539.

-Castillo PE, Younts TJ, Chávez AE, Hashimotodani Y. Endocannabinoid signaling and synaptic function. Neuron 2012; 76(1):70-81.

-Chung YC, Bok E, Huh SH, Park JY, Yoon SH, Kim SR, et al. Cannabinoid receptor type 1 protects nigrostriatal dopaminergic neurons against MPTP neurotoxicity by inhibiting microglial activation. J. Immunol. 2011; 187(12):6508-6517.

-Cline, E. N., Bicca, M. A., Viola, K. L., and Klein, W. L. The amyloid-b oligomer hypothesis: beginning of the third decade. J. Alzheimers Dis. 2018; 64, S567–S610.

-Colton CA, Chernyshev ON, Gilbert DL, Vitek MP. Microglial contribution to oxidative stress in Alzheimer's disease. Ann. N.Y. Acad. Sci. 2000; 899(1):292-307.

-Coronel R, Palmer C, Bernabeu-Zornoza A, Monteagudo M, Rosca A, Zambrano A, et al. Physiological effects of amyloid precursor protein and its derivatives on neural stem cell biology and signaling pathways involved. Neural. Regen. Res. 2019; 14(10):1661-1671.

-De la Vega, R., Zambrano, A. Alzheimer. Circunvalación del Hipocampo [Internet]. Hipocampo.org. Oct 2013 [Retrieved Dec 16, 2020]. Available from: https://www.hipocampo.org/alzheimer.asp/.

-Delcambre GH, Liu J, Herrington JM, Vallario K, Long MT. Immunohistochemistry for the detection of neural and inflammatory cells in equine brain tissue. PeerJ 2016; 4:e1601.

-Derocq J, Segui M, Marchand J, Le Fur G, Casellas P. Cannabinoids enhance human B-cell growth at low nanomolar concentrations. FEBS Lett. 1995; 369(2-3):177-182.

-Derocq JM, Jbilo O, Bouaboula M, Segui M, Clere C, Casellas P. Genomic and functional changes induced by the activation of the peripheral cannabinoid receptor CB2 in the

promyelocytic cells HL-60. Possible involvement of the CB2 receptor in cell differentiation. J. Biol. Chem. 2000; 275(21):15621-15628.

-DeWitt DA, Perry G, Cohen M, Doller C, Silver J. Astrocytes regulate microglial phagocytosis of senile plaque cores of Alzheimer's disease. Exp. Neurol. 1998; 149(2):329-340.

-Di Marzo V, Bifulco M, De Petrocellis L. The endocannabinoid system and its therapeutic exploitation. Nature reviews Drug discovery 2004; 3(9):771-784.

-Di Marzo V, Stella N, Zimmer A. Endocannabinoid signalling and the deteriorating brain. Nature Reviews Neuroscience 2015; 16(1):30-42.

-Dirikoc S, Priola SA, Marella M, Zsurger N, Chabry J. Nonpsychoactive cannabidiol prevents prion accumulation and protects neurons against prion toxicity. J. Neurosci. 2007; 27(36):9537-9544.

-Duffy SS, Hayes JP, Fiore NT, Moalem-Taylor G. The cannabinoid system and microglia in health and disease. Neuropharmacology 2021; 190:108555.

-Dzamba D, Harantova L, Butenko O, Anderova M. Glial cells–The key elements of Alzheimer s disease. Current Alzheimer Research 2016; 13(8):894-911.

-Dzyubenko E, Gottschling C, Faissner A. Neuron-glia interactions in neural plasticity: contributions of neural extracellular matrix and perineuronal nets. Neural Plast 2016; 2016.

-Eikelenboom P, Stam F. Immunoglobulins and complement factors in senile plaques. Acta Neuropathol. 1982; 57(2-3):239-242.

-El Falougy H, Kubikova E, Benuska J. The microscopical structure of the hippocampus in the rat. Bratisl. Lek. Listy. 2008; 109(3):106.

-Eljaschewitsch E, Witting A, Mawrin C, Lee T, Schmidt PM, Wolf S, et al. The endocannabinoid anandamide protects neurons during CNS inflammation by induction of MKP-1 in microglial cells. Neuron 2006; 49(1):67-79.

-Escartin C, Guillemaud O, Carrillo-de Sauvage M. Questions and (some) answers on reactive astrocytes. Glia 2019; 67(12):2221-2247.

-Esposito G, De Filippis D, Steardo L, Scuderi C, Savani C, Cuomo V, et al. CB1 receptor selective activation inhibits β-amyloid-induced iNOS protein expression in C6 cells and subsequently blunts tau protein hyperphosphorylation in co-cultured neurons. Neurosci. Lett. 2006; 404(3):342-346.

-Facchinetti F, Del Giudice E, Furegato S, Passarotto M, Leon A. Cannabinoids ablate release of TNFα in rat microglial cells stimulated with lypopolysaccharide. Glia 2003;41(2):161-168.

-Farina C, Aloisi F, Meinl E. Astrocytes are active players in cerebral innate immunity. Trends Immunol. 2007; 28(3):138-145.

-Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, et al. Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. Mol. Pharmacol. 1995; 48(3):443-450.

-Fezza F, Bari M, Florio R, Talamonti E, Feole M, Maccarrone M. Endocannabinoids, related compounds and their metabolic routes. Molecules 2014; 19(11):17078-17106.

-Frautschy SA, Yang F, Irrizarry M, Hyman B, Saido TC, Hsiao K, et al. Microglial response to amyloid plaques in APPsw transgenic mice. Am. J. Pathol. 1998; 152(1):307-317.

-Frost GR, Li Y. The role of astrocytes in amyloid production and Alzheimer's disease. Open biology 2017; 7(12):170228.

-Fujise N, Hunziker W, Heizmann C, Kosaka T. Distribution of the calcium binding proteins, calbindin D-28K and parvalbumin, in the subicular complex of the adult mouse. Neurosci. Res. 1995; 22(1):89-107.

-Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, et al. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. European journal of biochemistry 1995; 232(1):54-61.

-Gardner B, Zu LX, Sharma S, Liu Q, Makriyannis A, Tashkin DP, et al. Autocrine and paracrine regulation of lymphocyte CB2 receptor expression by TGF-β. Biochem Biophys Res. Commun. 2002; 290(1):91-96.

-George S, Rönnbäck A, Gouras GK, Petit GH, Grueninger F, Winblad B, et al. Lesion of the subiculum reduces the spread of amyloid beta pathology to interconnected brain regions in a mouse model of Alzheimer's disease. Acta neuropathologica communications 2014; 2(1):1-8.

-Glass M, Northup JK. Agonist selective regulation of G proteins by cannabinoid CB(1) and CB(2) receptors. Mol. Pharmacol. 1999; 56(6):1362-1369.

-Glenner, G. G., and Wong, C. W. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem. Biophys. Res. Commun. 1984; 120, 885–890.

-Goldman S. Glia as neural progenitor cells. Trends Neurosci 2003; 26(11):590-596.

-Golovyashkina, N., Penazzi, L., Ballatore, C., Smith, A. B. III., Bakota, L., and Brandt, R. Region-specific dendritic simplification induced by Ab, mediated by tau via dysregulation of microtubule dynamics: a mechanistic distinct event from other neurodegenerative processes. Mol. Neurodegener. 2015; 10:60. doi: 10.1186/s13024-015-0049-0

-Gómez del Pulgar T, Velasco G, Guzmán M. The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. Biochem. J. 2000; 347(2):369-373.

-Gomez-Nicola D, Fransen NL, Suzzi S, Perry VH. Regulation of microglial proliferation during chronic neurodegeneration. J. Neurosci. 2013; 33(6):2481-2493.

-Gomez-Nicola D, Perry VH. Microglial dynamics and role in the healthy and diseased brain: a paradigm of functional plasticity. Neuroscientist 2015; 21(2):169-184.

-Gong J, Onaivi ES, Ishiguro H, Liu Q, Tagliaferro PA, Brusco A, et al. Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. Brain Res. 2006; 1071(1):10-23.

-Grabert K, Michoel T, Karavolos MH, Clohisey S, Baillie JK, Stevens MP, et al. Microglial brain region-dependent diversity and selective regional sensitivities to aging. Nat. Neurosci. 2016; 19(3):504-516.

-Griffin G, Wray EJ, Tao Q, McAllister SD, Rorrer WK, Aung M, et al. Evaluation of the cannabinoid CB2 receptor-selective antagonist, SR144528: further evidence for cannabinoid CB2 receptor absence in the rat central nervous system. Eur. J. Pharmacol. 1999; 377(1):117-125.

-Guénette SY. Astrocytes: a cellular player in Aβ clearance and degradation. Trends Mol. Med. 2003; 9(7):279-280.

-Guerrero-Alba R, Barragán-Iglesias P, González-Hernández A, Valdez-Moráles EE, Granados-Soto V, Condés-Lara M, et al. Some prospective alternatives for treating pain: the endocannabinoid system and its putative receptors GPR18 and GPR55. Frontiers in pharmacology 2019; 9:1496.

-Gutiérrez-Rodríguez A, Bonilla-Del Río I, Puente N, Gómez-Urquijo SM, Fontaine CJ, Egaña-Huguet J, et al. Localization of the cannabinoid type-1 receptor in subcellular astrocyte compartments of mutant mouse hippocampus. Glia 2018; 66(7):1417-1431.

-Guzmán M, Sánchez C, Galve-Roperh I. Control of the cell survival/death decision by cannabinoids. Journal of molecular medicine 2001; 78(11):613-625.

-Guzmán M, Sánchez C, Galve-Roperh I. Cannabinoids and cell fate. Pharmacol Ther 2002; 95(2):175-184.

-Hampson AJ, Grimaldi M, Axelrod J, Wink D. Cannabidiol and (-)Delta9-tetrahydrocannabinol are neuroprotective antioxidants. Proc Natl Acad Sci U S A 1998; 95(14):8268-8273.

-Han J, Kesner P, Metna-Laurent M, Duan T, Xu L, Georges F, et al. Acute cannabinoids impair working memory through astroglial CB1 receptor modulation of hippocampal LTD. Cell 2012; 148(5):1039-1050.

-Hansen DV, Hanson JE, Sheng M. Microglia in Alzheimer's disease. J. Cell Biol. 2018; 217(2):459-472.

-Harris E, Witter MP, Weinstein G, Stewart M. Intrinsic connectivity of the rat subiculum: I. Dendritic morphology and patterns of axonal arborization by pyramidal neurons. J. Comp. Neurol. 2001; 435(4):490-505.

-Hartlage-Rübsamen M, Zeitschel U, Apelt J, Gärtner U, Franke H, Stahl T, et al. Astrocytic expression of the Alzheimer's disease β-secretase (BACE1) is stimulus-dependent. Glia 2003; 41(2):169-179.

-Hashimotodani Y, Ohno-Shosaku T, Kano M. Endocannabinoids and synaptic function in the CNS. Neuroscientist 2007; 13(2):127-137.

-Haspula D, Clark MA. Cannabinoid receptors: An update on cell signaling, pathophysiological roles and therapeutic opportunities in neurological, cardiovascular, and inflammatory diseases. International journal of molecular sciences 2020;21(20):7693.

-Heneka MT, Rodríguez JJ, Verkhratsky A. Neuroglia in neurodegeneration. Brain Res. Rev. 2010; 63(1-2):189-211.

-Hirayama A, Okoshi Y, Hachiya Y, Ozawa Y, Ito M, Kida Y, et al. Early immunohistochemical detection of axonal damage and glial activation in extremely immature brains with periventricular leukomalacia. Clin. Neuropathol. 2001; 20(2):87-91.

-Hou L, Liu Y, Wang X, Ma H, He J, Zhang Y, et al. The effects of amyloid-β 42 oligomer on the proliferation and activation of astrocytes in vitro. In Vitro Cellular & Developmental Biology-Animal 2011; 47(8):573.

-Howlett AC. The cannabinoid receptors. Prostaglandins Other Lipid Mediat. 2002; 68:619-631.

-Hu Q, Klatt GM, Gudmundsrud R, Ottestad-Hansen S, Verbruggen L, Massie A, et al. Semi-quantitative distribution of excitatory amino acid (glutamate) transporters 1–3 (EAAT1-3) and the cystine-glutamate exchanger (xCT) in the adult murine spinal cord. Neurochem. Int. 2020; 140:104811.

-Hyman BT, Van Hoesen GW, Damasio AR, Barnes CL. Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. Science 1984; 225(4667):1168-1170.

-Insausti R, Amaral DG. Hippocampal formation. The human nervous system 2004; 2:871-914.

-Ishihara Y, Fukuda T. Immunohistochemical investigation of the internal structure of the mouse subiculum. Neuroscience 2016; 337:242-266.

-Itagaki S, McGeer P, Akiyama H, Zhu S, Selkoe D. Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. J. Neuroimmunol. 1989; 24(3):173-182.

-Ito TA, Larsen JT, Smith NK, Cacioppo JT. Negative information weighs more heavily on the brain: the negativity bias in evaluative categorizations. J. Pers. Soc. Psychol. 1998; 75(4):887.

-Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. Trends Pharmacol. Sci. 2009; 30(10):515-527.

-Jankowsky JL, Zheng H. Practical considerations for choosing a mouse model of Alzheimer's disease. Molecular neurodegeneration 2017; 12(1):1-22.

-Jean-Gilles L, Gran B, Constantinescu CS. Interaction between cytokines, cannabinoids and the nervous system. Immunobiology 2010; 215(8):606-610.

-Jimenez-Blasco D, Busquets-Garcia A, Hebert-Chatelain E, Serrat R, Vicente-Gutierrez C, Ioannidou C, et al. Glucose metabolism links astroglial mitochondria to cannabinoid effects. Nature 2020; 583(7817):603-608.

-Kamphuis W, Orre M, Kooijman L, Dahmen M, Hol EM. Differential cell proliferation in the cortex of the APPswePS1dE9 Alzheimer's disease mouse model. Glia 2012; 60(4):615-629.

-Kamphuis W, Middeldorp J, Kooijman L, Sluijs JA, Kooi E, Moeton M, et al. Glial fibrillary acidic protein isoform expression in plaque related astrogliosis in Alzheimer's disease. Neurobiol. Aging 2014; 35(3):492-510.

-Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M. Endocannabinoid-mediated control of synaptic transmission. Physiol. Rev. 2009; 89(1):309-380.

-Kaplan BL. The role of CB1 in immune modulation by cannabinoids. Pharmacol. Ther. 2013; 137(3):365-374.

-Karperien A, Ahammer H, Jelinek H. Quantitating the subtleties of microglial morphology with fractal analysis. Frontiers in cellular neuroscience 2013; 7:3.

-Kato S, Gondo T, Hoshii Y, Takahashi M, Yamada M, Ishihara T. Confocal observation of senile plaques in Alzheimer's disease: senile plaque morphology and relationship between senile plaques and astrocytes. Pathol. Int. 1998; 48(5):332-340.

-Katona I, Urban GM, Wallace M, Ledent C, Jung KM, Piomelli D, et al. Molecular composition of the endocannabinoid system at glutamatergic synapses. J. Neurosci. 2006; 26(21):5628-5637.

-Katona I, Freund TF. Multiple functions of endocannabinoid signaling in the brain. Annu Rev. Neurosci. 2012; 35:529-558.

-Kettenmann H, Hanisch U, Noda M, Verkhratsky A. Physiology of microglia. Physiol. Rev. 2011; 91(2):461-553.

-Klegeris A, Bissonnette CJ, McGeer PL. Reduction of human monocytic cell neurotoxicity and cytokine secretion by ligands of the cannabinoid-type CB2 receptor. Br. J. Pharmacol. 2003; 139(4):775-786.

-Koppensteiner, P., Trinchese, F., Fà, M., Puzzo, D., Gulisano, W., Yan, S., et al. Time-dependent reversal of synaptic plasticity induced by physiological concentrations of oligomeric Ab42: an early index of Alzheimer's disease. Sci. Rep. 2016; 6:32553.

-Laird FM, Cai H, Savonenko AV, Farah MH, He K, Melnikova T, et al. BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. J. Neurosci. 2005; 25(50):11693-11709.

-Lee SF, Newton C, Widen R, Friedman H, Klein TW. Differential expression of cannabinoid CB2 receptor mRNA in mouse immune cell subpopulations and following B cell stimulation. Eur. J. Pharmacol. 2001; 423(2-3):235-241.

-Lee SH, Foldy C, Soltesz I. Distinct endocannabinoid control of GABA release at perisomatic and dendritic synapses in the hippocampus. J. Neurosci. 2010; 30(23):7993-8000.

-Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC. Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J. Neurosci. 1995; 15(3 Pt 1):1835-1853.

-Lein, E.S. et al. Genome-wide atlas of gene expression in the adult mouse brain, Nature 2007; 445: 168-176.

-Leng F, Edison P. Neuroinflammation and microglial activation in Alzheimer disease: where do we go from here? Nature Reviews Neurology 2021; 17(3):157-172.

-Leuba G, Wernli G, Vernay A, Kraftsik R, Mohajeri MH, Saini KD. Neuronal and nonneuronal quantitative BACE immunocytochemical expression in the entorhinohippocampal and frontal regions in Alzheimer's disease. Dement. Geriatr. Cogn. Disord. 2005; 19(4):171-183.

-Li Y, Zhou Y, Danbolt NC. The rates of postmortem proteolysis of glutamate transporters differ dramatically between cells and between transporter subtypes. Journal of Histochemistry & Cytochemistry 2012; 60(11):811-821.

-Lin L, Yihao T, Zhou F, Yin N, Qiang T, Haowen Z, et al. Inflammatory regulation by driving microglial M2 polarization: neuroprotective effects of cannabinoid receptor-2 activation in intracerebral hemorrhage. *Frontiers in immunology* 2017; 8:112.

-Lindberg O, Mårtensson G, Stomrud E, Palmqvist S, Wahlund L, Westman E, et al. Atrophy of the posterior subiculum is associated with memory impairment, tau-and Aβ pathology in non-demented individuals. Frontiers in aging neuroscience 2017; 9:306.

-López A, Aparicio N, Pazos MR, Grande MT, Barreda-Manso MA, Benito-Cuesta I, et al. Cannabinoid CB 2 receptors in the mouse brain: Relevance for Alzheimer's disease. Journal of neuroinflammation 2018; 15(1):1-11.

-Lorente de Nó R. Studies on the structure of the cerebral cortex. II. Continuation of the study of the ammonic system. Journal für Psychologie und Neurologie 1934.

-Lu H, Mackie K. An introduction to the endogenous cannabinoid system. Biol. Psychiatry 2016; 79(7):516-525.

-Lu Y, Anderson HD. Cannabinoid signaling in health and disease. Can. J. Physiol. Pharmacol. 2017; 95(4):311-327.

-Mackie K, Hille B. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proc. Natl. Acad. Sci. U.S.A 1992; 89(9):3825-3829.

-Maeda J, Ji B, Irie T, Tomiyama T, Maruyama M, Okauchi T, et al. Longitudinal, quantitative assessment of amyloid, neuroinflammation, and anti-amyloid treatment in a living mouse model of Alzheimer's disease enabled by positron emission tomography. J. Neurosci. 2007; 27(41):10957-10968.

-Mandrekar-Colucci S, Landreth GE. Microglia and inflammation in Alzheimer's disease. CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders) 2010; 9(2):156-167.

-Marchalant Y, Brownjohn PW, Bonnet A, Kleffmann T, Ashton JC. Validating antibodies to the cannabinoid CB2 receptor: antibody sensitivity is not evidence of antibody specificity. Journal of Histochemistry & Cytochemistry 2014; 62(6):395-404.

-Maresz K, Carrier EJ, Ponomarev ED, Hillard CJ, Dittel BN. Modulation of the cannabinoid CB2 receptor in microglial cells in response to inflammatory stimuli. J. Neurochem. 2005; 95(2):437-445.

-Mark LP, Daniels DL, Naidich TP, Hendrix LE. Limbic connections. AJNR Am. J. Neuroradiol. 1995; 16(6):1303-1306.

-Marsicano G, Moosmann B, Hermann H, Lutz B, Behl C. Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1. J. Neurochem. 2002; 80(3):448-456.

-Marsicano G, Goodenough S, Monory K, Hermann H, Eder M, Cannich A, et al. CB1 cannabinoid receptors and on-demand defense against excitotoxicity. Science 2003; 302(5642):84-88.

-Martin E, Boucher C, Fontaine B, Delarasse C. Distinct inflammatory phenotypes of microglia and monocyte-derived macrophages in Alzheimer's disease models: effects of aging and amyloid pathology. Aging cell 2017; 16(1):27-38.

-Martín-Moreno AM, Reigada D, Ramirez BG, Mechoulam R, Innamorato N, Cuadrado A, et al. Cannabidiol and other cannabinoids reduce microglial activation in vitro and in vivo: relevance to Alzheimer's disease. Mol. Pharmacol. 2011; 79(6):964-973.

-Maruszak A, Żekanowski C. Mitochondrial dysfunction and Alzheimer's disease. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 2011; 35(2):320-330.

-Massoud F, Léger GC. Pharmacological treatment of Alzheimer disease. The Canadian Journal of Psychiatry 2011; 56(10):579-588.

-Matsumoto N, Kitanishi T, Mizuseki K. The subiculum: unique hippocampal hub and more. Neurosci. Res. 2019; 143:1-12.

-McAllister SD, Glass M. CB1 and CB2 receptor-mediated signalling: a focus on endocannabinoids. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA) 2002; 66(2-3):161-171.

-McAlpine CS, Park J, Griciuc A, Kim E, Choi SH, Iwamoto Y, et al. Astrocytic interleukin-3 programs microglia and limits Alzheimer's disease. Nature 2021; 595(7869):701-706.

-McCoy KL, Matveyeva M, Carlisle SJ, Cabral GA. Cannabinoid inhibition of the processing of intact lysozyme by macrophages: evidence for CB2 receptor participation. J. Pharmacol. Exp. Ther. 1999; 289(3):1620-1625.

-McGeer PL, McGeer EG. The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Research Reviews. 1995; 21(2):195-218.

-Mecha M, Carrillo-Salinas F, Feliú A, Mestre L, Guaza C. Microglia activation states and cannabinoid system: Therapeutic implications. Pharmacol. Ther. 2016; 166:40-55.

-Medeiros R, LaFerla FM. Astrocytes: conductors of the Alzheimer disease neuroinflammatory symphony. Exp. Neurol. 2013; 239:133-138.

-Metna-Laurent M, Marsicano G. Rising stars: Modulation of brain functions by astroglial type-1 cannabinoid receptors. Glia 2015; 63(3):353-364.

-Meyer-Luehmann M, Spires-Jones TL, Prada C, Garcia-Alloza M, De Calignon A, Rozkalne A, et al. Rapid appearance and local toxicity of amyloid-β plaques in a mouse model of Alzheimer's disease. Nature 2008; 451(7179):720-724.

-Min R, Di Marzo V, Mansvelder HD. DAG lipase involvement in depolarization-induced suppression of inhibition: does endocannabinoid biosynthesis always meet the demand? Neuroscientist 2010; 16(6):608-613.

-Molina-Holgado F, Molina-Holgado E, Guaza C, Rothwell NJ. Role of CB1 and CB2 receptors in the inhibitory effects of cannabinoids on lipopolysaccharide-induced nitric oxide release in astrocyte cultures. J. Neurosci. Res. 2002; 67(6):829-836.

-Monory K, Massa F, Egertová M, Eder M, Blaudzun H, Westenbroek R, et al. The endocannabinoid system controls key epileptogenic circuits in the hippocampus. Neuron 2006; 51(4):455-466.

-Monory K, Polack M, Remus A, Lutz B, Korte M. Cannabinoid CB1 receptor calibrates excitatory synaptic balance in the mouse hippocampus. J. Neurosci. 2015; 35(9):3842-3850.

-Moreira Paula I, Zhu Xiongwei, Liu Quan, Honda Kazuhiro, Suedlak Sandra L, Harris Peggy L et al . Compensatory responses induced by oxidative stress in Alzheimer disease. Biol. Res. [Internet]. 2006 [Retrieved Sep 07, 2021] ; 39(1): 7-13. Available from: http://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0716-97602006000100002&lng=es. http://dx.doi.org/10.4067/S0716-97602006000100002.

-Mrak RE, Griffinb WS. The role of activated astrocytes and of the neurotrophic cytokine S100B in the pathogenesis of Alzheimer's disease. Neurobiology of aging. 2001; 22(6):915-22.

-Mrak RE, Griffin WST. Glia and their cytokines in progression of neurodegeneration. Neurobiol Aging 2005; 26(3):349-354.

-Mufson EJ, Mahady L, Waters D, Counts SE, Perez SE, DeKosky S, et al. Hippocampal plasticity during the progression of Alzheimer's disease. Neuroscience 2015; 309:51-67.

-Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. Nature 1993; 365(6441):61-65.

-Nagele RG, D'Andrea MR, Lee H, Venkataraman V, Wang H. Astrocytes accumulate Aβ42 and give rise to astrocytic amyloid plaques in Alzheimer disease brains. Brain Res. 2003; 971(2):197-209.

-Nagele RG, Wegiel J, Venkataraman V, Imaki H, Wang K, Wegiel J. Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. Neurobiol. Aging 2004; 25(5):663-674.

-Nakagawa Y, Chiba K. Role of microglial m1/m2 polarization in relapse and remission of psychiatric disorders and diseases. Pharmaceuticals 2014; 7(12):1028-1048.

-Navarrete M, Araque A. Endocannabinoids mediate neuron-astrocyte communication. Neuron 2008; 57(6):883-893.

-Navarrete M, Araque A. Endocannabinoids potentiate synaptic transmission through stimulation of astrocytes. Neuron 2010; 68(1):113-126.

-Navarro G, Borroto-Escuela D, Angelats E, Etayo Í, Reyes-Resina I, Pulido-Salgado M, et al. Receptor-heteromer mediated regulation of endocannabinoid signaling in activated microglia. Role of CB1 and CB2 receptors and relevance for Alzheimer's disease and levodopa-induced dyskinesia. Brain Behav. Immun. 2018; 67:139-151.

-Nicoll JA, Weller RO. A new role for astrocytes: β-amyloid homeostasis and degradation. Trends Mol. Med. 2003; 9(7):281-282.

-Núñez E, Benito C, Pazos MR, Barbachano A, Fajardo O, González S, et al. Cannabinoid CB2 receptors are expressed by perivascular microglial cells in the human brain: an immunohistochemical study. Synapse 2004; 53(4):208-213.

-Oakley H, Cole SL, Logan S, Maus E, Shao P, Craft J, et al. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. J. Neurosci. 2006; 26(40):10129-10140.

-Oblak AL, Lin P, Kotredes KP, Pandey R, Garceau D, Williams HM, et al. Comprehensive Evaluation of the 5xFAD Mouse Model for preclinical testing applications: A MODEL-AD Study Comprehensive Evaluation of the 5xFAD Mouse Model. Frontiers in Aging Neuroscience 2021; 13:431.

-Okello A, Edison P, Archer HA, Turkheimer FE, Kennedy J, Bullock R, et al. Microglial activation and amyloid deposition in mild cognitive impairment: a PET study. Neurology 2009; 72(1):56-62.

-Okere CO, Kaba H. Heterogenous immunohistochemical expression of microglia-specific ionized calcium binding adaptor protein (Iba1) in the mouse olfactory bulb. Brain Res. 2000; 877(1):85-90.

-Olabarria M, Noristani HN, Verkhratsky A, Rodríguez JJ. Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. Glia 2010; 58(7):831-838.

-O'Mara SM, Commins S, Anderson M, Gigg J. The subiculum: a review of form, physiology and function. Prog. Neurobiol. 2001; 64(2):129-155.

-Olmos-Alonso A, Schetters ST, Sri S, Askew K, Mancuso R, Vargas-Caballero M, et al. Pharmacological targeting of CSF1R inhibits microglial proliferation and prevents the progression of Alzheimer's-like pathology. Brain 2016; 139(3):891-907.

-Onaivi ES, Ishiguro H, GONG J, Patel S, Perchuk A, Meozzi PA, et al. Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain. Ann. N.Y. Acad. Sci. 2006; 1074(1):514-536.

-Onaivi ES, Ishiguro H, Gong J, Patel S, Meozzi PA, Myers L, et al. Brain neuronal CB2 cannabinoid receptors in drug abuse and depression: from mice to human subjects. PLoS one 2008; 3(2):e1640.

-Orre M, Kamphuis W, Osborn LM, Jansen AH, Kooijman L, Bossers K, et al. Isolation of glia from Alzheimer's mice reveals inflammation and dysfunction. Neurobiol. Aging 2014; 35(12):2746-2760.

-Ortiz-Sanz C, Gaminde-Blasco A, Valero J, Bakota L, Brandt R, Zugaza JL, et al. Early effects of Aβ oligomers on dendritic spine dynamics and arborization in hippocampal neurons. Frontiers in Synaptic Neuroscience 2020; 12:2.

-Peñasco, S., Rico-Barrio, I., Puente, N., Gómez-Urquijo, S. M., Fontaine, C. J., Egaña-Huguet, J., Achicallende, S., Ramos, A., Reguero, L., Elezgarai, I., Nahirney, P. C., Christie, B. R., & Grandes, P. Endocannabinoid long-term depression revealed at medial perforant path excitatory synapses in the dentate gyrus. Neuropharmacology 2019; 153:32-40.

-Perea G, Navarrete M, Araque A. Tripartite synapses: astrocytes process and control synaptic information. Trends. Neurosci. 2009; 32(8):421-431.

-Perry VH, Holmes C. Microglial priming in neurodegenerative disease. Nature Reviews Neurology 2014; 10(4):217-224.

-Pertwee RG. Endocannabinoids and their pharmacological actions. Endocannabinoids: Springer; 2015; 1-37.

-Petrocellis LD, Cascio MG, Marzo VD. The endocannabinoid system: a general view and latest additions. Br. J. Pharmacol. 2004; 141(5):765-774.

-Piomelli D. The molecular logic of endocannabinoid signalling. Nature Reviews Neuroscience 2003; 4(11):873-884.

-Puente N, Bonilla-Del Río I, Achicallende S, Nahirney PC, Grandes P. High-resolution immunoelectron microscopy techniques for revealing distinct subcellular type 1 cannabinoid receptor domains in brain. Bio-protocol 2019; 9(2):e3145-e3145.

-Querfurth H.W., LaFerla F.M. Alzheimer's disease. N. Engl. J. Med. 2010; 362:329–344.

-Rajmohan V, Mohandas E. The limbic system. Indian J Psychiatry 2007 Apr;49(2):132-139.

-Ramirez BG, Blazquez C, Gomez del Pulgar T, Guzmán M, de Ceballos ML. Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. J. Neurosci. 2005; 25(8):1904-1913.

-Rapino C, Tortolani D, Scipioni L, Maccarrone M. Neuroprotection by (endo) cannabinoids in glaucoma and retinal neurodegenerative diseases. Current neuropharmacology 2018; 16(7):959-970.

-Renau-Piqueras J, Zaragoza R, De Paz P, Baguena-Cervellera R, Megias L, Guerri C. Effects of prolonged ethanol exposure on the glial fibrillary acidic protein-containing intermediate filaments of astrocytes in primary culture: a quantitative immunofluorescence and immunogold electron microscopic study. J. Histochem. Cytochem. 1989; 37(2):229-240.

-Research models: Alzheimer´s Disease. 5xFAD; 3xTg; htau; Tg2576 [Internet]. Alzforum; c1996–2021 FBRI LLC. [Retrieved Aug 17, 2021]. From: http://www.alzforum.org/research-models/alzheimers-disease/.

-Ribeiro R, Wen J, Li S, Zhang Y. Involvement of ERK1/2, cPLA2 and NF-κB in microglia suppression by cannabinoid receptor agonists and antagonists. Prostaglandins Other Lipid Mediat. 2013; 100:1-14.

-Rodriguez J, Olabarria M, Chvatal A, Verkhratsky A. Astroglia in dementia and Alzheimer's disease. Cell Death & Differentiation 2009; 16(3):378-385.

-Rönnbäck A, Sagelius H, Bergstedt KD, Näslund J, Westermark GT, Winblad B, et al. Amyloid neuropathology in the single Arctic APP transgenic model affects interconnected brain regions. Neurobiol. Aging 2012; 33(4):831. e11-831. e19.

-Rossner S, Lange-Dohna C, Zeitschel U, Perez-Polo JR. Alzheimer's disease β-secretase - BACE1 is not a neuron-specific enzyme. J. Neurochem. 2005; 92(2):226-234.

-Rozenfeld R, Devi LA. Regulation of CB1 cannabinoid receptor trafficking by the adaptor protein AP-3. The FASEB Journal 2008; 22(7):2311-2322.

-Salvi V, Sozio F, Sozzani S, Del Prete A. Role of atypical chemokine receptors in microglial activation and polarization. Frontiers in aging neuroscience 2017; 9:148.

-Sánchez MG, Ruiz-Llorente L, Sánchez AM, Díaz-Laviada I. Activation of phosphoinositide 3-kinase/PKB pathway by CB1 and CB2 cannabinoid receptors expressed

in prostate PC-3 cells. Involvement in Raf-1 stimulation and NGF induction. Cell Signal 2003; 15(9):851-859.

-Sarlus H, Heneka MT. Microglia in Alzheimer's disease. J. Clin. Invest. 2017; 127(9):3240-3249.

-Savchenko VL, McKanna JA, Nikonenko IR, Skibo GG. Microglia and astrocytes in the adult rat brain: comparative immunocytochemical analysis demonstrates the efficacy of lipocortin 1 immunoreactivity. Neuroscience. 2000; 96(1):195-203.

-Schatz AR, Lee M, Condie RB, Pulaski JT, Kaminski NE. Cannabinoid receptors CB1 and CB2: a characterization of expression and adenylate cyclase modulation within the immune system. Toxicol. Appl. Pharmacol. 1997; 142(2):278-287.

-Schmitt A, Asan E, Puschel B, Kugler P. Cellular and regional distribution of the glutamate transporter GLAST in the CNS of rats: nonradioactive in situ hybridization and comparative immunocytochemistry. J. Neurosci. 1997; 17(1):1-10.

-Scuderi C, Facchinetti R, Steardo L, Valenza M. Neuroinflammation in Alzheimer's Disease: Friend or Foe? The FASEB Journal 2020; 34(S1):1-1.

-Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 2001.

-Selkoe DJ, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO molecular medicine 2016; 8(6):595-608.

-Shaffer LM, Dority MD, Gupta-Bansal R, Frederickson RC, Younkin SG, Brunden KR. Amyloid β protein (Aβ) removal by neuroglial cells in culture. Neurobiol. Aging 1995; 16(5):737-745.

-Shankar, G. M., Bloodgood, B. L., Townsend, M., Walsh, D. M., Selkoe, D. J., and Sabatini, B. L. Natural oligomers of the Alzheimer amyloid- b protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J. Neurosci. 2007; 27, 2866–2875.

-Shapiro LA, Wang L, Ribak CE. Rapid astrocyte and microglial activation following pilocarpine-induced seizures in rats. Epilepsia 2008; 49:33-41.

-Shen Z, Li X, Bao X, Wang R. Microglia-targeted stem cell therapies for Alzheimer disease: A preclinical data review. J. Neurosci. Res. 2017; 95(12):2420-2429.

-Sheng WS, Hu S, Min X, Cabral GA, Lokensgard JR, Peterson PK. Synthetic cannabinoid WIN55, 212-2 inhibits generation of inflammatory mediators by IL-1β-stimulated human astrocytes. Glia 2005; 49(2):211-219.

-Simchowicz T. Histologische und histopathologische Arbeiten über die Grosshirnrinde mit besonderer Berücksichtigung der pathologischen Anatomie der Geisteskrankheiten

Histologische Studien über die senile Demenz. Gustav Fischer Verlag, Jena, Germany 1911; 267-444.

-Sinha D, Bonner TI, Bhat NR, Matsuda LA. Expression of the CB1 cannabinoid receptor in macrophage-like cells from brain tissue: immunochemical characterization by fusion protein antibodies. J. Neuroimmunol. 1998; 82(1):13-21.

-Sinor AD, Irvin SM, Greenberg DA. Endocannabinoids protect cerebral cortical neurons from in vitro ischemia in rats. Neurosci. Lett. 2000; 278(3):157-160.

-Smit T, Deshayes NA, Borchelt DR, Kamphuis W, Middeldorp J, Hol EM. Reactive astrocytes as treatment targets in Alzheimer's disease—Systematic review of studies using the APPswePS1dE9 mouse model. Glia 2021; 69(8):1852-1881.

-Sokolowski K, Corbin JG. Wired for behaviors: from development to function of innate limbic system circuitry. Frontiers in molecular neuroscience 2012; 5:55.

-Song H, Stevens CF, Gage FH. Astroglia induce neurogenesis from adult neural stem cells. Nature 2002; 417(6884):39-44.

-Soto-Mercado V, Mendivil-Perez M, Jimenez-Del-Rio M, Velez-Pardo C. Multi-Target Effects of the Cannabinoid CP55940 on Familial Alzheimer's Disease PSEN1 E280A Cholinergic-Like Neurons: Role of CB 1 Receptor. J Alzheimer's Dis. 2020(Preprint); 1-20.

-Spires, T. L., Meyer-Luehmann, M., Stern, E. A., McLean, P. J., Skoch, J., Nguyen, P. T., et al. Dendritic spine abnormalities in amyloid precursor protein transgenic mice demonstrated by gene transfer and intravital multiphoton microscopy. J. Neurosci. 2005; 25, 7278–7287.

-Stefano GB, Liu Y, Goligorsky MS. Cannabinoid receptors are coupled to nitric oxide release in invertebrate immunocytes, microglia, and human monocytes. J. Biol. Chem. 1996; 271(32):19238-19242.

-Stella N. Endocannabinoid signaling in microglial cells. Neuropharmacology 2009; 56:244-253.

-Stella N. Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. Glia 2010; 58(9):1017-1030.

-Straiker A, Mackie K. Cannabinoids, electrophysiology, and retrograde messengers: challenges for the next 5 years. The AAPS journal 2006; 8(2):E272-E276.

-Suárez I, Bodega G, Ramos JA, Fernandez-Ruiz JJ, Fernandez B. Neuronal and astroglial response to pre- and perinatal exposure to delta-9-tetra- hydrocannabinol in the rat substantia nigra. Dev. Neurosci. 2000; 22(4):253-263.

-Sugiura T, Kondo S, Kishimoto S, Miyashita T, Nakane S, Kodaka T, et al. Evidence that 2-arachidonoylglycerol but not N-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. J. Biol. Chem. 2000; 275(1):605-612.

-Tahir SK, Trogadis JE, Stevens JK, Zimmerman AM. Cytoskeletal organization following cannabinoid treatment in undifferentiated and differentiated PC12 cells. Biochemistry and Cell Biology 1992; 70(10-11):1159-1173.

-Talarico G, Trebbastoni A, Bruno G, de Lena C. Modulation of the cannabinoid system: a new perspective for the treatment of the Alzheimer's disease. Current neuropharmacology 2019; 17(2):176-183.

-Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, et al. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society 1991; 30(4):572-580.

-Thal DR, Schultz C, Dehghani F, Yamaguchi H, Braak H, Braak E. Amyloid β-protein (Aβ)-containing astrocytes are located preferentially near N-terminal-truncated Aβ deposits in the human entorhinal cortex. Acta Neuropathol. 2000; 100(6):608-617.

-Thompson PM, Hayashi KM, de Zubicaray G, Janke AL, Rose SE, Semple J, et al. Dynamics of gray matter loss in Alzheimer's disease. J. Neurosci. 2003; 23(3):994-1005.

-Thompson PM, Hayashi KM, Dutton RA, Chiang MC, Leow AD, Sowell ER, et al. Tracking Alzheimer's disease. Ann. N.Y. Acad. Sci. 2007; 1097:183-214.

-Twitchell W, Brown S, Mackie K. Cannabinoids inhibit N-and P/Q-type calcium channels in cultured rat hippocampal neurons. J. Neurophysiol. 1997; 78(1):43-50.

-Ueda N, Tsuboi K, Uyama T, Ohnishi T. Biosynthesis and degradation of the endocannabinoid 2-arachidonoylglycerol. Biofactors 2011; 37(1):1-7.

-Unger JW. Glial reaction in aging and Alzheimer's disease. Microsc. Res. Tech. 1998; 43(1):24-28.

-Van Groen T, Wyss JM. Extrinsic projections from area CA1 of the rat hippocampus: olfactory, cortical, subcortical, and bilateral hippocampal formation projections. J. Comp. Neurol. 1990; 302(3):515-528.

-Van Hoesen GW, Hyman BT. Hippocampal formation: anatomy and the patterns of pathology in Alzheimer's disease. Prog. Brain. Res. 1990; 83:445-457.

-Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, et al. Identification and functional characterization of brainstem cannabinoid CB2 receptors. Science 2005; 310(5746):329-332.

-Verkhratsky A, Olabarria M, Noristani HN, Yeh C, Rodriguez JJ. Astrocytes in Alzheimer's disease. Neurotherapeutics 2010; 7(4):399-412.

-Verkhratsky A, Nedergaard M. Physiology of astroglia. Physiol. Rev. 2018; 98(1):239-389.

-Verkhratsky A, Parpura V, Rodriguez-Arellano JJ, Zorec R. Astroglia in Alzheimer's disease. Neuroglia in Neurodegenerative Diseases: Springer; 2019; p. 273-324.

-Waksman Y, Olson JM, Carlisle SJ, Cabral GA. The central cannabinoid receptor (CB1) mediates inhibition of nitric oxide production by rat microglial cells. J. Pharmacol. Exp. Ther. 1999; 288(3):1357-1366.

-Walter L, Dinh T, Stella N. ATP induces a rapid and pronounced increase in 2-arachidonoylglycerol production by astrocytes, a response limited by monoacylglycerol lipase. J. Neurosci. 2004; 24(37):8068-8074.

-Wilson RI, Nicoll RA. Endocannabinoid signaling in the brain. Science 2002; 296(5568):678-682.

-Wyss-Coray T, Mucke L. Inflammation in neurodegenerative disease—a double-edged sword. Neuron 2002; 35(3):419-432.

-Wyss-Coray T, Loike JD, Brionne TC, Lu E, Anankov R, Yan F, et al. Adult mouse astrocytes degrade amyloid-β in vitro and in situ. Nat. Med. 2003; 9(4):453-457.

-Yiangou Y, Facer P, Durrenberger P, Chessell IP, Naylor A, Bountra C, et al. COX-2, CB2 and P2X7-immunoreactivities are increased in activated microglial cells/macrophages of multiple sclerosis and amyotrophic lateral sclerosis spinal cord. BMC neurology 2006; 6(1):1-14.

-Yin Z, Raj D, Saiepour N, Van Dam D, Brouwer N, Holtman IR, et al. Immune hyperreactivity of Aβ plaque-associated microglia in Alzheimer's disease. Neurobiol. Aging 2017; 55:115-122.

-Zhang J, Hoffert C, Vu HK, Groblewski T, Ahmad S, O'Donnell D. Induction of CB2 receptor expression in the rat spinal cord of neuropathic but not inflammatory chronic pain models. Eur. J. Neurosci. 2003; 17(12):2750-2754.

-Zhang HY, Gao M, Liu QR, Bi GH, Li X, Yang HJ, et al. Cannabinoid CB2 receptors modulate midbrain dopamine neuronal activity and dopamine-related behavior in mice. Proc. Natl. Acad. Sci. U.S.A. 2014; 111(46):E5007-15.

-Zhang M, Zhong L, Han X, Xiong G, Xu D, Zhang S, et al. Brain and Retinal Abnormalities in the 5xFAD Mouse Model of Alzheimer's Disease at Early Stages. Frontiers in Neuroscience 2021; 858.

-Zhao J, O'Connor T, Vassar R. The contribution of activated astrocytes to Aβ production: implications for Alzheimer's disease pathogenesis. Journal of neuroinflammation 2011; 8(1):150.

-Zou S, Kumar U. Cannabinoid receptors and the endocannabinoid system: signaling and function in the central nervous system. International journal of molecular sciences 2018; 19(3):833.

CB$_1$ eta CB$_2$ hartzaile kannabinoideen kokapen gliala Alzheimer gaixotasunaren sagu-eredu batean

DOKTOREGO TESIA

Itziar Terradillos Irastorza

ITZIAR TERRADILLOS IRASTORZA-k

aurkeztutako doktorego tesia

Zuzendariak:

PEDRO ROLANDO GRANDES MORENO irak. dok.

IZASKUN ELEZGARAI GABANTXO irak. dok.

Neurozientzietako Saila

Medikuntza eta Erizaintza Fakultatea

Euskal Herriko Unibertsitatea (UPV/EHU) Leioa, 2021

1. LABURPENA

Alzheimer gaixotasuna (*Alzheimer´s disease*; AD) adinekoen artean eman ohi den endekapenezko gaixotasun ohikoena da, baita dementzia motarik sarriena ere, kognizio eta oroimen asaldurak sortzen dituena. Oraingoz ez da lortu gaixotasun horren patogenesia argitzea, nahiz eta aldaketa patologiko asko deskribatu diren, hala nola neuroinflamazioa eta oxidazio-estresa. ADren síntoma klinikoak agertu aurretik, nerbio-sistema zentralean (*central nervous system*; CNS), zelula mailan gertatu ohi den aldaketarik esanguratsuena neuronen heriotza da. Hala ere, gainerako zelula-motetan ere aldaketa garrantzitsuak aurkitu izan dira. Oraingoz ez da lortu gaixotasun horren patogenesia argitzea.

Azken hamarkada hauetan lortu diren aurrerapen biokimiko eta farmakologikoen artean garrantzitsuenetariko bat giza-hartzaile kanabinoideen identifikazioa, hauek osasun eta gaixotasun egoeran duten eginkizunarekin batera, izan da. Laborategi ugari saiatu izan dira hartzaile hauen erabilgarritasuna argitzen AD kasuetan, ekintza neurobabesleak dituztelako eta kannabinoideek mikrogliaren aktibazioan eta Aβ-ren neurotoxikotasuna saihesteko jokatu ohi duten funtzioa dela eta. Ondorioz, bereziki interesgarria da Sistema Endokanabinoidearen (*endocannabinoid system*; SE) azterketa egikaritzea ADren garapenean zehar. Endokanabinoide tonuan gertatzen diren alterazioek hartzaile kanabinoide nagusien (CB$_1$R eta CB$_2$R) adierazpen edo/eta funtzio aldaketekin erlazioa gordetzen dute sarritan. Hala eta guztiz ere, CB$_1$R-ak ADean duen adierazpen aldaketei dagokienez, emaitza kontraesankorrak lortu izan dira, eta CB$_2$R-en ikerketa oraindik eta konplexuagoa suertatzen da hartzaile honen kontrako antigorputz espezifikoen gabezia dela eta. Astrozitoek mikrogliarekin modu estuan elkar-eragiten dute, eta zelula mota biek ADean gertatu ohi den gliosi erreaktiboan parte hartzen dute. Horrela, Aβ duten agregatuek hanturazko erantzun kronikoa eragiten dute, ondorioz mikroglia eta astrozitoen aktibazioa ekarriko duena, azken biek plaka amiloideak inguratu eta haiek inbadituko dituztelarik. Ondorio bezala sortzen den gliosi erreaktibo horrek ezohiko morfología bat sortuko du CNSean, astrozito eta mikrogliaren ugaritzea azalduko duena. Aldaketa histopatologiko hauek onuragarriak diruditen arren, zelula glialen eragin kaltegarriak edo hutsalak izan litezkeenak ez dira argitu. Horrez gain, gaur egun hartzaile kanabinoideek zelula glialengan duten kokapen eta adierazpenaren inguruan datu gutxi dauzkagu.

Doktorego tesi honetan aurkezten dugun hipotesia hurrengoa da: gliako CB$_1$Rren eta CB$_2$Rren adierazpen eraldatua topatuko dugu ADren sagu eredu baten subikuluan, astrozito eta mikrogliarengan gertatu ohi diren aldaketa subzelularren ondorioz, eta eremu hori plaken pilaketa handia aurkezten duen garun-eremua izanik.

Jada plakak eta kalte neuronala azaltzen zuten 6.5-7.5 hilabetetako adina zuten CB2$^{EGFP/f/f}$/5xFAD saguen subikuluko mikroglia eta astrozitoak ikertu izan ditut, kontrol-taldeko CB2$^{EGFP/f/f}$ saguekin batera (López et al., 2018). Sagu hauek ere EGFP adierazten dute CB$_2$R genaren promotore berdinaren pean. CB2$^{EGFP/f/f}$/5xFAD eta CB2$^{EGFP/f/f}$ saguak, Julian Romero doktoreak (Zientzia Esperimentalen Fakultatea, Francisco de Vitoria Unibertsitatea, Pozuelo de Alarcón) eskaini zizkigun, gure lankidetza proiektuaren alorrean. Erretxinan murgildu aurreko immuno-urre eta immuno-peroxidasaren mikroskopia elektronikorako metodo bikoitza erabiliz, astrozitoetako eta mikrogliako CB$_1$R adierazpena eta mikrogliako CB$_2$R adierazpena kuantifikatu ditut.

CB$_1$R-en kasuan, CB$_1$R-positibo ziren mikroglia-prozesuen kopurua eta mikroglia-prozesu guztiekiko suposatzen zuten portzentaiaren handitzea azaldu ziren. Horrekin batera, CB2$^{EGFP/f/f}$/5xFAD saguengan agertzen ziren mikroglia urre partikulak normalizatutako azalerarekiko ere ugaritu ziren. Mutante hauek ere CB$_1$R-en dentsitatean gutxitze bat erakutsi zuten, nahiz eta hori mikroglia-prozesuen perimetroaren hazkundearekin loturik zegoen. Astrozitoen kasuan, aldaketarik ez zen hauteman: Prozesu CB$_1$R-positibo kopuruan edo/eta portzentaian, astrozitoetako CB$_1$R inmunopartikula kopuruan, ezta astrozitoetako CB$_1$R-prozesuen dentsitatean ere. Dena dela, CB$_1$Rren partikulen kopuruan prozesu astrozitiko bakoitzeko hazkunde bat nabaritu zen. Horrekin guztiak, CB$_1$R adierazpenak CB2$^{EGFP/f/f}$/5xFAD saguen subikuluko mikroglia erreaktiboan gora egiten duela adierazten du. Astrozitoetan, aldiz, konstante mantentzen da, CB$_1$R partikulen ugaritzea astrozitoen prozesu erreaktiboen perimetroaren hazkundearekiko proportzionala delarik.

CB$_2$R-en kasuan, hurrengo ezaugarrietan hazkunde bat nabaritu zen: GFP-positibo ziren prozesuen kopuru eta portzentaian, mikrogliako prozesu bakoitzeko GFP partikulen kopuruan prozesuaren azalerarekiko eta GFP partikula mikroglialen kopuruan azalera normalizatu bakoitzeko. Ondorioz, CB$_2$R-ren adierazpen areagotua ikusi zen CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan.

Gliaren erreaktibitatearen eta ADean gertatzen den mikroglia eta astrozitoetako CB$_1$Rren eta CB$_2$Rren adierazpenaren arteko erlazioa jakiteak gaixotasun horren fisiopatologian sistema endokanabinoideak izan dezakeen eginkizuna ulertzen lagunduko liguke.

-**Aβ:** Beta-amiloide

-**ABC:** Abidina-biotina-peroxidasa konplexua

-**AC:** Adenilato-ziklasa

-**AD:** Alzheimer gaixotasuna

-**AEA:** Arachidonoil-etanolamina edo anandamida

-**AICD:** APP-aren zelula barruko domeinua

-**APP**: Amiloidearen proteina prekurtsorea

-**BACE1:** β-sekretasa edo β-lekuko amiloidearen proteina aintzindaria

-**BBB:** Hesi hematoentzefalikoa

-**BDNF:** Garunetik eratorritako faktore neurotrofikoa

-**BSA:** Behi-serumaren albumina

-**CA:** Cornu Ammonis

-**CA1:** Cornu Ammonis 1

-**CB₁⁻ᐟ⁻:** CB_1R-knockout

-**CB₂⁻ᐟ⁻:** CB_2R-knockout

-**CB₁R:** 1 motako hartzailea kannabinoidea

-**CB₂R:** 2 motako hartzaile kannabinoidea

-**CNS:** Nerbio sistema zentrala

-**DAB:** Diaminobentzidina

-**DAG:** Diazilglizerola

-**DAGL:** Diazilglizerol lipasa

-**DAMPs:** Arriskuei asoziaturiko patroi molekularrak

-**DG:** Hortz bihurgunea

-**EC:** Kortex entorrinala

-**ECBs:** Endokannabinoideak

-**EGFP:** Indartutako proteina fluoreszente berdea

-**ERK:** Zelula-kanpoko seinaleen erregulazio kinasa

-**ES**: Sistema endokannabinoidea

-**EtOH:** Etanola

-**FAAH:** Gantz-azidoen amida hidrolasa

-**FAD:** Familiako Alzheimer gaixotasuna

-**GFAP:** Zuntz proteina azido gliala

-**GFP:** Proteina fluoreszente berdea

-**GLAST:** 1 motako glutamato aspartatoaren garraiatzailea

-**GPCRs:** G proteinei akoplatutako hartzaile metabotropikoak

-**GPR18:** G proteinei akoplatutako 18 hartzaile metabotropikoa

-**GPR55:** G proteinei akoplatutako 55 hartzaile metabotropikoa

-**hAPP:** Giza amiloidearen proteina aitzindaria

-**HIV:** Giza immunoeskasiaren birusa

-**H$_2$O$_2$:** Hidrogeno peroxidoa

-**Iba1:** 1 motako kaltzio ionizatua finkatzeko molekula egokitzailea

-**IL-1β:** Interleuzina-1 beta

-**IL-4:** Interleuzina-4

-**IL-10**: Interleuzina-10

-**iNOS:** Oxido nitriko sintasa induzigarria

-**IP3:** Inositol trifosfato

-**IRES:** Txertatutako barneko erribosoma-sarreraren sekuentzia

-**LPS:** Lipopolisakarido

-**LRP1:** Lipoproteina hartzaileari lotutako proteina1

-**LS:** Sistema linbikoa

-**MAPK:** Mitogenoek aktibatutako proteina kinasa

-**MHCII:** II. motako histokonpatibilitate-konplexu nagusia

-**NAPE:** N-azil-fosfatidiletanolamina

-**NAPE-PLD:** NAPErentzako selektiboa den D fosfolipasa

-**NArPE:** N-aracadonoil-fosfatidiletanolamina

-**NFT**: Haril neurofibrilarrak

-**NGF:** Nerbio-hazkundearen faktorea

-**NO:** Oxido nitrikoa

-**NT:** Neurotransmisorea

-**NT-3:** Neurotrofina 3

-**NT-4/5:** Neurotrofina 4/5

-**ObR:** Leptina hartzailea

--**OH:** Hidroxiloa

-**O^{2-}:** Superoxidoa

-**PAMPs:** Patogenoei asoziaturiko patroi molekularrak

-**PB:** Fosfato disoluzio tanpoia

-**PBS:** Gatz fosfatozko disoluzio indargetzailea

-**PE:** Fosfatidiletanolamina

-**PI:** Fosfatidilinositol

-**PI3K:** Fosfatidilinositol 3-kinasa

-**PKA:** A kinasa proteina

-**PKB:** B kinasa proteina

-**PLC:** C fosfolipasa

-**PLD:** D fosfolipasa

-**PPAR-α:** Peroxisomen proliferazio bidez aktibaturiko hartzailea α

-**PPAR- γ:** Peroxisomen proliferazio bidez aktibaturiko hartzaileak γ

-**PreS:** Presubikulua

-**ROS:** Oxigeno espezie erreaktiboak

-**RT:** Giro tenperatura

-**RT-PCR:** Alderantzizko transkripzioa duen polimerasaren kate-erreakzioa

-**SPs:** Xafla senilak

-**SUB:** Subikulua

-**TBS 1X:** Trisekin tanponatutako gatz-disoluzioa

-**TGF-β:** Hazkunde faktore eraldatzailea beta

-**THC:** Delta-9-tetrahidrokannabinol

-**TNF-α:** Tumore-nekrosiaren faktorea-α

-**TRPV1:** Potentzial aldakorreko V1 kanale hartzailea

-**UTR**: Itzuli gabeko eremua

-**VSCCs:** Boltai menpeko Ca^{2+} kanalak

-5xFAD: Familiako Alzheimer gaixotasunaren bost mutazio

-2-AG: 2-arakidonilglizerola

-τ: Tau

2. SARRERA

Alzheimerren gaixotasuna (*Alzheimer Disease*; AD) nahasmendu neurodegeneratibo sarriena da adineko pertsonen artean, eta dementziaren kausa ohikoena (Glenner eta Wong, 1984; Verkhratsky et al., 2019). Auguste Deter izan zen 1906an Alois Alzheimer doktoreak ADez diagnostikatu zuen lehen gaixoa. Zahartzaro aurreko dementzia progresiboa zuen eta denborarekin oroimena galdu zuen; portaeraren beste ondorio batzuk ere izan zituen, 55 urte zituela hil zelarik. Garunaren autopsian zilar-tindaketaren bidez xafla senilak (SPs) eta haril neurofibrilarrak (*Nerurofibrillary tangles*; NFT) zeudela ikus zitekeen, baita garunaren bolumen-txikiagotze garrantzitsu bat ere (berrikuspenerako Mufson et al., 2015).

ADren ezaugarri nagusiak oroimenaren eta gaitasun kognitiboen narriadura, dementzia, eguneroko bizitzako jardueren disfuntzioa eta portaeraren alterazioak dira. Gaixotasun honen patogenesia oraindik ez dago argi; izan ere, aldaketa patologiko ugari antzeman dira AD duten pazienteen post mortem garunetan (Medeiros eta LaFerla, 2013). Beta-amiloide (*beta-amyloid*; Aβ) peptidoen agregazioak eta deposizioak, SPs-ren eraketarekin lotuta, eta tau (*Tau*; τ) proteinaren hiperfosforilazioak, NFTen sorrerarekin lotuta, gaixotasunaren garapenean eginkizun garrantzitsua dutela proposatu da. Hala ere, badakigu beste mekanismo batzuk daudela ADren patogenesian inplikatuta. Horien artean daude sinapsien disfuntzioa, inflamazioa, oxidazio-estresa, mitokondrien metabolismoaren alterazioak edo neurona kolinergikoen galera (berrikuspenerako Moreira et al., 2006; berrikuspenerako Querfurth eta LaFerla, 2010; berrikuspenerako Bloom, 2014; Bobkova eta Vorobyov, 2015). Beraz, AD faktore anitzeko gaixotasun esporadiko gisa aurkezten da gehienetan (berrikuspenerako Frost eta Li, 2017).

SPs-ak zelulaz kanpoko metaketak dira, neurita distrofiko eta endekatuez, mikrogliaz eta astrozito erreaktiboz osatuak daudenak, baina, batez ere, Aβ peptidoz, hau da, amiloidearen proteina aintzindaren *(Amyloid precursor protein*; APP) katabolismoaz osatutakoa, eta zuntzexkak eta metaketak osatzen dituena. SPs-ren hiru mota nagusi bereizten dira. Alde batetik, plaka lausoak, depositu amiloide ez-zuntzkarak dituztenak, neuropila aldatzen ez dutenak eta erantzun glialik eragiten ez dutenak, eta, beraz, narriadura kognitiborik eragiten ez dutenak. Beste alde batetik, erdigune trinko bat duten plaka amiloideak dituztenak (De la Vega eta Zambrano, 2013). Azkenik, plaka trinko edo neuritikoak dituztenak, SPs ere deitzen direnak, nukleo trinko batekin eta APPrekiko immunopositiboak diren neurita distrofikoen presentziarekin (APP[+]), ADrekiko espezifikoak eta izaera toxikodunak direnak, astrozito eta mikroglia aktibatua dituztelarik (De la Vega eta Zambrano, 2013; berrikuspenerako Selkoe eta Hardy, 2016).

APP proteolisiaren bi prozesu deskribatu dira: ez-amiloidogenikoa eta amiloidogenikoa (1. irudia). Ez-amiloidogenikoa den bidean, α-sekretasa entzimak APP zatitzen du, zati disolbagarri bat (sAPPα) eta beste C83 zati bat askatuz. Zati horren gainean ere egiten du lan γ-sekretasak, eta p3 eta APParen zelula barruko domeinua (*APP intracellular domain*; AICD) sortzen ditu. AICD zitoplasman askatu eta nukleora zuzentzen da, zenbait generen transkripzioa eginez (berrikuspenerako Querfurth eta LaFerla, 2010; berrikuspenerako Coronel et al., 2019). SAPPα peptido disolbagarri bat da, ondoren organismoak erraz ezabatzen duena. Bide amiloidogenikoan, β-sekretasak edo β-lekuko amiloidearen proteina aintzindariak (*β-site amyloid precursor protein 1*; BACE1) zati disolbagarri bat (sAPPβ) eta C99 karboxi-terminal zatia askatzen ditu. C99 zati hori, zelularen mintz-barnean dagoen γ-secretasak zatitzen du, AICD eta Aβ peptidoak (Aβ 1-40 eta Aβ 1-42) osatuz (De la Vega eta Zambrano, 2013; Coronel et al., 2019).

Gainera, Aβ espezieak γ-secretasaren jardudueraren gaineko atzera-eragile negatibo gisajarduten du. ADean, APPren β-sekretasa edo BACE1ren bidezko eta γ-sekretasaren ondoz ondoko zatiketa gailentzen da, Aβ peptido disolbaezina (Aβ 1-42) eratuz, ondoren neuronek iraizten dutena (berrikuspenerako Querfurth eta LaFerla, 2010; De la Vega eta Zambrano, 2013; berrikuspenerako Frost eta Li, 2017). Jarraian, glia zelulak (astrozitoak eta mikroglia), arrakastarik gabe, Aβ 1-42 ezabatzen saiatzen dira, hantura prozesu bat eraginez, eta horrek, Aβ 1-42-aren beraren efektu toxikoarekin batera, kalte neuronala eragiten du.
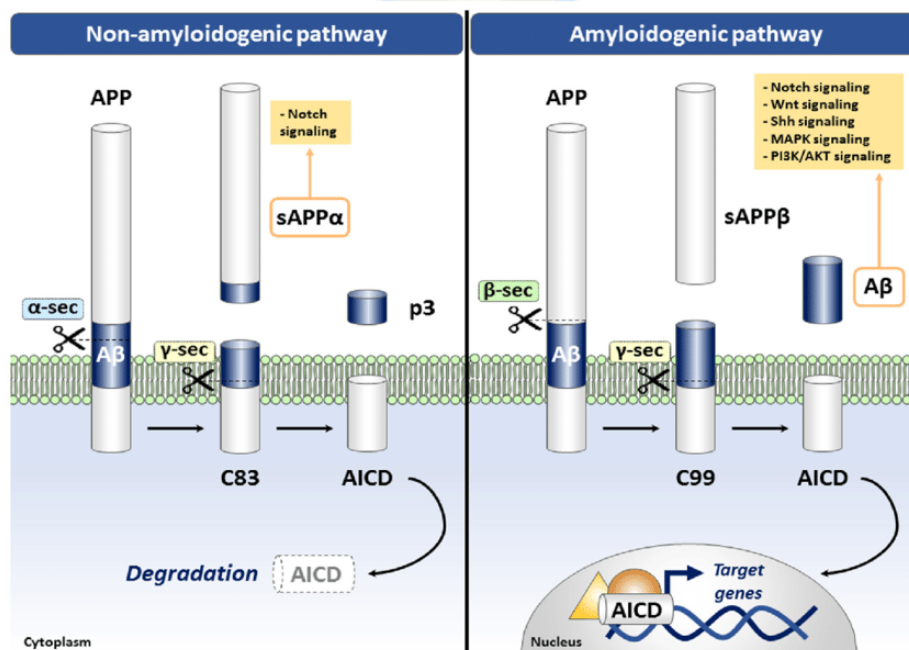
1. irudia. APPren proteolisi-prozesuak. APP proteolisiaren bi prozesu deskribatu dira, bata amiloidogenikoa eta bestea ez-amiloidogenikoa (De Coronel et al., 2019).

NFTei dagokienez, endekapen neurofibrilarra osatzen dute eta τ proteinaren zelula barneko gordailuen ondorioz sortzen dira. τ arauzko proteinak zitoeskeleto neuronala osatzen duten mikrotubuluak behar bezala lotuta mantentzen dituzten "zubiak" eratzen ditu, baina ADean (neurri batean Aβ 1-42-aren eragin toxikoaren ondorioz) proteinaren hiperfosforilazio anormal bat gertatzen da. Proteinen hiperfosforilazioak ubikuitinari lotutako zuntz helikoidal parekatuak eratzen ditu, eta horrek mikrotubuluak eta neurofilamentuak suntsitzea eragiten du, zitoeskeletoa hautsiz eta degenerazio neurofibrilarra eraginez (De la Vega eta Zambrano, 2013; berrikuspenerako Selkoe eta Hardy, 2016).

Bi lesio mota horiek (SPsak eta NFTak) adineko pertsona osasuntsuen garunean ere aurki daitezke. Diagnostiko histopatologikoa benetan zehazten duen kopurua eta kokapena dira, eta kopurua eta dentsitatea paziente horien dementziaren intentsitatearekin zuzenean erlazionaturik daude (De la Vega eta Zambrano, 2013).

Hasiera goiztiarreko ADean, giza amiloidearen proteina aitzindariaren (*Human amyloid precursor protein*; hAPP) prozesamendu proteolitiko anormal bat gertatzen da, hAPPa edo 1 edo 2 presenilinak (γ-sekretasaren unitate katalitikoa osatzen duten mintz-zeharreko proteinak) kodetzen dituzten geneen mutazioen ondorioz. Horrek, hAPPtik abiatutako Aβren sintesi eta zelulaz-kanpoko metaketa areagotu egiten ditu (berrikuspenerako Wyss-Coray eta Mucke, 2002; berrikuspenerako Selkoe, 2001). ADaren sagu-ereduetan *in vivo* egindako azterketek agerian uzti dute Aβ metaketek eragin toxiko zuzena dutela neuronetan, eta horrek sinplifikazio dendritikoa eragiten du, arantza dendritikoen galera eta distrofia neuritikoa eraginez, baita inflamazioa eta heriotza zelularra ere (Spires et al., 2005; Meyer-Luehmann et al., 2008; berrikuspenerako Cline et al., 2018). Horrela, Aβ oligomerikoarekiko esposizioak sinapsi-disfuntzioa eragiten du AD goiztiarrean, arantza dendritikoen dentsitatea murriztuz (Shankar et al., 2007), baita morfologian aldaketak eraginez ere (Shankar et al., 2007; Ortiz-Sanz et al., 2020). Fenomeno horiek sare sinaptikoetako aldaketekin lotuta daude (Shankar et al., 2007), eta horrek, aldi berean, eragina dauka AD duten pazienteen narriadura kognitiboaren mailan (Terry et al., 1991). ADeko patologia goiztiarrean dementzia agertzen denean, garun-funtzioa eta kognizioa mantentzeko ahaleginean, neuronen antolamenduak birmoldaketa ahalbidetzen du, konpentsazio-mekanismo gisa. Hau da, hipokanpoak, erdialdeko loki-lobuluan kokatuta dagoen eta memoria-zirkuituan funtsezkoa den egitura, eta ADren lehen urratsetan kaltetuta dagoenak, zelulaz-kanpoko Aβ metaketa patologikoaren eta zelula-barneko NFT gordailuen ondorioz, birmoldaketa sinaptiko eta intraneuronaleko erantzun-adaptatibo bat jartzen du martxan (berrikuspenerako Mufson et al., 2015). Aldaketa

horiek heriotza neuronala eta SPs-ren eraketa baino lehen gertatzen dira ADeko lehen urratsetan (Terry et al., 1991). Beraz, Aβ-ak efektu bikoitzak susta ditzake, dendriten konplexutasuna eta arantzen dentsitatea modu akutuan handitzen baititu (Ortiz-Sanz et al., 2020). Horrela, morfologian eta dentsitate dendritikoan gertatzen diren aldaketak ADren ezaugarri patologiko goiztiarrak dira (berrikuspenerako Bakota eta Brandt, 2016), eta aurkikuntza berriek iradokitzen dute arantza dendritikoen plastikotasunak dementziaren aurkako erresilientzia kognitiboa eragin dezakeela gaixotasunaren etapa horietan (Boros et al., 2017). Modu horretan, gaixotasunaren progresioan aldi "latentea" edo ezkutua deskribatzen da, burmuinean konpentsazio-mekanismoen parte hartzearekin erlazioa daukana, aldi baterako edo etengabe prozesu neuroendekatzaileetatik babesteko gai dena. Uste da garuneko mekanismo konpentsatzaile/egokitzaile horiek zenbait garun-sistemaren funtzionamendu txarrari erantzunez aktibatzen direla: antioxidatzailea, neurotrofikoa, neurotransmisoreena (*Neurotransmitter*; NT), immunologikoa, besteak beste (Bobkova eta Vorobyov, 2015). Hala ere, Aβ-rekiko esposizio luzeak, ezinbestean, mikroegiturazko aldaketak eragiten ditu sinapsian, eta, ondorioz, NTen askapena, plastikotasun sinaptikoaren porrota eta memoriaren galera areagotzen dira (Koppensteiner et al., 2016). Arantza dendritikoetan ematen diren aldaketa horiek eta neuronen galerak modu espezifikoan gertatzen dira eskualdearen arabera (Golovyashkina et al., 2015).

Dena den, ADren fisiopatologia ulertzetik urrun dago, eta, ahalegin zientifiko biomediko handiak egin diren arren, gaur egun eskuragarri dauden tratamenduek sintomak iragankorki eta partzialki hobetzea besterik ez dute lortzen, eta gaixotasunaren progresioan atzerapen txiki bat baino ez dute eskaintzen (berrikuspenerako Massoud eta Leger, 2011; Beauquis et al., 2013). Adinarekin erlazionatutako patologia neuroendekatzaile kroniko hau asko hedatzen ari da gizarte modernoan, eta adierazpen klinikoak agertu baino askoz lehenago hasten dela ikusi da (Bobkova eta Vorobyov, 2015). Gero eta froga gehiagok iradokitzen dute SPs-ak eta NFTak ez direla ADeko lehen aldaketa patologikoak (Beauquis et al., 2013).

Alde batetik, gero eta froga gehiagok iradokitzen dute oxidazio-estresak garrantzi handia duela gaixotasunaren fisiopatologian. Zelula barneko oxidazio oreka hertsiki doituta dago, eta, beraz, AD duten pazienteen neuronetan konpentsazio-mekanismoak erregulatzeko seinaleztapen-bide ugari aktibatzea espero da. Oxidazio-estresa gaixotasunaren hasieran gertatzen da, ezaugarri patologikoak (SPs-ak eta NFTak) garatu baino askoz lehenago. Horrela, ADren lehen urratsetan, oxidazio-estresaren mailak areagotu egiten dira, nagusiki mitokondriek eta trantsizioko metalek osatutako oxigeno espezie erreaktiboen (*Reactive oxygen species*; ROS) arteko elkarreraginaren ondorioz. Horrek neuronan erantzun moldagarriak eragiten ditu, hala nola MAP kinasa aktibatzea, Aβ metatzea eta τ proteinaren

hiperfosforilazioa, kaltea edo/eta heriotza neuronala saihesteko. Hala ere, AD aurrera doan heinean eta ROS mailak etengabe handitzen doazen heinean, metal-Aβ konplexua eta τ proteina hiperfosforilatuaren ezabapena gaineztuta ikusiko da bere neurrigabeko sorrera dela eta. Horrek SPs-en eta NFTen kontrolik gabeko hazkuntza ekarriko du, baita ROS sorrera handitzea ere, kalte neuronala eta ondorengo heriotza neuronala eraginez (berrikuspenerako Moreira et al., 2006). Beraz, Aβ eta τ proteina hiperfosforilatuaren metaketak erantzun konpentsatzaileak eta ondorengo egokitzapenak direla hipotetizatzen da, gaixotasunaren etapa goiztiarrean zelula neuronalak kalte oxidatibotik babesteko (berrikuspenerako Moreira et al., 2006). Gainera, Aβ peptidoaren forma toxikoek Ca^{2+}-aren sarrera eragin dezakete neuronetan, oxidazio-estresagatik edo mintzean poro oligomerikoa eratuz. Horrek neuronak eszitotoxikotasun eta apoptosiarekiko kalteberak izatea eragiten du. Beraz, ADak neuronek Ca^{2+}-aren sarrera, fluxua eta zelula barneko banaketa erregulatzeko duten gaitasuna galzorian jartzen du (berrikuspenerako Bezla vanny eta Mattson, 2008).

Bestalde, gero eta froga gehiagok iradokitzen dute gliak eragindako inflamaziozko erantzun immunitarioa ADren neurofisiologiaren osagai garrantzitsua dela. Astrozitoak eta aktibatutako mikrogliak SPs-ekin eta endekapen neurofibrilarra jasaten duten zelulekin duten lotura estuak eta zitokina disolbagarrien askapenak, inflamazio-prozesuek ADren patogenesian paper garrantzitsua bete dezaketela iradokitzen dute (Unger, 1998; Mrak eta Griffin, 2005; Dzamba et al., 2016).

Doktorego tesi honetan mikroglian eta astrozitoetan agertzen diren aldaketa patologikoak aztertzen dira, familiako Alzheimer gaixotasunaren bost mutazio (*five familial Alzheimer's disease mutations*; 5xFAD) dituzten sagu transgenikotan (1. taula). 5xFAD sagu transgenikoa 2006an garatu zen, eta APP eta PSEN1 gehiegizko adierazpenak ditu 5xFAD mutazioengatik: hAPP hiru mutaziogatik APP transgenean (695) [suediar (K670N, M671L), Florida (I716V) eta London (V7171) mutazioak], eta giza PSEN1 bi mutaziorengatik PSEN1 transgenean (M146L eta L286V) (Oakley et al., 2006). 5xFAD lerroa sortzetiko C57BL/6J hondo genetikoan eratu zen, eta metaketa amiloidea, gliosia eta galera neuronal jarraitua ditu (Oakley et al., 2006; Oblak et al., 2021; Zhang et al., 2021), urritasun kognitiboekin eta motorrekin batera, giza ADren ezaugarri asko bilduz. Hala ere, 5xFAD ereduan ez dira NFTak agertzen (Oakley et al., 2006).

2.2. Astrozitoak:

ADean sintoma klinikoak agertu aurretik nerbio-sistema zentralean gertatzen diren aldaketa zelularren artean, heriotza neuronala da esanguratsuena. Hala ere, astroglia zelule

garuneko homeostasia mantentzeko funtzioa dutenez, erlazio handia daukate gaixotasunaren gertatzen diren aldaketekin ere (Verkhratsky et al., 2019). Dementzia duten gaixoen burmuinetako astrozitoen aldaketa patologikoa Alois Alzheimerrek deskribatu zuen lehen aldiz (Simchowicz, 1911). SPs-etan kokatzen ziren zelula glial ugari hauteman zituen (Simchowicz, 1911). Ondoren, gaixotasunaren azken urratsetako giza ehunen *post-mortem* analisiek astrogliosi erreaktibo orokorra erakutsi izan dute, SPs-etan astrozitoen barneratzea agertzen delarik (Nagele et al., 2003; Nagele et al., 2004; Rodríguez et al., 2009; Olabarria et al., 2010; Verkhratsky et al., 2019).

Astrogliosi erreaktiboa, beraz, ADren ezaugarri morfologiko arketipiko bat da, giza ehunetan zein gaixotasunaren animalia-ereduen burmuin isolatuetan behatu izan dena (Nagele et al., 2004; Rodríguez et al., 2009; Heneka et al., 2010; Beauquis et al., 2013; berrikuspenerako Verkhratsky et al., 2013,).

Astrozitoak, garuneko zelula motarik ugarienak dira, gai grisaren oinarriaren izanik, garunaren sistema homeostatikoaren elementu nagusiak dira. Garuneko mikroarkitekturari forma ematen diote, neurona-glia-odol-hodi unitateak eratzen dituzte, hesi hematoentzefalikoa (*Blood-brain barrier*; BBB) erregulatzen dute, CNSren mikrogiroa kontrolatzen dute eta eraso askoren aurkako defentsa gisa jarduten dute. ADren eredu transgenikoetan, astrozito erreaktibo hipertrofikoek SPs-k inguratzen dituzte, eta atrofia astrogliala ikusten da garuneko parenkima osoan zehar. Horrek azal lezake plastikotasun sinaptikoan agertzen diren aldaketa goiztiarrak eta alterazio neuroendekatzaile larrien aurretik garatzen diren asaldura kognitiboak (berrikuspenerako Rodríguez et al., 2009). Gainera, ebidentzia berriek ADeko etapa goiztiarrak Aβ agregatuen inguruko astrozitoen metaketarekin erlazionaturik daudela adierazten dute. Horrek, lesio hauek astrozitoak erakartzen dituzten molekula kimiotaktikoak jariatzen dituztela iradokitzen du (Wyss-Coray et al., 2003). Gertakari horrek alterazioak eragiten ditu konektibitate sinaptikoan, NTen homeostasian eta eszitotoxikotasuna areagotzearen ondoriozko heriotza neuronalean (Wyss-Coray et al., 2003; Verkhratsky et al., 2010). SPs-ak inguratzen dituzten astrozito erreaktiboak gaixotasunaren inflamazio-prozesuan inplikatuta daudela dirudi ere (Medeiros eta LaFerla, 2013). *In vitro* ikerketa osagarriek erakutsi dutenez, SPs-en inguruko astrozitoak xafla horien eraketan parte hartzen dute eta tokian tokiko inflamazio erantzunean. Glia zelula horiek Aβ 1-42 –ak aktibatzen ditu, eta horrek interleuzina-1 beta (*interleukin-1 beta*; IL-1β) bezalako zenbait zitokina edo beste produktu toxiko batzuk jariatzea eragiten du (Nagele et al., 2004; berrikuspenerako Heneka et al., 2010; berrikuspenerako Rodríguez et al., 2009; Hou et al., 2011; Beauquis et al., 2013). Bada, ebidentzia horrek astrozitoek Aβ-k eragindako

garuneko toxizitate zelularraren modulazioan funtsezko eginkizuna betetzen dutela iradokitzen du (Beauquis et al., 2013).

ADren eredu transgeniko batean (3XTg-AD) (1. taula), astrozito erreaktibo hipertrofikoak SPs-ak inguratzen deskribatu dira, baina zelula astroglialen atrofia ikusi da garuneko gainontzeko parenkiman ere. Astrozitoen atrofia goiztiarra patologikoki garrantzitsua izan daiteke FADren aurrera egitean (baita FADren antzeko patologia erreproduzitzen duten ADko animalia-ereduetan ere). Aldaketa atrofikoak bigarren mailako erdialdeko eta urruneko prozesu meheekin lotzen dira, eta, ondorioz, astrogliaren adarkadura orokorraren murriztea dakar, eta, beraz,ondorioz, estaldura sinaptiko gliala murriztu egin daiteke (berrikuspenerako Rodríguez et al., 2009).

Beste ikerlan batean, sagu-eredu bera erabili zen (3xTg-AD) (1. taula), eta Adinak eta ADren baldintzek astroglialren zelula dentsitate orokorrari ez ziotela eragiten erakusten zuen, hau da, ez zahartzeak ezta ADren patologiak ere ez dira erlazionatzen zelula-galerarekin. Hala eta guztiz ere, ADdun burmuinean, zuntz proteina azido glialaren (*Glial fibrillary acidic protein*; GFAP) markaketarekiko astrozito positiboen atrofia orokor bat deskribatu da, bai bihurgune horzdunean (*Dentate gyrus*; DG), bai *Cornu Ammonis* 1ean (*Cornu Ammonis 1*; CA1). Atrofia hori DGn GFAP-profil positiboen azalerak eta bolumenak behera egin dutelako igertzen da, gaixotasunaren lehen urratsean, 6 hilabetetik aurrera. GFAP-positibo diren astrozitoen antzeko hipotrofia bat ere deskribatu da CA1 eskualde hipokanpalean 18 hilabetetako adinean. Hala, atrofia seinaleak deskribatu dira plakaren ertzetatik 50 µm baino gehiagora dauden astrozitoetan (soma plakaren ertzetik 50 µm baino gutxiagora duten zelula guztiak plakari lotuta daudela jotzen da), hala nola, somen bolumen gutxitua eta prozesu nagusien kopuru, adarkadura, azalera eta bolumen globalaren murrizpen orokorra. Hala ere, SPs-ei lotutako astrozitoek erreaktibotasun-seinale argiak dituzte: hipertrofia, prozesu lodituak, gorputz zelular handituak eta GFAPrako profil positiboen bolumenaren eta azaleraren gehikuntza orokorra. Beraz, aldaketa bereizgarriak ikus daitezke astrozitoen morfologian, SPs-ekin duten harremanaren arabera (Olabarria et al., 2010).

1. taula. Alzheimer gaixotasunaren sagu transgenikoen ereduak. Eredu bakoitzak gaixotasunaren hainbat ezaugarri erakusten ditu: plakak (berdea), harilak (horia), galera neuronala (more iluna), gliosia (urdin argia), galera sinaptikoa (more argia), LTP/LTD aldaketak (urdin iluna) eta asaldura kognitiboa (laranja) adin desberdinetan (http://www.alzforum.org/research-disease/alzheimerase).
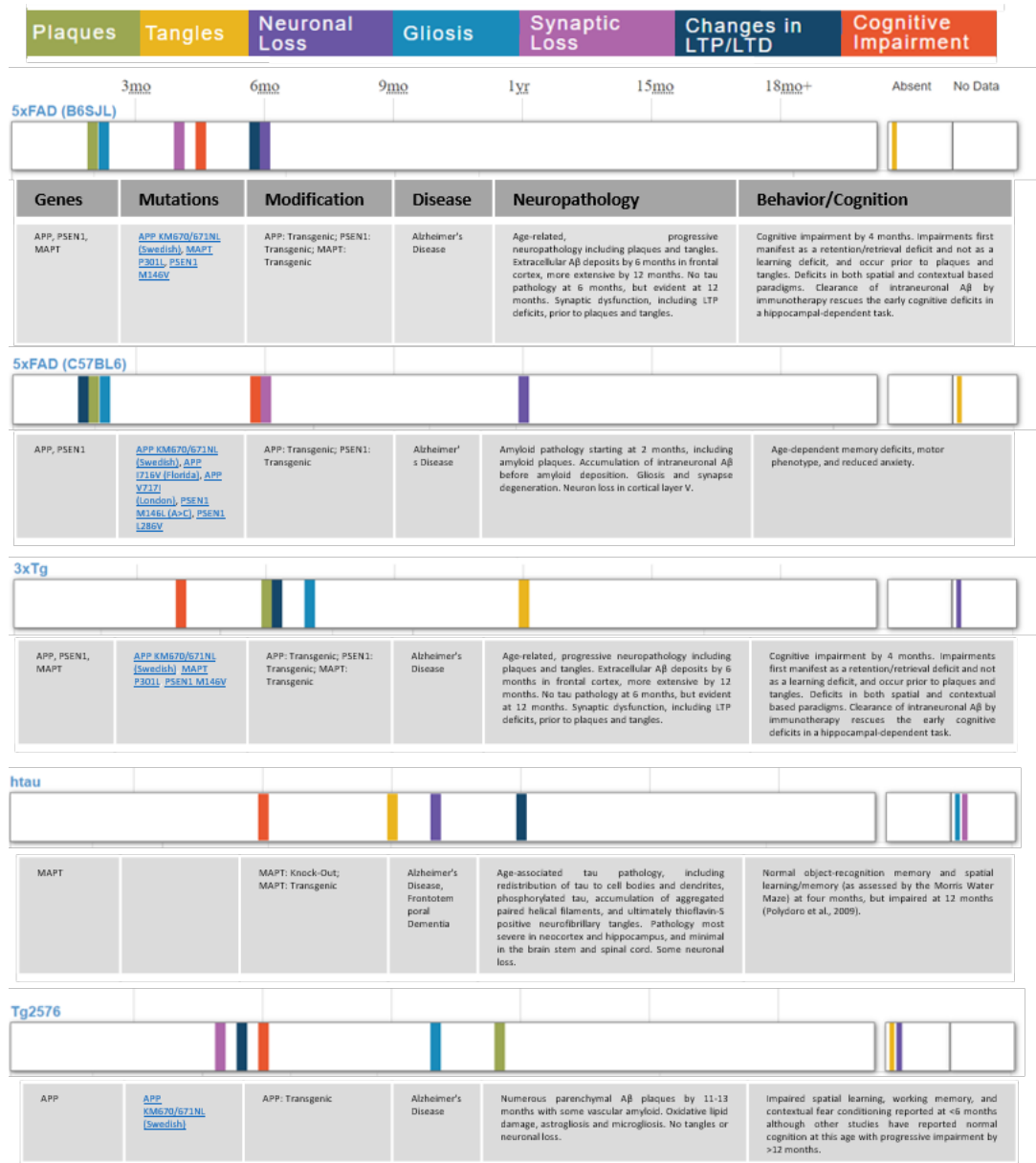
Gainera, zenbait azterlanek frogatu dute astrozitoek zelula-barneko Aβ kantitate nabarmenak pilatu ditzaketela (Nagele et al., 2003; Thal et al., 2000). SPs-ekin hertsiki lotutako astrozito batzuetan, Aβ-ren metaketa ikusten da antigorputz espezifikoekin (Nagele et al., 2003; berrikuspenerako Rodríguez et al., 2009). Duela 15 urte inguru, Aβ–ren garbiketa eta degradazioan astrogliaren parte-hartzea iradoki zen, baina alderdi hau oraindik ez dago argi (Guénette berrikusketarako, 2003; Nicoll eta Weller, 2003; Verkhratsky et al., 2019).

Horrela, APP mutantea adierazten zuen AD Tg2576 sagu transgenikoaren eredu batean (1. taula), SPs-ekin lotzen ziren astrozito erreaktiboek zinkaren mendeko neprilisina metalloendopeptidasa adierazten zutela ikusi zen, Aβ degradatzeko gai zen entzima (Apelt et al., 2003). Ikerketa gehiago behar dira astrozitoek Aβ zelula barnean edo zelulaz kanpo degradatzen duten zehazteko, eta burmuinetik Aβ ezabatzea astrozitoen funtzio homeostatiko arrunten artean dagoen jakiteko (Wyss-Coray et al., 2003). Zenbait ikerlanek astrozitoak Aβ-ren deuseztapenean inplikatzen dituzte (Shaffer et al., 1995; Wyss-Coray et al., 2003), baina beste batzuek, ordea, mikrogliaren bidezko SPs-ren fagozitosia inhibitzen dutela erakusten dute. Gaur egun, oraindik ez dago argi Aβ inguruan kokatzen diren astrozito erreaktiboek funtzio neurobabesleak edo suntsitzaileak dituzten (DeWitt et al. 1998; Wyss-Coray et al. 2003).

Aβ-ren sintesia batez ere neuronekin lotzen bada ere, astrozitoak prozesu horretan inplikatuta daudela adierazten duten hainbat datu daude (berrikuspenerako Frost eta Li, 2017). Aβ-ren ekoizpenean, astrozitoen ekarpena ere ez dago erabat ezaugarrituta. Denbora luzez, neuronak Aβ-ren iturri nagusia zirela uste izan zen, honen ekoizpen-entzimak adierazten dituztelako (Laird et al., 2005; Verkhratsky et al., 2019). Dirudienez, astrozito osasuntsuek ez dituzte entzima horiek adierazten, baina estres kroniko edo baldintza neuroinflamatoriopean dauden astrozitoetan, horien adierazpena eragin daiteke (Blasko et al., 2000; Leuba et al., 2005; Zhao et al., 2011; Orre et al., 2014; berrikuspenerako Frost eta Li, 2017; Verkhratsky et al., 2019). Horrela, β-secretasa edo BACE1-en adierazpena antzeman da (Hartlage-Rübsamen et al., 2003; para reviación Rossner et al., 2005; Verkhratsky et al., 2019) hAPP mutantea adierazten duten AD sagu-ereduetako astrozito erreaktiboetan (Hartlage-Rübsamen et al., 2003; berrikuspenerako Rossner et al., 2005; Verkhratsky et al., 2019) aurkitu da.

Horrez gain, badira β-secretasa edo BACE1 gizakien eta karraskarien burmuin-neuronetan adierazten direla egiaztatzen duten frogak, baina baita adineko Tg2576 sagu transgenikoen garuneko SPs inguruko astrozito erreaktiboetan ere (1. taula) (Hartlage-Rübsamen et al., 2003; Rossner et al., 2005). Gainera, gliosi kronikodun animalia-ereduetan eta AD duten pazienteen garunetan BACE1 adierazpena detektatu izan da astrozito erreaktiboetan (Brugg et al., 1995; berrikuspenerako Frost eta Li, 2017).

Era berean, arratoien astrozitoetan APPa eta bere ARNm-aren adierazpena hauteman da, eta baita zitokina proinflamatorio anitzen presentzia ere. Badirudi azken hauek sagu-garuneko astrozitoen APP mailak erregulatzen dituztela. ADren testuinguru neuroinflamatorioan, astrozito erreaktiboek atseden egoeran daudenak baino APP maila

altuagoak adierazten dituztela esan nahi du horrek, eta, beraz, Aβ gehiago sor dezaketela (Brugg et al., 1995; berrikuspenerako Frost eta Li, 2017). Oro har, astrozito erreaktiboek Aβ ekoizteko behar diren hiru osagaien maila altuagoak dituzte: APP, BACE1 eta γ-secretasa (berrikuspenerako Frost eta Li, 2017). Horrek, astrozito erreaktiboek ADren garapenean paper garrantzitsua jokatzen dutela iradokitzen du (Hartlage-Rübsamen et al., 2003). Astrozitoen egoera modulatzeak, haien endekapena moteltzeak edo gelditzeak edo/eta erreaktibitate astrogliala erregulatzeak, ADren ibilbidea alda lezake, gaixotasuna atzeratuz edo narriadura kognitiboa aldatuz (Verkhratsky et al., 2019).

2.3. Mikroglia:

Burmuinak mikrogliaren mailak proliferazio eta apoptosiaren arteko oreka oso estuan mantentzen ditu (Askew et al., 2017). Karraskarien garunean, zelula glial guztien % 5 eta % 12-a suposatzen dute, eta gizakiengan % 0,5 eta % 16 artekoa (berrikuspenerako Gomez-Nicola eta Perry, 2015; berrikuspenerako Sarlus eta Heneka, 2017). Mikrogliak CNSren inflamazio prozesuan parte hartzen duten zelula nagusiak dira eta euren funtsezko eginkizuna CNSrekiko kaltea agertzen denean erantzutea da. Zelula mota hau azkar aktibatzen da CNSren lesio gehienen aurrean, eta kantitate handitan pilatzen da kaltea dagoen lekuetan. Fenomeno hau, mikrogliosi erreaktiboa deitua, CNSren ia patologia guztien ezaugarri bereizgarria da, traumatismoak, garun odol-hodietako istripuak, inflamazioa, autoimmunitatea eta AD bezalako neuroendekapenezko gaixotasunak barne (Ajami et al., 2007).

Mikroglia CNSren parenkiman dagoen ehun-makrofagoen populazio espezializatu bat den arren, urteetan zehar "garunaren makrofagoa" bezala ezagutu izan dena, zelula mikroglialek funtzio garrantzitsu asko dituzte CNSren garapenean, funtzioan eta konponketan, antolaketa sinaptikoan, neuronen kitzikagarritasunean, hondakinen ezabaketan edo euskarri trofikoan (berrikuspenerako Gómez-Nicola eta Perry, 2015; Grabert et al., 2016).

Mikroglia zelulak zaintza funtzioa eginez, garuneko mikro-giroa etengabe eskaneatzen dute CNSren homeostasiaren asaldura txikien bila, eta gai da zirkulazio sistemikotik datozen substantzia neurotoxikoen edo hantura-bitartekarien presentzia detektatzeko, BBBrekin harreman estuan baitaude. Mikroglia fagozitikoak berehala antzeman eta ezaba ditzakete kaltetutako neuronak edo neurona apoptotikoak, inguruko zelulen lesioa ekidinez eta zelula-populazio espezifikoen berriztapen-tasa eusten lagunduz. Gainera, mikrogliaren gaitasun fagozitikoa bereziki garrantzitsua da garapenean (inausketa- mikroglia), sinapsi supernumerarioak bide neuronal espezifikoetan ezabatzen lagun baitezakete. Mikrogliak

11

sinapsiak ere modulatzen ditu, neuronen jardueran eraginez (mikroglia neuromodulatzailea) (berrikuspenerako Gomez-Nicola eta Perry, 2015).

Mikroglia zelulek sentsore molekular gisa jarduten duten hartzaileen sorta zabala adierazten dute. Horri esker, kalte exogenoa edo endogenoa antzeman dezakete eta erantzun immunea abiarazi (berrikuspenerako Sarlus eta Heneka, 2017). Beraz, mikroinguruneko alterazioen sentsore aktibo gisa jarduten dute, eta gai dira homeostasi tisularra berrezartzeko erantzun-espektro zabal baten bidez (berrikuspenerako Gomez-Nicola eta Perry, 2015). Homeostasiaren galerak edo ehunen aldaketek hainbat prozesu mikroglial dinamikoen agerpena eragiten duten zenbait baldintza agertzea eragiten dute, besteak beste, gainazalaren fenotipoan aldaketak gertatzea, bitartekari zelularren jariaketa, morfologia zelularraren aldaketak eta erantzun proliferatiboak ("egoera aktibatua" deitzen dena) (berrikuspenerako Sarlus eta Heneka, 2017). Horrela, inguruneak funtsezko eragina izango du fenotipo mikroglialaren konfigurazioan (Grabert et al., 2016) eta, ondorioz, transkriptoma mikrogliala eskualde batetik bestera heterogeneoa da CNSean. Kontuan hartuta mikrogiroa ez dela uniformea garuneko eskualde guztietan, neuronen azpimotak, NTen jariapen profilak, hemodinamikak eta metabolismoko aldaketek eragina izan dezakete tokiko fenotipo mikroglialean, eta horrek agerian uzten du berriz ere haren aniztasun funtzionala (Grabert et al., 2016; berrikuspenerako Sarlus eta Heneka, 2017). Beraz, hainbat fenotipo morfologiko eta funtzional erakusten dituzten plastikotasun handiko zelulak dira. Bi aktibazio-kategoria deskribatu dira (2. irudia): klasikoa (M1) eta alternatiboa (M2). Zehazki, interferoi-γ edo lipopolisakaridoarekin (*lipopolysaccharide*; LPS) kitzikatzeak, mikrogliaren aktibazioa sustatzen du M1 fenotiporantz, zeinak inflamazio-bitartekariak askatzen baititu: zitozinak, hala nola tumore-nekrosiaren faktorea (*Tumour necrosis facto*r-α; TNF-α) eta interleuzina-1β (*Interleukin-1β*; IL-1β), kimiozinak, redox molekulak, proteina koestimulatzaileak eta II. motako histokonpatibilitate-konplexu nagusia (*Major histocompatibility complex type II*; MHCII). Bitartekari horiek hantura areagotzen dute zeluletan eta ehunetan lesioak eraginez. Aldiz, interleuzina-4 (*Interleukin-4*; IL-4) eta interleuzina-10 (*Interleukin-10*; IL-10) bezalako zitokinek, M2 egoera polarizatua sustatzen dute. Mikroglia mota horrek jarduera antiinflamatorioa duten zitozinak sintetizatzen ditu, hala nola, hazkunde faktore eraldatzaile beta-ren (*Transforming growth factor beta*; TGF-β) eta IL-10, propietate neurobabesleak dituztenak. Hanturaren aurkako bitartekari horiek garuna bere onera ekartzen laguntzen dute, zelula-hondarrak ezabatzen dituztelako, tokiko hantura murrizten dutelako eta ehunen berreskurapenean parte hartzen dutelako. Baldintza neuroinflamatorioetan, mikroglia zelulek morfologia ameboidea hartzen dute eta M1 fenotipo klasikoa edo M2 alternatiboa hartzen

dute, tokiko ingurunearen izaeraren arabera (berrikuspenerako Nakagawa eta Chiba, 2014; berrikuspenerako Salvi et al., 2017).
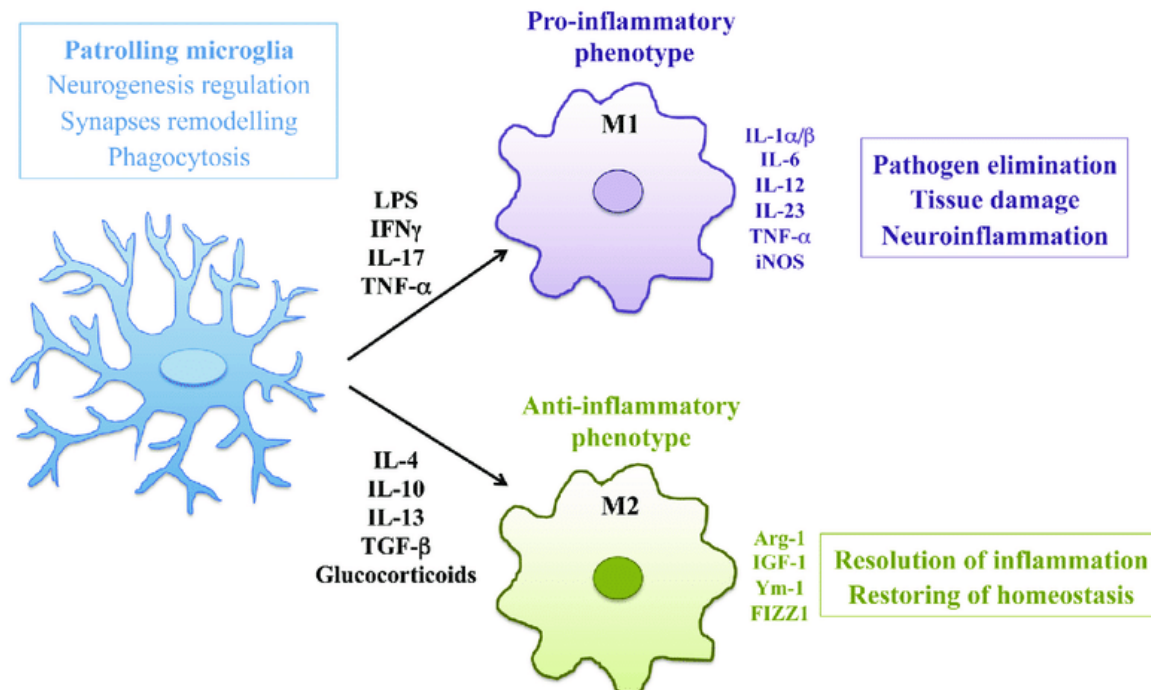
2. irudia. Mikrogliaren aktibazioa eta polarizazioa atseden-baldintzetan eta neuroinflamazioan. Mikrogliaren egoera funtzional ezberdinak adierazten dira, hauei loturiko morfologia eta fenotipoarekin (De Salvi et al., 2017).

Aktibazio mikrogliala eta haren M1/M2 polarizazioa CNSren hainbat kalte mota ezberdinetan ikusi da (Lin et al., 2017). Hala ere, ikerketa askok frogatu dute mikrogliak M1 eta M2 fenotipoen markatzaileak aldi berean adieraz ditzakeela (Almolda et al., 2015), eta horrek fenotipo mistoak daudela iradokitzen du (berrikuspenerako Sarlus eta Heneka, 2017).

Irudi-teknikek "kieszentzia adarkatuaren" eta "ameboide aktibatuaren" morfologia mikroglialen ikuspegi tradizionala indartu dute. Hala ere, mikroglia oso dinamikoa da, funtzio ugari eta morfologia aldakorrak dituelarik. Ziklatu, migratu, izurtu, fagozitatu eta prozesu fin eta lodiak hedatzen edo biltzen ditu. Horrela, mikrogliak betetzen duen hiru dimentsioko espazioa etengabe aldatzen da bere prozesu zelularrak migratu eta mugitzen dituzten heinean, forma biribil sinple batetik forma konplexu adarkatu batera igaroz, punta lausoak edo are adarkatuagoak dituzten bigarren eta hirugarren mailako adarrak garatuz (Karperien et al., 2013). Trantsizio hau oso azkarra izan daiteke, edo modu berean iraun dezake urteetan zehar (Colton et al., 2000). CNS helduan duen morfologia malgu hori oinarri hartuta, mikrogliaren forma ez-adarkatuak eta tartekoak "aktibatutzat", "erreaktibotzat" edo "tartekotzat" jotzen

dira, nabarmentzen diren prozesu sendo gutxi edo askorekin. Mikroglia-mota hori funtzio immunoinflamatorioa betetzeko aktibatzen da, lesioa izan den lekuetan biltzen edo erreklutatzen delarik eta beste zelula batzuen aktibazioa, proliferazioan, fagozitosian, hondakinak deuseztatzean eta sendatze prozesuan parte hartzen duelarik. Erabat adarkatutako formetan, mikrogliak aktiboki parte hartzen du funtsezko funtzio fisiologikoetan, neuronen funtzionamendu egokia bermatzen duten zelula zaindari gisa jardunez, substantzia neurotrofikoz hornituz, NTak eta hormonak erregulatuz, mina bitartekotuz, neuronak kalteetatik babestuz eta mikroinguruneko aldaketei erantzunez. Beren zeregin ugariak burutzen dituzten bitartean, zelula mikroglial adarkatu horiek aldatu egiten dira eta modu askotara mugitzen dira (berrikuspenerako Karperien et al., 2013). ADan, hainbat erreakzio mikroglial gertatzen dira gaixotasunaren etapa ezberdinetan (berrikuspenerako Sarlus eta Heneka, 2017). Horrela, garun osasuntsuan ez bezala, mikroglia ugaritzen da eta SPs-en inguruan pilatzen da, hauen eliminazioa sustatzeko (berrikuspenerako Gomez-Nicola eta Perry, 2015). Neurodegenerazioari eta Aβ metaketari erantzunez, mikrogliak *priming* izeneko aktibazio-egoera hartzen du (berrikuspenerako Perry eta Holmes, 2014). ADan aktibatutako mikrogliak inflamazio-markatzaileen adierazpena handitzen du, hala nola CD14, IL-1β, p40, CCL-3, CCL-4, CXCL-1 eta oxido nitriko sintasa induzigarria (*inducible nitric oxide synthase*; iNOS) (Martin et al., 2017). Era berean, jarduera mikrogliala asko areagotzen dela deskribatu da ADko sagu-ereduetan (1. taula) (Kamphuis et al., 2012; Olmos-Alonso et al., 2016) eta gaixotasuna duten pazienteen post-mortem garuneko laginetan (Gómez-Nicola et al., 2013). Beraz, aktibatutako mikrogliak, immunoglobulinekin eta konplementuaren osagaiekin batera, lotura estua dauka Aβ metaketekin AD duten pazienteen garunetan eta sagu-ereduetan (1. taula) (Eikelenboom eta Stam, 1982; Frautschy et al., 1998; berrikuspenerako Sarlus eta Heneka, 2017).

Mikrogliaren eta Aβ-ren arteko harremanaren ondoren, mikrogliaren patroiak ezagutzen dituzten hartzaileekin (*pattern recognition receptors*; PRRs) elkartzen da Aβ-a, sortzetiko immunitatearen hartzaile zelularrak direnak, arriskuei edo patogenoei asoziaturiko patroi molekularrei (*danger- or pathogen-associated molecular patterns*; DAMPs or PAMPs) erantzuteko diseinatutakoak. Hauek atsedenean dagoen mikroglia aktibatzea eragiten dute. Mikroglia aktibatuak askatutako zitokinek Aβ-ren erakartzea eta fagozitosia areagotzen dute. Hala ere, mikrogliaren epe luzeko aktibazioak proliferazioa eta inflamazio kronikoa bultzatzen ditu, eta horrek neurotoxikotasuna eta neuroendekapena eragiten ditu eta Aβ-ren fagozitosia eteten du. Beraz, ADren patogenesian, mikrogliaren aktibazioak zeregin bikoitza izan dezake: 1), mikrogliaren aktibazio akutuak Aβ-aren metaketa murriztea eragin dezake fagozitosia eta eliminazioaren bidez; 2) mikrogliaren aktibazio kronikoak neurotoxikotasuna

eta sinapsien endekapena bultzatu ditzake hainbat ur-jauzi prozesu proinflamatorio pizteagatik (berrikuspenerako Sarlus eta Heneka, 2017).

2.4. Sistema endokannabinoidea:

Sistema endokannabinoidea (*endocannabinoid system*; ES) sistema neuromodulatzaile garrantzitsua da, molekula lipidikoz, hartzaile espezifikoz eta haien sintesi eta degradazioaz arduratzen diren hainbat entzimaz osatua (berrikuspenerako Lu eta Mackie, 2016; berrikuspenerako Talarico et al., 2019). Endokannabinoide (*endocannabinoids*; ECBs) nagusiak arachidonoil-etanolamina edo anandamida (*arachidonoylethanolamine*; AEA) eta 2-arakidonilglizerola (*2-arachidonoylglycerol*; 2-AG) eikosanoideak dira. Alde postsinaptikoan sintetizatzen dira mintz fosfolipidoetatik abiatuta, eta mezulari atzerakoi gisa funtzionatzen dute hartzaile kannabinoide presinaptikoetan (berrikuspenerako Aso eta Ferrer, 2014; berrikuspenerako Pertwee, 2015; Monory et al., 2015; berrikuspenerako Lu eta Mackie, 2016). ESak modu negatiboan erregulatzen dute zenbait NTen jarduraren araberako askapena, eta horrek zirkuitu neuronalen kitzikagarritasunean eragiten du (berrikuspenerako Katona eta Freund, 2012; Monory et al., 2015). ECBs-ak ez dira zeluletan biltegiratzen eta besikuletatik askatzen, aitzitik, "eskariaren arabera" sortzen dira (Min et al., 2010). Oro har, datu farmakologikoek eta elektrofisiologikoek erakutsi dute Ca^{2+}-aren sarrerak ECBs-en sintesia eragin dezakeela, baita hartzaile metabotropiko batzuen aktibazioa ere (Petrocellis et al., 2004). Sintetizatu ondoren, endokannabinoide hauek despolarizatutako neurona postsinaptikotik askatzen dira, eta 1 motako kannabinoide hartzaile (*cannabinoid receptor type 1*; CB₁R) presinaptikoa aktibatzen dute, NTen askapena txikituz terminal presinaptiko inhibitzaile edo kitzikatzaileetatik (berrikuspenerako Hashimotodani et al., 2007). Hala, mekanismo horren bidez, ESak aldi baterako eta modu iraunkorrean zelula presinaptikoen NTen askapena doitzen du (berrikuspenerako Ueda et al., 2011). Beraz, endokannabinoideek sinapsi kitzikatzaile eta inhibitzaileetan plastikotasun-formak epe labur eta luzera burutzeko duten modu nagusia seinaleztapen atzerakaria da. Hala ere, seinaleztapen endokannabinoidea hasieran uste zena baino konplexuagoa eta erabiltzen dituen mekanismoetan anitzagoa da (berrikuspenerako Castillo et al., 2012).

ECBs-en sintesi- eta degradazio-bideek arreta handia erakarri dute aurkitu zirenetik (berrikuspenerako Ueda et al., 2011). 2-AG eta AEA erabat bide desberdinetan sortu eta degradatzen dira, nahiz eta egiturazko antzekotasunak eta antzekotasun funtzionalak izan (berrikuspenerako Ueda et al., 2011).

2-AGren kasuan, CNSean AEA baino askoz ugariagoa dena (berrikuspenerako Hashimotodani et al., 2007), fosfatidilinositol (*phosphatidylinositol*; PI) C fosfolipasa (*phospholipase C*; PLC)/diazilglizerol lipasa (*diacilglicerol lipasa*; DAGL) sintesi bidea da ziurrenik bide garrantzitsuena (berrikuspenerako Ueda et al., 2011). Boltai-menpeko Ca^{2+} kanalak (*voltage-sensitive Ca_2^+ channels*; VSCCs) eta $G_{q/11}$-ri lotutako hartzaile metabotropikoak aldi berean aktibatu ondoren, PLC aktibatu egiten da eta diazilglizerola (*diacylglicerol*; DAG) sortzen du PIetik aurrera. DAG hau, orduan, 2-AG bihurtzen da DAGLren bidez, zelula-barneko Ca^{2+} maila altuek estimulatuko dutena (Min et al., 2010). Beraz, despolarizazio neuronal handiak, boltai-menpeko Ca^{2+} kanalak aktibatuko ditu, neurona postsinaptikoetan Ca^{2+}-aren sarrera sustatuko duena, zelula-barneko Ca^{2+} kontzentrazioa handitzea eraginez (berrikuspenerako Hashimotodani et al., 2007). CB_1Rrekin gertatzen den bezala, DAGLren kokapena eta adierazpen-mailak ezberdinak dira garunaren eskualde bakoitzean, eta horrek 2-AG bidez burututako transmisio kitzikatzaile edo inhibitzailearen ezabatze atzerakariaren atalasea erregulatuko luke. Gainera, jakina da ESk plastikotasun sinaptikoa modulatzen duela hipokanpoan, bereziki CB_1Rk eta 2-AGk funtsezko zeregina betetzen dute, garun helduetan gertatzen den oroimenaren prozesamenduan duten parte-hartze funtzionala azpimarratzekoa delarik (berrikuspenerako Kano et al., 2009; Peñasco et al., 2019).

AEA N-aziletanolamina taldeko lipido bat da. Fosfolipidoen menpekoa den bide batez biosintetizatzen da. Zehazki, dagozkien N-azil-fosfatidiletanolaminen (*N-acyl-phosphatidylethanolamine*; NAPE) hidrolisi entzimatikotik (berrikuspenerako Piomelli, 2003).

AEAren kasuan, N-aracadonoil-fosfatidiletanolaminaren (*N-arachadonoyl-phosphatidylethanolamine*; NArPE) bidez sintetizatzen da, azido arakidonikoaren eta mintzari lotutako fosfatidiletanolaminaren (*phosphatidylethanolamine*; PE) loturaz sortzen dena. NAPEen sintesia, NArPE barne, PEren fosfolipidoen gantz azil talde baten amina primarioaren N posiziorako transferentziaren bidez gertatzen da, kaltzioaren mendekoak edo independenteak diren N-aziltransferasa batzuek bideratuta. Jarraian, AEA NArPEtik askatzen da fosfolipasen bidezko hidrolisiaren bidez (berrikuspenerako Biringer, 2021), bereziki NAPErentzako selektiboa den D fosfolipasa (*phospholipase D*; PLD) entzimaren (*NAPE-selective PLD*; NAPE-PLD) bidez, mintzeko beste fosfolipidoekiko afinitate gutxi daukana. NAPEren presentzia burmuinean, barrabiletan eta leukozito murinoetan konfirmatu da. Gainera, NAPE eta AEA konposatuen antzeko banaketa aurkitu zen garunaren eremu desberdinetan, bien arteko aitzindari/produktu erlazioa CNSean berretsi zuena (Bisogno et al., 1999; berrikuspenerako Petrocellis et al., 2004). Hartzaile kannabinoidea aktibatu

ondoren, endokannabinoideak hidrolisi entzimatikoaren bidez degradatu egiten dira (berrikuspenerako Lu eta Anderson, 2017). AEA gantz-azidoen amida hidrolasak (*fatty acid amide hydrolase*; FAAH) hidrolizatzen du neurona postsinaptikoetan, edo prozesu konplexuago bat, eikosanoideen biosintesi-makineria inplikatzen duena, jartzen da martxan (berrikuspenerako Biringer, 2021). Bestalde, 2-AGa monoazilglizerol lipasak (*monoacylglycerol lipase*; MAGL) hidrolizatzen du nagusiki neurona presinaptikoetan (berrikuspenerako Lu eta Anderson, 2017; berrikuspenerako Talarico et al., 2019).

ESren hartzaile nagusien artean ezagunenak CB_1R eta 2 motako hartzaile kannabinoidea (*Cannabinoid receptor type 2*; CB_2R) dira. Beste hartzaile kanabinoide batzuk ere topatu dira, hala nola, potentzial aldakorreko V1 kanal hartzailea (*transient receptor potential channel V1*; TRPV1), α eta γ motako peroxisomen proliferazio bidez aktibaturiko hartzaileak (*peroxisome proliferator-activated receptors* α and γ; PPAR-α eta PPAR-γ, hurrenez hurren), G proteinari loturiko 55. hartzailea (*G protein-coupled receptor 55*; GPR55) eta G proteinari loturiko 18. hartzailea (*G protein-coupled receptor 18*; GPR18) (berrikuspenerako Stella, 2009; berrikuspenerako Izzo et al., 2009; berrikuspenerako Stella, 2010; berrikuspenerako Fezza et al., 2014; berrikuspenerako Rapino et al., 2018; berrikuspenerako Guerrero-Alba et al., 2019).

Klonatu eta karakterizatu zen lehen hartzaile cannabinoidea CB_1R izan zen 1991n, eta bigarrena, CB_2R, 1993an identifikatu zen lehen aldiz. Bere banaketa oso ezberdina da, CB_1R adierazpen handia duelako CNSean, CB_2R askoz maila baxuagoan adierazten den bitartean. Horrek CB_1R kannabinoide exogenoen psikoaktibitatearen eta CNSeko endokannabinoideen ekintza fisiologikoen erantzule nagusia dela esan nahi du. CB_1R-knockout (*cannabinoid receptor type 1-knock out*; $CB1^{-/-}$) saguekin eta CB_1Rren antagonista espezifikoekin egindako azterketek gertakari hau baieztatu egin dute (Munro et al., 1993; berrikuspenerako Di Marzo et al., 2004; berrikuspenerako Stella, 2009; berrikuspenerako Zou eta Kumar, 2018).

Bi hartzaileak (CB_1R eta CB_2R) mintz-zeharreko zazpi domeinutako hartzaileen superfamiliakoak dira eta G proteinei akoplatutako hartzaile metabotropikoak (*metabotropic G protein-coupled receptors*; GPCRs) dira (berrikuspenerako McAllister eta Glass, 2002; Sánchez et al., 2003). CB_1R-ak $G_{i/o}$ proteinei akoplatuta daude eta zenbait ioi-kanaleen eta bigarren mezularien jarduera modulatzen dute (berrikuspenerako Straiker eta Mackie 2006; berrikuspenerako Stella 2010). Gainera, funtzio zelularretan hainbat efektu hauteman dira, hartzailearen estimulazio motaren arabera. Adibidez, milisegundo edo segundoetako CB1R neuronalaren aktibazio akutuak neurotransmisioa murrizten du eta berezko kitzikagarritasuna kontrolatzen du (Mackie eta Hille, 1992; berrikuspenerako Stella, 2010). Hala ere, minutu

edo ordutako etengabeko aktibazioak, zelula barneko seinaleak estimulatzen ditu, jarduera entzimatikoa eta gene espezifikoen adierazpena aldatzen dituztenak (Marsicano et al., 2003; berrikuspenerako Stella, 2010).

CB_2R ere G_i proteinei lotzen zaie, baina ez G_o proteinei ziurrenik (Glass eta Northup 1999; Munro et al. 1993; berrikuspenerako Stella, 2010). Proteina-egituraren % 44-a partekatzen du CB_1Rrekin, baina profil farmakologiko eta adierazpen-patroi desberdinak adierazten ditu (Felder et al., 1995; berrikuspenerako Stella, 2010).

Jakina da ECBs-en bidezko hartzaile kannabinoideen aktibazioak zelula barneko seinaleen transdukzio bide anitzen modulazioa eragiten duela (berrikuspenerako McAllister eta Glass, 2002; Sánchez et al., 2003). Horrela, CB_1R eta CB_2R 2-AG edo AEA bidez estimulatzen direnean, $G_{i/o}$ proteinen aktibazioaren bidezko zelula barneko seinaleztapen-gertaerak sortzen dira (berrikuspenerako Di Marzo et al., 2004; berrikuspenerako Jean-Gilles et al., 2010). Horrek hainbat ondorio dauzka, adenilato-ziklasaren (*adenylate cyclase*; AC) inhibizioa bezala, eta ondorioz, adenosina monofosfato zikliko (*cyclic adenosine monophosphate*; cAMP)/protein kinase A (*protein kinase A*; PKA) fosforilazio-bidea inaktibatuko da, mitogenoek aktibatutako proteina kinasa (*mitogen-activated protein kinase*; MAPK) estimulatzea eta zelula-kanpoko seinaleen erregulazio kinasa (*extracellular signal-regulated kinase*; ERK) aktibatzea. Zelula barruko gertaera horiek komunikazio sinaptikoa modulatu dezakete, entzima-jarduera aldatu eta gene-adierazpena erregulatu, besteak beste (berrikuspenerako Guzmán et al., 2002; berrikusketarako Piomelli, 2003; berrikusketarako Di Marzo et al., 2004; berrikusketarako Stella, 2010). CB_1R-ak, halaber, N eta P/Q motako boltai menpeko Ca^{2+} kanalak (*N- and P/Q-type voltage-sensitive Ca_2^+ channels*; VSCCs) inhibitzen ditu eta K^+ kanalak aktibatzen ditu (berrikuspenerako Guzmán et al., 2002). Gainera, $G_{i/o}$ proteinetako βγ azpiunitateen bidezko PLC-γ-ren aktibazioak, fosfatidilinositol 3-kinasaren (*phosphatidylinositol 3-kinase*; PI3K) estimulazioa eragiten du, ondoren inositol trifosfatoaren (*inositol triphosphate*; IP3) sintesia eragingo duena, zelula-barnerako Ca^{2+} mobilizazioa sustatuz (berrikuspenerako Guzmán et al., 2002; berrikuspenerako Petrocellis et al., 2004). Gainera, CB_1R-ak proteina konplexuagoen fosforilazio-ur-jauziak eragiten dituela proposatu da, PI3K eta B kinasa proteinaren (*protein kinase B*; PKB) bidez (Gómez del Pulgar et al., 2000; Di Marzo et al., 2004).

Beraz, kannabinoideek CB_1Rren bidez gauzatzen dituzte CNSean dituzten ondorio gehienak (Gómez del Pulgar et al., 2000). Hartzaile kannabinoide ugariena delarik, hipokanpoan, kortexean, garuntxoan eta oinaldeko nukleoetan maila altuetan adierazten delarik, garuneko funtzio garrantzitsuak erregulatuz, hala nola, kognizioa eta oroimena,

emozioak, kontrol motorra, elikadura eta minaren pertzepzioa. CB_1R G proteinei loturiko hartzaile ugariena dela kontsideratzen da ugaztunen garunean (berrikuspenerako Piomelli, 2003). Bere adierazpena, nagusiki, neurona eta glia zelulaletan gertatzen da (berrikuspenerako Wilson eta Nicoll, 2002). Zehazki, mikroskopia elektronikoko ikerketek frogatu dutenez, bukaera presinaptikoetan CB_1R ugari dago, baina egitura postsinaptikoetan eta glian ere kokatzen da. CB_1R baldintza fisiologikoetan adierazten duten sinapsien artean, interneurona inhibitzaile GABAergikoek hartzailearen askoz ere eduki handiagoa izaten dute sinapsi glutamatergiko kitzikatzaileek baino (Monory et al., 2006; Katona et al., 2006; berrikuspenerako Katona eta Freund, 2012; berrikuspenerako Lu eta Mackie, 2016). Astrozitoetan, CB_1Rren adierazpena txikiagoa da (Gutiérrez-Rodríguez et al., 2018). Mikroskopia elektroniko bidez egindako ikerlanetan, behatutako CB_1Rren markaketaren % 56 inguru bukaera GABAergikoetan ikusi izan ziren, % 12 bukaera glutamatergikoetan eta % 6 astrozitoetan. Gainerako immunopartikulak mitokondrietan (% 15) edo zelula mikroglial, lisosoma, endosoma eta abar izan zitezkeen beste konpartimentu batzuetan kokatu ziren (Bonilla-Del Río et al., 2019, 2020). Gainera, sinapsi inhibitzaileetan CB_1Rren adierazpenaren aberastasun hori asko aldatzen da garuneko eskualdeen arabera (berrikuspenerako Howlett, 2002; berrikuspenerako Stella, 2009). Interneurona mota ezberdinak ere euren CB_1Rren edukiari dagokionez ezberdinak dira, adibidez, saski-zelula perisomatikoak eta Schaffer-en albo zuntzekin erlazionaturiko zelula dendritiko inhibitzaileak. Hau bat dator sinapsi horietan endokannabinoide bidezko plastikotasun sinaptikoaren eraginkortasunaren ezberdintasunarekin (Lee et al. 2010; berrikuspenerako Katona eta Freund, 2012).

Zelula mota bakoitzerako CB_1Rren delezioa duten animalia-eredu transgenikoak tresna baliagarriak dira leku espezifikoetan seinaleztapen endokannabinoideak duen sare-aktibitatea eta portaeran duen ekarpena argitzeko (Marsicano et al., 2003; Monory et al., 2006; berrikuspenerako Katona eta Freund, 2012).

In situ hibridazioak, immunozitokimikak eta auto-erradiografiak CB_2R barean, timoan, amigdaletan, hezur-muinean, arean, makrofago/monozito esplenikoen prestakinetan, mastozitoetan, odol periferikoko leukozitoetan, eta baita hazkuntza zelularretako zenbait zelula-immunitariotan (berrikuspenerako Howlett, 2002). CB_2R, hartzaile periferiko gisa identifikatu zen hasieran makrofagoetan. Ondoren, CB_2Rren adierazpena deskribatu zen garunean, alderantzizko transkripzioa zuen polimerasaren kate-erreakzioaren (*reverse transcription-polymerase chain reaction*; RT-PCR), *in situ* hibridazioaren eta immunohistokimikaren bidez. Hala ere, eztabaida handia dago hartzaile horrek CNSean duen presentziari buruz. Azterketa immunohistokimiko batzuek CB_2R garuneko neurona

osasuntsuetan dagoela adierazten dute (Van Sickle et al., 2005; Gong et al., 2006), baina ez zituzten kontuan hartu immunotindaketaren espezifikotasuna ebaluatzeko beharrezkoak ziren kontrol negatiboak, bereziki CB_2R knockout saguak (Gong et al., 2006; Onaivi et al., 2006, berrikuspenerako Stella., 2008). Hala ere, beste ikerketa batzuek CB_2Rren presentzia gliara mugatzen dute, mikrogliara zehazki. Gainera, mikrogliaren adierazpena aktibazio zelularraren mailaren araberakoa dela proposatu da (Carlisle et al., 2002; Maresz et al., 2005; López et al., 2018). Horrela, kalte neuronala aktibazio mikroglialarekin eta fenotipo proinflamatorioetara aldatzearekin erlazionatzen da, erradikal askeak eta toxinak askatzera eta CB_2Rren adierazpenaren goranzko erregulaziora daramatenak. Gainera, mikroglia aktibatuan adierazitako CB_2R-ak migrazio mikrogliala erregulatzen du eta polarizazio mikrogliala hanturaren aurkako fenotiporantz bultzatzen du (Lin et al., 2017). Horrela, kaltetutako neuronek eta lesioek estimulatutako astrozitoek sortutako endokannabinoideek mikroglian adierazitako CB_2Ren gainean jarduten dute, zelulak fenotipo antiinflamatorio batera bideratuz (berrikuspenerako Stella, 2009). CB_2R adierazpena nabarmen handitzen da neuroinflamazioa ematen den baldintza patologikoetan, hala nola AD, esklerosi anizkoitza, Down sindromea edo giza immunoeskasiaren birusak (human immunodeficiency virus; HIV) eragindako entzefalitisa (berrikuspenerako Benito et al., 2008; López et al., 2018). Hala ere, CB_2R markatzeko antigorputz espezifikoren gabeziak, zalantzan jartzen ditu emaitza horiek (Gong et al., 2006; berrikuspenerako Atwood eta Mackie, 2010; Marchalant et al., 2014; López et al., 2018). Azkenik, zelula mikroglialek CB_1R ere adieraz dezakete, eta haien aktibazioak zelula horietan mikrogliaren bidezko immunitatearekin lotutako funtzio espezifikoak erregulatzen ditu (berrikuspenerako Stella, 2010).

Western blot eta immunozitokimikaren azterketek CB_2Rren presentzia adierazi dute giza astrozitoetan (Sheng et al., 2005). Gainera, AD duten pazienteen *post-mortem* garunetako SPs-ei asoziaturiko astrozitoetan CB_2Rren presentzia deskribatu da (Benito et al., 2003; berrikuspenerako Kano et al., 2009). Hala ere, zenbait azterlanek CB_2R adierazpena zelula mikroglialetara mugatu zuten (Benito et al., 2003, 2008; López et al., 2018). Astrozitoetako CB_1Rak haien funtzio metabolikoetan parte hartzen du. Adibidez, CB_1Rren aktibazioak hazkuntza zelularretako arratoi-astrozitoetan, garun-energiaren hornaduran inplikatutako glukosaren oxidazio-tasa eta zetogenesia handitzen ditu (Blazquez et al., 1999; berrikuspenerako Stella, 2010). Hau astrozitikoetako mitokondrien CB_1Rren presentziaren ondorioz izan daiteke, astrozitoetan CB_1Rren presentzia ez baita soilik mintz zitoplasmatikoan deskribatzen. Horrela, hipokanpoaren, bekoki-aurreko kortexaren, kortex piriformearen eta accumbens nukleoaren astrozitoen mintz mitokondrialean CB_1Rren presentzia ere antzeman da, zelula glial horiek funtzionala den CB_1R mitokondriala ere

badutela frogatuz. Horren aktibazioak disolbagarria den ACren aktibitatea eta PKAren menpeko fosforilazioa murriztu egiten dituela deskribatu da. Ondorioz, astrozitoen CB_1R mitokondrialaren aktibazioak glukosaren metabolismoari eta laktatoaren ekoizpenari eragiten die, eta horrek funtzio neuronalengan eta portaerarengan eragina dauka (Jiménez-Blasco et al., 2020). Halaber, CB_1R astrozitikoak leptina-hartzailearen (*leptin receptor*; ObR) adierazpen eta funtzio optimoei eusteko ezinbesteko eginkizun metabolikoa duela frogatu da (Bosier et al., 2013). Horretaz gain, CB_1R astrozitikoak zelula horien inflamazio-bitartekariak sortzeko gaitasuna moteltzen du (Sheng et al., 2005; berrikuspen baterako, Stella, 2010).

Gainera, hipokanpoko astrozitoek adierazitako CB_1Rak aktibatzeak, Ca^{2+} mobilizazioa bultzatzen du barne-biltegietatik, eta astrozito zelulen barneko ioi horren handipenak glutamatoaren askapena emendatzen du, eta, azken batean, NMDA hartzaileak aktibatzen ditu neurona piramidaletan (Navarrete eta Araque, 2008). Beraz, CB_1R astrozitikoak metabolismo energetikoa erregulatzeko eta neurona-glia elkarrekintzetan bitartekari izateko duen gaitasuna dela eta, badirudi eginkizun garrantzitsua duela funtsezko funtzio erregulatzaile anitzetan (berrikuspenerako Stella, 2010).

Oro har, bai astrozitoek eta bai mikrogliak hartzaile kannabinoideak aktibitatearen menpeko modu batean adierazten dituztela argudiatu daiteke (berrikuspenerako Bilkei-Gorzo, 2012), eta beraien aktibazioak zelula horien bereizketa, funtzioak eta bideragarritasuna erregulatu ditzake (berrikuspenerako Stella, 2010).

2.4.1. SISTEMA ENDOKANNABINOIDEA ETA ALZHEIMER GAIXOTASUNA:

ADren aurkako gaur egungo terapien eraginkortasuna mugatua da, eta horrek gaixotasunaren prozesuari aurre hartzeko edo atzeratzeko eragile berriak garatzea beharrezkoa dela agerian uzten du. ESak neuroendekapenbidean gertatzen diren prozesu patologiko nagusiak moldatzeko gaitasuna duela ikusi da. Ondorioz, gaixotasun horri aurre egiteko estrategia berritzaile batean bihurtu da (berrikuspenerako Aso eta Ferrer, 2014). ESak CB_1Rren menpeko funtzio neurobabeslea duela dirudi. Izan ere, $CB_1^{-/-}$ saguetan heldutasuneko ikaskuntzaren errendimenduan gainbeheraren azkartzea hauteman da (Bilkei-Gorzo et al., 2005), aipatutako eragin neurobabesle horien gabeziarekin erlazionaturik egon daitekeena (Sinor et al., 2000; Bilkei-Gorzo et al., 2005). ADean, hartzaile kannabinoideen adierazpenean gertatzen diren aldaketak gaixotasunaren egoeraren araberakoak izan daitezke. Ildo horretan, CB_1Rek eta CB_2Rek adierazpen-eredu desberdinak dituzte: ADren lehen faseetan hipokanpoko eta bekoki-lobuluko CB_1Rek erakusten dute aktibitate mailarik altuena, baina badirudi gaixotasuna garatu ahala murriztu egiten dela. Hala ere, CB_2Raren

adierazpena handitzen da gaixotasunaren etapa aurreratuetan, neuroinflamazioa nabariagoa denean eta mikroglia eta astrozitoak aktibatzen direnean (berrikuspenerako Di Marzo et al., 2015; berrikuspenerako Talarico et al., 2019).

Kannabinoideek ADren patogenesian gertatzen diren hainbat prozesuetan eragiteko duten ahalmena dela eta, hala nola, oxidazio-estresa, Aβ eta τ proteinen metabolismoa, inflamazioa, disfuntzio mitokondriala eta eszitotoxikotasuna, estrategia terapeutikotzat jotzen dira (Casarejos et al., 2013; berrikuspenerako Talarico et al., 2019). Hala, glutamatoaren askapen presinaptikoa inhibitzean (Hampson et al., 1998; Marsicano et al., 2003; Monory et al., 2006) eta boltai menpeko Ca^{2+} kanalak blokeatzean (Mackie eta Hille, 1992; Twitchell et al., 1997), ECBs-ek eszitotoxikotasunaren kontrako efektu neurobabeslea dute (Sinor et al., 2000; Marsicano et al., 2002).). Gainera, in vitro eta in vivo ikerketek zenbait konposatu kannabinoidek Aβ-ren kotrako eragin neurobabeslea dutela frogatu dute. Hartzaile kannabinoideen estimulazio zuzenak (2-AG bidez) edo endokannabinoideen entzima degradatzaileen inhibizioak (MAGL), Aβ-ren eliminazioa errazten dutela ikusi da, in vitro eta in vivo. Badirudi ekintza horiek hartzaile kannabinoideen bidez gertatzen direla, CB_1Rren edo CB_2Rren modulazioak edo inhibizioak kannabinoide tratamendu horren eragina arintzen baitu. Gainera, kannabinoideekin tratamendua egin ondoren, Aβ-ren proteina garraiatzaileak, lipoproteina hartzaileari lotutako 1 proteina (*lipoprotein receptor-related protein 1*; LRP1), garunean eta plasman gora egiten du, Aβ BBBren bidez garunetik periferiara igarotzeak azalduko lukeena (Bachmeier et al., 2013; berrikuspenerako Talariko et al., 2019).

Ikerketa askok frogatu dute, halaber, funtzio mitokondriala hondatuta dagoela ADren lehen urratsetan, eta okerrera egiten duela gaixotasunaren progresioarekin batera (berrikuspenerako Maruszak eta Żekanowski, 2011; berrikuspenerako Talarico et al., 2019). Gainera, garuneko metabolismoa murriztu egiten da ADean, eta energia gutxiago ekoizten da. Horrela, zelulen ahalmen glukolitiko mugatuak fosforilazio oxidatibo aerobiko mitokondriala handitzera eramaten du, behar energetikoak asetzeko. Hala ere, fosforilazio oxidatiboa erradikal aske endogeno eta toxikoen iturri garrantzitsua da, hala nola hidrogeno peroxidoa (*hydrogen peroxide*; H_2O_2), hidroxiloa (*hydroxyl*; -OH) eta superoxidoa (*superoxide*; O^{2-}) (berrikuspenerako Moreira et al., 2006; berrikuspenerako Talarico et al., 2019). Ildo horretan, ECBs-ek ROS produkzioa ekidin dezakete eta oxido nitrikoa (*nitric oxide*; NO) murriztu dezakete, iNOS adierazpena inhibituz. Efektu horrek, era berean, NOren menpeko τ proteinaren hiperfosforilazioaren inhibizio esanguratsua eragiten du, kontzentrazioaren menpe. Beraz, kannabinoideek paper garrantzitsua bete dezakete τ-ren hiperfosforilazioan. Izan ere, Aβ-z estimulatutako C6 arratoi-gliomako zeluletan egindako in vitro azterketek fenomeno hori CB_1Raren aktibazio selektibo baten bidez gertatzen dela

frogatu dute (Esposito et al., 2006; berrikuspenerako Talarikorako et al., 2019). Badaude beste ikerketa batzuk 9 tetrahidrokannabinolaren (*Δ9-tetrahydrocannabinol*; THC) bidez, konplexu mitokondrial I-aren zati den, NADH oxidasa mitokondrialaren aktibitatea murrizten dela adierazten dutenak. Hala ere, kannabinoideen efektu mitokondrial horiek kontzentrazioaren araberakoak dira (Athanasiou et al., 2007).

Era berean, kannabinoideek aktibazio mikrogliala murriztean Aβ-ak eragindako neuroendekapena prebenitzen dutela frogatu da. Mikroglian adierazitako CB_1R eta CB_2R inplikatuta daude ekintza horretan. Horrenbestez, hartzaile hauek ROSen eraketa prebenituz neuroinflamazioa inhibitzeko eta mikrogliaren zitokinen askapena inhibitzeko duten eginkizuna adierazi izan da (Ramírez et al., 2005; Martín-Moreno et al., 2011; Casarejos et al., 2013; berrikuspenerako Talarico et al., 2019).
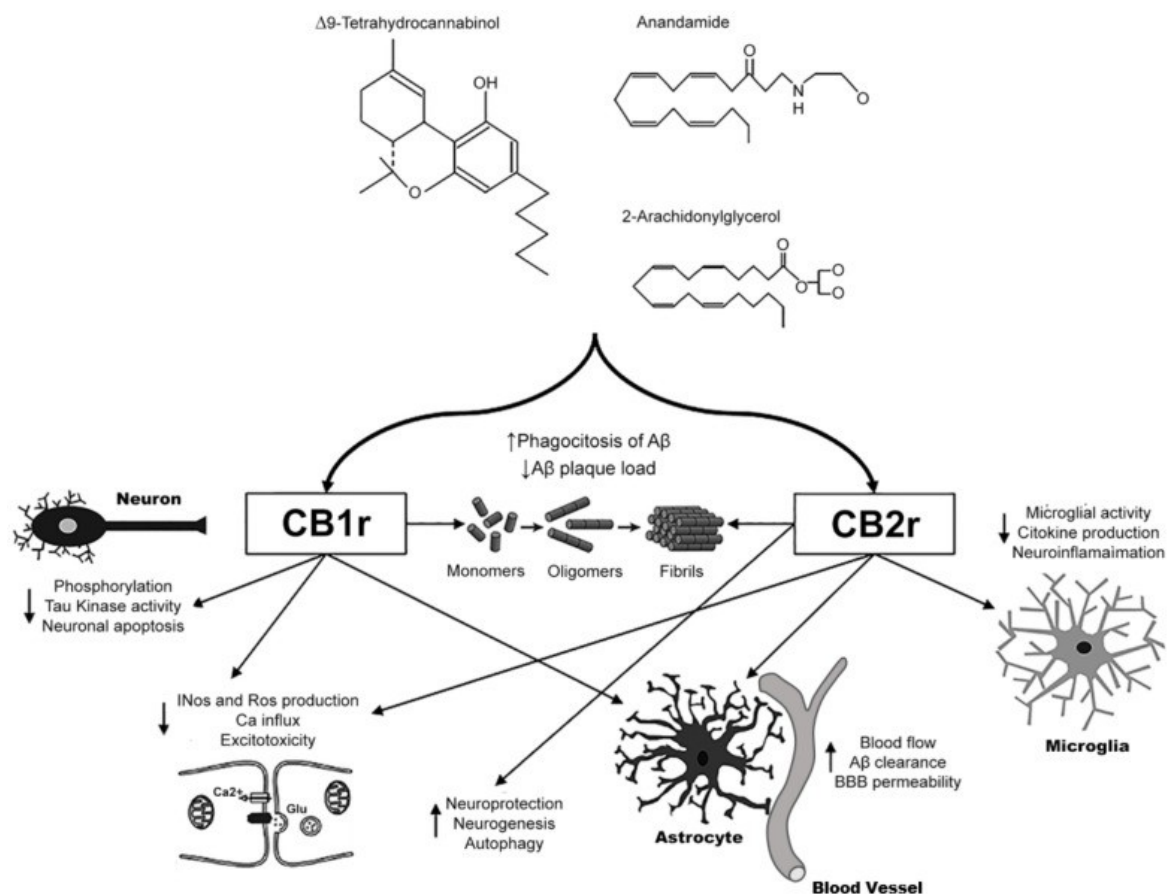
3. irudia. Sistema kannabinoideak ADean dituen ondorio onuragarrien eskema.
Kannabinoideekin egindako tratamenduek ADren prozesu ugari modulatu ditzakete, hala nola, Aβ eta τ prozesamendua, neuroinflamazioa, aktibazio mikrogliala, disfuntzio mitokondriala eta eszitotoxikotasuna (Talarico et al.-etik, 2019).

2.5. SISTEMA LINBIKOA:

Garunaren sistema linbikoak (*Limbic system*; LS) ornodun espezie guztien biziraupenerako funtsezko portaerak arautzen ditu, baita gizakienak ere. Emozionalki, sozialki edo motibazioz garrantzitsuak diren kanpoko seinaleak edo estimuluak testuinguruaren eta espeziearekiko espezifikoak diren jokabide egokien multzo espezifiko batekin lotzen ditu. Portaera horietako asko ikaskuntzaren bidez eta esperientziarekin sendotzen dira, baina gehienak sortzetikoak dira, eta horrek esan nahi du aldez aurretik ikasi gabe agertzen direla. Gizabanakoaren edo kumeen biziraupenean eta espeziearen hedapenean oinarritutako sortzetiko portaera horien artean daude gorteatzea, amaren zaintza, defentsa eta hierarkia sozialaren ezarpena. Portaera horiek erregulatzen dituzten zirkuituen aktibazioa periferian hasten da zentzumen-estimuluekin, hala nola ukimenarekin, entzumenarekin eta karraskarietan bereziki, usainmenarekin. Ondoren, estimulu horiek, burmuinean, egitura mugatu multzo batean, amigdala eta hipotalamoa barne, prozesatzen dira. Animalia batek gizarte- edo ingurumen-seinaleak antzemateko edo behar bezala prozesatzeko gaitasunik ez badu, ezohiko jokabidea izango du, , eta eraso eta/edo harrapari arriskua areagotzen du. Konexio horien oinarrizko neuroanatomia ondo ezarrita dagoen arren, oraindik ez dakigu informazioa sortzetiko zirkuituen barruan nola prozesatzen den (berrikuspenerako Sokolowski eta Corbin, 2012).

LSeko aldaketa endekatzaileek paper garrantzitsua betetzen dute ADren sorreran (Van Hoesen eta Hyman, 1990). ADeko lehen gertaera neuroendekatzaileak kortex transentorrinalean agertzen dira, eta, ondoren, kortex entorrinalera (*Enthorrinal cortex*; EC) eta hipokanpokora hedatzen dira. Geroago, prozesu neuroendekatzaile hori loki lobulu, bekoki eta pareta lobuluetan zehar hedatzen da (Thompson et al., 2003; 2007; Verkhratsky et al., 2019). Gaixotasunaren azken urratsetan, garuneko parenkimaren atrofia orokor bat ematen da, neurona eta kontaktu sinaptikoen galera nabarmenarekin. Atrofia horrek gai zuria eta grisa barne hartzen ditu (Simchowicz et al., 1911; Verkhratsky et al., 2019). Hipokanpo eraketa bereziki kaltetua dago ADean, patologiaren foku garrantzitsu bat delarik (Van Hoesen eta Hyman, 1990).

2.5.1. HIPOKANPO ERAKETA:

Hipokanpo eraketa giza burmuinaren loki lobuluan kokatuta dago. Egitura zitoarkitektoniko ezberdinen multzoz osatuta dago: DG; hipokanpoa bera edo Cornu Ammonis (CA); CE, karraskarietan bereziki, erdialde- eta albo-azpieremuetan banatzen dena; subikulua (4. irudia) (berrikuspenerako O 'Mara et al., 2001; berrikuspenerako Rajmohan eta

Mohandas, 2007). Egitura horiek taldekatzeko arrazoi nagusia, gehienetan norabide bakarrekoak diren konexioez lotuta daudela da, entitate funtzional bat osatzen dutelarik. Hala ere, bere osagaiak egile ezberdinen arabera aldatzen dira (Insausti eta Amaral, 2004).

- *HIPOKANPOA:*

CA edo hipokanpoa egitura geruzatua da (arkikortexa), oriens geruza, piramide-geruzak, erradiodun geruzak eta lacunosum-molekulare geruzek osatutakoa, CA3 eskualdean geruza luzidoa gehitzen zaio . Zitoarkitekturaren eta konektibitatearen desberdintasunei dagokienez, hipokanpoa lau eremutan bana daiteke (1934an Lorente de Nó-k izendatuak): CA1, CA2, CA3 eta CA4. CA4-a CA-ren zatia den edo egitura independentea denari buruzko desadostasuna dago. Hipokanpoak alveusarekin egiten du muga, finbria osatzeko elkartzen den zuntz geruza fin bat, eta, ondoren, fornixaren oinarekin, hipokanpo eraketaren bide eferente nagusia dena. Fornixaren oinak bateratzen dira fornixaren gorputza osatzeko, eta, ondoren, hipotalamoaren gorputz mamilarretara iristen diren fornixaren zutabeak eratzeko (berrikuspenerako Rajmohan eta Mohandas, 2007; El Falougy et al., 2008).

- *SUBIKULUA:*

DG eta CA-en fisiologia eta funtzioak sakonki aztertuak izan diren bitartean, subikuluak arreta gutxi jaso du (berrikuspenerako O 'Mara et al., 2001), hipokanpo eraketaren sarrera- eta irteera-eskualde garrantzitsua izan arren (4. irudia). Egitura hori funtsezkoa da ikaskuntzaren prestakuntzaren eta oroimenaren zirkuitu neuralean (Ishihara eta Fukuda, 2016). Informazio ugari (espazioa, denbora, etab.) prozesatzen da CA1 eremuan eta eraldeko/alboko CEean, eta, gero, bi eskualdeek subikuluarekin sinapsia egiten dute, zeinak aktiboki aldatzen eta integratzen baititu sarrera horiek. Gainera, zelula piramidal subikularrek plastikotasun sinaptikoko hainbat forma adierazten dituzte eta zirkuitu errepikariak osatzen dituzte (berrikuspenerako Matsumoto et al., 2019).
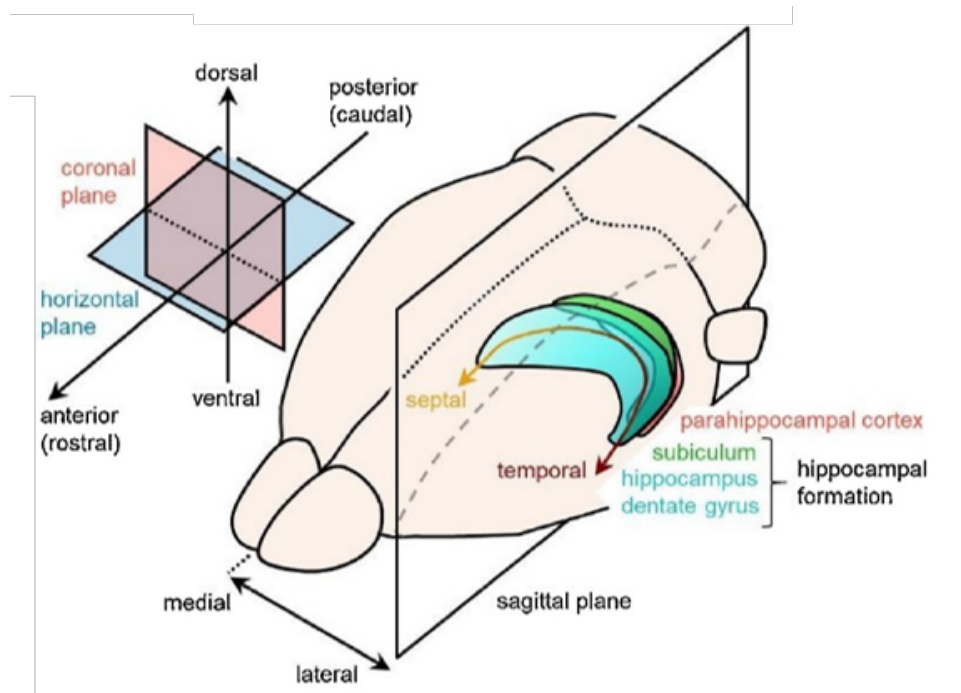
4. irudia. Arratoiaren subikuluaren anatomia (Matsumoto et al., 2019). **Hipoknpo** eraketa berde eta urdinez adierazita dago, eta hipokanpo-alboko kortexa gorriz. Hipokanpo eraketan hipokanpoa eta DG sartzen dira, urdinez agertzen direnak, eta subikulua berdez. Hiru erreferentzia-ardatz irudikatu dira dagozkien ebaketa planoekin: aurre-atzekoa (aurpegi-buztenetakoa), , bizkar-sabeletakoa, eta erdialde-alboetakoa, koroa plano, horizontala eta gezi planoekiko elkarzutak direnak, hurrenez hurren. Karraskarien C formako hipokanpo eraketan, septutik gertuen dagoen muturra, septuko poloa deritzo, eta loki lobuluaren ondoko muturra, aldiz, loki poloa (berrikuspenerako Matsumoto et al., 2019).

Subikulu konplexua hiru azpi-eremutan banatzen da: subikulua bera, subikulu-aurreko eremua (*presubiculum*; PreS) eta subikulu-alboko eremua. Hainbat azpibanaketa adibideak existitzen dira. Adibidez, Lorente de Nó-k (1934) CA1 eremuaren eta subikuluaren arteko eskualde bat deskribatu zuen, prosubikulua deitutakoa, baina gaur egun ikerlari askok trantsizio eskualde bat dela esaten dute eta ondorioz ez dela eskualde bereizi bat bezala definitu behar. Brodmannek (1909) hainbat espezietan banatuta agertzen zen eskualde bat ere deskribatu zuen: post-eskualdea edo retrosubicularis eremua; autore berriagoek, eskualde hau PreSaren zatitzat har daitekeela iradokitzen dute. Geroago, Van Groenek eta Wyssek post-subikuluaren ideiaren alde egin zuten berriro, subikulu konplexuaren laugarren eskualde gisa (Van Groen eta Wyss, 1990; O 'Mara et al., 2001). Beraz, subikulu konplexuaren azpibanaketak eztabaidagarriak izan dira azken mendean.

Subikuluaren zatirik handiena, PreS eta hipokanpoaren CA1 eskualdearen artean dago, septuko eremuan esplenio-atzeko kortexaren ondoko eremu batekin (Ishihara eta Fukuda, 2016). Gutxi ezagutzen da subikuluaren barne egiturari buruz, eta oraindik ere eztabaidagarria izaten jarraitzen du. Subikulua, alokortexaren bereizgarria den hiru

geruzetako egitura bat da, ECren neokortexeko ohiko sei geruzen aldean. Subikuluaren hiru geruza nagusiak hurrengoak dira: molekula-geruza, CA1 eremuko lacunosum-moleculare eta erradiodun geruzekin jarraia dena; zelula piramidalen hedatutako geruza, neurona nagusien somak dauzkana; geruza polimorfikoa (berrikuspenerako O 'Mara et al., 2001). Molekula-geruza hipokanpoaren ildotik gertu dago; piramide-geruza molekula-geruzaren eta geruza polimorfikoaren artean dago; geruza polimorfikoa alveusetik gertuago dago eta CA1-eko oriens geruzaren jarraia da (berrikuspenerako Matsumoto et al., 2019). Molekula-geruza eta polimorfikoa azaleko geruza eta geruza sakona ere deitzen dira, hipokanpoaren ildotik eta alveusetik duten hurbiltasunaren arabera, hurrenez hurren. Bestalde, "hurbileko" eta "urruneko" terminoak CA1 eskualdetik gertuen dagoen subikuluaren eskualdea eta PreS eskualdetik gertuen dagoen subikuluaren eskualdea izendatzeko erabiltzen dira, hurrenez hurren. Bost geruza bereizi dira hurbileko subikuluan, neurona handiagoak eta dentsitate zelular txikiagoak dituztenak, batez ere erdialdeko geruzetan eta geruza sakonetan. Urruneko subikulua , ordea, soma tamaina homogeneoagoa eta zelula-dentsitate handiagoa duten neurona txikiagoek osatzen dute (Ishihara eta Fukuda, 2016). Sailkapen horrez gain, ikerketa immunohistokimikoek are gehiago ezaugarritu dute subikuluaren barne-egitura, zitoarkitektura diferentzialean eta kalbindinarentzako immunorreaktibitatean oinarrituta (Fujise et al., 1995; berrikuspenerako Matsumoto et al., 2019).

Subikuluko zelula mota nagusia zelula piramidala da. CA1-eko zelula piramidalek baino albo zuntz axonal gutxiago erakusten dute, eta garun-eremu gutxi batzuetara soilik proiektatzen dute. Neurona horiek, ordea, alveusera iristen diren albo axoiak dituzte, eta, aldi berean, hainbat bukaera sinaptiko dauzkate, bai zelula piramidalen geruzan bai dendrita apikaletan (Harris et al., 2001; berrikuspenerako Matsumoto et al., 2019). Subikuluko piramide-geruzako paketatze zelularra CA1-ekoa baino lausoagoa da (berrikuspenerako O 'Mara et al., 2001). Horretaz gain, zelula piramidalekin nahasten diren neurona txikiago asko interneurona subikulartzat hartzen dira, baina ez dira asko ezaugarritu (berrikuspenerako O 'Mara et al., 2001; berrikuspenerako Matsumoto et al., 2019).

2.5.2. HIPOKANPO ERAKETAREN KONEXIOAK:

ECak hipokanpo eraketara dituen proiekzioak banandua daude: III. geruzako neurona-entorrinalak CA1 eta subikulura proiektatzen dute, eta II. geruzako zelula izar itxurakoak, berriz, DG, CA2 eta CA3-ra. Erdialdedko CE II. geruzako zelula piramidalek (uharte-zelulak) ere CA1-era proiektatzen dute eta, neurri txikiagoan, subikulura (berrikuspenerako Matsumoto et al., 2019).

Bide zulatzailea hipokanpoaren bide nagusitzat hartzen da, non CEko II eta III geruzetako neurona glutamatergikoetatik abiatzen diren zuntzak subikulutik igaro eta DGeko pikor-zelulen geruzara iristen dira. Ondoren, zuntz goroldiotsu glutamatergikoak DGtik CA3ko zelula piramidaletara hedatzen dira. Gero, CA3ren zuntz eferente batzuk fimbriara proiektatzen duten arren, CA3ko axoi askok Schafferren albo zuntzak igortzen dituzte CA1ko dendrita ipsilateraletara (erradiodun geruza eta *oriens* geruza). Azkenik, CA1etik zuntzak alveusera, finbriara eta ondoren fornixera hedatzen dira. Gainera, subikuluak CA1 eskualdetik proiekzio garrantzitsuen sarrera masibo bat ere jasotzen du. Horrenbestez, subikulua CA1 eta EC eremuengatik inerbatuta dago (O 'Mara et al., 2001; Ishihara eta Fukuda, 2016; berrikuspenerako Matsumoto et al., 2019).

Hipokanpoan hiru sistema eferente eratzen dira: komisura-aurreko fornix-zuntzak, fornixarenak ez diren zuntzak eta komisura-atzeko fornix-zuntzak. Komisura-aurreko fornix-zuntzak CA geruzan eta subikuluan sortzen dira, eta finbria, oina eta fornixaren gorputzaren barruan abiatzen dira. Fornixarenak ez diren zuntzak hipokanpotik zuzenean proiektatzen dute eremu entorrinalera, atzeko zingulu-kortexra, esplenio-atzeko kortexera eta amigdalara. Komisura-atzeko zuntzak, nagusiki, gorputz mamilarretan amaitzen dira, baina zuntz batzuk, talamoaren aurreko nukleoetara ere proiektatzen dute, bukaera ildaska oheko nukleoak eta hipotalamoaren sabel-erdialdeko nukleoa. CAko zuntzak septuko nukleoetan bakarrik amaitzen dute. Subikulu-zuntzak, aldiz, accumbens nukleora, aurreko usaimen nukleora, septuko nukleoetara, komisura-aurreko hipokanpora, erdialdeko bekoki kortexera eta bihurgune zuzenera banatzen dira. Proiekzio-eredu zabal horrek, bai subikuluak eta bai CA1 eskualdeak hipokanpoaren irteeraren antolaketan funtsezko eginkizuna betetzen dutela esan nahi du (Mark et al., 1995; berrikuspenerako Rajmohan eta Mohandas, 2007; Ishihara eta Fukuda, 2016; berrikuspenerako Matsumoto et al., 2019).

ADean, subikuluko proeikzio-neuronak bereziki kaltetuta daude. Hipokanpoko irteera bideen kalteek hipokanpo eraketaren deskonexioa eta isolamendua eragiten dute (Hyman et al., 1984). 5xFAD saguen burmuinean, Aβ-ren metaketak (eta gliosia) bi hilabeterekin hasten direla deskribatu da, eta metaketa maila altuetara heltzen dira, batez ere subikuluan eta kortexeko geruza sakonetan (Oakley et al., 2006). Horrela, patologia lehen aldiz subikuluan agertzen da, eta patroi espezifiko bati jarraituz konektatutako garuneko eskualde linbikoetara hedatzen da, 3-15 hilabetetan zehar (Rönnbäck et al., 2012; George et al., 2014). Horrela, Aβ-ren metaketak aurreko eremuetatik neurona-proiekzioak jasotzen dituzten eskualdeei sekuentzialki eragiten die. ADren neuropatologiak bide neuronaletan zehar izaten duen espazio- eta denbora-progresioa aldez aurretik hainbat ikerlanek iradoki dute, baina azpian dauden mekanismoak oraindik ez daude argi. Gaixotasunak, beraz, subikuluko sarrerak

28

jasotzen dituzten garuneko eskualdeei eragiten die, funtzionalki modu sekuentzialean konektaturik daudenak, ezagutzen diren konexio anatomikoen araberako ordena batean, hala nola, retroesplenio-atzeko kortexa, gorputz mamilarrak eta talamoa (5. irudia) (Rönnbäck et al., 2012). Narriadura kognitibo maila desberdinak dituzten AD duten pazienteen azterketetan, gaixotasunaren lehen faseetatik hasita, atrofia baten prebalentzia ikusten zen subiku-aurre eta subikuak osatutako konplexuan. Aurkikuntza hori bat dator AD duten pazienteei egindako behaketa neuropatologikoekin, eta, ziur asko, bide zulatzailearen endekapen larria islatzen du, eremu subikularrean barrena sartzen baita ECetik DGrainoko ibilbidean. Beraz, azterlan horien arabera, atrofia-patroi ez-homogeneoa zegoen ADeko pazienteen hipokanpoari dagokionez, gaixotasunaren lehen faseetatik aurrerako faseetan prebalentea eta begi-bistakoa den subikulu-aurre/subikulu konplexuaren eragin nagusiarekin (Carlesimo et al., 2015; Lindberg et al., 2017). Beraz, subikuluko kalte neuronalaren eta aldaketa glialen azterketa funtsezkoa da ADen patologia ulertzeko, sistema horrek garuneko funtzio anitzen erregulazioan duen inplikazio garrantzitsua dela eta (berrikuspenerako Matsumoto et al., 2019).
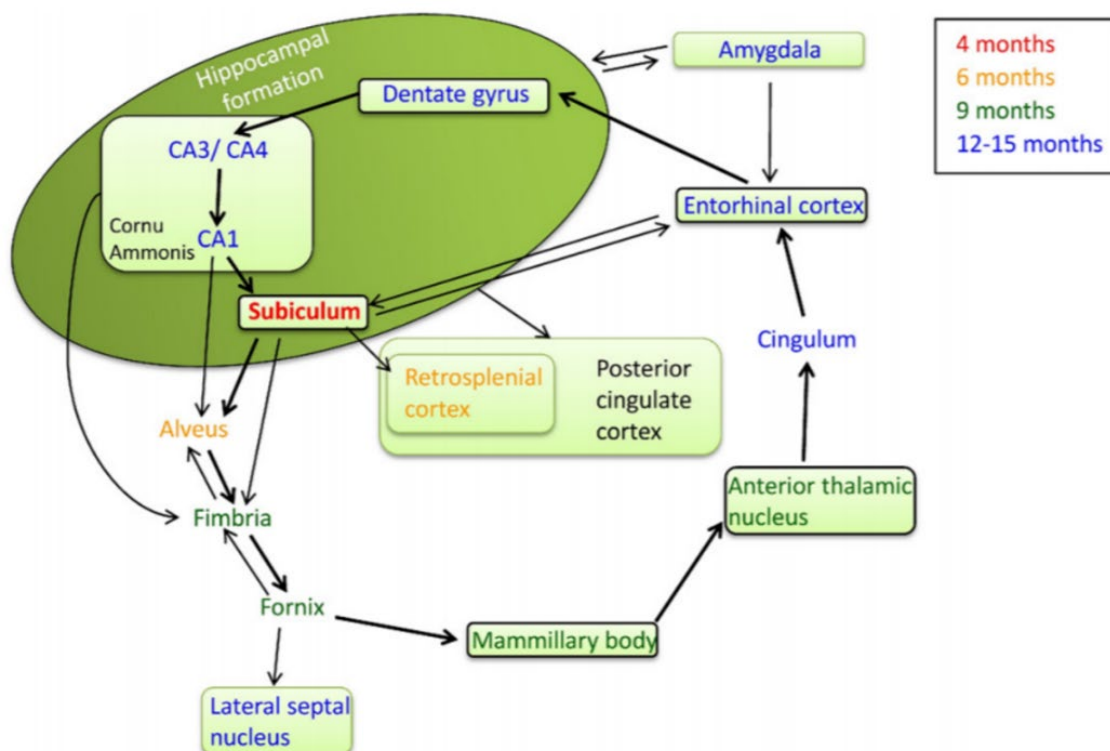
5. irudia. Amiloide lausoaren gordailuaren progresioaren eskema Alzheimer gaixotasunaren sagu-eredu batean (TgAPParc). Garuneko eskualdeen arteko lotura anatomiko garrantzitsuak geziz adierazita daude, eta testuaren koloreak zehazten du amiloide lausoaren lehen gordailuak ze adinetan detektatzen diren (eraldatuta Rönnbäck et al., 2012).

3. LAN HIPOTESIA

AD zuten pazienteen post-mortem garunetan eta animalien ereduetan aldaketa patologiko ugari detektatu izan dira (Medeiros and LaFerla, 2013). Alterazio hauen artean Aβ peptidoen agregazio eta pilaketa topa dezakegu, SPs-en eraketarekin, τ proteinaren hiperfosforilazioarekin, NFTen sorrerarekin, neuronen heriotzarekin, sinapsietako funtzionamendu okerrarekin, hanturarekin, oxidazio-estresarekin eta mitokondriako metabolismoaren alterazioekin lotuta (berrikuspenerako Moreira et al., 2006; berrikuspenerako Querfurth and LaFerla, 2010; berrikuspenerako Bloom, 2014; Bobkova eta Vorobyov, 2015). Hala ere, oraindik ere ADren patogenesia ez da argitzea lortu. Azken urteotan, hantura zelulek ADean izan dezaketen eragina ikertu izan da, eta badirudi mekanismo patologiko ugari badaudela tarteko. Honekin lotuta, zelula glialetan zentratutako atal berri bat sortzen ari da ikerketa arloan.

Mikroglia, CNSeko hantura-zelula nagusia da, eta ezinbesteko eginkizuna dauka CNSari eragindako erasoekiko erantzunaren aurrean (Ajami et al., 2007).

Gaixotasunaren prozesuan zehar aktibatzen direnean, mikrogliako zelulek Aβ pilaketa gutxitzen dute fagozitosi eta garbiketaren bitartez, baina ur-jauzi proinflamatorio ugari ere aktibatzen dituzte, erantzun neurotoxikoan eta sinapsien endekapenean ere parte hartuz (berrikuspenerako Sarlus eta Heneka, 2017).

Astrozitoak kalte handiena jasan duten guneetan erreaktiboago bihurtzen direla ematen du, SPs-ak inguratuz (berrikuspenerako Rodriguez et al., 2009). Gainera, gaixotasunaren prozesu inflamatorioan parte hartzen dutela dirudi (Medeiros eta LaFerla, 2013).

Gaur egun AD tratatzeko dauzkagun baliabide terapeutikoen eraginkortasuna mugatua da. Hori dela eta, bereziki beharrezkoa da endekapen neuronala saihestu edo atzeratzen dituzten agente berriak lortzea (berrikuspenerako Massoud eta Leger, 2011; Beauquis et al., 2013). ES neuromodulazioan parte hartzen duen sistema garrantzitsua da (berrikuspenerako Talarico et al., 2019; berrikuspenerako Lu eta Mackie, 2016) eta ADeko patogenesiaren barne dauden hainbat prozesuetan behartu izan den kanabinoideen eragina ikusita, gaixotasunaren tratamenduan euren erabilera posible izan zitekeela pentsa dezakegu (berrikuspenerako Talarico et al., 2019). Hala ere, gutxi dakigu gaur egun ADean zelula glialetan ematen diren hartzaile kanabinoideen adierazpen eta kokapenaren inguruan. Doktoregoko tesi honetan planteatzen dugun hipotesia hurrengoa da: Glia zeluletako CB₁R eta CB₂R-en adierazpenean alterazioak agertzen direla ADeko sagu eredu baten subikuluan (SPs-pilaketek bereziki afektatu ohi dute garun eremua), mikroglia eta astrozitoetan gertatzen diren zelula-mailako

aldaketen ondorioz. Beraz, ADean ematen den gliaren erreaktibitatearen eta mikroglia eta astrozitoetako CB_1Rren eta CB_2Rren adierazpenaren arteko erlazioa jakiteak gaixotasun honen fisiopatologian ES-ak izan dezakeen eginkizuna ulertzen lagungarria izango litzateke.

4. HELBURUAK

Nire doktorego tesiaren helburu nagusia ADeko sagu eredu batean CB_1R eta CB_2R kannabinoideen adierazpen gliala ikertzea zen, bereziki subikuluko erreaktibitate astrozitikoa eta mikrogliala. Baita CB_1Rren kokapena astroglian eta mikroglian eta CB_2Rren kokapena mikroglian.

Subikuluan ardaztu nintzen 1) SPs kopuru handiena metatzen duelako; 2) erreakzio glial garrantzitsua erakusten duelako; 3) endekapenezko ezaugarri gehien erakusten dituen eskualdeetako bat delako; eta 4) oroimenaren eraketan inplikatuta dagoelako.

Helburu espezifikoak honako hauek ziren:

1. Morfologia mikrogliala aztertzea.
AD-ak eragiten al die prozesu mikroglialen azalerari, perimetroari eta kopuruari?

2. Astrozitoen morfologia ikertzea.
AD-ak aldatzen al ditu prozesu astrozitikoen azalera, perimetroa eta kopurua?

3. Mikroglian dagoen CB_1Rren kokapena aztertzea.
AD-ak ba al du eraginik mikrogliaren CB_1Rren adierazpenean?

4. Astrozitoetan CB_1R non kokatzen den aztertzea.
AD-ak aldatzen al du CB_1Rren adierazpena astrozitoetan?

5. CB_2Rren adierazpena mikroglian deszifratzea.
AD-ak eragina al du zelula mikroglialetako CB_2Rren adierazpenean?

Helburu horiek lortzeko, $CB2^{EGFP/f/f}/5xFAD$ saguetan eta $CB2^{EGFP/f/f}$ kontrol-saguetan mikroskopia immunoelektronikoa erabili nuen.

5. MATERIAL ETA METODOAK

5.1. ADIERAZPEN ETIKOA:

Animaliak zaindu eta erabiltzeko protokoloak Euskal Herriko Unibertsitateko Animalien Ongizaterako Etika Batzordeak onartu zituen (M20/2015/093), eta bat etorri ziren Europako Erkidegoen Kontseiluaren 2010eko irailaren 22ko Zuzentarauarekin (2010/63/EB) eta Espainiako araudiarekin (53/2013 Errege Dekretua, BOE, 2013/02/08). Animalien kopurua eta sufrimendua gutxitzeko ahaleginak egin ziren.

5.2. ESPERIMENTAZIORAKO ANIMALIAK:

Lan hau bost AD (5xFAD) mutazio adierazten dituzten CB2$^{EGFP/f/f}$ sagu transgenikoekin egin zen. Julián Romero Paredes doktoreak (Universidad Francisco de Vitoria, Madril) eman zituen lankidetza proiektuaren alorrean. CB2$^{EGFP/f/f}$ saguak Genoway-ren instalazioetan sortu ziren (Lyon, Frantzia), saguaren cnr2 genearen 3' itzuli gabeko eremuan (*untranslated region*; UTR) txertatutako barneko erribosoma-sarreraren sekuentzia (*internal ribosomal entry site sequence*; IRES) baten ondoren indartutako proteina fluoreszente berdearen (*enhanced green fluorescent protein*; EGFP) gene erreportaria sartzean datzan fokalizazio-estrategia baten bidez. Honen emaitza, gene erreportariaren (EGFP) adierazpena saguaren cnr2 genearen sustatzaile endogenoaren kontrolpean ematea da, eta baita CB$_2$H proteinaren mRNA bizistronikoaren transkripzio berdina ematea ere. Gainera, sagu horiek AGaren bost mutazio familiar (5xFAD) adierazten dituzte aldi berean. C57BL/6J jatorria duten 5xFAD saguak Jackson laborategian erosi ziren (Harbor taberna, Main, AEB). 5xFAD saguak CB2$^{EGFP/f/f}$ saguekin parekatu ziren gutxienez bost belaunalditan zehar, aldibereko adierazpena zuten CB2$^{EGFP/f/f}$/5xFAD saguak sortzeko (López et al., 2018). 5xFAD ereduak ez dirudi endekapen neurofibrilarra eragiten duen mutazioa adierazten duenik (Oblak et al., 2021), baina again zeharka gerta liteke endekapen neuronalaren eta Aβ 1-42 metaketen bidez.

Proteina berde fluoreszentearen (*green fluorescent protein*; GFP) kontrako antigorputzaren espezifikotasuna frogatzeko, CB$_2$R-knockout (cannabinoid receptor type 2-knock out; *CB2$^{-/-}$*) saguak erabili ziren, CB$_2$R adierazten ez dutenak, eta ondorioz GFP ezta (8. irudia; 9 a,b irudiak).

Tesi honetan, 6,5 eta 7,5 hilabete arteko sagu arren eskualde subikularrak erabili ziren, ikusgaiak ziren metaketa amiloideekin eta neurita distrofikoekin. Astrozitoak 5,596.477 μm^2-tan aztertuak izan ziren 5 sagu CB2$^{EGFP/f/f}$-tan eta 7,681.429 μm^2-tan 7 sagu CB2$^{EGFP/f/f}$/5xFAD-tan. Honez gain, prozesu mikroglialen azalera, perimetroa eta kopurua 7,900.338 μm^2-tan neurtu ziren 7 sagu CB2$^{EGFP/f/f}$-tan eta 10,792.572 μm^2-tan 10 sagu CB2$^{EGFP/f/f}$/5xFAD-tan. CB$_1$H mikroglian aztertzeko, 6,344.998 μm^2 hartu ziren 5 CB2$^{EGFP/f/f}$

saguetan eta 7,268.343 μm^2 7 CB2$^{EGFP/f/f}$/5xFAD saguetan. Azkenik, mikroglian CB$_2$R aztertzeko, 3,524 μm^2 aztertu ziren 3 CB2$^{EGFP/f/f}$ saguetan eta 4,078 μm^2 3 CB2$^{EGFP/f/f}$/5xFAD saguetan.

5.3. GARUN-EHUNAREN GORDETZEA:

Saguak ketaminaz/xilazinaz anestesiatu ziren (100 mg/10 mg/kg gorputz-pisuko, peritoneo barneko injekzioa), eta ondoren ezkerreko bentrikuluaren bidez bihotzean zeharreko perfusioa egin zen giro-tenperaturan (Room temperature; RT). Lehenik, gatz fosfatozko disoluzio indargetzaileaz (*phosphate buffered saline*; PBS) 0,1 M (pH 7,4) 20 segundoz, eta, ondoren, % 4-ko formaldehidoz osatutako soluzio finkatzaileaz (paraformaldehidoz despolimerizatu berria), % 0,2-ko azido pikrikoaz eta % 0,1-eko glutaraldehidoaz PBS 0,1 M (pH 7,4) disoluzioan, 10-15 minutuz. Ondoren, garunak garezurretik atera ziren eta disoluzio finkatzailean post-finkatu ziren astebetez, gutxi gorabehera, 4°C-tan. Ostean, 1:10-era diluitutako soluzio finkatzailean biltegiratu ziren, 4°C-tara ,% 0,025-eko azida sodikoarekin, behar bezala babesteko. Garunak 50 μm-tara sekzio koronaletan moztu ziren bibratomo batean, eta 12 edo 24 putzudun zelula-hazkuntzako plaketan biltegiratu ziren, 1 mL fosfatozko tanpoi disoluzioa (*phosphate buffer*; PB) 0,1 M (pH 7,4) eta % 0,025-eko azida sodikoarekin 4 ºC-tan.

5.4. ABIDINA-BIOTINA PEROXIDASA METODOA ARGI-MIKROSKOPIARAKO:

Protokolo hau erabili izan da:

1. 50 μm-tako eremu subikularreko bibratomarekin ebakitako garunaren sekzio koronarioen eremu subikularrak hautatu ziren. 12 edo 24 putzudun zelula-hazkuntzako plaketan bildu ziren, eta PB 0,1 M-tan (7,4 pH) mantendu ziren.

2. Sekzioak aurreinkubatu egin ziren blokeo-soluzioarekin, Trisekin tanponatutako gatz-disoluzioan (*1X Tris-buffered saline*; TBS 1X) (7,4 pH) disolbatutako % 10eko behi-serum albuminarekin (*Bovine serum albumin; BSA*), % 0,1-eko azida sodikoarekin eta % 0,5eko X-100 tritoiarekin (7,4 pH) 30 minutuz RTan..

3. Ondoren, blokeo soluzioan prestatutako CB$_1$Rren kontrako (1:100), GFParen kontrako (1:500), 1 motako kaltzio ionizatua finkatzeko molekula egokitzailearen kontrako (*ionized calcium-binding adapter molecule 1*; Iba1) (1:500) edo 1 motako glutamato aspartatoaren garraiatzailearen kontrako (*Glutamate aspartate transporter 1*; GLAST) (0,3 μg/ml) antigorputzak (2. taula) egun batean RTan edo 2 egunetan 4°C-tara inkubatu ziren lehuntasunez irabiatuz.

4. Sekzioak % 1eko BSA-dun eta % 0,5eko X-100 tritodun TBS 1X-ean garbitu ziren 30 minutuz, gehiegizko antigorputzak kentzeko (3 x 1 min eta 2 x 10 min).

5. Ondoren, disoluzio garbitzailean diluituriko antigorputz sekundario biotinilatu batekin inkubatu ziren, indietako untxiaren aurka (1:200, Biotin-SP-AffiniPure Goat Anti-Guinea Pig), arratoiaren aurka (1:200, Biotin-SP-AffiniPure Goat Anti-Rat IgG) edo untxiaren aurka (1:200, Biotin-SP-AffiniPure Donkey Anti-Rabbit IgG), RTan ordubetez irabiagailuan.

6. Sekzioak % 1eko BSAdun eta % 0,5eko Tritoi X-100-dun TBS 1X-en garbitu ziren (3 x 1 min eta 2 x 10 min).

7. Ondoren, ehuna abidina-biotina peroxidasa konplexuarekin (*avidin-biotin-peroxidase complex*; ABC) inkubatu zen (1:50 avidin-biotin peroxidase complex, Elite, Vector Laboratories, Burlingame, ca, AEB), garbiketa-soluzioan prestatuta, ordubetez RTra.

8. Laginak garbitu ziren BSArekin % 1ean eta X-100 tritoiarekin % 0,5ean, TBS 1X-en disolbaturik (3 x 1 min) eta azkenik PB 0,1M-en (pH 7,4) disolbatutako X-100 tritoiarekin % 0,5ean (2 x 10 min).

9. Markatzea % 0,05eko diaminobentzidina (*diaminobenzidine*; DAB) kromogenoarekin errebelatu zen, PB 0,1 M-tan (pH 7,4) disolbaturik, X-100 tritoiarekin % 0,5 ean eta % 0,01eko hidrogeno peroxidoarekin (*hydrogen peroxide*; H_2O_2), 3,5 minutuz RTra.

10. Ondoren, sekzioak PB 0,1 M-en (pH 7,4) garbitu ziren, X-100 tritoiarekin % 0,5ean (3 x 1 min eta 2 x 10 min).

11. Ehun-sekzioak porta gelatinizatuetan muntatu ziren.

12. Lehortu ondoren, deshidratatu egin ziren etanol graduatuan (50°, 70°, 96° eta 100°) 5 minutuz.

13. Ebakidurak xilenoarekin garbitu ziren (3 x 5 min).

14. Portak DPXz estali ziren.

15. Ehun subikularraren azterketa eta argazkiak egin ziren Zeiss AxioCam HRc kamera bati akoplatutako Zeiss AxioCam argi-mikroskopio batekin.

5.5. ERRETXINAN MURGILDU AURREKO IMMUNO-URRE ETA IMMUNO-PEROXIDASAREN METODO BIKOITZA MIKROSKOPIA ELEKTRONIKORAKO:

Erabilitako protokoloa gure laborategiak argitaratu du (Puente et al., 2019):

1. Lau eta bost sekzio bitarte aukeratu ziren garun bakoitzeko, hipocanpo-kaudal eta subikulua zituztenak, eta plaka berri batean jarri ziren.

2. Blokeo-soluzio batean (1 mL/kikara) aurreinkubatu ziren, %10eko BSAz, % 0,02ko saponinaz eta % 0,1eko azida sodikoaz TBS 1X-en (7,4 pH), 30 minutuz irabiagailuan (300 rpm) RTra.

3. Ondoren, ehuna bi antigorputz primarioz inkubatu zen (2. taula): indietako untxian egindako CB$_1$Raren kontrako antigorputz poliklonala 1:100-ean diluitua (Frontier Institute Co, ltd) edo arratoian egindako GFParen kontrako antigorputz monoklonala, 1:500ean diluitua (Nacalai), untxian egindako GLASTen kontrako antigorputz poliklonalarekin (0.3 µg/mL; Gifted by Prof. Niels Christian Danbolt) konbinatuta, edo/eta untxian egindako Iba1en kontrako antigorputz poliklonalarekin (FUJIFILM Wako Pure Chemical Corporation) 1:500era diluituta. Disoluzioak % 10eko BSA, % 0,1eko sodio azidoa eta % 0,004eko saponina zuen TBS 1Xn. Inkubazioa irabiagailu batean egin zen 2 egunez, 4 °C-an.

4. Ondoren, bost garbiketa egin ziren (3 x 1 min eta 2 x 10 min), % 1eko BSAdun TBS 1X-ekin, antigorputz soberakina kentzeko.

5. Antigorputz sekundarioen inkubaziorako (2. taula): sekzioak 1,4 nm-ko urrearekin konjugatutako ahuntzean egindako indietako untxiaren kontrako IgG antigorputzarekin (Fab' fragment, 1:100, Nanoprobes Inc, Yaphank, NY, AEB) edo 1,4 nm-ko urrearekin konjugatutako ahuntzean egindako arratoiaren kontrako IgG antigorputzarekin (Fab' fragment, 1:100, Nanoprobes Inc, Yaphank, NY, AEB) inkubatu ziren. Untxiaren aurkako IgG antigorputz biotinilatuarekin ere inkubatu zen (1:200, Biotin-SP-AffiniPure Donkey Anti-Rabbit IgG). Dena % 1eko BSAdun eta % 0,004ko saponinadun TBS 1X-en disolbaturik, irabiagailu batean, 4 orduz RTan.

6. Ehuna % 1eko BSAdun TBS 1X-en garbitu egin zen (3 x 1 min eta 2 x 10 min) RTan irabiagailu batean, gehiegizko antigorputza sakonki kentzeko.

7. Ondoren sekzioak garbitzeko disoluzioan prestatutako ABCrekin (1:50) inkubatu ziren (1 ml/putzu), 1,5 orduz giro-tenperaturan.

8. Atalak % 1eko BSAdun TBS 1X-ekin garbitu ziren (3 x 1 min, 1 x 10 min), eta garbitze-soluzio berean mantendu ziren gau osoan zehar, 4 ºC-an, irabiatuz.

9. Hurrengo egunean, ehuna % 1eko glutaraldehidoarekin post finkatu zen TBS 1X-n (1 mL/putzu) 12 minutuz RTan.

10. Ondoren, sekzioak ur destilatu bikoitzean garbitu ziren (3 x 10 min bakoitza).

11. Saio-hodietara transferitu ziren.

12. Urrezko partikulen intentsifikazioa egin zen zilarrareikin, HQ Silver kitarekin (Nanoprobes Inc., Yaphank, NY, AEB; 1 mL/hodi) iluntasunean, 12 minutuz RTan.

13. Intentsifikazioaren ondoren, sekzioak beirazko bialetara aldatu ziren (15 mL, 3 x 5 cm) eta hiru aldiz garbitu ziren ur destilatu bikoitzean (1 min bakoitza) iluntasunean.

14. PB 0,1 M (pH 7,4) erabiliz hainbat aldiz garbitu ziren ilunpean 30 minutuz.

15. Antigorputz biotinilatua % 0,05eko DABarekin errebelatu zen, % 0,01eko hidrogeno peroxidoarekin eta % 0,5eko X-100 tritoiarekin, PB 0,1 M-en (pH 7,4) (1 mL/putzu) 3,5

minutuz RTan, eta, ondoren, zenbait garbiketa burutu ziren PB 0,1 M-en (pH 7,4) (3 x 1 min eta 2 x 10 min).

16. Sekzioak osmifikatu egin ziren (osmio tetroxidoa % 1ekin PB 0,1 M-ean (pH 7,4) (1 mL/bialeko) iluntasunean 20 minutuz.

17. PB 0,1 M disoluzioarekin garbitu ziren (pH 7,4) (3 x 1 min eta 2 x 10 min).

18. Ondoren, sekzioak deshidratatu egin ziren EtOH-n (% 50, % 70, % 96; 5 min/bakoitza), eta ondoren EtOH-n % 100ean (3 x 5 min) (1 mL/bialeko).

19. Propileno oxidoan garbitu ziren (3 x 5 min) (1mL/bialeko).

20. Sekzioak 1:1 propileno oxido/Epon 812 erretxinean murgildu ziren (1 ml/bialeko) irabiagailu batean, gau osoan zehar RTan.

21. Ondoren, Epon 812 erretxina hutsean (1 ml/bialeko) murgildu ziren, gutxienez 2 orduz RTan.

22. Ondoren, ehuna bi beirazko porten artean jarri ziren, aluminio-paperean bilduta, eta berogailuan utzi ziren, 60 ºC-an, bi egunez.

23. Erretxinan sartutako sekzioak aluminio-paperetik bereizi ziren eta subikuluko eremua moztu zen.

24. Ondoren, ehuna erretxinazko molde batean itsatsi zen, 1 μm-ko lodiera duen ebakidura bat mozteko histo-diamantezko hortz batekin (Diatome AEB) ultramikrotomo batean, eta % 1eko toluidina-urdinez tindatu zen.

25. Intereseko eremura iristean, 50 nm-ko lodierako sekzioak moztu ziren ultra diamantezko hortz batekin, eta nikelezko saretan bildu ziren.

26. Saretak gutxienez bi orduz kanpaian lehortu ondoren, sekzioak berunezko zitratoarekin (% 2,5) kontrastatu ziren (tanta bat/sareta bakoitzeko) 20 minutuz RTan.

27. Azkenik, ur destilatu bikoitzean garbitu ziren, 1 tanta/sareta (5 x 10 min bakoitza).

28. Tindatutako saretak transmisiorako mikroskopio elektroniko baten bidez aztertu ziren (JEOL JEM 1400 Plus, Kanada), eta mikroskopio elektronikoan txertatutako Hamamatsu FLASH kamera digital batekin argazkiak egin zitzaizkion ehunari. Erreferentzia-puntu anatomikoak hartu ziren subikuluaren eremua aurkitzeko.
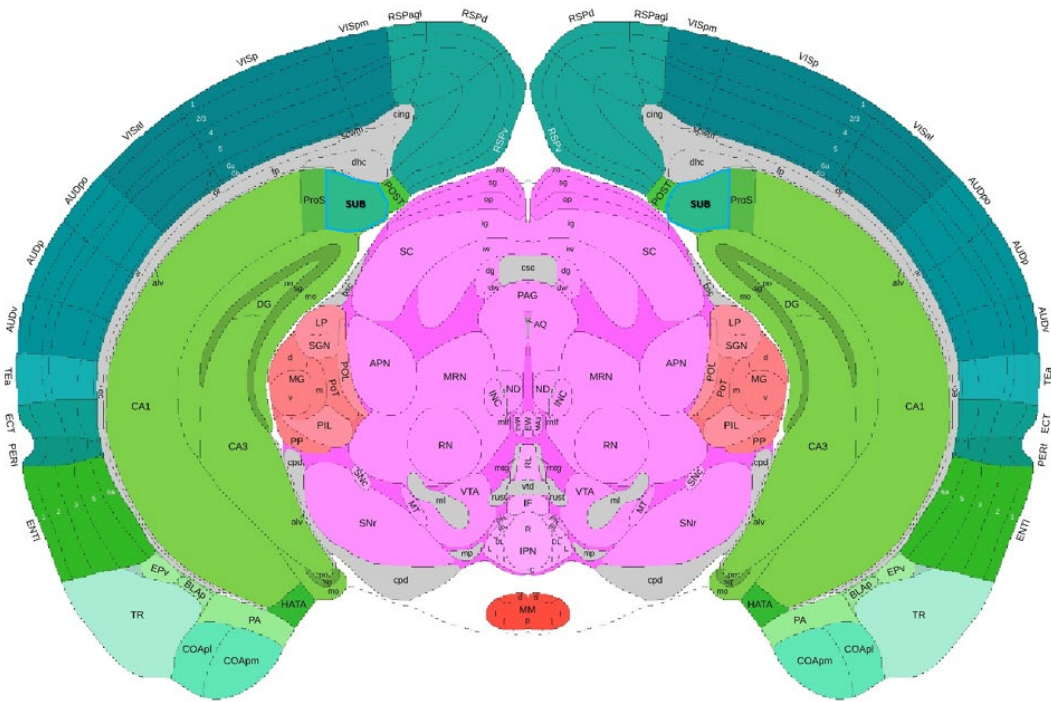
6. irudia. Sagu baten garunaren ebakidura koronala (Allen Mouse Brain Atlas-etik eraldatua; © 2004 Allen Institute for Brain Science). Allen Mouse Brain Atlas. Eskuragarri hemen: atlas.brain-map.org. Subikulua urdinez delineatuta dago (SUB).
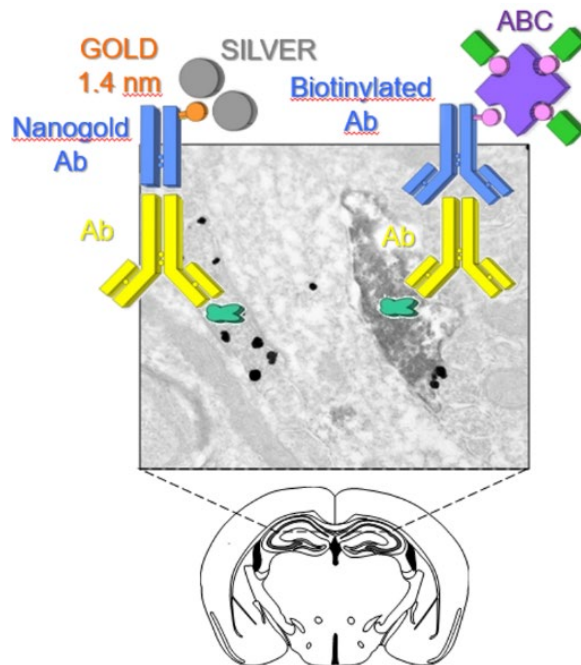

7. irudia. Transmisioko mikroskopia elektronikorako immunomarkaketa bikoitzeko metodoa (Puente et al., 2019-ren baimenarekin egokitua).
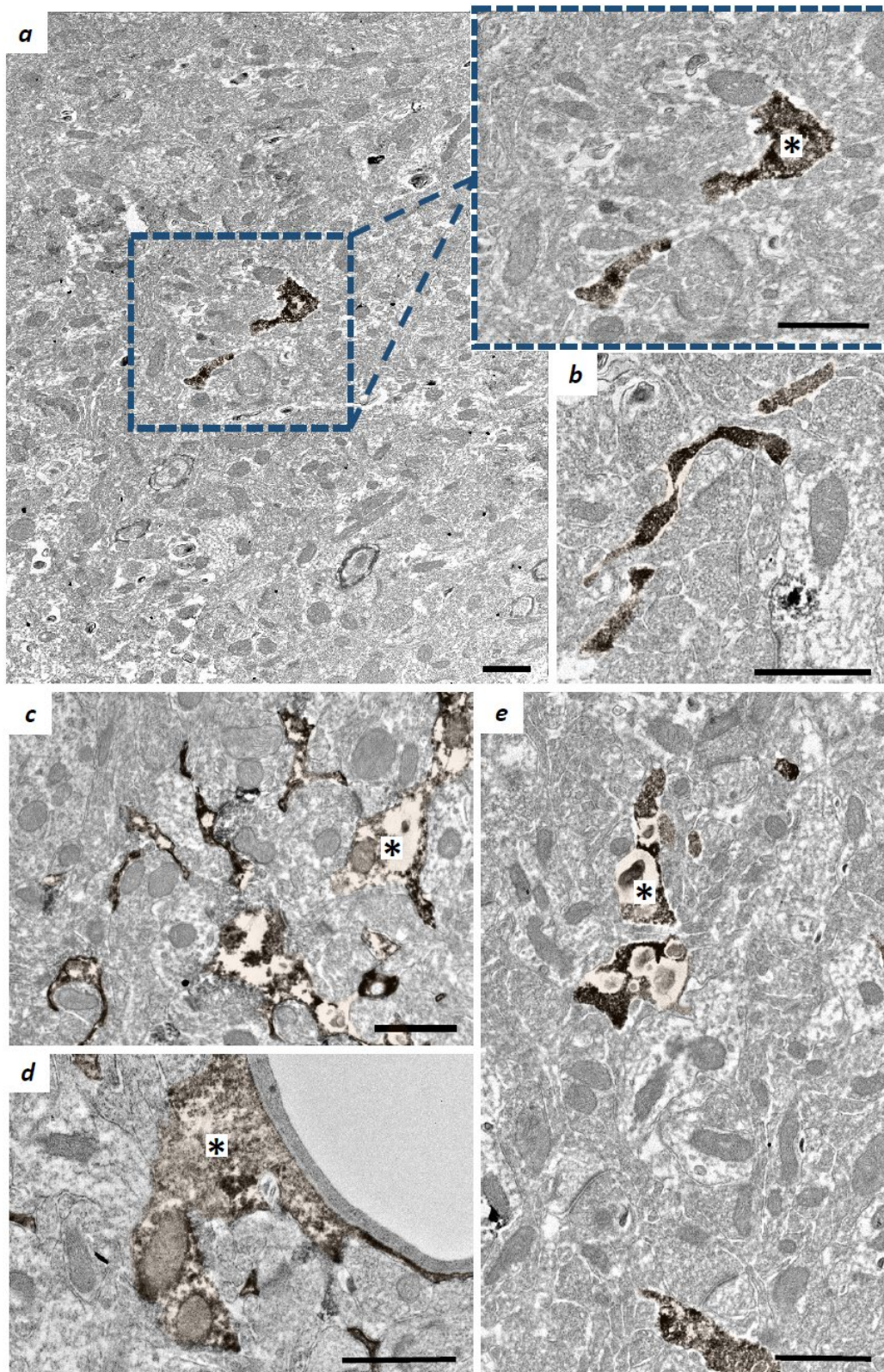
5.6. ANTIGORPUTZAK:

Esperimentuak baldintza berdinetan egin ziren. Gainera, kontrol negatiboak erabili ziren, antigorputz primarioak alde batera utziz. Honetaz gain, CB_1Raren kontrako antigorputza $CB_1^{-/-}$ garunean frogatu zen (9 c, d, e irudiak) eta GFPren kontrako antigorputza $CB_2^{-/-}$ saguetan (8. irudia; 9a, b irudiak).

Azkenik, erabilitako beste antigorputzen espezifikotasuna zehatz-mehatz berretsi zen aurreko ikerketetan (Bjørnsen et al., 2007; Delcambre et al., 2016).
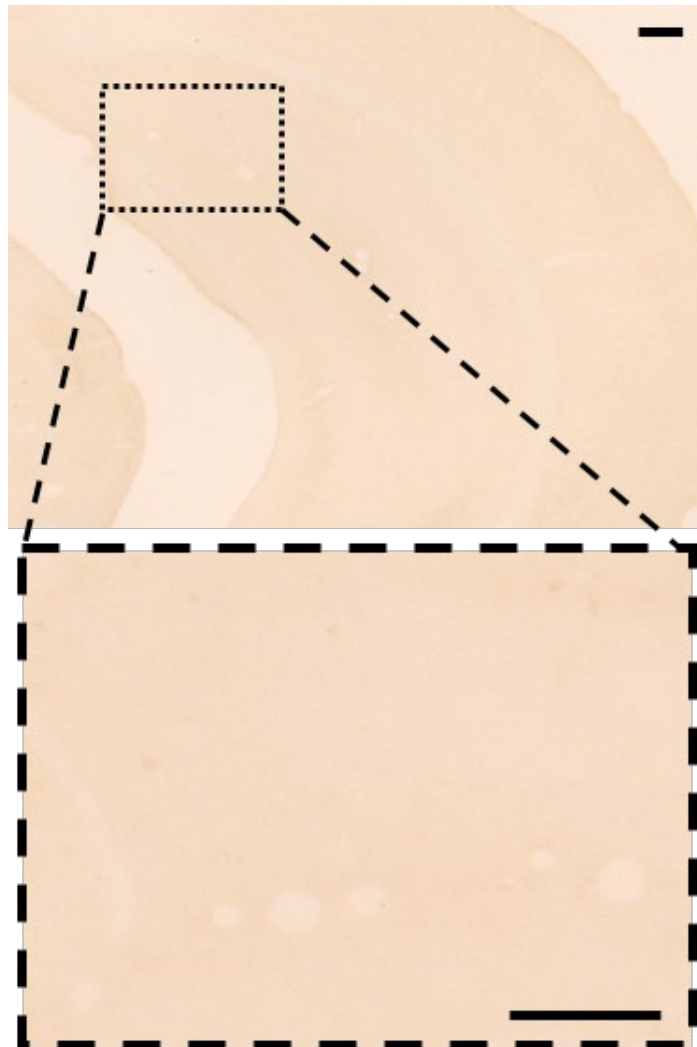
8. irudia. CB2$^{-/-}$ saguaren garuna, GFP kontrako antigorputzak erabiliz. Abidina-biotina peroxidase metodoa argi-mikroskopiarako. Subikuluan ez da tindaketa-zeinu espezifikorik nabaritzen (handittako eremua markoztuta). Eskala barrak: 200 μm.

9. irudia. 1 edo 2 motatako hartzaile kannabinoide ezabatudun subikuluak. Mikroskopia elektronikorako immunogold eta immunoperoxidasaren metodo bikoitza. Antigorputzak CB$_2^{-/-}$ (a, b) eta CB1$^{-/-}$ (c, d, e) saguetan frogatu ziren. (a, b) Soilik ikusten da GFP/CB$_2$ hondoa Iba1en (DAB, marroia, *) eta GFP/CB$_2$ren (urrea) aldi bereko markaketa erakusten duten irudietan. Hondar ez-espezifikoa ere ikusten da GLAST (DAB, marroia, *) eta CB$_1$R (urrea) aldibereko markaketa duten irudietan (c, d), eta Iba1 (DAB, marroia, *) eta CB$_1$R (urrea) markaketa konbinatuen (e) irudietan. Eskala barrak: 1 µm.

46

2. taula. Erabilitako antogorputzen xehetasunak.

ANTIGORPUTZA	FABRIKATZAILEA	OSTALARIA	KONTZENTRAZIOA
CB_1Raren kontrako antigorputz poliklonala; CB_1-GP-Af530; AB_2571593	Frontier Institute Co., ltd	Indietako untxia	(1:100)
A522 (EAAT1 [GLAST])-en kontrako antigorputz poliklonala; Ab#314; AB_2314561	Gifted by Prof. Niels Christian Danbolt University of Oslo	Untxia	(0.3 µg/mL)
Iba1en kontrako antigorputz poliklonala; 019-19741	FUJIFILM Wako Pure Chemical Corporation	Untxia	(1:500)
GFPren kontrako antigorputz monoklonala; GF090R	Nacalai	Arratoia	(1:500)
Indiako untxiaren kontrako ahuntzean egindako Biotin-SP-AffiniPure IgGa (H+L); poliklonala; AB_2337394	Jackson Immuno Research	Ahuntza	(1:200)
Untxiaren kontrako astoan egindako Biotin-SP-AffiniPure IgGa (H+L); poliklonala; AB_2340593	Jackson Immuno Research	Astoa	(1:200)

Arratoiaren kontrako ahuntzean egindako Biotin-SP-AffiniPure IgGa (H+L); poliklonala; AB_2338179	Jackson Immuno Research	Ahuntza	(1:200)
Indiako untxiaren kontrako ahuntzean egindako 1.4 nm urrez-konjugatutako Nanogold®-Fab´ IgGa (H+L) #2055	Nanoprobes	Ahuntza	(1:100)
Arratoiaren kontrako ahuntzean egindako 1.4 nm urrez-konjugatutako Nanogold®-Fab´ IgGa (H+L) #2008	Nanoprobes	Ahuntza	(1:100)

5.7. CB_1 HARTZAILEAREN ETA GFPREN MARKAKETAREN KUANTIFIKAZIOA ETA EBALUAZIO ESTATISTIKOA:

Lagin guztien artean markaketa homogeneoa bermatzeko, espezimen bakoitzaren sekzioaren azaleraren lehen 1,5 µm-ak baino ez ziren jaso (antigorputzaren barneratzea ez da fidagarria sakonera horretatik haratago). Interesekoak ziren eremuei ausaz atera zitzaizkien argazkiak subikuluaren barruan, kalte larriak erakusten zituztelarik, neurita distrofiko eta terminal sakabanatu ugarirekin, eta prozesu astrozitiko eta mikroglialekin ere. Zelula-gorputz mikroglialak edo astrozitikoak zituzten mikrografia elektronikoak baztertu ziren, haien tamaina handiak balio okerrak eman zitzakeelako. Astrozitoetako CB_1Rak GLASTentzako immunoerreaktiboak ziren prozesu astrozitikoetan ebaluatu ziren. Mikrogliako CB_1Rak Iba1entzat immunopositiboak ziren mikroglia-prozesuetan ikertu ziren. Ondoren, CB_1Rrako markatutako konpartimentu zelularren proportzioa kalkulatu zen. Etiketatzea positibotzat jo zen, gutxienez CB_1R immunopartikula bat aztertutako konpartimentuko mintzetik 30 nm-ren barruan zegoenean. CB_1Raren dentsitatea (mintzeko partikulak/µm) ere zehaztu zen konpartimentu positiboetan CB_1R immunopartikulak zenbatuta. Mintzen luzeerak (perimetroak) Image-J (NIH; RRID: SCR_003070) softwarearekin neurtu ziren.

CB_2Ra aztertu zen Iba1arekiko mikroglia-prozesu positiboetan. Urrezko GFP partikulak zenbatu ziren, eta mintzean (mintzetik 30 nm-ra gehienez) edo zitosolean (30 nm-tik gorako

distantzian) kokatzen ziren bereiztu zen. Bereizketa hori egin da animalia-eredu horretan proteina hori (GFP) non kokatzeko joera duen den zehazten saiatzeko, ez baitu zertan egon behar CB_2Rren leku berean zelularen barruan.

Balio guztiak batez bestekoa ± bataz bestekoaren errore estandarra (standard error of the mean; S.E.M.) gisa adierazi ziren estatistikako software pakete bat erabiliz (GraphPad Prism 5, GraphPad Software Inc., San Diego, AEB). Normaltasun froga (Kolmogorov-Smirnov normaltasun froga) beti aplikatu zen estatistika probak egin aurretik. Datuak bataz besteko ez-parametrikoen edo parametrikoen bidez aztertu ziren: Mann-Whitneyren U froga edo parekatu gabeko Studenten t froga (* P<0,05).

Irudietan kontraste eta distira doikuntza txikiak egin ziren Image-J softwarea (NIH; RRID: SCR_003070), Adobe Photoshop eta Gimp erabiliz.

6. EMAITZAK

6.1. Argi-mikroskopiaren bidezko gliaren morfologiaren deskribapena CB2$^{EGFP/F/F}$/5xFAD saguen subikuluan.
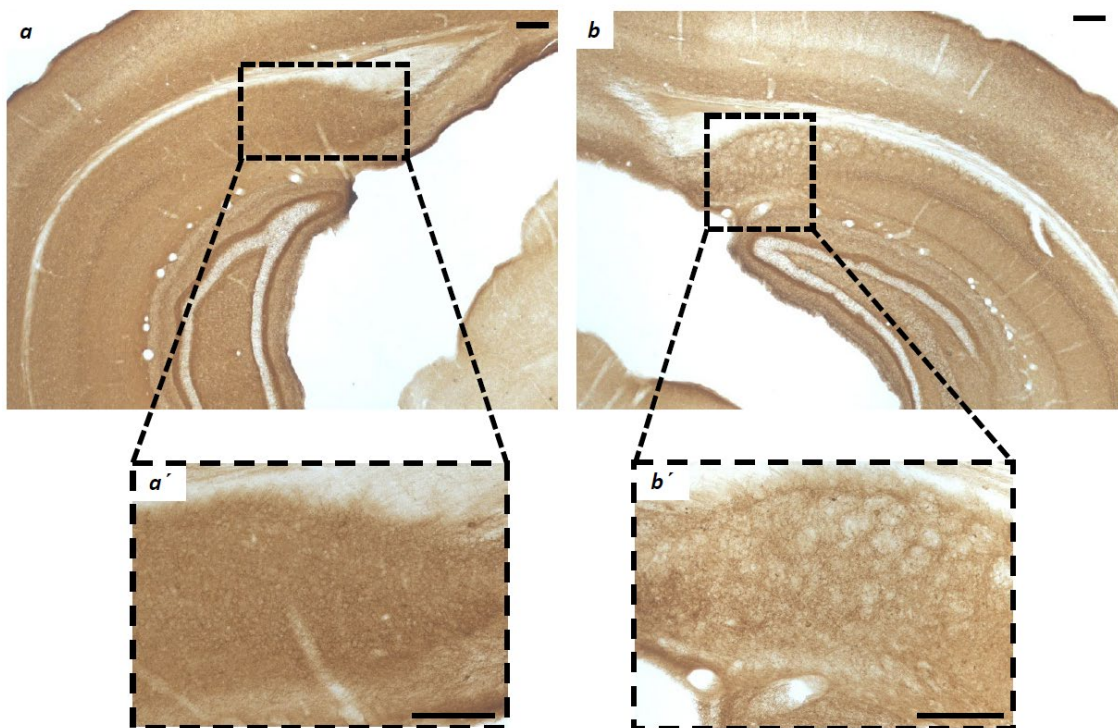
CB2$^{EGFP/f/f}$/5xFAD saguen subikuluko zelula glialetan gertatzen diren aldaketen ikuspegi orokorra izateko, CB2$^{EGFP/f/f}$ saguekin alderatuta, mikrogliaren eta astrozitoen Iba1 eta GLAST markaketak bistaratu ziren, hurrenez hurren, argi-mikroskopiarako abidina-biotina peroxidasa metodoa erabiliz. Bi zelula glialen tindaketa-dentsitateen aldaketak hauteman ziren (10. irudia). Atzemandako mikroglia zelulek CB2$^{EGFP/f/f}$ saguetan baino (10a, a´ irudiak) askoz eremu handiagoa hartzen zuten CB2$^{EGFP/f/f}$/5xFAD saguetan (10b, b' irudiak). Hazkunde horrek zerikusia zuen, antza, bai zelula mikroglialen kopuruarekin, bai haien prozesuen lodierarekin, eta horrek aktibazio mikrogliala zegoela iradokitzen du. Astrozitoen kasuan, CB2$^{EGFP/f/f}$ saguen GLAST tindaketa (10c, c' irudiak) baino biziagoa zen CB2$^{EGFP/f/f}$/5xFAD saguetan ikusitakoa (10d, d' irudiak), hipertrofia astrozitikoarekin bateragarria. Aurkikuntza hauek mikroskopia elektronikoaren bidez sakonkiago aztertu ziren.
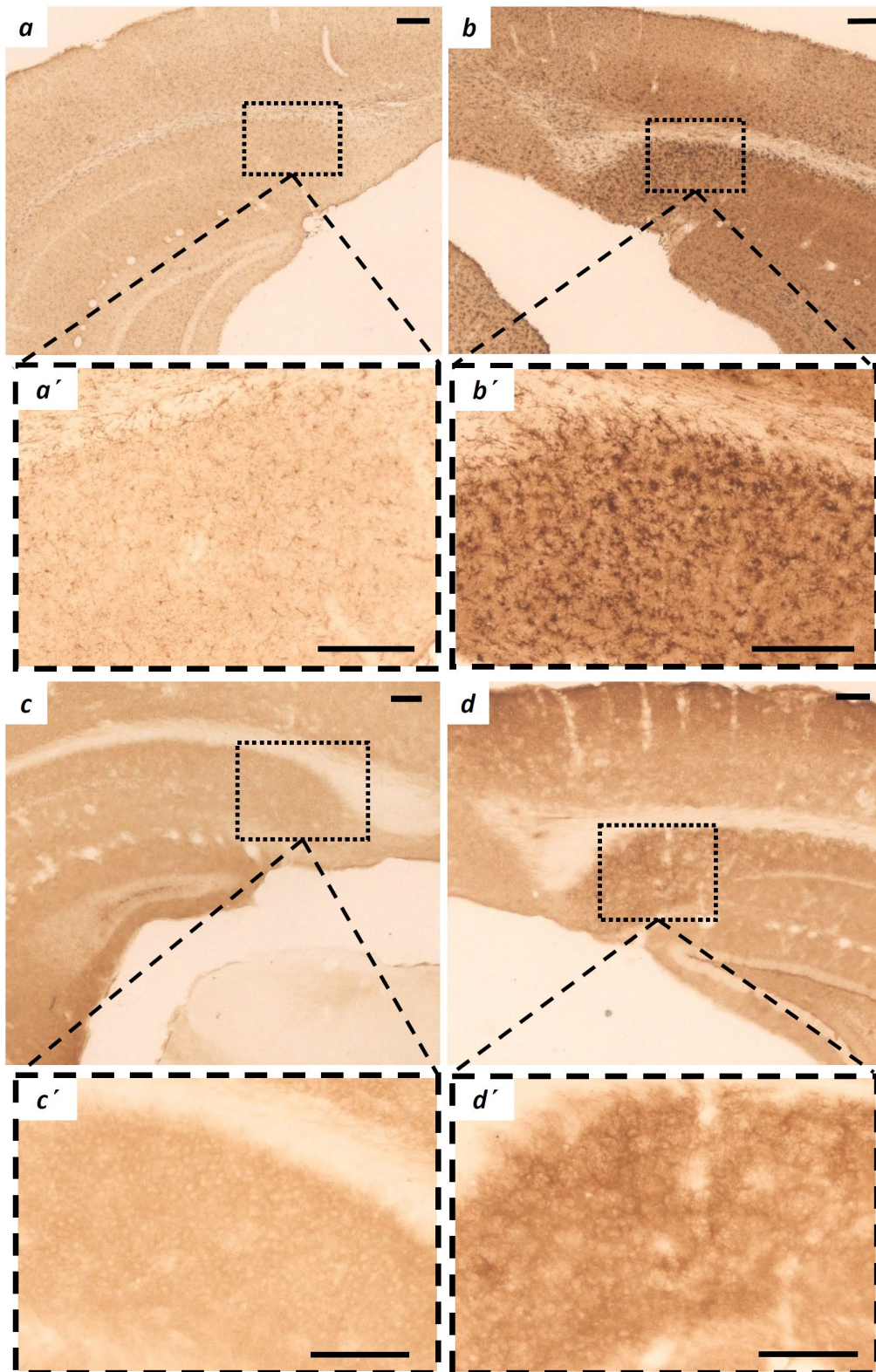
10. irudia. Iba1 eta GLAST CB2$^{EGFP/f/f}$ eta CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan. Argi-mikroskopiarako abidina-biotina peroxidasaren metodoa. CB2$^{EGFP/f/f}$ (a, a') mikroglian behatutako Iba1en tindatze subikularra nabarmen handitzen da zabaldutako (b, b') CB2$^{EGFP/f/f}$/5xFAD mikroglian. CB2$^{EGFP/f/f}$ saguetan (c, c') behatutako GLAST tindaketa ere indartsuagoa da CB2$^{EGFP/f/f}$/5xFAD saguetan (d, d'). Eskala barrak: 200 µm.

6.2. Argi-mikroskopiaren bidezko CB$_1$ hartzailearen adierazpenaren deskribapena Alzheimer familiarraren gaixotasunaren sagu-eredu baten subikuluan.

CB$_1$Raren adierazpenean gertatzen diren aldaketei buruzko ikuspegi orokorra izateko, CB2$^{EGFP/f/f}$/5xFAD saguen subikulu eremuan, CB2$^{EGFP/f/f}$ saguekin alderatuta, ehuna CB$_1$Rren kontrako antigorputzaren bidez tindatu zen (2. taula), argi-mikroskopiarako abidina-biotina peroxidasa metodoa erabiliz. Markaketan aldaketa nabaritu zen CB2$^{EGFP/f/f}$/5xFAD saguetan (11b, b' irudiak) CB2$^{EGFP/f/f}$ saguekin alderatuta (11a, a' irudiak), adabaki itxuraduna. Bazirudien markaketa gutxiagoko eremuak zeudela, SPen presentziarekin bat etor zitekeena, eta haien inguruan markaketa biziagoa zuten eremuak zeuden (11b, 11b' irudiak). CB$_1$Raren adierazpenaren aldaketa horiek sakonago aztertu ziren mikroskopia elektronikoaren bidez, bai mikroglian eta bai astrozitoetan.
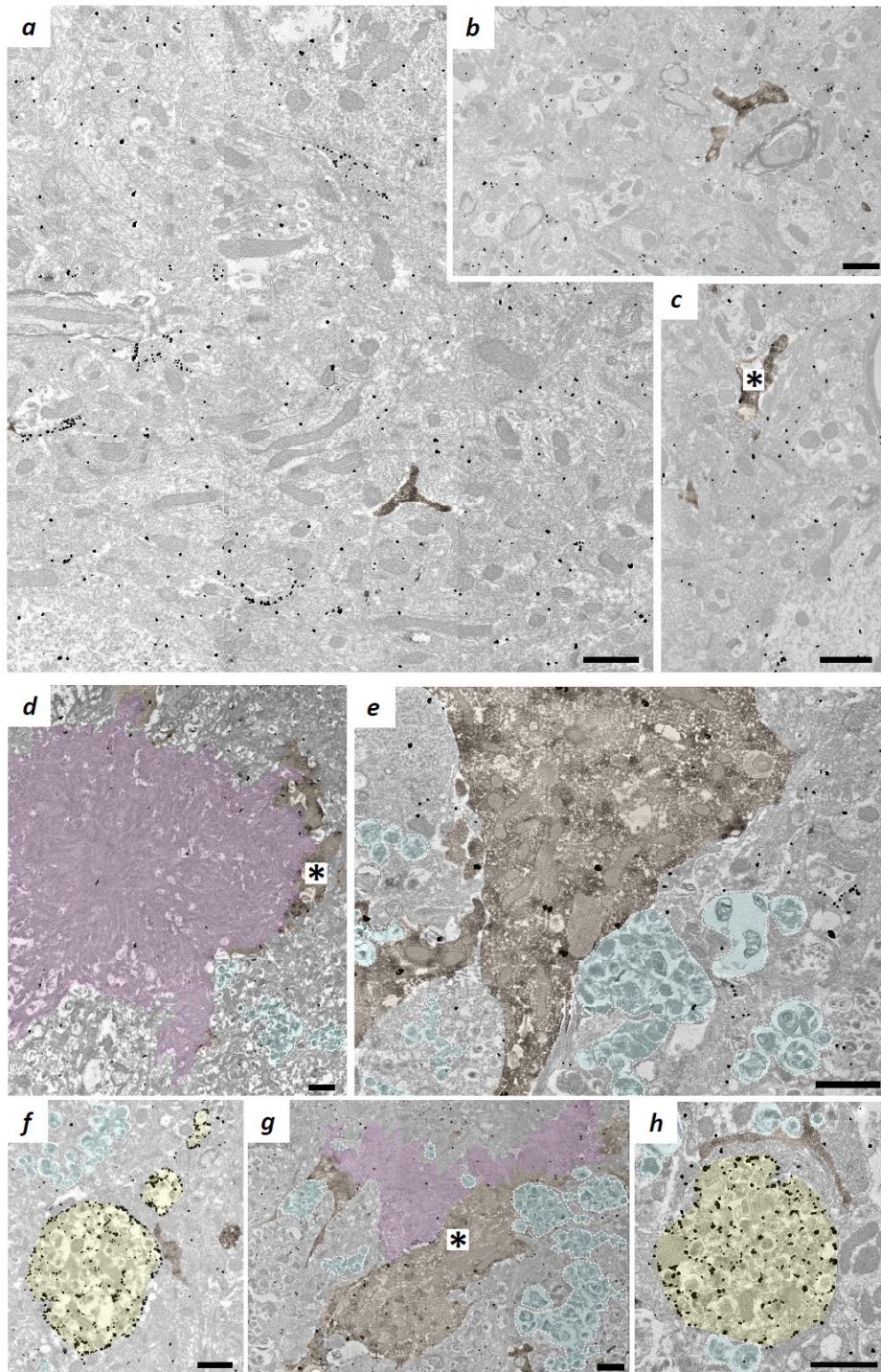

11. irudia. CB1R immunotindaketa CB2$^{EGFP/f/f}$ eta CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan. CB2$^{EGFP/f/f}$ saguen subikuluan (a, a') behatutako CB$_1$R tindaketa, irregularra bilakatzen da CB2$^{EGFP/f/f}$/5xFAD saguetan (b, b'), eta badirudi irregulartasun horiek lesioak dauden lekuekin bat datozela. Eskala barrak: 200 µm.

6.3. ALDAKETAK MIKROGLIA-PROZESUEN MORFOLOGIA ETA KOPURUAN CB2$^{EGFP/F/F}$/5xFAD SAGUEN SUBIKULUAN.

Lehenik eta behin, mikrogliaren morfologia aztertu nuen. DAB immunotindaketa erabili izan zen Iba1 zuten prozesu mikroglialak identifikatzeko (12. irudia). Kontroletan, prozesu mikroglial sakabanatuak baino ez ziren ikusi. CB2$^{EGFP/f/f}$/5xFAD saguetan, berriz, plakak inguratzen zituzten Iba1erako positibo ziren prozesu ugari hauteman ziren (Fig. 12 d and g) eta neurita distrofikoak (Fig. 12 d-h).

Gainera, CB2$^{EGFP/f/f}$/5xFAD saguetan mikroglia-prozesuen azaleraren handipen esanguratsua behatu zen (0.3229 ± 0.05282 μm^2; ***$p < 0.0001$; 13a irudia) CB2$^{EGFP/f/f}$ saguekin alderatuz (0.1000 ± 0.01754 μm^2). Aldaketa hori 100 μm^2-tara normalizatutako lagin bakoitzeko azalera osoan ere ikusgaia zen μm^2 (CB2$^{EGFP/f/f}$/5xFAD: 2.074 ± 0.5156 μm^2; ***$p < 0.0001$; CB2$^{EGFP/f/f}$: 0.3485 ± 0.06955 μm^2; 13b irudia). Gainera, mikroglia-luzapenen perimetroaren handipen esanguratsua ere ikusi zen CB2$^{EGFP/f/f}$/5xFAD saguetan (2.200 ± 0.1248 μm; ***$p < 0.0001$) CB2$^{EGFP/f/f}$ saguekin alderatuz (1.260 ± 0.1036 μm; 13c irudia). Hazkunde hori 100 μm^2-ko lagin normalizatu bakoitzeko mikroglia-prozesu guztien perimetroan ere nabaritu izan zen (15.73 ±1.929 μm CB2$^{EGFP/f/f}$/5xFAD saguetan; 4.334 ± 0.5345 μm CB2$^{EGFP/f/f}$ saguetan; ***$p < 0.0001$; 13d irudia). Azkenik, aldaketa nabarmenak hauteman ziren mikroglia-prozesuen kopuruan ere (7.260 ± 0.6304 CB2$^{EGFP/f/f}$/5xFAD saguetan; 3.413 ± 0.4092 CB2$^{EGFP/f/f}$ saguetan; ***$p < 0.0001$; 13e irudia)

12. irudia. Mikrogliaren bistaratze ultraestrukturala CB2$^{EGFP/f/f}$ eta CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan. Erretxinan murgildu aurreko immuno-urre (CB$_1$R) eta immuno-peroxidasaren (Iba1) metodo bikoitza mikroskopia elektronikorako. Mikroglia-prozesuek Iba1en tindaketa erakusten dute (DAB immunobiltegiak, marroia, *). CB2$^{EGFP/f/f}$ kontrolean, elementu mikroglial mehe gutxi batzuk antzeman daitezke (a, b, c). Hala ere, CB2$^{EGFP/f/f}$/5xFAD saguen mikroglia erreaktiboaren prozesu lodiak agertzen dira (d, e, f, g, h). Plakak (arrosa; d, g) eta neurita distrofiko ugari (urdina; d, e, f, g, h) ere identifikatzen dira. Ikus daiteke CB$_1$R partikulak daudela mintzean eta zitosolen bi mutanteen mikroglia-prozesuetan, eta bereziki ugariak direla ustez endekatuta dauden CB2$^{EGFP/f/f}$/5xFAD saguen terminal inhibitzaileetan (horia; f, h). Eskala barrak: 1 μm.
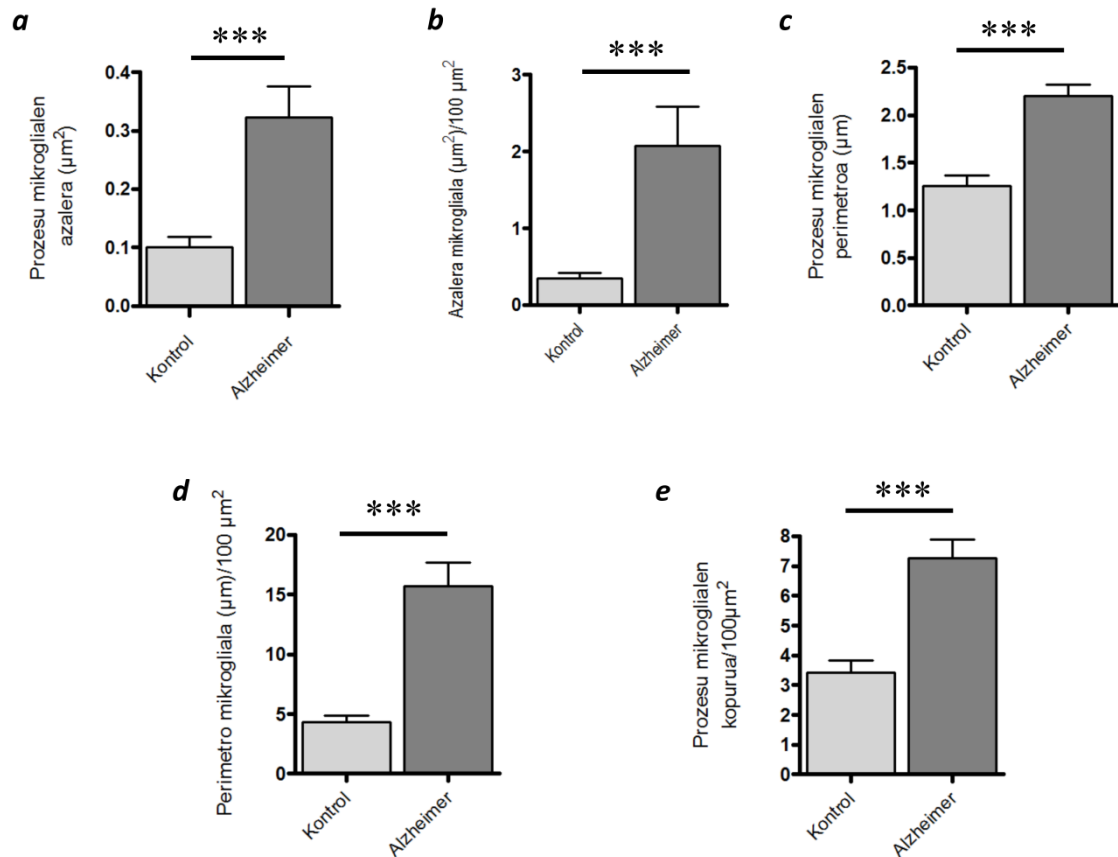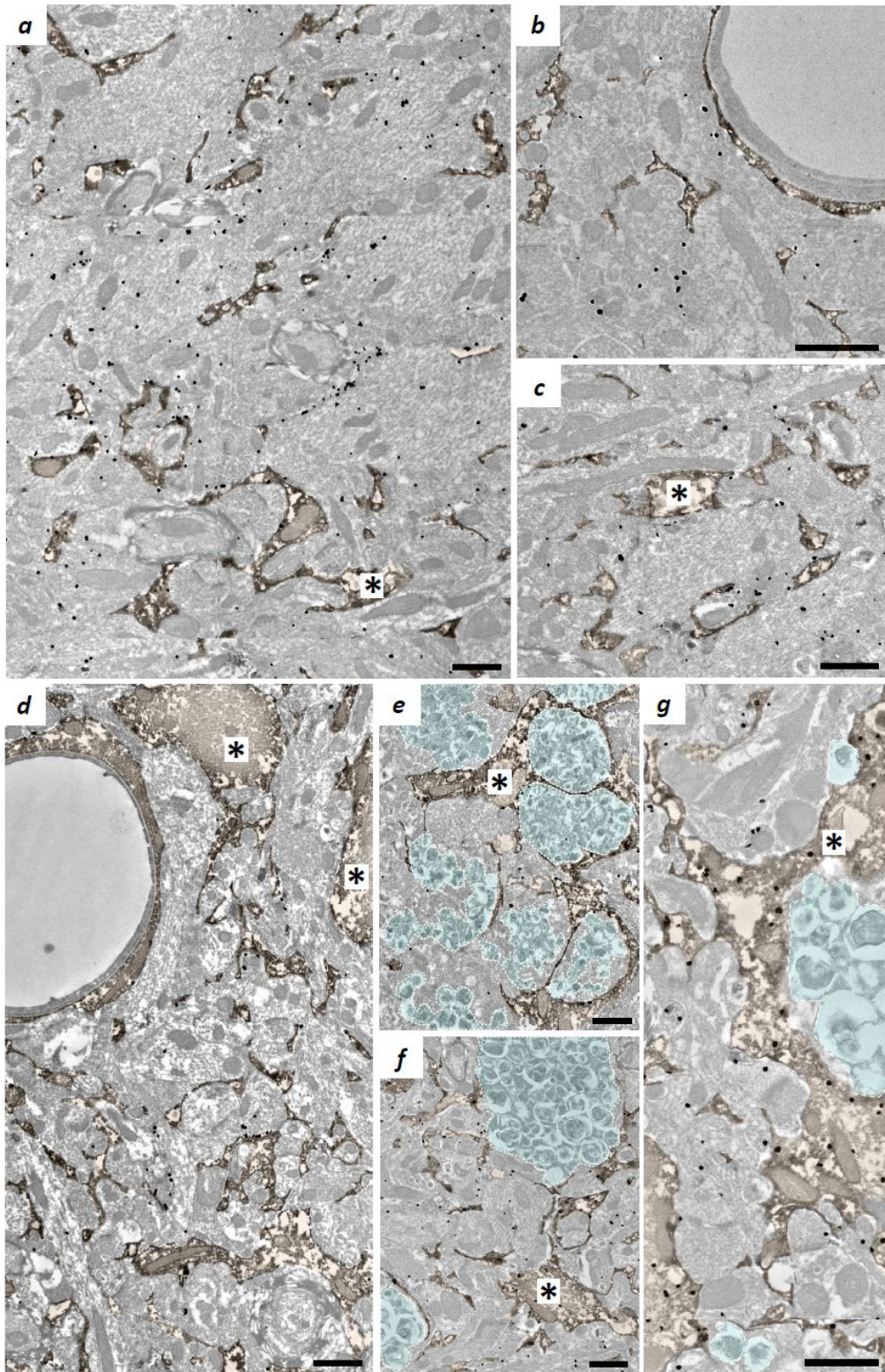
13. irudia. CB2^EGFP/f/f (kontrola) eta CB2^EGFP/f/f/5xFAD (Alzheimer) saguen subikuluko mikroglia-ultraegituraren kuantifikazioa eta estatistikak. (a) Mikroglia prozesuen azalera. (b) Mikrogliaren azalera 100 μm^2-tara normalizatuta. (c) Mikroglia prozesuen perimetroa. (d) Mikrogliaren perimetroa 100 μm^2-tara normalizatuta. (e) 100 μm^2-tako mikroglia-prozesuen kopurua. Datuak froga ez-parametrikoen edo parametrikoen bidez aztertu ziren. (Mann–Whitney U-test edo Student's t-froga). Mann–Whitney U-test edo Student's t-froga. $p < 0.05$*; $p < 0.01$**; $p < 0.001$***; $p < 0.0001$****. Balio guztiak batez bestekoa $\pm$ S.E.M. gisa adierazita daude.

6.4. ALDAKETAK CB2$^{EGFP/F/F}$/5xFAD SAGUEN SUBIKULUKO ASTROGLIA-PROZESUEN MORFOLOGIAN ETA KOPURUAN.

GLAST-DAB markaketa erabiliz, CB2$^{EGFP/f/f}$ eta CB2$^{EGFP/f/f}$/5xFAD saguen subikuluko prozesu astrozitikoen azalera, perimetroa eta kopurua aztertu ziren (14. irudia).

Prozesu astrozitikoen azalera nabarmen handitu zen (CB2$^{EGFP/f/f}$/5xFAD: 0.2598 ± 0.01853 μm^2; CB2$^{EGFP/f/f}$: 0.1565 ± 0.006515 μm^2; ***$p < 0.0001$; 15a irudia). Hala ere, ez zen alde nabarmenik hauteman lagin bakoitzeko prozesu astrozitikoek okupatutako azaleran 100 μm^2-ko azalera normalizatuan (CB2$^{EGFP/f/f}$/5xFAD: 8.993 ± 0.8664μm^2; CB2$^{EGFP/f/f}$: 7.415 ± 0.6552 μm^2; p: 0.1711; 15b irudia). Prozesu astrozitikoen perimetroa ere nabarmen handitu zen (CB2$^{EGFP/f/f}$/5xFAD: 2.833 ± 0.08486 μm; CB2$^{EGFP/f/f}$: 2.116 ± 0.04741 μm; ***$p < 0.0001$; 15c irudia). Hala ere, lagin bakoitzeko prozesu astrozitikoen perimetro osoan ere ez da alderik hauteman (CB2$^{EGFP/f/f}$/5xFAD: 99.89 ± 8.087 μm; CB2$^{EGFP/f/f}$: 100.1 ± 6.811 μm; p: 0.9820; 15d irudia.). Emaitza horiekin bat etorriz, prozesu astrozitikoen kopurua nabarmen jaitsi zen CB2$^{EGFP/f/f}$/5xFAD saguetan (35.52 ± 2.661 100 μm^2-ko), CB2$^{EGFP/f/f}$ saguekin alderatuta (47.33 ± 2.709 100 μm^2-ko; **p: 0.0036; 15e irudia).

14. irudia. Astroglia CB2$^{EGFP/f/f}$ eta CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan. Erretxinan murgildu aurreko immuno-urre (CB$_1$R) eta immuno-peroxidasaren (GLAST) metodo bikoitza mikroskopia elektronikorako. CB2$^{EGFP/f/f}$ saguetan (a, b, c) behatutako astrozito-prozesu GLAST-positboak (DAB immunobiltegiak, marroia, *) lodiagoak dira CB2$^{EGFP/f/f}$/5xFAD saguetan (d, e, f, g). Erreparatu neurita distrofikoen inguruan kokatzen diren elementu astrozitikoetan AD saguan (urdina; e, f, g). Eskala barrak: 1 µm.
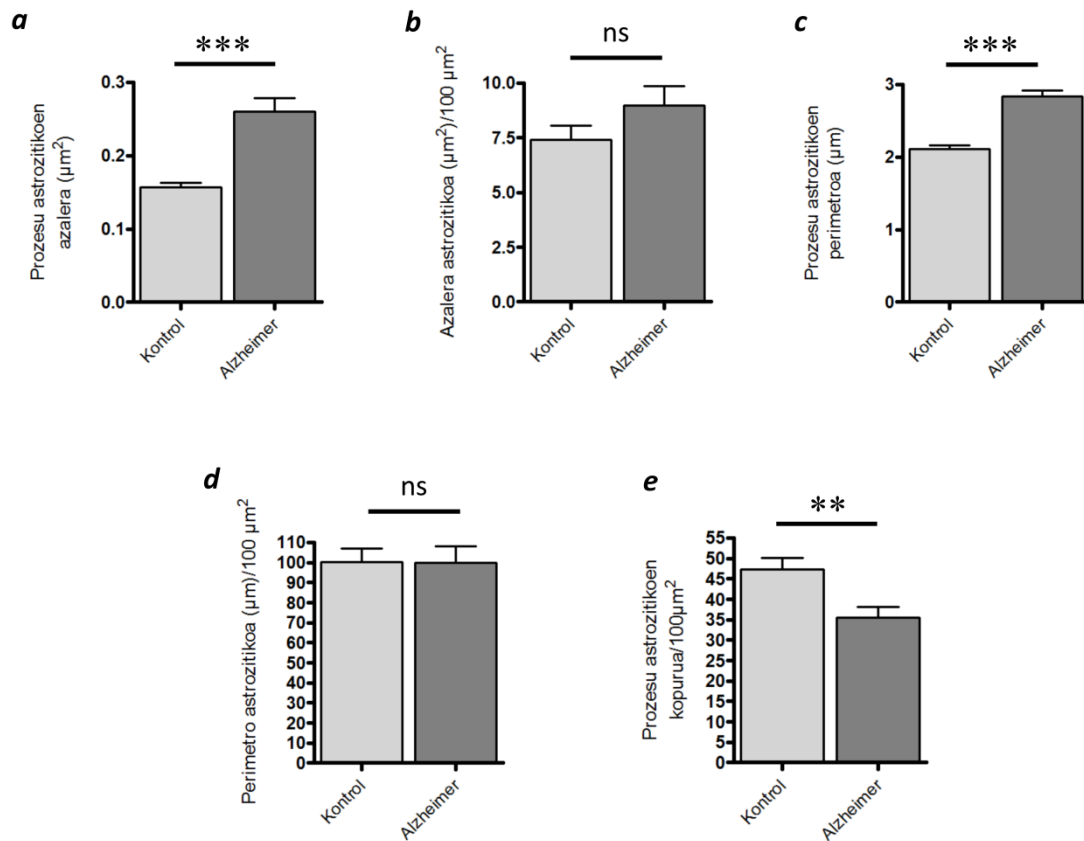
15. irudia. Astrozitoen parametro morfologikoen kuantifikazioa eta ebaluazio estatistikoak CB2$^{EGFP/f/f}$ (kontrol) eta CB2$^{EGFP/f/f}$/5xFAD (Alzheimer) saguen subikuluan. (a) Prozesu astrozitikoen azalera. (b) 100 µm^2-ko eremu normalizatuarekiko astrozito-azalera. (c) Prozesu astrozitikoen perimetroa. (d) 100 µm^2-ko eremu normalizatuarekiko perimetro astrozitikoa. (e) Prozesu astrozitikoen kopurua 100 µm^2-tan. Datuak froga ez-parametrikoen edo parametrikoen bidez aztertu ziren. (Mann–Whitney U-test edo Student's t-froga). Mann–Whitney U-test edo Student's t-test. $p < 0.05$*; $p < 0.01$**; $p < 0.001$***; $p < 0.0001$****. Balio guztiak batez bestekoa ± S.E.M. gisa adierazita daude.

6.5. CB$_1$ HARTZAILEAREN LOKALIZAZIOA CB2$^{EGFP/F/F}$/5xFAD SAGUEN SUBIKULUAN.

CB$_1$R immunopartikulak aztertu ziren mikroglia-prozesuen mintzetan, bai CB2$^{EGFP/f/f}$ saguetan, bai CB2$^{EGFP/f/f}$/5xFAD saguetan (12, 16 irudiak). CB2$^{EGFP/f/f}$/5xFAD saguetan (0.9942 ± 0.1259) saguetako prozesu microglial CB$_1$-positiboen hazkunde esanguratsua ikusi zen, CB2$^{EGFP/f/f}$ saguekin alderatuta (0.3254 ± 0.07758; ***p <0.0001; 17a irudia). Gainera, CB2$^{EGFP/f/f}$/5xFAD saguetan prozesu microglial CB$_1$-positiboen portzentaia esanguratsuki handitzen zela behatu zen (% 6.27 ± 1.15; CB2$^{EGFP/f/f}$: % 3.79 ± 2.10; **p: 0.0033; 17b irudia). Hala ere, mikroglia-prozesu positiboetako CB$_1$R-aren dentsitatea nabarmenki murriztu zen CB2$^{EGFP/f/f}$/5xFAD saguetan (69.44 ± 7,577 partikula/mintzezko 100 µm-ko; CB2$^{EGFP/f/f}$: 135.5 ± 24.78 partikula/ mintzezko 100 µm-ko; **p: 0.0023; 17c irudia). Gainera, 100 µm^2-ko azalerarekiko behatutako CB$_1$R partikula mikroglialen guztizko kopuruan ezberdintasun nabarmenak hauteman ziren CB2$^{EGFP/f/f}$/5xFAD saguen (1.31 ± 0.18) eta CB2$^{EGFP/f/f}$ saguen (0.48 ± 0.13 partikula; ***p: 0.0009; 17d irudia) artean. Azkenik, ez da alderik ikusi bi mutanteen artean mikroglia-prozesu bakoitzeko CB$_1$R immunopartikula kopuruan (CB2$^{EGFP/f/f}$/5xFAD: 1.333 ± 0.08347 partikula; CB2$^{EGFP/f/f}$: 1.450 ± 0.2112 partikula; p: 0.7736; 17e irudia).
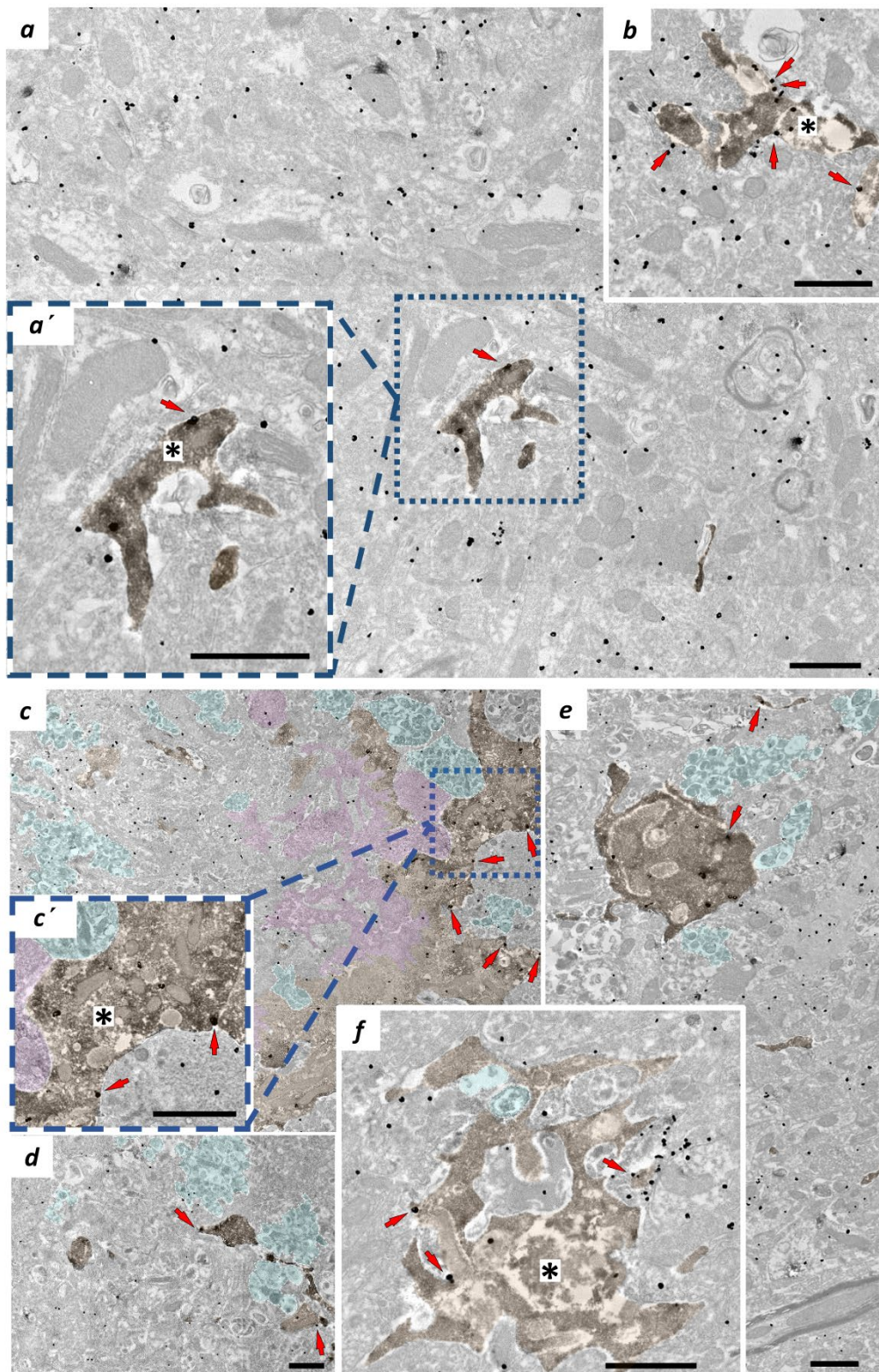
16. irudia. CB₁R-aren lokalizazioa mikroglia-konpartimentuetan, CB2$^{EGFP/f/f}$ eta CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan. Erretxinan murgildu aurreko immuno-urre (CB₁R) eta immuno-peroxidasaren (Iba1) metodo bikoitza mikroskopia elektronikorako. CB₁R-aren urrezko partikula batzuk (gezi gorriak) ikusten dira mikroglia-mintzetan (DAB immunobiltegiak, marroia, *), bai CB2$^{EGFP/f/f}$ saguetan (a, b) eta bai CB2$^{EGFP/f/f}$/5xFAD saguetan (c, c', d, e, f). Plakak (arrosa) (c) eta neurita distrofikoak (urdina; c, c', d, e, f) daude CB2$^{EGFP/f/f}$/5xFAD saguetan. Eskala barrak: 1 μm.
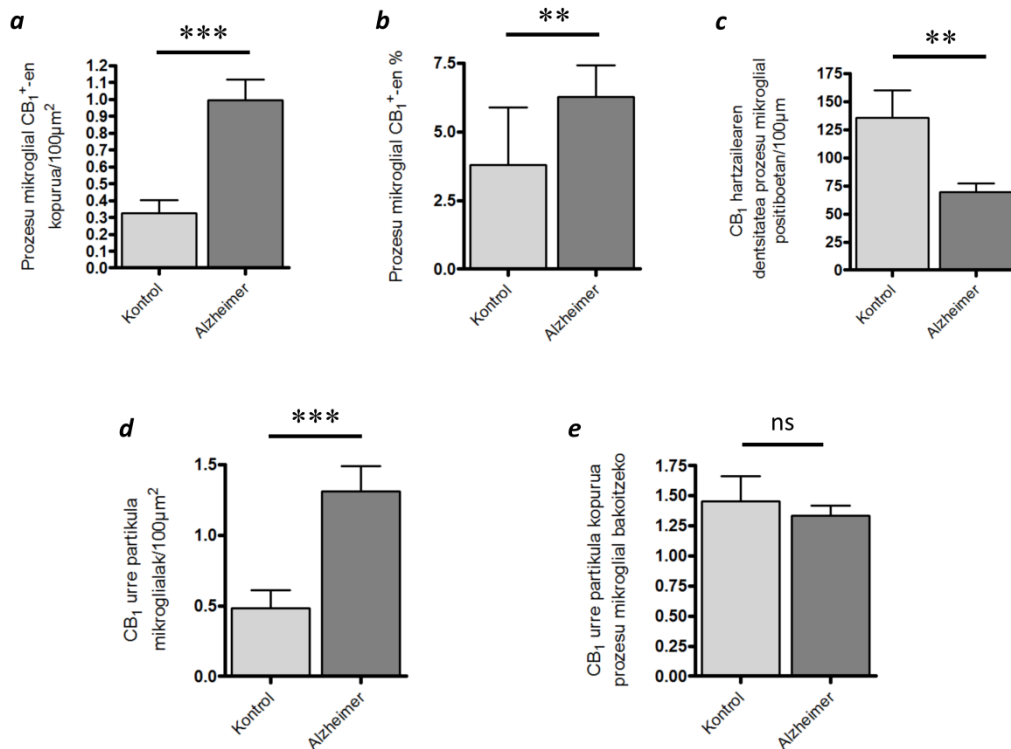
17. irudia. CB$_1$R mikroglialaren lokalizazioaren analisi estatistikoa CB2$^{EGFP/f/f}$ eta CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan. (a) Prozesu CB$_1$-positiboen kopurua 100μm^2-ko. (b) Prozesu CB$_1$R-positiboen ehunekoa. (c) CB$_1$R-ren dentsitatea mikroglia-prozesu positiboetan, 100μm-rekiko. (d) CB$_1$R-aren partikula mikroglialak 100 μm^2-ko. (e) CB$_1$R-ren markaketa elementu mikroglial bakoitzeko. Datuak froga ez-parametrikoen edo parametrikoen bidez aztertu ziren. (Mann–Whitney U-test edo Student's t-froga). Mann–Whitney U-test edo Student's t-froga. $p < 0.05$*; $p < 0.01$**; $p < 0.001$***; $p < 0.0001$****. Balio guztiak batez bestekoa ± S.E.M. gisa adierazita daude.

6.6. CB2 HARTZAILEAREN LOKALIZAZIOA CB2$^{\text{EGFP/F/F}}$/5XFAD SAGUEN SUBIKULUAN.

CB$_2$R-ren sustatzaile beraren pean GFP adierazten duten CB2$^{\text{EGFP/f/f}}$ eta CB2$^{\text{EGFP/f/f}}$/5xFAD saguak erabili nituen CB$_2$R-aren kokapena ikertzeko mikroglian (18, 19 irudiak). Hala ere, nahiz eta bi proteinak sustatzaile beraren pean sintetizatu, ez daude elkarrekin lotuta, eta, beraz, ez daude nahitaez zelularen barruko leku beretan kokatu behar. Hau esanda, argi-mikroskopioan ez zen GFP markaketa (CB$_2$R) behatu CB2$^{\text{EGFP/f/f}}$ saguetan (18a, a´ irudiak), CB2$^{\text{EGFP/f/f}}$/5xFAD saguetan immunotindatze punteatua (18b, b´ irudiak) adierazi zelarik. Ondoren, mikroskopio elektronikoan aztertu zen nola banatzen zen GFP-a mintzetan eta konpartimentu zitosolikoetan. Iba1-ekiko mikroglia-prozesu immunopositiboek GFP-a (CB$_2$R) adierazten zuten bai CB2$^{\text{EGFP/f/f}}$ eta bai CB2$^{\text{EGFP/f/f}}$/5xFAD saguetan (19. irudia). Analisi estatistikoak erakutsi zuenez, zenbatutako prozesu-mikroglial GFP-positiboen kopurua nabarmenki handitzen zen CB2$^{\text{EGFP/f/f}}$/5xFAD saguetan (0.7126 ± 0.2311) CB2$^{\text{EGFP/f/f}}$ saguekin (0.1648 ± 0.07686) alderatuz (*p: 0.0176; 20a irudia). Gainera, prozesu-mikroglial GFP-positiboen portzentaiak handipen esanguratsua aurkezten zuen CB2$^{\text{EGFP/f/f}}$/5xFAD saguetan (% 16.71 ± 3.664) CB2$^{\text{EGFP/f/f}}$ saguekin konparatuta (% 5.430 ± 2.631; *p: 0.0191; 20b irudia). Horretaz gain, GFP partikulen kopurua prozesu mikroglialen azalerarekiko askoz handiagoa izan zen CB2$^{\text{EGFP/f/f}}$/5xFAD (1.238 ± 0.2534) saguetan CB2$^{\text{EGFP/f/f}}$ saguekin alderatuz (0.6962 ± 0.4138; *p: 0.0467; 20c irudia). Mikroglia-prozesuetako GFP partikulen kopurua 100 μm^2-ekiko estatistikoki handiagoa izan zen CB2$^{\text{EGFP/f/f}}$/5xFAD saguetan (0.8343± 0.2962) CB2$^{\text{EGFP/f/f}}$ saguetan baino (0.1648± 0.07686; *p: 0.0176; 20d irudia). Nabarmentzekoa da CB2$^{\text{EGFP/f/f}}$/5xFAD saguetan mikroglia-mintzetan aurkitutako GFP immunopartikulen ehunekoa (% 77.22 ± 11.40) nabarmen handiagoa izan zela zitosolean kokatutako proportzioa baino (% 22.78 ± 11.40; *p: 0.0106; 20e irudia). CB2$^{\text{EGFP/f/f}}$ saguen kasuan, % 100-eko GFP partikulak mikroglia-prozesuen mintzean kokatzen ziren.
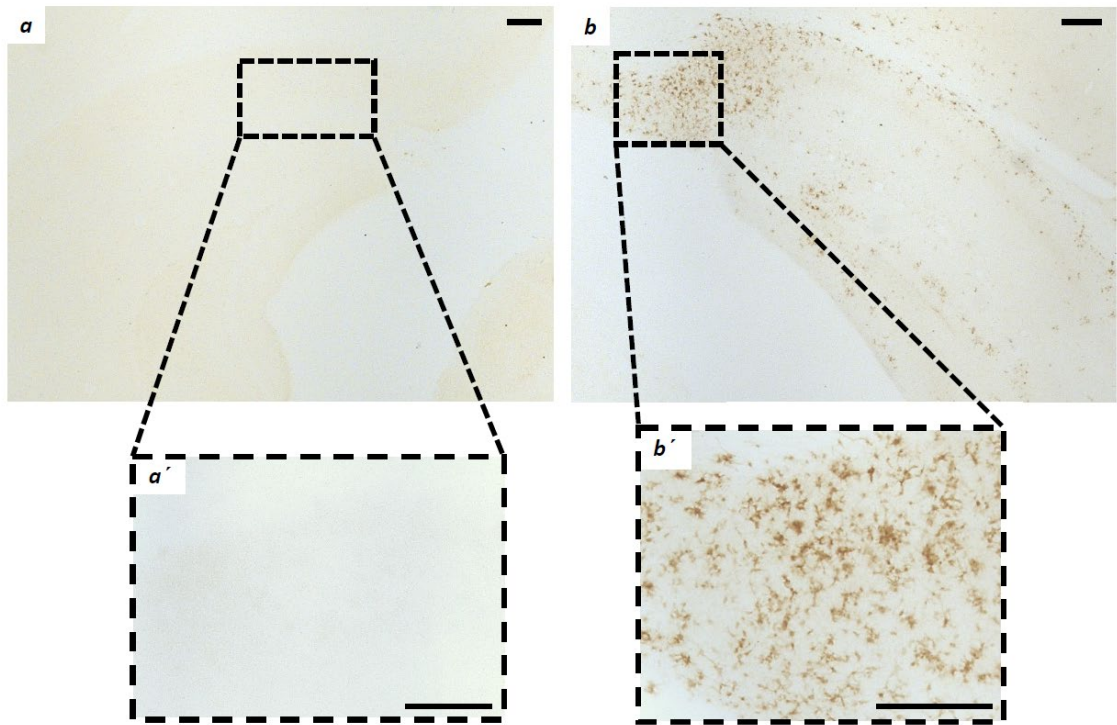
18. irudia. GFP immunotindaketa CB2$^{EGFP/f/f}$ eta CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan. CB2$^{EGFP/f/f}$ (a, a´) saguetan ez da tindaketarik ikusten; hala ere, GFP immunotindaketa hipokanpoan sakabanatuta agertzen da, baina subikuluan metatuta agertzen da CB2$^{EGFP/f/f}$/5xFAD saguetan (b, b') Eskala barrak: 200 µm.
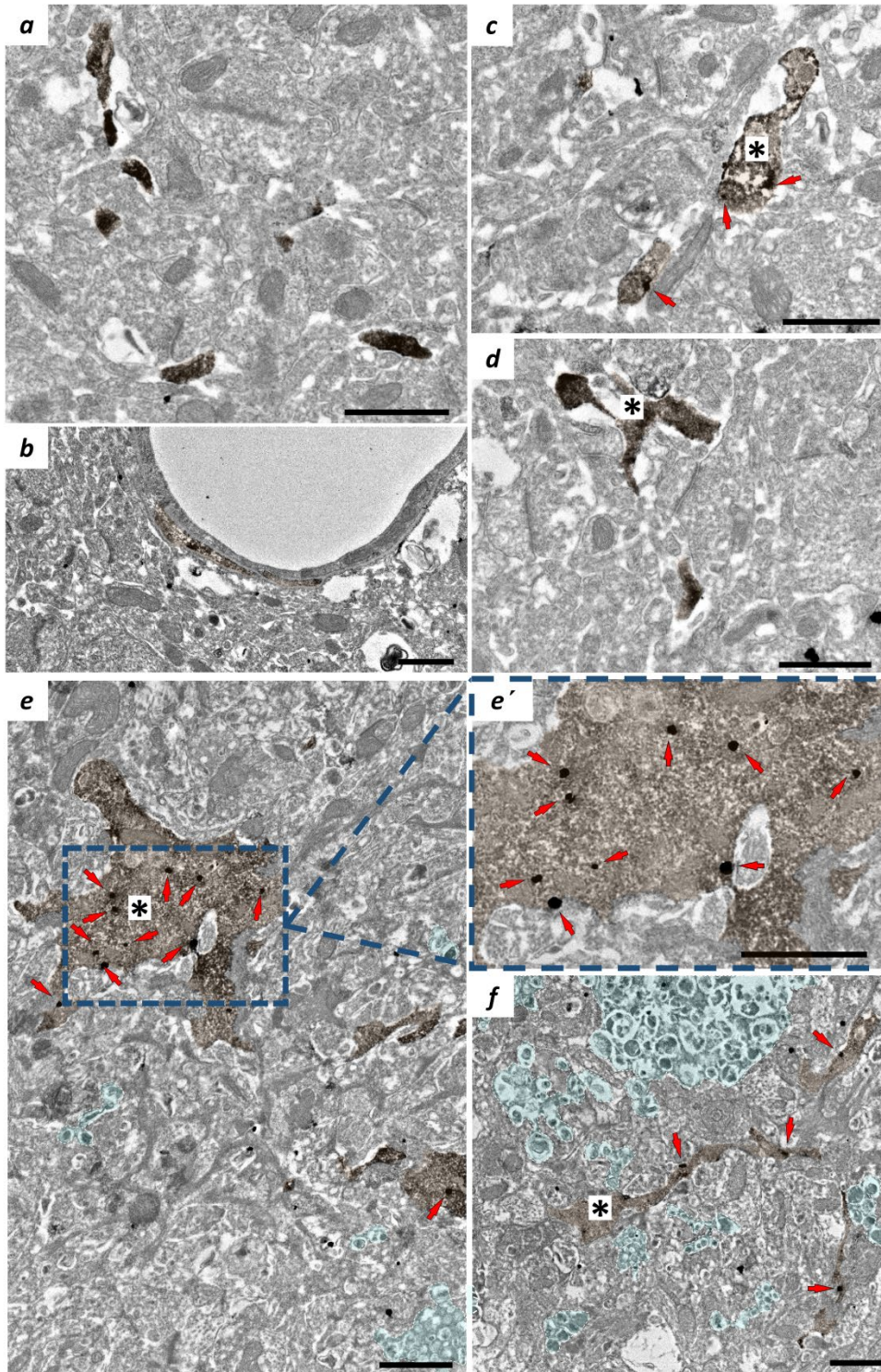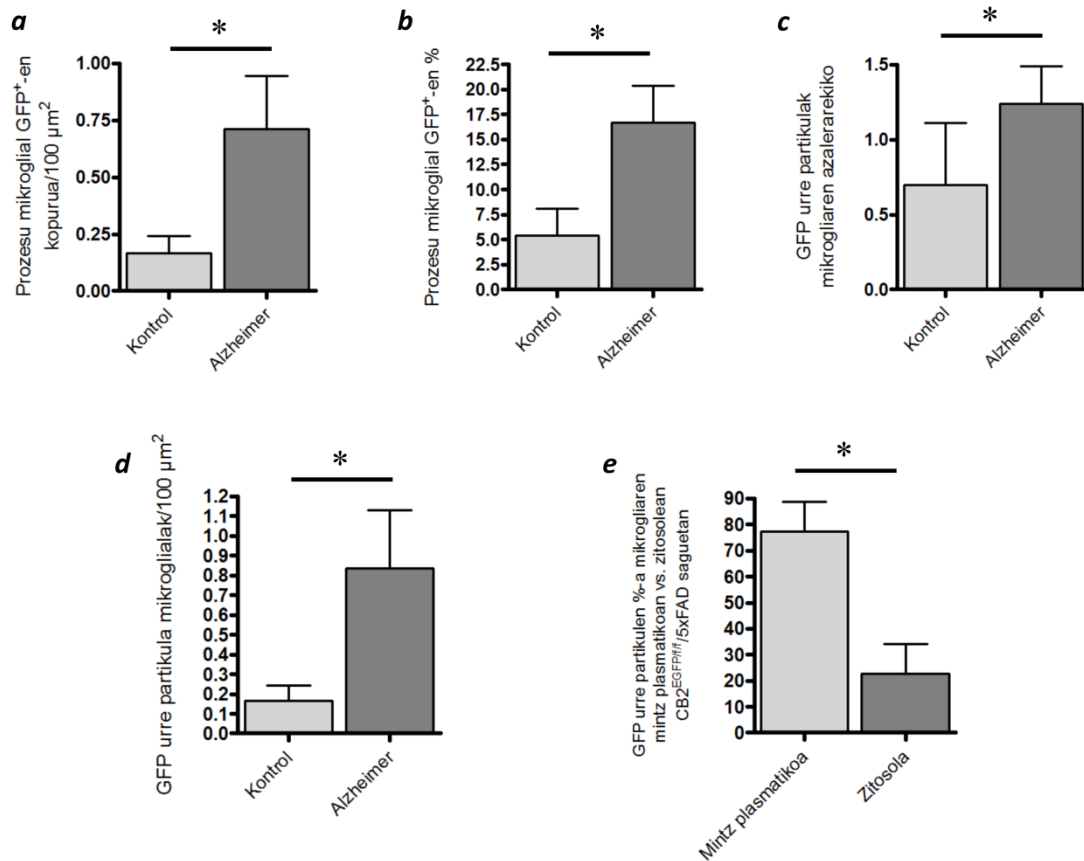
19. irudia. Mikrogliako GFP lokalizazioa CB2$^{EGFP/f/}$ eta CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan. Erretxinan murgildu aurreko immuno-urre (GFP) eta immunoperoxidasaren (Iba1) metodo bikoitza mikroskopia elektronikorako. GFP partikulak (gezi gorriak) Iba1-entzat positiboak diren mikroglia-elementuetan kokatzen dira (DAB immunobiltegiak, marroia, *). CB2$^{EGFP/f/f}$ saguetan (a, b, c, d), GFP-a bakarrik mintzean aurkitzen da (geziak, c). CB2$^{EGFP/f/f}$/5xFAD saguetan, GFP partikulak bai mintzean bai zitosolean (e, e', f) daude. Neurita distrofikoak (urdina) CB2$^{EGFP/f/f}$/5xFAD saguetan behatzen dira (e, f). Eskala barrak: 1 µm.

20. irudia. GFP mikroglialen (CB₂R) lokalizazioari buruzko analisi estatistikoak CB2^EGFP/f/f eta CB2^EGFP/f/f/5xFAD saguen subikuluan. (a) Mikroglia-prozesu GFP-positiboen kopurua 100µm²-ko. (b) Mikroglia-prozesu GFP positiboen ehunekoa. (c) Azalera mikroglialeko GFP partikulak. (d) GFP mikroglialen immunopartikulak, 100 µm²-ekiko. (e) GFP partikulen ehunekoa mikroglia mintzean eta zitosolean CB2^EGFP/f/f/5xFAD. Datuak froga ez-parametrikoen edo parametrikoen bidez aztertu ziren. (Mann–Whitney *U*-test edo Student's *t*-froga). Mann–Whitney *U*-test edo Student's *t*-froga. $p < 0.05$*; $p < 0.01$**; $p < 0.001$***; $p < 0.0001$****. Balio guztiak batez bestekoa ± S.E.M. gisa adierazita daude.

67

6.7. CB$_1$ hartzailearen lokalizazio astrogliala CB2$^{EGFP/F/F}$/5xFAD saguen subikuluan.

Alzheimer gaixotasunak neurita distrofikoak eta SP-ak inguratzen dituzten astrozito erreaktiboen CB$_1$R adierazpenean nola eragiten duen zehazteko, CB2$^{EGFP/f/f}$ eta CB2$^{EGFP/f/f}$/5xFAD saguen subikulua aztertu zen. Mikroskopio elektronikoan, CB$_1$R prozesu astrozitikoen mintzetan (GLAST positiboa) lokalizatu ziren bi mutanteetan (21. irudia), gure laborategiak aurretik jakinarazi duen bezala (Han et al., 2012; Bosier et al., 2013; Gutiérrez-Rodríguez et al., 2018; Bonilla-Del Río et al., 2019, 2021). Ez dira ezberdintasun esanguratsurik hauteman astrozito-prozesu CB$_1$R-positiboen kopuruan bi sagu taldeen artean (100 μm^2 bakoitzeko, CB2$^{EGFP/f/f}$: 8.661 ± 0.8977 prozesu; CB2$^{EGFP/f/f}$/5xFAD: 7.967 ± 1.224 prozesu; *p*: 0.3094; 22a irudia). Gainera, prozesu astrozitiko CB$_1$R-positiboen ehunekoa antzekoa izan zen estatistikoki CB2$^{EGFP/f/f}$/5xFAD saguetan (% 21.24 ± 2.37) eta CB2$^{EGFP/f/f}$ saguetan (% 17.75 ± 1.21; *p*: 0.2303; 22b irudia). Era berean, ez zen alde nabarmenik ikusi CB$_1$R-aren dentsitatean prozesu astrozitiko positiboetan (CB2$^{EGFP/f/f}$/5xFAD: 57.64 ± 2.105 partikula; CB2$^{EGFP/f/f}$: 59.83 ± 2.324; *p*: 0.2209; 22c irudia), ez lagin bakoitzeko CB$_1$ partikula astrozitikoen kopuruan (CB2$^{EGFP/f/f}$/5xFAD: 12.78 ± 2.174 partikula; CB2$^{EGFP/f/f}$: 11.63 ± 1.265; *p*: 0.6716; 22d irudia). Hala ere, prozesu astrozitiko positiboetako CB$_1$R partikulen kopurua nabarmenki handiagoa izan zen CB2$^{EGFP/f/f}$/5xFAD saguetan (1.603 ± 0.05081 partikula/prozesu) CB2$^{EGFP/f/f}$ saguetan baino (1.343 ± 0.03909; ***p*: 0.0005; 22e irudia).
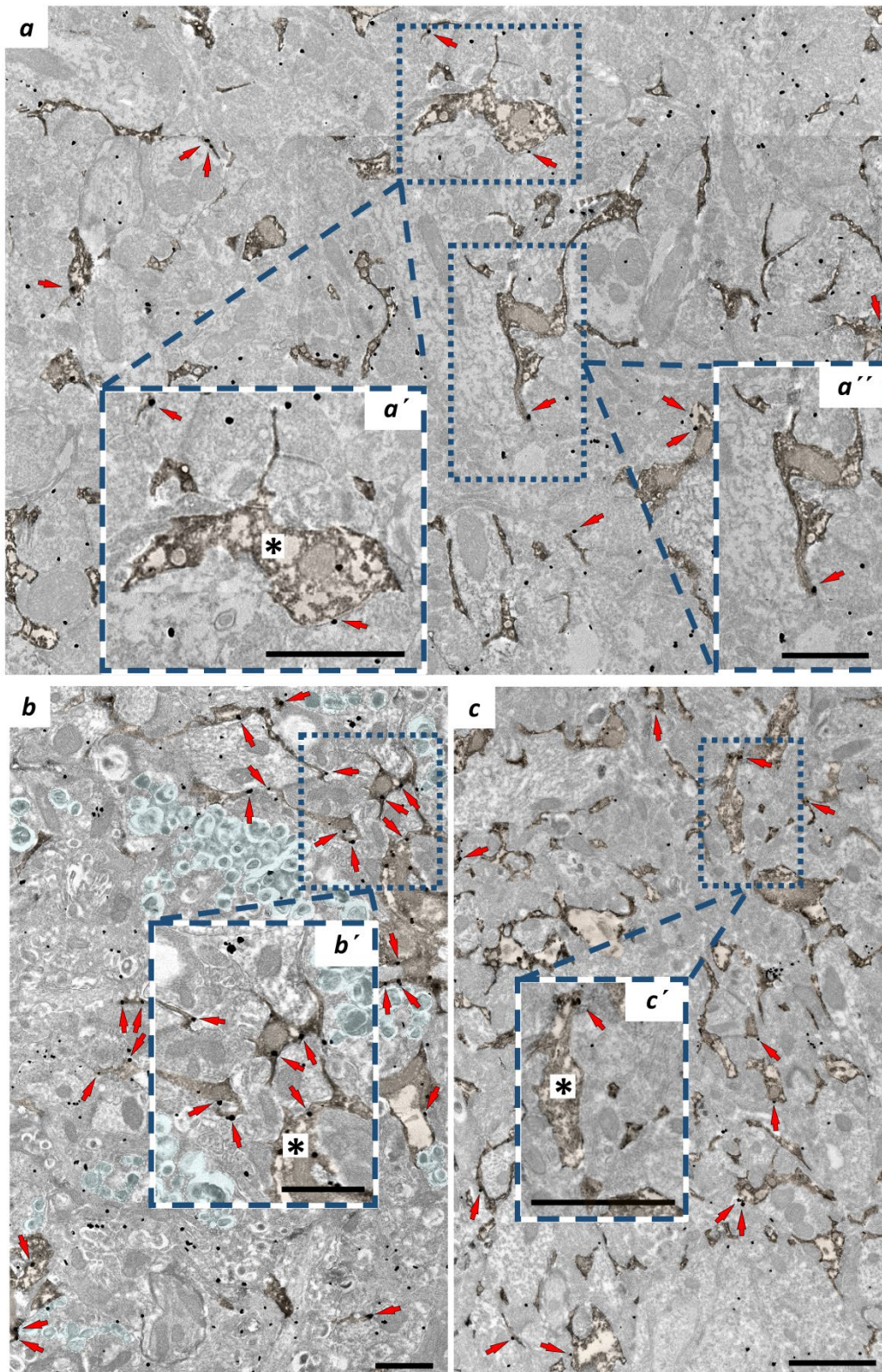
21. irudia. CB₁R astroglialaren lokalizazioa CB2$^{EGFP/f/f}$ eta CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan. Erretxinan murgildu aurreko immuno-urre (CB₁R) eta immuno-peroxidasaren (GLAST) metodo bikoitza mikroskopia elektronikorako. CB₁R partikulak (gezi gorriak) CB2$^{EGFP/f/f}$(a) eta CB2$^{EGFP/f/f}$/5xFAD (b, c) saguen prozesu astrozitiko GLAST-positiboen (DAB immunobiltegiak, marroia, *) mintz plasmatikoetan kokatzen dira. Beha itzazu neurita distrofikoak (urdina) CB2$^{EGFP/f/f}$/5xFAD saguetan (b). Eskala barrak: 1 µm.
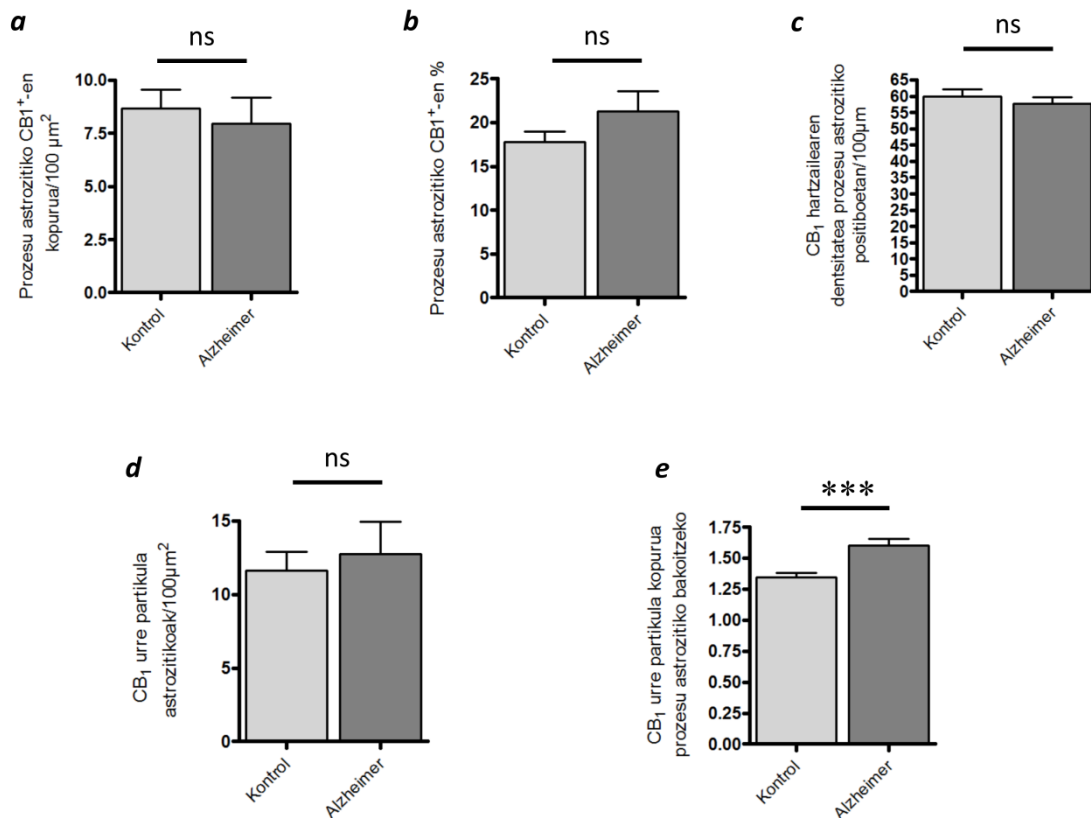
22. irudia. CB$_1$R-aren lokalizazioaren ebaluazio estatistikoa CB2$^{EGFP/f/f}$ eta CB2$^{EGFP/f/f}$/5xFAD saguen astrozito subikularretan. (a) Prozesu astrozitikoen CB$_1$R-positiboen kopurua 100 μm^2-tan. (b) Prozesu astrozitikoen CB$_1$R-positiboen ehunekoa. (c) CB$_1$R dentsitatea prozesu astrozitiko positiboetan 100 μm-ko. (d) CB$_1$R urre partikula astrozitikoak 100 μm^2-ko. (e) CB$_1$R partikulak prozesu astrozitiko bakoitzeko. Datuak froga ez-parametrikoen edo parametrikoen bidez aztertu ziren. (Mann–Whitney U-test edo Student's t-froga). Mann–Whitney U-test edo Student's t-froga. $p < 0.05$*; $p < 0.01$**; $p < 0.001$***; $p < 0.0001$****. Balio guztiak batez bestekoa ± S.E.M. gisa adierazita daude.

7. EZTABAIDA

Nire doktorego tesian, cnr2 genearen kontrolpean EGFP adierazten duten sagu transgenikoak eta Alzheimer gaixotasun familiarreko 5 mutazio (5xFAD) zituzten saguen arteko gurutzaketaren bidez lortutako ADren sagu eredua (CB2$^{EGFP/f/f}$/5xFAD) erabili izan dut, eta bere CB2$^{EGFP/f/f}$ kontrolak (López et al., 2018). Sagu horiek guztiak Julian Romero doktoreak eta bere laborategiak (Zientzia Esperimentalen Fakultatea, Francisco de Vitoria Unibertsitatea, Pozuelo de Alarcón) eskaini zizkiguten, gure lankidetza proiektuaren alorrean.

AD eta haren azpiko patogenesia aztertzeko sagu-eredu ugari sortu dira (1. taula). Eredu horiek funtsezkoak izan dira gaixotasunaren garapena eta progresioa ulertzeko, gaixotasunaren mekanismoak eta arrisku-faktoreak ezagutzeko bezalaxe (berrikuspenerako Jankowsky eta Zheng, 2017; Oblak et al., 2021). Hala ere, giza ADeko gertakizunekin alderatuz, animalien ereduetan ezaugarri patologikoen maila mugatua da. Hori dela eta, beharrezkoa izan da ADeko sagu-ereduen karakterizazio egoki bat egitea, sendagai diseinuan eta tratamenduen garapenean duten balio traslazionala hobetzeko (Oblak et al., 2021). Ildo horretan, fenotipatuaren azterketa sakon bat egin zen, 5xFAD saguak kumaldi bereko mutaziorik gabeko kideekin (WT) alderatuz. Transkriptomikan, elektroentzefalograman, *in vivo* irudi tekniketan, karakterizazio biokimikoan eta portaera-ebaluazioetan izandako aldaketak aztertu ziren. Ikerketaren arabera, 5xFAD saguek giza AD islatzen dute (1) gorputz-hauskortasunaren eta ezohiko funtzio motorraren, (2) sexuan oinarritutako geneen egoera eta adierazpenetako asalduren, (3) immunitate-sistemak gaixotasunaren garapenean duen parte-hartzean eta (4) amiloidosiaren testuinguruetan. Hori dela eta, ADri buruzko azterlanetan animalia-eredu hau erabiltzea gomendatzen da (Oblak et al., 2021).

Subikuluan jarri nuen arreta, hipokanpoaren irteera-fluxuaren kontrolean estrategikoki kokatuta baitago (berrikuspenerako Mark et al., 1995; berrikuspenerako Rajmohan eta Mohandas, 2007; Ishihara eta Fukuda, 2016; berrikuspenerako Matsumoto et al., 2019). Aldi berean, ADean subikuluma garun-eskualde kaltetuenetariko bat da, eta lehenetarikoa, eta eragiten duen hipokanpoaren deskonexioa dela eta defizit kognitiboak agertzen dira (Hyman et al., 1984; Oakley et al., 2006; López et al., 2018).

Mikroglia eta astrozitoak aztertu ditut CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan (6.5 eta 7.5 hilabete bitartekoak), adin horretarako plakak eta kalte neuronalak jada garatuak zituztelarik. Aβ-dun agregatuek hantura erantzun kronikoa eragiten dute, mikrogliaren eta astrozitoen aktibazioa eragiten duena, besteak beste (Benito et al., 2003). Horrela, plakak mikroglia aktiboz (Yin et al., 2017; berrikuspenerako Smit et al., 2021) eta astrozito

erreaktiboz (Kamphuis et al., 2014; Kato et al., 1998; berrikuspenerako Smit et al., 2021) inguratuta eta inbadituta daude. Astrozitoak ezagunak dira mikrogliarekin duten elkarrekintza estuagatik, eta bi zelula hauek ADko gliosi erreaktiboan inplikatuta daude (McAlpine et al., 2021; berrikuspenerako Smit et al., 2021). Gliosi erreaktibo honek astrozitoen eta mikrogliaren morfologia anormala eta ugaritzea eragiten du. Prozesu neurodegeneratiboetan gertatzen diren aldaketa histopatologiko horiek zelula glialen jarduera onuragarria, kaltegarria edo garrantzirik gabekoa islatzen duten oraindik ez dago argi (berrikuspenerako Hansen et al., 2018). Ikerketa gehienek neuroinflamazioa, ondorio positibo zein negatiboekin, ADko etaparik goiztiarrenean hasten dela adosten dute. Garunaren immunitate-sistemak proteinen ezohiko metaketa estimulu kaltegarritzat hartzen du eta, defentsa gisa, erreakzio fisiologiko bat hasten du zelula glialak aktibatuz eta molekula proinflamatorioak askatuz. Hala ere, prozesu horiek denboran luzatzen direnean, neuronen disfuntzioa eta zelulen heriotza eragin dezakete, gaixotasunaren progresioa bultzatuz (berrikuspenerako Sarlus eta Heneka, 2017).

Gainera, badirudi ADean mikrogliaren eta astrogliaren arteko komunikazioa onuragarria ematen dela. Adibidez, Aβ metaketak detektatzean, mikrogliak IL-3Rα-ren adierazpena handitzen du, IL-3-ren hartzaile espezifikoa (CD123 bezala ere ezagutzen dena). Astrozitoek, era berean, IL-3 berez jariatzen dute, eta IL-3Rα-a hartzaileei lotzerakoan, mikrogliak erantzun bat abiatzen du, mikrogliaren programazio transkripzionala, morfologikoa eta funtzionala eragiten duena. Beraz, zelula hauen inmunitate erantzun akutuko programa bat jartzen da martxan, mugikortasun handiagoa, agregatzeko ahalmen gehiago eta Aβ eta τ agregatuak ezabatzeko ahalmen handiagoa ematen diena. Bi zelula glial hauen arteko elkarlanaren ondorioz sortutako aldaketa hauek, ADren patologia eta asaldura kognitiboa mugatuko lituzkete (McAlpine et al., 2021).

Erreaktibitate gliala behatu nuen bai mikroglian bai astrozitoetan, beraien prozesuen azalera eta perimetroaren handipenarekin. Mikroskopio elektronikoan, halaber, CB2$^{\text{EGFP/f/f}}$/5xFAD saguetan plaken eta neurita distrofiko ugarien presentzia hauteman nuen, eta horrek are gehiago berresten du animalia-eredu honek ADren fisiopatologia aztertzeko duen erabilgarritasuna. Nire azterlanean lortutako emaitzek, ADan glia-zelula horien prozesuetan gertatzen diren azaleraren, perimetroaren eta kopuruaren aldaketak zehazten lagundu dute, zitoarkitektura subikularrean aldaketa garrantzitsuak daudela frogatuz, erreaktibotasun mikroglial eta astrozitiko nabarmenarekin eta heriotza neuronal ugarirekin (Scuderi et al., 2020).

7.1.1. Alzheimer gaixotasunean subikuluan gertatzen diren aldaketa mikroglialak

Nire tesian, CB2$^{EGFP/f/f}$/5xFAD saguetan prozesu mikroglialen kopurua, azalera eta perimetroa nabarmen handitzen direla frogatu dut. Beraz, ADdun sagu-mutante horien subikuluan aktibazio mikroglial esanguratsua dago 6.5-7.5 hilabeteko adinean. Mikrogliak ADdun garunean eragindako hantura-erantzuna dokumentatzen duten froga ugari daude. Zelula mikroglialak, normalean, ehunak mantentzeaz eta garunaren zaintza immunitarioaz arduratzen dira. ADean amiloidearen metaketak dibertsitate mikrogliala pizten du, aktibazio mikroglialaren eragiten duelako, eta aktibazio horrek zelulen plastikotasuna eta estatu aktiboetara igarotzeko gaitasuna islatzen ditu (Cameron eta Landreth, 2010; berrikuspenerako Hansen et al., 2018). Hala, erreakzio mikroglial ugari ikus daitezke gaixotasunaren hainbat etapetan (berrikuspenerako Sarlus eta Heneka, 2017). PET teknikan oinarritutako gizakiekin egindako azterketek, gaitasun kognitiboen murrizketaren eta aktibazio mikroglialaren arteko korrelazioa erakutsi dute narriadura kognitibo arina duten pazienteetan (berrikuspenerako Blasko et al., 2004; Cagnin et al., 2007; Okello et al., 2009). AD duten pazienteen *post-mortem* laginetan (Gomez-Nicola et al., 2013) eta gaixotasunaren sagu-ereduetan, mikroglialen proliferazioa tinko korrelazionatzen da aktibazio mikroglialarekin eta plaken gordailuekin (berrikuspenerako Blasko et al., 2004; Maeda et al., 2007; Kamphuis et al., 2012; Olmos-Alonso et al., 2016). Beraz, zelula mikroglialen izaera dinamikoak eta hauen aniztasun fenotipikoak, agerian uzten du ADean zelula mota hauek karakterizatzeko dagoen beharra (berrikuspenerako Mandrekar-Colucci eta Landreth, 2010). Azken ikerketek adierazten dutenaren arabera, ADeko sagu-ereduek aldaketak erakusten dituzte aktibazio mikroglialean, gaixotasunaren progresioari erantzunez. Gainera, zelula mikroglialen kopurua eta horien tamaina handitzen dira inguratzen duten plakaren tamainaren arabera, modu zuzenki proportzionalean. Plaka amiloideen hurbileko zonaldeetan mikrogliak ere gora egiten du, amiloide gordailuen periferian zelula hauen metatzea ere ematen delarik. ADeko sagu-transgenikoen ereduetan, plakari lotutako zelula mikroglialek euren prozesuak hedatuz Aβ gordailuak barne hartzen dituzte, eta lotura horren bidez plakaren dinamika erregulatu dezakete (berrikuspenerako Mandrekar-Colucci eta Landreth, 2010; berrikuspenerako Kettenmann et al., 2011). Honela, Aβ oligomeroen metaketarekin, mikroglia azkar aktibatzen da estimulu kaltegarri hauek ezabatzeko. Hala ere, *in vivo* ikerketek mikrogliaren funtzio onuragarriak gaixotasuna aurrera joan ahala pixkanaka murrizten direla iradokitzen dute, mikrogliaren fenotipo "toxiko" bat agertzen delarik. Gainera, saguetan egindako ikerketa esperimental ezberdinek zenbait mekanismoen bitartez neuronen galera eragiten duen disfuntzio mikrogliala deskribatzen dute, hala nola, Aβren

metatzea amiloidearen eliminazioaren murrizpena eman ondoren, hantura zitokinen gehiegizko jariaketa, mikrogliak bideratutako sinapsien fagositosia edo τ duten exosoma kaltegarrien askatzea (berrikuspenerako Shen et al., 2017). Beraz, mikrogliaren ugaritzea eta aktibatzea plaka amiloideen inguruan horiek ezabatzeko ahaleginetan, ADren gertakizun bereizgarri bat da (berrikuspenerako Gomez-Nicola eta Perry, 2015). Aldiz, mikrogliaren narriadura eta Aβ-rekiko erantzun mikroglialen asaldurak ADrekiko arrisku handiagoarekin lotzen dira (berrikuspenerako Hansen et al., 2018).

7.1.2. Alzheimer gaixotasunean subikuluan gertatzen diren aldaketa astroglialak

Astrozitoak garuneko zelularik ugarienak dira (berrikuspenerako Blasko et al., 2004) eta funtsezkoak dira neuronen eta, azken batean, garunaren funtzionamendu egokirako (berrikuspenerako Verkhratsky eta Nedergaard, 2018; berrikuspenerako Smit et al., 2021). Zelula-populazio honek CNSean duen garrantzia kontuan hartuta, nire doktorego tesian aztertutako sagu-ereduen astrozitoetan gertatutako aldaketak aztertu nahi izan genituen. CB2$^{EGFP/f/f}$/5xFAD saguetan prozesu astrozitiko GLAST-positiboen azalera eta perimetroa nabarmenki handitu zirela behatu nuen, kopurua nabarmenki murriztu zelarik. Hau da, CB2$^{EGFP/f/f}$/5xFAD saguetan prozesu astrozitiko gutxiago baina handiagoak zeuden, CB2$^{EGFP/f/f}$ saguekin alderatuta.

Konpartimentu astrozitikoak maila ultraestrukturalean argi identifikatzeko, arratoiaren EAAT1-ren C-terminaleko 522-541 hondareak itu dituen anti-A522 (EAAT1 [GLAST]) antigorputza (Ab#314) erabili zen (Hu et al., 2020), Niels Christian Danbolt irakasleak (Osloko Unibertsitatea) eskainia. GLAST astrozitoetan selektiboki adierazten da, eta nire saiakuntzek erakutsi dute GLAST astrozitoetara mugatuta dagoela eta zelulen barruan kokatzen dela, nerbio-terminaletan markaketarik ez delarik antzematen, aurretik deskribatu bezala (Lehre et al., 1995; Schmitt et al., 1997). GLASTen adierazpenean aldeak egon daitezke karraskarien eta gizakien artean, baina meta-analisi azterketek erakutsi zuten garraiatzailearen banaketa oso antzekoa dela bi espezieetan (Li et al., 2012). Beraz, antigorputz honen erabilera astrozitoak markatu eta aldaketa astrozitikoak aztertzeko estrategia ona dela frogatu da ADko ereduan.

Astrozitoen ikuspuntua izugarri aldatu da azken urteotan, zelula laguntzaileen kontzeptutik hasiz zelula multifuntzionalen kontzepturaino, neuronei informazioa prozesatzeko zereginetan lagunduz. Hala, astrozitoek garunaren egitura-arkitektura antolatzeaz ez ezik, komunikazio eta plastikotasunean ere parte hartzen dute

(berrikuspenerako Blasko et al., 2004). Indar eta jarduera sinaptikoa modulatzeaz gain, positiboki eta negatiboki erregulatzen dituzte neurogenesia eta glukogenesia giza garun helduaren eremu neurogenikoetan, zelula aitzindari egoiliarretatik abiatuta (Song et al., 2002; berrikuspenerako Goldman, 2003), astrozitoen, zelula endotelialen eta zelula ependimarioen arteko elkarrekintza parakrinoen bidez (berrikuspenerako Goldman, 2003; Cambier et al., 2005). Gainera, astrozitoek paper garrantzitsua betetzen dute hantura-prozesuetan (Wyss-Coray et al., 2003; berrikuspenerako Blasko et al., 2004). Horretaz gain, beste funtzio batzuk betetzen dituzte: homeostasi ionikoa mantentzea, aminoazido kitzikatzaileen maila estrazelularrak erregulatzea, glutamatoaren kasuan bezala, akoplamendu baskularra, plastikotasun sinaptikoa, zirkuituen eraikuntza, sinapsiak ordeztea, hondakinak ezabatzea edo goragoko funtzioak, hala nola, lo-esnatze zikloa erregulatzea, elikagaien ahoratzea edo oroimena (berrikuspenerako Blasko et al., 2004).

Astrozitoen itxura ezberdina da gaixorik dagoen garunean, zelula mota honek garuneko homeostasiaren alterazio ia guztiei erantzuten baitie, astrogliosi bezala ezagutzen diren aldaketa morfologiko eta molekular garrantzitsuen bidez (berrikuspenerako Escartin et al., 2019). Adibidez, nerabezaroko alkohol kontsumo-eredu batean, aldaketa morfologiakoak deskribatuak izan dira hipokanpo helduan, azalera handiagoa eta prozesu-kopuru murriztuarekin, nerabezaroan EtOH eraginpean egon ondoren (Bonilla-Del Río et al., 2019). AD-an, erreaktibitate astroglialak Aβ gordekinekin asoziaturiko bitarteko zuntzen proteinen adierazpena eta zelulen hipertrofia eragiten ditu (berrikuspenerako Escartin et al., 2019; berrikuspenerako Smit et al., 2021). Mikrogliaren kasuan ez bezala, astrozitoak gai dira Aβ garbitu eta degradatzeko opsonina edo zitokina bezalako inolako estimulurik edo/eta bitartekorik gabe (Bard et al., 2000; Wyss-Coray et al., 2003; berrikuspenerako Blasko et al., 2004). Gainera, astrozito erreaktiboek mikrogliak askatzen dituen antzeko zitokinak eta hazkunde-faktoreak jariatu ditzakete (McGeer eta McGeer, 1995), eta neuronentzako substantzia trofikoak askatu ditzakete, hala nola, nerbio-hazkundearen faktorea (nerve growth factor; NGF) (Aguado et al., 1998), S100β seinalizazio molekula neutotrofikoa (Mrak eta Griffin, 2001, 2005), garunetik eratorritako faktore neurotrofikoa (brain-derived neurotrophic factor; BDNF) (Hock et al., 2000), neurotrofina 3 (neurotrophin 3; NT-3) (Blondel et al., 2000) edo neurotrofina 4/5 (neurotrophin 4/5; NT-4/5). Substantzia horiek askatzeak, aminoazido kitzikatzaileen edo bide antioxidatzaileen metabolismoan inplikatutako molekula garraiatzaileekin eta entzimekin batera, neuronak eta beste garuneko zelula batzuk babesten lagungarria izan daiteke, toxikoak izan daitezkeen substantzien ekoizpena kontrolatuz (berrikuspenerako Blasko et al., 2004).

Laburbilduz, erreaktibitate astrozitikoarekin lotutako aldaketa funtzionalek eragin zuzena izan dezakete komunikazio sinaptikoan eta sare neuronalaren funtzioan, eta horrek narriadura kognitiboan eragina izan dezake (berrikuspenerako Escartin et al., 2019; berrikuspenerako Smit et al., 2021).

7.2. HARTZAILE KANNABINOIDEAK ALZHEIMER GAIXOTASUNAGATIK ERAGINDAKO SUBIKULUKO GLIA-ZELULETAN

CB_1R eta CB_2R kannabinoide nagusien adierazpena aztertzeko mikroglian eta astroglian ADean, nire doktorego tesian $CB2^{EGFP/f/f}$/5xFAD eta $CB2^{EGFP/f/f}$ saguen subikuluak erabili ziren. CB_1R eta CB_2R adierazpenek AD duten saguen zelula glialetan aldaketa nabarmenak jasaten zituztela izan ziren egindako aurkikuntza nagusiak. Horretaz gain, CB_1Raren lokalizazioa subikuluko mikroglia-prozesuetan kontroletan eta plaka amiloideak eta neurita distrofikoak estuki inguratuz AD ereduaren subikuluan, mota honetako zelula glialetan hartzaile honen presentziari buruz aurretik egindako iradokizunak babesten ditu (ikus beherago).

Zelula glialetan dagoen CB_1Raren eta CB_2Raren adierazpen baxua zehaztasunez detektatu ahal izan zen mikroskopia immunoelektronikoaren bidez. Izan ere, aldez aurretik frogatu izan da CB_1R astrozitikoen lokalizaziorako immuno-urre eta immuno-peroxidasaren mikroskopia elektronikorako metodo bikoitza bide ezin hobea dela (Gutiérrez-Rodríguez et al., 2018; Puente et al., 2019). Gainera, lan honetan egin nituen esperimentuek mikroskopia elektronikorako metodo bikoitzaren protokolo konbinatu hori CB1R eta GFP mikroglian kokatzeko optimoa zela erakutsi zuten. CB_1Rren eta CB_2Rren adierazpen mikroglialaren gehikuntza antzeman nuen, eta CB_1Rren dentsitatearen mantentzea astrozitoetan, nahiz eta haien prozesuek erreaktibitatea pairatu. Mikrogliaren CB_1Rren eta CB_2Rren adierazpena aldatu egiten da fenotipo mikroglialaren eta aktibazio-profilaren arabera (berrikuspenerako Stella, 2010). CB_2R-ak modu selektibo eta oparoan adierazten dira NPen inguruko mikroglian (berrikuspenerako Bedse et al., 2015), eta cannabinoideek CB_1Rren adierazpen genikoa handitzen dute CB_2Rren bidez (berrikuspenerako Haspula eta Crark, 2020), beraz, CB_2Raren adierazpenaren handipenak CB_1Rren adierazpenean eragin dezake lesioen alboan. Beraz, badirudi mikrogliak egoera proinflamatorioan bi hartzaile kannabinoideen adierazpena handitzen duela, erreaktibitate gliala murrizteko mekanismo autoerregulatzaile gisa (berrikuspenerako Benito et al., 2008). CB_1Rren eta CB_2Rren adierazpenaren handipenak eragin babeslea izan dezake ADean.

7.2.1. CB2^{EGFP/F/F} ETA CB2^{EGFP/F/F}/5xFAD SAGUEN SUBIKULUKO MIKROGLIAREN CB₁ HARTZAILE KANNABINOIDEAK

Zelula mikroglialek CB_1R-ak adierazten dituztenaren ebidentziak daude (berrikuspenerako Stella, 2009; Ribeiro et al., 2013), eta horiek kannabinoideek zelula horietan dituzten ondorioetako batzuk bitartekatzen dituzte (berrikuspenerako Kaplan, 2013). Horrela, mikroglia atsedenean dagoenean, endokannabinoideak hartzaile honengana zuzenduko lirateke nagusiki (Navarro et al., 2018). Hala ere, mikrogliak ia ez ditu (adieraztekotan) CB_1R (eta CB_2R) adierazten atseden egoeran. Egia esan, CB_1Rren aurkako antigorputz espezifikoek ez zuten CB_1R detektatzeko ahalmenik garun osasauntsuko zelula mikroglialetan. Hala ere, CB_1R zenbait espezietako mikroglia-kultiboetan behatu zen, baina ez gizakienean (Stefano et al., 1996; Sinha et al., 1998; Waksman et al., 1999; Carlisle et al., 2002; Molina-Holgado et al., 2002; Facchinetti et al., 2003; Klegeris et al., 2003; Walter et al., 2003).

Sarreran aipatu dudan bezala, CB_1Rren adierazpena oso altua da terminal inhibitzaileetan baldintza normaletan (Gutiérrez-Rodríguez et al., 2017; berrikuspenerako Katona eta Freund, 2012; berrikuspenerako Lu eta Mackie, 2016),), baxua terminal kitzikatzaileetan (Gutiérrez-Rodríguez et al., 2017; Katona et al., 2006; Monory et al., 2006) eta are baxuagoa astrozitoetan (Gutiérrez-Rodríguez et al., 2018). Gure laborategiak aurreko azterlanetan aurkeztu duen moduan, CB_1Rren markaketaren ∼ % 56 terminal gabergikoetan dago, ∼ % 12 terminal glutamatergikoetan, ∼ % 6 astrozitoetan eta ∼ % 15 mitokondrietan (Gutiérrez-Rodríguez et al., 2018; Bonilla-Del Río et al., 2019). Aipatzekoa da CB_1Rren immunopartikulen ∼ % 11 beste leku batzuetan kokatu zirela (Gutiérrez-Rodríguez et al., 2018; Bonilla-Del Río et al., 2019), lisosoma/endosometan koatutako CB_1Rekin bat etor daitekeena (Rozenfeld eta Devi, 2008) edo mikroglia bezalako beste konpartimentu zelular batzuetan.

Lan honetan ADko baldintzetan, aktibazio mikroglial nabarmenarekin, CB_1Rren adierazpenean gertatzen diren aldaketak saiatu naiz argitzen. Subikulua behatu izan dut Iba1-en kontrako antigorputza erabiliz, azpipopulazio mikroglial mota guztiak markatzen dituelarik (Ito et al., 1998; Okere eta Kaba, 2000; Hirayama et al., 2001; Shapiro et al., 2008), eta CB2^{EGFP/f/f} saguen mikroglia mintzetan CB_1Rren presentzia ikusi dut. Aurkikuntza hauek bat datoz gure laborategiak berriki izan dituen aurretiazko ebidentziekin, mikroskopia elektronikorako bereizmen handiko teknika immunozitokimikoak ere erabiliz. CB_1Raren kokapena prozesu mikroglialen ∼ % 9an (hondoa kenduta) topatu izan da CX3C 1

hartzailearen adierazpenaren pean mikroglian EGFP adierazten duen sagu transgeniko baten hipokanpoan (argitaratu gabe). Portzentaia baxua da, egoera patologikoan aldatu daitekeena.

Era berean, CB2$^{EGFP/f/f}$/5xFAD saguetan CB1Rarekiko positiboak ziren prozesu mikroglialen kopuruan eta portzentaian handipen esanguratsua ere topatu nuen CB2$^{EGFP/f/f}$ saguekin alderatuta. Hala ere, ez zen aldaketa esanguratsurik egon prozesu mikroglialetako CB1R-en partikulen kopuruan, baina CB1R dentsitatea nabarmen murriztu zen prozesu mikroglialen perimetro-igoeraren ondorioz. Gainera, prozesu mikroglial CB1R-positibo gehiago zeuden, eta horrek CB2$^{EGFP/f/f}$/5xFAD saguetan prozesu mikroglial gehiago zeudela CB1Rren eraginpean iradokitzen du. Gure laborategiaren aurretiko emaitzek, 10 hilabeteko WT saguen subikuluan eta plaka amiloideak dituzten saguetan, CB1R-ak prozesu mikroglialen % 15-20an daudela adierazten dute. Ehuneko hori handiagoa da nire emaitzek 6.5-7.5 hilabeteko saguetan erakusten zutena baino, izan ere, CB2$^{EGFP/f/f}$/5xFAD saguetan CB1R-prozesu positiboen proportzioa ~ % 7 zen. Ezberdintasun hori zehatz-mehatz aztertu beharko litzateke, degenerazioak aurrera egin ahala CB1Rren adierazpenak sagu-eredu horretan gora egiten duela iradokitzen baitu. Aldi berean, CB2R gehiago adierazten da ADko etapa aurreratuetan, neuroinflamazioa nabariagoa denean (ikus aurrerago; berrikuspenerako Di Marzo et al., 2015; berrikuspenerako Talarico et al., 2019).

CB1R adierazpenak gora egiten du AD bezalako gaixotasun inflamatorio eta neurodegeneratibo askotan (berrikuspenerako Bisogno eta Di Marzo, 2010; Ribeiro et al., 2013). Aktibazio mikroglialak ere 2-AGren eta AEAren ekoizpena nabarmen handitzea eragiten du, atsedenean dagoen mikrogliarekin alderatuta. ECBs horiek, era berean, CB1R eta CB2R gehiago aktibatzen dituzte, baita horien seinaleztapeneko ur-jauziak ere, mikrogliaren hanturaren aurkakoa eta babeslea den fenotipoa anplifikatuz (berrikuspenerako Mecha et al., 2016; berrikuspenerako Duffy et al., 2021). CB1Rek zelula mikroglialen funtzioa arautzen duten modua eztabaidagarria da (berrikuspenerako Stella, 2010). Ildo horretan, CB1R mikroglialak aktibatzeak inhibitu egiten du liposakaridoek-induzitutako NO askapena (Stefano et al., 1996; Waksman et al., 1999) eta MPTPak-induzitutako oxidatzaileen ekoizpena (Chung et al., 2011), besteak beste (berrikuspenerako Kaplan, 2013). Zenbait ikerketek, CB1R-ak eta CB2R-ak elkarreragin dezaketela ere proposatu dute, heteromeroak eratuz, eta hauen egitura kuaternarioa mikroglia atsedenean edo aktibatuta dagoen arabera aldatuko litzateke (Navarro et al., 2018). Horrek azalduko luke CB1R eta CB2R dauzkaten eragin ezberdinak modulazio immunean, aktibazio mikroglialean eta neuroinflamazioari aurre egiterakoan (berrikuspenerako Kaplan, 2013; berrikuspenerako Stella, 2010; Navarro et al., 2018).

7.2.2. CB2^{EGFP/F/F} ETA CB2^{EGFP/F/F}/5xFAD SAGUEN SUBIKULUKO MIKROGLIAREN CB₂ HARTZAILE KANNABINOIDEAK

CB₂R-ei dagokienez, garun osasuntsuan soilik ARNm pixka bat antzeman da, eta horrek iradokitzen du hartzaile hau atsedenean dagoen mikroglian ez dela asko adierazten (Munro et al., 1993; Derocq et al., 1995; Galiègue et al., 1995; Schatz et al., 1997; Griffin et al., 1999; McCoy et al., 1999; Sugiura et al., 2000; Carlisle et al., 2002). Izan ere, CB₂R periferikotzat hartzen zen barean eta giza leukozitoetan zuen espresio oso altuagatik (Munro et al., 1993; Galiègue et al., 1995). Duela urte batzuk, CB₂R giza zerebeloaren substantzia zuriaren zelula mikroglial peribaskularretan aurkitu zen (Núñez et al., 2004). Kultiboetako mikrogliak ere CB₂R adierazten du (Carlisle et al., 2002; Facchinetti et al., 2003; Klegeris et al., 2003; Walter et al., 2003) patogeno eta zitokina batzuek erregulatuta (Carayon et al., 1998; Waksman et al., 1999, Derocq et al., 2000; Lee et al., 2001; Gardner et al., 2002). Gainera, proliferazio-tasa altua duten zelula mikroglialen lerroek CB₂R adierazten dute (Walter et al., 2003; Carrier et al., 2004). Garunean, mikrogliak baldintza jakin batzuetan adierazten da CB₂R. Adibidez, min neuropatikoko ereduetan bizkarrezur-muineko mikroglian CB₂R handitzen da, baina hau ez da ikusten hanturazko min kroniko periferikoko eredu batean (Zhang et al., 2003). Era berean, mikrogliaren CB₂Rren gehikuntza ematen da hanturan (Maresz et al., 2005) eta AD edo esklerosi anizkoitza duten pazienteen garun-ehunean aktibatutako mikroglian, batez ere lesioen lekuetan (Benito et al., 2003; Yiangou et al., 2006). Baita tumoreen inguruko mikroglian (berrikuspenerako Guzmán et al., 2001) eta hartutako immunoeskasiaren sindromearen dementziaren tximino-eredu baten mikroglia aktibatuan (Benito et al., 2005). CNSean baldintza basaletan CB₂R-ak aurkitzeko lehen saiakerek porrot egin zuten, CB₂R-ak baldintza patologikoetan soilik ikus zitezkeelako, lehen deskribatu den bezala. Hala ere, CB₂R mikroglian ez ezik (berrikuspenerako Cabral et al., 2008; berrikuspenerako Atwood eta Mackie, 2010) neuronetan ere detektatu zen (Van Sickle et al., 2005; Zhang et al., 2014). Hala ere, zalantza handiak daude CNSeko CB₂Rren kokapenari buruz, CB₂Rren aurkako antigorputz espezifikorik ez dagoelako (berrikuspenerako Atwood eta Mackie, 2010; berrikuspenerako Lu eta Mackie, 2016), eta horrek garunean CB₂Rren banaketa frogatzea eragozten du. Muga hori gainditzeko, azken aldian sagu-lerro berrietan oinarritutako estrategia genetiko berriak garatu dira, CB₂R-ak garunean nola kokatzen diren argitzeko, nire doktorego-tesian erabilitakoa bezalakoa. Sagu-eredu horretan, GFParen adierazpena detekzio-mailen azpitik zegoen (López et al., 2018). Hala ere, egileek ikusi zuten CB2^{EGFP/f/f}/5xFAD saguetan garuneko hainbat eskualdetan, plaka neuritikoak zeuden lekuekin bat etortzen zela. Bestalde, GFParen adierazpena (CB₂R) plaka neuritikoetatik

gertuko zelula mikroglialetara mugatuta zegoen. Garrantzitsua da nabarmentzea GFParen adierazpen-eredu hori (CB2R) ez zela ikusi $CB_2^{-/-}$/5xFAD saguetan (López et al., 2018).

Aurreko behaketa horiekin bat, nire doktorego tesian CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan prozesu mikroglial GFP-positiboen kopuruaren eta proportzioaren igoera esanguratsua ematen dela frogatu dut, eta baita GFP mikroglialaren partikula kopurua ere. Patologiarik gabeko saguen (CB2$^{EGFP/f/f}$) subikuluko mikroglian CB_2Rren detekzioa Interesgarria da ere. Honek CB_2Rren adierazpen basala dagoela burmuin osasuntsuko mikroglian iradokitzen du. Bestela, baliteke adierazpen hori CB2$^{EGFP/f/f}$ kontrol-saguen adinari lotutako zahartze-zeinu goiztiarrak adieraztea (6.5-7.5 hilabete).

CB_2R adierazpena areagotu egiten da aktibatutako mikroglian baldintza neuropatologiko eta neuroinflamatorio batzuen aurrean. Kaltetutako neuronek eta lesioek estimulatutako astrozitoek sortutako ECBs-ek mikroglian adierazitako CB_2R aktibatzen dute (berrikuspenerako Stella, 2009). Horrela, zelula horien funtzio immunitarioak erregulatzen dituzte, proliferazio eta migrazio mikrogliala estimulatuz eta, aldi berean, TNFα edo erradikal askeak bezalako faktore neurotoxikoak murriztuz (Walter et al., 2003; Carrier et al., 2004; Ramirez et al., 2005; Eljaschewitsch et al., 2006; Dirikoc et al., 2007; Lin et al., 2017) horrela mikrogliak murriztuz lesioen ondoko ondorio kaltegarriak. Oro har, nire doktorego tesian behatutako zelula mikroglialen CB_2Rren areagotzea, konpentsazio-mekanismo bat izan liteke ADrekin erlazionatutako hantura- eta neurodegenerazio-mekanismoen ondorio negatiboak arintzeko. Zelula mikroglialetan CB_2R-en adierazpena handitzeak perspektiba berriak irekitzen ditu, bai hartzaile horrek CNSean duen eginkizun funtzionalari dagokionez, bai tratamendu antiinflamatorio berriak garatzeko dituen eragin terapeutikoei dagokienez (berrikuspenerako Benito et al., 2008).

7.2.3.   CB2$^{EGFP/F/F}$ ETA CB2$^{EGFP/F/F}$/5xFAD SAGUEN SUBIKULUKO ASTROGLIAREN CB$_1$ HARTZAILE KANNABINOIDEAK

NPs-en inguruan astrozitoetan gertatzen diren aldaketa morfologikoak kontuan hartuta, CB_1R astrozitikoaren adierazpena ikertzea beharrezkoa dirudi, hartzaile honek funtzio garrantzitsuak betetzen baititu astrozitoetan. Adibidez, CB_1Rak glukosaren oxidazio eta zetogenesiaren tasak handitzen ditu (Blazquez et al., 1999; berrikuspenerako Stella, 2010), euren hanturaren bitartekariak sortzeko gaitasuna modulatzen du (Sheng et al., 2005; berrikuspenerako Stella, 2010) edo endokannabinoideek bideratutako neurona-astrozito komunikazioan parte hartzen du (Navarrete and Araque, 2008). Gainera, FAAH maila altuak aurkitu dira NPen inguruko astrozitoetan (Benito et al., 2003; berrikuspenerako Bedse et al.,

2015; berrikuspenerako Abate et al., 2021). Ebidentzia guzti horiek ESren aldaketak ADren lesioetatik hurbilen dauden astrozitoetan gertatzen direla iradokitzen dute.

Astrozito erreaktiboek ADren patogenesiari egiten dioten ekarpena zein den zehazteko, beharrezkoa da haien aldaketa funtzionalen ondorioak ulertzea, azkenean dementziara daramatenak (berrikuspenerako Smit et al., 2021). Horrela, CB2$^{EGFP/f/f}$/5xFAD saguen astrozito subikularretan CB$_1$R adierazpena ebaluatu zen. CB2$^{EGFP/f/f}$/5xFAD saguetan, astrozito-prozesu bakoitzeko CB$_1$Rren partikula kopuruaren handipen esanguratsua behatu nuen., baina ez zen alde nabarmenik egon CB2$^{EGFP/f/f}$ saguekin CB$_1$R partikulen kopuruan, ezta prozesu CB$_1$R-positiboen kopuruan edo ehunekoan ere. Bi sagu transgeniko en arteko CB$_1$Rren dentsitatean ere ez nuen alde nabarmenik ikusi. Beraz, badirudi prozesu astrozitiko erreaktiboen perimetroa handitu ahala, CB$_1$R-ek ere gora egiten dutela, hartzailearen dentsitatea eta adierazpena aldatu gabe mantenduz. Horrela, bada, CB$_1$Ren adierazpenean aldaketa adaptatiboak gertatu ziren, prozesu astrozitiko gutxiago zeudelako, baina handiagoak, eta CB$_1$R gehiago zeukatenak mintzetan. Beraz, plaken inguruan erreaktibitate astrozitiko argia zegoen arren, CB$_1$R-etan ez zeuden aldaketarik. Hala ere, CB$_1$Rren adierazpena astrozitoetan, hauek neurita distrofikoekiko edo/eta SP-ekiko duten distantziaren arabera aldatzen den argitzear dago. Azkenik, interesgarria litzateke ES-ko beste osagai batzuk ikertzea CB2$^{EGFP/f/f}$/5xFAD saguen astrozitoetan.

Astrozitoen hanturak disfuntzio astrogliala dakar (berrikuspenerako Adermark eta Bowers, 2016) eta astrozitoen bitarteko zuntzetan aurkitzen den GFAParen alterazioa eragiten du (Renau-Piqueras et al., 1989). Astrozitoek hantura erantzunean parte hartzen dute molekula proinflamatorioak askatzeko gaitasuna dutelako (berrikuspenerako Farina et al., 2007). Molekula horiek murriztu egin daitezke CB1R astroglialen gainean jarduten duten ECBs-en erreakzio antiinflamatorioen bidez (berrikuspenerako Metna-Laurent eta Marsicano, 2015). Gainera, baliteke handitutako prozesu astrozitikoak eraginkorrak ez izatea neuronen jarduerari erantzunez sortutako ECBs hautemateko, kannabinoideek sinapsietan eragindako gliotransmisoreen eskuragarritasuna arriskuan jarriz (Han et al. 2012; Araque et al., 2014). Beraz, hantura astrozitikoa dela eta, zentzuzkoa da CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan behatutako plaken eta neurita distrofikoen inguruko astrogliaren erreakzio antiinflamatorioaren asaldura espero izatea. Gainera, NT-en garbiketan eta gliotransmisioan gerta daitezken alterazioak direla eta, plastikotasun sinaptikoan defizitak eman daitezke (berrikuspenerako Dzyubenko et al., 2016) eta, ondorioz, garuneko disfuntzioa eragin. Zehazteke dago asaldura astrozitikoak CB2$^{EGFP/f/f}$/5xFAD saguetan GLAST adierazpenaren aldaketekin korrelazioan dauden.

CB$_1$R astrozitikoek, tonu endokannabinoide basalarekin batera, garun funtzioetan paper garrantzitsua jokatzen dute, hala nola plastikotasun sinaptikoan, oroimenean eta portaeran (Han et al., 2012; Navarrete eta Araque, 2008; berrikuspenerako Perea et al., 2009; Navarrete eta Araque, 2010). Adibidez, astrozitoetako CB$_1$R-ek lan memoria espazialaren defizitean eta kannabinoideekiko esposizio akutuaren ondoren hipokanpoaren CA3-CA1 sinapsietan emandako in vivo LTDan bitartekari dira (Han et al., 2012). Dirudienez, CB$_1$R astrozitikoaren adierazpena hainbat faktorek erregulatzen dute, beste hartzaile kannabinoideren bat faltan duten animalia transgenikoetan edo beste baldintza ezberdinetan alda daitekeelako. Adibidez, TRPV1 knock out saguek CB$_1$R dentsitatea nabarmenki murrizturik dute astrozitoetan (Egaña-Huguet et al., 2021). Gainera, THCaren injekzio akutuaren ondoren, gure laborategiak CB$_1$R astroglialaren gorakada nabarmena deskribatu du (Bonilla-Del Río et al., 2021). Horretaz gain, gehikuntza horrek zelula horien morfologiari eragin diezaioke nolabait. Izan ere, THCarekiko esposizio akutuaren ondoren prozesu astrozitikoen azalera murrizten dela ere deskribatu da (Bonilla-Del Río et al., 2021), eta horrek garun-funtzioari eta portaerari eragin diezaieke (Tahir et al., 1992; Suárez et al., 2000).

Oro har, morfologia astroglialaren aldaketek eta haren hantura-ondorioek narriadura kognitiboa eragin dezakete. Disfuntzio astroglialek ADean izan ditzakeen ondorioak ez dira oso ezagunak (berrikuspenerako Verkhratsky eta Nedergaard, 2018; berrikuspenerako Smit et al., 2021), nahiz eta funtzio astrozitikoan eta neurona eta astrozitoen arteko elkarrekintzetan zentratutako azterlanak argitaratzen ari diren (Escartin et al., 2019; berrikuspenerako Smit et al.). Horrek, zalantzarik gabe, astrozitoek osasunean eta gaixotasunean duten eginkizuna hobeto ulertzea ekarriko du, eta hori funtsezkoa da ADren patogenesiaren espektro osoa ulertzeko. Informazio horri esker, astrozitoei zuzendutako terapia berritzaileak garatu ahal izango dira, zelula hauek eragindako kalteari aurre egiteko, edo, kaltea oraindik larriegia eta itzulezina ez denean, horiek konpontzeko edo/eta berreskuratzeko (berrikuspenerako Smit et al., 2021).

7.2.4. KANNABINOIDEETAN OINARRITUTAKO POTENTZIAL TERAPEUTIKOA ALZHEIMER GAIXOTASUNEAN

Kannabinoideek jarduera pleiotropikoa dute, eta ADren patogenesian inplikatutako zenbait prozesutan eragiten dute aldi berean (berrikuspenerako Aso eta Ferrer, 2014). Horrela, kannabinoideek ondorio onuragarriak izan ditzakete Aβ agregazioa murriztuz, τ proteinaren hiperfosforilazioa inhibituz eta ROSen eraketa inhibituz, besteak beste (Casarejos et al., 2013; berrikuspenerako Aso eta Ferrer, 2014; berrikuspenerako Talarico et al., 2019;

Soto-Mercado et al., 2020). Doktorego tesi honetan ikusi diren aldaketarik esanguratsuenak, CB_1R eta CB_2R mikroglialen handipenak dira, AD tratatzeko kannabinoideetan oinarritutako terapien itu potentzialtzat har daitezkeenak. Kannabinoideek mikroglian duten eraginari dagokionez, oraindik deszifratzear dago, baina oso itxaropentsua dirudi (Esposito et al., 2006; berrikuspenerako Talarico et al., 2019). Beraz, efektu neurobabesle eta antiinflamatorioen konbinazioarekin aktibazio gliala kontrolatzeko gai diren espektro zabaleko konposatuak, ADerako ikuspegi terapeutiko berri gisa har daitezke kontuan. Hala ere, zelula glialei zuzendutako bitartekari terapeutikoen eragina, efektu kaltegarrien indargabetzearen eta, aldi berean, garunaren defentsa-mekanismo onuragarrien mantentzearen arteko oreka egokiaren araberakoa da (berrikuspenerako Hansen et al., 2018; berrikuspenerako Scuderi et al., 2020).

Doktorego tesi lan honek, beraz, zelula astrozitikoetan eta mikroglialetan gertatutako aldaketa morfologikoak eta CB$_1$Rren adierazpena bi zelula mota hauetan karakterizatzeko balio izan du, 6.5 eta 7.5 hilabete bitarteko 5xFAD sagu-eredu batean. Gainera, CB2$^{EGFP/f/f}$ eta CB2$^{EGFP/f/f}$/5xFAD ereduei esker, CB$_2$Rren adierazpen mikrogliala karakterizatu ahal izan dugu. Glia mailan bi hartzaile horiek oso garrantzitsuak dirudite patologia honetan zehar, eta kontuan har daitezke ADeko itu terapeutiko gisa, gaur egun tratamendu eraginkorrik ez duen gaixotasuna delarik.

8. ONDORIOAK

Hona hemen nire doktorego tesiaren ondorioak:

1. CB2$^{EGFP/f/f}$/5xFAD saguaren subikuluko mikrogliak prozesuen azaleran, perimetroan eta kopuruan handipena aurkezten du.

2. CB2$^{EGFP/f/f}$/5xFAD saguaren subikuluko astrozitoek adarkaduren kopuruaren murrizpena adierazten dute, baina adar horien azalera eta perimetroa handitu egin da. Hala, azalera eta perimetroa konstante mantentzen dira laginaren azalerean zehar.

3. CB$_1$Rrekiko immunopositiboak diren mikroglia-prozesuen kopurua eta proportzioa nabarmen handitzen dira CB2$^{EGFP/f/f}$/5xFAD saguaren subikuluan, baina prozesu mikroglial bakoitzeko CB$_1$Rren partikulen kopurua konstante mantentzen da. Horrek, CB2$^{EGFP/f/f}$/5xFAD saguetan ematen den prozesuen perimetroaren handitzearekin batera, CB$_1$R mikroglialaren dentsitatea murriztea dakar.

4. Mikroglia-prozesu GFP-positiboen kopurua eta portzentaia esanguratsuki handiagotzen dira CB2$^{EGFP/f/f}$/5xFAD saguaren subikuluan. Era berean, GFP partikula kopurua mikroglia prozesu bakoitzaren azalerarekino eta laginaren azalerarekiko nabarmen handitzen dira. Beraz, CB$_2$R mikroglialaren igoera dago CB2$^{EGFP/f/f}$/5xFAD saguan.

5. Prozesu astrozitiko bakoitzeko CB$_1$Rren partikula kopurua handitu egiten da, baina CB$_1$Rren dentsitatea konstante mantentzen da CB2$^{EGFP/f/f}$/5xFAD saguen subikuluan. Beraz, CB$_1$Rren adierazpena ez da aldatzen ADeko sagu eredu honetan.

6. Oro har, doktorego tesi honek frogatu du CB$_1$Rren eta CB$_2$Rren adierazpenak aldatzen direla CB2$^{EGFP/f/f}$/5xFAD saguetan, eta aldaketa horiek AD-ak eragindako narriadura kognitiboan eragina izan dezaketela.

9. BIBLIOGRAFIA

-Abate G, Uberti D, Tambaro S. Potential and Limits of Cannabinoids in Alzheimer's Disease Therapy. Biology 2021;10(6):542.

-Adermark L, Bowers MS. Disentangling the role of astrocytes in alcohol use disorder. Alcoholism: Clinical and Experimental Research 2016; 40(9):1802-1816.

-Allen Mouse Brain Atlas [Internet]. Allen Institute for Brain Science; 2004 [Kontsulta: 2021-06-09]. Eskuragarri: atlas.brain-map.org/.

-Aguado F, Ballabriga J, Pozas E, Ferrer I. TrkA immunoreactivity in reactive astrocytes in human neurodegenerative diseases and colchicine-treated rats. Acta neuropathologica. 1998; 96(5):495-501.

-Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. Nat. Neurosci. 2007;10(12):1538-1543.

-Almolda B, de Labra C, Barrera I, Gruart A, Delgado-Garcia JM, Villacampa N, et al. Alterations in microglial phenotype and hippocampal neuronal function in tablw mice with astrocyte-targeted production of interleukin-10. Brain Behav. Immun. 2015; 45:80-97.

-Apelt J, Ach K, Schliebs R. Aging-related down-regulation of neprilysin, a putative β-amyloid-degrading enzyme, in transgenic Tg2576 Alzheimer-like mouse brain is accompanied by an astroglial upregulation in the vicinity of β-amyloid plaques. Neurosci. Lett. 2003; 339(3):183-186.

-Araque A, Carmignoto G, Haydon PG, Oliet SH, Robitaille R, Volterra A. Gliotransmitters travel in time and space. Neuron 2014; 81(4):728-739.

-Askew K, Li K, Olmos-Alonso A, Garcia-Moreno F, Liang Y, Richardson P, et al. Coupled proliferation and apoptosis maintain the rapid turnover of microglia in the adult brain. Cell reports 2017; 18(2):391-405.

-Aso E., Ferrer I. Cannabinoids for treatment of Alzheimer's disease: moving toward the clinic. Frontiers in pharmacology 2014; 5:37.

-Aso E, Ferrer I. CB2 cannabinoid receptor as potential target against Alzheimer's disease. Frontiers in neuroscience 2016; 10:243.

-Athanasiou A, Clarke AB, Turner AE, Kumaran NM, Vakilpour S, Smith PA, et al. Cannabinoid receptor agonists are mitochondrial inhibitors: a unified hypothesis of how cannabinoids modulate mitochondrial function and induce cell death. Biochem. Biophys. Res. Commun. 2007; 364(1):131-137.

-Atwood BK, Mackie K. CB2: a cannabinoid receptor with an identity crisis. Br. J. Pharmacol. 2010; 160(3):467-479.

-Bachmeier C, Beaulieu-Abdelahad D, Mullan M, Paris D. Role of the cannabinoid system in the transit of beta-amyloid across the blood–brain barrier. Molecular and Cellular Neuroscience 2013; 56:255-262.

-Bakota, L., and Brandt, R. Tau biology and Tau-directed therapies for Alzheimer's disease. Drugs 2016; 76, 301–313.

-Bard F, Cannon C, Barbour R, Burke RL, Games D, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K. Peripherally administered antibodies against amyloid β-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nature medicine. 2000; 6(8):916-9.

-Beauquis J, Pavía P, Pomilio C, Vinuesa A, Podlutskaya N, Galvan V, et al. Environmental enrichment prevents astroglial pathological changes in the hippocampus of APP transgenic mice, model of Alzheimer's disease. Exp. Neurol. 2013;239:28-37.

-Bedse G, Romano A, Cianci S, Lavecchia AM, Lorenzo P, Elphick MR, et al. Altered expression of the CB1 cannabinoid receptor in the triple transgenic mouse model of Alzheimer's disease. J. Alzheimer's Dis. 2014; 40(3):701-712.

-Bedse G, Romano A, Lavecchia AM, Cassano T, Gaetani S. The role of endocannabinoid signaling in the molecular mechanisms of neurodegeneration in Alzheimer's disease. J. Alzheimer's Dis. 2015;43(4):1115-1136.

-Benito C, Nunez E, Tolon RM, Carrier EJ, Rabano A, Hillard CJ, et al. Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. J. Neurosci. 2003; 23(35):11136-11141.

-Benito C, Kim WK, Chavarria I, Hillard CJ, Mackie K, Tolon RM, et al. A glial endogenous cannabinoid system is upregulated in the brains of macaques with simian immunodeficiency virus-induced encephalitis. J. Neurosci. 2005; 25(10):2530-2536.

-Benito C, Tolon R, Pazos M, Nunez E, Castillo A, Romero J. Cannabinoid CB2 receptors in human brain inflammation. Br. J. Pharmacol. 2008; 153(2):277-285.

-Bezprozvanny I, Mattson MP. Neuronal calcium mishandling and the pathogenesis of Alzheimer's disease. Trends Neurosci. 2008; 31(9):454-463.

-Bilkei-Gorzo A, Racz I, Valverde O, Otto M, Michel K, Sastre M, et al. Early age-related cognitive impairment in mice lacking cannabinoid CB1 receptors. Proc. Natl. Acad. Sci. U.S.A. 2005; 102(43):15670-15675.

-Bilkei-Gorzo A. The endocannabinoid system in normal and pathological brain ageing. Philosophical Transactions of the Royal Society B: Biological Sciences 2012; 367(1607):3326-3341.

-Biringer RG. The rise and fall of anandamide: processes that control synthesis, degradation, and storage. Mol. Cell Biochem. 2021; 476(7):2753-2775.

-Bisogno T, Berrendero F, Ambrosino G, Cebeira M, Ramos J, Fernandez-Ruiz J, et al. Brain regional distribution of endocannabinoids: implications for their biosynthesis and biological function. Biochem. Biophys. Res. Commun. 1999; 256(2):377-380.

-Bisogno T, Di Marzo V. Cannabinoid receptors and endocannabinoids: role in neuroinflammatory and neurodegenerative disorders. CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders) 2010; 9(5):564-573.

-Bjørnsen L, Eid T, Holmseth S, Danbolt N, Spencer D, De Lanerolle N. Changes in glial glutamate transporters in human epileptogenic hippocampus: inadequate explanation for high extracellular glutamate during seizures. Neurobiol. Dis. 2007; 25(2):319-330.

-Blasko I, Veerhuis R, Stampfer-Kountchev M, Saurwein-Teissl M, Eikelenboom P, Grubeck-Loebenstein B. Costimulatory effects of interferon-γ and interleukin-1β or tumor necrosis factor α on the synthesis of Aβ1-40 and Aβ1-42 by human astrocytes. Neurobiol. Dis. 2000; 7(6):682-689.

-Blasko I, Stampfer-Kountchev M, Robatscher P, Veerhuis R, Eikelenboom P, Grubeck-Loebenstein B. How chronic inflammation can affect the brain and support the development of Alzheimer's disease in old age: the role of microglia and astrocytes. Aging cell 2004; 3(4):169-176.

-Blázquez C, Sánchez C, Daza A, Galve-Roperh I, Guzmán M. The stimulation of ketogenesis by cannabinoids in cultured astrocytes defines carnitine palmitoyltransferase I as a new ceramide-activated enzyme. J. Neurochem. 1999; 72(4):1759-1768.

-Blondel O, Collin C, McCarran WJ, Zhu S, Zamostiano R, Gozes I, Brenneman DE, McKay RD. A glia-derived signal regulating neuronal differentiation. Journal of Neuroscience. 2000; 20(21):8012-20.

-Bloom GS. Amyloid-β and tau: the trigger and bullet in Alzheimer disease pathogenesis. JAMA neurology 2014; 71(4):505-508.

-Bobkova N, Vorobyov D - Institute of Cell Biophysics, Russian Academy of Sciences, 142290 Pushchino, Moscow Region,,Russia. The brain compensatory mechanisms and Alzheimer's disease progression: a new protective strategy. Neural Regen. Res. 2015; 10(5):696-697.

-Bonilla-Del Río I, Puente N, Peñasco S, Rico I, Gutiérrez-Rodríguez A, Elezgarai I, et al. Adolescent ethanol intake alters cannabinoid type-1 receptor localization in astrocytes of the adult mouse hippocampus. Addict. Biol. 2019; 24(2):182-192.

-Bonilla-Del Río I, Puente N, Mimenza A, Ramos A, Serrano M, Lekunberri L, et al. Acute Δ9-tetrahydrocannabinol prompts rapid changes in cannabinoid CB1 receptor immunolabelling and subcellular structure in CA1 hippocampus of young adult male mice. J. Comp. Neurol. 2021; 529(9):2332-2346.

-Boros, B. D., Greathouse, K. M., Gentry, E. G., Curtis, K. A., Birchall, E. L., Gearing, M., et al. Dendritic spines provide cognitive resilience against Alzheimer's disease. Ann. Neurol. 2017; 82, 602–614.

-Bosier B, Bellocchio L, Metna-Laurent M, Soria-Gomez E, Matias I, Hebert-Chatelain E, et al. Astroglial CB1 cannabinoid receptors regulate leptin signaling in mouse brain astrocytes. Molecular metabolism 2013; 2(4):393-404.

-Brodmann K. Vergleichende Lokalisationslehre der Grosshirnrinde in ihren Prinzipien dargestellt auf Grund des Zellenbaues. : Barth; 1909.

-Brugg B, Dubreuil YL, Huber G, Wollman EE, Delhaye-Bouchaud N, Mariani J. Inflammatory processes induce beta-amyloid precursor protein changes in mouse brain. Proc. Natl. Acad. Sci. U.S.A. 1995; 92(7):3032-3035.

-Cabral G, Raborn E, Griffin L, Dennis J, Marciano-Cabral F. CB2 receptors in the brain: role in central immune function. Br. J. Pharmacol. 2008; 153(2):240-251.

-Cagnin A, Kassiou M, Meikle SR, Banati RB. Positron emission tomography imaging of neuroinflammation. Neurotherapeutics 2007; 4(3):443-452.

-Cambier S, Gline S, Mu D, Collins R, Araya J, Dolganov G, et al. Integrin αvβ8-mediated activation of transforming growth factor-β by perivascular astrocytes: an angiogenic control switch. The American journal of pathology 2005; 166(6):1883-1894.

-Cameron B, Landreth GE. Inflammation, microglia, and Alzheimer's disease. Neurobiol. Dis. 2010; 37(3):503-509.

-Carayon P, Marchand J, Dussossoy D, Derocq J, Jbilo O, Bord A, et al. Modulation and functional involvement of CB2 peripheral cannabinoid receptors during B-cell differentiation. Blood, The Journal of the American Society of Hematology 1998; 92(10):3605-3615.

-Carlesimo GA, Piras F, Orfei MD, Iorio M, Caltagirone C, Spalletta G. Atrophy of presubiculum and subiculum is the earliest hippocampal anatomical marker of Alzheimer's disease. Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring 2015; 1(1):24-32.

-Carlisle S, Marciano-Cabral F, Staab A, Ludwick C, Cabral G. Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. Int. Immunopharmacol 2002; 2(1):69-82.

-Carrier EJ, Kearn CS, Barkmeier AJ, Breese NM, Yang W, Nithipatikom K, et al. Cultured rat microglial cells synthesize the endocannabinoid 2-arachidonylglycerol, which increases proliferation via a CB2 receptor-dependent mechanism. Mol. Pharmacol. 2004; 65(4):999-1007.

-Casarejos MJ, Perucho J, Gomez A, Munoz MP, Fernandez-Estevez M, Sagredo O, et al. Natural cannabinoids improve dopamine neurotransmission and tau and amyloid pathology in a mouse model of tauopathy. J. Alzheimer's Dis. 2013; 35(3):525-539.

-Castillo PE, Younts TJ, Chávez AE, Hashimotodani Y. Endocannabinoid signaling and synaptic function. Neuron 2012; 76(1):70-81.

-Chung YC, Bok E, Huh SH, Park JY, Yoon SH, Kim SR, et al. Cannabinoid receptor type 1 protects nigrostriatal dopaminergic neurons against MPTP neurotoxicity by inhibiting microglial activation. J. Immunol. 2011; 187(12):6508-6517.

-Cline, E. N., Bicca, M. A., Viola, K. L., and Klein, W. L. The amyloid-b oligomer hypothesis: beginning of the third decade. J. Alzheimers Dis. 2018; 64, S567–S610.

-Colton CA, Chernyshev ON, Gilbert DL, Vitek MP. Microglial contribution to oxidative stress in Alzheimer's disease. Ann. N.Y. Acad. Sci. 2000; 899(1):292-307.

-Coronel R, Palmer C, Bernabeu-Zornoza A, Monteagudo M, Rosca A, Zambrano A, et al. Physiological effects of amyloid precursor protein and its derivatives on neural stem cell biology and signaling pathways involved. Neural. Regen. Res. 2019; 14(10):1661-1671.

-De la Vega, R., Zambrano, A. Alzheimer. Circunvalación del Hipocampo [Internet]. Hipocampo.org. Oct 2013 [Kontsulta: 2020-12-16]. Eskuragarri: https://www.hipocampo.org/alzheimer.asp/.

-Delcambre GH, Liu J, Herrington JM, Vallario K, Long MT. Immunohistochemistry for the detection of neural and inflammatory cells in equine brain tissue. PeerJ 2016; 4:e1601.

-Derocq J, Segui M, Marchand J, Le Fur G, Casellas P. Cannabinoids enhance human B-cell growth at low nanomolar concentrations. FEBS Lett. 1995; 369(2-3):177-182.

-Derocq JM, Jbilo O, Bouaboula M, Segui M, Clere C, Casellas P. Genomic and functional changes induced by the activation of the peripheral cannabinoid receptor CB2 in the promyelocytic cells HL-60. Possible involvement of the CB2 receptor in cell differentiation. J. Biol. Chem. 2000; 275(21):15621-15628.

-DeWitt DA, Perry G, Cohen M, Doller C, Silver J. Astrocytes regulate microglial phagocytosis of senile plaque cores of Alzheimer's disease. Exp. Neurol. 1998; 149(2):329-340.

-Di Marzo V, Bifulco M, De Petrocellis L. The endocannabinoid system and its therapeutic exploitation. Nature reviews Drug discovery 2004; 3(9):771-784.

-Di Marzo V, Stella N, Zimmer A. Endocannabinoid signalling and the deteriorating brain. Nature Reviews Neuroscience 2015; 16(1):30-42.

-Dirikoc S, Priola SA, Marella M, Zsurger N, Chabry J. Nonpsychoactive cannabidiol prevents prion accumulation and protects neurons against prion toxicity. J. Neurosci. 2007; 27(36):9537-9544.

-Duffy SS, Hayes JP, Fiore NT, Moalem-Taylor G. The cannabinoid system and microglia in health and disease. Neuropharmacology 2021; 190:108555.

-Dzamba D, Harantova L, Butenko O, Anderova M. Glial cells–The key elements of Alzheimer s disease. Current Alzheimer Research 2016; 13(8):894-911.

-Dzyubenko E, Gottschling C, Faissner A. Neuron-glia interactions in neural plasticity: contributions of neural extracellular matrix and perineuronal nets. Neural Plast 2016; 2016.

-Eikelenboom P, Stam F. Immunoglobulins and complement factors in senile plaques. Acta Neuropathol. 1982; 57(2-3):239-242.

-El Falougy H, Kubikova E, Benuska J. The microscopical structure of the hippocampus in the rat. Bratisl. Lek. Listy. 2008; 109(3):106.

-Eljaschewitsch E, Witting A, Mawrin C, Lee T, Schmidt PM, Wolf S, et al. The endocannabinoid anandamide protects neurons during CNS inflammation by induction of MKP-1 in microglial cells. Neuron 2006; 49(1):67-79.

-Escartin C, Guillemaud O, Carrillo-de Sauvage M. Questions and (some) answers on reactive astrocytes. Glia 2019; 67(12):2221-2247.

-Esposito G, De Filippis D, Steardo L, Scuderi C, Savani C, Cuomo V, et al. CB1 receptor selective activation inhibits β-amyloid-induced iNOS protein expression in C6 cells and subsequently blunts tau protein hyperphosphorylation in co-cultured neurons. Neurosci. Lett. 2006; 404(3):342-346.

-Facchinetti F, Del Giudice E, Furegato S, Passarotto M, Leon A. Cannabinoids ablate release of TNFα in rat microglial cells stimulated with lypopolysaccharide. Glia 2003;41(2):161-168.

-Farina C, Aloisi F, Meinl E. Astrocytes are active players in cerebral innate immunity. Trends Immunol. 2007; 28(3):138-145.

-Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, et al. Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. Mol. Pharmacol. 1995; 48(3):443-450.

-Fezza F, Bari M, Florio R, Talamonti E, Feole M, Maccarrone M. Endocannabinoids, related compounds and their metabolic routes. Molecules 2014; 19(11):17078-17106.

-Frautschy SA, Yang F, Irrizarry M, Hyman B, Saido TC, Hsiao K, et al. Microglial response to amyloid plaques in APPsw transgenic mice. Am. J. Pathol. 1998; 152(1):307-317.

-Frost GR, Li Y. The role of astrocytes in amyloid production and Alzheimer's disease. Open biology 2017; 7(12):170228.

-Fujise N, Hunziker W, Heizmann C, Kosaka T. Distribution of the calcium binding proteins, calbindin D-28K and parvalbumin, in the subicular complex of the adult mouse. Neurosci. Res. 1995; 22(1):89-107.

-Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, et al. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. European journal of biochemistry 1995; 232(1):54-61.

-Gardner B, Zu LX, Sharma S, Liu Q, Makriyannis A, Tashkin DP, et al. Autocrine and paracrine regulation of lymphocyte CB2 receptor expression by TGF-β. Biochem Biophys Res. Commun. 2002; 290(1):91-96.

-George S, Rönnbäck A, Gouras GK, Petit GH, Grueninger F, Winblad B, et al. Lesion of the subiculum reduces the spread of amyloid beta pathology to interconnected brain regions in a mouse model of Alzheimer's disease. Acta neuropathologica communications 2014; 2(1):1-8.

-Glass M, Northup JK. Agonist selective regulation of G proteins by cannabinoid CB(1) and CB(2) receptors. Mol. Pharmacol. 1999; 56(6):1362-1369.

-Glenner, G. G., and Wong, C. W. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem. Biophys. Res. Commun. 1984; 120, 885–890.

-Goldman S. Glia as neural progenitor cells. Trends Neurosci 2003; 26(11):590-596.

-Golovyashkina, N., Penazzi, L., Ballatore, C., Smith, A. B. III., Bakota, L., and Brandt, R. Region-specific dendritic simplification induced by Ab, mediated by tau via dysregulation of microtubule dynamics: a mechanistic distinct event from other neurodegenerative processes. Mol. Neurodegener. 2015; 10:60. doi: 10.1186/s13024-015-0049-0

-Gómez del Pulgar T, Velasco G, Guzmán M. The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. Biochem. J. 2000; 347(2):369-373.

-Gomez-Nicola D, Fransen NL, Suzzi S, Perry VH. Regulation of microglial proliferation during chronic neurodegeneration. J. Neurosci. 2013; 33(6):2481-2493.

-Gomez-Nicola D, Perry VH. Microglial dynamics and role in the healthy and diseased brain: a paradigm of functional plasticity. Neuroscientist 2015; 21(2):169-184.

-Gong J, Onaivi ES, Ishiguro H, Liu Q, Tagliaferro PA, Brusco A, et al. Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. Brain Res. 2006; 1071(1):10-23.

-Grabert K, Michoel T, Karavolos MH, Clohisey S, Baillie JK, Stevens MP, et al. Microglial brain region-dependent diversity and selective regional sensitivities to aging. Nat. Neurosci. 2016; 19(3):504-516.

-Griffin G, Wray EJ, Tao Q, McAllister SD, Rorrer WK, Aung M, et al. Evaluation of the cannabinoid CB2 receptor-selective antagonist, SR144528: further evidence for cannabinoid CB2 receptor absence in the rat central nervous system. Eur. J. Pharmacol. 1999; 377(1):117-125.

-Guénette SY. Astrocytes: a cellular player in Aβ clearance and degradation. Trends Mol. Med. 2003; 9(7):279-280.

-Guerrero-Alba R, Barragán-Iglesias P, González-Hernández A, Valdez-Moráles EE, Granados-Soto V, Condés-Lara M, et al. Some prospective alternatives for treating pain: the endocannabinoid system and its putative receptors GPR18 and GPR55. Frontiers in pharmacology 2019; 9:1496.

-Gutiérrez-Rodríguez A, Bonilla-Del Río I, Puente N, Gómez-Urquijo SM, Fontaine CJ, Egaña-Huguet J, et al. Localization of the cannabinoid type-1 receptor in subcellular astrocyte compartments of mutant mouse hippocampus. Glia 2018; 66(7):1417-1431.

-Guzmán M, Sánchez C, Galve-Roperh I. Control of the cell survival/death decision by cannabinoids. Journal of molecular medicine 2001; 78(11):613-625.

-Guzmán M, Sánchez C, Galve-Roperh I. Cannabinoids and cell fate. Pharmacol Ther 2002; 95(2):175-184.

-Hampson AJ, Grimaldi M, Axelrod J, Wink D. Cannabidiol and (-)Delta9-tetrahydrocannabinol are neuroprotective antioxidants. Proc Natl Acad Sci U S A 1998; 95(14):8268-8273.

-Han J, Kesner P, Metna-Laurent M, Duan T, Xu L, Georges F, et al. Acute cannabinoids impair working memory through astroglial CB1 receptor modulation of hippocampal LTD. Cell 2012; 148(5):1039-1050.

-Hansen DV, Hanson JE, Sheng M. Microglia in Alzheimer's disease. J. Cell Biol. 2018; 217(2):459-472.

-Harris E, Witter MP, Weinstein G, Stewart M. Intrinsic connectivity of the rat subiculum: I. Dendritic morphology and patterns of axonal arborization by pyramidal neurons. J. Comp. Neurol. 2001; 435(4):490-505.

-Hartlage-Rübsamen M, Zeitschel U, Apelt J, Gärtner U, Franke H, Stahl T, et al. Astrocytic expression of the Alzheimer's disease β-secretase (BACE1) is stimulus-dependent. Glia 2003; 41(2):169-179.

-Hashimotodani Y, Ohno-Shosaku T, Kano M. Endocannabinoids and synaptic function in the CNS. Neuroscientist 2007; 13(2):127-137.

-Haspula D, Clark MA. Cannabinoid receptors: An update on cell signaling, pathophysiological roles and therapeutic opportunities in neurological, cardiovascular, and inflammatory diseases. International journal of molecular sciences 2020;21(20):7693.

-Heneka MT, Rodríguez JJ, Verkhratsky A. Neuroglia in neurodegeneration. Brain Res. Rev. 2010; 63(1-2):189-211.

-Hirayama A, Okoshi Y, Hachiya Y, Ozawa Y, Ito M, Kida Y, et al. Early immunohistochemical detection of axonal damage and glial activation in extremely immature brains with periventricular leukomalacia. Clin. Neuropathol. 2001; 20(2):87-91.

-Hou L, Liu Y, Wang X, Ma H, He J, Zhang Y, et al. The effects of amyloid-β 42 oligomer on the proliferation and activation of astrocytes in vitro. In Vitro Cellular & Developmental Biology-Animal 2011; 47(8):573.

-Howlett AC. The cannabinoid receptors. Prostaglandins Other Lipid Mediat. 2002; 68:619-631.

-Hu Q, Klatt GM, Gudmundsrud R, Ottestad-Hansen S, Verbruggen L, Massie A, et al. Semi-quantitative distribution of excitatory amino acid (glutamate) transporters 1–3 (EAAT1-3) and the cystine-glutamate exchanger (xCT) in the adult murine spinal cord. Neurochem. Int. 2020; 140:104811.

-Hyman BT, Van Hoesen GW, Damasio AR, Barnes CL. Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. Science 1984; 225(4667):1168-1170.

-Insausti R, Amaral DG. Hippocampal formation. The human nervous system 2004; 2:871-914.

-Ishihara Y, Fukuda T. Immunohistochemical investigation of the internal structure of the mouse subiculum. Neuroscience 2016; 337:242-266.

-Itagaki S, McGeer P, Akiyama H, Zhu S, Selkoe D. Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. J. Neuroimmunol. 1989; 24(3):173-182.

-Ito TA, Larsen JT, Smith NK, Cacioppo JT. Negative information weighs more heavily on the brain: the negativity bias in evaluative categorizations. J. Pers. Soc. Psychol. 1998; 75(4):887.

-Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. Trends Pharmacol. Sci. 2009; 30(10):515-527.

-Jankowsky JL, Zheng H. Practical considerations for choosing a mouse model of Alzheimer's disease. Molecular neurodegeneration 2017; 12(1):1-22.

-Jean-Gilles L, Gran B, Constantinescu CS. Interaction between cytokines, cannabinoids and the nervous system. Immunobiology 2010; 215(8):606-610.

-Jimenez-Blasco D, Busquets-Garcia A, Hebert-Chatelain E, Serrat R, Vicente-Gutierrez C, Ioannidou C, et al. Glucose metabolism links astroglial mitochondria to cannabinoid effects. Nature 2020; 583(7817):603-608.

-Kamphuis W, Orre M, Kooijman L, Dahmen M, Hol EM. Differential cell proliferation in the cortex of the APPswePS1dE9 Alzheimer's disease mouse model. Glia 2012; 60(4):615-629.

-Kamphuis W, Middeldorp J, Kooijman L, Sluijs JA, Kooi E, Moeton M, et al. Glial fibrillary acidic protein isoform expression in plaque related astrogliosis in Alzheimer's disease. Neurobiol. Aging 2014; 35(3):492-510.

-Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M. Endocannabinoid-mediated control of synaptic transmission. Physiol. Rev. 2009; 89(1):309-380.

-Kaplan BL. The role of CB1 in immune modulation by cannabinoids. Pharmacol. Ther. 2013; 137(3):365-374.

-Karperien A, Ahammer H, Jelinek H. Quantitating the subtleties of microglial morphology with fractal analysis. Frontiers in cellular neuroscience 2013; 7:3.

-Kato S, Gondo T, Hoshii Y, Takahashi M, Yamada M, Ishihara T. Confocal observation of senile plaques in Alzheimer's disease: senile plaque morphology and relationship between senile plaques and astrocytes. Pathol. Int. 1998; 48(5):332-340.

-Katona I, Urban GM, Wallace M, Ledent C, Jung KM, Piomelli D, et al. Molecular composition of the endocannabinoid system at glutamatergic synapses. J. Neurosci. 2006; 26(21):5628-5637.

-Katona I, Freund TF. Multiple functions of endocannabinoid signaling in the brain. Annu Rev. Neurosci. 2012; 35:529-558.

-Kettenmann H, Hanisch U, Noda M, Verkhratsky A. Physiology of microglia. Physiol. Rev. 2011; 91(2):461-553.

-Klegeris A, Bissonnette CJ, McGeer PL. Reduction of human monocytic cell neurotoxicity and cytokine secretion by ligands of the cannabinoid-type CB2 receptor. Br. J. Pharmacol. 2003; 139(4):775-786.

-Koppensteiner, P., Trinchese, F., Fà, M., Puzzo, D., Gulisano, W., Yan, S., et al. Time-dependent reversal of synaptic plasticity induced by physiological concentrations of oligomeric Ab42: an early index of Alzheimer's disease. Sci. Rep. 2016; 6:32553.

-Laird FM, Cai H, Savonenko AV, Farah MH, He K, Melnikova T, et al. BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. J. Neurosci. 2005; 25(50):11693-11709.

-Lee SF, Newton C, Widen R, Friedman H, Klein TW. Differential expression of cannabinoid CB2 receptor mRNA in mouse immune cell subpopulations and following B cell stimulation. Eur. J. Pharmacol. 2001; 423(2-3):235-241.

-Lee SH, Foldy C, Soltesz I. Distinct endocannabinoid control of GABA release at perisomatic and dendritic synapses in the hippocampus. J. Neurosci. 2010; 30(23):7993-8000.

-Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC. Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J. Neurosci. 1995; 15(3 Pt 1):1835-1853.

-Lein, E.S. et al. Genome-wide atlas of gene expression in the adult mouse brain, Nature 2007; 445: 168-176.

-Leng F, Edison P. Neuroinflammation and microglial activation in Alzheimer disease: where do we go from here? Nature Reviews Neurology 2021; 17(3):157-172.

-Leuba G, Wernli G, Vernay A, Kraftsik R, Mohajeri MH, Saini KD. Neuronal and nonneuronal quantitative BACE immunocytochemical expression in the entorhinohippocampal and frontal regions in Alzheimer's disease. Dement. Geriatr. Cogn. Disord. 2005; 19(4):171-183.

-Li Y, Zhou Y, Danbolt NC. The rates of postmortem proteolysis of glutamate transporters differ dramatically between cells and between transporter subtypes. Journal of Histochemistry & Cytochemistry 2012; 60(11):811-821.

-Lin L, Yihao T, Zhou F, Yin N, Qiang T, Haowen Z, et al. Inflammatory regulation by driving microglial M2 polarization: neuroprotective effects of cannabinoid receptor-2 activation in intracerebral hemorrhage. *Frontiers in immunology* 2017; 8:112.

-Lindberg O, Mårtensson G, Stomrud E, Palmqvist S, Wahlund L, Westman E, et al. Atrophy of the posterior subiculum is associated with memory impairment, tau-and Aβ pathology in non-demented individuals. Frontiers in aging neuroscience 2017; 9:306.

-López A, Aparicio N, Pazos MR, Grande MT, Barreda-Manso MA, Benito-Cuesta I, et al. Cannabinoid CB 2 receptors in the mouse brain: Relevance for Alzheimer's disease. Journal of neuroinflammation 2018; 15(1):1-11.

-Lorente de Nó R. Studies on the structure of the cerebral cortex. II. Continuation of the study of the ammonic system. Journal für Psychologie und Neurologie 1934.

-Lu H, Mackie K. An introduction to the endogenous cannabinoid system. Biol. Psychiatry 2016; 79(7):516-525.

-Lu Y, Anderson HD. Cannabinoid signaling in health and disease. Can. J. Physiol. Pharmacol. 2017; 95(4):311-327.

-Mackie K, Hille B. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proc. Natl. Acad. Sci. U.S.A 1992; 89(9):3825-3829.

-Maeda J, Ji B, Irie T, Tomiyama T, Maruyama M, Okauchi T, et al. Longitudinal, quantitative assessment of amyloid, neuroinflammation, and anti-amyloid treatment in a living mouse model of Alzheimer's disease enabled by positron emission tomography. J. Neurosci. 2007; 27(41):10957-10968.

-Mandrekar-Colucci S, Landreth GE. Microglia and inflammation in Alzheimer's disease. CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders) 2010; 9(2):156-167.

-Marchalant Y, Brownjohn PW, Bonnet A, Kleffmann T, Ashton JC. Validating antibodies to the cannabinoid CB2 receptor: antibody sensitivity is not evidence of antibody specificity. Journal of Histochemistry & Cytochemistry 2014; 62(6):395-404.

-Maresz K, Carrier EJ, Ponomarev ED, Hillard CJ, Dittel BN. Modulation of the cannabinoid CB2 receptor in microglial cells in response to inflammatory stimuli. J. Neurochem. 2005; 95(2):437-445.

-Mark LP, Daniels DL, Naidich TP, Hendrix LE. Limbic connections. AJNR Am. J. Neuroradiol. 1995; 16(6):1303-1306.

-Marsicano G, Moosmann B, Hermann H, Lutz B, Behl C. Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1. J. Neurochem. 2002; 80(3):448-456.

-Marsicano G, Goodenough S, Monory K, Hermann H, Eder M, Cannich A, et al. CB1 cannabinoid receptors and on-demand defense against excitotoxicity. Science 2003; 302(5642):84-88.

-Martin E, Boucher C, Fontaine B, Delarasse C. Distinct inflammatory phenotypes of microglia and monocyte-derived macrophages in Alzheimer's disease models: effects of aging and amyloid pathology. Aging cell 2017; 16(1):27-38.

-Martín-Moreno AM, Reigada D, Ramirez BG, Mechoulam R, Innamorato N, Cuadrado A, et al. Cannabidiol and other cannabinoids reduce microglial activation in vitro and in vivo: relevance to Alzheimer's disease. Mol. Pharmacol. 2011; 79(6):964-973.

-Maruszak A, Żekanowski C. Mitochondrial dysfunction and Alzheimer's disease. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 2011; 35(2):320-330.

-Massoud F, Léger GC. Pharmacological treatment of Alzheimer disease. The Canadian Journal of Psychiatry 2011; 56(10):579-588.

-Matsumoto N, Kitanishi T, Mizuseki K. The subiculum: unique hippocampal hub and more. Neurosci. Res. 2019; 143:1-12.

-McAllister SD, Glass M. CB1 and CB2 receptor-mediated signalling: a focus on endocannabinoids. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA) 2002; 66(2-3):161-171.

-McAlpine CS, Park J, Griciuc A, Kim E, Choi SH, Iwamoto Y, et al. Astrocytic interleukin-3 programs microglia and limits Alzheimer's disease. Nature 2021; 595(7869):701-706.

-McCoy KL, Matveyeva M, Carlisle SJ, Cabral GA. Cannabinoid inhibition of the processing of intact lysozyme by macrophages: evidence for CB2 receptor participation. J. Pharmacol. Exp. Ther. 1999; 289(3):1620-1625.

-McGeer PL, McGeer EG. The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Research Reviews. 1995; 21(2):195-218.

-Mecha M, Carrillo-Salinas F, Feliú A, Mestre L, Guaza C. Microglia activation states and cannabinoid system: Therapeutic implications. Pharmacol. Ther. 2016; 166:40-55.

-Medeiros R, LaFerla FM. Astrocytes: conductors of the Alzheimer disease neuroinflammatory symphony. Exp. Neurol. 2013; 239:133-138.

-Metna-Laurent M, Marsicano G. Rising stars: Modulation of brain functions by astroglial type-1 cannabinoid receptors. Glia 2015; 63(3):353-364.

-Meyer-Luehmann M, Spires-Jones TL, Prada C, Garcia-Alloza M, De Calignon A, Rozkalne A, et al. Rapid appearance and local toxicity of amyloid-β plaques in a mouse model of Alzheimer's disease. Nature 2008; 451(7179):720-724.

-Min R, Di Marzo V, Mansvelder HD. DAG lipase involvement in depolarization-induced suppression of inhibition: does endocannabinoid biosynthesis always meet the demand? Neuroscientist 2010; 16(6):608-613.

-Molina-Holgado F, Molina-Holgado E, Guaza C, Rothwell NJ. Role of CB1 and CB2 receptors in the inhibitory effects of cannabinoids on lipopolysaccharide-induced nitric oxide release in astrocyte cultures. J. Neurosci. Res. 2002; 67(6):829-836.

-Monory K, Massa F, Egertová M, Eder M, Blaudzun H, Westenbroek R, et al. The endocannabinoid system controls key epileptogenic circuits in the hippocampus. Neuron 2006; 51(4):455-466.

-Monory K, Polack M, Remus A, Lutz B, Korte M. Cannabinoid CB1 receptor calibrates excitatory synaptic balance in the mouse hippocampus. J. Neurosci. 2015; 35(9):3842-3850.

-Moreira Paula I, Zhu Xiongwei, Liu Quan, Honda Kazuhiro, Suedlak Sandra L, Harris Peggy L et al . Compensatory responses induced by oxidative stress in Alzheimer disease. Biol. Res. [Internet]. 2006 [Kontsulta: 2021-09-07] ; 39(1): 7-13. Eskuragarri: http://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0716-97602006000100002&lng=es. http://dx.doi.org/10.4067/S0716-97602006000100002.

-Mrak RE, Griffinb WS. The role of activated astrocytes and of the neurotrophic cytokine S100B in the pathogenesis of Alzheimer's disease. Neurobiology of aging. 2001; 22(6):915-22.

-Mrak RE, Griffin WST. Glia and their cytokines in progression of neurodegeneration. Neurobiol Aging 2005; 26(3):349-354.

-Mufson EJ, Mahady L, Waters D, Counts SE, Perez SE, DeKosky S, et al. Hippocampal plasticity during the progression of Alzheimer's disease. Neuroscience 2015; 309:51-67.

-Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. Nature 1993; 365(6441):61-65.

-Nagele RG, D'Andrea MR, Lee H, Venkataraman V, Wang H. Astrocytes accumulate Aβ42 and give rise to astrocytic amyloid plaques in Alzheimer disease brains. Brain Res. 2003; 971(2):197-209.

-Nagele RG, Wegiel J, Venkataraman V, Imaki H, Wang K, Wegiel J. Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. Neurobiol. Aging 2004; 25(5):663-674.

-Nakagawa Y, Chiba K. Role of microglial m1/m2 polarization in relapse and remission of psychiatric disorders and diseases. Pharmaceuticals 2014; 7(12):1028-1048.

-Navarrete M, Araque A. Endocannabinoids mediate neuron-astrocyte communication. Neuron 2008; 57(6):883-893.

-Navarrete M, Araque A. Endocannabinoids potentiate synaptic transmission through stimulation of astrocytes. Neuron 2010; 68(1):113-126.

-Navarro G, Borroto-Escuela D, Angelats E, Etayo Í, Reyes-Resina I, Pulido-Salgado M, et al. Receptor-heteromer mediated regulation of endocannabinoid signaling in activated microglia. Role of CB1 and CB2 receptors and relevance for Alzheimer's disease and levodopa-induced dyskinesia. Brain Behav. Immun. 2018; 67:139-151.

-Nicoll JA, Weller RO. A new role for astrocytes: β-amyloid homeostasis and degradation. Trends Mol. Med. 2003; 9(7):281-282.

-Núñez E, Benito C, Pazos MR, Barbachano A, Fajardo O, González S, et al. Cannabinoid CB2 receptors are expressed by perivascular microglial cells in the human brain: an immunohistochemical study. Synapse 2004; 53(4):208-213.

-Oakley H, Cole SL, Logan S, Maus E, Shao P, Craft J, et al. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. J. Neurosci. 2006; 26(40):10129-10140.

-Oblak AL, Lin P, Kotredes KP, Pandey R, Garceau D, Williams HM, et al. Comprehensive Evaluation of the 5xFAD Mouse Model for preclinical testing applications: A MODEL-AD Study Comprehensive Evaluation of the 5xFAD Mouse Model. Frontiers in Aging Neuroscience 2021; 13:431.

-Okello A, Edison P, Archer HA, Turkheimer FE, Kennedy J, Bullock R, et al. Microglial activation and amyloid deposition in mild cognitive impairment: a PET study. Neurology 2009; 72(1):56-62.

-Okere CO, Kaba H. Heterogenous immunohistochemical expression of microglia-specific ionized calcium binding adaptor protein (Iba1) in the mouse olfactory bulb. Brain Res. 2000; 877(1):85-90.

-Olabarria M, Noristani HN, Verkhratsky A, Rodríguez JJ. Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. Glia 2010; 58(7):831-838.

-O'Mara SM, Commins S, Anderson M, Gigg J. The subiculum: a review of form, physiology and function. Prog. Neurobiol. 2001; 64(2):129-155.

-Olmos-Alonso A, Schetters ST, Sri S, Askew K, Mancuso R, Vargas-Caballero M, et al. Pharmacological targeting of CSF1R inhibits microglial proliferation and prevents the progression of Alzheimer's-like pathology. Brain 2016; 139(3):891-907.

-Onaivi ES, Ishiguro H, GONG J, Patel S, Perchuk A, Meozzi PA, et al. Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain. Ann. N.Y. Acad. Sci. 2006; 1074(1):514-536.

-Onaivi ES, Ishiguro H, Gong J, Patel S, Meozzi PA, Myers L, et al. Brain neuronal CB2 cannabinoid receptors in drug abuse and depression: from mice to human subjects. PLoS one 2008; 3(2):e1640.

-Orre M, Kamphuis W, Osborn LM, Jansen AH, Kooijman L, Bossers K, et al. Isolation of glia from Alzheimer's mice reveals inflammation and dysfunction. Neurobiol. Aging 2014; 35(12):2746-2760.

-Ortiz-Sanz C, Gaminde-Blasco A, Valero J, Bakota L, Brandt R, Zugaza JL, et al. Early effects of Aβ oligomers on dendritic spine dynamics and arborization in hippocampal neurons. Frontiers in Synaptic Neuroscience 2020; 12:2.

-Peñasco, S., Rico-Barrio, I., Puente, N., Gómez-Urquijo, S. M., Fontaine, C. J., Egaña-Huguet, J., Achicallende, S., Ramos, A., Reguero, L., Elezgarai, I., Nahirney, P. C., Christie, B. R., & Grandes, P. Endocannabinoid long-term depression revealed at medial perforant path excitatory synapses in the dentate gyrus. Neuropharmacology 2019; 153:32-40.

-Perea G, Navarrete M, Araque A. Tripartite synapses: astrocytes process and control synaptic information. Trends. Neurosci. 2009; 32(8):421-431.

-Perry VH, Holmes C. Microglial priming in neurodegenerative disease. Nature Reviews Neurology 2014; 10(4):217-224.

-Pertwee RG. Endocannabinoids and their pharmacological actions. Endocannabinoids: Springer; 2015; 1-37.

-Petrocellis LD, Cascio MG, Marzo VD. The endocannabinoid system: a general view and latest additions. Br. J. Pharmacol. 2004; 141(5):765-774.

-Piomelli D. The molecular logic of endocannabinoid signalling. Nature Reviews Neuroscience 2003; 4(11):873-884.

-Puente N, Bonilla-Del Río I, Achicallende S, Nahirney PC, Grandes P. High-resolution immunoelectron microscopy techniques for revealing distinct subcellular type 1 cannabinoid receptor domains in brain. Bio-protocol 2019; 9(2):e3145-e3145.

-Querfurth H.W., LaFerla F.M. Alzheimer's disease. N. Engl. J. Med. 2010; 362:329–344.

-Rajmohan V, Mohandas E. The limbic system. Indian J Psychiatry 2007 Apr;49(2):132-139.

-Ramirez BG, Blazquez C, Gomez del Pulgar T, Guzmán M, de Ceballos ML. Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. J. Neurosci. 2005; 25(8):1904-1913.

-Rapino C, Tortolani D, Scipioni L, Maccarrone M. Neuroprotection by (endo) cannabinoids in glaucoma and retinal neurodegenerative diseases. Current neuropharmacology 2018; 16(7):959-970.

-Renau-Piqueras J, Zaragoza R, De Paz P, Baguena-Cervellera R, Megias L, Guerri C. Effects of prolonged ethanol exposure on the glial fibrillary acidic protein-containing intermediate filaments of astrocytes in primary culture: a quantitative immunofluorescence and immunogold electron microscopic study. J. Histochem. Cytochem. 1989; 37(2):229-240.

-Research models: Alzheimer´s Disease. 5xFAD; 3xTg; htau; Tg2576 [Internet]. Alzforum; c1996–2021 FBRI LLC. [Retrieved Aug 17, 2021]. From: http://www.alzforum.org/research-models/alzheimers-disease/.

-Ribeiro R, Wen J, Li S, Zhang Y. Involvement of ERK1/2, cPLA2 and NF-κB in microglia suppression by cannabinoid receptor agonists and antagonists. Prostaglandins Other Lipid Mediat. 2013; 100:1-14.

-Rodriguez J, Olabarria M, Chvatal A, Verkhratsky A. Astroglia in dementia and Alzheimer's disease. Cell Death & Differentiation 2009; 16(3):378-385.

-Rönnbäck A, Sagelius H, Bergstedt KD, Näslund J, Westermark GT, Winblad B, et al. Amyloid neuropathology in the single Arctic APP transgenic model affects interconnected brain regions. Neurobiol. Aging 2012; 33(4):831. e11-831. e19.

-Rossner S, Lange-Dohna C, Zeitschel U, Perez-Polo JR. Alzheimer's disease β-secretase - BACE1 is not a neuron-specific enzyme. J. Neurochem. 2005; 92(2):226-234.

-Rozenfeld R, Devi LA. Regulation of CB1 cannabinoid receptor trafficking by the adaptor protein AP-3. The FASEB Journal 2008; 22(7):2311-2322.

-Salvi V, Sozio F, Sozzani S, Del Prete A. Role of atypical chemokine receptors in microglial activation and polarization. Frontiers in aging neuroscience 2017; 9:148.

-Sánchez MG, Ruiz-Llorente L, Sánchez AM, Díaz-Laviada I. Activation of phosphoinositide 3-kinase/PKB pathway by CB1 and CB2 cannabinoid receptors expressed in prostate PC-3 cells. Involvement in Raf-1 stimulation and NGF induction. Cell Signal 2003; 15(9):851-859.

-Sarlus H, Heneka MT. Microglia in Alzheimer's disease. J. Clin. Invest. 2017; 127(9):3240-3249.

-Savchenko VL, McKanna JA, Nikonenko IR, Skibo GG. Microglia and astrocytes in the adult rat brain: comparative immunocytochemical analysis demonstrates the efficacy of lipocortin 1 immunoreactivity. Neuroscience. 2000; 96(1):195-203.

-Schatz AR, Lee M, Condie RB, Pulaski JT, Kaminski NE. Cannabinoid receptors CB1 and CB2: a characterization of expression and adenylate cyclase modulation within the immune system. Toxicol. Appl. Pharmacol. 1997; 142(2):278-287.

-Schmitt A, Asan E, Puschel B, Kugler P. Cellular and regional distribution of the glutamate transporter GLAST in the CNS of rats: nonradioactive in situ hybridization and comparative immunocytochemistry. J. Neurosci. 1997; 17(1):1-10.

-Scuderi C, Facchinetti R, Steardo L, Valenza M. Neuroinflammation in Alzheimer's Disease: Friend or Foe? The FASEB Journal 2020; 34(S1):1-1.

-Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 2001.

-Selkoe DJ, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO molecular medicine 2016; 8(6):595-608.

-Shaffer LM, Dority MD, Gupta-Bansal R, Frederickson RC, Younkin SG, Brunden KR. Amyloid β protein (Aβ) removal by neuroglial cells in culture. Neurobiol. Aging 1995; 16(5):737-745.

-Shankar, G. M., Bloodgood, B. L., Townsend, M., Walsh, D. M., Selkoe, D. J., and Sabatini, B. L. Natural oligomers of the Alzheimer amyloid- b protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J. Neurosci. 2007; 27, 2866–2875.

-Shapiro LA, Wang L, Ribak CE. Rapid astrocyte and microglial activation following pilocarpine-induced seizures in rats. Epilepsia 2008; 49:33-41.

-Shen Z, Li X, Bao X, Wang R. Microglia-targeted stem cell therapies for Alzheimer disease: A preclinical data review. J. Neurosci. Res. 2017; 95(12):2420-2429.

-Sheng WS, Hu S, Min X, Cabral GA, Lokensgard JR, Peterson PK. Synthetic cannabinoid WIN55, 212-2 inhibits generation of inflammatory mediators by IL-1β-stimulated human astrocytes. Glia 2005; 49(2):211-219.

-Simchowicz T. Histologische und histopathologische Arbeiten über die Grosshirnrinde mit besonderer Berücksichtigung der pathologischen Anatomie der Geisteskrankheiten Histologische Studien über die senile Demenz. Gustav Fischer Verlag, Jena, Germany 1911; 267-444.

-Sinha D, Bonner TI, Bhat NR, Matsuda LA. Expression of the CB1 cannabinoid receptor in macrophage-like cells from brain tissue: immunochemical characterization by fusion protein antibodies. J. Neuroimmunol. 1998; 82(1):13-21.

-Sinor AD, Irvin SM, Greenberg DA. Endocannabinoids protect cerebral cortical neurons from in vitro ischemia in rats. Neurosci. Lett. 2000; 278(3):157-160.

-Smit T, Deshayes NA, Borchelt DR, Kamphuis W, Middeldorp J, Hol EM. Reactive astrocytes as treatment targets in Alzheimer's disease—Systematic review of studies using the APPswePS1dE9 mouse model. Glia 2021; 69(8):1852-1881.

-Sokolowski K, Corbin JG. Wired for behaviors: from development to function of innate limbic system circuitry. Frontiers in molecular neuroscience 2012; 5:55.

-Song H, Stevens CF, Gage FH. Astroglia induce neurogenesis from adult neural stem cells. Nature 2002; 417(6884):39-44.

-Soto-Mercado V, Mendivil-Perez M, Jimenez-Del-Rio M, Velez-Pardo C. Multi-Target Effects of the Cannabinoid CP55940 on Familial Alzheimer's Disease PSEN1 E280A Cholinergic-Like Neurons: Role of CB 1 Receptor. J Alzheimer's Dis. 2020(Preprint); 1-20.

-Spires, T. L., Meyer-Luehmann, M., Stern, E. A., McLean, P. J., Skoch, J., Nguyen, P. T., et al. Dendritic spine abnormalities in amyloid precursor protein transgenic mice demonstrated by gene transfer and intravital multiphoton microscopy. J. Neurosci. 2005; 25, 7278–7287.

-Stefano GB, Liu Y, Goligorsky MS. Cannabinoid receptors are coupled to nitric oxide release in invertebrate immunocytes, microglia, and human monocytes. J. Biol. Chem. 1996; 271(32):19238-19242.

-Stella N. Endocannabinoid signaling in microglial cells. Neuropharmacology 2009; 56:244-253.

-Stella N. Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. Glia 2010; 58(9):1017-1030.

-Straiker A, Mackie K. Cannabinoids, electrophysiology, and retrograde messengers: challenges for the next 5 years. The AAPS journal 2006; 8(2):E272-E276.

-Suárez I, Bodega G, Ramos JA, Fernandez-Ruiz JJ, Fernandez B. Neuronal and astroglial response to pre- and perinatal exposure to delta-9-tetra- hydrocannabinol in the rat substantia nigra. Dev. Neurosci. 2000; 22(4):253-263.

-Sugiura T, Kondo S, Kishimoto S, Miyashita T, Nakane S, Kodaka T, et al. Evidence that 2-arachidonoylglycerol but not N-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. J. Biol. Chem. 2000; 275(1):605-612.

-Tahir SK, Trogadis JE, Stevens JK, Zimmerman AM. Cytoskeletal organization following cannabinoid treatment in undifferentiated and differentiated PC12 cells. Biochemistry and Cell Biology 1992; 70(10-11):1159-1173.

-Talarico G, Trebbastoni A, Bruno G, de Lena C. Modulation of the cannabinoid system: a new perspective for the treatment of the Alzheimer's disease. Current neuropharmacology 2019; 17(2):176-183.

-Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, et al. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society 1991; 30(4):572-580.

-Thal DR, Schultz C, Dehghani F, Yamaguchi H, Braak H, Braak E. Amyloid β-protein (Aβ)-containing astrocytes are located preferentially near N-terminal-truncated Aβ deposits in the human entorhinal cortex. Acta Neuropathol. 2000; 100(6):608-617.

-Thompson PM, Hayashi KM, de Zubicaray G, Janke AL, Rose SE, Semple J, et al. Dynamics of gray matter loss in Alzheimer's disease. J. Neurosci. 2003; 23(3):994-1005.

-Thompson PM, Hayashi KM, Dutton RA, Chiang MC, Leow AD, Sowell ER, et al. Tracking Alzheimer's disease. Ann. N.Y. Acad. Sci. 2007; 1097:183-214.

-Twitchell W, Brown S, Mackie K. Cannabinoids inhibit N-and P/Q-type calcium channels in cultured rat hippocampal neurons. J. Neurophysiol. 1997; 78(1):43-50.

-Ueda N, Tsuboi K, Uyama T, Ohnishi T. Biosynthesis and degradation of the endocannabinoid 2-arachidonoylglycerol. Biofactors 2011; 37(1):1-7.

-Unger JW. Glial reaction in aging and Alzheimer's disease. Microsc. Res. Tech. 1998; 43(1):24-28.

-Van Groen T, Wyss JM. Extrinsic projections from area CA1 of the rat hippocampus: olfactory, cortical, subcortical, and bilateral hippocampal formation projections. J. Comp. Neurol. 1990; 302(3):515-528.

-Van Hoesen GW, Hyman BT. Hippocampal formation: anatomy and the patterns of pathology in Alzheimer's disease. Prog. Brain. Res. 1990; 83:445-457.

-Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, et al. Identification and functional characterization of brainstem cannabinoid CB2 receptors. Science 2005; 310(5746):329-332.

-Verkhratsky A, Olabarria M, Noristani HN, Yeh C, Rodriguez JJ. Astrocytes in Alzheimer's disease. Neurotherapeutics 2010; 7(4):399-412.

-Verkhratsky A, Nedergaard M. Physiology of astroglia. Physiol. Rev. 2018; 98(1):239-389.

-Verkhratsky A, Parpura V, Rodriguez-Arellano JJ, Zorec R. Astroglia in Alzheimer's disease. Neuroglia in Neurodegenerative Diseases: Springer; 2019; p. 273-324.

-Waksman Y, Olson JM, Carlisle SJ, Cabral GA. The central cannabinoid receptor (CB1) mediates inhibition of nitric oxide production by rat microglial cells. J. Pharmacol. Exp. Ther. 1999; 288(3):1357-1366.

-Walter L, Dinh T, Stella N. ATP induces a rapid and pronounced increase in 2-arachidonoylglycerol production by astrocytes, a response limited by monoacylglycerol lipase. J. Neurosci. 2004; 24(37):8068-8074.

-Wilson RI, Nicoll RA. Endocannabinoid signaling in the brain. Science 2002; 296(5568):678-682.

-Wyss-Coray T, Mucke L. Inflammation in neurodegenerative disease—a double-edged sword. Neuron 2002; 35(3):419-432.

-Wyss-Coray T, Loike JD, Brionne TC, Lu E, Anankov R, Yan F, et al. Adult mouse astrocytes degrade amyloid-β in vitro and in situ. Nat. Med. 2003; 9(4):453-457.

-Yiangou Y, Facer P, Durrenberger P, Chessell IP, Naylor A, Bountra C, et al. COX-2, CB2 and P2X7-immunoreactivities are increased in activated microglial cells/macrophages of multiple sclerosis and amyotrophic lateral sclerosis spinal cord. BMC neurology 2006; 6(1):1-14.

-Yin Z, Raj D, Saiepour N, Van Dam D, Brouwer N, Holtman IR, et al. Immune hyperreactivity of Aβ plaque-associated microglia in Alzheimer's disease. Neurobiol. Aging 2017; 55:115-122.

-Zhang J, Hoffert C, Vu HK, Groblewski T, Ahmad S, O'Donnell D. Induction of CB2 receptor expression in the rat spinal cord of neuropathic but not inflammatory chronic pain models. Eur. J. Neurosci. 2003; 17(12):2750-2754.

-Zhang HY, Gao M, Liu QR, Bi GH, Li X, Yang HJ, et al. Cannabinoid CB2 receptors modulate midbrain dopamine neuronal activity and dopamine-related behavior in mice. Proc. Natl. Acad. Sci. U.S.A. 2014; 111(46):E5007-15.

-Zhang M, Zhong L, Han X, Xiong G, Xu D, Zhang S, et al. Brain and Retinal Abnormalities in the 5xFAD Mouse Model of Alzheimer's Disease at Early Stages. Frontiers in Neuroscience 2021; 858.

-Zhao J, O'Connor T, Vassar R. The contribution of activated astrocytes to Aβ production: implications for Alzheimer's disease pathogenesis. Journal of neuroinflammation 2011; 8(1):150.

-Zou S, Kumar U. Cannabinoid receptors and the endocannabinoid system: signaling and function in the central nervous system. International journal of molecular sciences 2018; 19(3):833.