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URB597 Administration decreases Neuroinflammation and promotes Autophagy in Alzheimer's Disease models

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INDEX

ABSTRACT

In recent years, much attention has been focused on the endocannabinoid system as a therapeutic target for neurodegenerative diseases, including Alzheimer's disease (AD). The first endocannabinoid to be characterized was anandamide (N - arachidonoyl ethanolamine, AEA), which is hydrolyzed in postsynaptic neurons by the fatty acid amide hydrolase (FAAH). Notably, the post-mortem examination of brains from AD patients has revealed several alterations of the endocannabinoid system reporting a decrease in cortical AEA levels and a parallel increase in FAAH activity. On this ground, by using both cellular and a transgenic mouse model of AD, we aimed at determining whether pharmacological inhibition of FAAH via URB597 administration may affect microglia polarization and provide beneficial effects in restoring autophagic process and reducing amyloid-β (Aβ) aggregates, which are all neuropathological features of AD. The evaluation of morphological and major markers of microglial activation (iNOS and ARG-1) showed the ability of URB597 to revert microglial activation towards an anti-inflammatory condition, which was associated with the decrease of pro-inflammatory cytokines and an increase of anti-inflammatory cytokines together with the restoration of key factors involved in autophagic processes. These data were confirmed *in vivo* in the Tg2576 mice where the broad-spectrum action of endocannabinoids ranging from the anti-inflammatory action to the restoration of autophagic flux was demonstrated by the increase of proteins such as ATG7, Beclin1, LC3 and activation of ULK1 signaling following mTOR decrease after URB597 treatment. Moreover, treatment with URB597 decreases the expression of BACE1 through the modulation of CB2 receptors, leading to the reduction of amyloid plaques.

Taken together, these data suggest that inhibition of FAAH can drive the polarization of microglia towards an anti-inflammatory phenotype and demonstrate that the modulation of the endocannabinoid system in AD, via URB597 administration, could be a potential and effective therapy not only to fight neuroinflammation but also to regulate the autophagic machinery.

INTRODUCTION

Many neurodegenerative diseases are accompanied by microglia-mediated inflammatory processes (Labzin et al., 2018). Microglia cells, belonging to innate immunity, arise from primitive yolk sac macrophages (Ginhoux & Prinz, 2015; Nayak et al., 2014; Tay et al., 2017) They are characterized by different activation states: M1 phenotype induced by toxins, LPS, releasing pro-inflammatory cytokines; M2 phenotype releasing anti-inflammatory cytokines that are involved in repair processes and counteract neuroinflammation. Inflammatory processes induce the production of endocannabinoids (eCB) as a means of recruiting microglia expressing cannabinoid receptors with a pro-homeostatic effect (Mecha et al., 2016). Endocannabinoids system (ECBS) have pleiotropic effects on the complex development of neurodegenerative diseases like AD (Cooray et al., 2020). The accumulation of protein aggregates in AD is associated to the dysfunction of autophagy leading to increased oxidative stress, neuroinflammation, and brain atrophy (Heckmann et al., 2020; Lachance et al., 2019; Ichimiya et al., 2020). Thus, dysfunction of the autophagy machinery contributes to AD pathogenesis. However, few studies have evaluated the dysfunction of autophagy on microglia activation (Plaza-Zabala et al., 2017). Microglial hyperactivation reacting with misfolded proteins such as amyloid-beta peptide (Aβ), increases inflammatory milieu that induces neuronal apoptosis (Hansen et al., 2018). Several studies have indicated that eCB levels and metabolic enzymes change during the AD progression (Benito et al., 2007; Jung et al., 2012) and that inhibition of the main enzyme that hydrolyzes AEA, the fatty acid amide hydrolase (FAAH), shows a possible neuroprotective role against oxidative stress, inflammation, and excitotoxicity (Hwang et al., 2010; Jain et al., 2022).

Alzheimer’s disease (AD)

Alzheimer's disease (AD) is the most common dementia cause associated with neuroinflammation and neurodegeneration. AD was described for the first time in 1907 by Aloysius Alzheimer (Bondi et al., 2017). The disease early symptoms include memory loss, spatial and temporal disorientation, and cognitive deficits.

The classic pathophysiological hypothesis ascribes the disorder to Aβ accumulation at the extracellular level, with aggregation of hyperphosphorylated tau protein intracellularly (Soria Lopez et al., 2019). Macroscopically, the brain of AD patients shows cortical atrophy whereas, at microscopical level, amyloid senile plaques around neuronal cells, and intra-neuronal neurofibrillary tangles constituted of hyperphosphorylated tau protein (Selkoe, 2001, 2011). Aβ is the result of proteolysis of amyloid precursor protein (APP), a transmembrane protein involved in synaptic function (Hefter et al., 2020). Sequential cleavage of APP occurs by two different processes, the nonamyloidogenic and the amyloidogenic proteolytic pathways. In the nonamyloidogenic way, the APP is hydrolyzed by α-secretase on the transmembrane strand (Esch et al., 1990), producing a distinct APP-soluble variant (sAPPα) and a shorter C-terminal fragment, which is successively hydrolyzed by γ-secretase to produce peptide P3, a non-toxic Aβ fraction (Haass et al., 1993).

By contrast, within the amyloidogenic pathway, APP receives first proteolytic cleavage by β-secretase 1 (BACE1) and consequently by γ-secretase, resulting in the generation of a 37-49 aminoacidic fragment, Aβ (Fig.1), the major player in "the amyloid cascade hypothesis" through its potential to form the amyloid plaques typical of AD brains (G. Chen et al., 2017; Holtzman et al., 2011). Aβ monomers assemble to constitute oligomers, protofibrils, and amyloid fibrils. The oligomers, being smaller and soluble, propagate, during different stages of AD, all over the brain, generating neurotoxicity, neuroinflammation, oxidative stress until neuronal apoptosis. (G. Chen et al., 2017).

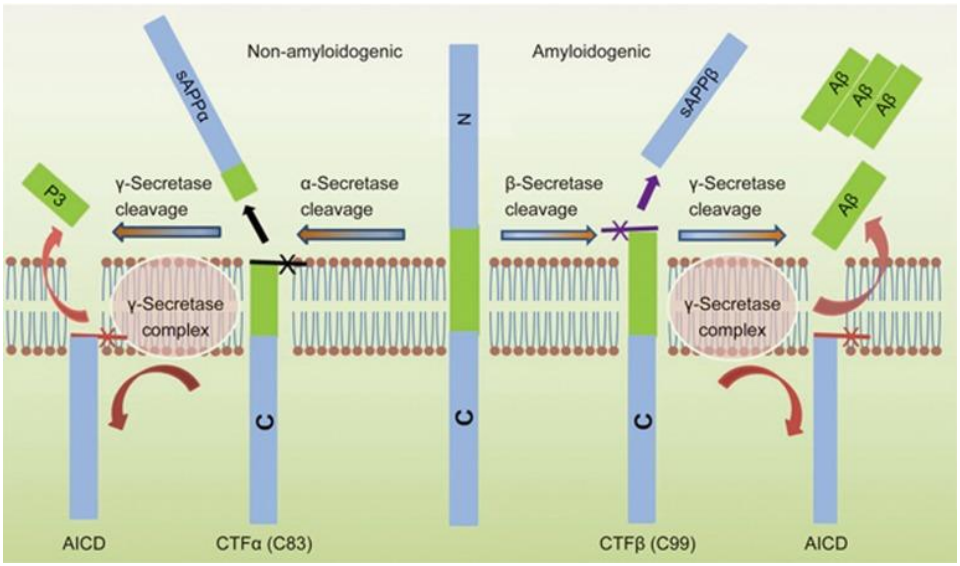


Fig.1 – (Chen et al., 2017) APP proteolysis is via the nonamyloidogenic pathway and via the amyloidogenic pathway. α-secretase performs the first proteolytic cleavage forming a fragment that is then cleaved by γ-secretases thus forming extracellular P3. The amyloidogenic pathway occurs through sequential cleavage by β- and γ-secretases.

Current therapeutic approaches

AD is a multifactorial disease, and its molecular basis is still unclear. It has been shown that aging, infections, nutritional status, genetic and environmental factors contribute to the onset of AD, and several pathogenetic hypotheses in addition to the amyloid cascade have been posited. One of these is the "cholinergic" hypothesis, according to which Aβ oligomers play a role in cholinergic transmission by interfering with the release and uptake of acetylcholine, which is responsible for AD pathogenesis including memory and other cognitive functions, thus contributing to the degeneration of cholinergic neurons (Breijyeh & Karaman, 2020).

However, despite decades of intensive research, no therapies aimed at delaying or resolving the progression of AD are currently available. The most common pharmacological treatment, focused on improving symptomatology, is focused on inhibitors of acetylcholinesterase enzyme, such as donepezil, galantamine and rivastigmine, and memantine, an N-methyl-D-aspartate (NMDA) receptor antagonist (Munoz-Torrero, 2008).

In addition, Aβ aggregation exacerbates the neuroinflammatory milieu and cytotoxicity and, because of this, anti-inflammatory therapies, show promise in lowering neurodegeneration (Ali et al., 2019).

On this ground, the investigation of new therapeutic targets for AD appears to be of utmost importance, in terms of minimizing Aβ production and promoting Aβ clearance.

ENDOCANNABINOID SYSTEM

In recent years, increasing research has been focused on a network of pro-homeostatic mediators that operate extensively in the entire body: the endocannabinoid system (ECBS). Interest in ECBS was generated by the study of some endogenous compounds that can bind to the same receptors targeted by exogenous psychoactive drugs derived from *Cannabis sativa* (Devane et al., 1992). Ancient civilization healers considered, *Cannabis sativa* and its psychotropic effects, a therapeutic compound. The most characterized compounds found in the Cannabis plant are Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Mechoulam & Gaoni, 1965; Mechoulam & Shvo, 1963). Recent literature has demonstrated the therapeutic effects of compounds derived from *Cannabis* flowers (Alexander, 2016). The molecular knowledge developed about ECBS up to now is derived from the investigation of these naturally exogenous cannabinoids. The most well-known eCB in the literature are derived from arachidonic acid and consist of anandamide (AEA) and 2-arachidonoylglycerol (2-AG), hydrolyzed by fatty acid amide hydrolase (FAAH), forming arachidonic acid and ethanolamine, and monoacylglycerol lipase (MAGL), respectively (Fig.2) (Armeli et al., 2021; Cooray et al., 2020). AEA is synthesized from N-acyl-phosphatidylethanolamine (NAPE). 2-AG is produced from diacylglycerol (DAG) (Cooray et al., 2020). The ECBS consists of a network of a lipid-based neuromediators that master and control cognitive and physiological processes. The ECBS signaling network is made up of Cannabinoid Receptor 1 (CB1) and Cannabinoid Receptor 2 (CB2). They are inhibitory G-protein-coupled receptors (GPCRs). They interfere with adenylyl cyclase and calcium channels. In the central nervous system (CNS), CB1 are mainly found on neurons, while CB2 are localized

primarily in microglia (Lu & Mackie, 2021). eCB are produced "on demand" in the post-synaptic terminals of neurons and follow a retrograde pathway to reach receptors at the pre-synaptic region. Their secretion is modulated by Calcium (Ca^{2+}) influx (Cooray et al., 2020).

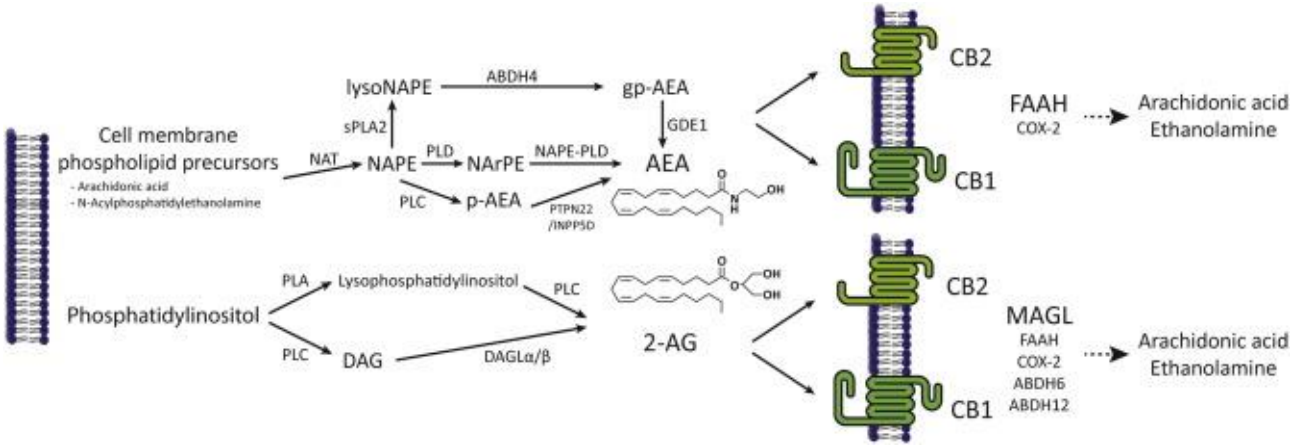


Fig.2 - (Mecha et al., 2016) Signaling of endocannabinoids. The eCB are lipids synthesized from membrane precursors. AEA is degraded by FAAH, while 2-AG is degraded by MAGL, giving arachidonic acid and ethanolamide as end products.

Indeed, in AD, CB2 activation is associated with the reduction of pro-inflammatory cytokines from microglia cells (Grieco et al., 2021; X. Li et al., 2019). APP/PS1 mice, expressing the mutant form of presenilin and amyloid precursor protein, have reduced activity of microglia cells when treated with CB2 agonists, in parallel AD CB2 knockout mice have increased Aβ levels (Aso et al., 2013; Koppel et al., 2013).

There are other receptors that cooperate with the neuroprotective action of ECBS, such as the metabotropic receptors GPR55, GPR119, GPR18, and the transient receptor of vanilloid potential (TRPV1), that is involved in the functioning of synaptic terminals (Cristino et al., 2006, 2008; Edwards, 2014). TRPV1 together with CB2 counteracts microglial hyperactivation. In addition, the eCB activation of two other receptors, PPARα and PPARγ, counteracts the inflammatory events typically seen in neurodegenerative diseases (Villapol, 2018).

Neuroinflammation and microglial activation in Alzheimer's disease

Neuroinflammation plays a predominant role in neurodegenerative diseases: patients with AD have increased levels of inflammation mediators. The acute or chronic neuroinflammatory process, caused by insults such as trauma, toxins, ischemia, or CNS pathology, is associated to an immune response. The cells of innate immunity in the CNS are glial cells, such as astrocytes and microglia (Leng & Edison, 2021).

Microglia are innate immune cells whose pro-homeostatic features are analogous to those of macrophages. Microglial activity is the immediate defensive line against insults to the CNS, through a cytokine-mediated inflammatory mechanisms that are characterized by multiple responses including phagocytosis, migration and release of bioactive molecules (Ginhoux & Prinz, 2015; Soulet & Rivest, 2008). Microglial responses are associated with morphological changes: resting microglia have a small soma with long branches sensing changes in the surrounding microenvironment. Instead, in the presence of a phlogogenic stimulus, they become activated and their soma increases in size, assuming a ameboid morphology (Kettenmann et al., 2013). The stages of microglia activation include the classic M1-type phenotype, which is associated with the production of pro-inflammatory cytokines such as IL-1β and TNF-α, and the alternative M2-type phenotype, associated with the production of anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β (Fig. 3) (Kwon & Koh, 2020; Leng & Edison, 2021). In AD, activated microglia demonstrates increased proliferation and enhanced expression of M1 phenotype-associated markers such as CD36, MHC-II, and iNOS, while M2 phenotype express reparative genes that enhance repair and healing, such as Arg-1. (Qin et al., 2021; Sarlus & Heneka, 2017). Acute-type damage to the CNS activates microglia initially by polarizing it toward an M1 phenotype, whereas polarization microglia toward an anti-inflammatory M2 phenotype, occurs when resolution of the damage takes place (Nakagawa & Chiba, 2015). In contrast, chronic inflammation, a hallmark of neurodegenerative disorders, induces hyperactivation of microglia towards a pro-inflammatory polarization that causes cytotoxicity and apoptosis, exacerbating a pre-existing inflammatory condition (De Caris et al., 2020).

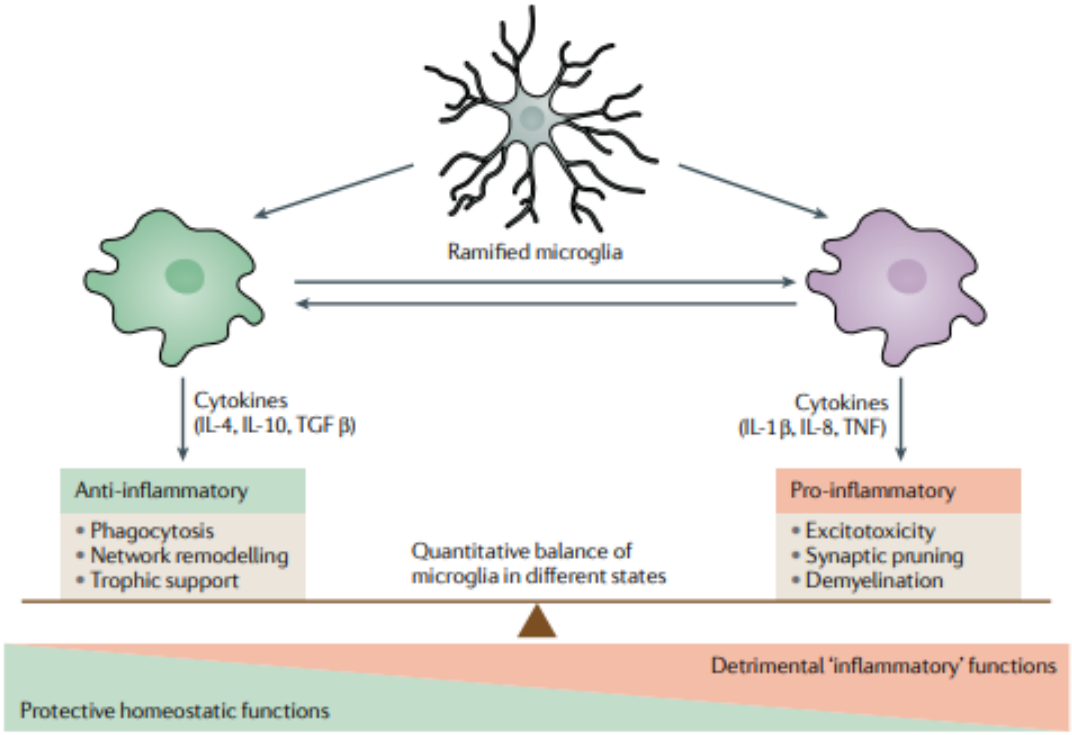


Fig.3 - (Leng & Edison, 2021) Phenotypes of microglia. Microglial responses are associated with morphological changes: the stages of microglia activation include the classic M1-type phenotype, which is associated with the production of pro-inflammatory cytokines and M2-type phenotype, associated with the production of anti-inflammatory cytokines.

In AD neuropathology, β-amyloidosis, attracts microglia cells around the plaques, compacting and isolating them and forming a defensive barrier that prevents further accumulation of Aβ. However, as mentioned earlier, by reacting with misfolded proteins such as Aβ, microglial hyperactivation increases inflammatory milieu promoting neuronal death. Aβ aggregates interact with Toll-like receptors (TLRs) and the NRLP3 inflammasome, increasing astrogliosis and the release of pro-inflammatory cytokines like TNFα, IL-1β (Hansen et al., 2018). In mice where both the inflammasome and TLRs were genetically silenced there was less Aβ accumulation and better cognitive performance, confirming the hypothesis that chronic inflammation further aggravates the course of AD (Fig. 4) (Heneka et al., 2015). In advanced stages of AD, Aβ interacts with microglial receptors TLR4 and TLR6 increasing microglial proliferation and pro-inflammatory cytokine release. In

light of these premises, chronic microglial activation can accelerate the course of AD (Qin et al., 2021, p. 2).

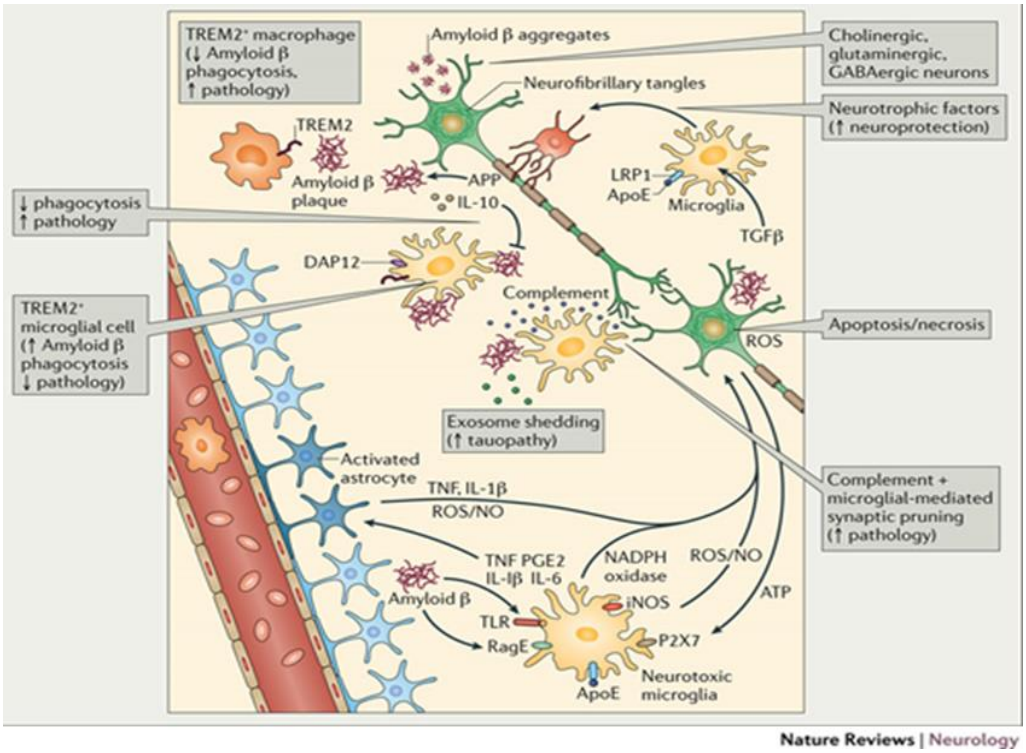


Fig.4 - (Dendrou et al., 2016) Neuroinflammation : in AD, microglial cells secrete pro-inflammatory cytokines.

Investigating the endocannabinoid system in neurodegenerative diseases

For those conditions characterized by chronic inflammation, such as AD and Parkinson's Disease (PD), some authors describe microglial cell activity as a "double-edged sword," because its neuroprotective activity facilitates Aβ and α-synuclein clearance, but the persistent accumulation of misfolding protein during the course of diseases also contributes to hyper-activate M1-polarized microglia leading to the additional accumulation of these peptides. There are limited data in the literature on the relationship between ECBS and morphological changes associated with the different states of microglia activation. Microglia cells produce eCB and possess CB2, which following stimulation, helps microglia to revert from M1 phenotype to the M2 phenotype. It supports the possibility of the therapeutic power of cannabinoids (Cooray et al., 2020). As yet there are no drugs that target inflammatory-mediated

modulation of microglia, the ECBS may be considered in this scenario as a new area for therapeutic intervention in neurodegenerative diseases (Cooray et al., 2020).

Analysis of tissue specimens from AD patients have shown that CB2 receptors are most highly expressed on microglia associated with amyloid plaques (Benito et al., 2003).

Stimulation of CB2 receptors in transgenic mouse models of AD improved cognitive performance (Aso et al., 2013). In addition, in *in vitro* models of AD, CB2 stimulation also reduced the release of pro-inflammatory cytokines (Ehrhart et al., 2005; López et al., 2018). Stimulation of CB2 receptors in microglial cells has been hypothesized to play an anti-inflammatory role by reducing neuronal degeneration (Pazos et al., 2004).

In a different post-mortem study on brains from AD patients, the cortical levels of AEA were decreased together with an increase of FAAH levels (Benito et al., 2007; Jung et al., 2012).

Modulation of ECBS could act in a broad spectrum on neuroinflammation stimulating the clearance of Aβ (Tönnies & Trushina, 2017).

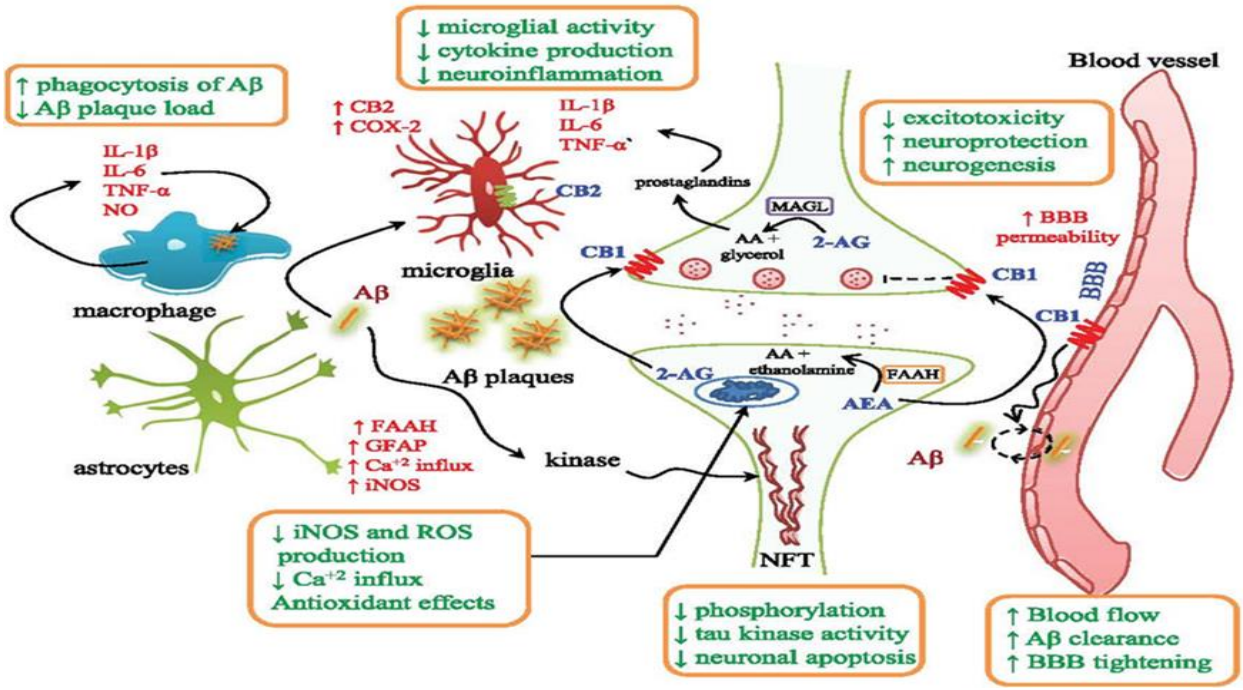


Fig.5 - (Bedse et al., 2014) Neuroprotective effects of cannabinoid treatment in AD: increased clearence of phosphorylated tau and Aβ, decreased of pro-inflammatory mediators, oxidative stress, and excitotoxicity.

AUTOPHAGIC PROCESS IN ALZHEIMER'S DISEASE

Few recent studies have evaluated the impact that impaired autophagy can have on microglial phenotype (Plaza-Zabala et al., 2017). The accumulation of pathological protein aggregates occurring in neurodegenerative diseases such as AD is related to the dysfunction of autophagy that controls protein catabolism (Z. Zhang et al., 2021). In a 2005 study, electron microscopy analyses demonstrated abnormal accumulation of immature autophagosomes in neurons of AD patients (Nixon et al., 2005). In the same year, Yu et al. showed that the onset of amyloid plaques follows the accumulation of immature autophagosomes in neurons (Yu et al., 2005). Recently, the downregulation of several proteins involved in autophagic processes in AD has been reported (Heckmann et al., 2020; Lachance et al., 2019). Autophagy dysfunction results in increased reactive oxygen species (ROS) correlated with reduced mitochondrial function, increased genome instability, with subsequent activation of pro-apoptotic processes (Ichimiya et al., 2020). Based on these data, it has been hypothesized that dysfunction of the autophagy process may contribute to the development of AD.

Autophagy is a pro-homeostatic process involving protein metabolism that engages both degradation and recycling of misfolded proteins and malfunctioning organelles. There are 3 types of autophagy mechanisms: 1) microautophagy, where cargoes are phagocytosed by lysosomal membranes; 2) chaperone-mediated autophagy (CMA) mediated by chaperone protein HSPA8 and co-chaperones; 3) and macroautophagy.

Macroautophagy is the most studied process; it is stimulated by cellular stressors such as cellular nutrient deficiency, protein accumulation, and inflammation. (Z. Zhang et al., 2021). Autophagosome formation involves ATG proteins (autophagy-related genes) (Lamb et al., 2013) and is partitioned into 4 steps: initiation, nucleation, fusion and degradation (Kiriyama & Nochi, 2015).

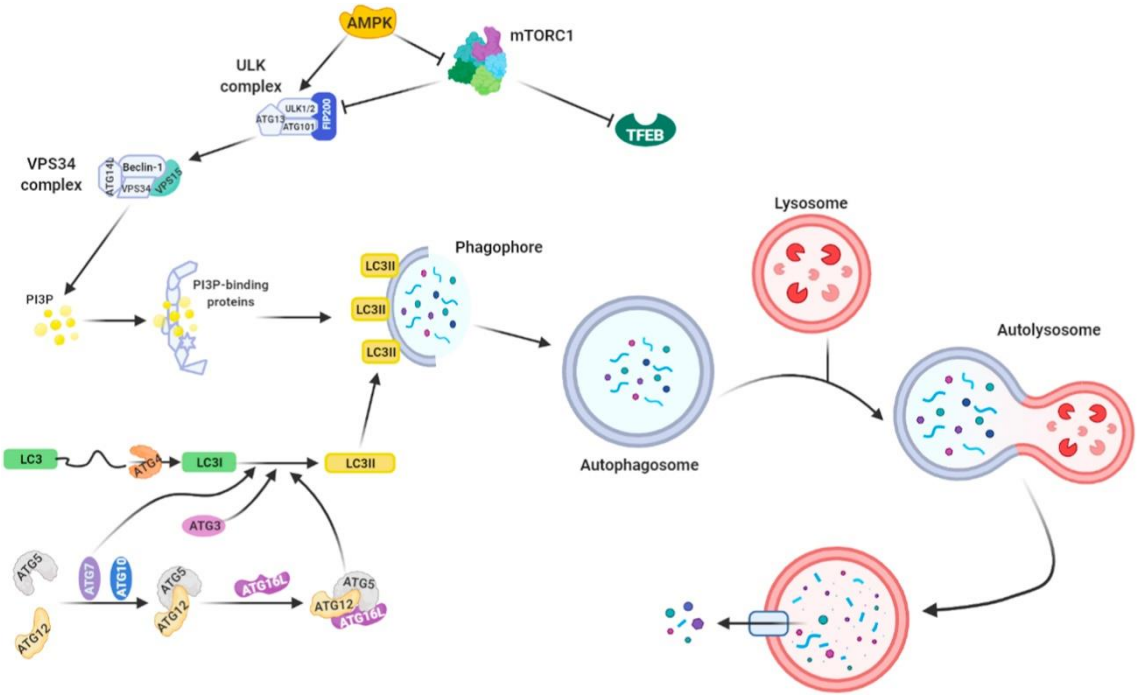


Fig.6 - (Z. Zhang et al., 2021) Representation of Autophagy Flux.

The start of the process is inhibited by mTOR (mammalian target of rapamycin). Deregulation of mTOR signaling has been implicated in many neurodegenerative diseases. mTOR forms two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2); mTORC1 inhibits catabolic pathways such as autophagy. mTORC1 is a downstream PI3K/AKT signaling pathway, and its activity is controlled by a signaling network involving Ras/Raf/MEK/ERK. In physiological conditions, nutrient deprivation inhibit mTORC1 activity and induce

autophagy. The initiation of autophagy by ULK1 is reciprocally regulated by mTORC1 and AMPK: AMPK activates ULK1; when the nutrients are sufficient, mTOR prevents the interaction between ULK1 and AMPK and thus the activation of ULK1. Autophagy initiation is also mediated by the VPS34 complex where Beclin-1 plays a key role: AMPK, through phosphorylation of Beclin-1, promotes its dissociation from Bcl-2, and the autophagy process can start (J. Kim et al., 2011; Y. C. Kim & Guan, 2015; Z. Zhang et al., 2021). After autophagy onset, ATG7 recruits all other proteins related to autophagy and the formation of the phagophore begins, which enlarges to become an autophagosome (Mizushima et al., 2003). Autophagosome formation is completed by MAP1LC3/LC3 (ATG8 in yeast), which is subjected to proteolytic cleavage by ATG4 generating LC3-I. The C-terminal glycine residue of LC3-I binds the phosphatidylethanolamine (PE), leading to the formation of LC3-II (Tanida et al., 2004). LC3-II binds to the autophagosome membrane. Autophagosomes are later fused with the lysosomal membrane to make autolysosomes (Kiriyama & Nochi, 2015; Z. Zhang et al., 2021). Beclin1 is decreased in the brains of mouse models and in AD patients, with greater accumulation of immature autophagosomes and Aβ (Kiriyama & Nochi, 2015). In mouse models of AD, mTOR inhibition reduces Aβ peptide levels and improves cognitive performance (Dello Russo et al., 2013; Sierra et al., 2013; Yamamoto & Yue, 2014). Cell components that are destined to the autophagosome are "marked" with ubiquitin, which is identified by selective receptors, such as p62/SQSTM1, that "confiscate" them and carry them to the autolysosome (Lamark et al., 2017).

Oxidative stress is involved in AD by promoting the deposition of Aβ (Z. Chen & Zhong, 2014). Autophagy has been shown to be strongly correlated with oxidative stress, through the direct interaction between p62 and Keap1 (Jiang et al., 2015). Low nutrient levels activate autophagy to restore homeostasis through the degradation of macromolecules to provide nutrients. Deregulation of autophagy in AD results in accumulation of Aβ (Q. Li et al., 2017). Recently, it has been shown that these two cellular pathways directly intersect at the level of p62, which through interaction with Keap1, promotes translocation into the nucleus of Nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 activates the transcription of antioxidant genes deputed to

cytoprotection from oxidative stress through upregulation of anti-oxidant enzymes (Fig.7).

The p62-Nrf2 pathway and autophagy are both involved in the oxidative stress and innate immunity response; dysregulation of these pathways is associated with human disease-pathogenic mechanisms (Ichimura et al., 2013; Jiang et al., 2015).

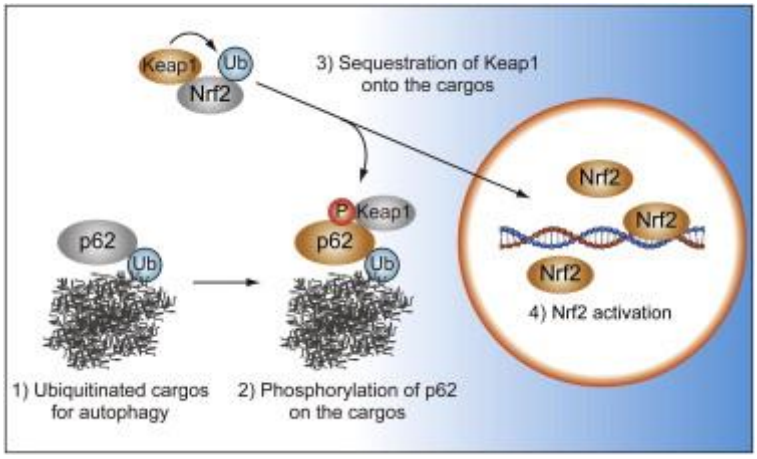


Fig. 7 - (Ichimura et al., 2013) p62 activates Nrf2 Pathway.

Autophagy is implicated in aging and neurodegeneration. Autophagy malfunctioning has been studied predominantly in neurons but is also present in microglia by affecting innate immune responses such as phagocytosis and neuroinflammation. Few recent studies have evaluated the role that impaired autophagy plays on the microglial phenotype (Plaza-Zabala et al., 2017). Microglia phagocytose and degrade toxins and Aβ protein accumulations sharing similarities with autophagy (Plaza-Zabala et al., 2017; Sierra et al., 2013). Phagocytic activity of microglial cells is compromised in the brains of mice and patients with epilepsy and AD (Abiega et al., 2016; Hickman et al., 2008; Mawuenyega et al., 2010; Solé-Domènech et al., 2016). A 2013 study found that a decrease of Beclin-1, both *in vitro* and *in vivo*, was combined with a lower clearance of Aβ by microglia (Lucin et al., 2013). Modulation of microglial autophagy may influence neuroinflammation in aging and neurodegenerative diseases (Plaza-Zabala et al., 2017). Further investigation of the relationship between autophagy and microglia in the CNS is needed, and will help the development of molecular knowledge of mechanistic processes of autophagy for the development of new preventive therapeutic strategies for AD. Autophagy dysfunction increases pro-inflammatory response in microglia

that leads to chronic inflammation. Therefore, restoration of autophagy can reverse the polarization of microglia towards the M2 phenotype (Zubova et al., 2022). This evidence suggests that the functionality of the autophagic process is most important for the progression of AD and modulation of autophagy could be a potential therapeutic target for AD.

URB597: a pharmacological tool to achieve FAAH inhibition

The "Fatty acid amide hydrolase" (FAAH) is an integral membrane serine amide hydrolase isolated in 1996, and the major hydrolase involved in the metabolism of eCB and other endogenous ligands that cooperate with anti-inflammatory activity such as N-palmitoylethanolamine (PEA) and N-oleoylethanolamine (OEA) (Cravatt et al., 1996, p. 199; Mileni et al., 2008; Rafiei & Kolla, 2021).

FAAH is expressed in the CNS, in the neocortex, hippocampus, amygdala, and cerebellum (Waleh et al., 2002). A very recent study in 2021 proved the increased activity of FAAH in AD combined with reduced methylation of its gene promoter (Rafiei & Kolla, 2021).

FAAH expression is associated with amyloid plaques and its inhibition increases the availability and tone of AEA, which activates the ECBS receptor system, achieving a neuroprotective role by reducing the formation of Aβ-rich neuritic plaques (Hwang et al., 2010; Jain et al., 2022). In addition, modulation of FAAH activity is associated with a reduction of pro-inflammatory mediators and oxidative stress (Jain et al., 2022).

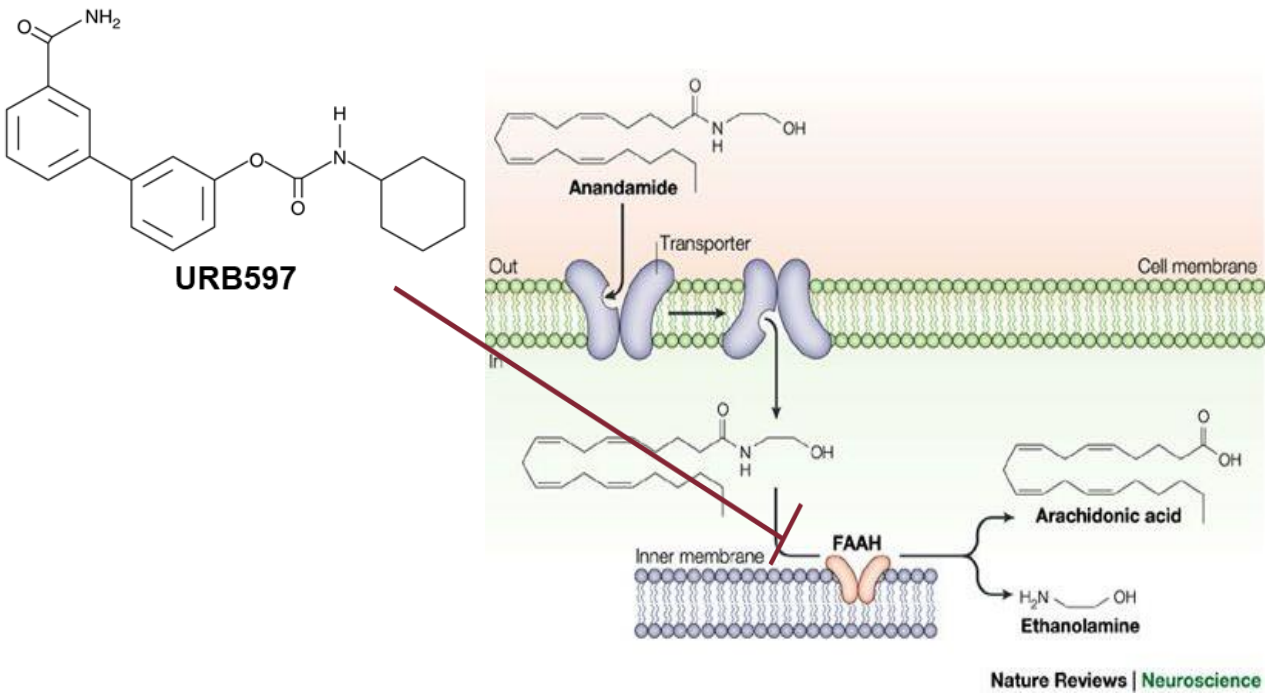
Activated microglia synthesizes eCB such as AEA and upregulates the expression of CB2 receptors, which promote a protective microglial phenotype by reducing the production of pro-inflammatory factors (Duffy et al., 2021).

The key pharmacological tool used in this work is the URB597, a cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester (Mor et al., 2004; Piomelli et al., 2006). URB597, an aryl ester of archylcarbamic acid, is capable to achieve the inhibition of FAAH enzyme activity via carbamylation of the nucleophilic residue

Ser241 (Fegley et al., 2005). A 2007 study showed that administration of URB597 reduces nociceptive response in mouse models of pain (Russo et al., 2007).

In a recent study, mouse models of PD treated with URB597 showed better behavioral performance than untreated mice, and lower microglial hyperactivity (Viveros-Paredes et al., 2019). Although some cannabinoids have shown efficacy in selected experimental animal models, their potential cannabimimetic effects also elicit several doubts about their clinical implementation (Naidu et al., 2010). The ECBS is rapidly emerging as a potential pharmacological target for different neurological diseases.

Fig. 8 - Modified from (Piomelli, 2003) AEA is hydrolyzed by FAAH. URB597 inhibits the activity of FAAH



AIM

Neurodegenerative diseases are becoming the main causes of dementia: 1.8% is represented by PD while AD represents 12% with the highest rate of incidence in the world population (Cooray et al., 2020). AD is characterized by a potent inflammatory profile due to the accumulation of Aβ peptides and the consequent hyperactivation of microglia cells (Eikelenboom & Veerhuis, 1996). These features are exacerbated by the malfunction of autophagic processes that accelerate accumulation of Aβ (Z. Zhang et al., 2021).

Today there is no definitive therapy for AD. For this reason, the study of new therapeutic targets is extremely important. In recent decades, much attention has been focused on the ECBS for its neuroprotective impacts (Cooray et al., 2020). ECBS is deregulated in AD patients, with a decrease of AEA and an increase in the activity of FAAH (Benito et al., 2007; Jung et al., 2012). Studies in transgenic AD models have shown cognitive recovery following modulation of the ECBS (Bisogno & Di Marzo, 2008). The purpose of this study is to evaluate how modulation of the ECBS through pharmacological treatment with URB597 can induce an anti-inflammatory effect by influencing microglial polarization towards an M2 phenotype and whether it may restore autophagic flux leading to a reduction of amyloid plaques.

The study was carried out both *in vitro* and *in vivo*. *In vitro*, by taking advantage from murine microglia BV-2 cells that were pretreated with URB597 with later exposure to Aβ. Here, we evaluated the impact of FAAH inhibition on cytoskeletal reorganization and on mRNAs expression of genes involved both in inflammation and autophagy.

The transgenic Tg2576 mouse model of AD overexpresses a mutant form of APP and develops accumulation of soluble Aβ in cortical and hippocampal amyloid plaques (Hsiao, 1998). Tg2576 mice were treated with URB597, and then we evaluated different inflammation mediators and autophagic mechanisms.

MATERIALS AND METHODS

In vitro Analysis

In vitro analyses relating to anti-inflammatory activity of URB597 were recently published in a 2021 paper (Grieco et al., 2021) and carried out in collaboration with the Laboratory of Professor Mancini Department of Experimental Medicine, Sapienza University of Rome and Dr. Tiziana Bisogno, Endocannabinoid Research Group, Institute of Translational Pharmacology, CNR.

Preparation of the stock solution of Aβ25-35 and URB597

As described by Grieco et al. 2021, URB597 [3- (3-carbamoylphenyl) phenyl] N-cyclohexylcarbamate came from Selleck Chemicals (Selleck Chemicals, Houston, TX, USA). The peptide-amyloid fragment (Aβ25-35) was synthesized by conventional solid phase (Colombo et al., 2009). Aβ25-35 was dissolved in phosphate-buffered sterile saline, pH 7.4 (PBS) at a concentration of 1 mM. To induce aggregation, the solution was incubated in a sonicator bath with ice for 30 min. After treatment, the solution was stored at -20°C until use. URB597 was dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 1 mM (Grieco et al., 2021).

Cell cultures and treatments

Mouse microglia cell line (BV2), kindly provided by Dr. Mangino, Sapienza University of Rome, was seeded in Dulbecco's Modified Eagle's Medium High Glucose (DMEM, D5671-500ML, Sigma Aldrich, St Louis, MO, USA) containing 5% Fetal Bovine Serum (FBS, F7524, Sigma Aldrich, St Louis, MO, USA), 1% L-glutamine and 1% of penicillin-streptomycin, 1% non essential aminoacids and 1% sodium pyruvate (Sigma Aldrich, St Louis, MO, USA), at 37 ◦C in a humidified atmosphere with 5% CO2. Cells were plated at an appropriate density according to each experimental setting and after 24 h treated with Aβ25–35 30 μM in presence or absence of URB597 5 μM. The cells were pre-treated with URB597 for 4h before adding the Aβ25–35.

Cell viability assays: Trypan blue exclusion test

BV-2 cells were seeded onto 48-well plates at a density of 3×10^3/well. After treatments, cells were detached with 1× Tripsin-EDTA (AU-L0940-100, Aurogene, Rome, Italy) and 10 μL of the cell suspension were mixed with 10 μL of Trypan blue solution (25-900-CI, Corning, Glendale, Arizona) cell counts were performed using a

Burker's chamber. Both living cells and dead cells were counted. The results are shown as percent viability of the treated groups relative to the control, which was considered 100%.

Real-time quantitative PCR analysis

Total RNA was extracted from control and treated BV-2 cells using the miRNeasy Micro kit (Qiagen, Hilden, Germany) and quantified with NanoDrop One/OneC (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR (qPCR) was performed for each sample in triplicate on an Applied Biosystems 7900HT Fast real-rime PCR system (Applied Biosystem, Cheshire, UK) using the SDS2.1.1 program (Applied Biosystem, Foster City, CA, USA) using the Power SYBR® Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA). Primers for real-time PCR amplification were designed with UCSC GENOME BROWSER (http://genome,cse.ucsc.edu/; University of California, Santa Cruz) (Table 1). The sequences of the primer pairs were matched via BLASTn to the genome sequence to identify the positions of the primers relative to the exons. The comparative threshold cycle (CT) method was used to analyze the real-time PCR data. The target quantity, normalized with respect to the endogenous reference of the 18S rDNA primers (ΔCT) and relative to the calibrant of the untreated control (ΔΔCT), was calculated with Equation $2^{-\Delta\Delta CT}$.

GENE	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
mIL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
mIL-6	CGGAGAGGAGACTTCACAGAGGA	TTTCCACGATTTCCCAGAGAACA
mTGF-β	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
mIL-10	GCCCTTTGCTATGGTGTCCTTTC	TCCCTGGTTTCTCTTCCCAAGAC
mR18s	AAATCAGTTATGGTTCCTTTGGTC	GCTCTAGAATTACCACAGTTATCCAA
mARG1	ATGTGCCCTCTGTCTTTTAGGG	GGTCTCTCACGTCATACTCTGT
miNOS	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
mLC3	TTCTTCCTCCTGGTGAATGG	GTCTCCTGCGAGGCATAAAC
mBeclin1	TGGGGAGGTTAGGATTTGGGA	GAGCCGTAGGGTGGAAAGC
mNrf2	TCTGAGCCAGGACTACGACG	GAGGTGGTGGTGGTGTCTCTGC
mp62	CCTTGCCCTACAGCTGAGTC	CCACACTCTCCCCCACATTC
mATG7	CAATGAGATCTGGGAAGCCATAA	AGGTCAAGAGCAGAAACTTGTTGA
mBDNF	GTGTGACAGTATTAGCGAGTGG	GCAGCCTTCCTTGGTGTAAC

Table 1. List of primer couples generated for qPCR.

Immunofluorescence analysis

As described by Grieco et al. 2021, cells grown on 12 mm glass coverslips in 24-well plate at a density of 15×10^3 cells/well, were fixed after treatments with 4% paraformaldehyde for 30 min, followed by treatment with 0.1 M glycine in PBS for 20 min. A total of 0.1% Triton X-100 in PBS was added for 5 min to allow permeabilization. To analyze cytoskeletal actin organization, cells were labeled with rhodamine-conjugated phalloidin (TRITC-phalloidin - Sigma-Aldrich, St. Louis, MO, USA) for 45 min. For the detection of M1/M2 polarization markers, cells were incubated with primary antibodies rabbit polyclonal IgG anti-iNOS (dil. 1:100—D6B65, Cell Signaling Technology, Danvers, MA, USA), or rabbit polyclonal IgG anti-Arg-1 (dil. 1:50—D4E3M, Cell Signaling Technology, Danvers, Ma, USA), and subsequently with anti-rabbit Alexa Fluor 488 secondary antibodies. Finally, the cells were marked with DAPI to highlight the nucleus. The fluorescence signal was analyzed using an Axio Observer inverted microscope, equipped with the ApoTome System (Carl Zeiss Inc., Ober Kochen, Germany). Cell area was quantified with ImageJ software (Grieco et al., 2021).

Statistical Analyses

Data were expressed as the mean values ± standard deviations (SD) or mean values ± SEM from at least three independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey's post hoc test comparison and using Unpaired Student t test (GraphPad Software Inc., San Diego, CA, USA). All results were considered statistically significant with $p < 0.05$.

In vivo study

Animals

To study the modulation of ECBS through FAAH inhibition in AD, in collaboration with the laboratory of Dr Coccurello at Fondazione Santa Lucia (FSL-Rome), we used Tg2576 transgenic mice, expressing high levels of mutated human APP (Swedish K670N/M671L mutation) and showing several cognitive deficits and brain accumulation of amyloid plaques (Hsiao et al., 1998). Tg2576 mice are heterozygous for the APP-K670N/M671L transgene and are obtained by crossing hemizygous males (Tg2576-F0) with females of C57BL/6J/SJL-F0 hybrid mice. The latter are

obtained by crossing wild type SJL males with wild type C57BL/6J females. Housing conditions (3-4 animals per cage) in pathogen-free facilities were controlled (temperature 22 °C, light/12-hour dark cycles, humidity 60 ± 5%) with ad libitum access to food and water. All experiments were approved by the Italian Ministry of Health (Legislative Decree No. 682/ 2016).

Fourteen-month-old Tg2576 and wild-type (WT) mice were used. Genotyping was performed to confirm the presence of the human mutant APP DNA sequence by PCR. Mice were genotyped between 20 and 25 days of age by tail biopsy analysis. Tail tissues were collected to extract genomic DNA, according to standard techniques; 10 ng of DNA per sample was amplified by polymerase chain reaction (PCR) performed according to the protocol of Maccarrone et al., 2018 (Maccarrone et al., 2018). In rodents, intraperitoneal injection was used as the route of administration of URB597 (S2631, Selleck Chemicals, USA). The compound (10mg/kg) was administered every 24 hours for a total of 8 administrations (8 days). Mice were assigned to treatment by vehicle or URB937. URB597 was dissolved in a vehicle composed of 5% PEG (polyethylene glycol), 5% Tween-20 and 80% saline (10 mg/kg). At the end of the treatment, animals were sacrificed 2 hours after the last injection.

Immunohistochemistry

Animals for immunohistochemical analysis were perfused intracardially with phosphate-buffered-saline (PBS) 0.1 mol/liter pH7.4 using a 30 mL syringe to remove blood contamination and then with 4% paraformaldehyde (PFA) solution in PBS. Next, the brains were carefully removed from the skull and post-fixed in 4% PFA solution for 16 h overnight at +4 ◦C and then passaged in 30% sucrose solution in 0.1 mol/liter phosphate buffer for 48-72 h until equilibration. Brains were frozen by immersion in cold isopentane for 3 min before being sealed in vials and stored at -80 ◦C until use. Frozen sections (10 μm thickness) were obtained with a Leica cryostat and placed on BDH slides (Milan, Italy) and stored at -20 ◦C.

Sections were defrosted at the time of analysis and 3 washes in PBS were performed. Blocking of endogenous peroxidases was done in methanol 3% H2O2 for 12 minutes. After 3 washes in PBS, non-specific sites were blocked with Blocking Serum (Scytek AFN600 Ultratek HRP Polyvalent, Utah, USA) for 12 minutes. Three washes in PBS

were performed, and the sections were covered with primary antibodies diluted in PBS and BSA 0.1% O.N. at 4°C. The sections were washed in PBS 3 times by 5 min and were covered with biotinylated secondary antibody (Scytek AFN600 Ultratek HRP Polyvalent, Utah, USA), anti-Ms or anti-Rb according to origin, for 12 min. The excess was removed by 3 washes in PBS by 5 min the reaction was revealed by a tertiary antibody (Scytek AFN600 Ultratek HRP Polyvalent, Utah, USA) conjugated to a catalyzing enzyme (peroxidase) that reacted with the DAB chromogen (Scytek ACH500 DAB Substrate Kit, Utah, USA), forming an insoluble colored precipitate visible under a light microscope. The nuclei were highlighted with hematoxylin for 5 min and stained under running water for 10 min to remove the excess. The sections were left to dry at RT and were closed with glass coverslip and aqueous mounting. The percentage of positive cells were measured using Nikon's universal software platform, NIS-Elements.

Primary Antibody:

- Anti-iNOS (1:100, NB300-605 Novus Biological, Centennial, CO, USA)
- Anti-ARG-1 (1:100, AB-84248, Immunological Sciences, Rome, Italy)
- Anti-BACE1 (1:300, ab183612, Abcam, Cambridge, UK)
- Anti-PSD95 (1:100, MAB-10681, Immunological Sciences, Rome, Italy)
- Anti-GFAP (1:300, MAB-12029, Immunological Sciences, Rome, Italy)
- Anti-P62/SQSTM1 (1:100, sc-28359, Santa Cruz, Dallas, TX)
- Anti-LC3I-II (1:100, PM036MS, MBL, Japan)
- Anti-Beclin-1 (1:100, AB-82599, Immunological Sciences, Rome, Italy)
- Anti-mTOR (1:100, AB-84433, Immunological Sciences, Rome, Italy)
- Anti-ULK1 (1:100, AB-84102, Immunological Sciences, Rome, Italy)
- Anti-CBII (1:50, ab3561, Abcam, Cambridge, UK)
- Anti-ATG7 (1:100, AB-83978, Immunological Sciences, Rome, Italy)

Congo red method

We examined the presence of amyloid plaques by the congo red method. The sections were defrosted, and the nuclei were highlighted with hematoxylin for 5 min, staining under running water was performed for 10 min. After that, the sections were covered

with Congo Red solution 1% aqueous (Electron Microscopy Sciences 26090-25, Hatfield, PA) for 1h and washed with PBS, staining under running water was performed for 5 min The sections were left to dry at RT and were closed with glass coverslip and aqueous mounting. The area (μm^2) and number of plaques was assessed by Nikon's universal software platform, NIS-Elements.

Western Blot (WB)

At the end of treatments, the animals were deeply anesthetized, and brains were quickly removed after decapitation. Cerebral cortex and hippocampus were quickly dissected and stored at -80 °C to perform WB analysis.

Homogenates of cortex and hippocampus were obtained with Ripa Lysis Buffer with 1 mM Na_3VO_4, 1 mM PMSF and proteases inhibitors (sc-24948, Santa Cruz, Dallas, TX). The homogenates were sonicated (72% AMPL) for 15 seconds and centrifuged for 10 minutes at 12,000 rpm at 4°C to remove the pellet. Protein quantification was performed by Pierce BCA protein assay kit (23227, ThermoScientific, Rockford, USA). Samples were prepared for WB under reducing conditions with Laemmli 4x (161-0747, BioRad, California, USA) and β-mercaptoethanol (A1108,0100, ITW Reagents, Monza MI, Italy). Samples were boiled at 98°C for 10 min.

Acrylamide gel was prepared at 10% or 15% depending on the protein considered, where the samples were loaded for the 1x run buffer run that was carried out at 90V and 100V. The proteins were transferred to nitrocellulose membrane in 1X 20% Methanol transfer buffer for 70 minutes, 90V cold.

Proteins were highlighted with ponceau red, the membrane was rinsed in 1X TTBS, and nonspecific sites were blocked with 5% nonfat milk in 1X TTBS. Four 10-min washes in TTBS1X were performed, and the membrane was covered with primary antibody (1:1000) dissolved in 5% milk or 3% BSA, O.N. 4°C. The housekeeping used is GAPDH (AB-81594, Immunological Sciences, Rome, Italy).

The secondary (HRP Conjugated Goat Anti-Mouse, IS20400, Immunological Sciences, Rome, Italy - HRP Conjugated Goat Anti-Rabbit, bs0295G-HRP, Bioss Massachusetts, USA) was kept for 1h at RT dissolved in 2.5% milk. Five 10 min washes were performed, and the membrane was developed at BioRad ChemiDoc XRS+ with ImageLab software, with ECL Chemiluminescence system (ECL-2001

West PICO PLUS substrate, Immunological Sciences, Rome, Italy). Densitometry was analyzed by ImageJ.

ELISA Assay

To distinguish the LC3-II form from the LC3-I form, LC3-II antigen was analyzed by ELISA assay. Homogenates of cortex and hippocampus from untreated and vehicle/URB597-treated Tg2576 and WT mice were prepared and analyzed according to Autophagy ELISA Kit (LC3-II Quantitation, Cell Biolabs, INC, San Diego, CA, USA).

Statistical Analyses

Data were expressed as the mean values ± standard deviations (SD) or mean values ± SEM from at least three independent experiments. Statistical analyses were performed using Unpaired Student t test (GraphPad Software Inc., San Diego, CA, USA). All results were considered statistically significant with $p < 0.05$.

RESULTS

URB597 significantly reduces the activity of FAAH on BV-2 cells

We first evaluated, in collaboration with Dr. Tiziana Bisogno, CNR, whether URB597 indeed modulates FAAH activity in BV-2 cells via the radioligand anandamide. FAAH enzymatic activity was studied by using membranes prepared from BV-2 cells treated with vehicle or pre-treated with 5 μM URB597 for 4 h and incubated with 30 μM Aβ25-35 for 24 h. Membrane preparations were then incubated with [14C]-AEA. The [14C]-Ethanolamine produced from [14C]-AEA hydrolysis was measured by scintillation counting of the aqueous phase after the extraction of the incubation mixture, with two volumes of CHCl3/CH3OH (1:1, v/v); the activity was expressed as percentage of the maximum effect observed in the absence of treatments. Aβ25-35 had no effect on FAAH activity at 24 hours. URB597 inhibits the activity of FAAH (Fig.9) (Grieco et al., 2021).

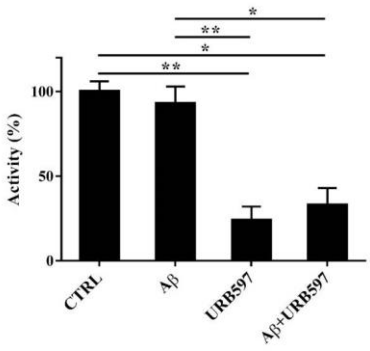


Fig. 9 - (Grieco et al., 2021) FAAH activity in BV-2 cells pre-treated with 5 μM URB597 for 4 h and incubated with 30 μM Aβ25–35 for 24 h. Data are reported as mean ± SEM of three independent experiments using one-way analysis of variance (ANOVA) with Tukey's post hoc test comparison * $p < 0.05$, ** $p < 0.01$.

URB597 does not exhibit cytotoxic effects in BV-2 cells

Trypan blue assay performed at 24 h on BV-2 cells revealed that addition of Aβ to cell culture significantly reduced the percentage of live cells and significantly increased the percentage of dead cells compared with untreated cells (CTRL). URB597-treated cells showed no difference from CTRL, confirming the non-cytotoxicity of the drug, as already described in our 2021 paper (Grieco et al., 2021). Combined Aβ+URB597 treatment significantly increased the number of live cells

compared with Aβ treatment and significantly decreased the percentage of dead cells (Fig.10).

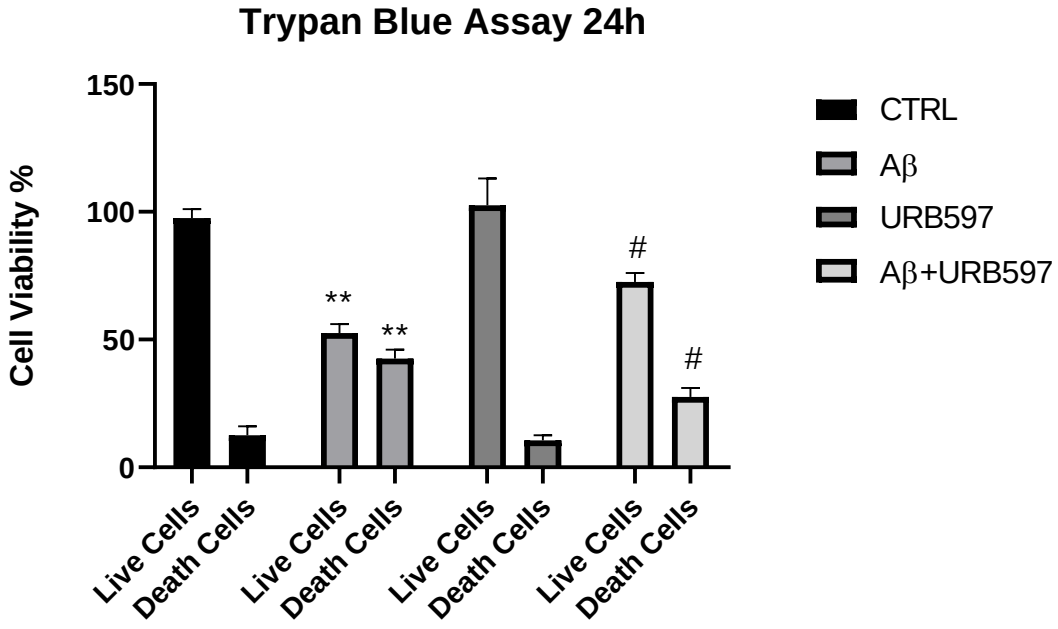
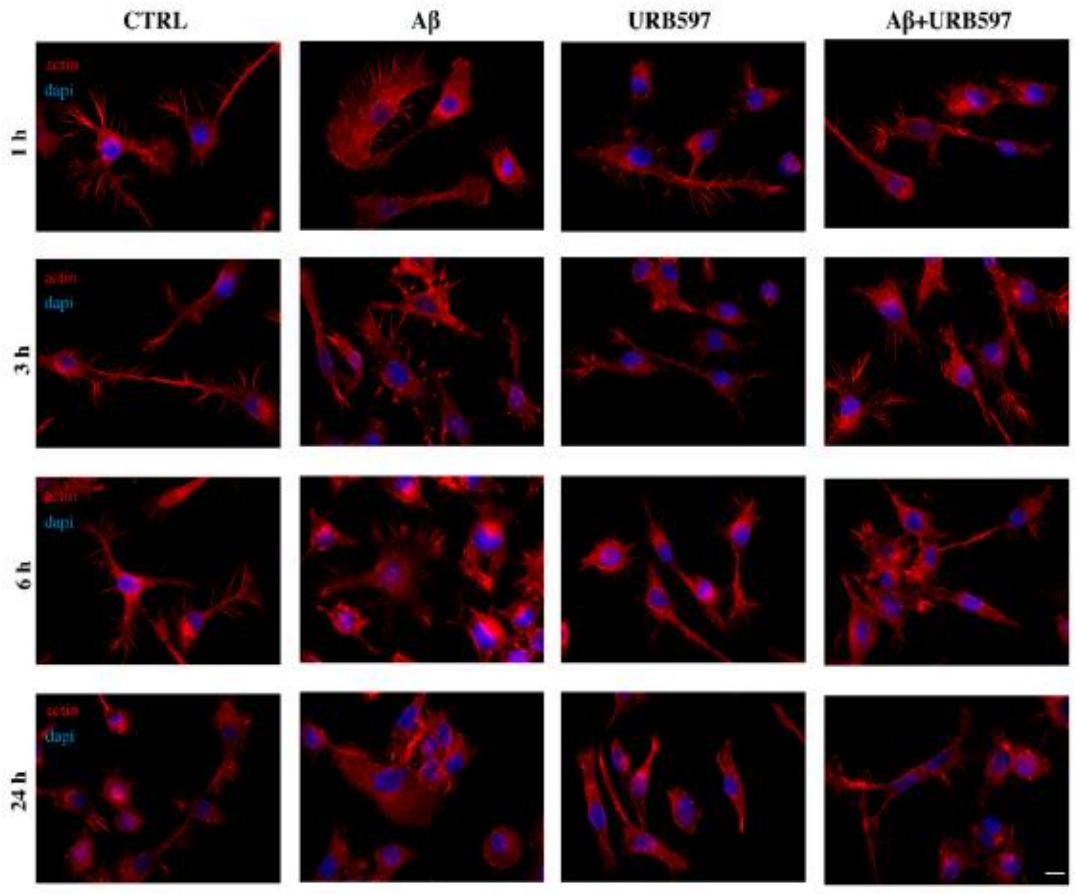


Fig. 10 - Trypan blue exclusion test. Effects of Aβ25–35 in the presence or absence of URB597 on BV-2 cells. Cell count was determined in BV-2 cells exposed for 24 h to 30 μM of Aβ25–35 in the presence or absence of 5 μM of URB597 and expressed as cell death (cell death/cell death + living cell). Data are reported as percentage versus CTRL (*) and versus Aβ (#). The values are the mean ± SEM of triplicate determination from independent experiments using Unpaired Student t test. * $p < 0.05$, ** $p < 0.01$.

URB597 counteracts changes of phenotype induced by Aβ25–35 in BV-2 cells

Microglia cells react very early to CNS injury; their activation state, as mentioned previously, is associated with morphological changes. Microglia responding to an exogenous challenge takes an amoeboid phenotype. We evaluated the effects of URB597 on microglia morphology by rhodamine-conjugated phalloidin (TRITC-phalloidin) staining to highlight actin. We pretreated BV-2 cells with 5 μM of URB597 for 4 h and then we incubated the cells with 30 μM of Aβ25-35 (1 h, 3 h, 6 h, 24 h). The untreated cells (CTRL) expressed a resting type morphology, with small and elongated soma and many branches. Following an inflammatory stimulus

with Aβ25-35 the cells increased their somatic area with few ramifications. URB597-treated cells appeared to have a morphology comparable to CTRL. In the combined treatment with URB597 and Aβ25-35 the BV-2 showed a phenotype similar to that obtained with URB597 alone or to the untreated cells. These results have been confirmed by measuring the area expressed in μm^2 at 24 h (Fig 11). Treatment with Aβ25-35 increased the area of the cellular soma of microglia cells, as expected for a phlogogenic stimulus, whereas treatment with URB597 reduced the area to values comparable to those of CTRL cells (Grieco et al., 2021).



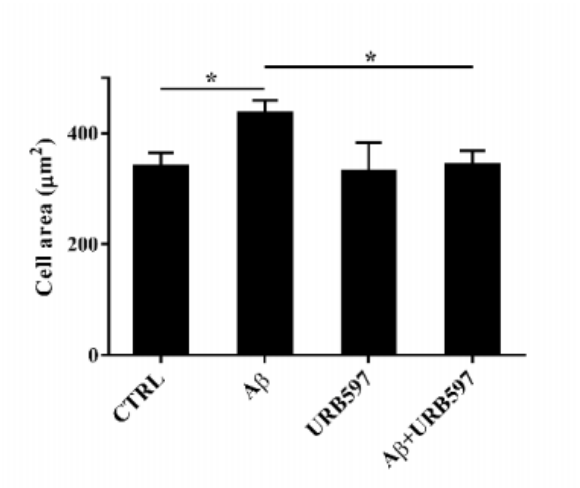


Fig. 11 - (Grieco et al., 2021) Morphological analysis performed by F-actin stain. Analysis of BV-2 cell morphology by rhodamine-conjugated phalloidin (TRITC-phalloidin) staining to highlight actin and 4′, 6-diamidino-2-phenylindole (DAPI) to detect the nucleus, after pre-treatment with 5 μM URB597 for 4 h and incubated with 30 μM Aβ25–35 for 1 h, 3 h, 6 h, and 24 h. Cell areas were quantified at 24 h using Image J software. Data were reported as mean ± SD of at least three independent experiments using one-way analysis of variance (ANOVA) with Tukey's post hoc test comparison. * $p < 0.05$. Bar: 20 μm.

FAAH inhibition increases Arginase-1 mRNA expression and decreases iNOS mRNA expression in BV-2 cells

The M1 pro-inflammatory phenotype of microglia is associated with the expression of inducible nitric oxide synthase (iNOS), while the M2 anti-inflammatory phenotype is linked to the expression of arginase 1 (ARG-1). To work out the point of whether URB597 reverts microglia under resting conditions or helps microglia to switch the phenotype towards the M2 phenotype in the presence of Aβ, we assessed the mRNA expression of the M1 and M2 phenotypic markers by RT-PCR. The figure shows (Fig.12) that BV-2 cells treated with Aβ only express significantly increased iNOS mRNA and simultaneously reduced ARG-1 mRNA compared with CTRL. Treatment with URB597 shows no significant difference compared with CTRL, but cells pretreated with 5 μM URB597 for 4 h and incubated with 30 μM Aβ25-35 for 24h show a significant decrease in iNOS mRNA expression and a significant increase in ARG-1 mRNA expression.

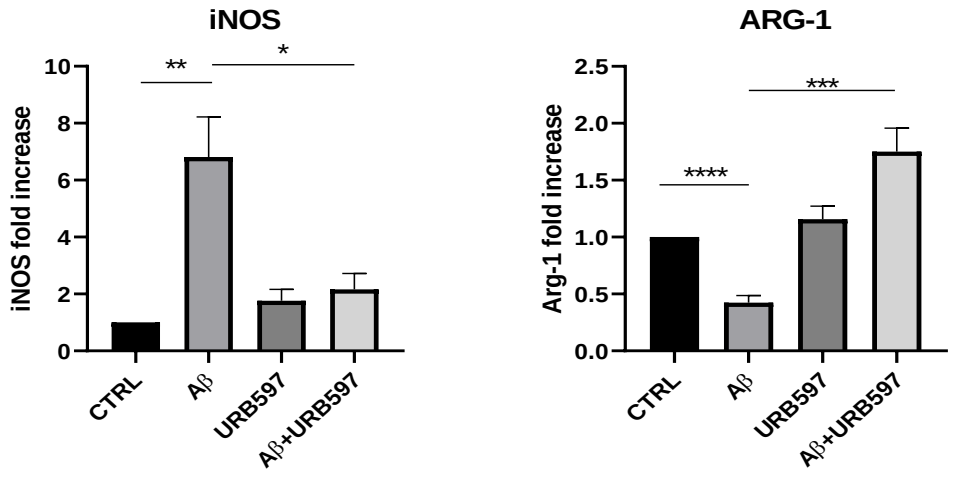


Fig. 12 - mRNA expressions of M1 (iNOS) and M2 (ARG-1) markers were evaluated in BV-2 after pre-treatment with 5 μM URB597 for 4 h and incubated with 30 μM Aβ25–35 by qRT-PCR at 24h. Data are shown as mean ± SD from three independent experiments performed in triplicate. Expression profiles were determined using the 2−ΔΔCT method. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

URB597 drives microglia to M2 phenotype in presence of Aβ25-35

To confirm the data obtained by RT-PCR, we evaluated the protein expression of iNOS and ARG-1 by immunofluorescence. LPS and IL-4 were considered as positive controls for iNOS and ARG-1 markers, respectively. BV-2 cells stimulated with Aβ25-35 for 24 h showed a significant increase in iNOS expression, as reported in the graph where the ratio of fluorescence intensity to the number of cells per field was evaluated. In contrast, Aβ25-35 induced a significant reduction in ARG-1 expression compared with CTRL (Fig. 13). Treatment with URB597 did not show significant changes in the expression of both iNOS and ARG-1, while BV-2 cells pretreated with 5 μM of URB597 for 4 hours and incubated with 30 μM of Aβ25-35 for 24h, showed a decrease in the expression of iNOS and an increase in ARG-1 compared with Aβ25-35-treated cells. URB597 confirmed its anti-inflammatory effect in microglial cells (Grieco et al., 2021)**.**

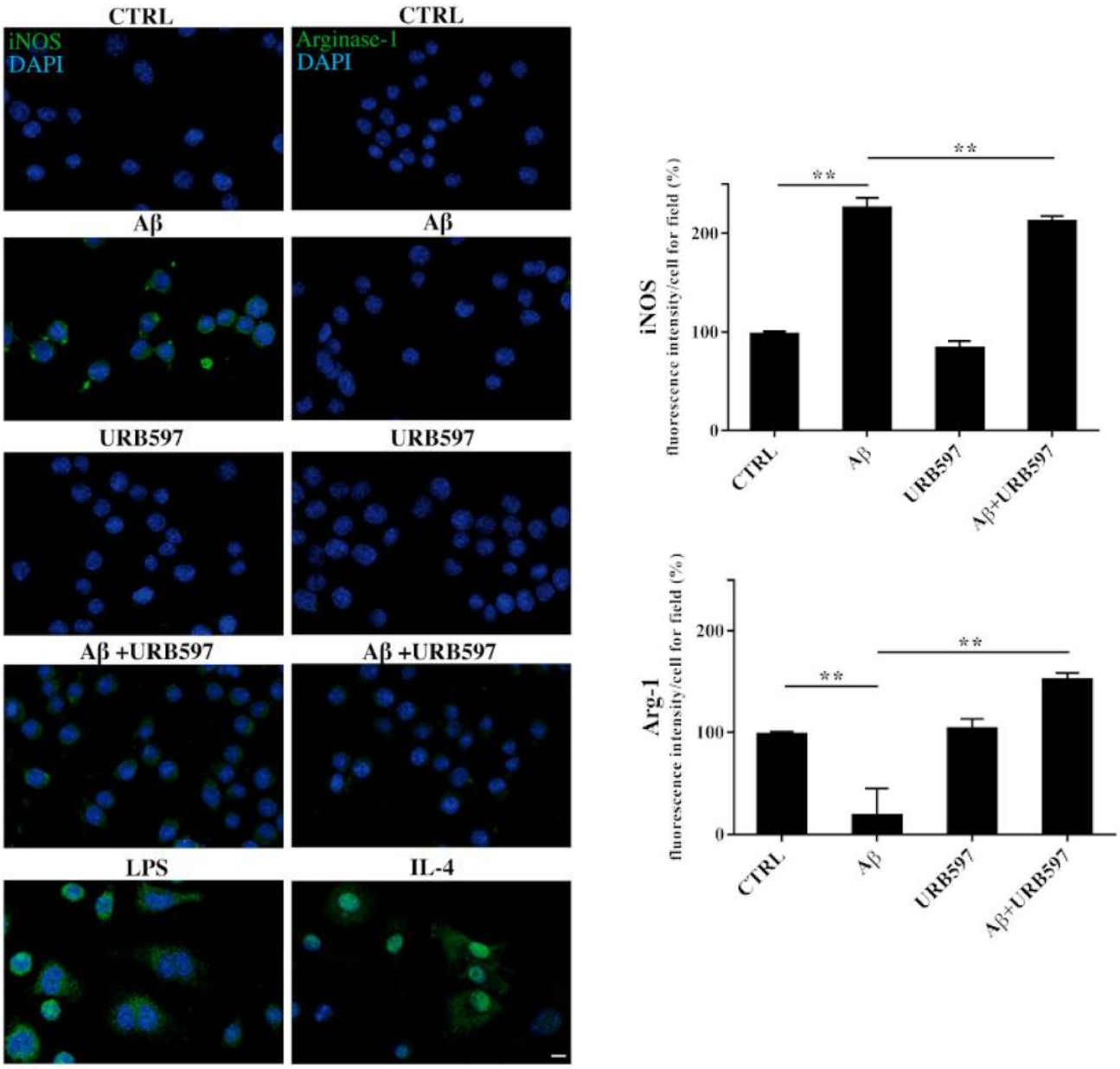


Fig. 13 - (Grieco et al., 2021) Immunofluorescence analysis of iNOS and ARG-1 polarization markers. BV-2 cells were treated with Aβ 30 μM in the presence or absence of 5μM URB597 for 24 hours. LPS and IL-4 were used as positive controls. Quantization of fluorescence signal intensity by ImageJ software. The results are the SD average of three independent experiments using one-way analysis of variance (ANOVA) with Tukey's post hoc test comparison. ** $P < 0.01$ vs Aβ. Bar 20 μm.

URB597 reduces the expression of IL-1β and Il-6 mRNAs and increases the expression of TGF-β and IL-10 mRNAs

The pro-inflammatory microglial phenotype M1, induced by bacterial components (e.g., LPS) or CNS damage produce the release not only of enzymes involved in oxidative stress (e.g., iNOS), but also of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-alpha, thus increasing the cytotoxic status. By contrast, the anti-inflammatory microglial phenotype M2 plays a role in inflammation resolution via the release of anti-inflammatory cytokines, such as IL-4, IL- 10, and TGFβ as well as through the expression of antioxidant factors, such as Nrf2 (Scipioni et al., 2022).

Here, we demonstrated the ability of URB597 to reduce the release of pro-inflammatory cytokines such as IL-1β, TNF-α and to increase the release of anti-inflammatory cytokines such as TGF-β and IL-10 in BV-2 cells, either in lack or in the presence of Aβ.

Moreover, it was evaluated the expression of IL-1β and IL-6 mRNA levels, as well as levels of TGF-β and IL-10, by qPCR at 3h and 24h. The results reported in Fig. 14 demonstrate that IL-1β and IL-6 mRNAs increase 3h after Aβ-treatment compared to the control. Treatment with URB597 alone did not induce cytokine mRNAs expression. On the other hand, a reduction of pro-inflammatory cytokines was observed following treatment with URB597 + Aβ at 3h as compared to the stimulation with Aβ only. No significant changes in the mRNAs expression of both IL-1beta and IL-6 were evident at 24h. On the other hand, the expression of IL-10 at 3h increased significantly following treatment with Aβ, although also the addition of URB597 increased the mRNA expression of IL-10. TGF-β mRNA expression did not show significant changes at 3h after treatment. At 24h, the expression of IL-10 and TGF-β increased significantly in the presence of URB597 compared to control as well as in the combined URB597 and Aβ co-treatment with treatment with as compared to Aβ alone. URB597 decreased the expression of anti-inflammatory cytokines in the

short term, considering their early kinetics, and, simultaneously, induced the secretion of anti-inflammatory cytokines 24 hours after treatment.

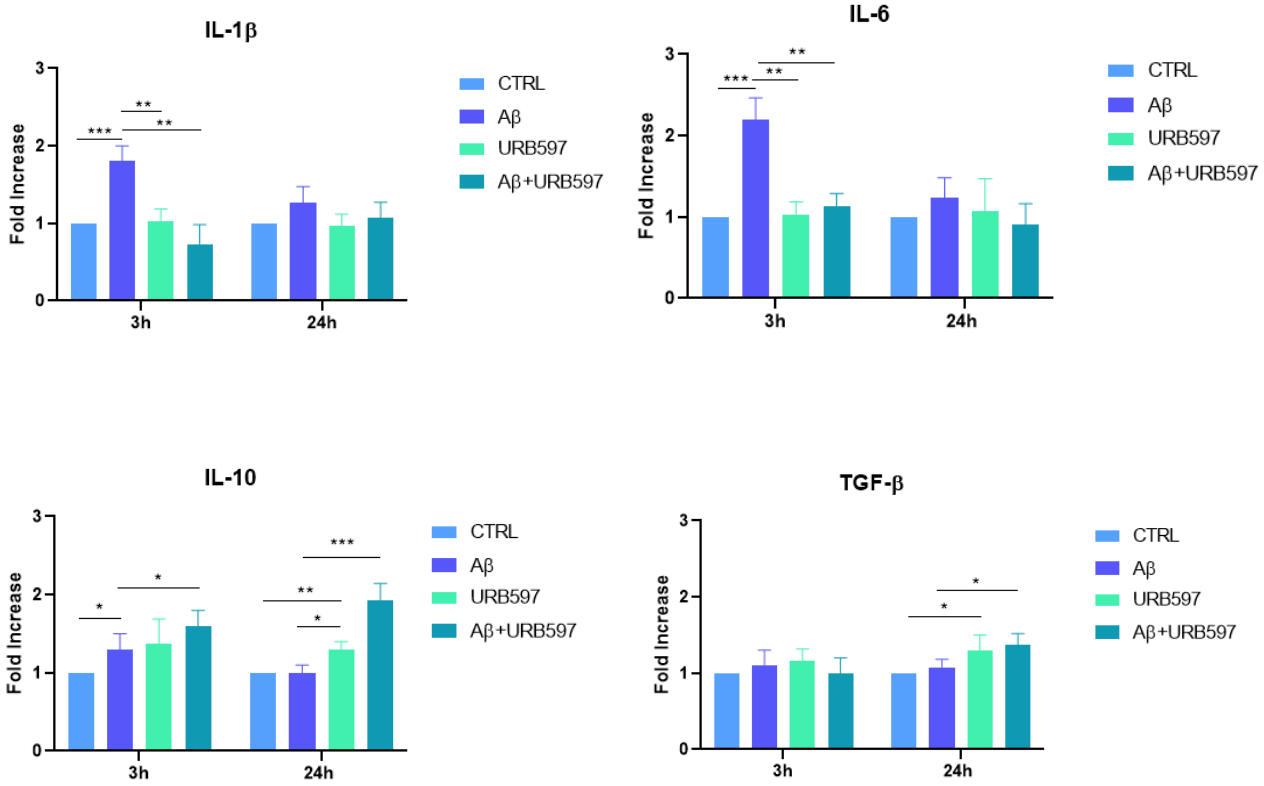


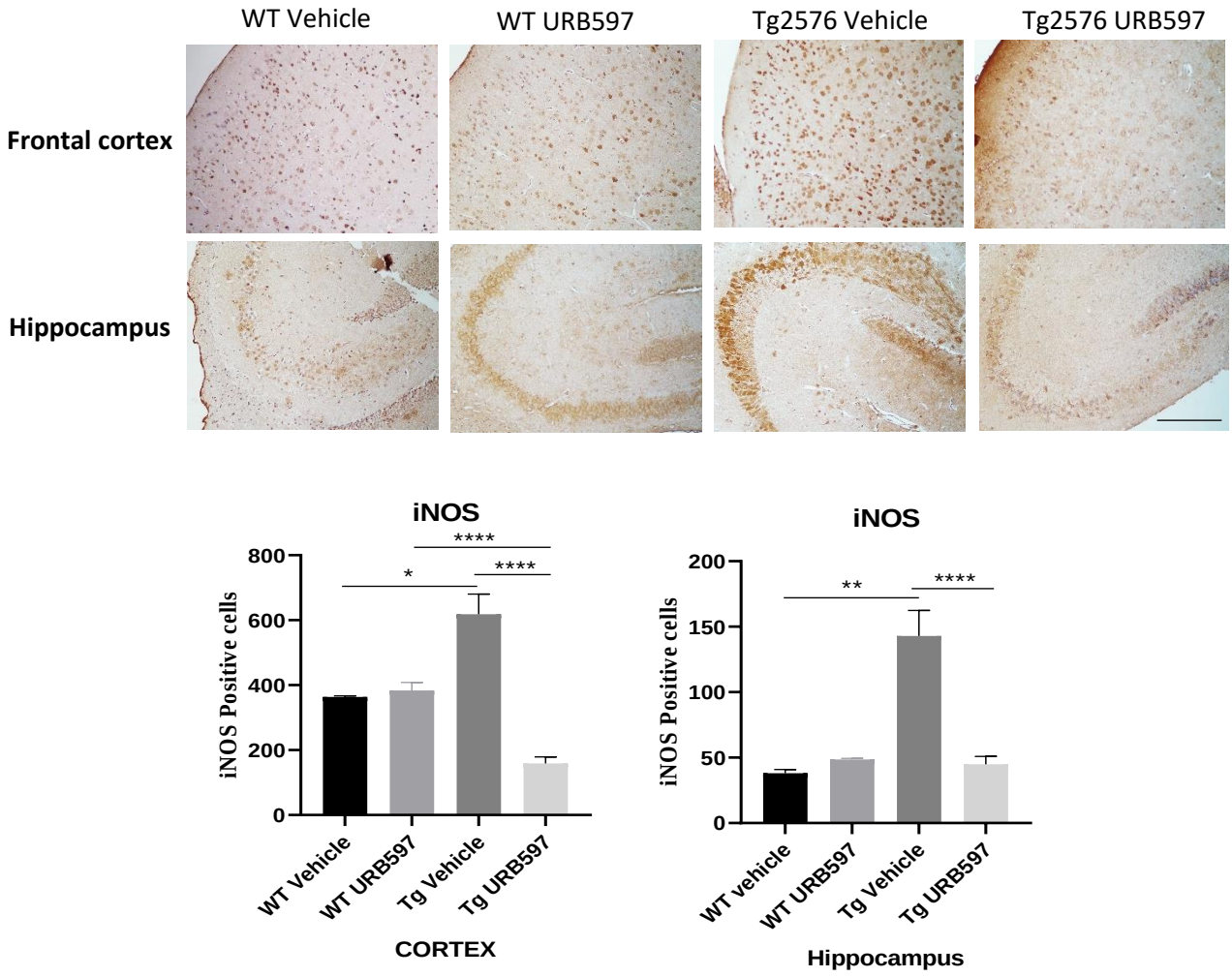
Fig. 14 - The mRNA expression of different inflammatory cytokines such as IL-1 β, IL-6, TGF-β, and IL-10, monitored by qPCR and normalized to 18S ribosome subunit. Data are shown as mean ± SD from three independent experiments performed in triplicate. Expression profiles were determined using the 2−ΔΔCT method. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Immunohistochemical detection of M1/M2 markers in prefrontal cortex and hippocampus from URB597-treated Tg2576 mice

Tg2576 mice treated with URB597 for 8 consecutive days were compared with vehicle-only treated Tg2576 mice and WT groups. We evaluated by immunohistochemistry the expression of microglia phenotypic markers iNOS and ARG-1. The quantification was performed on the basis of cell counts using Nikon NIS element Software highlights a significant increase in iNOS positive cells and a statistically significant reduction of ARG-1 positive cells, both within pre-frontal cortex and hippocampus, in Tg2576 mice treated with vehicle only, confirming the

pronounced inflammatory milieu typical of AD. Tg2576 mice treated with URB597 were found to have significantly decreased iNOS positive cells in the cortex and hippocampus as compared to vehicle-treated Tg2576 mice, and their number was comparable to that of WT. At prefrontal cortex level, a significant reduction was detected compared to the WT treated with URB597 (Fig.15 A). In addition, we found an increased expression of ARG-1 within both prefrontal cortex and hippocampus in URB597-treated Tg2576 mice compared to both vehicle-treated Tg2576 mice and WT (Fig.15 B). These data overlap with previous *in vitro* analyses confirming that FAAH inhibition by treatment with URB597 significantly reduces neuroinflammation by stimulating an anti-inflammatory activity.

A **iNOS**



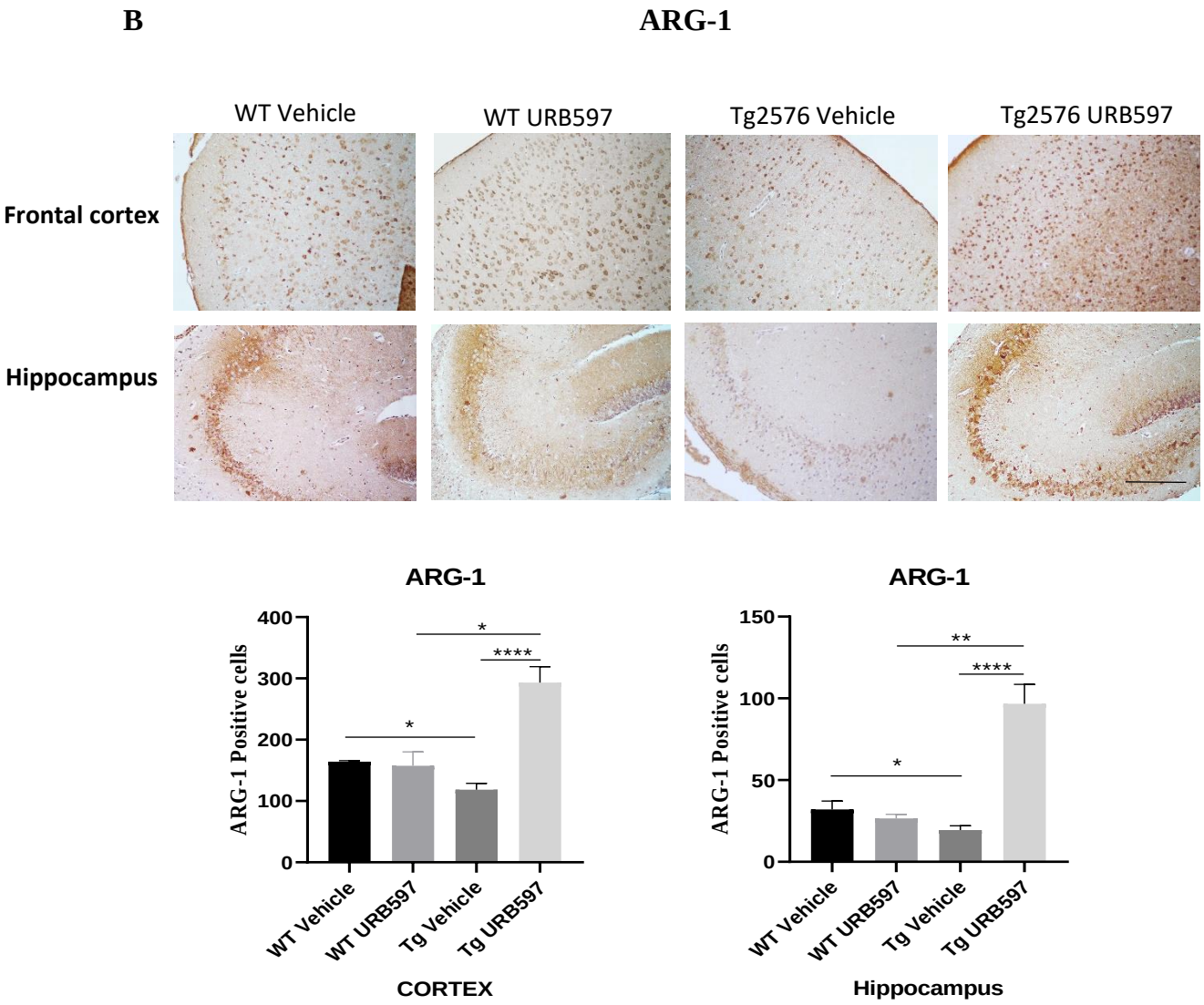
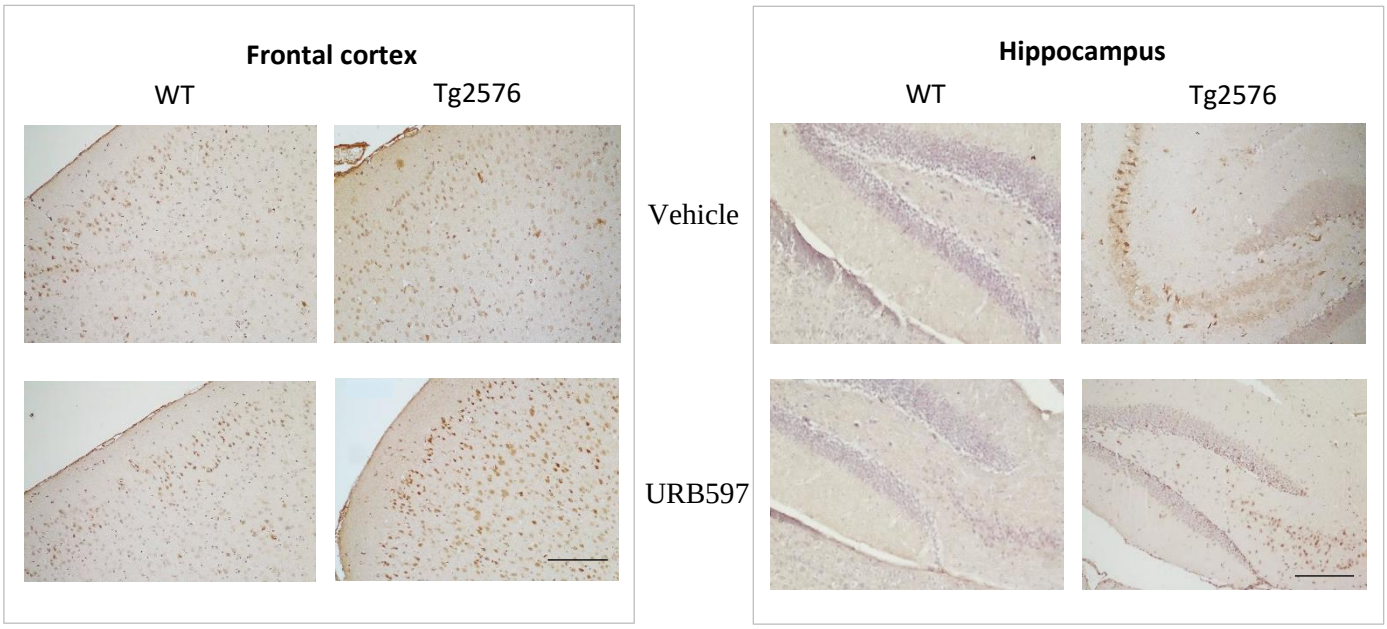


Fig. 15 - immunohistochemical analysis in sagittal sections of cortex and hippocampus (10x) in vehicle-treated WT and Tg2576 experimental groups and WT and Tg2576 treated with URB597 intraperitoneally for 8 days. The images show decreased iNOS marking (A) and increased ARG-1 (B) marking in tg2576 mice treated with URB597 confirmed by quantification of the expression of the two M1/M2 markers shown in the graphs. Analysis was performed by considering 4 10x magnification fields for both hippocampus and cortex, and values were expressed as mean ± SD of the total positive cells expressed in both brain regions from 6 independent experiments for each experimental group using Unpaired Student t test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

URB597 administration changed the expression of CB2 receptors

In AD patients the expression of CB2 receptors was found positively associated with the accrual of amyloid plaques, thus confirming the involvement of the ECBS and microglia in the inflammatory processes underlying AD. There is now much evidence that CB2 receptors expression is higher in advanced stages of AD (Scipioni et al., 2022). Here, we evaluated by immunohistochemical analysis the expression of CB2 receptors in the prefrontal cortex and hippocampus of URB597-treated Tg2576 and WT mice as well as in vehicle-treated Tg2576 and WT mice. The images and quantitative analysis shown in the graphs demonstrate a nonsignificant increase in CB2 receptor expression in the cortex of vehicle-treated Tg2576 mice, while URB597-treated Tg2576 mice showed a significant increase in CB2 expression as compared with vehicle-treated WT and Tg2576 mice (Fig. 16).

By contrast, in the hippocampal area we found a significant increase of CB2 receptor expression in vehicle-treated Tg2576 mice compared with WT mice and a significant decrease in URB597-treated Tg2576 mice.



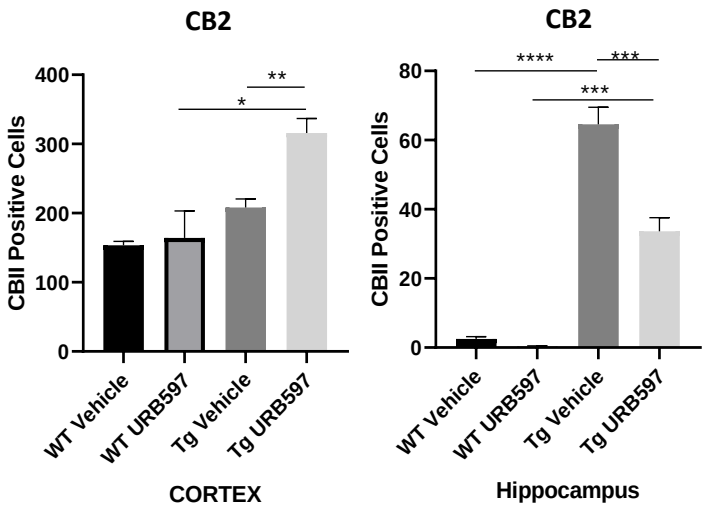


Fig.16 - immunohistochemical analysis (10x) in sagittal sections of vehicle-treated WT and Tg2576 experimental groups and WT and Tg2576 treated with URB597 intraperitoneally for 8 days. The images show CB2 positive cells in cortex and hippocampus. Analysis was performed by considering 4 10x magnification fields for both hippocampus and cortex, and values were expressed as mean ± SD of the total positive cells expressed in both brain regions from 6 independent experiments for each experimental group using Unpaired Student t test. * $p < 0.05$, ** $p < 0.01$, *** $p< 0.001$; **** $p < 0.0001$.

URB597 administration decreased the expression of BACE1 in cortex and hippocampus from Tg2576 mice

BACE1 (beta-site amyloid precursor protein cleavage enzyme 1) was first characterized in 1999 (Vassar et al., 1999). As mentioned earlier, BACE1 is the enzyme that activates the amyloid pathway by executing the proteolytic cleavage of APP that generates all forms of Aβ. Modulation of BACE1 expression, increased in AD patients, possibly may prevents he accumulation of amyloid plaques (Hampel et al., 2021).

Here, we evaluated the impact of URB597 treatment on BACE1 expression (Fig 17). The results showed a statistically significant increase in BACE1 expression, both in cortex and hippocampus, in vehicle-treated Tg2576 mice as compared with vehicle-treated WT. Tg2576 mice treated with URB597, instead, showed a significant decrease in BACE1 levels as compared with vehicle-treated Tg2576 mice.

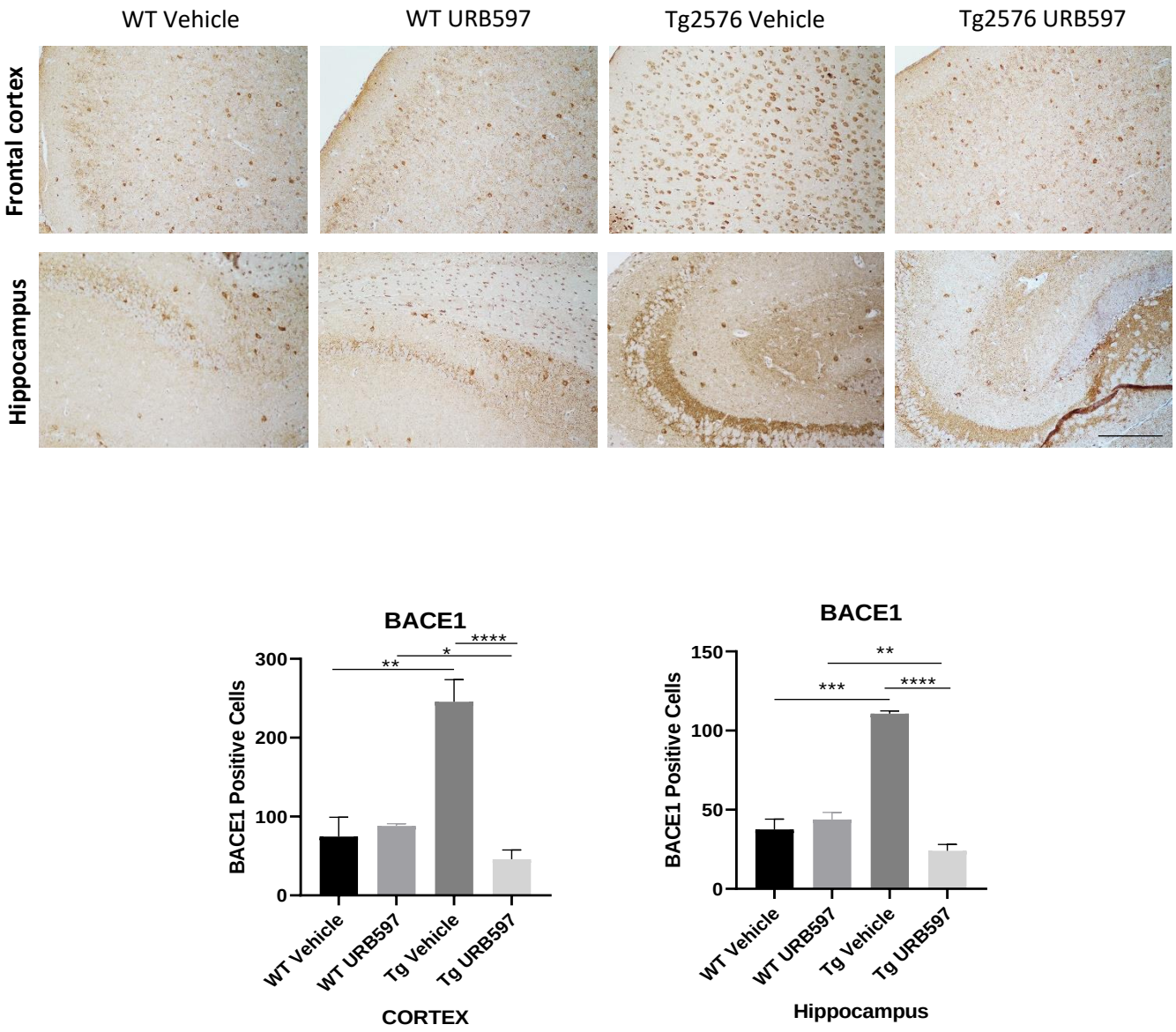
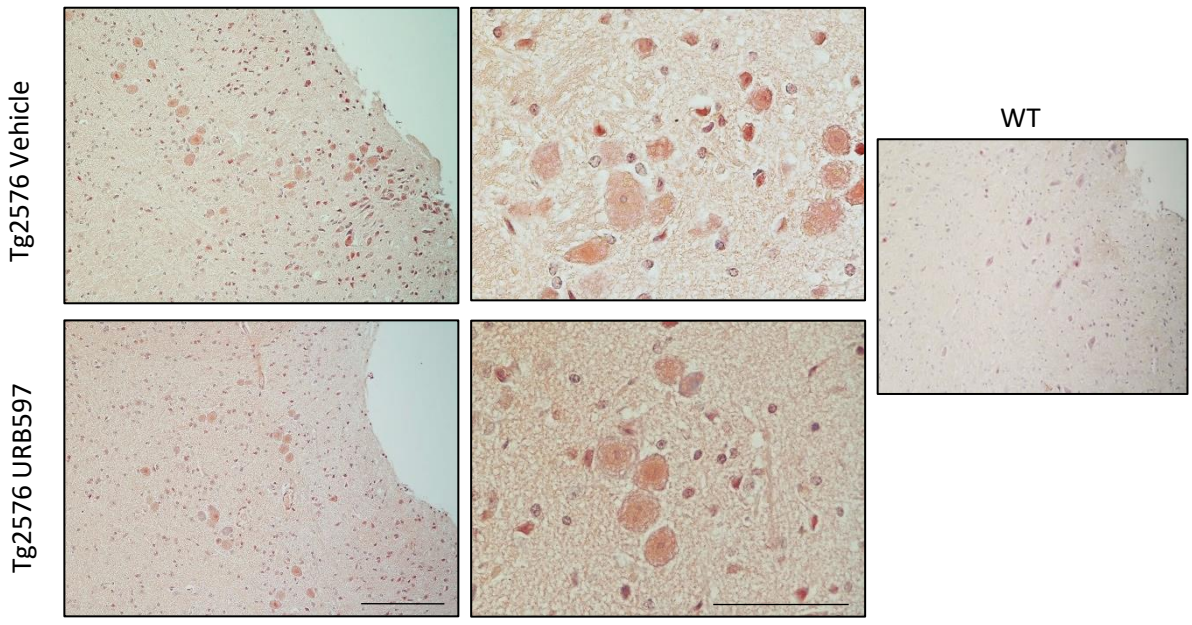


Fig. 17 -immunohistochemical analysis (10x) in sagittal sections of vehicle-treated WT and Tg2576 experimental groups and WT and Tg2576 treated with URB597 intraperitoneally for 8 days. The images show BACE1 positive cells in cortex and hippocampus. Analysis was performed by considering 4 10x magnification fields for both hippocampus and cortex, and values were expressed as mean ± SD of the total positive cells expressed in both brain regions from 6 independent experiments for each experimental group using Unpaired Student t test. * $p < 0.05$, ** $p < 0.01$, *** $p< 0.001$; **** $p < 0.0001$.

URB597 administration reduced β-amyloid plaques

To evaluate the beneficial effect of URB597 on the accumulation of amyloid plaques, we analyzed the number and area of plaques by using the congo red procedure. As reported, Tg2576 mice between 6 and 14 months of age show cognitive deficits and first signs of brain atrophy; after 14 months of age, they begin to develop extensive extracellular accumulations of Aβ (Lesné et al., 2006). We treated Tg2576 mice at 14 months of age and we showed that early plaque accumulation occurs more in the midbrain region. In fact, one of the early features of AD is degeneration of cholinergic fibers that start from the basal forebrain and innervate the hippocampal and neocortical areas (Orta-Salazar et al., 2014). Figure 18 shows that in Tg2576 mice treated with URB597 there are less amyloid plaques (number and area of plaques), and the significant reduction as compared to plaques present in Tg2576 mice treated with vehicle only.



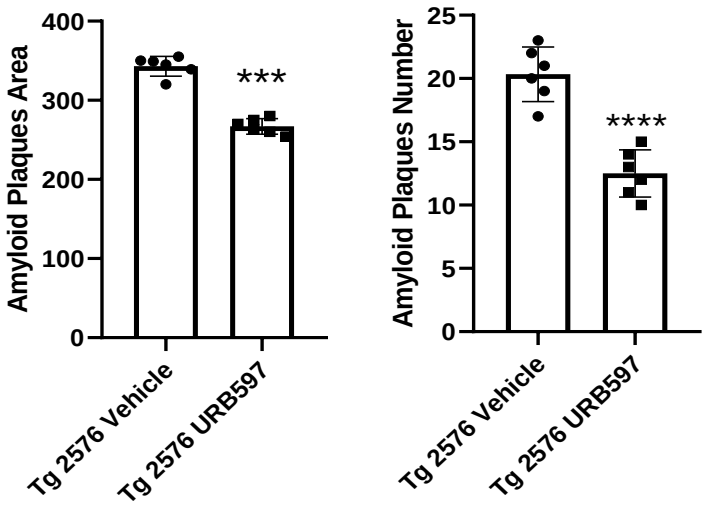


Fig 18 - Congo Red analysis (10x and 40x) in sagittal sections of vehicle-treated Tg2576 and Tg2576 treated with URB597 intraperitoneally for 8 days. The images show amyloid plaques. Analysis was performed by considering 4 10x (number) and 40x (area) magnification fields, the area was expressed in μm^2 and values were expressed as mean ± SD of the total positive cells expressed in both brain regions from 6 independent experiments for each experimental group using Unpaired Student t test *** $p < 0.001$; **** $p < 0.0001$.

URB597 increases the expression of PSD95

In AD the Aβ oligomers interfere with synaptic transmission by disrupting NMDA receptors with Ca^{2+} and glutamate influx resulting in mitochondria malfunction and synaptic dysfunction. Post-synaptic density protein 95 (PSD95) is a key factor in synaptic plasticity, and there is much evidence showing a decrease of PSD95 during aging and, to a more extent, in neurological disorders such as AD (Bustos et al., 2017; Tu et al., 2014).

Figure 19 shows that treatment with URB597 significantly increased the expression of PSD95 in both cortex and hippocampus from Tg2576 mice as compared with Tg2576 mice treated with vehicle only.

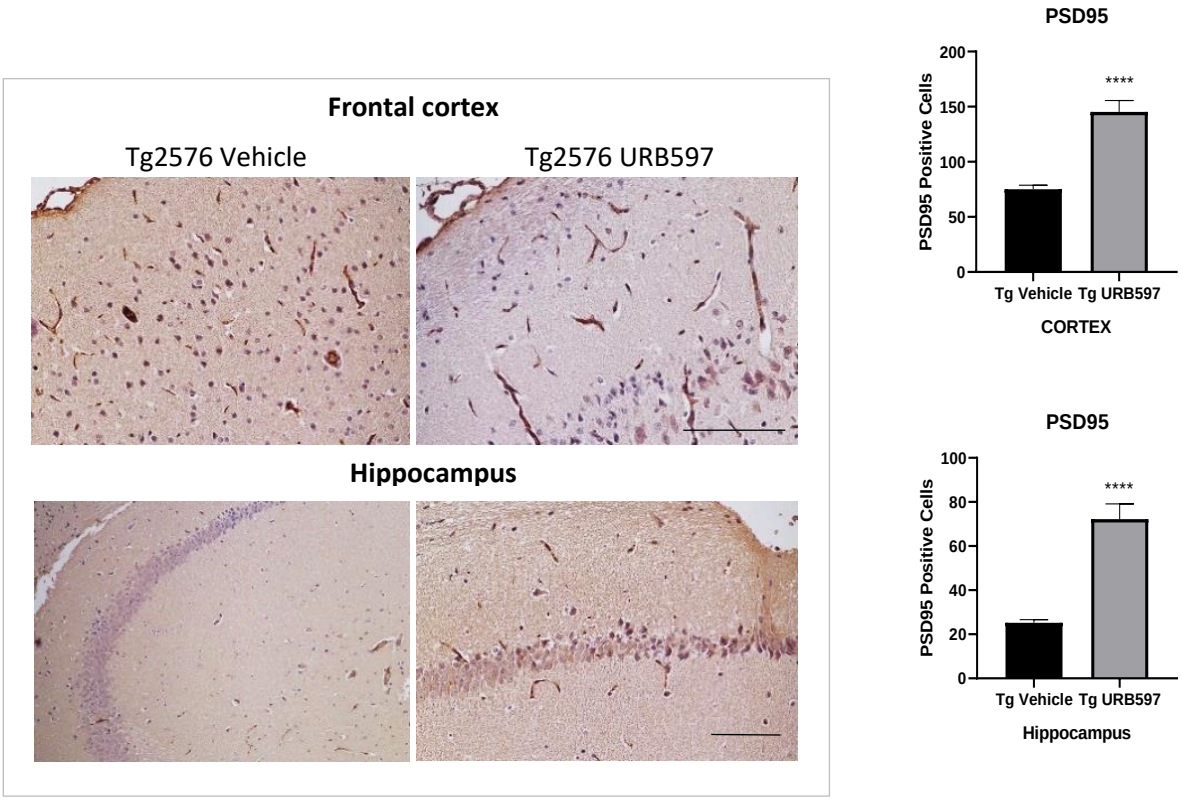


Fig. 19 - immunohistochemical analysis (20x and 10x) in sagittal sections of vehicle-treated Tg2576 and Tg2576 treated with URB597 intraperitoneally for 8 days. The images show PSD95 positive cells in cortex and hippocampus. Analysis was performed by considering 4 10x magnification fields for both hippocampus and cortex, and values were expressed as mean ± SD of the total positive cells expressed in both brain regions from 6 independent experiments for each experimental group using Unpaired Student t test. **** $p < 0.0001$.

URB597 administration reduced astrogliosis in the prefrontal cortex from Tg2576 mice

As mentioned above, astrogliosis is well detected in AD (Fakhoury, 2018). We then investigated the presence of astrocytes in cortex and hippocampus from URB597-treated Tg2576 mice, as detected by the staining of the astrocyte marker GFAP. Figure 20 shows a significant astrocyte reduction in cortical areas compared with the corresponding areas of Tg2576 mice treated with vehicle only. In the hippocampal areas, the results showed no significant differences in GFAP expression; the values are comparable.

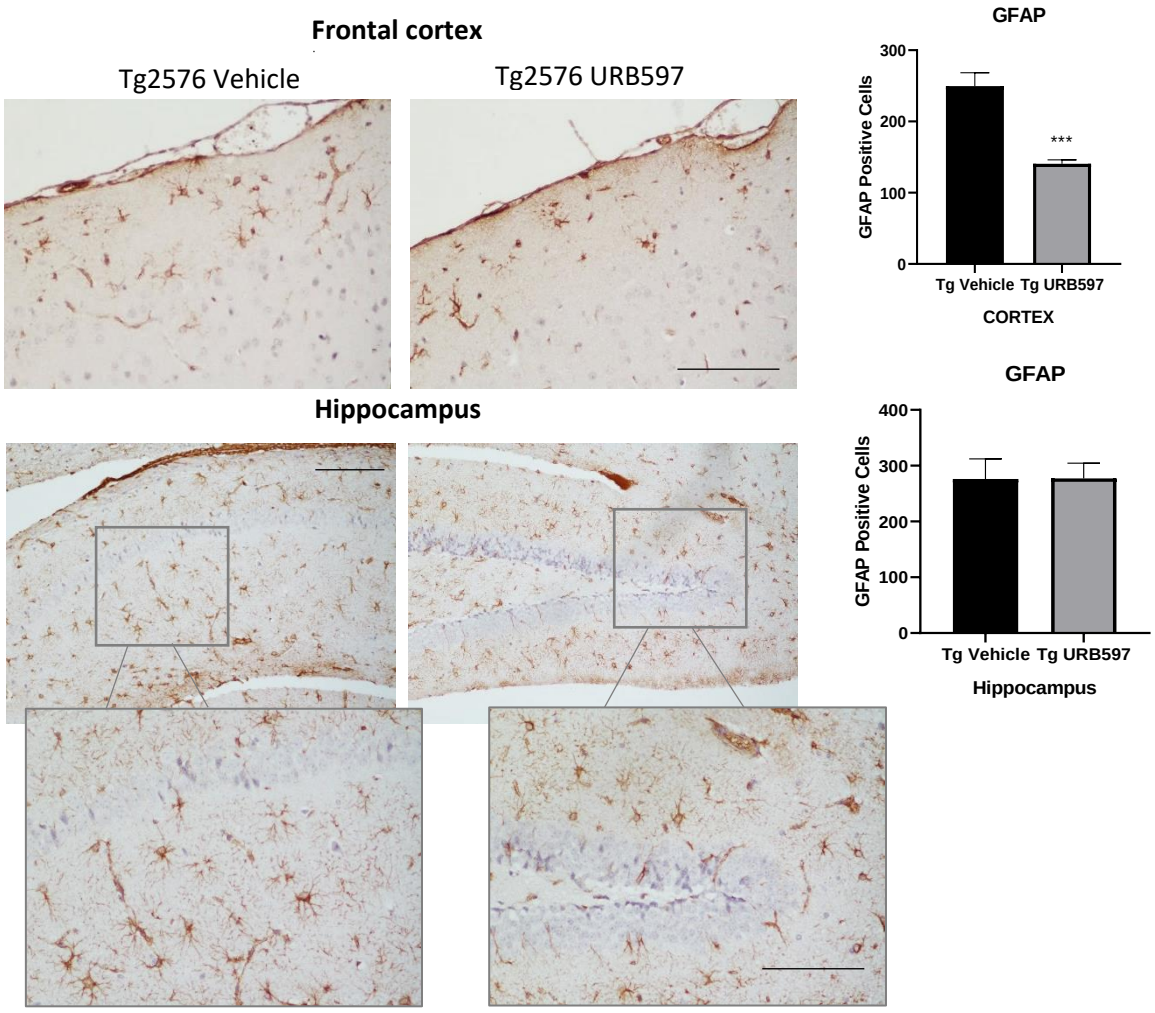


Fig. 20 - immunohistochemical analysis (20x and 10x) in sagittal sections of vehicle-treated Tg2576 and Tg2576 treated with URB597 intraperitoneally for 8 days. The images show GFAP positive cells in cortex and hippocampus. Analysis was performed by considering 4 10x magnification fields for both hippocampus and cortex, and values were expressed as mean ± SD of the total positive cells expressed in both brain regions from 6 independent experiments for each experimental group using Unpaired Student t test. *** $p < 0.001$.

URB597 administration restored autophagic pathways

Here, we analyzed by RT-PCR the mRNA expression of major factors involved in autophagic flux in BV2 cells treated with URB597 either in the presence or in lack of Aβ.

The panels in Fig. 21 show the mRNA expression of ATG7, Beclin1, p62 and LC3 at 6h and 24h after treatment. ATG7 and Beclin1 induced autophagy, and LC3 is the main autophagosomal marker, and p62 transports cargo to be eliminated at the

autophagosome. All 4 factors showed a significant reduction in mRNA expression in the presence of Aβ compared with BV2 CTRL cells, showing a negative influence exerted by Aβ on autophagy. Treatment with URB597 alone significantly increased the mRNA expression of ATG7, Beclin1, LC3 and p62, and combined Aβ+URB597 treatment significantly increased mRNAs expression compared with CTRL cells. These results are superimposable at both 6h and 24h, thus demonstrating that URB597 increased mRNA expression of factors involved in autophagy flux and therefore its potential involvement in recovery of impaired autophagy in AD.

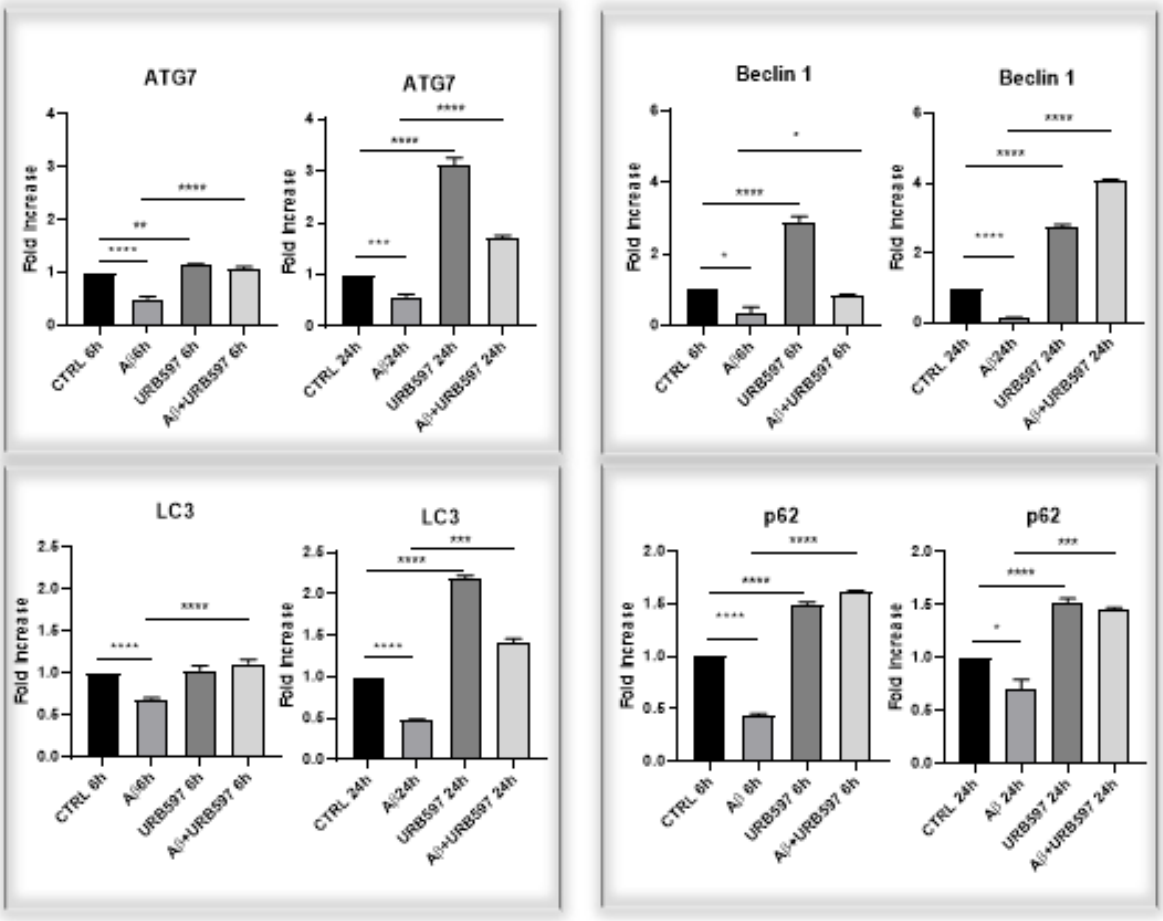


Fig. 21 - mRNA expressions were evaluated by qRT-PCR at 6 and 24h of ATG7, Beclin1, LC3 and p62. Data are shown as mean ± SD from three independent experiments performed in triplicate. Expression profiles were determined using the 2−ΔΔCT method. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$

FAAH inhibition increased Nrf2 mRNA expression

The canonical activation of Nrf2, following increase of ROS, regulates p62 gene expression, influencing autophagy. This circuit is deregulated in neurodegenerative diseases by exacerbating oxidative stress and inflammation (W. Zhang et al., 2021)**.** We evaluated by RT-PCR Nrf2 mRNA expression in URB597-treated or untreated BV2 cells either in lack or in the presence of Aβ. Following 3h Aβ treatment, Nrf2 mRNA did not show significant variation, whereas following URB597 addition, we detected a slight significant increase in Nrf2, and a strong significant increase with the combined treatment (i.e., URB597 + Aβ). At 6h after treatment, we found (Fig. 22) a significant reduction in Nrf2 mRNA expression in the presence of Aβ compared with CTRL cells, while following URB597 treatment Nrf2 mRNA expression increased significantly in the presence of Aβ. Under stress conditions URB597 promoted the increased of Nrf2 mRNA expression (Fig.22).

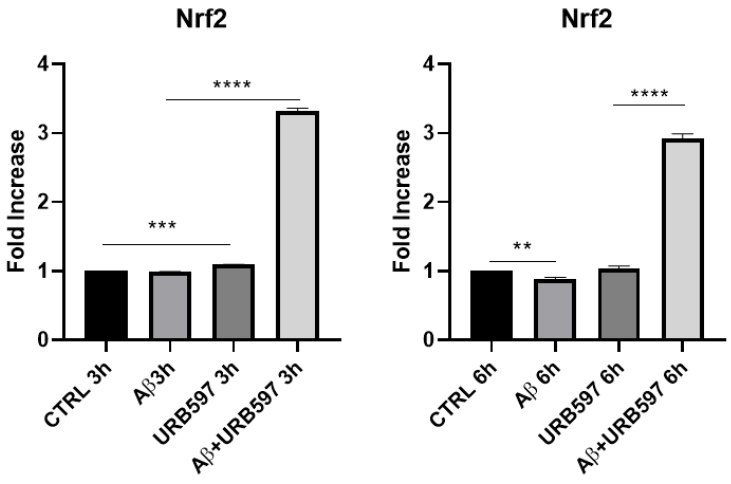


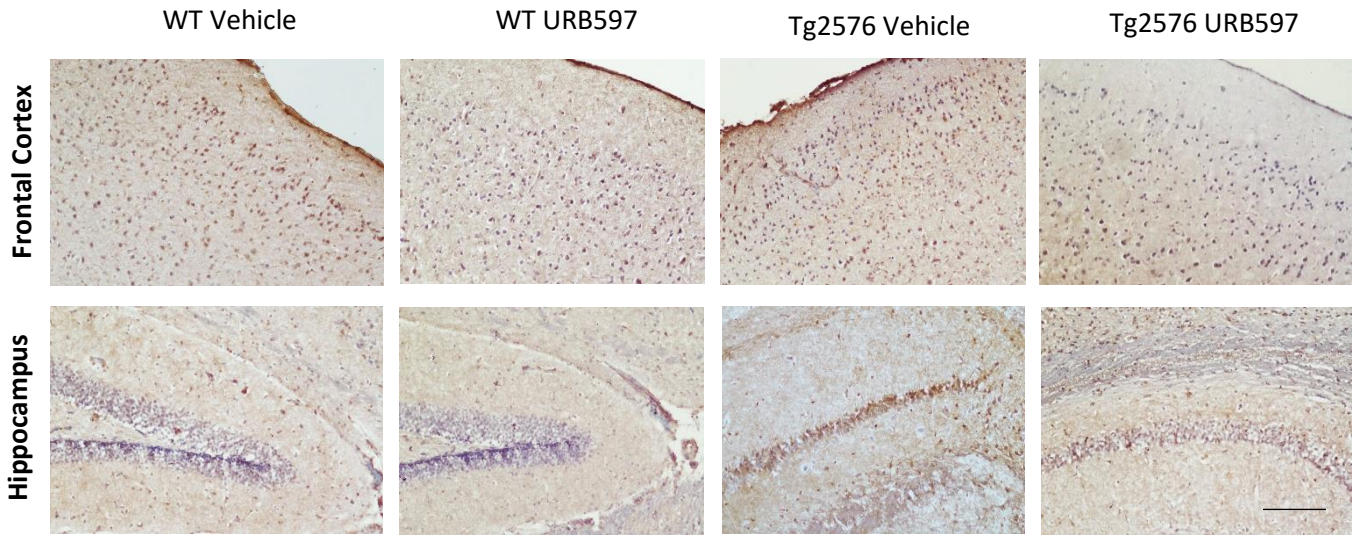
Fig. 22 - mRNA expression of Nrf2 was evaluated by qRT-PCR at 3 and 6h. Data are shown as mean ± SD from three independent experiments performed in triplicate. Expression profiles were determined using the 2−ΔΔCT method. ** $p < 0.01$, **** $p < 0.0001$.

URB597 administration decreased mTOR expression in Tg2576 mice

The kinase mTOR inhibits autophagy and its signaling is deregulated in AD (Y. C. Kim & Guan, 2015; Switon et al., 2017).

Here, we evaluated mTOR expression in sagittal sections from Tg2576 and WT mice treated with URB597, as compared with mice groups treated with vehicle alone. The analysis showed a significant increase in mTOR expression in both cortex and hippocampus from Tg2576 mice treated with vehicle alone, as compared with WT mice. In contrast, in Tg2576 mice treated with URB597 there was a significant reduction of mTOR expression compared with vehicle-treated Tg2576 mice (values comparable to WT). Moreover, URB597 reduced the expression of the major inhibitor of autophagy: mTOR (Fig. 23).

mTOR



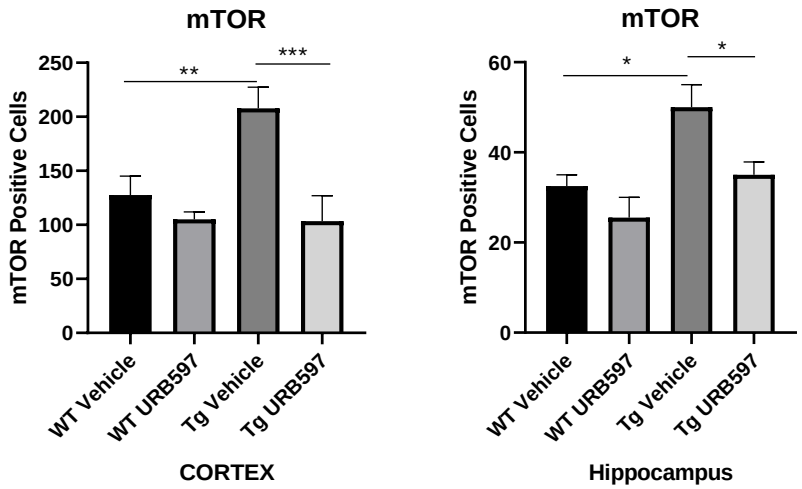
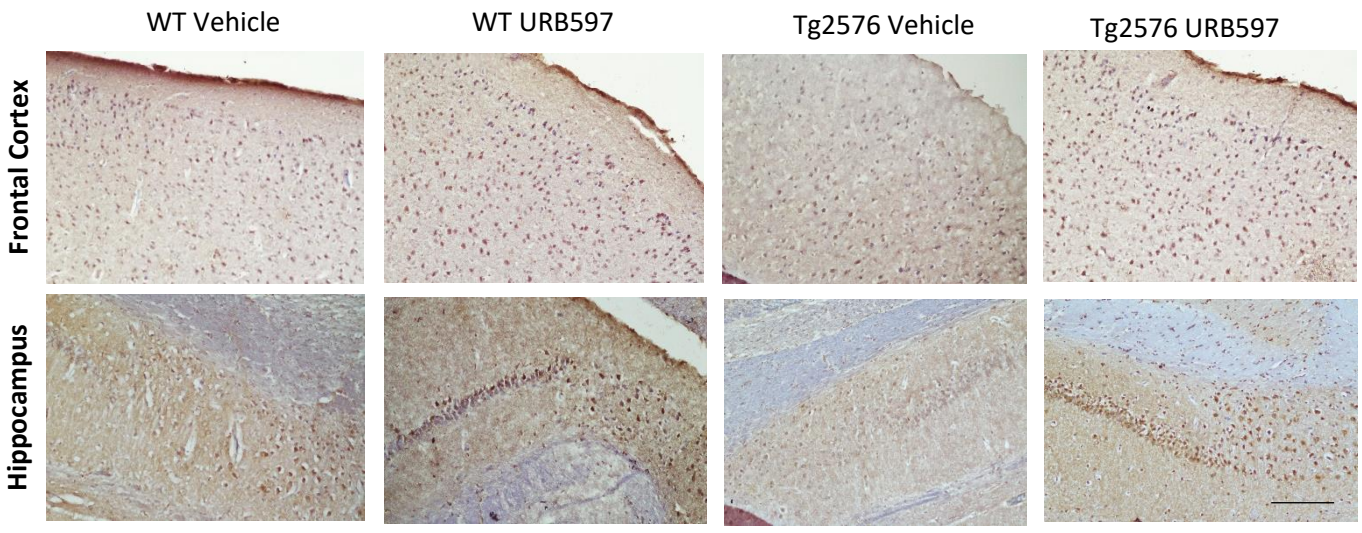


Fig. 23 - immunohistochemical analysis (10x) in sagittal sections of vehicle-treated WT and Tg2576 experimental groups and WT and Tg2576 treated with URB597 intraperitoneally for 8 days. The images show mTOR positive cells in cortex and hippocampus. Analysis was performed by considering 4 10x magnification fields for both hippocampus and cortex, and values were expressed as mean ± SD of the total positive cells expressed in both brain regions from 6 independent experiments for each experimental group using Unpaired Student t test. * $p < 0.05$, ** $p < 0.01$, *** $p< 0.001$.

URB597 administration increased ULK1 expression in Tg2576 mice

ULK1 kinase regulation is involved in the activation of the autophagic process (J. Kim et al., 2011). We evaluated the expression of ULK1 in the cortex and hippocampus from Tg2576 mice treated with URB597 compared with WT and Tg2576 groups treated with vehicle only. Our results show (Fig. 24) that the expression of ULK1 in Tg2576 mice was significantly reduced compared with WT mice in both regions considered. Expression levels of ULK1 were restored in Tg2576 mice treated with URB597 and were comparable to those observed in WT mice.



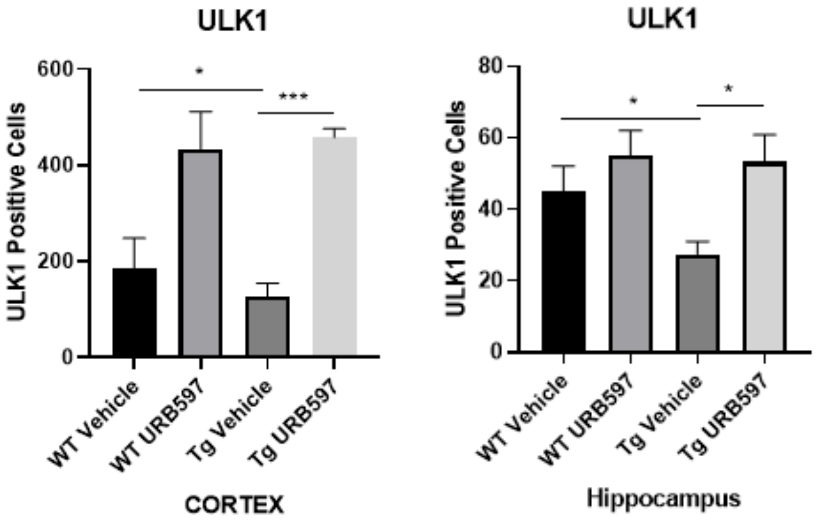
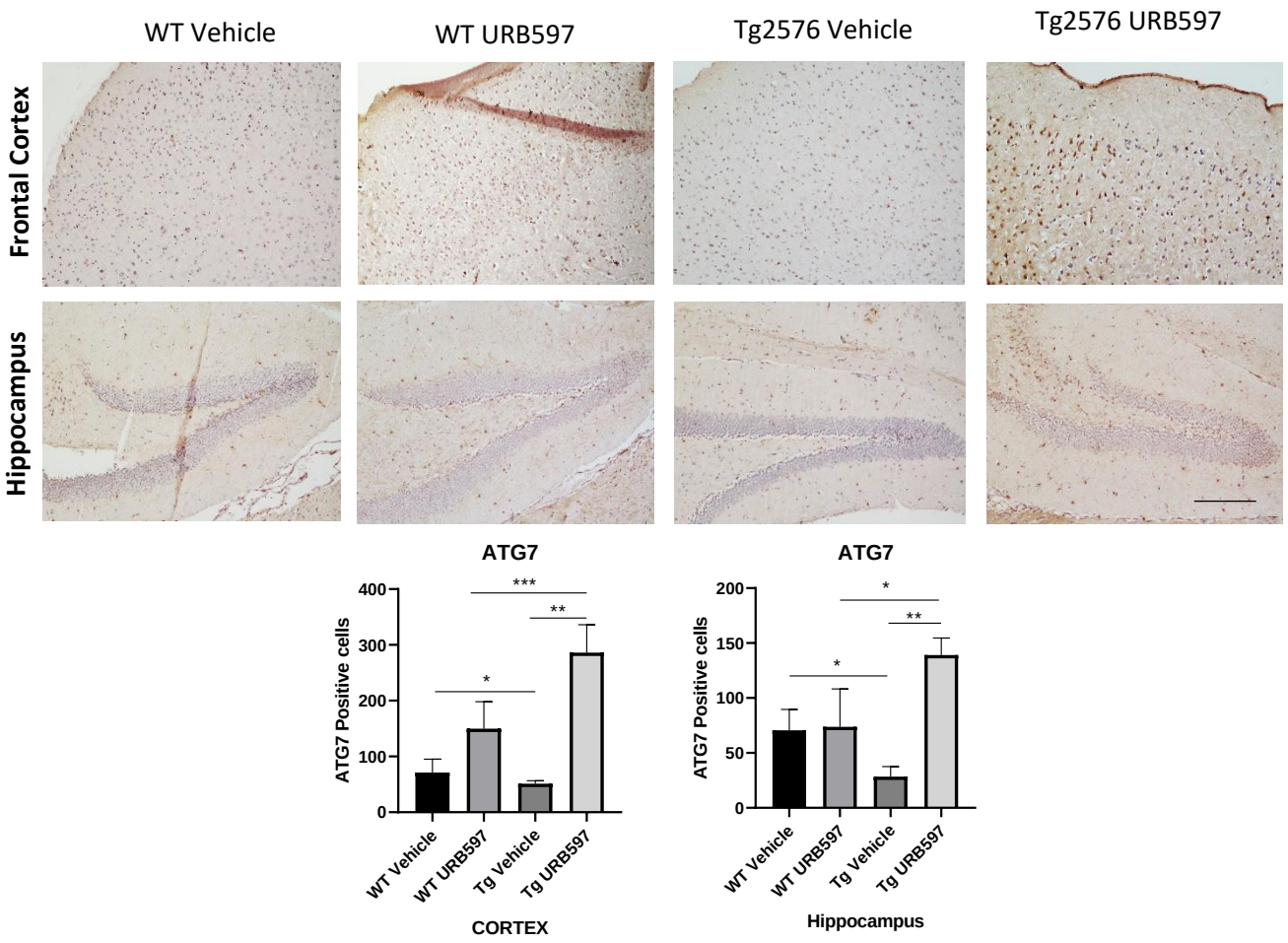


Fig. 24 - immunohistochemical analysis (10x) in sagittal sections of vehicle-treated WT and Tg2576 experimental groups and WT and Tg2576 treated with URB597 intraperitoneally for 8 days. The images show ULK1 positive cells in cortex and hippocampus. Analysis was performed by considering 4 10x magnification fields for both hippocampus and cortex, and values were expressed as mean ± SD of the total positive cells expressed in both brain regions from 6 independent experiments for each experimental group using Unpaired Student t test. * $p < 0.05$, **$p< 0.01$, *** $p< 0.001$.

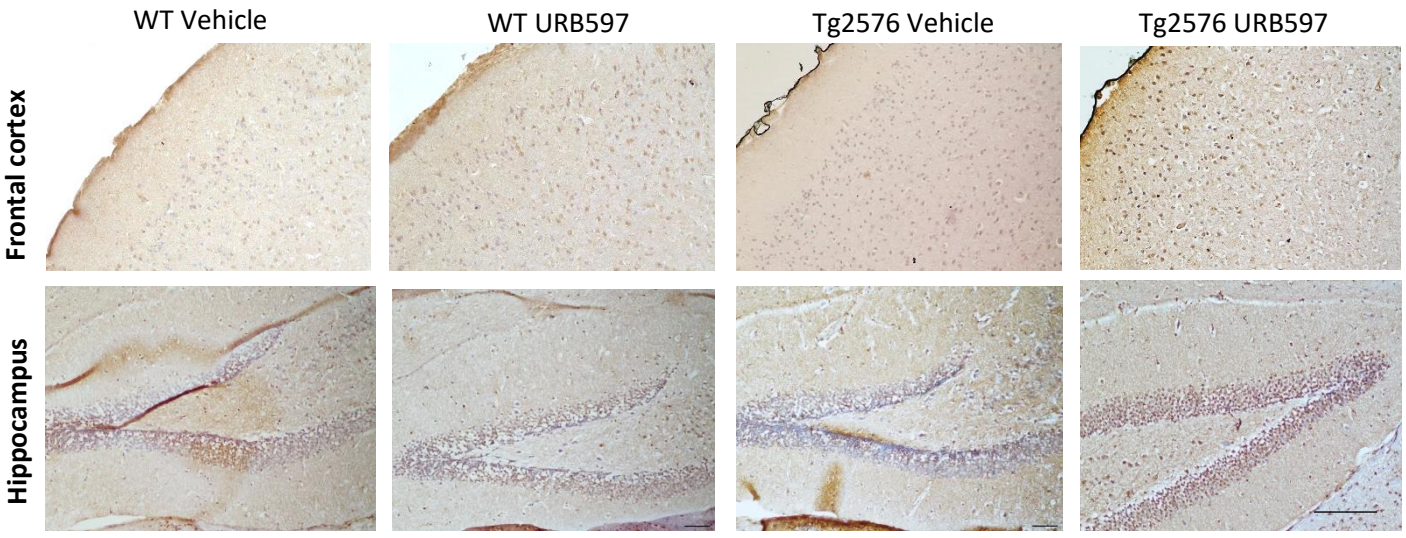
URB597 administration modulated ATG7 and Beclin1 expression in cortex and hippocampus from Tg2576 mice

We evaluated the protein expression of Beclin1 and ATG7 in Tg2576 mice treated with URB597 or with vehicle-only and we compared them to WT groups undergoing the same treatment. Both in hippocampus and cortex of vehicle-only treated Tg2576 mice was shown a significant reduction in ATG7 and Beclin1 expression compared to WT mice. Our data confirm the alteration of proteins involved in the induction of autophagy. After treatment with URB597, Tg2576 mice showed a significant increase in the expression of Beclin1 and ATG7 compared with both Tg2576 mice treated with vehicle only and WT mice (Fig.25).

A **ATG7**



B **BECLIN1**



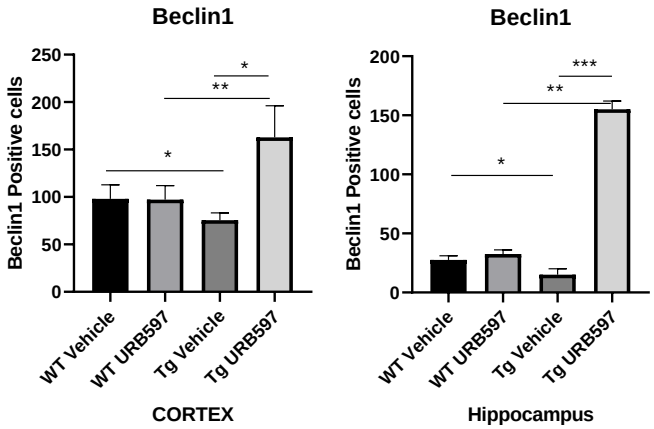
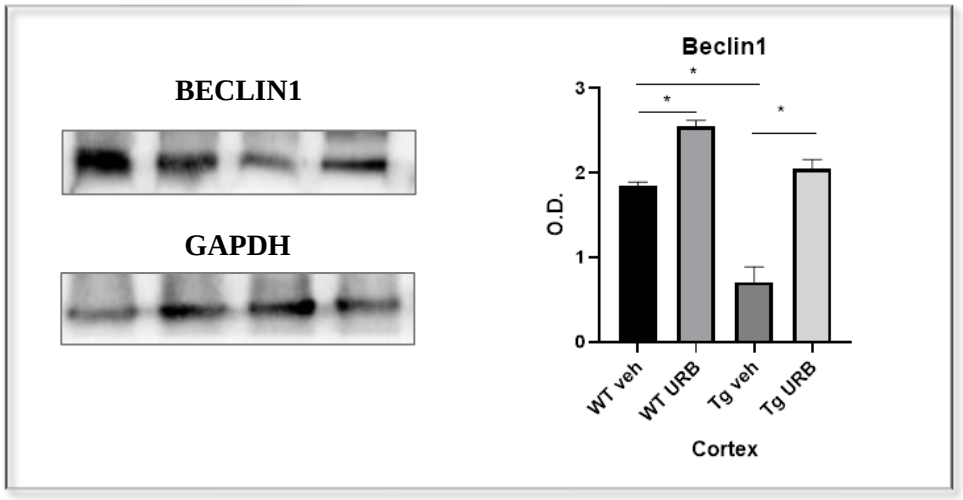
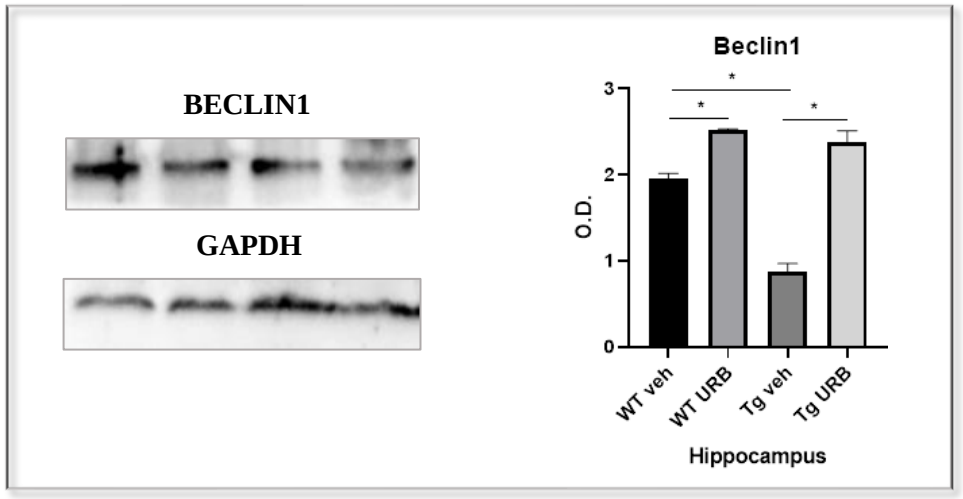


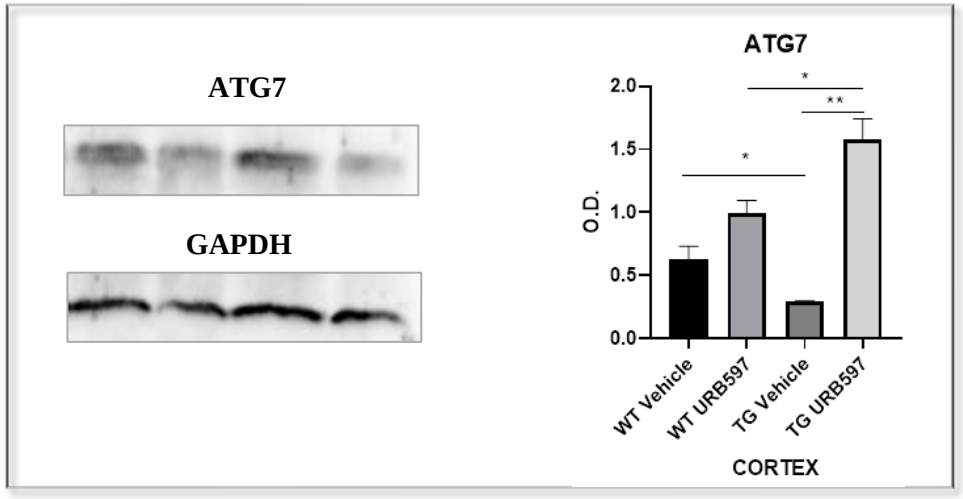
Fig. 25 - immunohistochemical analysis (10x) in sagittal sections of vehicle-treated WT and Tg2576 experimental groups and WT and Tg2576 treated with URB597 intraperitoneally for 8 days. The images show ATG7 (A) and Beclin1 (B) positive cells in cortex and hippocampus. Analysis was performed by considering 4 10x magnification fields for both hippocampus and cortex, and values were expressed as mean ± SD of the total positive cells expressed in both brain regions from 6 independent experiments for each experimental group using Unpaired Student t test. * $p < 0.05$, **$p< 0.01$, *** $p< 0.001$.

URB597 administration increased the expression of Beclin1 and ATG7 in cortex and hippocampus from Tg2576 mice

WB analyses were performed on cortex and hippocampus homogenates obtained from URB597 or vehicle only-treated Tg2576 mice or from WT. A significant decrease in both Beclin1 and ATG7was detected in the Tg2576 vehicle-treated mice, compared to WT and a significant increase in the URB597-treated Tg2576 mice, with protein levels comparable to those observed in WT.


BECLIN1
GAPDH
Beclin1
O.D.
WT veh
WT URB
Tg veh
Tg URB
Cortex


BECLIN1
GAPDH
Beclin1
O.D.
WT veh
WT URB
Tg veh
Tg URB
Hippocampus


ATG7
GAPDH
ATG7
O.D.
WT Vehicle
WT URB597
TG Vehicle
TG URB597
CORTEX

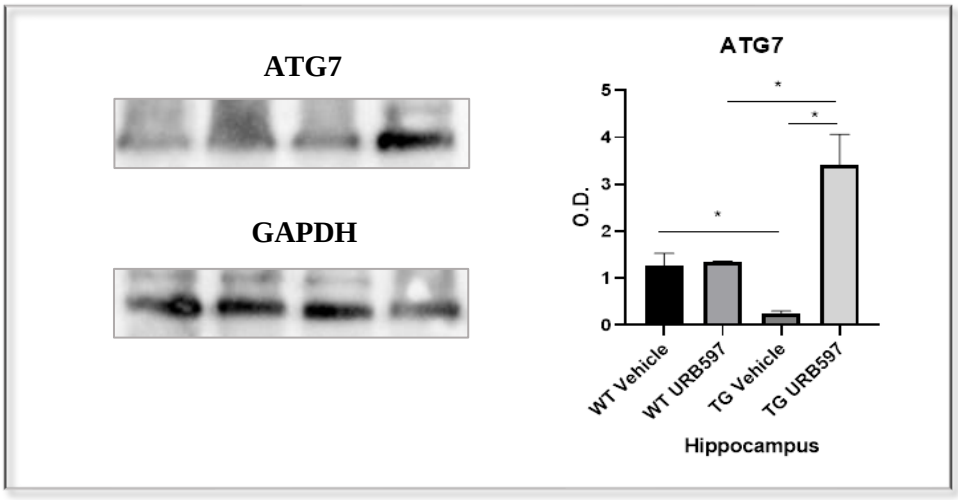
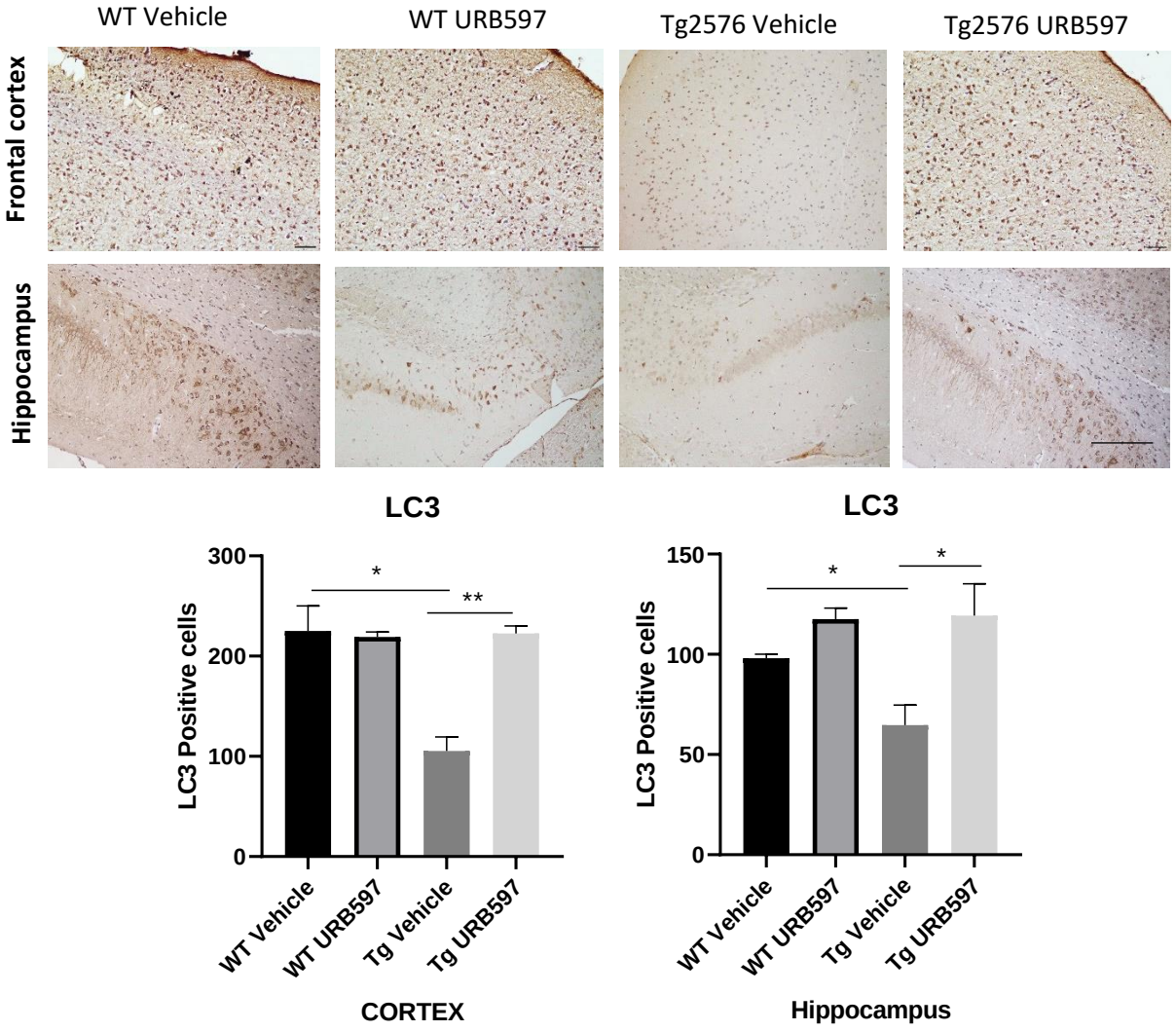


Fig. 26 – Western Blot analysis on cortex and hippocampal homogenates collected from URB597-treated Tg2576 mice and compared with vehicle Tg2576 mice and WT experimental groups. The images show Beclin1 and ATG7 O.D. Values were expressed as mean ± SD from 5 independent experiments for each experimental group using Unpaired Student t test. * $p < 0.05$, **$p< 0.01$.

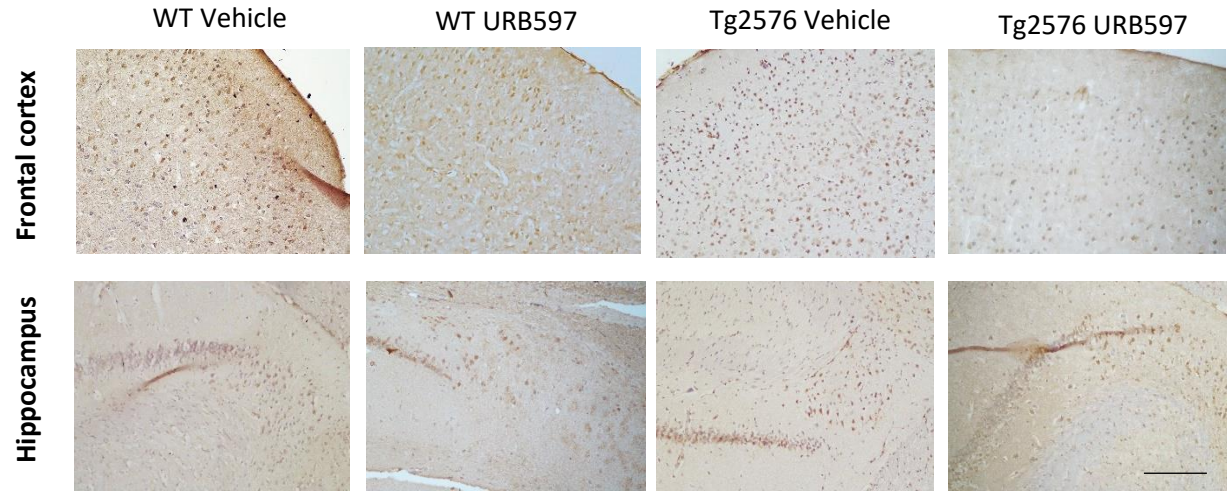
FAAH inhibition increased the expression of LC3 and decreased the expression of p62 in Tg2576 mice

To monitor autophagic flux, we also evaluated the expression of LC3I-II and p62. The expression of these proteins was assessed sagittal sections obtained from frontal cortex and hippocampus of Tg2576 mice treated with URB597 and compared with Tg2576 treated with vehicle and WT treated with the same conditions. The results (Fig. 27A) showed a reduced amount of LC3I-II in Tg2576 mice treated with vehicle only compared to WT, while after treatment with URB597 the amount of LC3I-II increased significantly, restoring values detected in WT in both brain regions. In parallel, we found a significant increase in the p62 expression in hippocampus of vehicle Tg2576 mice compared with WT (Fig.27 B), and a small increase in prefrontal cortex. After treatment with URB597, Tg2576 mice showed a significant reduction in p62 expression compared with Tg2576 mice treated with vehicle only. Further semi-quantitative analysis by WB is in progress to evaluate the ratio of LC3I to LC3II to make an estimate of the actual increase in the number of autophagosomes.

A **LC3 I-II**



B **p62**



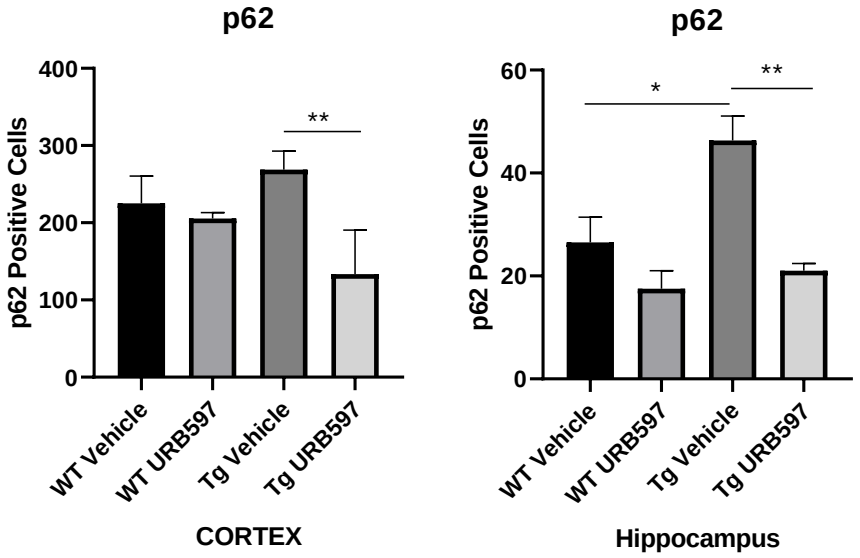


Fig. 27 - immunohistochemical analysis (10x) in sagittal sections of vehicle-treated WT and Tg2576 experimental groups and WT and Tg2576 treated with URB597 intraperitoneally for 8 days. The images show LC3I-II (A) and p62 (B) positive cells in cortex and hippocampus. Analysis was performed by considering 4 10x magnification fields for both hippocampus and cortex, and values were expressed as mean ± SD of the total positive cells expressed in both brain regions from 6 independent experiments for each experimental group using Unpaired Student t test. * $p < 0.05$, **$p< 0.01$.

Administration of URB597 increases the expression of LC3-II in Tg2576 mice

The LC3 protein is synthesized as a precursor (proLC3), which is cleaved to form LC3-I with an exposed C-terminal glycine and ,by conjugation the phosphatidylethanolamine (PE) is modified into a membrane-bound protein, LC3-II, which has been characterized as an autophagosome marker in mammalian cells (X. Li et al., 2020).

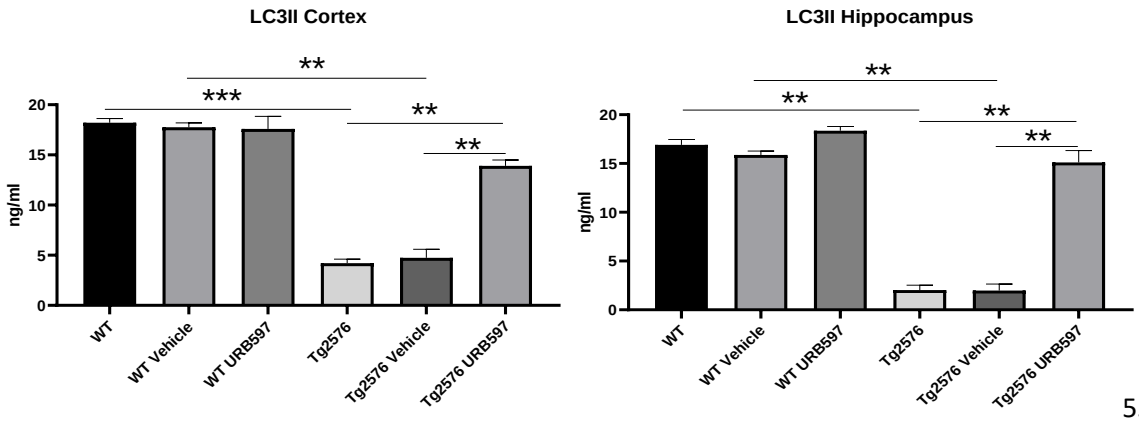


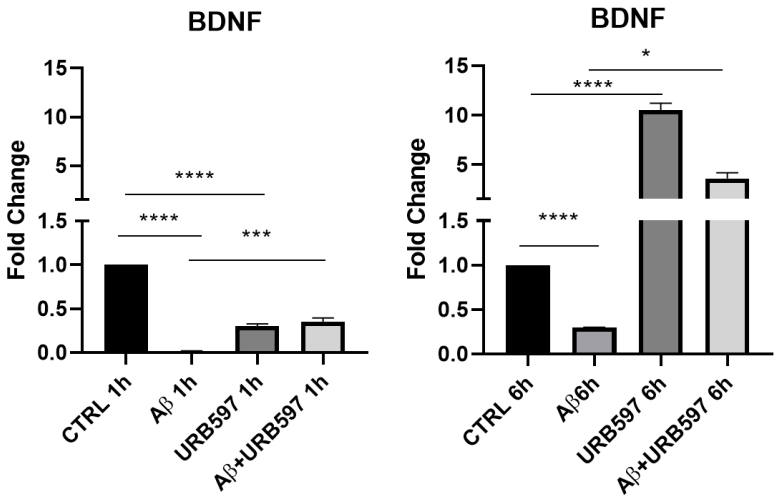
Fig. 28 LC3 II ELISA assay on homogenates of cortex and hippocampus from untreated and vehicle/URB597-treated Tg2576 and WT mice. Data are shown as mean ± SD from three independent experiments performed in duplicate using Unpaired Student t test. **p< 0.01, ***p<0.001.

Our results show a significant reduction in LC3II concentration in homogenates of both cortex and hippocampus from Tg2576 mice and vehicle-treated Tg2576 mice compared with WT mice groups. LC3II expression increases significantly in URB597-treated Tg2576 mice in both cortex and hippocampus compared with untreated and vehicle-treated Tg2576 mice.

FAAH inhibition increased mRNA expression of BDNF in BV-2 cells

BDNF promotes neuroprotection and modulates synaptic interactions critical for cognition and memory by increasing survival and inducing neuronal regeneration (Kowiański et al., 2018; Palasz et al., 2020). BDNF deficiency in AD contributes to neurodegeneration (Z.-H. Wang et al., 2019). We evaluated whether URB597 treatment is able to affect the mRNA expression of BDNF in the presence and absence of Aβ in BV2 cells. As shown in Figure 28, there was a significant reduction in BDNF mRNA expression in the presence of Aβ compared with untreated cells (CTRL) at both 1h and 6h after treatment. After 1h, URB597 significantly increased BDNF mRNA expression in the presence of Aβ compared with BV2 cells treated only with Aβ. 6h after treatment BDNF mRNA expression increased significantly in both URB597-treated cells, compared with CTRL cells, and in Aβ+URB597 treatment.

Fig.29 mRNA expression of BDNF was evaluated by qRT-PCR at 1 and 6h. Data are shown as mean ± SD from three independent experiments performed in triplicate. Expression profiles were determined using the 2−ΔΔCT method. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$



DISCUSSION

ECBS can affect immune responses by inhibiting the production of pro-inflammatory cytokines by microglia (Kendall & Yudowski, 2016). However, little is known about the relationship between the ECBS and microglial polarization. Our study investigated the role played by the ECBS in the modulation of microglial activation following the inhibition of FAAH activity. Moreover, the polarization of microglia is associated with cytoskeletal changes (Gordon & Martinez, 2010). Alterations in cytoskeletal organization are a hallmark of AD (Aguilar et al., 2017). Following a stimulus, microglia is activated and express an amoeboid phenotype (Hanisch & Kettenmann, 2007). Stimulation with Aβ in BV2 cells shifted morphology towards an amoeboid phenotype but URB597 treatment restored the typical phenotype of microglia in resting conditions. To better understand whether URB597 reverts microglia towards an anti-inflammatory phenotype or maintains microglia in a resting condition, we examined the expression of iNOS, associated with an M1 phenotype, and the expression of ARG-1, associated with the M2 phenotype. Arg-1 is an enzyme that decreases inflammation through arginine-dependent biosynthesis of tissue-regenerating polyamines, and through downregulation of NO levels, which is by competing with iNOS for the availability of their common substrate, arginine (Chauhan et al., 2021; Scipioni et al., 2022). Interestingly, URB597 reduced iNOS expression after Aβ challenge while increased the expression of ARG-1, according to Grieco et al., 2021. Furthermore, in the presence of Aβ, URB597 treatment reduced the mRNA expression of pro-inflammatory cytokines, such as IL-6 and IL-1β, and stimulated the production of anti-inflammatory cytokines, such as TGF-β and IL-10. These results are in agreement with the experiments by Tanaka et al. (2019), who showed that treatment with URB597 in LPS-stimulated BV-2 cells suppresses the expression of several pro-inflammatory genes (Tanaka et al., 2019). Our data demonstrate that FAAH inhibition by URB597 is able to drive microglial polarization toward the anti-inflammatory M2 phenotype (Grieco et al., 2021).

To further corroborate such evidence, we performed an immunohistochemical analyses on Tg2576 mice treated *in vivo* with URB597 compared with a vehicle-only treated group and WT groups. Primarily, we evaluated the expression of iNOS and

ARG-1 in neocortical and hippocampal regions. In AD, the increase in iNOS-positive cells is well-known. (Xie et al., 2021). Our data show that after UB597 treatment there is in Tg2576 mice a substantial decrease in iNOS and parallel increase in ARG-1, thus confirming the data obtained *in vitro*. The anti-inflammatory effect of URB597 was also demonstrated in a 2020 paper where FAAH inhibition had a protective effect in airway inflammation in LPS-induced inflammation in mice (Abohalaka et al., 2020).

Modulation of the ECBS and CB2 receptors stimulation appears to provide an anti-inflammatory effect in AD, although the regulatory mechanisms of this process are not yet fully understood. CB2 stimulation increases ARG-1 secretion and impacts microglia to acquire the anti-inflammatory phenotype (Carlisle et al., 2002; Komorowska-Müller & Schmöle, 2020). Our data show a significant increase in CB2 receptor expression in the cortical areas from Tg2576 mice treated with URB597 compared with WT and Tg2576 mice treated with vehicle. Interestingly, in the hippocampal area we found an opposite situation: a significant increase in CB2 in the Tg2576 compared with WT experimental groups and a significant decrease in their expression in Tg2576 mice treated with URB597. Early neurobiological changes in AD have been shown to involve the entorhinal cortex and hippocampus (Frisoni et al., 2010); a reduction of microglial cells in hippocampal areas can be hypothesized as we previously demonstrated where URB597 treatment reduced Aβ-induced BV-2 cell migration (Grieco et al., 2021). In cortical areas, which are affected in the more advanced stages of the disease, FAAH inhibition could induce a sustained receptor stimulation resulting in CB2 upregulation in microglia cells.

A 2021 study in rats showed that modulation of CB2 receptors can decrease neuroinflammation only in the hippocampus, but not in the cortex, a finding associated with the selective increase of CB2 receptor expression observed in the hippocampus (Karan et al., 2021).

BACE1 is a proteolytic enzyme required for the formation of the amyloidogenic form of Aβ. In AD, the increase in BACE1 supports the hypothesis of the important role of this enzyme in the initiation of the amyloid cascade (Hampel et al., 2021). Both in the hippocampus and in the cortex we observed a decrease of the

expression of BACE1 after URB597 treatment in Tg2576 mice. Hence, inhibition of FAAH significantly reduces the expression of BACE1, corroborating the pro-homeostatic pleiotropic action of ECBS.

Next, we further investigate this result at the light of the formation of amyloid plaques. The increase of BACE1 in AD leads to the increase in the formation of oligomers that compact in neuritic plaques with high-density Aβ accumulation (Cervellati et al., 2021). Our data show that treatment with URB597 reduces the number and area of amyloid plaques in Tg2576 mice at 14 months of age where an accumulation of amyloid plaques in the midbrain takes place. In fact, at the onset of AD pathology, the degeneration of the cholinergic fibers, that innervate the hippocampal and neocortical areas, starts from the basal forebrain (Orta-Salazar et al., 2014). Aβ plaques affect cholinergic deficits in AD, and cholinergic synapses in the CNS are critical for cognitive functions such as memory and learning and play a key role in age-related neurological disorders such as AD (Hampel et al., 2018). PSD-95 is an important protein that regulates synaptic organization and the activity of NMDA and AMPA receptors, and Aβ accumulation in AD negatively affects the expression of PSD95 (Tu et al., 2014).

It was recently shown that the increase in PSD-95 has protective effects at the synaptic level against the influence of Aβ (Dore et al., 2021). Our data suggest the restoration of PSD95 signaling after treatment with URB597 in TG2576 mice, confirming the broad-spectrum pro-homeostatic action of the ECBS.

A 2015 study showed that chronic treatment with CB2 agonist in mice increased excitatory synaptic transmission. Moreover, these effects were blocked by a CB2 antagonist (J. Kim & Li, 2015, p. 2).

Astrogliosis is a hallmark of AD, and together with the hyperactivation of microglia contribute to neuronal death (Liddelow et al., 2017; Reichenbach et al., 2019). Our analysis of astrocyte marker GFAP showed that treatment with URB597 reduced astrogliosis in prefrontal cortex. In the hippocampal areas no differences emerged on astrogliosis after treatment with URB597, in contrast to a 2015 study

where reduction of astrocytes in the dentate gyrus was demonstrated in URB597-treated mice (Rivera et al., 2015).

Notably, few works have highlighted the influence of ECBS on autophagy in neurodegenerative diseases (Bedse et al., 2015; Maroof et al., 2013; Shao et al., 2014). The innovative aspect of this study is the analysis of the recovery of autophagy flux after treatment with URB597. The accumulation of Aβ peptide in AD is affected by autophagy (Uddin et al., 2018)**.** It was previously shown that CB2 knockout mice in experimental autoimmune encephalomyelitis (EAE) reduced the expression of LC3-II and Beclin-1 ratio with increased inflammatory status. In contrast, CB2 stimulation appears to have an opposite effect by restoring autophagic processes and by inhibiting BV2 cells hyperactivation (Shao et al., 2014).This suggests that there is a correlation between modulation of the ECBS and autophagy in neurological diseases such as AD, but further investigation will be needed to understand the mechanisms underlying these processes. After demonstrating the beneficial effect of URB597 treatment against neuroinflammation, we performed experiments to confirm the pleiotropic effect of the ECBS on autophagic processes.

In different mouse models of AD, decreased expression of ATG7 is found in both cortical and hippocampal areas. Inhibition of ATG7 increases the levels of Aβ and phosphorylated tau protein. In addition, both post-mortem analyses of AD patients and mouse models of AD, show reduced Beclin1 levels that resulted to be associated with increased levels of Aβ. The reduction of Beclin1 results in an increased pro-inflammatory response of microglia (Uddin et al., 2018).

Our data clearly show that in microglia cells the mRNA expression of the key factors involved in the total autophagy flux, such as Beclin1, ATG7, LC3, and p62 is decreased in the presence of Aβ while is increased 24h after URB597 treatment. We confirmed these data showing a reduction in the proteins involved in autophagic flux, but after treatment with URB597 an increase of Beclin1, ATG7 and LC3 in both prefrontal cortex and hippocampus was detected. The restoration of autophagic processes after ECBS stimulation is also confirmed by our results showing an increased expression of LC3-II, suggesting the increase of mature autophagosomes.

In contrast, an increase in p62 expression in vehicle-treated Tg2576 mice and a decrease in p62 after URB597 treatment are observed. Reduced levels of p62 are associated with induction of autophagic flux (Bjørkøy et al., 2009). Semi-quantitative analysis by WB further confirmed, in homogenates of cortex and hippocampus from Tg2576 mice, the increased expression of ATG7 and Beclin-1 following treatment with URB597.

p62 links autophagy to Nrf2 pathways that counteract oxidative stress through the activation of transcription of antioxidant enzyme genes. In addition, Nrf2 regulates p62 gene expression, influencing autophagy. This pathway is deregulated in AD by exacerbating oxidative stress and inflammation (W. Zhang et al., 2021)**.** Our data demonstrate that treatment with URB597 in the presence of Aβ in BV-2 cells stimulates the increased of Nrf2 mRNA expression. Also, in a very recent study, an activation of Nrf2 signaling pathways was demonstrated following treatment with URB597 in a model of cerebral ischemia (D.-P. Wang et al., 2022).

The mTOR pathway is one of the most promising target for modulating autophagy in AD. Decreased mTOR expression increases Aβ clearance (Uddin et al., 2018). Our data show that Tg2576 mice have elevated mTOR expression associated with reduced ULK1 in prefrontal cortex and hippocampus. After treatment with URB597 the expression levels of mTOR are significantly reduced, while the expression of ULK1 is increased.

Data on the restoration of autophagy after treatment with URB597 are in contrast with two recent studies that reported a therapeutic potential for URB597 in the treatment of chronic brain neuroinflammation where excessive autophagy was evident. Treatment with URB597 relieved autophagy and inflammation in mTOR-dependent manner (Su et al., 2019; D. Wang et al., 2017). A modulatory role by URB597 on autophagic processes could be hypothesized.

On the other hand, it was also shown that the synthetic CB2 agonist HU-308 can promote autophagy (Shao et al., 2014). In addition, Sativex ® , a drug combining THC and CBD, has been shown to reduce Aβ deposition in the hippocampus and

cerebral cortex in a mouse model of tauopathy through the activation of autophagy (Casarejos et al., 2013).

Deficiency of BDNF activity is considered a hallmark of AD contributing to neurodegeneration and increased inflammatory status, also increasing inappropriate cleavage of APP (Z.-H. Wang et al., 2019). Our data confirm reduced BDNF mRNA expression in Aβ-stimulated BV2 cells and increased mRNA expression of BDNF after URB597 treatment, corroborating the role of ECBS in neuronal trophism. Finally, it should be noted that the effect of BDNF on neuronal differentiation can be inhibited by CB1 or CB2 blockade (Ferreira et al., 2018).

CONCLUSIONS

In conclusion, our data demonstrate that inhibition of FAAH activity by URB597 administration is a treatment able to drive microglial polarization toward the anti-inflammatory M2 phenotype. Indeed, URB597 selectively inhibits eCB degradation, modulates microglia activation and promotes autophagic processes, ultimately reducing the presence of amyloid plaques associated with the decreased expression of BACE1. Such multiple and pleiotropic effect attributable to the pharmacological inhibition of FAAH activity are also linked to a substantial increase of Aβ clearance. Hence, the modulation of ECBS confirms all its potential as a promising therapeutic target for AD management, and in particular our study supports the view that the suppression of FAAH activity can trigger multiple anti-inflammatory, neuroprotective and pro-autophagy mechanisms to fight the multifactorial etiology of this high debilitating neurodegenerative disease.

Further analysis

Several studies have shown that phytochemicals such as the polyphenolic nutraceutical resveratrol, found in foods, are able to promote cell survival through multiple mechanisms, including reduction of oxidative stress, suppression of inflammation, and induction of autophagy. Resveratrol is able to act through activation of endocannabinoid receptors (Armeli et al., 2021). Therefore, it will be interesting to evaluate the action of nutraceutical in BV-2 microglial cells in the presence of an inflammatory stimulus (LPS/Aβ) to analyze the anti-inflammatory and antioxidant effect and the induction of autophagy by plant-derived compounds.

Modulation of endocannabinoid tone shows pleiotropic activity, targeting in parallel several processes that play key roles in AD, including Aβ clearance, neuroinflammation, excitotoxicity, mitochondrial dysfunction, oxidative stress, and autophagic process.

Therefore, because of these widespread properties, targeting the ECBS could represent a reliable opportunity to progress toward an effective therapy against AD. In addition, cannabinoids could represent a safe and low-cost therapy due to their natural origin and low side effect profile. Among the derivatives of *Cannabis sativa*, CBD has attracted interest because of its lack of psychotropic effects (Devinsky et al., 2014; Patricio et al., 2020). The compound has been tested in the treatment of Parkinson's-associated psychosis and Multiple Sclerosis (Furgiuele et al., 2021) with promising results and has shown neuroprotective effects due to both its anti-inflammatory and antioxidant properties (Chagas et al., 2014; Lastres-Becker et al., 2005).

Successful cannabinoid therapy in AD could increase the possibility of slowing exponential degenerative progression to dementia.

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