Prenatal inhibition of the soluble epoxide hydrolase by the pharmacological agent TPPU as a possible target for Alzheimer's disease therapy

Final Grade Project (TFG) of
'Biochemistry and Molecular Biology' Degree

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The Final Grade Project (TFG) of 'Biochemistry and Molecular Biology' Degree of **Alba Irisarri Martinez** entitled:

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LIST OF ABBREVIATIONS

AD Alzheimer's Disease
APOE Apolipoprotein E
APP Amyloid Precursor Protein
ASD Autism Spectrum Disorder
Aβ Amyloid β
CKs Cytokines
CTF99 C-Terminal Fragment 99
CTX Cortex
DHET Dihydroxyeicosatrienoic acid
DI Discrimination Index
EETs Epoxyeicosatrienoic acids
EGR1 Early Growth Response Protein 1
ELISA Enzyme-Linked ImmunoSorbent Assay
EPHX2 Epoxide Hydrolase 2
FAD Familial Alzheimer's Disease
GFAP Glial Fibrillary Acidic Protein
hAPP human APP
HC Hippocampus
Iba1 Ionized Calcium Binding Adaptor Molecule 1
IEG Immediate Early Gene
IF Intermediate Filaments
IL1β Interleukin 1 Beta
IL6 Interleukin 6
LOAD Late Onset Alzheimer's Disease
MAP Microtubule-Associated Protein
MAP2 Microtubule-Associated Protein 2
MAP2c Microtubule-Associated Protein 2c
MIA Maternal Immune Activation
NFTs Neurofibrillary Tangles
NOR Novel Object Recognition Test
OF Open Field
OLT Object Location Test
ORT Object Recognition Test
PSN1 Presenilin 1
PSN2 Presenilin 2
qPCR quantitative Polymerase Chain Reaction
sEH soluble Epoxide Hydrolase
sEHi soluble Epoxide Hydrolase inhibitor
TBP TATA-box Binding Protein
TNFα Tumor Necrosis Factor Alpha
TPPU (1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea
TREM2 Triggering Receptor Expressed on Myeloid cells 2
WB Western Blot
WT Wild-Type

ABSTRACT

Population is becoming older, as life expectancy is increasing, and with it, age-related neurodegenerative diseases have become the greatest health-care challenge to this day. More than 47 million people are estimated to suffer from dementia worldwide, from which 60-80% of the cases correspond to Alzheimer's disease (AD). One of the main factors contributing to the development and progressiveness of AD is the neuroinflammation, caused as a response to the characteristic amyloid aggregations and neurofibrillary tangles present in AD brains. More and more studies are focusing now its target in inflammatory signaling pathways, as for the case of the soluble epoxide hydrolase (sEH). This enzyme is responsible for transforming anti-inflammatory epoxyeicosatrienoic acids (EETs) to their corresponding diols, dihydroxyeicosatrienoic acids (DHETs), which promote the inflammatory micro-environment.

This study aims to determine to which extent the prenatal inhibition of the sEH by the pharmacological agent TPPU has a neuroprotective effect on the offspring. For doing so, identification of molecular markers related to AD pathology and synaptic plasticity, and its correlation with improvements in behavior are being analyzed. Behavioral tests are carried out using two-months-old 5xFAD mouse model for AD, and for molecular analysis, corresponding cortical and hippocampal tissues are preserved. Identification and quantification of proteins is done by using Western blot analysis and gene expression by quantitative PCR assay.

Preliminary results obtained suggest that there could be a possible effect of the prenatal treatment with TPPU in tau associated pathology and inflammatory markers, whereas amyloid pathology and neuronal plasticity have not been shown to be affected. Further studies should be performed with older mice, as at this age amyloid deposits seem not to be enough to start the aberrant glial response. More research on how prenatal administration of TPPU could protect the offspring is needed, as precise mechanisms are still unknown.

Keywords: soluble epoxide hydrolase (sEH), TPPU, neuroinflammation, Alzheimer's disease (AD), mouse model.

INTRODUCTION

1. Neurodegenerative Diseases

Neurodegenerative diseases, also known as neurodegenerative disorders, are characterized by the progressive and irreversible neuronal dysfunction, which finally causes the death of neurons. More specifically, they involve the loss of the most vulnerable populations of neurons, which differ from the static neuronal loss caused by metabolic or toxic disorders (1). They can be classified according to three different criteria: primary clinical features (such as dementia, which is the most prevalent disease globally with an estimated incidence of 9.33% (2)), anatomic distribution of neurodegeneration and principal molecular abnormality (1). The abnormal conformation of proteins explains the vast majority of specific patterns found in this type of pathologies, commonly disorders such as amyloidosis, tauopathies, α-synucleinopathies and TDP-43 proteinopathies (1).

2. Alzheimer's Disease (AD)

Alzheimer's Disease (AD) is the main cause of dementia worldwide and one of the greatest health-care challenges of the 21st century (3). It was first described as a progressive neurodegenerative disease by Alois Alzheimer in 1906, characterized since then by initial memory impairment and cognitive decline. However, when AD progresses, behavioral and psychological symptoms can also appear, as well as an alteration in visuospatial orientation and the motor system itself (4). Despite having observed better brain health care lately (3), there is currently no therapeutic curative treatments for the disease. Most of the employed drugs for alleviating symptoms caused by the disease are nowadays acetyl-cholinesterase inhibitors (AChEIs), which increase acetylcholine availability, and memantine, an alternative treatment to reduce L-glutamate excitatory neurotoxicity (5). For psychiatric symptoms, and depending on the stage the patient is found, antidepressants and antipsychotics are normally used (5).

It was estimated that more than 47 million people suffered from dementia in 2016, from which 60% to 80% of the cases corresponded to AD (4). A study carried out in 2005 by a group commissioned by Alzheimer Disease International, which was responsible for estimating the incidence in 14 World Health Organization regions, suggested that North America and Western Europe have the highest prevalence of dementia at age 60, followed by Latin America and China (6). More specifically, in Southern European countries such as Spain, prevalence was estimated to be 6.88% (7).

2.1 Causes

Alzheimer's disease can be developed as a result of genetic mutations, which are known as familial Alzheimer's disease (FAD). Specifically, they can occur in three possible genes: amyloid precursor protein (APP), presenilin 1 (PSN1) and presenilin 2 (PSN2). However, these type of Alzheimer's cases are the less common ones (estimated to be 1% or even less) (8). Abnormal processing of APP is responsible for Aβ peptides' accumulation, and so, risk factors contributing to its metabolism can be determinant for aggravating the disease. PSEN1/2 also take part in its processing, as they assemble to secretases responsible for it, and consequently, they can also generate big deposition of peptides (9). A person with these types of genetic abnormalities will usually experience symptoms early-onset, potentially as soon as at the age of 30 (8). Another gene associated to AD deterioration is the Apolipoprotein E (APOE), in this case, related to cholesterol homeostasis (9).

Apart from this exception, the rest of the cases are normally related to aging (8). Late onset AD (LOAD) is considered to be the most common one. This is sporadically developed (4), although multiple factors are known to be risky for developing this kind of chronic disease in addition to advanced age, like sex (7) or family history (8). With regard to the first one, age is the most relevant risk factor for AD worsening (9). The more advanced in age, the higher the estimated prevalence becomes for a person to suffer AD. However, getting older does not mean a person will develop AD, as age as a unique factor is not enough to cause it (8). In addition, incidence also resulted to be related to sex, as it was found to be higher in women than in men: 13.25 cases per 1000 person-years versus 7.02 cases per 1000 person-years (7). Finally, it is also believed that people who have a first-degree relative suffering from the disease have a higher risk to develop it, even though the degree of the genetic link varies among individuals (8).

2.2 *Pathological Diagnosis*

Pathological diagnosis for this multifactorial disease is still based mainly on the same microscopic features that were defined by Alois Alzheimer: the presence of extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) (4). In addition, these features can lead to changes in the structure and mass of the brain, contributing to the hippocampal volume loss and decreased glucose metabolism (4). All in all, the main negative lesions presented in AD brains are synaptic and neuronal losses (10).

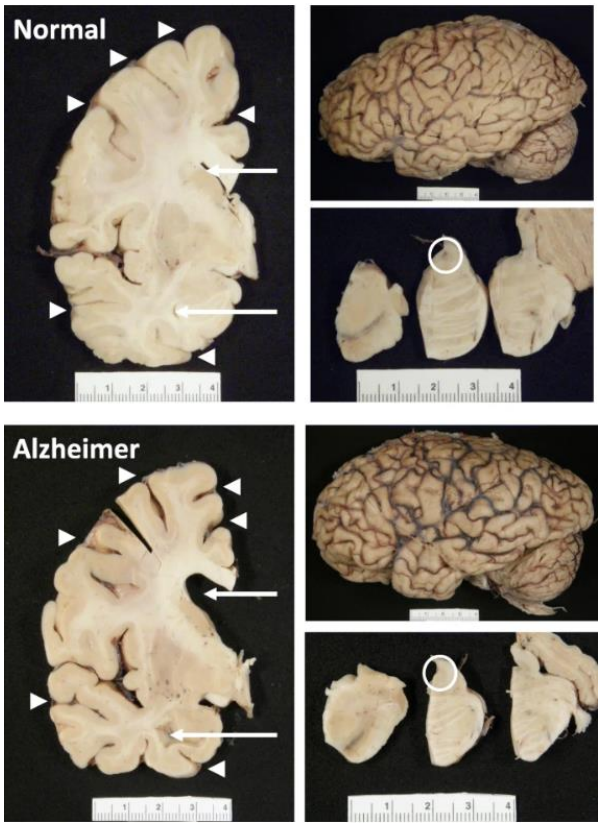


Figure 1. Comparison of the anatomy of a normal brain and an AD brain: loss of mass and volume. Figure taken from Deture MA and Dickson DW, BioMed Central Ltd, 2019 (4).

As mentioned before, the accumulation of Aβ peptides is one of the main features of AD. Those peptides are formed by the abnormal processing pathway or amyloidogenic pathway catalyzed by β- and γ-secretases of the amyloid precursor protein (APP), which results in toxic metabolites observed in AD brains (Aβ40-42). APP is firstly processed by β-secretase, resulting in APPβ being released to the extracellular space and a C-terminal fragment 99 (CTF99), which is finally cleaved by γ-secretase, responsible for Aβ peptide accumulation (11,12).

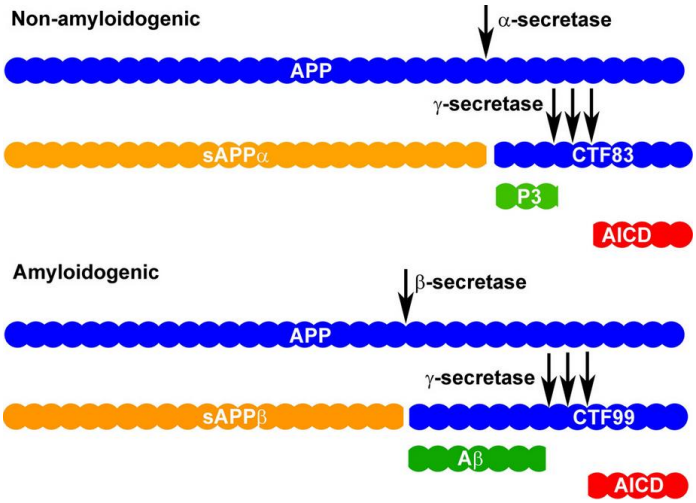


Figure 2. APP processing pathways: non-amyloidogenic and amyloidogenic pathways. Figure taken from Wilkins HM and Swerdlow RH, Elsevier Inc, 2017 (11).

Apart from these amyloid plaques, AD has been found to be a tauopathy. Tau is a microtubule-associated protein located primarily in the axon of neurons, taking part in axonal, and so neuronal, stabilization. When delocalized to the somatodendritic compartment, it gets hyperphosphorylated and misfolded, causing the aggregations responsible of neurofibrillary tangles and neuropil threads (10).

Not only has AD been characterized for being an amyloid and tau pathology, but also for being a neuroinflammatory disease, in which microglia activation and reactive astrocytes have the leading role.

Microglia is known as the principal macrophage type present in the central nervous system (CNS) parenchyma, where other neuroglial cells such as astrocytes and oligodendrocytes can also be found (13). Even though early microglia activation is beneficial for protecting the brain from Aβ accumulation, their response to a prolonged brain injury and to abnormal protein aggregations can lead to an acceleration and exacerbation of the disease (13). This happens as a result of the ability of the microglia to adapt polarized phenotypes; this first activation of the microglia is known as the classical activation, considered as the pro-inflammatory M1 phenotype, whereas the alternative activation or acquired deactivation of it corresponds to the immunosuppressive M2 phenotype (14). Aβ-induced pro-inflammatory cytokines (CKs) and agents, such as IL-1β and TNF-α (13), cause astrocytes to become reactive, and consequently, neurotoxic in AD brains (4). In fact, not only do these pro-inflammatory CKs reduce microglial Aβ clearance ability (15), but they also promote Aβ deposition by increasing it under inflammatory conditions (16).

Additionally, astrocytes are specialized glial cells, five times more abundant than neurons. When responding to brain injury or other types of threats, a process known as reactive astrogliosis is activated, which is responsible for this pathological effect of inflammation in the brain (17). The process is defined as progressive changes that increase gradually the reactivity of astrocytes altering gene expression and cellular function; from mild to moderate reactivity, to severe diffuse reactive astrogliosis, until finally reaches compact glial scar formation (17).

Even though amyloid deposition has an early plateau (10), chronic inflammation has been observed to be linked to both onset and progression of AD (18), indicating that a linear increase of glial response is correlated to neurofibrillary degeneration (10). Actually, alterations in different proteins' expression level have been observed (18) such as for glial reactivity and CKs, for amyloid and tau pathology and for neural plasticity.

2.3 Protein alterations in AD brains

According to glial reactive proteins, glial fibrillary acidic protein (GFAP) is the most important intermediate filament protein in mature astrocytes, as well as a key component of their cytoskeleton during development (19). It is usually found to be highly expressed when responding to brain injury and CNS degeneration, and so in aged brain (19). These high levels of GFAP indicate that the process of reactive astrogliosis is being carried out, as astrocytes try to deal with brain damage, initially and most notably in hippocampal region (19). The increasing expression of GFAP cause a higher production of intermediate filaments (IF) by these enlarged and reactive astrocytes.

Another important glial protein highly expressed in AD brains is the ionized calcium binding adaptor molecule 1 (Iba1) (20). Iba1 is an EF hand calcium binding protein, which is expressed strictly to the monocytic lineage, microglia and macrophages (21). It has been shown to be a key protein, with bundling F-actin, in membrane ruffles and phagocytic cups in activated microglia (21).

Finally, the triggering receptor expressed on myeloid cells 2 (TREM2) is also a surface receptor strictly and highly expressed by microglia, who's binding to ligands, such as phospholipids and lipoproteins, activates its downstream signal transduction pathways (22). Consequently, microglial cells are activated, so that promotes protective phagocytosis and cell survival. However, different polymorphisms of TREM2 have been shown to be genetically associated with AD risk, and so an increased expression is expected in AD brains (22).

Regarding pro-inflammatory CKs, two of the main ones present in neuroinflammation are Interleukin 6 (IL6) and Tumor Necrosis Factor α (TNFα). Their function consists of promoting inflammatory responses, being in first place protective proteins against damage (16). However, in AD brains, it has been proved that they up-regulate one of the key enzymes that cleaves APP, the β-secretase. TNFα seems to increase the enzyme's mRNA protein transcription, resulting in a bigger amyloid formation (16). Moreover, both microglia and reactive astrocytes participate in the chronic liberation of pro-inflammatory CKs when constant stimulus of amyloid deposition persists for a long time, which causes an increment of these protein's levels in AD brains (16).

With regard to amyloid associated proteins, different fragments of amyloid beta peptide are targeted (epitopes 6E10 and 4G8) for study, as well as the CTF99 (CTF of APP of 99 amino acids), formed by the amyloidogenic processing of β-secretase. The two epitopes, 6E10 and 4G8, correspond to fragments from residue number 3 to 8 and, from residue number 17 to 24, respectively (23).

As for tau associated proteins' analysis, HT7, AT8 and Ser396 targets are usually the preferred ones to study, as these are the main phosphorylated sites of the protein. Tau is a phosphoprotein, and so, phosphorylation is the most important post-translational modification that undergoes and which determines its biological function (24). HT7, which is tau's protein fragment between residues 159 and 163, represents the total tau epitope for antibody recognition; AT8, corresponds to the epitopes associated with intracellular and extracellular filamentous tau (phosphorylated in residues Ser202 and Thr205); and, finally, phosphorylated Ser396, which is associated with intraneuronal fibrillar structures (25). In AD brains, hyperphosphorylated tau protein is the responsible for forming NFTs, that is why its levels are estimated to increase (24).

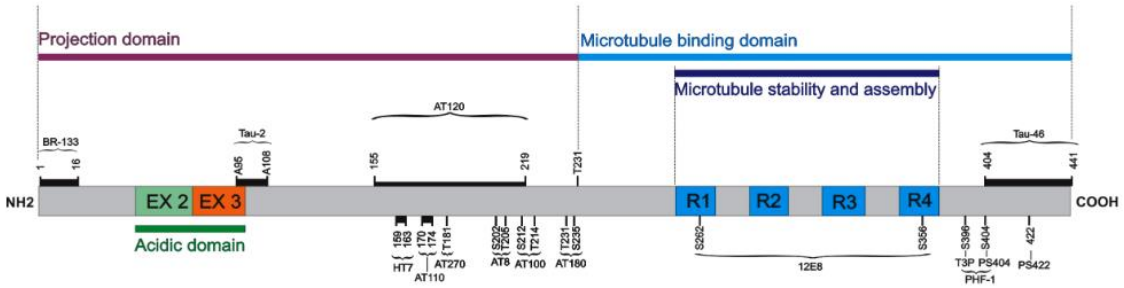


Figure 3. Main phosphorylation sites of Tau protein depending on each functional domain. Figure taken from Tudorică V et al., Rom J Morphol Embryol, 2017 (24).

Finally, early growth response protein 1 (EGR1) and microtubule-associated protein 2 (MAP2) protein's levels have been seen altered regarding neural plasticity in the brain (26,27).

EGR1 is an immediate early gene (IEG) and a zinc finger transcription factor, which means it is a mediator between genes and environment relations and a regulator of neural activity (26). In fact, it regulates many functions at the cellular level such as cell proliferation, apoptosis, cell growth and signal transduction. All of them can finally alter the expression of diverse genes related to every level of synaptic plasticity, from vesicular transport and release of neurotransmitters, to synaptic architecture, endocytosis, and protein degradation (26). In addition, EGR1 is induced when responding to hypoxia, brain injury or inflammation, all present in AD brains, but its transcriptional targets are still unclear (26).

MAP2, as Tau protein, is a microtubule-associated protein (MAP) found in neurons. Both of them share a carboxy-terminal domain containing microtubule-binding repeats (27) and it gives them the ability to perform their biological function: assembling and stabilizing microtubules by altering their dynamic instability. There are five possible isoforms of MAP2 whose activity vary at different stages of our lives. During neuronal development and for future adulthood, Tau protein is mainly localized at axons, while MAP2 is strictly found in cell bodies and dendrites (27). A difference between both of them is that MAP2 is also able to bind F-actin, which makes MAP2c isoform able to induce neurite initiation (27). Moreover, it has been observed that a decreased expression of MAP2c levels correlates with the reduction of cytoskeleton dynamics during neuronal maturation (28).

3. The Soluble Epoxide Hydrolase (sEH)

Epoxyeicosatrienoic acids (EETs) are synthetized from arachidonic acid (found particularly in high levels in the brain) by the activity of cytochrome P450 epoxygenases. Their function is mainly to act as paracrine and autocrine effectors in the cardiovascular system and kidneys (29). These products are known for participating in the mediation of vasodilatation, reducing inflammation and oxidative stress, and blocking pathological endoplasmic reticulum response (18). However, the soluble epoxide hydrolase (sEH), which is the enzyme responsible for catalyzing the hydrolysis to the corresponding diol, dihydroxyeicosatrienoic acid (DHET) (29), eliminates their beneficial effects (18).

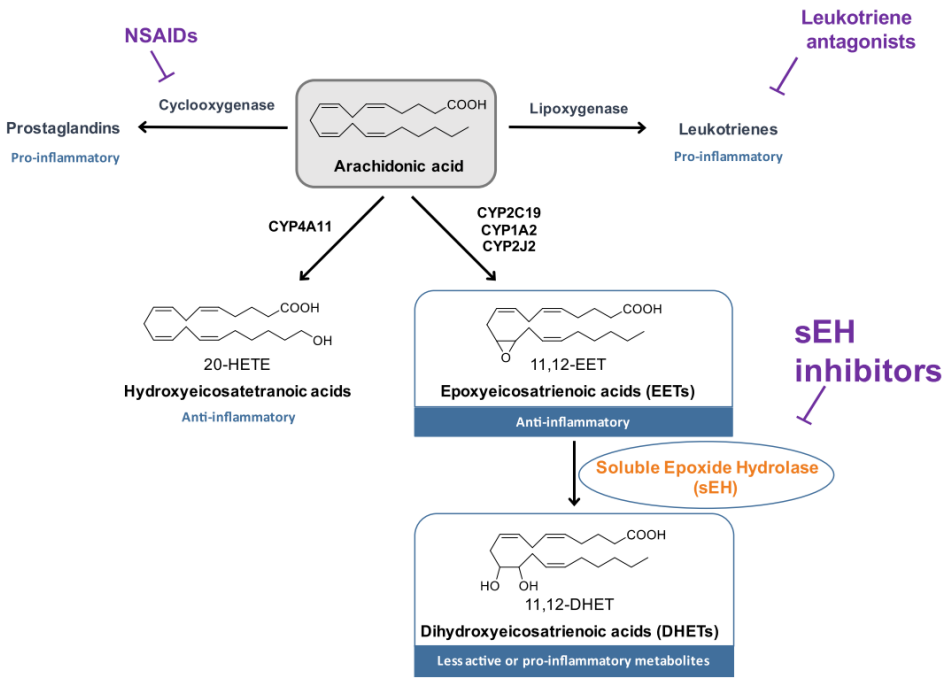


Figure 4. EETs enzymatic transformation to corresponding DHETs by sEH and the inflammatory effect. Figure taken from Griñán-Ferré C et al., Neurotherapeutics, 2020 (18).

The Human sEH, encoded by *EPHX2* (30), is a 62kDa enzyme formed by two domains separated by a short proline-rich linker. In fact, it is the C-terminal domain that contains the epoxide hydrolase activity responsible for the transformation of epoxides (31). In the case of the adult brain in mammals, low levels of sEH are found (29), where it is mainly expressed in lysosomes of astrocytes (30), as a homodimer in the intracellular environment (31). When converting EETs into DHET, they are mostly released to the extracellular fluid (29). However, the inhibition of the enzyme has been observed to be a prospering therapeutic target, as maintaining a higher level of EETs instead of DHETs could ensure an anti-inflammatory effect for the brain, and, as a consequence, a reduction in neuroinflammation and neurodegeneration in AD (18).

4. TPPU

(1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU) is a pharmacologic agent and a sEH inhibitor (sEHi) of both human and mouse sEH (IC_{50} = 3.7 and 2.8nM, respectively) (32) that is being studied as a possible future therapeutic agent for treating AD pathophysiology (30). This agent has been studied both *in vitro,* suggesting that it presents high absorption and metabolic stability, and *in vivo,* demonstrating that its blood concentration increases in a dose dependent manner within the period of administration through drinking water to rats (33). TPPU inhibits sEH, resulting in an increase of EETs, whose signaling pathway could be a key component for analyzing their vasodilator and anti-inflammatory effect. Furthermore, the increment of EETs/DHET ratio has been shown to be dependent of TPPU doses in all tissues tested, proving a systemic distribution ideal for future research (33).

TPPU is presented as a therapeutic strategy to restore normal Aβ processing by astrocytes (30), that once they become reactive, they even aggravate more neuroinflammation and increase the degeneration of neurons. In this way, progression of AD could be slowed, and early symptoms delayed.

5. *The 5xFAD Mouse Model and Behavioral Tests*

Animal models, and particularly mouse models, are the most relevant research tools for studying and finding new treatments for many diseases, commonly based on models for disease-causing mutations as FAD (34).

5xFAD corresponds to the most widely used mouse model of AD, which is based on the genetic association existing between early-onset familial forms of AD and Aβ42; the bigger the presence of Aβ42, the more it causes FAD (35). On the one hand, this type of models consists of transgenic expression of human APP (hAPP). APP protein, depending on the alternative splicing of its mRNA, can produce three different isoforms, and the one with 695 amino acids (APP695) in its final product is the isoform mutated in these mouse models. Actually, 5xFAD mouse models belong to the hAPP/PS1 models. Hence, not only do they carry pathological mutations in APP695, but they also present mutations in PSEN1 encoding for PS1. This way, it has been observed that crossed mice for both genes at the same time (as a single transgene), show an extensive plaque deposition and several behavioral deficits (34). In the case of the 5xFAD models, they are created by a combination of five FAD coexpressed mutations: Swedish, Florida (I716V) and London (V717I) mutations in hAPP gene, and the M146L and the L286V mutations in PS1 (35). The phenotypic results of the breeding mice include the expression of high levels of Aβ42 and cerebral amyloid plaques, causing cognitive deficits as a consequence. Moreover, unlike the rest of hAPP/PS1 models (34), they develop neuron loss as well as memory impairment in the Y-maze and memory deficits when ageing (35). These mice are breed in heterozygosis, namely a progenitor pair is formed by a 5xFAD mouse heterozygote for FAD mutations and a WT mouse. Mice of each litter are genotyped to identify 5xFAD and their WT siblings to be used in the experiments.

These transgenic mouse models used for AD experiments are ideal for carrying out different behavioral tests, so that the phenotypic features caused by the disease can be observed and measured. Usually, the tests performed for cognitive tasks are divided depending on which type of memory is being studied (36). Some of the tests included in the study of the working memory, novelty or activity are the open field test (OF), the object recognition test (ORT) and the object location test (OLT) (36).

The OF test was developed in 1934 for measuring emotionality in rodents (37). It consists on a locomotion test in which the motor function is studied by the total activity and spontaneous movements of the mouse (37). The field is divided into different quadrants, so that once the mouse is located in the center (first quadrant), all its moves (preferably to the periphery) can be monitored in order to examine its pattern of movement and anxiety (36). It is based on the fact that, as rodents show aversions to large, bright and open environments, when putting them into the center of the maze, they will have the feeling of openness and so movements responding to this fear will be the measure for its emotionality (37).

The ORT, also known as the novel object recognition test (NOR), is based on the ability and natural behavior of mice to explore and investigate novel objects instead of familiar ones. This kind of tests reflects the memory for learning and recognizing those mice innately present (36). It was first described in 1988 for rats, even though adaptation for mice has been also developed (38). It consists of three sessions: a habituation session, a training session and, finally, the test session. Firstly, training is performed by exposing mice to two identical objects. secondly, for the test session, one of the objects is changed for a novel one, so that based on

the principle of their ability to explore, mice are supposed to spend more time around the novel object (38).

Finally, the OLT provides a measure of hippocampus-dependent spatial memory (39). As for the case of the ORT, it is also based on the intrinsic preference of mice for exploring novelty. However, in this test, instead of varying the object, both of them remain the same but what changes is the location of one of them. In the training session, mice are exposed to two identical objects preferably in two corners of the maze. When carrying out the test, one of them is moved to another corner, so that following their instinct, mice are supposed to spend more time exploring the object in the novel location (39).

Taking into consideration the results obtained from the three behavioral tests, phenotypic differences between the 5xFAD mouse model and a wild-type (WT) mouse can be studied. This way, not only by conducting biochemical and molecular tests, but also observing their behavior, principal AD hallmarks can be found and investigated, so that the relationship between main proteins' pathways' alterations and its consequences in mice activity can be described.

BACKGROUND

This work has been a continuation of a previous project conducted by the 'Neurodegeneration and aging' research group, and so, the experimental design of it has been based on its results. The project aimed to find to which extent the use of the anti-inflammatory pharmacological agent TPPU in the prenatal period of mice could have a neuroprotective effect on the offspring. One of its main objectives was to analyze this neuroprotective function of TPPU in 5xFAD mouse model, by studying both cognitive effects and molecular markers of AD at different ages.

This work has supplemented the study from the project by performing the behavioral tests of the control study group left, which is why there has not been an analysis of the treatment in this case. In addition, molecular tests have been performed using the hippocampal and cortex tissue provided from the offspring of the treated mice used in this previous project, specifically from mice at the age of 2 months.

OBJECTIVES AND HYPOTHESIS

The general objective of this project is to analyze the molecular markers related to Alzheimer's disease pathology and synaptic plasticity in hippocampus tissue and cerebral cortex and its correlation with improvements on behavior. To this end, diverse molecular and behavioral tests are performed, in order to obtain relevant results that can help us to better understand how the disease is developed.

More specifically, the aim of this research is to study the molecular mechanisms of neuroprotection of a pharmacological agent, in this case, of the TPPU, by inhibiting the enzymatic activity of the sEH in the prenatal period, and so increasing the levels of anti-inflammatory EETs. Its effect will be studied in two-months-old transgenic mice for Alzheimer's disease, concretely in the 5xFAD mouse model.

Our hypothesis is that mice who have been treated with TPPU in the prenatal period will present less AD pathology as they get older; in other words, less amyloid beta accumulation, neurofibrillary tangles and, especially, neuroinflammation, when compared to the non-treated. These AD hallmarks are supposed to be more evident in transgenic 5xFAD mice, even though they could also appear in WT mice.

MATERIALS AND METHODS

To begin with, all the techniques explained on this work have been performed using two-months-old 5xFAD mouse model and corresponding wild-types. For behavioral tests, it has been analyzed a total number of 18 mice, whereas for molecular ones, the total number was 24. For these last tests, hippocampus and cortex tissue were extracted from mice, both divided into the right and the left side. More concretely, for performing Western Blot (WB) and quantitative Polymerase Chain Reaction (qPCR) right-side hippocampus and cortex were used, whereas only the left-side cortex for Enzyme-Linked ImmunoSorbent Assay (ELISA) technique. The total number of samples per group type was N=3 for WB and qPCR (both cortex and hippocampus). ELISA assay was only a preliminary trial.

All the research has been carried out based on three variables: sex, genotype and treatment. Differences between being male (M) or female (F), WT or 5xFAD model and control or TPPU treated have been analyzed, respectively. The following tables summarize the total samples used for each type of group.

Table 1. Total sample types used for behavioral tests.

Treatment	**Sex**	**Genotype**		**Total**
		WT	*5xFAD*	
TPPU				
Control	M	6	5	11
	F	6	1	7
				18

Table 2. Total sample types used for Western Blot. Same number of samples was used for hippocampus and cortex tissues.

Treatment	**Sex**	**Genotype**		**Total**
		WT	*5xFAD*	
TPPU	M	3	3	6
	F	3	3	6
Control	M	3	3	6
	F	3	3	6
				24

Table 3. Total sample types used for qPCR. Same number of samples was used for hippocampus and cortex tissues.

Treatment	**Sex**	**Genotype**		**Total**
		WT	*5xFAD*	
TPPU	M	3	3	6
	F	3	3	6
Control	M	3	3	6
	F	3	3	6
				24

1. *sEH inhibitor (TPPU) source*

The sEH inhibitor used as anti-inflammatory agent was (1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU), which was obtained from MedChemExpress (Monmouth Junction).

2. *Molecular Tests*

2.1 Western Blot (WB) analysis

Firstly, homogenization of the tissue and extraction of the proteins was done. On the one hand, for right hippocampus lysis, 100µl/well of ice-cold radioimmunoprecipitation (RIPA) buffer (pH 7.5) were used, containing 1% IGEPAL, 0.5% sodium deoxycholate and 0.1% SDS on PBS. The buffer was supplemented with 1mM sodium orthovanadate (NAVA), 5mM sodium fluoride (NaF), 2% SDS and protease inhibitor cocktail tablets (cOmplete, Roche) containing phenylmethylsulfonyl fluoride (PMSF). On the other hand, for right cortex tissue the volume of RIPA buffer used was 200µl for each well, and it was supplemented the same way as hippocampus samples.

Cell lysates were sonicated, and after centrifugating them (13.000xg, 10min, 4ºC), supernatant was collected. For determining the protein concentration of cell lysates, Bradford protein assay (Bio-Rad, Munchen, Germany) was performed. Absorbances were read at 595nm in the spectrophotometer of Multiskan specters (ThermoFisher Scientific).

This way, equal quantities of protein (40µg) were prepared and mixed with 6x DTT and distilled water, up to a total volume of 18µl. Samples were centrifugated, boiled in a dry bath for 5min, and centrifugated again. A final quantity of 40µg of protein were loaded in 10% or 15% polyacrylamide gel, depending on the size of the proteins to study. They were separated by SDS-PAGE electrophoresis at 100V for approximately 2h. Finally, gels were transferred into PVDF membranes of 0.45µm (Immobilon-P, Merck Millipore Ltd, Ireland) by electroblotting at 200mA during 90min. Membranes were blocked for an hour at room temperature with TBS-Tween (pH 7.4) containing 5% Blotting-Grade Blocker (nº 170-6404, Bio-Rad). Membranes were later incubated overnight at 4ºC on a rotating shaker in primary antibodies to different concentrations described in table 4 (diluted in the WestVision Block and Diluent SP-7000 (Vector Labs Inc., Burlingame, CA).

Table 4. List of antibodies, hosts and dilutions used for Western Blot analysis.

Target	Source	Host	Dilution
4G8	Covance (*SIG-39220*)	Mouse	1:1000
6e10	BioLegend (*803003*)	Mouse	1:1000
Actin	Sigma (*A5060*)	Rabbit	1:10000
APP-CTF	Covance (*SIG-39152*)	Mouse	1:1000
EGR1	Cell Signaling (*#4153*)	Rabbit	1:1000
GAPDH	Assay designs (*CSA-335*)	Mouse	1:2000
GFAP	Sigma (*G3893*)	Mouse	1:500
Iba1	Wako (*016-20001*)	Rabbit	1:2000
IL6	Cell Signaling (*#12912*)	Rabbit	1:1000
MAP2	Sigma (*M1406*)	Mouse	1:1000
p-Tau (S396)	Life Technologies (*44752G*)	Rabbit	1:1000
p-Tau AT8 (S202, T205)	Thermo Fisher(*MN1020*)	Mouse	1:1000

Tau HT7	Thermo Fisher (*MN1000*)	Mouse	1:1000
TNFα	Cell Signaling (*#3707*)	Rabbit	1:1000
β-Tubulin	Abcam (*ab21754*)	Mouse	1:1000

After incubation, membranes were washed (3 washes of 5min in TBS-Tween at room temperature) and stained with the correspondent peroxidase-conjugated (GE Healthcare) secondary antibody (1:2000) for 1h on shaking. Further three washes were done in TBS-Tween. For visualizing proteins, enhanced chemiluminescence (ECL) detection was used (ChemidocTM Imaging System, Bio-Rad, Hercules) and later, semi-quantitative levels of proteins were identified using Image Lab software (v3.0.1, Bio-Rad). Proteins were normalized compared to house-keeping controls, in this case, either actin, β-tubulin, GAPDH or total tau HT7.

2.2 quantitative Polymerase Chain Reaction (qPCR)

Extraction of total RNA from right hippocampus and cortex was carried out using mirVana™ mRNA Isolation Kit (Applied Biosystems) following manufacturer's protocol. RNA content was determined by the spectrophotometer (NanoDrop) at 260nm, and the quality was analyzed by the ratios A260/280 and A260/230. The ratios should be between 1.8 and 2 so that the purity of DNA is optimal. If A260/280 is below 1.6, it means the sample could be contaminated by proteins, and in case A260/230 < 1.6, it could be due to phenol contamination during the extraction procedure. In addition, samples with an RNA concentration smaller than 21ng/µl were concentrated using SpeedVac.

Reverse transcription from RNA to first-strand complementary DNA (cDNA) was performed using High-Capacity cDNA Reverse Transcription Kit (Life Technologies). The total volume of sample that was necessary for performing the assay was calculated for having a final concentration of 300ng of RNA, in case of hippocampus tissue, and 500ng of RNA, in case of cortex tissue. These volumes were adjusted with nuclease-free water up to 14.2µl, and for each sample 5.8µl of MasterMix were added so that the total volume was 20µl. Finally, the samples were put in the thermocycler (Analytikjena, FlexCycler) following the protocol (25ºC 10min; 37ºC 120min; 85ºC 5min; and 4ºC until wanted). When collecting the samples, they were diluted 1:4 with nuclease-free water and stored at -20ºC until use. The concentration obtained of cDNA was 3.75ng for hippocampus and 6.25ng for cortex tissue.

In the last step, each sample was analyzed in duplicate for each target in a 96-well optical plate. TaqMan probes (Applied Biosystems) described in table 5 were used to determine transcription levels of individual genes. For each well, 9µl of MasterMix containing 3.5µl H_2O, 5µl MasterMix buffer (Biotools) and 0.5µl TaqMan probe were mixed with 1µl of cDNA sample previously diluted 1:4. The reaction was performed on StepOnePlus RT-PCR System (Applied Biosystems) using the following protocol: 95ºC 5min; 40 cycles at 95ºC 10min; 60ºC 1min. Finally, TATA-Box-binding protein (Tbp) gene was used as housekeeping for normalizing the results obtained. For the analysis of relative gene expression of RT-PCR the $2^{-\Delta\Delta CT}$ method was used (40).

Table 5. List of TaqMan probes used for RT-PCR assay.

Gene	TaqMan Probe
Ephx2	Mm01313813_m1
Tbp	Mm00446971_m1
Tnf	Mm00443258_m1
Trem2	Mm00451744_m1

2.3 Enzyme-Linked ImmunoSorbent Assay *(ELISA)*

ELISA technique was carried out using Human Aβ42 ELISA Kit (Invitrogen, TermoFisher Scientific). For this last molecular test performed, only left-side cortex samples were used. For homogenization of cortex tissue (approximately 100mg of wet mass), 8 volumes of cold 5M guanidine-HCl in 50mM Tris (pH 8.0) were added by 50-100µl increments and mixed with a hand-held tissue homogenizer after each addition. The homogenate was put on the shaker at room temperature for 3-4h. After homogenizing, sample was diluted ten-fold with cold 1X PBS supplemented with 1X protease inhibitor cocktail tablet (cOmplete, Roche) and centrifugated (16.000xg, 20min, 4ºC). The supernatant (on ice) was diluted an additional 1:10 to 1:100 with Standard Diluent Buffer (Human Aβ42 ELISA Kit, Invitrogen) before performing the ELISA.

For ensuring that the dilution of the sample was correct, the standard curve was prepared by doing serial dilutions of the reconstituted Hu Aβ42 Standard to 2.000pg/ml (Human Aβ42 ELISA Kit, Invitrogen) with deionized water. The diluent composition of the standard curve was the same as for diluted experimental samples. Finally, 1X Anti-Rabbit IgG HRP solution (Human Aβ42 ELISA Kit, Invitrogen) was prepared, depending the final volume on the amount of 8-well strip used. Anti-Rabbit IgG HRP (100X) was diluted 1:100 in HRP diluent. For performing the ELISA, manufacturer's protocol was followed. Absorbance was read at 450nm in the spectrophotometer of Multiskan specters (Thermo fisher Scientific) to generate the standard curve.

In this case, as being a preliminary study with the technique, only homogenization of tissue and preparation of samples were completely done, as optimal dilution of samples (according to the standard curve of the kit) was not found.

3. Behavioral Tests

3.1 Animals

Behavioral tests have been performed for a total number of 18 mice, all of them two-months-old, from which 11 are males and 7 females. The study is done for control mice, in other words, for litters from females dosed with vehicle, which can differ by the genotype: WT or 5xFAD, as described in more detail in table 1. The exclusiveness of using only control groups for behavioral tests is due to the fact that this study is a continuation of a previous project non-completed yet (explained in 'Background' section).

The genetic background of these animals is C57BL/6J. 5xFAD male mice were bought from Jackson Laboratories (Maine, EE. UU) and B6SJLF1/J hybrid female mice from JANVIER LABS (Le Genest-Saint-Isle, France), following breeding directories from Jackson Lab. Each mouse's genotype was confirmed by PCR analysis of DNA extracted from ear punches. Wild type mice used as reference for the study were siblings of the heterozygous mice.

Animals were distributed and housed in Makrolon® cages (Techniplast, Buguggiatta, Italy), grouped depending on their genotype and sex. They had free access to food and water and a room temperature maintained at 22 ± 2 ºC. In addition, lighting was also controlled with 12 hours light/12 hours dark cycle. Animal housing and experimentation were performed at the

facilities of the Pharmacology Unit - Department of Pharmacology, Toxicology and Therapeutic Chemistry – of the Faculty of Pharmacy and Food Sciences from the University of Barcelona, Barcelona, Spain.

Approval for the study and experimental protocols was confirmed by the local animal experimentation ethics committee. All procedures were performed in accordance with approved Spanish guidelines and legislation concerning the protection of animals used for experimental and other scientific purposes and the European Commission Council Directive 86/609/EEC on this subject.

3.2 TPPU administration

TPPU was administrated to females orally within the drinking water during pregnancy and lactation, mixed with cyclodextrin 3% (Sigma-Aldrich) with the aim of improving its solubility. In this case, the control group was administrated the same dose of cyclodextrin within the water. Water consume was controlled every 3-4 days to ensure a constant diary dose of 5mg/kg of TPPU. The dose was decided based on the results of previous studies in which TPPU treatment was shown to be neuroprotective for these mouse models (18).

3.3 Experimental testing

3.3.1 Open Field (OF)

The open field maze, firstly designed and standardized for evaluating emotionality and motor activity in rodents, has been adapted for analyzing mice too (37). There are three measures for studying this: rearing (when mice raise the two legs for reincorporating), grooming (cleaning of the mice) and defecations and urine. By observing these, the exploratory activity, anxiety and emotionality of the animal can be studied, respectively. Furthermore, the latency of movement indicates the response known as freezing time, and it also measures anxiety at the first time in contact with a stressful environment.

Table 6. Variables measured for studying the exploring activity, anxiety and emotionality.

Exploring activity		**Anxiety**		**Emotionality**
Latency of Crossing	latC (s)	Latency of Movement	latM (s)	Defecations
Latency of Border	latB (s)	Latency of Grooming	latG (s)	Urine
Latency of Periphery	latP (s)	Number of Grooming	nG	
Latency of Rearing	latR (s)	Time of Grooming	tG (s)	
Number of Rearings	nR’			

In this case, each mouse is put in the center of the maze (a wood surface) of 50x50x20cm, which is divided into quadrants of 10x10cm. Once the mouse is in the first quadrant, and for 5 minutes long, a computerized filming system (SMART v3.0, Panlab S.A.) registers the path that it makes to measure the total distance covered and the ambulatory pattern.

This procedure is realized under controlled environmental conditions: adequate illumination, constant ventilation for minimizing external noises and a room temperature of 22ºC. Furthermore, with the aim of not interfering in mice behavior, between each mouse analyzed, the maze is cleaned with alcohol at 0.5%, so that there are no olfactory signals of territoriality, such as defecations and urine from the mouse studied before; and, before putting the mouse in the open field maze, it is moved to another cage for avoiding more stress.

3.3.2 Object Recognition Test (ORT)

For studying concretely hippocampal memory of mice, ORT measures the ability of exploration of novel objects in front of familiar ones (36,38). For doing so, three different sessions are performed.

Firstly, for the habituation session, which can last from one to seven days, the mouse is put for 10 minutes inside the box, where it will be carried out the test, but without any object, so that it habituates to the environment. This way, a reduction of motor activity can be observed.

The training session, which is considered to start at time zero (t=0h), consists of putting the mouse in the same box for 10 minutes with two identical objects (A + A) in both corners, as they prefer those to open spaces. For measuring the time it spends exploring each object, which in this case, as being identical it is expected to be similar, the object exploration is defined as the orientation of the nose to the object at a distance of less than 2cm.

Finally, for testing the mouse's ability for exploring novel objects (the test session), two hours later of performing the training (t=2h), one of the objects is changed for a novel one (object B). If it memorizes the old object (A), when changing it, it is supposed to spend more time observing the novel one, as it is the one it does not know yet. The following day, 24 hours later, the old object is changed for a novel one (object C) and so the one it was novel before, now becomes the old one (object B). Object B is moved to the corner where object A was located.

In all these sessions, boxes should be far away from each other, at least the maxim they can be for the mouse not to smell the one in the other box. Also, objects from both boxes should be in the same position.

The 10 minutes of test are divided into three intervals: 0-2 minutes, 0-5 minutes and, finally, 0-10 minutes. Doing so, it can be estimated the total time mice spend with each object for calculating the discrimination index (DI) of each interval:

$$DI = \frac{New\ Object\ (t) - Old\ Object(t)}{(New\ Object\ (t)\ + Old\ Object\ (t))}$$

If DI > 0.2, it means the mouse has remembered the training (the old object), and so it spends more time exploring the novel one (recognition memory).

As for OF maze, mice are firstly moved to a cage in the room where it will be carried out the test so they can habituate to the environment. Mice are recommended to be put inside the box looking to the opposite side of the objects. Once again, boxes and objects are cleaned with alcohol after testing a mouse and before putting the next one to avoid animal odor.

Table 7. Objects used for performing ORT test.

Object	**Boxes**	
	Box A	**Box B**
A	Culture flask	Jar
B	Jar	Culture flask
C	Lego	Lego

3.3.3 Object Location Test (OLT)

OLT is also a measure for analyzing to which extent mice remember spatial distribution of an object on a hippocampus-dependent manner (39). As for ORT, it is also divided into the same three sessions.

In this case, habituation session lasts one day, and the mouse is put in the box where the test will take place but without any object for 10 minutes, so it can habituate to the environment.

After habituating, the mouse is put in the box for the training for 5 minutes with two identical objects (A+A) in the corners. It is also considered to be object exploration when the orientation of the nose to the object is at a distance of less than 2cm.

To finish with, the test session is performed two hours later of the training (t=2h), and one of the objects is moved to a new position, a different corner, so the mouse is able to explore them for 5 minutes long.

The same way as for ORT, it can be measured the total time the mouse has spent exploring each object, so that by calculating the DI, it can be concluded if it has learnt the spatial location of each of them (DI > 0.2).

$$DI = \frac{New\ Position\ (t) - Old\ Position\ (t)}{(New\ Position\ (t) + Old\ Position\ (t))}$$

For OLT, same environmental conditions as for ORT are followed. However, different objects are used: the bigger jars (10x6cm) in 'Box A' and the smaller ones (6x3cm) in 'Box B'. Each pair of objects is maintained the same for both training and test sessions because it is not a novel object what is being studied, but a novel location.

4. Statistics

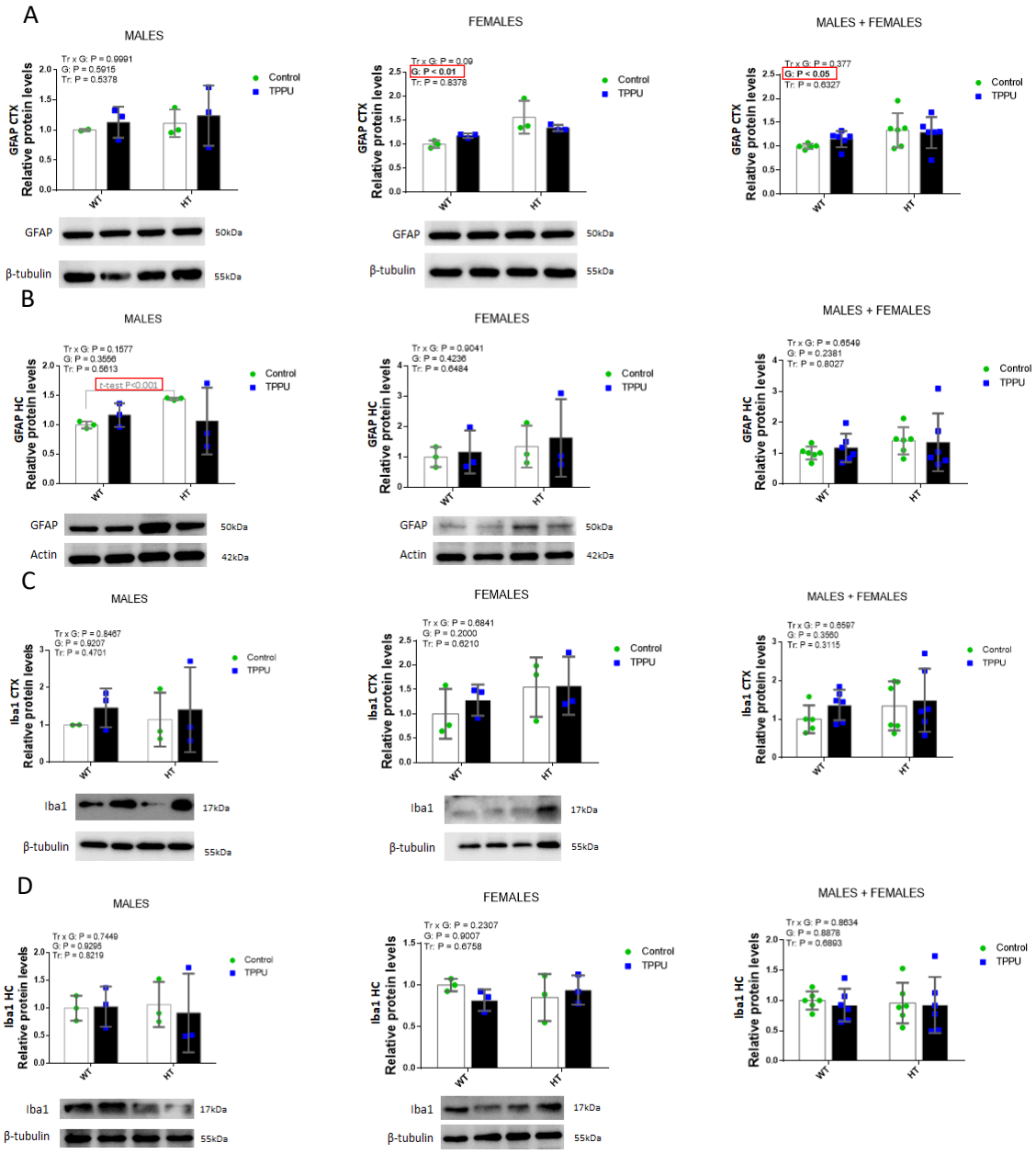
Results obtained from the different studies were analyzed by GraphPad Prism 6.01 package (GraphPad Software, Inc, La Jolla, CA). It was used a Student t test for significance or differences between two groups, and a one-way or two-way ANOVA (depending on the number of variables) followed by Fisher's LSD test for multiple comparisons. As being preliminary results, they are shown as mean ± SD, and differences were considered significant when $P<0.05$.

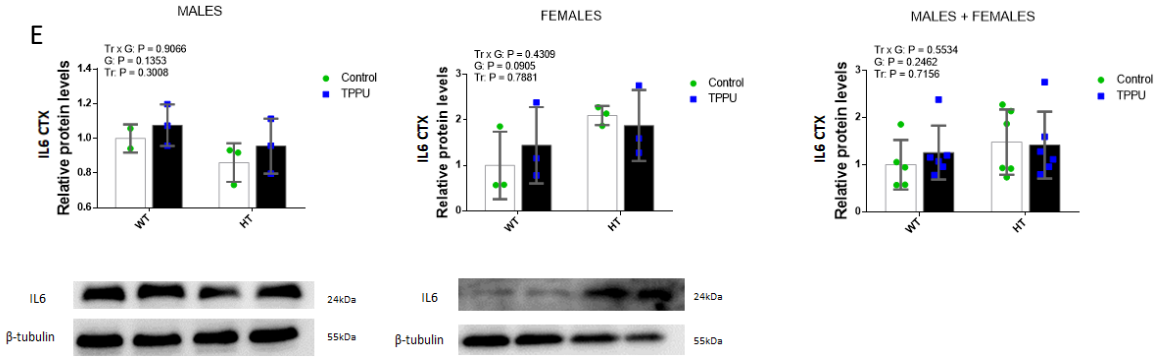
RESULTS

This *in vivo* study aims to determine whether the prenatal inhibition of the sEH by TPPU has a neuroprotective effect on the offspring. For doing so, results obtained from treated 5xFAD mouse model were compared with the control group, and at the same time, with the corresponding WT mice, to ensure the reliability of the AD mouse model. The analysis is performed based on the three study variables: treatment, genotype, and sex. There is need to highlight that, molecular tests are part of a preliminary study, which explains the N of 3 mice per group. On the contrary, behavioral tests are a supplement of a previous project, so treatment is not analyzed, and the N is more variable.

1. *Molecular markers of AD*

1.1 Alterations in protein expression: gliosis, tauopathy and neuronal plasticity

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IL6 CTX
Relative protein levels
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WT
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24kDa
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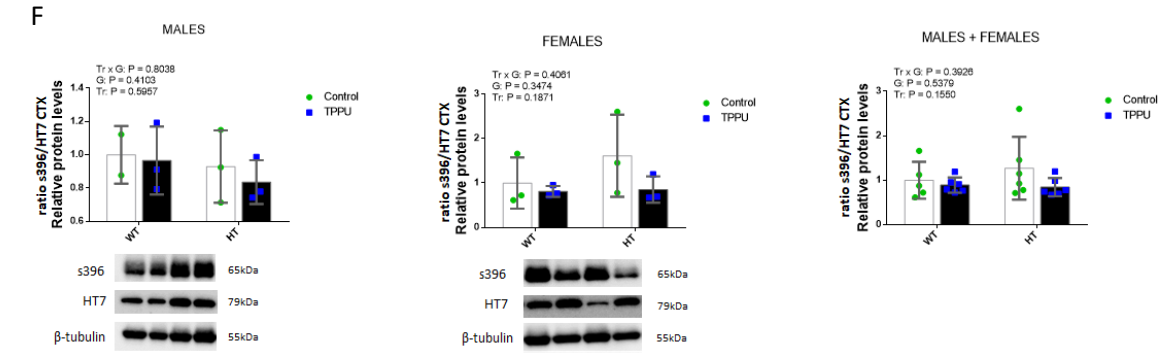
F
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Relative protein levels
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TPPU
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β-tubulin
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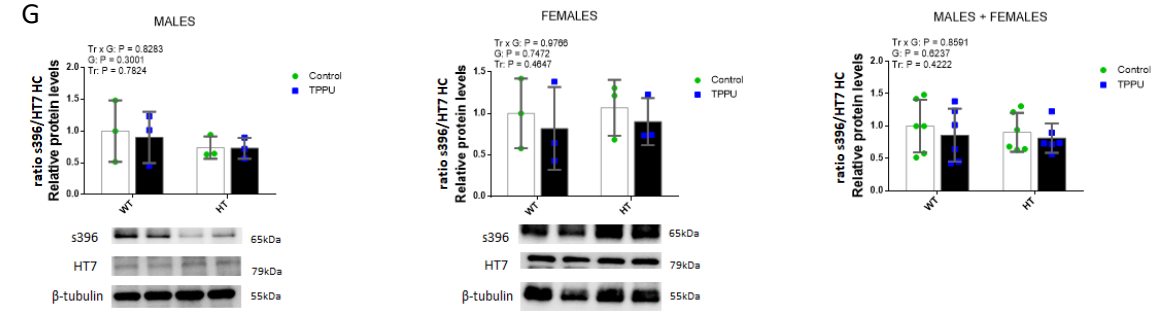
79kDa
55kDa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FEMALES
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ratio s396/HT7 HC
Relative protein levels
Control
TPPU
s396
HT7
β-tubulin
65kDa
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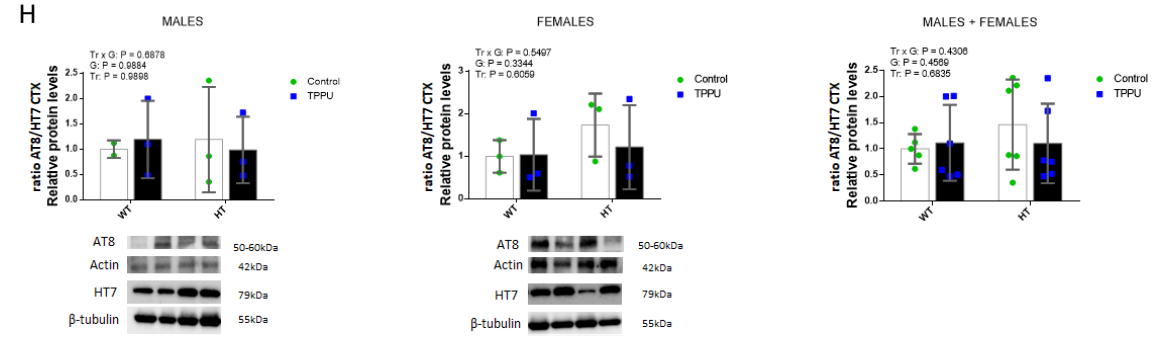
55kDa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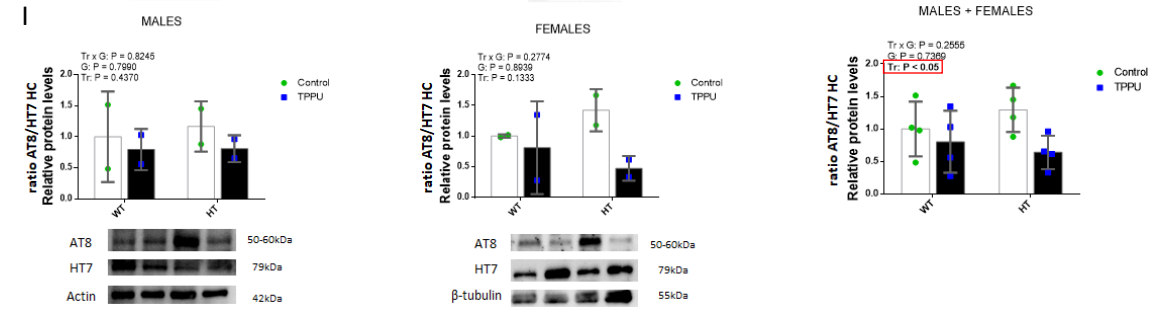
42kDa
79kDa
55kDa


I
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ratio AT8/HT7 HC
Relative protein levels
Tr: P < 0.05
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β-tubulin
50-60kDa
79kDa
42kDa
55kDa

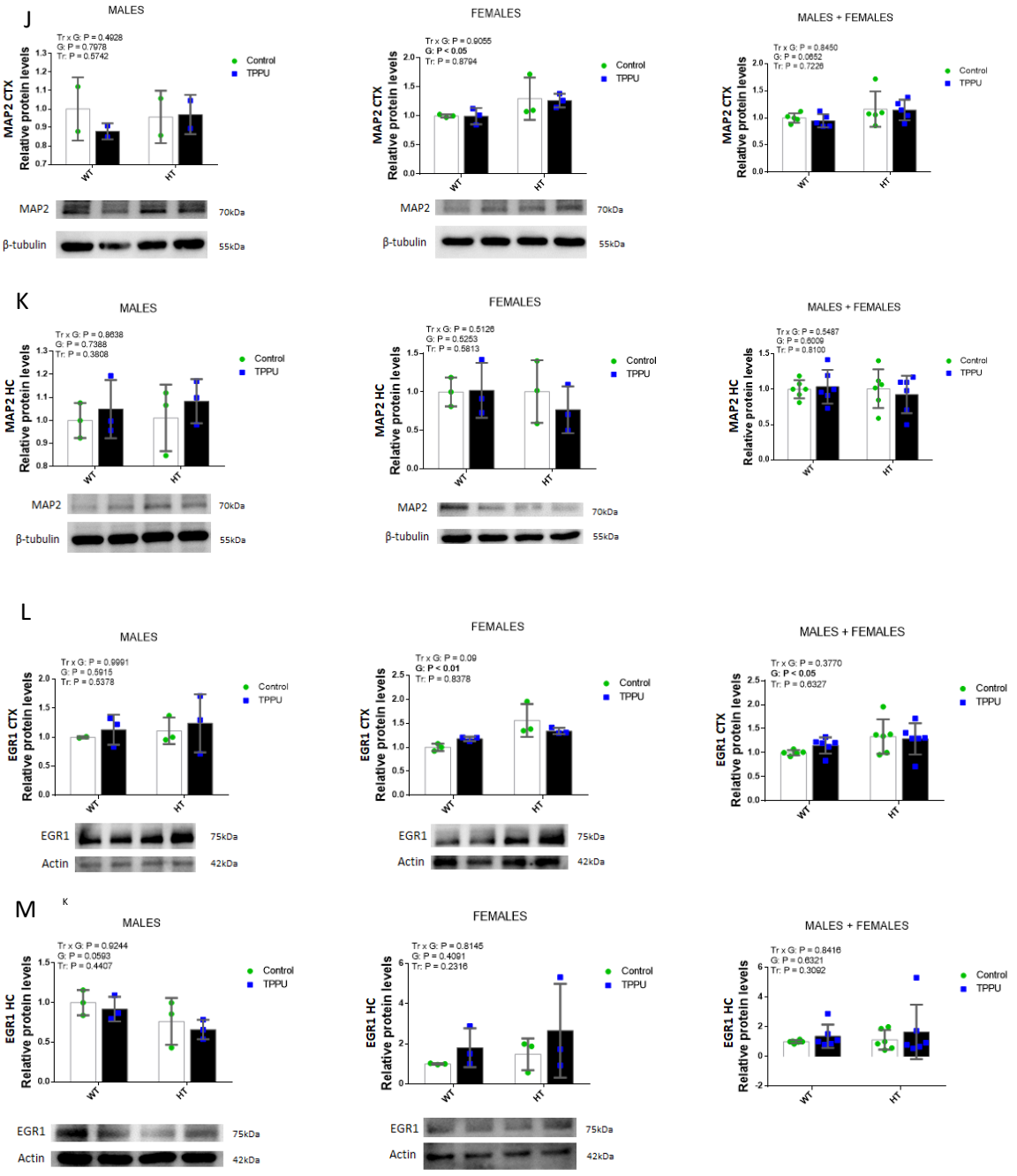

Figure 5. The neuroprotective effects of the inhibition of sEH by TPPU shown in Western Blot results. For each target protein, both cortex and hippocampal tissues are shown, respectively. Effects on glial reactivity are described by GFAP **(A, B)** and Iba1 **(C, D)**; on inflammation by IL6 **(E)**; on phosphorylated tau aggregations by both Ser396/HT7 **(F, G)** and AT8/HT7 **(H, I)** ratios; and, finally, on neuronal plasticity by MAP2 **(J, K)** and EGR1 **(L, M)**. A significant increase was found in the expression level of GFAP of 5xFAD mice for females and the sum of both sexes in cortical tissue, and for males in hippocampus. As for AT8/HT7 ratio, a clear effect of the treatment with TPPU is shown for hippocampal tissue, which makes its expression levels decrease for the sum of sexes. P-values for two-way ANOVA statistical analysis are indicated on the top of each graph: G, genotype main effect; Tr, treatment main effect; Tr x G, interaction effect. Significance level of effects are considered when P<0.05. Student *t*-test analysis was additionally applied for GFAP results to show significance between genotypes in control treated males. N=3 mice/group.

Based on the results of the WB analysis, it can be observed that as mice were only two months old, there were not much significant differences when studying the proteins related to gliosis and inflammation described in figure 5. However, there was a clear tendency on the expression levels of GFAP to be increased in 5xFAD mouse model, comparing to WT mice (mainly in cortex tissue of females and hippocampus of males). A bigger number of samples should be needed to reaffirm this tendency, as it seemed that if taking both sexes together, it still followed this trend (a significant increase was described in the cortex tissue of the sum of both sexes). Furthermore, as far as the study of hyperphosphorylated tau protein is concerned, described by the ratios of s396/HT7 and AT8/HT7 in figure 5, it is shown that TPPU had indeed a neuroprotective effect on AD brains: a well-defined tendency of a decrease on their expression could be seen in both genotypes for treated mice, being the difference significant in hippocampal tissue of both sexes for AT8/HT7 ratio. Finally, even though there were no evident results, neuronal plasticity could be improved by TPPU treatment, as if considering both sexes, it seemed to maintain or even increase the expression of MAP2 and EGR1.

Regarding amyloid aggregations, nor antibody worked on identifying the proteins responsible for causing the pathogenic depositions of Aβ, which are the C-terminal fragment (CT99), and the Aβ peptide as it should be detected by both epitopes 4G8 (residues from 17 to 24) and 6E10 (residues from 3 to 8). It can be concluded that mice may were too young to present Aβ deposits yet, or, at least, not enough to be identified. The following figure shows the WB analysis of the full APP, which had been correctly identified, and the theoretical sizes of the fragments that were supposed to be studied.

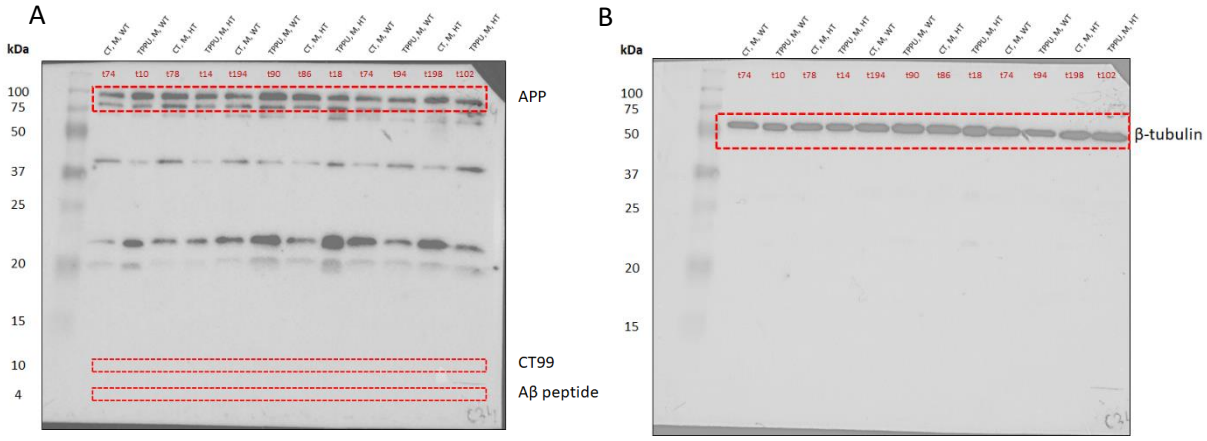

Figure 6. Western Blot analysis results for amyloid proteins are shown. Blot for full APP is shown at the top of the membrane (~100kDa) for each sample. At the bottom, the theoretically corresponding blots are marked for C-terminal fragment of amyloid precursor protein (APP) (CT99; ~10kDa) and for Aβ peptide (~4kDa) **(A)**. Corresponding β-tubulin blot is indicated for the membrane of study **(B)**. For both figures, loaded samples and respective study groups are indicated at the top. N=3 mice/group.

1.2 *TPPU treatment effects on inflammatory gene expression*

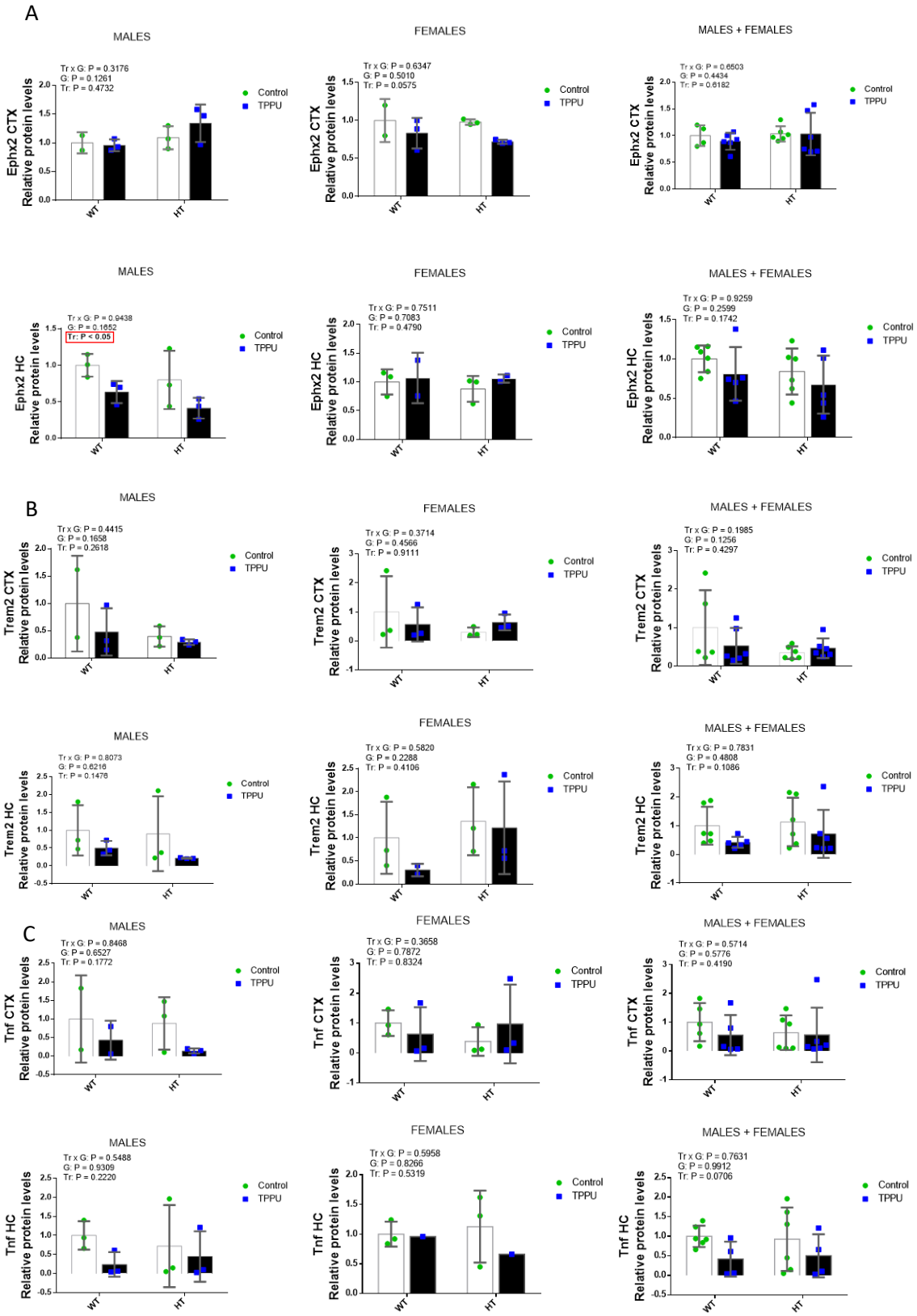

Figure 7. The neuroprotective effects of the inhibition of sEH by TPPU shown in qPCR results. Changes in the gene expression levels are described for *Ephx2* **(A)**, *Trem2* **(B)** and *Tnf* **(C)**. A significant effect of the treatment with TPPU can be seen in the hippocampal expression of *Ephx2* in males, as levels decrease for both genotypes. Two-way ANOVA statistical tests were performed for studying each gene expression and P-values are indicated at the top of the graphics: Tr, treatment main effect; G, genotype main effect; Tr x G, interaction effect. Significant differences are considered when P<0.05. N=3 mice/group.

Based on the results obtained from the qPCRs of the three genes, *Ephx2, Trem2* and *Tnf*, it can be observed a general reduction under the effect of the treatment with TPPU, as no matter the genotype, all of them presented a decreasing tendency on the expression level of treated mice. However, the difference was only significant in the expression of *Ephx2* of hippocampal tissue of males ($P<0.05$). In this case though, it was the gene expression what was being analyzed and not the activation of the enzyme. As for the study of both WB and qPCR, the number of samples per group should be bigger, so that preliminary results or some tendencies can be considered significant changes in the molecular markers.

1.3 Aβ42 level quantification

In the case of ELISA analysis, no data is shown, as except for the standard curve of the kit, no dilution was found appropriate for the samples of this project, and so, it should be further pursued.

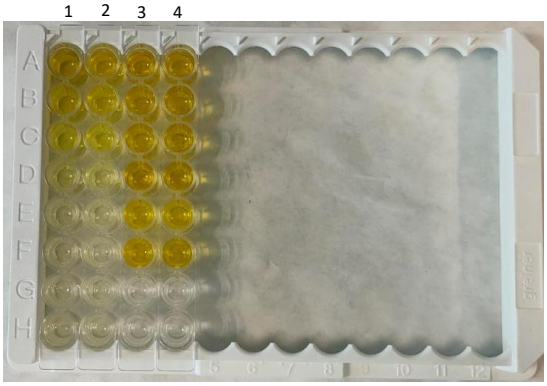

Figure 8. Standard curve used for ELISA assay. Serial dilutions of the reconstituted Hu Aβ42 Standard to 2.000pg/ml (Human Aβ42 ELISA Kit, Invitrogen) were prepared with deionized water for establishing the curve **(1, 2)**. No appropriate dilution of the study samples was found according to the curve **(3, 4)**.

2. *5xFAD mouse model behavior*

2.1 Exploratory activity, anxiety and emotionality

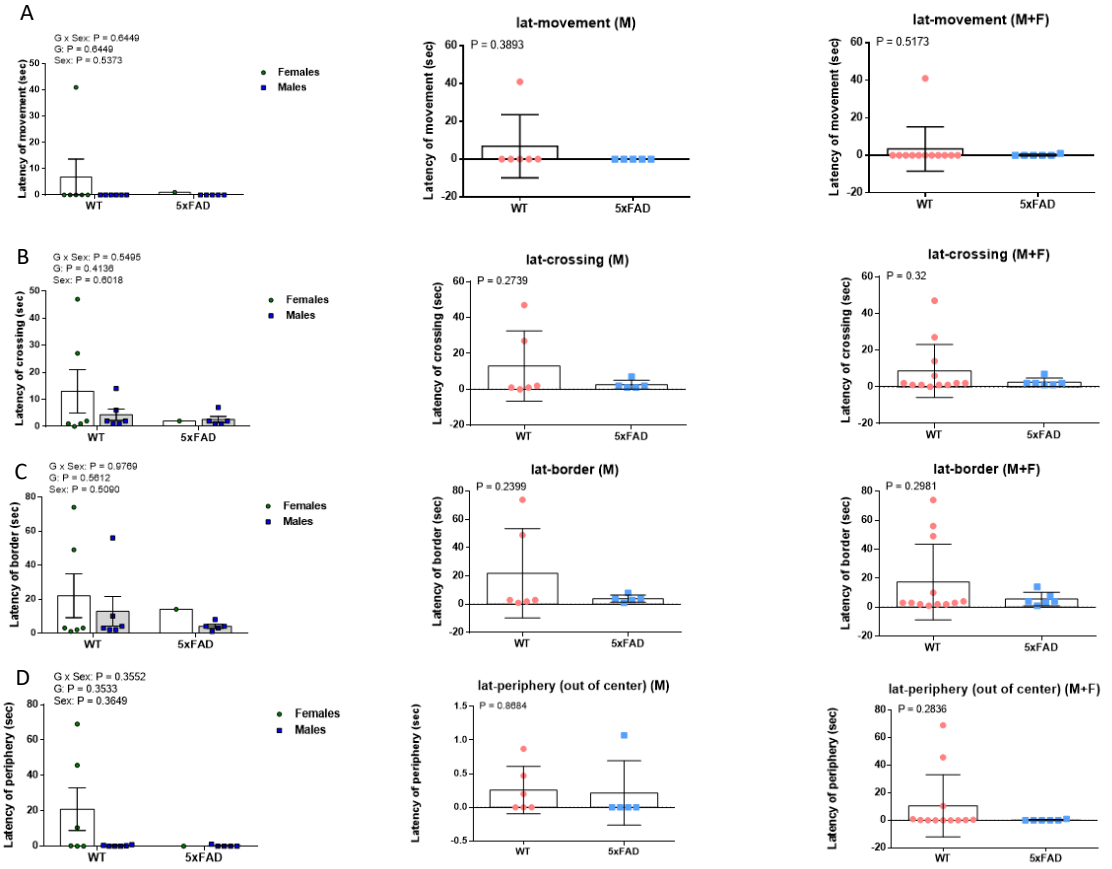

Figure 9. The neuroprotective effects of the inhibition of sEH by TPPU on motor activity of mice. Latency of movement **(A)**, latency of crossing **(B)**, latency of border **(C)** and latency of periphery **(D)** are shown. No significant differences are found, although all of them present a decrease on 5xFAD mouse model, due to a *freezing* phenomenon of a WT mouse **(A, B)**. P-values of two-way ANOVA analysis are indicated at the top of each graph: G, genotype main effect; Sex: sex main effect; G x Sex, interaction effect. Student *t*-test was performed for differences between two groups (WT vs 5xFAD) and P-values are indicated in each graph. M, males; M+F, sum of both sexes. N=6 mice for WT groups; N=1 for 5xFAD females; N=5 for 5xFAD males.

By observing figure 9, which corresponds to the study of motor activity of mice, it can be seen that due to the *freezing* of one of the WT mice (~40sec until it started to move), a notable increase was described in this group comparing to 5xFAD in all the latencies graphed. Apart from that and taking into account that all of them correspond to the control study group, no significant differences were found.

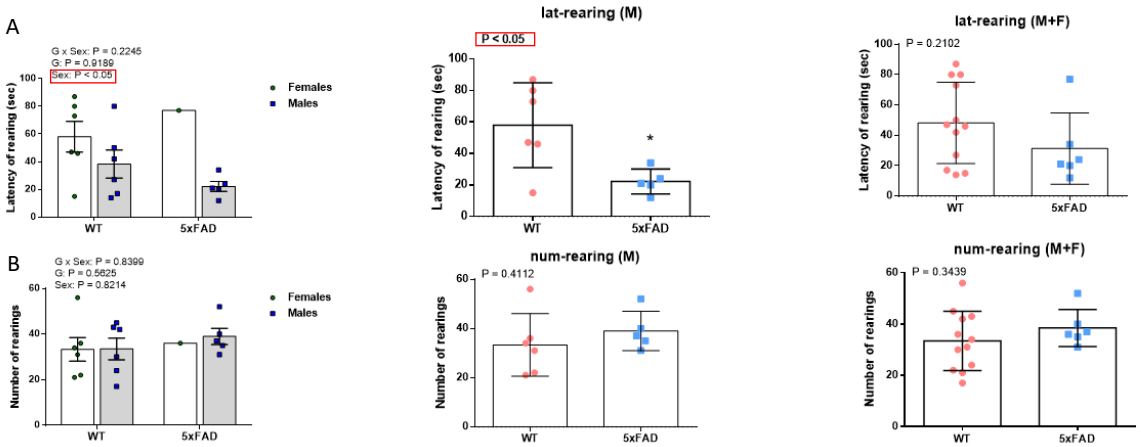

Figure 10. The neuroprotective effects of the inhibition of sEH by TPPU on exploratory activity of mice. The observation of rearing is described by its latency **(A)** and number **(B)**. A significant effect of the sex is shown in the latency of rearing, as females present a higher latency than males in both genotypes. In addition, a significant decrease on the latency of 5xFAD males is shown compared to WT mice. P-values of two-way ANOVA analysis are indicated at the top of each graph: G, genotype main effect; Sex: sex main effect; G x Sex, interaction effect. Student *t*-test was performed for differences between two groups (WT vs 5xFAD) and P-values are indicated in each graph. Significant differences are considered when P<0.05. M, males; M+F, sum of both sexes. N=6 mice for WT groups; N=1 for 5xFAD females; N=5 for 5xFAD males.

The study of the exploratory activity of mice was described by the rearing activity (both latency and number were measured). In the case of the latency, for WT mice seemed to be higher than for 5xFAD, being significantly different particularly in males (P<0.05); and when comparing both sexes by the two-way ANOVA analysis, the significant difference showed a decrease on males when comparing to females. However, the number seemed to be similar between the two groups, even though AD mouse model appeared to make some more rearings.

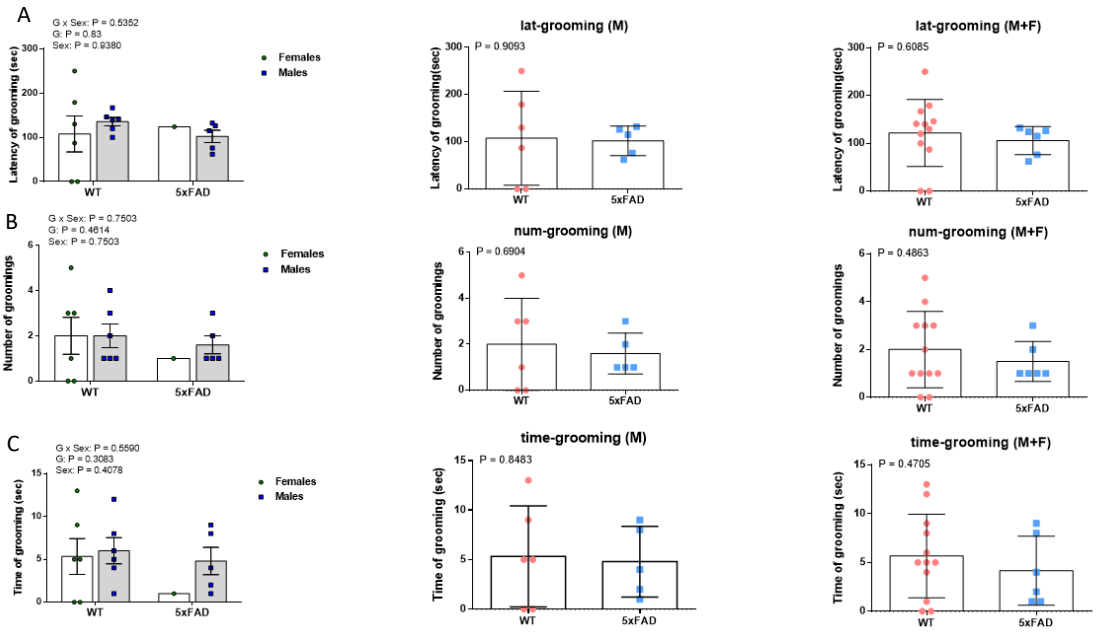

Figure 11. The neuroprotective effects of the inhibition of sEH by TPPU on anxiety of mice. The observation of grooming is described by the latency **(A)**, the number **(B)** and the time **(C)**. No significant changes are shown between study groups. P-values of two-way ANOVA analysis are indicated at the top of each graph: G, genotype main effect; Sex: sex main effect; G x Sex, interaction effect. Student *t*-test was performed for differences between two groups (WT vs 5xFAD) and P-values are indicated in each graph. M, males; M+F, sum of both sexes. N=6 mice for WT groups; N=1 for 5xFAD females; N=5 for 5xFAD males.

Anxiety in behavioral tests was described by the grooming (latency, number and time of grooming were measured). As it is observed in figure 11, no clear differences were apparently seen in this study group, apart from a general slightly decrease in 5xFAD mouse model.

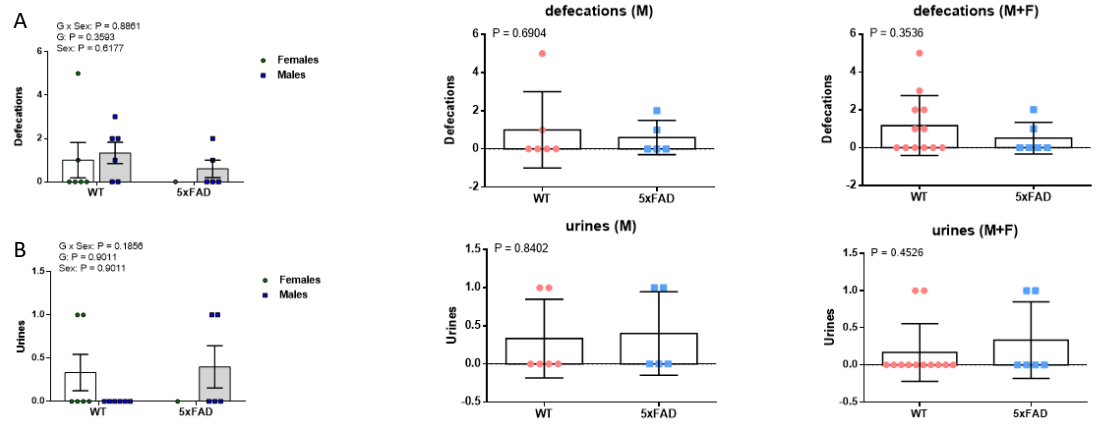

Figure 12. The neuroprotective effects of the inhibition of sEH by TPPU on emotionality of mice. The counting of defecations **(A)** and urines **(B)** is shown. No significant differences are shown. Whereas the number of defecations correspond to the real number counted on the OF test, urines are measured as 1 (presence) or 0 (absence) of urine. P-values of two-way ANOVA analysis are indicated at the top of each graph: G, genotype main effect; Sex: sex main effect; G x Sex, interaction effect. Student *t*-test was performed for differences between two groups (WT vs 5xFAD) and P-values are indicated in each graph. M, males; M+F, sum of both sexes. N=6 mice for WT groups; N=1 for 5xFAD females; N=5 for 5xFAD males.

Regarding the study of emotionality of mice, defecations and urines were counted. The bigger the presence of them, the more nervous mice appeared to be. In this case, both groups seemed to have similar results according to figure 12. No significant differences were found between the genotypes.

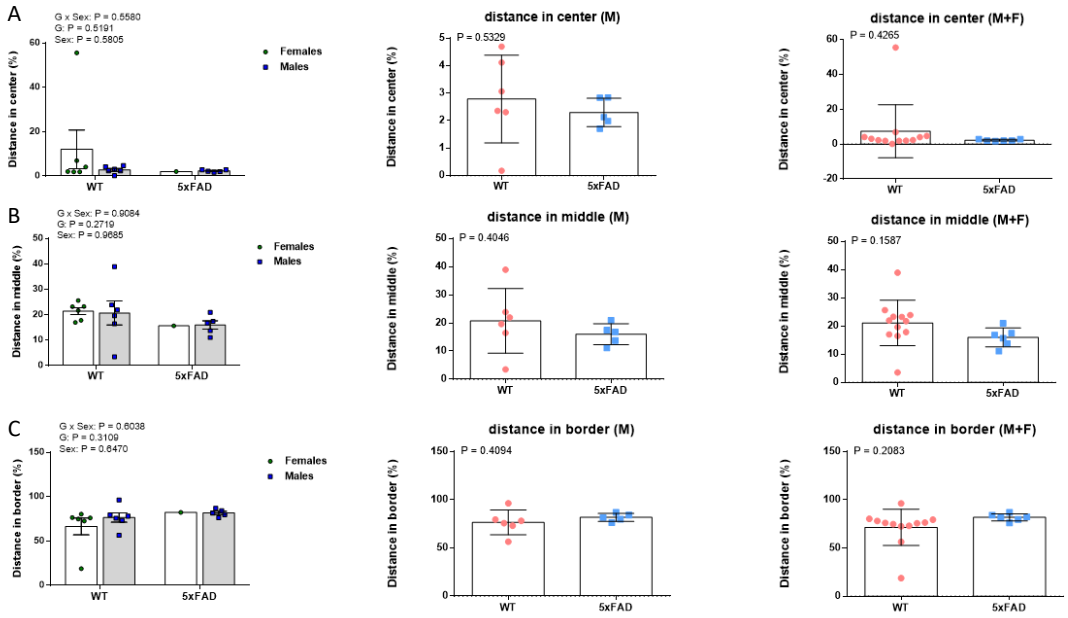

Figure 13. The percentage of the distance covered by the mice on each area of the maze is shown. Distance in the center **(A)**, in the middle **(B)** and in the border **(C)** are described. No significant differences are shown, even though it is observed that the less distance covered is in the central area. P-values of two-way ANOVA analysis are indicated at the top of each graph: G, genotype main effect; Sex: sex main effect; G x Sex, interaction effect. Student *t*-test was performed for differences between two groups (WT vs 5xFAD) and P-values are indicated in each graph. M, males; M+F, sum of both sexes. N=6 mice for WT groups; N=1 for 5xFAD females; N=5 for 5xFAD males.

Observing the distance percentage that mice had covered on each area in relation to the total distance of the maze, it can be concluded that the center was the one where they had been less, compared to the periphery (both middle and border areas). Between these last zones, there was no apparent difference on the distance run.

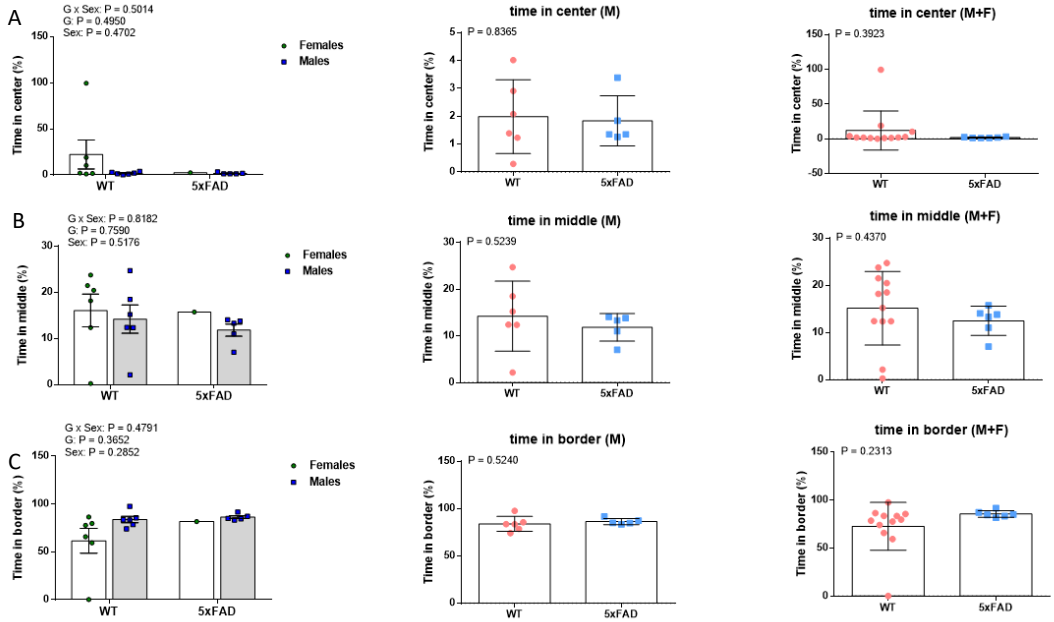

Figure 14. The percentage of the time covered by the mice on each area of the maze is shown. Time in the center **(A)**, in the middle **(B)** and in the border **(C)** are described. No significant differences are shown, even though it is observed that the less time spent is in the central area. P-values of two-way ANOVA analysis are indicated at the top of each graph: G, genotype main effect; Sex: sex main effect; G x Sex, interaction effect. Student *t*-test was performed for differences between two groups (WT vs 5xFAD) and P-values are indicated in each graph. M, males; M+F, sum of both sexes. N=6 mice for WT groups; N=1 for 5xFAD females; N=5 for 5xFAD males.

As for the measure of distance per area of the maze, the percentage of the time spent in each of them was calculated in relation to the total time. Once again, the area where the mice had spent less time was in the center, when comparing to both middle and border zones. These periphery zones were not significantly different between them neither.

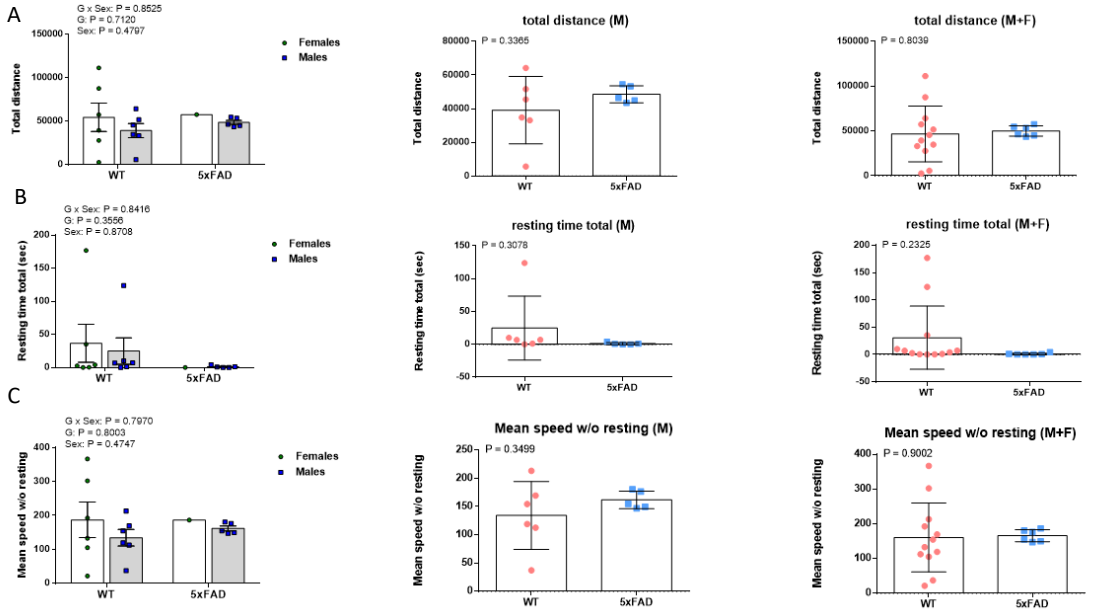

Figure 15. The mean speed of the mice, without considering the resting time, is shown. For calculating so, the total distance **(A)** and the total resting time **(B)** are measured, so that the mean speed can be described **(C)**. No significant differences are shown. P-values of two-way ANOVA analysis are indicated at the top of each graph: G, genotype main effect; Sex: sex main effect; G x Sex, interaction effect. Student *t*-test was performed for differences between two groups (WT vs 5xFAD) and P-values are indicated in each graph. M, males; M+F, sum of both sexes. N=6 mice for WT groups; N=1 for 5xFAD females; N=5 for 5xFAD males.

Finally, the last variable studied for observing the cognitive effects of inhibiting the sEH was the mean speed of mice during the test, without considering the resting time. For doing so, the total distance was measured as well as the total resting time they spent, so that mean could be obtained. Despite not being significant differences between genotypes, 5xFAD appeared to tend to run faster than WT mice, as they seemed not to rest in the whole test while making a bigger distance. This could be due to the willingness of escaping from the box, a sign of nervosity and anxiety that may present the AD mouse model.

2.2 Hippocampal-dependent recognition memory

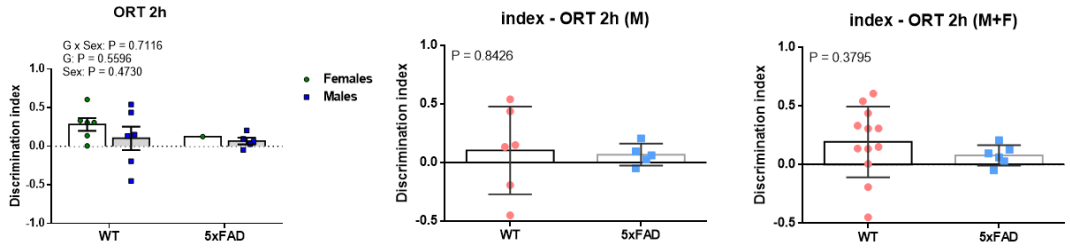

Figure 16. The neuroprotective effect of the inhibition of sEH by TPPU on the hippocampal-dependent recognition memory is described. Results from the test session at 2h of the training of the Object Recognition Test (ORT) are shown. There are no significant differences, although a decrease on the ability to recognize objects can be observed when comparing the 5xFAD mouse model to the WT mice in the three graphs. P-values of two-way ANOVA analysis are indicated at the top of each graph: G, genotype main effect; Sex: sex main effect; G x Sex, interaction effect. Student *t*-test was performed for differences between two groups (WT vs 5xFAD) and P-values are indicated in each graph. M, males; M+F, sum of both sexes. N=6 mice for WT groups; N=1 for 5xFAD females; N=5 for 5xFAD males.

The study of hippocampal-dependent recognition memory was done by performing the ORT test. Results shown in figure 16 correspond to the test session at 2h of doing the training. In this case, no significance differences were observed, but only a tendency on a reduction of the ability to memorize the object in 5xFAD mouse model, compared to the WT mice. In addition, females appeared to remember more than males, even though a bigger number of mice should be studied for determining so. Analysis of the data obtained from the test session at 24h is still being studied for further examination.

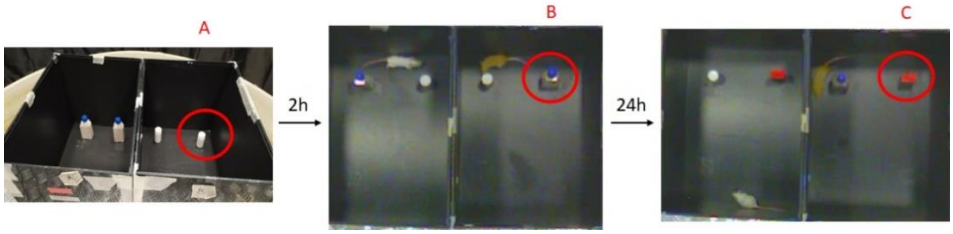

Figure 17. Sessions performed for the Object Recognition Test (ORT). In the first image, training session is shown, in which both objects were identical (A+A) so that mice could familiarize with them. 2h later of the training, the first test session was performed, in which one of the old objects was changed for a novel one (object B). Subsequently, after 24h, object B was replaced once again for a novel one (object C). Figures are taken from the videos recorded with the computerized filming system (SMART v3.0, Panlab S.A.) of two of the WT female mice.

2.3 Hippocampal-dependent location memory

As for the last test session of ORT, the data obtained from OLT test is still being processed. This way, hippocampal-dependent location memory will be further studied.

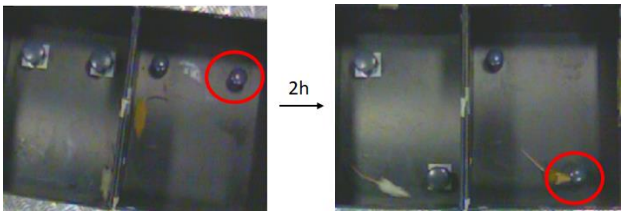

Figure 18. Sessions performed for the Object Location Test (OLT). In the first image, training session is shown, in which both objects were in the same position so that mice could familiarize with them. 2h later of the training, the first test session was performed, in which one of the objects was moved to the opposite corner, in other words, to a novel location. Figures are taken from the videos recorded with the computerized filming system (SMART v3.0, Panlab S.A.) of two of the WT female mice.

DISCUSSION

To this day, no approved treatment has been developed for neurodegenerative diseases, as it is the case of AD. Studies have focused on targeting different biomarkers that play a key role in the pathogenic pathways causing the development of the disease, such as Aβ42 or tau proteins. In fact, a very recent experimental treatment with the drug Aducanumab, that may decrease amyloid deposition in early phases of the disease, sheds some light on possible applications, although its protective effects were reported as very low yet (41,42). However, it is now that, not only these core proteins, but also interacting factors promoting its progressiveness are becoming more and more relevant in the search for an effective therapy.

AD has been shown to be a multifactorial disease, which apart from a possible genetic inheritance, can be developed as a result of various risk factors, predominantly related to age. In fact, prolonged inflammation associated to aging is one of the main factors involved in the development of the disease. This way, inhibition of sEH (enzyme responsible for the increase of pro-inflammatory DHETs) by pharmacological agents like TPPU is being studied as a possible target for alleviating this sustained inflammation, in this case, prenatally. The administration of prenatal TPPU is being shown to be effective at reducing neuroinflammatory factors contributing to other neurodegenerative diseases, such as autism spectrum disorder (ASD) (43,44). Prenatal treatment is being suggested as a therapy for the offspring after maternal immune activation (MIA) caused by prenatal environmental factors (43). Various studies have already suggested associations between MIA and neurodevelopmental disorders like ASD or schizophrenia (43). Despite the precise mechanisms remain still unknown (44), inhibition of maternal sEH seems to play a key role in improving pathogenesis of neurodegenerative disorders of the offspring. All in all, there are not many papers having studied the role of sEH in neurodegenerative diseases in the offspring (43), and that is why it is important to make a further research of it; following the lead of this work, and being the main cause of dementia worldwide, more research on its involvement in AD should be done.

Results from this work have been obtained from two-months-old mice, which explains why, as being so young, there have not been so many significant differences when studying mainly amyloid protein aggregations, and so, subsequent neuroinflammation. 5xFAD mouse model is generated to rapidly produce Aβ42 massive quantities, starting to accumulate them at 1.5 months; but it is at the age of 2 months that it begins to respond to these deposits by the process of gliosis (35). However, a significant difference in the expression levels of GFAP has been interestingly observed, the glial filament protein expressed highly in reactive astrocytes (19). Both in hippocampal and cortex tissues, there has been a clear increase of its expression in the AD mouse model when comparing to WT mice. This way, it seems the response to first plaques of amyloid is being started by astrocytes, as the tendency appears to be maintained. However, inflammation may have not started to be aberrant, as nor pro-inflammatory CKs (IL6, TNFα) are observed to have increased, neither other biomarkers of reactive microglia (Iba1, *trem2*). Despite this, it should be highlighted that a clear tendency has been shown in both gene expression studies of *tnf* and *trem2,* in which a reduction of both have been described when treated prenatally with TPPU. This suggests that even though it is too soon to find significant increments on AD mouse model, prenatal administration of TPPU in both genotypes appears to protect offspring at gene expression level.

Regarding tau pathology, and as a consequence of aberrant signaling pathways caused by Aβ deposits, hyperphosphorylation of tau protein was expected to form aggregates of NFTs, responsible for promoting even more the neuroinflammation. As far as results are concerned,

a well-defined tendency of TPPU treatment's effect can be observed. Both ratios describing tau pathology (Ser396/HT7 and AT8/HT7) have been shown to be decreased in treated mice, when compared to control. In fact, a significant difference can be observed in the hippocampal AT8/HT7 ratio of the sum of both sexes. In this case, 5xFAD mouse model was not supposed to develop NFTs, as this double-transgenic mouse model has not been reported to do so (35). This could explain why there has not been any significant differences between both genotypes, but only on the treatment. Furthermore, as response to amyloid plaques does not seem to be aberrant yet, it may has not impacted on tau phosphorylating pathways at all. This way, inhibition with TPPU may have had more impact on protecting tau from phosphorylating than preventing inflammatory response to start against Aβ42, as at first place, it has an acute protective role.

As for the last AD hallmark, neuronal plasticity is also damaged at early stages of the disease. Nevertheless, and according to the fact that these mice had not reached an aberrant state of neuroinflammation yet, no significant differences have been seen on the results obtained, but only a possible hypothesis that the treatment with TPPU could promote an increase of MAP2 and EGR1. The only significant difference has been seen when comparing both genotypes, as they have increased on the 5xFAD mouse model, but as mentioned before, supposedly due to the lack of activation of corresponding pathways at the age of 2 months.

Finally, considering *Ephx2* gene expression, there has been a significant decrease on treated mice. As commented before, it is not the enzyme activity that is being analyzed, but the gene expression of it, so that it measures the amount of protein present, not the catalyzing action. Being so, results suggest that treatment with TPPU has decreased the gene expression of sEH, which means it protects offspring from generating big amounts of pro-inflammatory DHETs.

Another important fact to mention is that, in most of the results presented, there is a clear trend of females showing more intense pathology than males, with regard to glial and inflammatory markers, tau and amyloid aggregates and neuronal plasticity. It has been shown in studies with the same 5xFAD mouse model, that females show a bigger accumulation of Aβ42 deposits from the very beginning (35), which promotes the impact on the rest of the factors mentioned before, as corroborated in this work.

Before concluding, and as cognitive effects in this work have been only studied for a control group of mice from a previous project, it is not possible to determine the effect of the treatment of TPPU on behavioral improvement. However, it can be affirmed by observing results that 5xFAD mouse model is a reliable model to study cognitive deficits of AD. Even though not finding many significant differences (only on the reduction of the latency of rearing), most results have shown an accordance with what theoretically should be found for this genotype: less ability to memorize objects, a higher mean speed and less exploratory activity (34,35).

To summarize, considering that this preliminary work has been performed with two-months-old mice and with a little number of samples (N=3), promising results could be achieved if studying older mice (4 or 6 months) and a bigger number of samples (at least N=6 for assays like qPCR or ELISA). In addition, more studies should be carried out in order to determine whether the prenatal inhibition of sEH does really play a key role in the cognition and behavior of the offspring. Many studies are focusing now its attention on targeting pathways contributing to the neuroinflammation involved in neurodegenerative diseases, and prenatal studies could be essential for developing therapies for directly affected offspring.

CONCLUSION

Neuroinflammation has been shown to be crucial for the development and progressiveness of neurodegenerative diseases, even though it is still not clear if it contributes as one more factor causing the pathology or as a consequence of it. What can be certainly concluded is that signaling pathways involved in prolonged inflammatory activation do really play a key role in their aggravation. In addition, and considering age-related diseases, the more advanced in age, the worse the molecular mechanisms start to work, promoting this sustained inflammation. Particularly in AD, amyloid aggregations and NFTs have been observed to be the main promoters of this activation.

In this case, the study of the prenatal inhibition of sEH by the pharmacological agent TPPU, which avoids the transformation of EETs to pro-inflammatory DHETs, has been shown to be a novel target for developing future AD therapies. However, despite having studied so far the mechanism of action of the enzyme, there is not enough research on how the prenatal inhibition of it could make offspring to be protected from suffering these type of disorders.

That is why, not only for AD, but also for the other neurodegenerative diseases, further studies should be carried out more focused on those inflammatory common pathways. Chronic inflammation is the most widely contributing factor to every kind of pathology, from neurodegenerative diseases to metabolic disorders. They all are defined as multifactorial diseases, because indeed not only inflammation on itself can cause the disease, but on the contrary it is the one that will perpetuate the wrong functioning of our defenses; and not only on ourselves but also on our descendants.

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