Disease-modifying treatment with I2 imidazoline receptor ligand LSL60101 in an Alzheimer's disease mouse model : A Comparative study with donepezil

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December 19, 2020

Abstract

Background and Purpose: The development of effective therapeutic strategies against Alzheimer's disease (AD) remains a challenge. I2 Imidazoline receptors (I2-IR) ligands have a neuroprotective role in AD. While co-treatment of acetylcholinesterase inhibitors with neuroprotective agents have shown better effects on the prevention of dementia. Here, we assessed the potential therapeutic effect of the I2-IR ligand LSL60101, donepezil and their combination in 5XFAD mice. Experimental Approach: 5XFAD female mice were treated with low doses of LSL60101 (1mg/kg/day), donepezil (1mg/kg/day), and donepezil plus LSL60101 (1+1mg/kg/day), during 4 weeks per os. Novel object recognition, Morris water maze, open field, elevated plus maze and three-chamber tests were employed to evaluate the cognitive and behavioural status of the mice after treatment. The effects of the treatments on AD-like pathology were assessed with immunohistochemistry, Western blot and qPCR. Key results: Chronic low-dose treatment with LSL60101 and donepezil reversed cognitive deficits and impaired social behaviour. LSL60101 treatment did not affect anxiety-like behaviour in contrast to donepezil. In the 5XFAD brains, LSL60101 and donepezil/LSL60101 treatments decreased Aβ-pathology and Tau hyperphosphorylation, and these alterations were accompanied by decreased microglia marker Iba-1 levels and increased Trem2 gene expression. LSL60601 and donepezil decreased glial fibrillary acidic protein (GFAP) astrocytic marker reactivity. However, only LSL60601 treatment significantly increased the levels of the synaptic markers post-density 95 (PSD95) and synaptophysin (SYN). Conclusion and implications: Our results suggest that chronic low dose treatment with selective I2-IR ligands can be an effective treatment for AD and provide insights into combination treatments of symptomatic and disease-modifying drugs

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Running Title: I2 Imidazoline receptor modulation as a potential therapeutic strategy against Alzheimer's Disease.

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WORD COUNT: Main text 6257 words (Introduction, methods, results and discussion) and 3513 words (excluding methods, references and figure legends)

ACKNOWLEDGEMENTS

This study was supported by Ministerio de Economía y Competitividad of Spain and FEDER

(PID2019-107991RB-I00, PID2019-106285RB-I00) and 2017SGR106 (AGAUR, Catalonia). The project leading to these results has received funding from "la Caixa" Foundation (ID100010434), under agreement CI18-00002. F.V. thanks the UB for APIF grant (UB2016); S. R.-A. to Generalitat de Catalunya, (2018FI-B-00227) and A. B. for APIF Grant Institute of Biomedicine (UB2018).

AUTHOR CONTRIBUTIONS

FV, CG-F and MP conceived the study, designed all the experiments, and interpreted the data; FV, CG-F performed the experiments and data analysis; all authors revised the manuscript draft. S R-A, AB and CE synthesized compounds tested; FV, CG-F and MP wrote, revised, and finalised the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for *Design & Analysis*, *Immunoblotting and Immunochemistry* and *Animal Experimentation* and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be available because of privacy or ethical restrictions.

ABSTRACT

Background and Purpose: The development of effective therapeutic strategies against Alzheimer's disease (AD) remains a challenge. I2 Imidazoline receptors (I2-IR) ligands have a neuroprotective role in AD. While co-treatment of acetylcholinesterase inhibitors with neuroprotective agents have shown better effects on the prevention of dementia. Here, we assessed the potential therapeutic effect of the I2-IR ligand LSL60101, donepezil and their combination in 5XFAD mice.

Experimental Approach : 5XFAD female mice were treated with low doses of LSL60101 (1 mg^{-1} $kg^{-1}day^{-1}$), donepezil (1 $mg^{-1}kg^{-1}$ day^{-1}), and donepezil plus LSL60101 (1+1 mg^{-1} $kg^{-1}day^{-1}$), during 4 weeks *per os* . Novel object recognition, Morris water maze, open field, elevated plus maze and three-chamber tests were employed to evaluate the cognitive and behavioural status of the mice after treatment. The effects of the treatments on AD-like pathology were assessed with immunohistochemistry, Western blot and qPCR.

Key results: Chronic low-dose treatment with LSL60101 and donepezil reversed cognitive deficits and impaired social behaviour. LSL60101 treatment did not affect anxiety-like behaviour in contrast to donepezil. In the 5XFAD brains, LSL60101 and donepezil/LSL60101 treatments decreased Aβ-pathology and Tau hyperphosphorylation, and these alterations were accompanied by decreased microglia marker Iba-1 levels and increased *Trem2* gene expression. LSL60601 and donepezil decreased glial fibrillary acidic protein (GFAP) astrocytic marker reactivity. However, only LSL60601 treatment significantly increased the levels of the synaptic markers post-density 95 (PSD95) and synaptophysin (SYN).

Conclusion and implications: Our results suggest that chronic low dose treatment with selective I2-IR ligands can be an effective treatment for AD and provide insights into combination treatments of symptomatic and disease-modifying drugs.

KEYWORDS: I2 Imidazoline receptors, β-amyloid, neuroinflammation, synaptic plasticity, donepezil, Alzheimer's disease

BULLET POINT SUMMARY

What is already known

I2-IR modulation by selective I2-IR ligands delivers neuroprotection in the central nervous system.

AchEI did not modify disease progressions, thereby a new drug for halting AD progression is needed.

What this study adds

LSL60101, an I2-IR ligand, treatment rescued 5XFAD mice from cognitive impairments and modified disease progression.

LSL60101 treatment provides greater effects on AD-hallmarks and synaptic plasticity than donepezil, even in combination.

Clinical significance

Chronic treatment with I2-IR ligands would constitute a relevant therapeutic disease-modifying strategy against AD.

INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia among the elderly and the most common irreversible and uncurable neurodegenerative disorder, clinically characterised by progressive behavioural disturbances and memory loss (Murray et al., 2011).$A\mu\psi\lambda o\iota\delta\,\beta$ (Aβ) plaques and neurofibrillary tangles consisting of hyperphosphorylated Tau (p-Tau) are two major neuropathological AD hallmarks, which lead to synaptic failure (Walsh and Selkoe, 2004; Selkoe, 2008; DeTure and Dickson, 2019). Moreover, the inflammatory response triggered by Aβ deposits and Tau hyperphosphorylation, among others and mediated by activated microglia and reactive astrocytes has a key role in the progression of AD. (Dickson and Rogers, 1992; MERAZ RIOS et al., 2013). Thus, targeting Aβ aggregation, p-Tau, and neuroinflammation has been proved so far, the main disease-modifying strategy for treating AD.

However, up to date, only symptomatic treatments, including the*acetylcholinesterase*inhibitors (AChEI) and the N-methyl-D-aspartate*receptor*antagonists are available for AD therapy. Those drugs showed modest symptomatic benefits on behaviour and cognition but they did not halt its progression(Grossberg, 2003; Mehta et al., 2012). Among AChEI, donepezil is clinically used for cognitive dysfunction in AD (Giacobini, 2000). Besides its main effects related to the enhancement of cholinergic transmission, donepezil has been demonstrated to exert the potential for disease pathway modifications in AD, including attenuation of Aβ load and anti-inflammatory properties *in vitro* and *in vivo* (Kim et al., 2014). However, at a clinical level, it lacks a curative effect, thereby the identification of new molecular targets for the development of treatments is crucial. In this context, to further enhance the non-cholinergic therapeutic effects of donepezil, a combination of donepezil with other neuroprotective agents could provide a novel approach to preserve the cognitive function and/or delay AD pathology.

I2 imidazoline receptors (I2-IR) are receiving growing attention due to the neuroprotective effects in the central nervous system (CNS) (Bousquet et al., 2020). In the brain, I2-IR are found in both neurons and glial cells (Regunathan et al., 1993; Olmos et al., 1994), and their modulation has been associated with neurodegenerative disorders, including AD (Ruiz et al., 1993). Most notably, the density of I2-IR was found increased in AD patients (Garcia-Sevilla et al., 1998). Several lines of evidence provided by our group demonstrated that selective I2-IR ligands protected against cognitive impairment ameliorating

AD pathological features related to APP processing, Tau hyperphosphorylation, neuroinflammation and oxidative stress (OS) processes, using well- established AD animal models (Abás et al., 2017; Griñán-Ferré et al., 2019; Abás et al., 2020; Vasilopoulou et al., 2020b). Likewise, *agmatine*, the proposed endogenous ligand for I2-IR, prevented cognitive deficits in Aβ 1-42 peptide injected mice and of note its effect was augmented and attenuated by I2-IR agonists and antagonists respectively (Kotagale et al., 2020). Collectively, this evidence supports the potential therapeutic effect of I2-IR ligands in AD.

Among the I2-IR ligands, the selective I2-IR ligand LSL60101 [2-(2-benzofuranyl)imidazole)] (Ki ratio for α_2/I2-receptors=286) has been associated with the induction of several central effects, such as acute hyperphagic effects (Menargues et al., 1994), inhibition of the development of opioid-induced tolerance and potentiation of morphine analgesia (Boronat et al., 1998). Interestingly, LSL60101 was shown to promote neuronal protection mediated by the induction of reactive astrocytes (Casanovas et al., 2000). However, the neuroprotective effect of LSL60101 on AD pathological conditions has not been reported.

In the present *in vivo* study, we explored the I2-IR ligand LSL60101 beneficial effects on the behavioural capabilities and cognitive impairments presented in AD, as well as on AD hallmarks, including neuroinflammation, glial reactivity and synaptic plasticity by using the 5XFAD mouse model, a widely accepted transgenic mouse model for early-onset AD. Additionally, the comparative effect with *donepezil*, considered a symptomatic AD treatment, was investigated alone and in combination therapy with the I2-IR ligand LSL60101 to decipher joint effects of both compounds in ameliorating AD pathology and molecular changes presented by 5XFAD mice.

2. METHODS

2.1 Animals

The 5XFAD mouse model is a well-characterised double transgenic APP/PSEN1 model, which co-expresses 5 familial AD mutations. This animal model incorporates AD pathological characteristics including early plaque formation and gliosis starting at 2 months, robust cognitive and behavioural deficits such as memory impairment, reduced anxiety and social disturbances starting at 4-5 months, and neuronal loss at 6 months. (Oakley et al., 2006; Landel et al., 2014; Griñán-Ferré et al., 2018). Thus, at the selected age of 7 months, 5XFAD mice provide a severe AD pathological landscape suitable for the evaluation of the drug effects.

In the present study, 5XFAD (n = 47) and Wild-Type (WT, n = 46) female mice (7-month-old) were used to perform behavioural and molecular analyses. Females were used because AD incidence is higher in women and few studies are available. WT animals were randomly divided into WT Control (**WT Ct**) (n=11), WT treated with donepezil (1 mg^{-1} kg^{-1} day^{-1}) (**WT Dp**) (n=12), LSL60101 (**WT LSL**) (1 mg^{-1} kg^{-1} day^{-1}) (n=12), and the co-treatment donepezil (1 $mg^{-1}kg^{-1}$ day^{-1}) and LSL60101 (1 mg^{-1} kg^{-1} day^{-1}) (**WT Dp+LSL**). 5XFAD mice were randomly divided into: 5XFAD Control (**5XFAD Ct**) (n=11), 5XFAD treated with Donepezil (1 mg^{-1} kg^{-1} day^{-1}) (**5XFAD Dp**) (n=12), LSL60101 (**5XFAD LSL**) (1 mg^{-1} kg^{-1} day^{-1}) (n=12), and the co-treatment Donepezil (1 $mg^{-1}kg^{-1}$ day^{-1}) and LSL60101 (1 mg^{-1} kg^{-1} day^{-1}(**5XFAD Dp+LSL**). The animals had free access to food and water and were kept under standard temperature conditions (22 ± 2°C) and 12-h/12-h light/dark cycles (300 lux/0 lux). Compounds were dissolved in 1.8% (2-hydroxypropyl)-β-cyclodextrin and administered through drinking water for 4 weeks. Control groups received water plus 1.8% (2-hydroxypropyl)-β-cyclodextrin during the treatment period. The sample size for the intervention was chosen following previous studies in our laboratory and using one of the available interactive tools (*http://www.biomath.info/power/index.html*). Moreover, the animal number mismatch among experimental groups was due to the exclusion of mice by death or ethical reasons according to the final point indicated in the approved protocol.

After 4 weeks of treatment, behavioural and cognitive tests were performed to study the effects of treatment on learning, memory, anxiety behaviour and social interaction (Fig. 1a). Weight and water consumption were controlled each week, and compounds concentrations were adjusted accordingly to reach the optimal dose until the euthanasia. All studies and procedures for the mouse behaviour tests, brain dissection and extractions followed the ARRIVE (Lilley et al., 2020) and standard ethical guidelines (European Commu-

nities Council Directive 2010/63/EU and Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research, National Research Council 2003) and were approved by Bioethical Committees from the University of Barcelona and the Government of Catalonia.

2.2 Behavioural tests

2.2.1 Novel Object Recognition Test (NORT)

A modification of NORT protocol was performed (Ennaceur and Delacour, 1988). In brief, mice were placed in a 90° two-arm (25 x 20 x 5 cm) black maze, with removable walls for easy cleaning and light intensity in mid-field was 30 lux. Before the memory, trials mice were habituated to the apparatus for 10 min for 3 days. On day 4, the animals were submitted to a 10 min acquisition trial, in which they were allowed to freely explore two identical objects located at the end of each arm (First trial-Familiarization). After 2h (for short-term memory evaluation) and 24h (for long-term memory evaluation) from the first trial, the mice were submitted to a 10 min retention trial, in which one of the two identical objects had been replaced by a novel one. The behaviour was recorded, and the time spent that the mice spent exploring the new object (TN) and the old one (TO) were measured manually. Exploration was defined as sniffing or touching the objects with nose and/or forepaws. The discrimination index (DI) was calculated as (TN-TO)/(TN+TO). To avoid object preference biases, objects were alternated. 70% EtOH was used to clean the arms and objects after each trial for the elimination of olfactory cues.

he Morris water maze test (Morris, 1984) was used to examine the learning and memory abilities of different subgroups of mice. The mice undergoing the visual platform training phase were first placed into the water from the opposite side of the visual platform and the elapse latency was recorded. Mice that could not find the platform within 60?seconds were guided to the visible platform. The elapse latency was recorded as 60?seconds and the mice remained for 30?seconds to adapt them to the experimental environment. The spatial reference memory test phase could be further divided into training period and exploration period. The mouse did not find the platform within 60?seconds, it was led to the hidden platform, the elapse latency was 60?seconds, and the mouse had to stay for 30?seconds. In addition, the path through which the mouse passed was observed. The exploration period was: 24?hours after the last training, following which the platform was removed from the pool. All mice were tested for 60?seconds, and the time they first crossed the hidden platform and the swimming path were recorded and analyzed

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2.2.2 Morris Water Maze (MWM)

The MWM test was performed as described previously (Grinan-Ferre et al., 2016) in an open circular pool, filled with water which temperature was maintained at 22 degC +- 1. The water surface was divided into four quadrants (Q1, Q2, Q3, and Q4) by two principal perpendicular axes, and five starting points were set (1,2,3,4). Four visual clues were placed on the walls of the tank (1,2,3,4). The animals' swimming paths were recorded, and the data were analysed with SMART version 3.0 software. On day 1, mice were placed individually into the pool, facing the wall, and allowed to swim for 60 seconds in order to be habituated to the experimental conditions. On day 2, a white platform was submerged 1.5 cm below the water level in the middle of the Q1 platform and the acquisition phase took place for 5 days. Each day the animals were submitted to five trials starting from the positions set in random order. At each trial mice were allowed to swim for 60 seconds and if not able find the platform within 60 seconds were guided to the visible platform. The mice remained for 30s onto the platform for spatial orientation. There was no resting phase between each trial and the subsequent one. 24h after the last training, a memory test was performed after the platform was removed from the pool in which the mice were tested for 60s. The distance to target, and the time spent in platform quadrant (Q1), among other parameters, were measured.

2.2.3 Open Field (OF)

Emotional alterations and locomotor activity were evaluated by the OF test using a white plywood apparatus (50 x 50 x 25 cm) as previously described (Archer, 1973; Grinan-Ferre et al., 2016).The apparatus' ground was divided into the center and peripheral area. Each individual was placed at the centre of the open field and allowed to explore the apparatus for 5 min. The apparatus was cleaned with 70% ethanol after between trials. The behaviour was recorded and later analysed with SMART ver. 3.0 software (Panlab). The locomotor activity of the mice calculated as the sum of total distance travelled in 5 min, the centre stay duration and the number of rearings were evaluated.

2.2.4 Elevated Plus Maze (EPM)

Animals were tested for anxiety-like behaviour by performing the EPM test, based on a previously described protocol (Walf and Frye, 2007). The EPM apparatus consisted of two open arms (30 x 5 x 15 cm) and two closed arms (30 x 5 x 15 cm). The mice were placed at the junction of the arms and allowed to explore the apparatus for 5 min freely. EPM apparatus was cleaned with 70% ethanol between tests. The behaviour was recorded and later analysed with SMART ver. 3.0 software (Panlab). Parameters recorded included the total distance travelled during the 5 min test, the time spent in open arms, closed arms, and centre, as well as the number of rearings.

2.2.5 Three-Chamber Test (TCT)

Social behaviour of the mice was evaluated by the TCT following a previously described protocol (Companys-Alemany et al., 2020). A box (15x15x20 com) divided into three equally dimensioned rooms with openings among them was used. The mice were submitted to 15-min trials. First, each mouse was placed in the centre of the box and allowed to explore the three chambers for 5 min (habituation). The entries to each room were measured manually. Afterwards, an intruder (same-sex and age) was placed in a metal cage at one of the rooms, and behaviour was recorded for 10 min. The time spent in each room and the time interacting with the intruder (e.g., sniffing, grooming) were measured manually. The TCT apparatus was cleaned with 70% ethanol between the trials to eliminate olfactory cues.

2.3 Brain processing

Mice were euthanised by cervical dislocation 3 days after the behavioural and cognitive tests were completed. The brains were immediately removed from the skulls, and the hippocampi were dissected, frozen and maintained at -80degC. For immunohistochemistry (IHC) experiments, mice were anesthetised (ketamine 100 $mg^{-1}kg^{-1}$ and xylazine 10 $mg^{-1}kg^{-1}$), intraperitoneally and then perfused with 4% paraformaldehyde

(PFA) diluted in 0.1 M phosphate buffer solution intracardially. Their brains were removed and postfixed in 4% PFA overnight at 4degC. Afterwards, the solutions were changed to PFA + 15% sucrose. Finally, the brains were frozen on powdered dry ice and stored at -80degC until sectioning.

2.4 Protein levels determination by Western blotting

For protein extraction, tissue samples were homogenised in lysis buffer containing phosphatase and protease inhibitors (Cocktail II, Sigma-Aldrich). Total protein levels were obtained, and protein concentration was determined by the method of Bradford. For WB, aliquots of 15 µg of hippocampal protein were used. Protein samples were separated by Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) (8-16%) and transferred onto Polyvinylidene difluoride (PVDF) membranes (Millipore). Afterwards, membranes were blocked in 5% bovine serum albumin (BSA) in 0,1% Tris-buffered saline - Tween20 (TBS-T) for 1h at room temperature, followed by overnight incubation at 4°C with the primary antibodies listed in Supplementary Table S1. Membranes were washed and incubated with secondary antibodies for 1h at room temperature. Immunoreactive proteins were viewed with a chemiluminescence-based detection kit, following the manufacturer's protocol (ECL Kit; Millipore) and digital images were acquired using a ChemiDoc XRS+ System (BioRad). Semi-quantitative analyses were carried out using ImageLab software (BioRad), and results were expressed in Arbitrary Units (AU), considering control protein levels as 100%. Protein loading was routinely monitored by immunodetection of glyceraldehyde-3-phosphate dehydrogenase (GADPH) or β-actin.

2.5 RNA extraction and gene expression determination

Total RNA isolation from hippocampal samples was performed using the TRIzol® reagent according to the manufacturer's instructions (Bioline Reagent). The yield, purity and quality of RNA were determined spectrophotometrically with a NanoDropND-1000 apparatus (Thermo Scientific) and an Agilent 2100B Bioanalyzer (Agilent Technologies). RNA samples with 260/280 ratios and RINs higher than 1.9 and 7.5, respectively, were selected. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed. Briefly, 2 µg of messenger RNA (mRNA) was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems).

SYBR® Green real-time PCR was performed using a Step One Plus Detection System (Applied-Biosystems) with SYBR® Green PCR Master Mix (Applied-Biosystems). Each reaction mixture contained 6.75 µL of complementary DNA (cDNA) (with a concentration of 2µg), 0.75 µL of each primer (with a concentration of 100nM), and 6.75 µL of SYBR® Green PCR Master Mix (2x).

The data were analysed utilising the comparative cycle threshold (Ct) ($\Delta\Delta$Ct) method, in which the levels of a housekeeping gene are used to normalize differences in sample loading and preparation. Normalization of expression levels was performed with β-actin. The primer sequences and TaqMan probes used in this study are presented in Supplementary Table S2. Each sample was analyzed in duplicate, and the results represent the n-fold difference in the transcript levels among different groups.

2.6. Glial immunohistochemical identification

Brain coronal sections of 30 µm were obtained (Leica Microsystems CM 3050S cryostat, Wetzlar, Germany) and kept in a cryoprotectant solution at -20°C until use. Free-floating slices were placed in a 24-well plate and washed with 0.01M PBS. Next, the free-floating sections were blocked with 0.1M PBS solution containing 1% BSA, 0,3% Triton X-100 for 20min at room temperature. Afterwards, slices were washed with PBS 0.01M two times for 5 min each and were incubated with the primary antibodies listed in Table X overnight at 4°C. The primary antibodies were diluted in a 0.1M PBS solution containing 1% BSA and 0.3% Triton x-100. On the following day, the coronal slices were washed with 0.1M PBS 0.1M 2 times for 5 min each and then incubated with the secondary antibodies listed in Supplementary Table S1 at room temperature for 1h. Later, the sections were washed 2 times for 5 min each with 0.1M PBS and were incubated with 5µM Hoechst staining solution (Sigma-Aldrich, St. Louis, MO) for 5 min in the dark at room temperature. After being washed, the slices were mounted using Fluoromount-G (EMS, USA).

2.7. Αμψλοιδ β πλαχυες ηιστολογψ

Aβ plaques were stained with Thioflavin-S. Brain coronal sections of 30 μm were obtained (Leica Microsystems CM 3050S cryostat, Wetzlar, Germany) and kept in a cryoprotectant solution at -20°C until use. Free-floating slices were placed in a 24-well plate and washed with 0.01M PBS for 5 min at room temperature to be rehydrated. Next, the brain sections were washed with 70% ethanol for 1 min followed by a wash with 80% ethanol for 1 min. The slices were then incubated with 0.3% Thioflavin-S (Sigma-Aldrich) solution for 15 min at room temperature in the dark. Afterwards, the samples were washed using 80%, 70% and 50% EtOH for 1 min each. Three 2-min washes with 0.1M PBS and the slices were mounted using Fluoromount-G (EMS, USA).

2.8 Image acquisition and analysis

Image acquisition was performed with a fluorescence laser microscope (Olympus BX51, Germany) using 4X, 10X, 20X objectives, and images were analysed using Imaje J software as previously described. For quantification of amyloid plaques similar and comparable histological areas were selected, focusing on the adjacent positioning of the whole cortical area and the hippocampus. The images were converted to 8-bit grayscale images, thresholder within the linear range and the number of particles (Analyse particle function 10-Infinity), as well as the percentage of area covered by Thioflavin-S (20X objective), was calculated and averaged from two different measurements per animal. Glial fibrillary acidic protein (GFAP) and Iba-1 stained images (10X) were acquired, maintaining constant exposure for all samples across single experiments. The fluorescence intensity of the positive cells was measured in DG, CA1 and CA3 areas of the hippocampus. Quantification was averaged from two to three different measurements.

2.9 Statistical Analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). Group size may vary according to power analysis and expertise of the authors regarding the behavioural tests (Griñan-Ferré et al., 2016; Griñán-Ferré et al., 2018) and statistical analysis was undertaken only for studies where each group size was at least n=5. Blinded analysis was performed for behavioural test.All data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was conducted using GraphPad Prism version 8 statistical software. All data were tested for normal distribution and equal variance. In the cognitive and behavioural studies, means were compared with two-way ANOVA. In molecular studies, means were compared with two-tailed Student's t-test (WT Control vs 5XFAD Control) or one-way ANOVA followed by Tukey's post hoc tests (5XFAD Control vs 5XFAD treated groups). Statistical significance was considered when P values were <0.05. Statistical outliers were determined with Grubbs' test and when necessary were removed.

2.10 Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.

RESULTS

I2-IR ligand LSL60101 and donepezil improve memory deficits in 5XFAD mice.

Short- and long-term working memory were evaluated by NORT. 7-month-old 5XFAD mice presented robust cognitive deficits when compared to WT. LSL60101 treatment resulted in a rapid and sustained recovery of cognitive function by increasing the DI in both 2h and 24h memory tests (Figure 1b-c). Donepezil enhanced but did not sustain memory function in 5XFAD mice, as a significant increase of the DI was found after 2h, but not at 24h memory test (Figure 1c). Co-treatment did not improve cognition in comparison with individual treatments (Figure 1b-c). Treatments had no significant effects on WTs cognitive performance (Figures 1b-c).

For spatial learning and memory evaluation, the MWM was performed. After 5 days of training, all experimental groups presented curves with progressively shorter path length on consecutive days. Of note, the path length to the platform was significantly decreased in LSL60101 treated 5XFAD mice when compared to 5XFAD controls (Figure 1d). In the probe trial, 5XFAD mice showed a decreased percentage of time spent in the platform quadrant while the mice spent significantly more time in the quadrant opposite to the platform (Figure 1e,1h). LSL60101 treatment significantly increased the time spent in the platform quadrant in the 5XFAD treated mice when compared to both vehicle and donepezil treated 5XFAD, whereas LSL60101 treatment had no effect on WTs (Figures 1e, 1f,1h). Neither donepezil nor co-treatment improved 5XFAD mice spatial memory (Figures 1d, 1e). Although WT treated mice performed better (Figures 1d,1f,1h). All treatments decreased the path length to the platform albeit not significantly, due to different performance of individual mice (Figure 1g).

I2-IR ligand LSL60101 does not affect behavioural and emotional disturbances in 5XFAD mice in contrast to donepezil.

We also investigated the effect of the treatments on the 5XFAD and WT mice anxiety-like behaviour by performing the OF and EPM tests. No differences in locomotor activity were observed among the WT and 5XFAD groups (Figure 2a). 5XFAD mice presented a significant increase in the time spent in the centre of the open field compared to WT mice (Figure 2b). No effect was observed on the WT mice behaviour after treatments. 5XFAD treated with donepezil but not with LSL60101 showed a significant decrease in the time spent in the centre compared to 5XFAD controls, reverting to the WT healthy phenotype (Figure 2b). Co-treatment LSL60101/donepezil displayed the same results that showed donepezil alone, in all parameters evaluated (Figure 2b and Supplementary table S3). Similarly, in the EPM, 5XFAD mice spent significantly more time in the open arms and less in the closed arms in comparison with age-matched WTs (Figures 2d, 2e). Donepezil had a positive effect by reverting the 5XFAD EPM parameters evaluated to those showed by WT group (Figures 2d-f, Supplementary table S4). I2-IR ligand treatment alone did not affect any of the EPM parameters studied, whereas co-treatment maintained the donepezil values. Treatments did not induce significant changes in EPM parameters evaluated in WT mice (Supplementary Table S4).

3.3 I2-IR ligand LSL60101 and donepezil ameliorate social deficits presented by 5XFAD mice.

To evaluate the effect of treatments on social behaviour, the mice were subjected to the TCT. No differences in the number of the entries to each chamber were determined in the habituation phase in any tested group (Figure 2g). In contrary mice spent more time in the intruder's chamber during the test phase in all experimental conditions (Figure 2h). When the social interaction was evaluated, 5XFAD mice spent significantly less time interacting with the intruder when compared to WT healthy control. All treatments improved social impairments in 5XFAD treated groups by increasing the time of interaction compared to the 5XFAD controls, but only 5XFAD treated with donepezil reached significance (Figure 2i), whereas for LSL60101 $p<0.06$ was calculated. WT treated mice presented no differences compared to WT controls.

3.4 I2-IP λιγανδ ΛΣΛ60101, βυτ νοτ δονεπεζιλ, ρεδυχες Αβ πλαχυες. Βψ χοντραστ, δονεπεζιλ/ΛΣΛ60101 αττενυατε Αβ πατηολογψ ιν 5ΞΦΑΔ μιχε.

The number of amyloid plaques in 5XFAD mice was assessed by histochemical staining with Thioflavin-S. Following treatment with LSL60101, there was a significant decrease in the total number and area of the plaques in 5XFAD mice when compared to the 5XFAD controls, demonstrating a neuroprotective function of I2-IR ligand regarding the senile plaque formation. 4-week-treatment with donepezil did not reduce the amyloid deposition significantly in 5XFAD mice (Figure 3a-3c). The protein levels of Aβ determined by WB tended to decrease in all treated groups without reaching significance (Figure 3d, 3f). Interestingly, alterations in the levels of proteins implicated in the APP processing showed complementary results in the combination of LSL60101 and donepezil treatment. In this line, the protein levels of C-terminal fragments (CTFs) were found significantly reduced in LSL60101 treated groups when compared to the 5XFAD controls while donepezil/LSL60101 treated group showed a higher decrease in CTFs compared with monotherapy (Figure 4a, 4g). The protein levels of phosphorylated amyloid precursor protein (p-APP) at Th668 were

significantly decreased only for the combination of donepezil/LSL60101 treated animals (Figure 4b). Soluble APPβ (sAPPβ) levels were found significantly decreased in donepezil/LSL60101 treated group, whereas soluble APPα (sAPPα) levels were found significantly increased after combination treatment when compared to 5XFAD controls or donepezil treated mice (Figure 4c, 4d, 4g). No differences in the gene expression of *APP* were found among the groups (Figure 4h). Regarding the levels of BACE1 *(β-σεςρεταση)* and ADAM10 (α-secretase), no significant differences were observed among the 5XFAD groups (Figures 4e-4g). However, when the gene expression and protein levels of enzymes implicated in amyloid degradation were studied, donepezil/LSL60101 treatment slightly increased gene expression of *insulin-degrading enzyme* (*Ide*) and *neprilysin*(*Nep*) (Figure 4i).

3.5 I2-IR ligand LSL60101 and combination with donepezil reduce Tau hyperphosphorylation in 5XFAD mice

Tau hyperphosphorylation, another major hallmark of AD, was evaluated in the hippocampus of the 5XFAD mice. I2-IR ligand LSL60101 and the co-treatment donepezil/LSL60101 decreased the Tau phosphorylation at the Ser404 and Ser396, diminutions that reached significance for the donepezil/LSL60101 treated 5XFAD mice. Of note, significant differences in p-Tau levels were found as well between the donepezil/LSL60101 5XFAD treated mice, and the donepezil or LSL60101 treated ones (Figure 3e, 3f).

3.6 Effects of LSL60101 on microglia activation and inflammatory markers expression.

In the AD brain, the formation of Aβ plaques leads to the activation of astrocytes and reactive gliosis. To examine changes in microglia reactivity, Iba-1 was determined by IHC experiments. Importantly, LSL60101 treatment resulted in a significant decrease in Iba-1 levels in the hippocampus of 5XFAD mice whereas donepezil did not affect Iba-1 immunoreactivity (Figure 5a-5d). Donepezil/LSL60101 combination did not modify the beneficial effect of LSL60101 by itself on microglia activation.

The gene expression of different inflammatory mediators was evaluated in the hippocampus of 5XFAD mice. In whole, 5XFAD mice presented an evident exacerbation of inflammatory response compared to WT mice, whereas treatments led to the upregulation of specific markers studied. Interestingly, significant increases or tendency to increase were determined in*Ιντερλευκιν 1β (Ιλ-1β),Ιντερλευκιν 6 (Ιλ-6),* and*Chemokine (C-C motif) ligand 12 (Ccl12)* markers in LSL60101 treated group, while donepezil alone had no significant effect on these markers (Figure 5e). Moreover, the gene expression of *Triggering receptor expressed on myeloid cells 2 (Trem2*) was increased significantly after treatment with the I2-IR ligand LSL60101 (Figure 5f).

3.6 LSL60101 effects on astroglial activation and synaptic dysfunction.

All treatments were able to attenuate astrogliosis in the hippocampus of 5XFAD brains by decreasing GFAP immunoreactivity in DG, CA1 and CA3 areas in 5XFAD mice groups in comparison with untreated mice (Figures 6a-d). Likewise, synaptic plasticity markers were evaluated by WB. Decreases in the protein levels of postsynaptic density protein 95 (PSD95) and synaptophysin (SYN) were determined in 5XFAD mice when compared to WT mice (Figures 6f-6h). I2-IR ligand LSL60101 increased PSD95 levels when compared to 5XFAD control (Figure 6f, 6h). SYN levels were found increased in LSL60101, and donepezil/LSL60101 treated 5XFAD mice, reaching significance only for the combination-treated group. Donepezil treatment was not able to significantly modify these markers (Figure 6g, 6h).

DISCUSSION

The identification of new targets for AD treatment is required due to the lack of effective disease treatment. At present, AChEI are one of the standard therapeutic options clinically available for AD patients; however, those treatments provide only symptomatic benefit in AD (Sinforiani et al., 2003; Rosini et al., 2014). Fortunately, the number of disease-modifying drugs targeting AD hallmarks such as aducanumab (BIIB037), which is currently in phase 3 trials, is increasing (Cummings et al., 2020). Combination therapies of symptomatic and disease-modifying drugs have centred attention due to the multifactorial origin of the disease, and most current clinical trials combine donepezil with novel neuroprotective drugs (Frölich et al., 2019).

However, it remains a challenge that must be addressed to unveil new strategies that could be more effective in disease-modifying treatment, rather than address symptoms (Schmitt et al., 2004).

Several studies have described the symptomatic effects of donepezil in animal models of dementia and AD, but few *in vivo* studies have evaluated donepezil neuroprotective effects regarding the disease-modifying actions of this compound alone or in combination (Jiangbo and Liyun, 2018; Krishna et al., 2020; Yang et al., 2020; Ongnok et al., 2021). Here, we studied the effect of chronic low doses of an I2IR ligand, donepezil and their combination.

In the light of our studies, we demonstrated for the first time the neuroprotective effects of selective I2-IR ligands in the senescence-accelerated mouse prone 8 (SAMP8), a mouse model of late-onset AD (Griñán-Ferré et al., 2019). LSL60101, a selective I2-IR ligand has been shown to induce several biological effects associated with I2-IR occupancy and most importantly neuroprotective effects in the CNS (Menargues et al 1994; Boronat et al., 1998; Casanovas et al., 2000; Sánchez-Blázquez et al., 2000).Therefore, it represents a suitable drug candidate to validate this receptor as a target for AD. Here, we demonstrated the efficacy of chronic low-dose I2-IR ligand LSL60101 treatment in comparison with donepezil by assessing the beneficial outcomes in a model of familial AD.

Cognitive abilities are the essential indicators to unveil pharmacological effects in AD. Firstly, chronic low-dose treatment with the I2-IR ligand LSL60101 or donepezil reversed the cognitive deficits presented by 7-month-old 5XFAD mice, without affecting WT mice in the NORT paradigm. However, in the spatial memory test, only LSL60101 showed improvements in memory. Likewise, 5XFAD exhibited improved social behaviour after LSL60101 or donepezil treatment. In agreement with these results, donepezil has been shown to improve social interactions in scopolamine-induced memory impairments in mice (Riedel et al., 2009) and in drug-trials for AD (Boada-Rovira et al., 2004). Nevertheless, the beneficial effect of an I2-IR ligand treatment on social interaction deficits has not been described previously.

By contrast, I2-IR ligand LSL60101 did not modify anxiety-like behaviour, albeit previous studies have shown the *in vivo*anxiolytic and anti-depressant-like effects induced by I2-IR ligands (Finn et al., 2003; Tonello et al., 2012). Interestingly, the absence of anti-depressant effect after treatment with LSL60101 in healthy rats was recently described (Hernández-Hernández et al., 2020) further supporting our results, since anxiety-like and depressive-like behaviour are strongly associated, sharing common molecular pathways (Gatt et al., 2009). In contrast, and according to literature (Fitzgerald et al., 2020), chronic treatment with donepezil showed beneficial effects on the anxiety-related disturbances exhibited by 5XFAD mice .

Recently, we reported that the amyloidogenic APP processing pathway was suppressed in SAMP8 and 5XFAD mice after treatment with novel I2-IR ligands, anticipating the role of I2-IR modulation in the Aβ biogenesis (Griñán-Ferré et al., 2019; Abás et al., 2020; Vasilopoulou et al., 2020b). Accordingly, in this study, we also demonstrated that chronic low-dose treatment with I2-IR ligand LSL60101 attenuated the amyloid plaque burden in 5XFAD mice. In addition, Aβ plaques reduction was accompanied by a decrease in CTFs and Aβ hippocampal protein levels, as well as favourable modifications in APP processing after treatment. Conversely, recently it was reported that the I2-IR ligand BU224 does not ameliorate Aβ amyloidosis in 5XFAD mice (Mirzaei et al., 2020), but improves memory. In contrast with LSL60101 like molecules, BU224 blocked the memory-enhancing effect of agmatine in memory deficits induced by $A\beta_{1\text{-}42}$ in mice (Kotagale et al., 2020). These discrepancies between I2-IR ligands can be explained by differences in compound administration conditions such as dose, time (sub-chronic*vs.* chronic) and route of administration. Thus, we hypothesise that low doses of LSL60101, as well as the chronicity of treatment, has a clear beneficial effect on amyloid burden because of differential characteristics among I2-IR ligands (Sánchez-Blázquez et al., 2000; Garau et al., 2013).

Several studies have demonstrated the effect of donepezil on Aβ pathology in AD models, reporting either beneficial changes (Dong et al., 2009; Takada-Takatori et al., 2019) or lack of effect (Ju and Tam, 2020). Here, low-dose donepezil treatment did not induce significant changes on neither Aβ plaques nor APP processing in 7-month-old 5XFAD. Of note, co-administration of I2-IR ligand LSL60101 with donepezil was shown to

have a greater effect on APP processing, as the co-treatment donepezil/LSL60101 induced an increase in Aβ degradation enzymes gene expression in 5XFAD mice, which was not determined in the other treated groups. To sum up, this is the first time that an I2-IR ligand was shown to be effective in reducing Aβ burden in *in vivo* mice model of AD.

Presence of p-Tau, another major AD hallmark, in the 5XFAD model is supported by previous studies suggesting that Tau pathology may be downstream from Aβ pathology (Blanchard et al., 2003; Saul et al., 2013). I2-IR ligand LSL60101 ameliorated Tau pathology in the hippocampus of 5XFAD mice. Interestingly, it was shown recently that chronic treatment with idazoxan, a mixed α2/I2 ligand, reduced p-Tau reversing cognitive deficits in AD mice, because of its α2 blockade action (Zhang et al., 2020). In this case, the effect of LSL60101 on Tau pathology can be attributed to its I2 selectivity, more than to the α2 one. Surprisingly, the p-Tau reduction reached significance in the 5XFAD mice treated with the combination of LSL60101 with donepezil demonstrating, in this case, a putative additive effect of the drugs on Tau pathology. Indeed, amelioration of Tau pathology has been induced in AD animal models both by donepezil (Yoshiyama et al., 2010) and by I2-IR ligand treatments (Griñán-Ferré et al., 2019; Vasilopoulou et al., 2020b). It is possible that the activation of distinct molecular pathways by the two molecules with different modes of actions resulted in a remarkable p-Tau reduction observed in the donepezil/LSL60101 treated mice group.

It is well-established that Aβ accumulation jointly with p-Tau increases microglial activation and inflammatory mediators' production in AD brains (Akiyama et al., 2000; Serrano-Pozo et al., 2011; Zhang and Jiang, 2015). On the one hand, chronic low-dose LSL60101 treatment reduced microgliosis in 5XFAD mice in contrast to the standard of care donepezil, explaining the decrease in the amyloid deposition that in turn would lead to a decrease in gliotic response after LSL60101 treatment. On the other hand, inflammatory gene expression increase (*Iλ-1β, Iλ-6,* and *Ccl12*) was observed after treatment with I2-IR ligand LSL60101 but not with donepezil. Interestingly, this is further supported by a significant upregulation of *Trem2* gene expression determined in the LSL60101 treated mice, further confirming the neuroinflammatory modulation by I2-IR ligand LSL60101 (Hwang et al., 2010; Griñán-Ferré et al., 2019; Vasilopoulou et al., 2020a). Furthermore, increased *Trem2* expression has been shown to reprogram microglia responsivity mediating microglial cytokine release, migration and clearance of Aβ deposits, ameliorating neuropathological and behavioural deficits of AD mouse models (Lee et al., 2018; Zhao et al., 2018).

It has been described that the I2-IR modulate the expression of astrocyte marker GFAP, especially considering their primary location in astrocytes (Regunathan et al., 1993; Olmos et al., 1994). GFAP diminution was observed in SAMP8 after chronic treatment with selective I2-IR ligands (Griñán-Ferré et al., 2019; Vasilopoulou et al., 2020b). In agreement with those results, chronic low-dose treatment with I2-IR ligand LSL60101 attenuated astrogliosis in 5XFAD mice. By contrast, it has been shown that chronic treatment with LSL60101 increased GFAP immunoreactivity (Alemany et al., 1995) resulting in reactive astrocytosis and preventing motoneuron cell death in neonatal rats (Casanovas et al., 2000). However, here, in a neurodegenerative landscape provided by the 5XFAD model, the diminution of GFAP reactivity ran in parallel with the attenuation of the Aβ pathology and microglial activation observed after LSL60101 and donepezil treatment, given further support to the beneficial effects of I2-IR ligand on mice behaviour. Ultimately, we demonstrated that chronic low-dose treatment with I2-IR ligand as well as donepezil enhanced synaptic plasticity, further supporting the cognitive and behavioural improvement induced by the LSL60101 in 5XFAD mice.

CONCLUSIONS

Collectively, we report that chronic low-dose treatment with I2-IR ligand LSL60101 reversed cognitive deficits in 5XFAD mice, changing AD neuropathological hallmarks, including glial activation and synaptic dysfunction. Strikingly, treatment with I2-IR ligand LSL60101 was found to exert more significant beneficial effects under neurodegenerative process caused by Aβ pathology than donepezil, However, combination treatment only showed discrete synergistic effects at the molecular level (e.g., tau hyperphosphorylation or synaptic plasticity), suggesting that increased dosage and/or duration of the treatment may be able to produce better effects on both behaviour and AD-hallmarks, targeting simultaneously pathological and symptomatic

reliefs. In conclusion, our findings demonstrate the therapeutic potential of the I2-IR for AD treatment as a disease-modifying single therapy and provide new insights for their efficacy.

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Figure 1. Effects of low-dose chronic treatment with I2-IR LSL60101, Donepezil and co-administration on cognitive status in 5XFAD and WT mice. (a) Scheme of experimental design. **Results of NORT:** Discrimination index calculated by using exploration time for novel and familiar object **(b)** in the short-term term memory test session (2h) (WT, n=10-12 per group; 5XFAD n=11-12 per group)**(c)** in the long-term memory test session (24h) (WT, n=10-12 per group; 5XFAD n=10-12 per group). **Results of MWM** : **(d)**Distance to target (platform) (cm) during the training session (WT n=10-11 per group; 5XFAD n=10-12 per group) **(e)** Quadrant preference in the test session as time (%) spent in each quadrant WT n=9-12 per group; 5XFAD n=10-12 per group) **(f)** Time (%) spent in platform quadrant in the test session (WT n=9-12 per group; 5XFAD n= 10-12 per group) **(g)** Distance to target (platform) (cm) in the test session (WT n=9-12 per group; 5XFAD n=10-12 per group) **(h)**Representative images of trajectory during memory test. Bars represent mean±SEM. one-way and two-way ANOVA with Tukey post hoc analysis, *P<0.05.

Figure 2. Effects of low-dose chronic treatments I2-IR LSL60101, Donepezil and co-administration on behavioral and social status in 5XFAD mice and WT controls. Results of OF: (a) Locomotor activity measured as distance travelled (cm) WT n=10-12 per group; 5XFAD n=11-12 per group**(b)** Time spent in the center (sec) (WT n=10-12 per group; 5XFAD n=9-11 per group) **(c)** number of rearings (WT n=11-12 per group; 5XFAD n=10-12 per group). **Results of EPM: (d)** Time (%) spent in Open Arms (WT n=11-12 per group; 5XFAD n=10-12 per group)**(e)** Time (%) spent in closed arms (WT n=11-12 per group; 5XFAD n=10-12 per group) **(f)** number of rearings (WT n= 11-12 per group; 5XFAD n=11-12 per group). **Results of TCT: (g)** Entries in chambers (A1, A2) in the habituation phase (n) (WT n=11-12 per group; 5XFAD n=11-12 per group) **(h)** Time in chambers (Empty, Stranger) during

the test session (WT n=10-11 per group; 5XFAD n=9-11 per group)**(i)** time of interaction with intruder (sec) in the test session (WT n=11-12 per group; 5XFAD n=10-11 per group). Bars represent mean±SEM. Student's t-test or two-way ANOVA with Tukey post hoc analysis, *P<0.05;

Figure 3. Effects of low dose chronic treatments I2-IR LSL60101, donepezil and co-administration on AD hallmarks in 5XFAD mice. (a) Histological images of amyloid plaques stained with Thioflavin-S and quantification of **(b)** number of the plaques in cortex and hippocampus (**c**) and area (%) covered by plaques in the DG area of the hippocampus in the 5XFAD mice. Representative Western blot and quantifications **(d-f)** for β-Amyloid p-Tau(Ser404) and p-Tau(ser396) in the hippocampus of 5XFAD mice. Values in bar graphs are adjusted to 100% for protein levels of the control WT control or the control 5XFAD. Bars represent mean±SEM. Student's t-test or one-way ANOVA with Tukey post hoc analysis, *P<0.05; n=5-6 per group.

Φιγυρε 4. Εφφεχτς οφ λοω δοσε χηρονις τρεατμεντς I2-IP ΛΣΛ60101, δονεπεζιλ ανδ χο-αδμινιστρατιον ον Αβ πατηολογψ ανδ ΑΠΠ προχεσσινγ ιν 5ΞΦΑΔ μιχε. Representative Western Blots and Quantifications for**(a-g)** CTFs, p-APP, sAPPβ, sAPPα, BACE 1, ADAM10 in the hippocampus of 5XFAD mice. Values in bar graphs are adjusted to 100% for protein levels of the control WT control or the control 5XFAD. Representative gene expression in the hippocampus of the 5XFAD mice for**(h)** *APP* and **(i)** *IDE, Nep.* Gene expression levels were determined by real-time PCR. Bars represent mean±SEM. Student's t-test or one-way ANOVA with Tukey post hoc analysis, *P<0.05; n=5-6 per group.

Figure 5. Effects of low dose chronic treatments I2-IR LSL60101, donepezil and co-administration on microgliosis and inflammatory markers in 5XFAD mice. (a) Representative images of GFAP immunostaining and quantification **(b-d)** in DG, CA1, CA3 areas of the hippocampus of the 5XFAd mice. Representative gene expression in the hippocampus of the 5XFAD mice for **(e)** *Ιλ-1β, Ιλ-6, ̃ζλ12,* **(f)**for *Trem2* . Gene expression levels were determined by real-time PCR. Bars represent mean±SEM. Student's t-test or one-way ANOVA with Tukey post hoc analysis, *P<0.05; n=5-6 per group.

Figure 6. Effects of low dose chronic treatments I2-IR LSL60101, donepezil and co-administration on astrogliosis and synaptic plasticity in 5XFAD mice. (a) Representative images of GFAP immunostaining and quantification **(b-d)** in DG, CA1, CA3 areas of the hippocampus of the 5XFAd mice. Representative western blot and quantifications**(f-h)** for PSD95 and SYN in the hippocampus of 5XFAD mice. Values in bar graphs are adjusted to 100% for protein levels of the control WT control or the control 5XFAD. Bars represent mean±SEM. Student's t-test or one-way ANOVA with Tukey post hoc analysis, *P<0.05; n=5-6 per group.

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