Gene knockout of 5-lipoxygenase rescues synaptic dysfunction and improves memory in the triple-transgenic model of Alzheimer’s disease

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INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder and the leading cause of dementia worldwide for which no effective treatments exist.1,2 Memory loss is the most prominent clinical aspect of AD, and it typically manifests before the development of overt brain pathologies. Although there is still debate on the actual contributors to the development of memory impairments, there is a consensus that alteration at the synaptic level, a phenomenon also known as synaptic dysfunction, is probably one of the most significant factors in the initial stages of memory loss.3,4

In the past decade, the development of transgenic mice has represented an invaluable tool for modeling diverse aspects of the AD phenotype. Although no model exactly and fully recapitulates the human disease, including synaptic dysfunction, memory impairments, and tau pathology.5 The 5-lipoxygenase (5LO) is a lipid-peroxidizing enzyme that inserts molecular oxygen into fatty acids leading to the biosynthesis of bioactive lipids, such as leukotrienes.6 The protein is widely expressed in the brain where its expression and activity increase in an age-dependent manner.7 Previous work showed that levels of SLO are elevated in AD brains,8 and its genetic absence or pharmacological blockade reduced Aβ levels and deposition in transgenic amyloid precursor protein (APP) mouse models.9,10 More recently, we demonstrated that SLO neuronal overexpression in the 3×Tg exacerbated their neuropathology.11 However, no data are currently available on the effect that SLO genetic deficiency has on the AD-like synaptic phenotype, which includes synaptic function, synaptic integrity, and cognition.

To address this issue, we used a genetic and a pharmacological approach by generating 3×Tg mice deficient for SLO and administering 3×Tg mice with a SLO inhibitor. Compared with controls, we found that even before the development of overt neuropathology, both animals manifested significant memory improvement, rescue of their synaptic dysfunction and amelioration of synaptic integrity. In addition, later in life, these mice had a significant reduction of Aβ and tau pathology. Our findings support a novel functional role for SLO in regulating synaptic plasticity and memory. They establish this protein as a pleiotropic contributor to the development of the full spectrum of the AD phenotype, making it a valid therapeutic target for the treatment of AD.

MATERIALS AND METHODS

All animal procedures were approved by the Institutional Animal Care and Usage Committee, in accordance with the US National Institutes of Health guidelines. The 3×Tg mice harboring a mutant APP (K670/671NL), a human mutant PS1 (M146V) knockin and tau (P301L) transgenes; 3×Tg wild type (WT) and mice genetically deficient for SLO (SLOKO) used in the study were reported previously.5,12 All the animals were backcrossed 10 times on the same genetic background. The SLOKO mice were crossed several times with 3×Tg mice to obtain founder animals (3×Tg/SLOKO), which were then crossed with each other and the animals from these crosses were used for the studies. They were kept in a pathogen-free environment.

Keywords: Alzheimer’s disease; amyloid beta; 5-lipoxygenase; memory; synapse; tau protein; transgenic mouse models

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environment, on a 12-h light/dark cycle and fed a normal chow and water ad libitum. Male and female mice were used throughout the studies. Animals underwent behavioral testing at two different age groups (6–8 months and 12–14 months). A separate group of 5-month old 3 × Tg mice were also randomized to receive zileuton (200 mg l⁻¹) or vehicle in their drinking water for a month. After this period, they underwent behavioral testing as described below, then killed for electrophysiology study. After killing, mice were perfused with ice-cold 0.9% phosphate-buffered saline containing EDTA (2 mmol l⁻¹) pH 7.4. Brain was removed, gently rinsed in cold 0.9% phosphate-buffered saline and immediately dissected in two halves. One half was immediately stored at −80°C for biochemistry; the other half was fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4 for immunohistochemical studies. The cortex and hippocampus were the two brain regions always used for biochemistry and immunohistochemistry studies, as also indicated in figure legends.

Behavioral tests

All the animals were handled for at least 3–4 days before testing. They were tested in random order and the experimenter conducting the tests was unaware of the genotype or treatment.

Y-maze

The Y-maze behavioral paradigm, a test widely used to assess working memory in rodents, was carried out as previously described. Briefly, each mouse was placed in the center of the Y-maze and allowed to freely explore the maze during a 5-min session for assessment of spontaneous alternating behavior. The sequence and total number of arms entered were video-recorded. Any entry into an arm was considered valid if all four paws entered the arm. An alternation was defined as three consecutive entries in three different arms (1,2,3 or 2,3,1, etc.). The percentage alternation score was calculated using the following formula: total alternation number/total number of entries − 2) × 100. Testing was always performed in the same room and at the same time to ensure environmental consistency.

Fear conditioning

Two weeks before killing, fear conditioning experiments were performed following the methods previously described. Briefly, on day 1 animals were placed into the conditioning chamber for 2 min before the onset of a sound, which in its last 2 s is paired with a foot shock. Mice were removed from the chamber 1 min after the shock and on day 2 tested for contextual and cued fear conditioning, which is typically a 24-h memory retention test. Conditioning is measured by testing the freezing behavior as the complete absence of movement, both during training as well as testing. The percentage time during which the mouse froze is calculated for analysis of context- and cued fear memory assessments. Tests were conducted in a conditioning chamber equipped with black methacrylate walls, a transparent floor, a speaker and grid floor (Start Fear System; Harvard Apparatus, Holliston, MA, USA).

Immunoblot analyses

Primary antibodies used in this paper are summarized in Table 1. Proteins were extracted from the hippocampus using a homogenization buffer containing 250 mm Tris base, 750 mm NaCl, 5% NP-40, 25 mm EDTA, 2.5% sodium deoxycholate, 0.5% sodium dodecyl sulfate and an EDTA-free protease and phosphatase inhibitors cocktail tablet (Roche Applied Science, Indianapolis, IN, USA), sonicated (exposed to 13 000 r.p.m. for 45 min at 4°C) and supernatants used for immunoblot analysis, as previously described. Total protein concentration was determined by using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Samples were electrophoretically separated using 10% Bis–Tris gels or 3–8% Tris–acetate gel (Bio-Rad, Richmond, CA, USA), according to the molecular weight of the target molecule, and then transferred onto nitrocellulose membranes (Bio-Rad). They were blocked with Odyssey blocking buffer for 1 h; and then incubated with primary antibodies overnight at 4°C. After three washing cycles with TBS, membranes were incubated with IRDye 800CW or IRDye 680CW-labeled secondary antibodies (LI-COR Bioscience, NE, USA) at 22°C for 1 h. Signals were developed with Odyssey Infrared Imaging Systems (LI-COR Bioscience). Actin was always used as an internal loading control.

Sarkosyl insolubility assay

The assay for insoluble tau was performed as previously described. Briefly, ultracentrifugation and sarkosyl extraction (30 min in 1% sarkosyl) was used to obtain soluble and insoluble fractions of tau. Insoluble fractions were washed one time with 1% sarkosyl, then immunoblotted with HT-7 antibody.

Biochemical analyses

Mouse brain homogenates were sequentially extracted first in RIPA (radioimmunoprecipitation assay) for the Aβ1–40 and 1–42 soluble fractions, then in formic acid for the Aβ1–40 and 1–42 soluble fractions and finally in formic acid for immunohistochemistry studies. The cortex and hippocampus were two brain regions always used for biochemistry and immunohistochemistry studies, as also indicated in figure legends.

Immunohistochemistry

Primary antibodies used in this study are listed in Table 2. Immunostaining was performed as reported previously. Briefly, serial 6-μm thick coronal sections were mounted on 3-aminopropyl triethoxysilane-coated slides. Every eighth section from the prior block was examined using unbiased stereological principles.

The sections for testing Aβ were deparaffinized, hydrated and pretreated with formic acid (1%) and subsequently with 3% H₂O₂ in methanol. The sections for testing total tau (HT-7), phospho-tau (PHF-1, PHF-13, AT8, AT100, and AT270), synaptophysin (SYP), post-synaptic density protein 95 (PSD-95) and microtubule-associated protein-2 (MAP-2) were deparaffinized, hydrated and subsequently pretreated with 3% H₂O₂ in methanol and then treated with citrate (10 mm) or IHC-Tek Epitope Retrieval Solution (IHC World, Woodstock, MD, USA) for antigen retrieval. Sections were blocked in 2% fetal bovine serum and then incubated with primary antibody overnight at 4°C. The following day, sections were incubated with biotinylated anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, CA, USA) and then developed by using the avidin–biotin complex method (Vector Laboratories) with 3,3-diaminobenzidine as a chromogen. Light microscopic images were used to calculate the area occupied by Aβ immunoreactivity, and the cell densities of GFAP (glial fibrillary acidic protein) and CD45-immunopositive reactions by using the software Image-Pro Plus for Windows version 5.0 (Media Cybernetics, Bethesda, MD, USA). The threshold optical density that discriminated staining from background was determined and held constant for all quantifications. The area occupied by Aβ immunoreactivity was measured by the software and divided by the total area of interest to obtain the percentage area of immunoreactivity.

Electrophysiology

Six-month-old mice (n = No. of slices/No. of animals): WT (n = 23/8); 3 × Tg (n = 21/7); 3 × Tg/SLOKO (n = 20/6); 3 × Tg plus zileuton (n = 5/2)). Mice were killed by rapid decapitation and the brains placed into ice-cold artificial cerebral spinal fluid (ACSF) in which sucrose (248 mm) was substituted for NaCl. Transverse hippocampal slices (400-μm thick) were cut using a Vibratome 3000 plus (Vibratome, Bannockburn, IL, USA) and placed in ACSF (124 nM NaCl, 2.5 mM KCl, 2.5 mM NaHCO₃, 2.5 mM CaCl₂, 2 mM MgSO₄, 10 mM dextrose and 26 mM NaHCO₃) at room temperature to recover for 1 h bubbled with 95% O₂/5% CO₂. Slices were transferred to a recording chamber (Warner Instruments, Hamden, CT, USA) and continuously perfused with ACSF at 1.5–2.0 ml min⁻¹ flow, bubbled with 95% O₂/5% CO₂ and maintained by an in-line solution heater (TC-324; Warner Instruments) at 32–34°C. We recorded field excitation postsynaptic potentials (EPSPs) from the CA1 stratum radiatum by using an extracellular glass pipette (3–5 MΩ) filled with ACSF. Schaffer collateral/commisural fibers in the stratum radiatum were stimulated with a bipolar tungsten electrode placed 200–300 μm from the recording pipette. Stimuli on intensities were chosen to produce a fEPSP with a maximum amplitude, based on an input/output (I/O) curve using stimulating intensities of 0–300 μA, in increments of 20 μA. Paired-pulse facilitation (PPF) experiments were performed using a pair of stimuli of the same intensity delivered 20, 50, 100, 200 and 1000 ms apart. Baseline was recorded for 20 mins before tetanization with pulses every 30 s. Long-term potentiation (LTP) at CA3–CA1 synapses was induced by four trains of 30 Hz stimulation delivered in 20 s intervals. Recordings were made every 30 s for 3 h following tetanization. The EPSP rise/slope (μV ms⁻¹) between 30% and 90% was measured offline using Clampfit 10.3.
Bonferroni multiple comparison tests were performed using Prism 5.0.

Genetic absence of 5LO ameliorates cognition

To assess the effect of 5LO genetic absence on behavior, mice were initially tested in the Y-maze at two different ages: 6–8 and 12–14 months old. As shown in Figure 1a, initially we did not notice any differences among the four groups of mice considered with regard to their general activity as assessed by the total number of arm entries for each group at both ages (Figure 1a). When we considered the number of alternations in the same test, we observed that 3 × Tg mice had a much lower number of alternations resulting in a significantly lower percentage at both ages, suggesting an improvement of their working memory (Figure 1b). Next, mice underwent contextual fear conditioning, which is a measure of 24-h retention memory for both the cued and the contextual form. No differences among the groups of mice were observed during the training session (not shown). When the four groups of mice were subjected to contextual fear conditioning, they did not manifest any significant differences (Figure 1c). In the cued phase of the conditioning, we observed that 5LOKO and WT mice exhibited similar levels of freezing at both ages. On the other hand, 3 × Tg mice had significant lower freezing percentages, which were normalized in the 3 × Tg/5LOKO mice (Figure 1d).

Genetic absence of SLO decreases brain Aβ level and deposition

Two weeks after completion of the last behavior tests (14 months of age), mice were killed, the brains harvested and assayed for Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition. In comparing the two groups, we observed that 3 × Tg mice genetically deficient for 5LO displayed a significant decrease in the amount of RIPA-soluble and -insoluble Aβ levels and deposition.
change, we assessed the steady-state levels of APP along with its cleavage products in the same samples. As shown in Figure 2c, no differences were detected for total APP, the \( \alpha \)-secretase (ADAM-10) and \( \beta \)-secretase (BACE-1) pathways between the two groups. By contrast, compared with controls, 3\( \times \)/C2Tg/5LOKO mice had a significant decrease in the steady-state levels of the four components of the \( \gamma \)-secretase complex (PS1, nicastrin, Pen-2 and APH-1), which was associated with lower levels of C-terminal fragments (Figures 2c and d).

Genetic knockout of 5LO modulates tau metabolism

We then examined the effect of 5LO knockout on tau metabolism. As shown in Figure 3, although we did not observe any change in levels of total soluble tau between the two groups, compared with the 3\( \times \)/C2Tg, the 3\( \times \)/C2Tg/5LOKO mice had a significant decrease at epitope S396, as recognized by the specific antibody PHF-13, at epitope S396/S404 as recognized by the specific antibody PHF-1 and at epitope S202/T205 as recognized by the specific antibody AT8 (Figures 3a and b). By contrast, no changes were detected for other phosphorylation sites as recognized by the antibodies AT180 (T231/S235) and AT270 (T181). Additionally, compared with 3\( \times \)/Tg we observed that 3\( \times \)/Tg/5LOKO mice had a significant reduction in the levels of insoluble tau (Figures 3c and d).

In accordance with the western blot results, immunohistochemical staining showed decreased somatodendritic accumulations of the phosphorylated epitopes recognized by the antibodies PHF-1, PHF-13 and AT-8 in the 3\( \times \)/Tg/5LOKO mice (ratios: PHF-13/tau = 0.57; PHF-1/tau = 0.64; AT8/tau = 0.52, respectively; Figure 3e).

To explore the molecular mechanism responsible for the hypophosphorylation of tau, we next assayed some of the kinases, which are considered major regulators of tau post-translational modification. In comparing the two groups of mice, we did not observe any significant differences in the levels of total and phosphorylated glycogen synthase kinase 3-\( \alpha \) (GSK3-\( \alpha \)) and GSK3-\( \beta \), c-Jun N-terminal kinase 2 (JNK2), total and phosphorylated p38 and total and phosphorylated stress-activated protein kinase (SAPK)/JNK (Figure 3f). However, we found that, compared with controls, 3\( \times \)/C2Tg/5LOKO mice had a statistically significant decrease in levels of Cdk5 kinase along with its coactivators p35 and p25 (Figures 3f and g).

Absence of SLO increases synaptic integrity

As changes in tau phosphorylation state have been implicated in alterations of synaptic integrity in AD, next we assessed this aspect of the 3\( \times \)/Tg mice phenotype. Compared with the control group, 3\( \times \)/Tg/5LOKO mice had a significant increase in the steady-state levels of two main synaptic proteins: post-synaptic density protein 95 and synaptophysin (Figures 4a and b). A similar result was also obtained when the dendritic protein microtubule-associated protein-2 was assayed (Figures 4a and b). These results were further confirmed in brain sections of the same mice when they were assessed by immunohistochemical analyses (Figure 4c).

Finally, we observed that, compared with the brain homogenates from 3\( \times \)/Tg, the ones from 3\( \times \)/Tg/5LOKO mice had a
significant decrease in GFAP and CD45 immunoreactivities, markers of astrocytes and microglia cell activation, respectively (Figures 4d and e).

Genetic knockout of 5LO ameliorates synaptic deficits
As the absence of 5LO in 3×/C2Tg-AD mice yielded an improvement in memory at a very early stage of their AD-like phenotype (6 months; before plaque and tangle pathology), we then explored its effect on synaptic function at this age. To this end, first we investigated basal synaptic transmission by generating I/O curves and measuring fEPSPs elicited in CA1 by stimulation of the Schaffer collaterals at increasing strength of stimulus intensities. As shown in Figure 5a, there were no differences observed in the I/O curves between any of the groups considered (WT, 3×/C2Tg, 3×/C2Tg/5LOKO).

Next, we measured short-term plasticity by examining PPF, which is due to an activity-dependent presynaptic modulation of transmitter release.16 Similar to the observation in the I/O curves, there were no differences in PPF between any of the groups analyzed (Figure 5b). Finally, we investigated LTP in the CA1 region of the hippocampus, which is thought to be a measure of neuronal plasticity and a major player in cognition.17 In this test, we found that, compared with WT, 3×Tg mice had a significant reduction in LTP responses. However, the genetic absence of 5LO in the 3×Tg mice completely restored the LTP responses to a level comparable with that of the WT mice (Figures 5c–e).

5LO pharmacological blockade improves memory and rescues synaptic deficits
To further confirm the involvement of the 5LO in the memory improvement and rescue of the pathological synaptic phenotype, 3×/C2Tg mice were randomized to receive zileuton, a selective and specific 5LO inhibitor in their drinking water for a month at a concentration we previously showed to completely block 5LO activation.10 At the end of this period, mice underwent memory assessment in the Y-maze as well as the fear conditioning paradigm. As shown in Supplementary Figure S2, first we observed that zileuton had no effect on the general motor activities of these mice. By contrast, we observed that compared with the vehicle group, 3×/C2Tg mice receiving zileuton had a significant increase in the number of alternations, suggesting an improvement of their working memory (Supplementary Figure S2). In the fear conditioning paradigm, although we did not observe any differences between the two groups in the training phase, compared with their controls 3×Tg mice receiving zileuton had a significant increase in the freezing percentage time in the cued phase, but no changes were

Figure 2. Genetic absence of S-lipoxygenase (5LO) reduces Aβ peptide levels of deposition in the brains of 3×Tg mice. (a) Radioimmunoprecipitation assay-soluble and formic acid-extractable Aβ1–40 and Aβ1–42 levels in cortex of 3×Tg and 3×Tg mice genetically deficient for 5LO (3×/C2Tg/5LOKO) mice at 14 months of age (n = 9 (5M, 4F) for 3×Tg and n = 10 (5M, 5F) for 3×Tg/5LOKO) (*P < 0.001). (b) Quantification of the area occupied with Aβ immunoreactivity in the brain cortices of the same group of mice (*P < 0.001). (c) Representative western blots of amyloid precursor protein (APP), ADAM-10, BACE-1, sAPPα, sAPPβ, C-terminal fragments (CTFs), PS1, Nicastrin, APH-1 and Pen-2 in the brain cortex homogenates from 14-month-old 3×Tg and 3×Tg/5LOKO mice. (d) Densitometric analyses of the immunoreactivities to the antibodies shown in the panel (*P < 0.01) (n = 4 (2M, 2F) for 3×Tg; n = 4 (2M, 2F) for 3×Tg/5LOKO). Values represent mean ± s.e.m.

Genetic knockout of 5LO ameliorates synaptic deficits
As the absence of 5LO in 3×/C2Tg-AD mice yielded an improvement in memory at a very early stage of their AD-like phenotype (6 months; before plaque and tangle pathology), we then explored its effect on synaptic function at this age. To this end, first we investigated basal synaptic transmission by generating I/O curves and measuring fEPSPs elicited in CA1 by stimulation of the Schaffer collaterals at increasing strength of stimulus intensities. As shown in Figure 5a, there were no differences observed in the I/O curves between any of the groups considered (WT, 3×Tg, 3×Tg/5LOKO).
Figure 3. 5-Lipoxygenase (5LO) modulates tau phosphorylation and metabolism in the brains of 3×Tg mice. (a) Representative western blot analyses for total tau (HT-7) and phosphorylated tau at residues S202/T205 (AT8), T231/S235 (AT180), T181( AT270), S396(PHF-13) and S396/S404(PHF-1) in the brain cortex homogenates of 3×Tg and 3×Tg mice genetically deficient for 5LO (3×Tg/5LOKO) mice at 14 months of age. (b) Densitometric analyses of the immunoreactivities to the antibodies shown in panel a (*P<0.01). (c) Representative western blot analysis of sarkosyl-soluble tau (HT-7) in the brain cortex homogenates from the same mice. (d) Densitometric analyses of the immunoreactivities shown in panel d (*P<0.001). (e) Representative immunohistochemical stainings for AT8, PHF-13, PHF-1 positive areas in the brain sections of 3×Tg and 3×Tg/5LOKO mice at 14 months of age. (f) Representative western blot analyses for CDK5, p35, p25, glycogen synthase kinase 3-α (GSK3-α), GSK3-β, p-GSK3-α, p-GSK3-β, p38,p-p38, stress-activated protein kinase/c-Jun N-terminal kinase 1 (SAPK/JNK1), SAPK/JNK2, p-SAPK/JNK1 and p-SAPK/JNK2 protein levels in the brain cortex homogenates from 3×Tg and 3×Tg/5LOKO mice at 14 months of age. (g) Densitometric analyses of the immunoreactivities to the antibodies from the previous panel (3×Tg, n=4 (2M, 2F); 3×Tg/5LOKO n=4 (2M, 2F)) (*P<0.001). Values represent mean±s.e.m.

Figure 4. Genetic absence of 5-lipoxygenase (5LO) ameliorates synaptic biomarkers and decreases neuroinflammation in 3×Tg mice. (a) Representative western blot analyses for synaptophysin (SYP), post-synaptic density protein 95 (PSD95) and microtubule-associated protein-2 (MAP2) in the brain cortex homogenates from wild-type (WT), 3×Tg and 3×Tg mice genetically deficient for 5LO (3×Tg/5LOKO) mice. (b) Densitometric analyses of the immunoreactivities from panel a (*P<0.001). (c) Representative immunohistochemical staining for SYP, PSD95 and MAP2 positive areas in the brain sections of WT, 3×Tg and 3×Tg/5LOKO mice. (d) Representative western blot analyses for GFAP and CD45 in the brain cortex homogenates from 3×Tg and 3×Tg/5LOKO mice. (e) Densitometric analyses of the immunoreactivities to the antibodies from the previous panel (3×Tg, n=4; 3×Tg/5LOKO; n=4), (*P<0.001). Values represent mean±s.e.m.
detected in the contextual phase of the conditioning (Supplementary Figure 2).

Next, mice were killed and their brains harvested for electrophysiology studies. First, we observed that 3 × Tg mice treated with zileuton did not differ from their controls in terms of basal synaptic transmission as measured by the fEPSPs or short-term plasticity by examining PPF (Figures 5a and b). However, analysis of the LTP responses demonstrated that pharmacological blockade of 5LO was sufficient to restore the impairments noticed in the 3 × Tg mice back to levels indistinguishable from WT animals (Figures 5c–e).

**DISCUSSION**

The data in the present paper unravel a new aspect of the neurobiology of 5LO by demonstrating its functional role in synaptic function and plasticity as well as memory. Together with the previous knowledge on this protein, they establish 5LO as a key player in the development of the full spectrum of the AD phenotype and as an important therapeutic target with true disease-modifying potential for the treatment of AD.

In recent years, there has emerged growing evidence suggesting that alterations in synaptic integrity and function are the earliest phenotypic manifestation during the evolution of AD pathogenesis. Thus, synaptic loss, as reflected by changes in synaptic markers, is a constant feature of early stage AD pathology and better correlates with clinical cognitive impairments than classical assessment of Aβ or tau brain lesions. However, the mechanisms involved in this phenomenon are still under investigation.

Recent developments in gene-targeted and transgenic mice represent an invaluable tool for modeling diverse aspects of the AD phenotype, including synaptic dysfunction and pathology. Notably, the 3 × Tg mice exhibit deficits in synaptic plasticity, such as LTP, which occur at an early age before extracellular Aβ deposition and tau pathology.5

Previously, we showed that genetic manipulation or pharmacological inhibition of the SLO modulates the phenotype of the APP transgenic mice.9,10 However, this mouse model represents a limited version of AD as it manifests mainly AD-like brain amyloidosis and lacks tau pathology. The availability of the 3 × Tg mice offered us the unique opportunity to better explore the biological relevance of this pathway in the context of AD pathogenesis. In the current paper, first we showed that genetic absence of SLO per se did not influence any of the memory tests performed in our study. Next, we observed that, compared with their controls, the 3 × Tg mice did not manifest any difference in general motor activities. By contrast, they had a significant improvement in their memory performance already at 6 months of age, as demonstrated in the Y-maze paradigm, which by recording spontaneous alternation behavior assesses working memory in rodents.11 Additionally, the same animals had an improvement in their learning memory ability, as assessed by the fear conditioning test. Thus, in this setting we observed that 3 × Tg mice performed significantly worse than the animals genetically deficient for the SLO in the cued recall, but not in the contextual paradigm, suggesting a possible amygdala involvement.

Consistent with these results, we observed that, compared with 3 × Tg, the 3 × Tg/SLOKO mice had a significant increase in three distinct protein markers of synaptic integrity (that is, synaptophysin, post-synaptic density protein 95 and microtubule-associated protein-2), suggesting an improvement of this important function for memory and learning secondary to SLO deficiency.

In association to these changes, we observed a significant reduction in Aβ levels and deposition, which, confirming our previous report, was secondary to an effect on the γ-secretase pathway.11 Besides the effect on the Aβ pathology, we also observed that SLO genetic absence had an influence on tau metabolism. Thus, we observed that genetic absence of SLO resulted in a significant decrease in phosphorylated tau epitopes as recognized by the immunoreactivity of the specific antibodies PHF-1, PHF13 and AT8, but not for the other two tested, AT180 and AT270, and confirmed that the cdk-5 kinase pathway but not the ones genetically deficient for the 5LO in the cued recall, observed that 3 × Tg mice performed significantly worse than the 3 × Tg/5LOKO mice had a significant increase in three distinct protein markers of synaptic integrity (that is, synaptophysin, post-synaptic density protein 95 and microtubule-associated protein-2), suggesting an improvement of this important function for memory and learning secondary to SLO deficiency.

To corroborate the involvement of the SLO in the improvement of the behavioral deficits we detected as early as at 6 months of age, we adopted a pharmacological approach by administering a selective SLO inhibitor to 5-month old 3 × Tg mice for a month. The dosage selected was based on our previous published work where we showed that zileuton at this concentration significantly reduced SLO activation up to 85%.10 Similar to the genetic study, we observed that 3 × Tg mice receiving the active

**Figure 5.** Genetic absence or pharmacological inhibition of 5-lipoxygenase (SLO) rescues synaptic dysfunction in 3 × Tg mice. (a) Input/output curves and representative field excitatory postsynaptic potentials (fEPSPs) at increasing stimulus strengths (0–300 μA) are shown for wild-type (WT), 3 × Tg, 3 × Tg mouse genetically deficient for SLO (3 × Tg/SLOKO) and 3 × Tg + zileuton mice at 6 months of age. (b) Mean fEPSP slopes as a function of interpulse interval between the first and second fEPSPs evoked at CA3-CA1 synapses in slices from the same mice at 20, 50, 100, 200 and 1000 ms in the same animals. (c) fEPSP slopes were recorded for 3h and expressed as the percentage of the pretetanus baseline in the same mice. (d) LTP magnitudes expressed as the percentages of baseline for 0–10 min post-tetanus (274.6 ± 3.4% for 3 × Tg; 272.7% ± 9.6% (n = 20 slices) for 3 × Tg/SLOKO; 268.5% ± 14.3% (n = 5 slices) for 3 × Tg + zileuton)). (e) For the same groups of mice, LTP magnitudes expressed as the percentages of baseline for 170–180 min post-tetanus (202.6% ± 6.5% for WT; 121.0% ± 3.4% for 3 × Tg; 197.1% ± 11.5% for 3 × Tg/SLOKO; 156.9% ± 6.9%; 199.5% ± 8.7% for 3 × Tg + zileuton) (*P < 0.0001). Values represent mean ± s.e.m.
drug had a significant amelioration of their working memory and conditioning learning.

Because of the early improvements in memory deficits we noticed in both the 3 x Tg mice genetically deficient for SLO and the ones administered with a SLO inhibitor, we next assessed the effects that both conditions had on synaptic function by implementing an electrophysiological approach.

First, we investigated basal synaptic transmission by measuring fEPSPs elicited in CA1 by stimulation of the Schaffer collaterals. We found that there were no differences in this parameter among the four groups of mice, suggesting that synaptic transmission is not altered in any of the conditions implemented. Next, we measured short-term plasticity by examining PPF, which is secondary to an activity-dependent presynaptic modulation of transmitter release.16 Similar to the observation for the basal synaptic transmission, there were no significant differences in PPF among any of the groups investigated. These results suggest that there is not an increase or decrease in the probability of transmitter release in any of these groups of mice.5,22

Finally, we assessed the LTP response, which is a type of plasticity that is thought to have a major role in learning and memory functions. As reported previously,13 there was a significant difference in LTP responses between the WT and 3 x Tg mice, with the latter showing deficits. However, the genetic absence of SLO in the 3 x Tg mice was sufficient to restore their LTP responses to a level comparable with the ones measured in the WT mice. The biological importance of this finding was further corroborated by the demonstration that SLO pharmacological blockade was also sufficient to rescue the abnormal synaptic phenotype of the 3 x Tg mice. Taken together, these data unravel a new aspect of the neurobiology of SLO pathway by demonstrating its functional role in synaptic function and plasticity as well as memory and learning.

Interestingly, the above described SLO-dependent beneficial effects on the LTP parameters are in line with previously reported enhancements of the GluR1/AMPA receptor phosphorylation by SLO genetic absence or pharmacological inhibition.11,22

Importantly, despite the fact that some of the SLO biological effects are mediated by an involvement of the Notch-secretion pathway, no alteration of the Notch signaling has been reported.10 This observation makes any potential Notch-related side effect secondary to a chronic SLO inhibition unlikely.

Considering the anti-Aβ and tau effect of 5-LO, our findings have important patho-physiological implications for AD as they establish this pathway as a major actor and contributor to all of the aspects of the disease phenotype. The new information by demonstrating the pleiotropic role of this protein in AD pathogenesis makes it not only a valuable pharmacological target, as SLO inhibitors are already under tested, but, most importantly, also represents a unique therapeutic opportunity with a true disease-modifying potential for the treatment of AD.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS
PFG and DP designed the study, developed the experimental design, performed data analyses and wrote the paper. PFG, JC, YBJ, JL and MS performed the experiments. PFG, MS and LGK designed and performed electrophysiology experiments. All the authors discussed the results and commented on the manuscript.

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