Pharmacologic Inhibition of 5-Lipoxygenase Improves Memory, Rescues Synaptic Dysfunction and Ameliorates Tau Pathology in a Transgenic Model of Tauopathy

Supplemental Information

Y-maze

The Y-maze apparatus consisted of three arms 32 cm (long) × 10 cm (wide) with 26-cm walls (San Diego Instruments). Each mouse was placed in the center of the Y-maze and allowed to explore freely through the maze over a 5-min session to assess spontaneous alternating behavior. The sequence and total number of arms entered were video-recorded. Any entry into an arm was considered legitimate 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 as previously described (1,2).

Morris Water Maze

The apparatus used was a white circular plastic tank (122 cm in diameter) with walls 76 cm high, filled with water maintained at 22° ± 2°C, which was made opaque by the addition of a nontoxic white paint, and inside had a removable, square (10 cm in side length) Plexiglas platform. The tank was located in a test room containing various prominent visual cues. Before the first trial of the first session, the mouse was placed for 10 seconds on the platform. Mice were trained to swim to the platform submerged 1.5 cm beneath the surface of the water and invisible to the mice while swimming. The platform was located in a fixed position, equidistant from the center and the wall of the tank. Mice were subjected to four training trials per day (inter-trial interval, 15 minutes). During each trial, mice were placed into the tank at one of four designated start points in a random order. Mice were allowed to find and escape onto the

submerged platform. If they fail to find the platform within 60 seconds, they were manually guided to the platform and allowed to remain there for 10 seconds. Mice were trained to reach the training criterion of 20 seconds (escape latency). To control for overtraining, probe trials were run for each group, both as soon as they reached group criterion and after all groups had reached criterion.

Mice were assessed in the probe trial 24 hours after the last training session and consisted in a 60-second free swim in the pool without the platform. Each animal's performance was monitored using the Any-Maze™ Video Tracking System (Stoelting Co., Wood Dale, IL) that provided data for the acquisition parameters (latency to find the platform and distance swam) and the probe-trial parameter (number of entries to the target platform zone and time in quadrants).

Western Blot Analyses

RIPA extracts from human and mouse brain homogenates were used for western blot analyses as previously described (1-3). Samples were electrophoresed on 10% Bis–Tris gels or 3–8% Tris–acetate gel (Bio-Rad, Richmond, CA), according to the molecular weight of the target molecule, transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA), and then incubated with appropriate primary antibodies as indicated in Table S1. After three washings with T-TBS, membranes were incubated with IRDye 800CW or IRDye 680CW-labeled secondary antibodies (LI-COR Bioscience, Lincoln, NE) at 22°C for 1 h. Signals were developed with Odyssey Infrared Imaging Systems (LI-COR Bioscience, Lincoln, NE). Beta-actin was always used as internal loading control.

Sarkosyl Insolubility Assay

The assay for insoluble tau was performed as previously described (4). Briefly, ultracentrifugation and sarkosyl extraction (30 min in 1% sarkosyl) was used to obtain soluble

and insoluble fractions of tau from brain homogenates. Insoluble fractions were washed one time with 1% sarkosyl, then immunoblotted with the HT7 antibody.

Immunohistochemistry

Immunostaining was performed as reported previously by our group (1-3). Briefly, serial coronal sections were mounted on 3-aminopropyl triethoxysilane (APES)-coated slides. Every eighth section from the habenular to the posterior commissure (8–10 sections per animal) was examined using unbiased stereological principles. The sections used for testing HT7, AT8, AT180, PHF-1, PHF-13, MC1, synaptophysin, PSD95, MAP2, GFAP, and CD45 were deparaffinized, hydrated and subsequently with 3% H_2O_2 in methanol, and then antigen retrieved with citrate (10 mM) or IHC-Tek Epitope Retrieval Solution (IHC world, Woodstock, MD). Sections were blocked in 2% fetal bovine serum before incubation with primary antibody overnight at 4°C (Wako Chemicals, Richmond, VA). After washing, sections were incubated with biotinylated anti-mouse IgG (Vector Lab, Burlingame, CA) and then developed by using the avidin-biotin complex method (Vector Lab, Burlingame, CA) with 3,3′-diaminobenzidine (DAB) as a chromogen.

5LO Activity Assay

Brain extracts were used to assay for LTB4 levels by using a specific LTB4 ELISA detection kit according to the instructions of the manufacturer (Enzo Life Sciences).

Cdk5 Activity Assay

Brain homogenates were rinsed with PBS once and lysed in buffer A (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L sodium chloride, 1% NP-40, .5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.02% sodium azide and freshly added protease inhibitors [100 µg/mL phenylmethysulfonyl fluoride and 1 µg/mL aprotinin]). After incubation on ice for 30 min,

samples were centrifuged at 12,000 *g* at 4°C for 20 min, and the supernatants collected, and incubated with anti-cdk5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 2 hours. Protein A agarose beads (50 µL) were then added and incubated for another hour. The immunoprecipitates were washed with lysis buffer and once with HEPES buffer. The kinase activity of the immunoprecipitated cdk5 was determined by using histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were incubated with 5 µg of histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA) in HEPES-buffered saline containing 15 mmol/L MgCl2, 50 µM adenosine triphosphate, 1 mmol/L dithiothreitol, and 1 µCi of [32P] adenosine triphosphate. After 30 min of incubation at 30°C, the reaction products were determined by a liquid scintillation counter.

Electrophysiology

Ten-month-old mice [(*n* = No. of slices/No. of animals): wild type (*n* = 15/4); htau (*n* = 14/3); htau plus zileuton (*n* = 13/3)] 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 (Bannockburn, IL, USA) and placed in ACSF (124 nM NaCl, 2.5 mM KCl, 2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 2 mM $MgSO_4$, 10 mM dextrose and 26 mM $NaHCO_3$) at room temperature to recover for 1 h bubbled with 95% O_2/5% CO_2. 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% O_2/5% CO_2 at room temperature. We recorded field excitatory postsynaptic potentials (fEPSPs) from the CA1 stratum radiatum by using an extracellular glass pipette (3–5 MΩ) filled with ACSF. Schaffer collateral/commissural fibers in the stratum radiatum were stimulated with a bipolar tungsten electrode placed 200–300 µm from the recording pipette. Stimulation intensities were chosen to produce a fEPSP that was 1/3 of the maximum amplitude, based on an input/output curve using stimulations of 0–300 µA, in increments of 20

μAs. Paired-pulse facilitation 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 minutes before tetanization with pulses every 30 s. Long-term potentiation at CA3–CA1 synapses was induced by four trains of 100 Hz stimulation delivered in 20 s intervals. Recordings were made every 30 s for 2 h following tetanization. The fEPSP rise/slope (mV ms−1) between 30% and 90% was measured offline using Clampfit 10.3 (Molecular Devices, LLC, Sunnyvale, CA, USA) and normalized to the mean rise/slope of the baseline. Slices were eliminated if an unstable baseline was produced or if the normalized rise/slope dropped >20–50 mV ms−1 in an approximately 10-min period. All the tests were performed by an experimenter who was unaware of the mouse genotypes and treatment.

Table S1. Antibodies used in the study.

Antibody	Immunogen	Host	Application	Source	Dilution
5LO	Human 5-LO aa 442-590	Mouse	WB	BD Transduct	1:300
HT7	aa 159-163 of human tau	Mouse	WB,IHC	Pierce	1:600
AT-8	Peptide containing phospho-S202/T205	Mouse	WB,IHC	Pierce	1:100
AT-180	Peptide containing phospho-T231/S235	Mouse	WB,IHC	Pierce	1:100
AT-270	Peptide containing phospho-T181	Mouse	WB,IHC	Pierce	1:500
PHF-13	Peptide containing phospho-Ser396	Mouse	WB,IHC	Cell Signaling	1:200
PHF-1	Peptide containing phospho-Ser396/S404	Mouse	WB,IHC	Dr. P. Davies	1:20
MC-1	Detecting conformational abnormality of tau	Mouse	IHC	Dr. P. Davies	1:20
GFAP	aa spinal cord homogenate of bovine origin	Mouse	WB,IHC	Santa Cruz	1:200
CD45	Mouse thymus or spleen	Rat	WB,IHC	BD Pharmingen	1:200
SYP (H-8)	aa 221-313 of SYP of human origin	Mouse	WB,IHC	Santa Cruz	1:500
PSD95 (7E3-1B8)	Purified recombinant rat PSD-95	Rabbit	WB,IHC	Thermo Scientific	1:400
P38	Peptide corresponding to human p38 MAPK sequence	Rabbit	WB	Cell Signaling	1:200
pP38	residues surrounding Thr180/Tyr182 of human p38 MAPK	Rabbit	WB	Cell Signaling	1:200
MAP2	Bovine brain microtubule protein	Rabbit	WB,IHC	Millipore	1:1000
GSK3α/β	aa 1-420 full length GSK-3β of Xenopus origin	Mouse	WB	Millipore	1:200
p-GSK3α/ β	aa around Ser21 of human GSK-3a.	Rabbit	WB	Cell Signaling	1:200
JNK2	aa of human JNK2	Rabbit	WB	Cell Signaling	1:300
SAPK/JNK	aa of recombinant human JNK2 fusion protein	Rabbit	WB	Cell Signaling	1:500
p-SAPK/JNK	p46 and p54 SAPK/JNK dually phosphorylated at threonine 183 and tyrosine 185	Rabbit	WB	Cell Signaling	1:200
Cdk5	aa C-terminus of Cdk5 of human origin	Rabbit	WB	Santa Cruz	1:1000
P35/25	aa C-terminus of p35 /25 of human origin	Rabbit	WB	Santa Cruz	1:400
PP2a	aa 295-309 of catalytic subunit of human protein phosphotase 2A. Clone 1D6	Mouse	WB	Millipore	1:200
Actin	aa C-terminus of Actin of human origin	Goat	WB	Santa Cruz	1:1000

IHC, immunohistochemistry; WB, western blot.

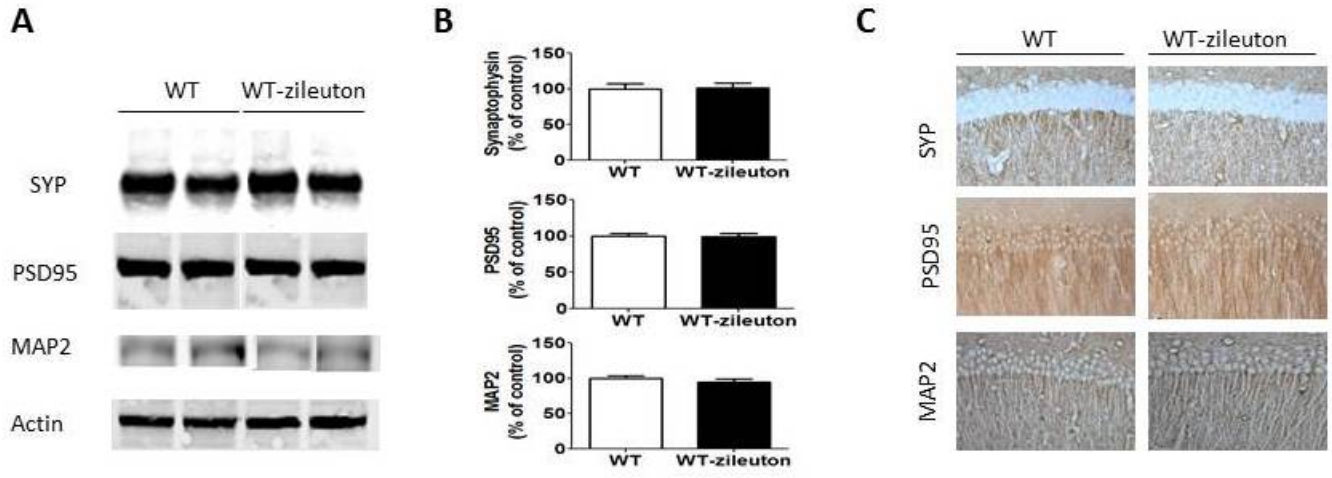

Figure S1. Zileuton treatment does not alter synaptic integrity in wild type mice. (**A**) Representative Western blot analyses for synaptophysin (SYP), postsynaptic density protein (PSD) 95 and microtubule associated protein (MAP)-2 in brain homogenates from cortices of wild type (WT) and WT-zileuton mice. (**B**) Densitometric analyses of the immunoreactivities from panel A. Results are mean ± SEM. (**C**) Representative images of immunohistochemical staining for SYP, PSD95 and MAP2 in the hippocampus of WT and WT-zileuton mice.

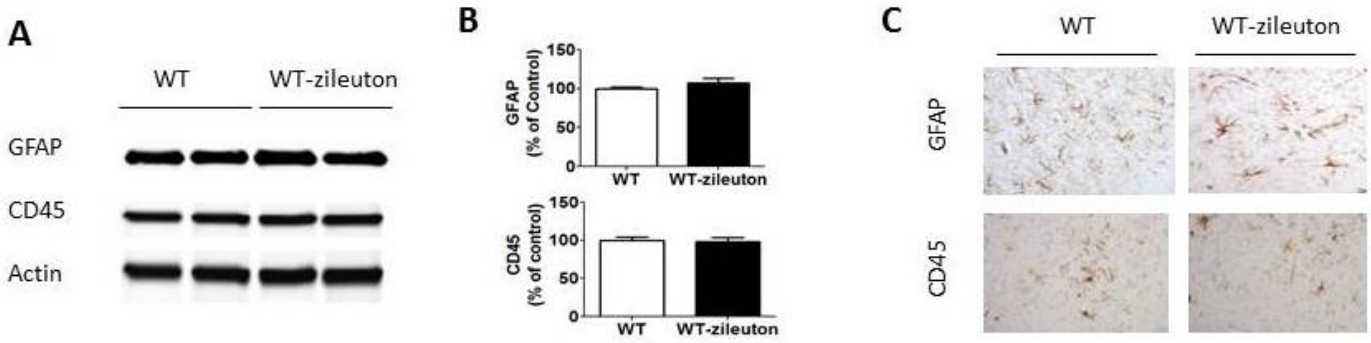

Figure S2. Pharmacological inhibition of 5LO does not influence neuroinflammation in wild type mice. (**A**) Representative Western blot analyses for glial fibrillary acidic protein (GFAP) and cluster domain (CD)45 in brain homogenates from wild type mice (WT) and WT-zileuton mice. (**B**) Densitometric analyses of the immunoreactivities to the antibodies from the previous panel. (**C**) Representative images of immunohistochemical staining in the hippocampus for GFAP and CD45 of WT and WT-zileuton mice.

Supplemental References

1. Giannopoulos PF, Chu J, Joshi YB, Sperow M, Li JG, Kirby LG, *et al.* (2014): Gene knockout of 5-lipoxygenase rescues synaptic dysfunction and improves memory in the triple-transgenic model of Alzheimer's disease. *Mol Psychiatry*. 19:511-518.
2. Giannopoulos PF, Chu J, Joshi YB, Sperow M, Li JG, Kirby LG, *et al.* (2013): 5-lipoxygenase activating protein reduction ameliorates cognitive deficit, synaptic dysfunction, and neuropathology in a mouse model of Alzheimer's disease. *Biol Psychiatry*. 74:348-356.
3. Chu J, Giannopoulos PF, Ceballos-Diaz C, Golde TE, Pratico D (2012): 5-Lipoxygenase gene transfer worsens memory, amyloid, and tau brain pathologies in a mouse model of Alzheimer disease. *Ann Neurol*. 72:442-454.
4. Andorfer C, Kress Y, Espinoza M, de Silva R, Tucker KL, Barde YA, *et al.* (2003): Hyperphosphorylation and aggregation of tau in mice expressing normal human tau isoforms. *J Neurochem*. 86:582-590.