Supporting online material

Material and Methods

Animals and ages: APP/PS1 transgenic animals[5] were obtained from The Jackson Laboratory (# 005864) on the C57/Bl6 background. NLRP3 deficient animals[27] (Millennium Pharmaceuticals) were backbred onto C57/Bl6 mice genotype to >99% C57/Bl6, which was confirmed by microsatellite analysis. Caspase-1 deficient mice were generated by BASF (Worcester, MA)[28]; these mice were subsequently obtained by Dr. Michael Starnbach (Harvard Medical School), who provided the mice for this study after 10 generations of backbreeding onto the C57/Bl6 background. All mice were housed under standard conditions at 22°C and a 12 h light-dark cycle with free access to food and water. Animal care and handling was performed according to the declaration of Helsinki and approved by the local ethical committees. The following animal groups were analyzed: WT, NLRP3$^{-/-}$, APP/PS1, APP/PS1/NLRP3$^{-/-}$, Caspase-1$^{-/-}$, APP/PS1/ Caspase-1$^{-/-}$.

Human tissue samples: Post-mortem brain material from histologically confirmed AD cases and age-matched controls who had died from non-neurological disease was from the Neurological Tissue Bank of the biobank from the Hospital Clinic-Institut d'Investigacions Biomèdiques August Pi i Sunyer (Barcelona, Spain). Samples from mild cognitive impairment (MCI) patients and early onset AD (EOAD) cases were obtained from the Banner Health collection (www.bannerhealth.com). Ages and post-mortem sampling times were similar between controls MCI, EOAD and AD cases. Post-mortem times across all cases varied from 1.5-5 h. Patients were 75 ±10 years old.

Behavioural phenotyping: *Morris Water Maze test*. Spatial memory testing was conducted in a pool consisting of a circular tank (Ø1 m) filled with opacified water at

24°C. The water basin was dimly lit (20-30 lux) and surrounded by a white curtain. The maze was virtually divided into four quadrants, with one containing a hidden platform (15x15 cm), present 1.5 cm below the water surface. Mice were trained to find the platform, orientating by means of three extra maze cues placed asymmetrically as spatial references. They were placed into the water in a quasi-random fashion to prevent strategy learning. Mice were allowed to search for the platform for 40 s; if the mice did not reach the platform in the allotted time, they were placed onto it manually. Mice were allowed to stay on the platform for 15 s before the initiation of the next trial. After completion of four trials, mice were dried and placed back into their home cages. Mice trained 4 trials per day for 8 consecutive days. For spatial probe trials, which were conducted 24 h after the last training session (day 9), the platform was removed and mice were allowed to swim for 30 s. The drop position was at the border between the 3rd and 4th quadrant, with the mouse facing the wall at start. Data are given as percent of time spent in quadrant Q1, representing the quadrant where the platform had been located, and compared to the averaged time the animals spent in the remaining quadrants. In the afternoon of the same day, a visual cued testing was performed with the platform being flagged and new positions for the start and goal during each trial. All mouse movements were recorded by a computerized tracking system that calculated distances moved and latencies required for reaching the platform (Noldus, Ethovision 3.1). *Open Field exploration*. Mice were placed in the center of the dimly lit (20-30 lux) chamber of the open field arena. Animal movements were tracked by an automatic monitoring system (Noldus Ethovision 3.1) for 5 min. The area was virtually divided into a center (square with 40 cm edge lengths), a corridor (7.5 cm along the walls) and four corner squares (10 cm edge lengths), which partly overlapped with the corridor area. The time spent in each area, horizontal and vertical activity, frequency of urination and defecation were

monitored. The experiment was repeated on three consecutive days. *Object recognition test*. The novel object recognition test was carried out according to a previously established protocol with minor changes[29]. Briefly, the test procedure consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the open field arena. Habituation was allowed for 10 min. One day later, during the training session, two identical objects (object A) were placed into the two opposing corners of the center area 30 cm apart from each other, and mice were allowed to explore the area and the objects for 10 min. The total time spent exploring both identical objects was recorded to examine place or object preference. Exactly 60 min or 1 day later, during the retention sessions, mice were placed back into the same arena in which one familiar (object A) and one novel object (object B) replacing the second object A were placed. Mice were then allowed to explore freely for a 5 min period of time and the time spent exploring each object was recorded. Exploration of the object was considered when the head of the animal was at least facing the object from a minimum distance of 1–2 cm or closer, but recording was cut as soon as mice turned their heads away from the previously investigated object. Time spent exploring the objects during trials was determined and is demonstrated as discrimination ratio (novel object interaction/total object interaction). The arena and all objects were thoroughly cleaned with 70% ethanol solution after each trial.

Electrophysiology. *Slice preparation*. Acute hippocampal transversal slices were prepared from 7-9 months-old WT, NLRP3$^{-/-}$, APP/PS1 or combined APP/PS1/ NLRP3$^{-/-}$ and APP/PS1/Casp-1$^{-/-}$ mice according to standard procedures. In brief, mice were deeply anesthetized and the brain was quickly transferred into ice-cold carbogenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) which contained 125 mM NaCl, 2 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgCl_2$, 26 mM $NaHCO_3$, 2 mM

$CaCl_2$, 25 mM glucose. Hippocampi were dissected and cut into 400 µm thick transverse slices with a vibratome (Leica, VT1200S). Slices were maintained in carbogenated ACSF at room temperature for at least 1.5 h before recording. Recordings were performed in a submerged recording chamber at 32°C. *Electrophysiology*. After placing the slices in the submerged recording chamber, field excitatory postsynaptic potentials (fEPSPs) were recorded in stratum radiatum of CA1 region with a borosilicate glass micropipette (resistance 3-15 MΩ) filled with 3 M NaCl at a depth of ~120-200 µm. Monopolar tungsten electrodes were used for stimulating the Schaffer collaterals at a frequency of 0.1 Hz. Stimulation was set to elicit a fEPSP with a slope of ~40% of maximum for LTP recordings and ~60% for LTD recordings. After 20 min baseline stimulation, LTP was induced by applying theta-burst stimulation (TBS). One burst consisted of 4 pulses at 100 Hz, repeated 10 times in a 200 ms interval. Three such bursts were used to induce LTP at 0.1 Hz. Basic synaptic transmission and presynaptic properties were analyzed via input-output-(IO) measurements and paired pulse facilitation. The IO-measurements were performed by application of a defined value (PPF) of current (25-250 µA in steps of 25 µA) and by adjusting the stimulus intensity to a certain current eliciting a fiber volley (FV) of desired voltage. PPF was measured by applying a pair of two stimuli in different inter-stimulus-intervals (ISI) ranging from 10, 20, 40, 80 to 160 ms. *Data analysis*. Data were collected, stored and analyzed with LABVIEW software (National Instruments, Austin, TX). The initial slope of fEPSPs elicited by stimulation of the Schaffer collaterals was measured over time, normalized to baseline, which was the mean response of the 20 minutes before TBS application and plotted as average ± SEM. Parameters leading to an exclusion of single experiments were (a) an unstable baseline (variability more than ± 10%) or (b) a large population spike after TBS application producing an artifactually large LTP. Analysis of the PPF data was

performed by calculation of the ratio of the slope of the second fEPSP divided by the slope of the first one. All data were recorded and analyzed in a blind fashion.

Tissue preparation: After completion of the behavioral testing, mice were deeply anesthetized and transcardially perfused with 15 ml phosphate-buffered saline. The brains were removed from the skull. One hemisphere was frozen immediately for biochemical analysis and the other was either fixed in 4 % paraformaldehyde or frozen over a mixture of dry ice and isopentane.

Brain protein extraction. Snap-frozen brain hemispheres were extracted as previously described[25]. Briefly, hemispheres were homogenized in PBS, 1 mM EDTA, 1 mM EGTA, 3 µl/ml protease inhibitor mix (Sigma, Munich, Germany). Homogenates were extracted in RIPA buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 % NP40, 0.5 % NaDOC, 0.1 % SDS), centrifuged at 100.000 x g for 30 min and the pellet containing insoluble Aβ was solubilized in 2 % SDS, 25 mM Tris-HCl, pH 7.5. In addition, the SDS-insoluble pellet was extracted with 70 % formic acid in water. Formic acid was removed using a speed vac (Eppendorf, Hamburg, Germany) and the resulting pellet was solubilized in 200 mM Tris-HCl, pH 7.5.

Immunohistochemistry. Free-floating 40-µm thick serial sections were cut on a vibratome (Leica, Wetzlar, Germany). Sections were stored in 0.1% NaN3, PBS. For immunohistochemistry, sections were treated with 50% methanol for 15 min. Then, sections were washed 3 times for 5 min in PBS and blocked in 3% BSA, 0.1% Triton-X100, PBS (blocking buffer) for 30 min followed by overnight incubation with the primary antibody in blocking buffer. Specificity controls were performed by staining with the secondary reagent and omission of the primary antibodies. Sections were washed three times in 0.1% Triton X-100, PBS and incubated with Alexa 488 or Alexa 594 conjugated secondary antibodies (1:500, Invitrogen, Karlsruhe, Germany) for 90 min, washed 3 times with 0.1% Triton X-100, PBS for 5 min. Sections were

mounted using Immomount (Thermo, Bonn, Germany). The following primary antibodies were used with respective concentrations: rabbit polyclonal anti-GFAP (1:800, Dako, Glostrup, Denmark), rat monoclonal anti-mouse CD11b (MCA711, 1:400, Serotec, Oxford, UK), rabbit polyclonal anti-Iba1 (1:200, Wako, Osaka, Japan), anti-ASC (1:200; AL177, AdipoGen, San Diego, CA), anti-NLRP3 (1:200; Cryo-2, AdipoGen, San Diego, CA) and anti-Fizz-1 (1:400 MAB1523, R&D Systems, Wiesbaden Germany). Fluorescence microscopy was done on an Olympus BX61 equipped with a spinning disk unit and images were processed in Cell^P 3.5 (Olympus, Hamburg, Germany) or on an A1-MP laser scanning microscope (Nikon, Düsseldorf, Germany) and images were processed using NIS-elements 4 (Nikon, Düsseldorf, Germany). Alternatively, cryosections (20 µm) were fixed in 4 % paraformaldehyde and immunostained using antibody IC16[30] against human Aβ1-15 (1:400) or a specific antiserum against $3NTyr^{10}$-$Aβ^{25}$ following the above described protocol.

Plaque histology. For thioflavin S staining, vibratome sections were rinsed in water, incubated in 0.01% thioflavin S in 50% ethanol and differentiated in 50% ethanol. Sections were analyzed using a BX61 microscope equipped with a disk spinning unit to achieve confocality (Olympus, Hamburg, Germany) or an A1-MP laser scanning microscope (Nikon, Düsseldorf, Germany). Image stacks were deconvoluted using Cell^P (Olympus, Hamburg, Germany). Quantitative assessment of plaque areas was done using the MBF-ImageJ 1.43m software bundle (NIH, Bethesda, MA). In brief, total plaque number and Aβ area fraction were calculated using the software ImageJ 1.43m with plugins from the WCIF ImageJ collection. In particular, images were normalized and an automatic thresholding based on the entropy of the histogram (“MaxEntropy”) were used to identify the plaques. Pictures were converted to a binary and the “fill holes” and “watershed” algorithm were applied. Finally, plaque

number, plaque area and average Aβ plaque size were calculated using the “analyze particles” plugin of ImageJ. The Aβ area fraction was determined by dividing total plaque area by the area of the microscopic field. For staining plaques with methoxy-X04, sections were washed with PBS, incubated with 10 µM methoxy-XO4 in 50% DMSO/50% NaCl (0.9%), pH 12 for 10 min and washed twice with PBS before continuing with immunohistochemistry.

Protein blotting. Samples were separated by 4-12 % NuPAGE (Invitrogen, Karlsruhe, Germany) using MES or MOPS buffer and transferred to nitrocellulose membranes. For caspase-1 blots, positive and negative controls were generated by precipitating supernatants from wild-type immortalized murine macrophages. For the negative control, cells were treated with 200 ng/ml lipopolysaccharide for 4 hr. For the positive control, cells were treated with 200 ng/ml lipopolysaccharide for 3 hr, followed by 10 µM nigericin for 1 h. APP and Aβ were detected using antibody 6E10 (Covance, Münster, Germany) and the c-terminal APP antibody 140 (CT15, gift from J. Walter, University of Bonn). IDE was blotted using antibody PC730 (Calbiochem, Darmstadt, Germany), caspase-1 using antibodies casp-1 clone 4B4.2.1 (gift from Genentech, San Francisco, CA) and a caspase-1 antibody raised in rabbit (gift from Gabriel Nuñez), neprilysin using antibody 56C6 (Santa Cruz, Heidelberg, Germany), tubulin using antibody E7 (Developmental Studies Hybridoma Bank, Iowa City, IA), BACE1 with antibody 2253 (ProSci Inc., Poway, CA), NOS2 using antibody 160862 (Cayman Chemicals), and β-actin using A2228 (Sigma, Munich, Germany) and 926-42212 (LI-COR Biosciences, Bad Homburg, Germany). Immunoreactivity was detected by enhanced chemiluminescence reaction (Millipore, Darmstadt, Germany) or near-infrared detection (Odyssey, LI-COR). Chemoluminescence intensities were analyzed using Chemidoc XRS documentation system (Biorad, Munich, Germany).

ELISA quantification of cerebral Aβ concentrations. Quantitative determination of Aβ was performed using an electrochemoluminescence ELISA for Aβ1-38, Aβ1-40 and Aβ1-42 (Meso Scale Discovery, Gaithersburg, MD, USA). Signals were measured on a SECTOR Imager 2400 reader (Meso Scale Discovery, Gaithersburg, MD, USA). For ELISA determination of $3NTyr^{10}$-Aβ, Mesocale L15XA 96 well plates were coated with 2 µg/ml of the monoclonal 3NTyr10-Aβ antibody 4A5E8 (own production) in PBS overnight at 4°C. Plates were blocked with 5 % blocker A (Meso Scale, Gaithersburg, MA), 0.1 % mouse gamma globulin (Rockland, Gilbertsville, PA). SDS and FA fractions from mouse brain were diluted in 1 % blocker A, 0.1 % mouse gamma globulin 1:25 and 1:100, respectively. 30 µl Samples were incubated for 4 h at RT, washed with tris wash buffer (Meso Scale, Gaithersburg, MA) and incubated with 0.25 µg/ml MSD-tagged antibody 4G8 (Meso Scale, Gaithersburg, MA) diluted in 1 % blocker A, 0.1 % mouse gamma globulin for 1 h at RT. Wells were washed with tris wash buffer and 150 µl of 2x read buffer (Meso Scale, Gaithersburg, MA) was added.

ELISA quantification of cerebral Il-1β concentrations. Quantitative determination of IL-1β was performed using the MLB00C ELISA for the determination of murine IL-1β according to the protocol of the supplier (R&D Systems, Wiesbaden, Germany).

Quantitative PCR. RNA was extracted from brain tissues using the RNAeasy Micro Kit (Qiagen). Total RNA was quantified spectrophotometrically and reversely transcribed into complementary DNA using the RevertAid First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. Real time qPCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). The TaqMan gene expression assay and TaqMan universal PCR master mix (Applied Biosystems) was used for PCR amplification and real-time detection of PCR products. PCRs were

carried out in 20 µl with 1 µl of the reversely transcribed product corresponding to 40 ng of total RNA, 1 µl of the gene expression assay mix and 10 µl of the master mix with the following temperature profile: 95 °C for 10 min and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. mRNA expression values were normalized to the level of GAPDH expression. The following probes from LifeTechnologies were used: GAPDH (Mm99999915_g1), FIZZ-1 (Mm00445109_m1), NOS2 (Mm00440485_m1), Arg-1 (Mm00475988_m1), IL-4 (Mm00445259_m1), IDE (Mm00473077_m1), for BACE1 detection the following set of primers was used forward: ACAACCTGAGGGGAAAGTCC, reverse: TACTACTGCCCGTGTCCACC. Analysis of the expression of the genes was performed using StepOne 2.2 software provided by Applied Biosystems.

Determination of Aβ containing plaque-associated microglia

For the determination of Aβ containing plaque-associated microglia, a double immunofluorescence staining for CD11b and Aβ was carried out using the above described antibodies. Fields of plaques were randomly selected in the cortex. Images were made in Cell-P with automatic illumination. The area of CD11b overlaying plaques was determined with the colocalization finder plugin in Image J 1.43m and corrected for total plaque area determined with the subroutine particle analysis after background subtraction equal for all images and binarization. Only plaques with a diameter smaller than 30 µm were included in the analysis. Per animal, a coverage of 50 to 200 plaques by microglia was determined. Animal number per group was 5.

Assessment of microglial functions *in vivo*: *In vivo Aβ phagocytosis assay*: Mice were intraperitonealy injected 3 h before sacrification with 10 mg/kg methoxy-XO4 (kindly provided by Dr. Alfons Verbruggen, Katholieke Universiteit Leuven) in 50% DMSO/50% NaCl (0.9%), pH 12. Mice were perfused with ice-cold PBS and the

brains were removed, chopped into pieces using scalpels and incubated in HBSS, 10 % FCS containing 0,144 mg/ml collagenase type IV for 1 h at 37°C. Homogenization was achieved by pipetting gently up and down using a 19G needle. The homogenate was filtered through a cell strainer (70 µm) and centrifuged at 155 x g at 4°C for 10 min without brake. The pellet was resuspended in 9 ml 70% Percoll in PBS and underlayered with ice-cold 10ml 37% Percoll in PBS and overlayered with 6 ml ice-cold PBS. The gradient was centrifuged at 800 x g at 4°C for 25 min without brake. Microglial cells were recovered from the 37/70% Percoll interphase, diluted with 3 vol. PBS and centrifuged at 880 x g at 4 °C for 25 min (Beckman Allegra) without brake. The pellet containing the microglial cells was resuspended in 200 µl PBS. For flow cytometry analysis, 50 µl of cells were diluted with 0.5 ml HBSS, centrifuged at 250 x g for 5 min at 4°C. Binding of antibodies to Fc-receptors was prevented by adding 1 µg Fc-block and incubating for 10 min on ice. Cells were taken up in 50 µl of primary antibody mix (CD11b-APC (1:100, BioLegend, Fell, Germany, #101212), CD45-FITC (1:100, eBioscience, San Diego, CA, #11-0451), CD36-PE, (1:100, eBioscience, San Diego, CA , #12-0361) and incubated for 30 min on ice. Cells were centrifuged at 250 xg for 5 min at 4 °C and resuspended in 200 µl HBSS. For control and compensation, corresponding isotype control antibodies were used. Cells were measured on a FACSCanto II (BD Bioscience, Heidelberg, Germany). For analysis, the CD11b$^+$ CD45$^+$ population was gated. WT mice injected with methoxy-XO4 were used to determine the methoxy-X04-threshold for non-phagocytosing cells and unstained WT cells were used to determine background fluorescence. For FACS, microglia were stained with CD11b alone and sorted using a FACSDiVa cell sorter (BD Bioscience, Heidelberg, Germany). For determination of total brain methoxy-X04 fluorescence, APP/PS1 and APP/PS1/NRLP3$^{-/-}$ were injected with methoxy-X04 as described above. After 3 h, brains were homogenized in PBS with 1 mM 4-(2-

Aminoethyl) benzenesulfonyl fluoride hydrochloride and 50 µl of the homogenate was measured at 368 nm excitation and 450 nm emission in a black 96 well plate using an infinite 200 plate reader (Tecan, Grödig, Austria).

Immunocytochemistry of sorted microglia. A subset of microglial cells that were isolated according to the procedure described above, were used to verify the uptake of methoxy-04 labelled Aβ by immunocytochemistry. Therefore, cells were brought onto glass slides by cytospin and subsequently fixed with 4% paraformaldehyde. Intracellular Aβ was visualized by double immunostaining for IC16[30] and either CD11b (MCA711; AbSerotec, Düsseldorf, Germany) to detect microglial boundaries or LAMP2 using antibody Abl-93 (Developmental Studies Hybridoma Bank, Iowa City, IO), to determine the intracellular localization of methoxy-X04 and IC16 positive Aβ. Inflammasome activation was visualized using the same cells and staining for CD11b and anti-ASC (AL177; AdipoGen, San Diego, CA).

DiOlistics and morphological analysis. Hippocampal neurons from wild type, APP/PS1, APP/PS1/NLRP3$^{-/-}$ and APP/PS1/Casp-1$^{-/-}$ mice were labelled using DiOlistic on acute slices. Briefly, the mice were anesthetized and decapitated, and the brain was quickly transferred into ice-cold carbogenated (95 % O2, 5 % CO2) artificial CSF. Hippocampi were dissected and cut into 400-µm-thick transversal slices with a vibratome (VT 1000S, Leica, Solms, Germany). Vibratome slices were immediately fixed in 4 % PFA overnight at 4 °C. Tungsten particles (50 mg; 1.7 µm in diameter; Bio-Rad, Munich, Germany) were spread on a glass slide, and 100 µl of dye solution prepared by dissolving 3 mg of lipophilic dye DiI (Invitrogen, Karlsruhe, Germany) in 100 µl of methylenechloride (Sigma-Aldrich, Munich, Germany). The dried dye-coated particles were removed from the glass slide, resuspended in 3 ml of distilled water, and sonicated. The dye solution was subsequently diluted 1:100. To improve the bead attachment, the tube walls were precoated with a solution of PVP

(polyvinyl-pyrrolidone) (stock: 0.05 mg/ml in ethanol; Bio-Rad), and the bullets were stored at room temperature. Dye-coated particles were delivered to the acute slices using a hand-held gene gun (Bio-Rad, Helios Gene Gun System). A membrane filter (3 μm; Millipore, Darmstadt, Germany) was inserted between the gene gun and the preparation to prevent clusters of large particles from landing on the tissue. After shooting, slices were kept in PBS for 3 d at room temperature to allow dye diffusion. The slices were postfixed with 4% PFA, washed, and mounted using an antifading water-based mounting medium (Biomeda, Foster City, CA). The spine density of pyramidal cells was measured for mid-apical dendrites. The selected dendrite segments were imaged using a LSM510 Meta confocal microscope (Zeiss, Oberkochen, Germany) using a 40x water-immersion objective and a zoom 4 and were z-sectioned at 0.5 μm. The number of spines was normalized per micrometer of dendritic length. The statistical analysis was performed using GraphPad Prism 4. All data shown are presented as mean SEM. The data obtained were compared between two different experimental conditions using a two-tailed Student t test. Asterisks indicate the significance levels as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.