Supplementary Materials for

Scanning ultrasound removes amyloid-β and restores memory in an Alzheimer’s disease mouse model

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This PDF file includes:

Methods
Fig. S1. Absence of brain damage after either repeated or short-term SUS treatment.
Fig. S2. Skeleton analysis of microglia.
Fig. S3. Increased Aβ uptake by microglial cells in the presence of albumin.
Fig. S4. Analysis of IDE and tau phosphorylation after SUS treatment in AD mice.
Fig. S5. Analysis of SUS-treated mice for inflammatory markers.
Fig. S6. Absence of astrogliosis but activation of microglia after acute ultrasound treatment in wild-type mice.
Legend for movie S1

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencetranslationalmedicine.org/cgi/content/full/7/278/278ra33/DC1)

Movie S1 (.mov format). High-resolution 3D reconstruction of a plaque imaged in a 40-µm section of a SUS-treated mouse.
Supplementary Methods

Behavioral testing - Y maze
The Y-maze was made of clear Plexiglas and had three identical arms (40 x 9 x 16 cm) 120° apart. The center platform was a triangle with 9 cm side-length. The room was illuminated by 70 lux. Mice were habituated to the testing room and the apparatus 24 h prior to testing by being in the maze for 5 min. On the day of testing mice were placed in one of the arms and allowed to explore the maze for 8 min. Arm entry was defined as having all four limbs inside one of the arms. Mice were videotaped and the videos were analyzed blind. The maze was cleaned with 70% ethanol between animals. The sequence of arm entries was used to obtain a measure of alternation, reflecting spatial working memory. The percentage alternation was calculated by the number of complete alternation sequences (i.e., ABC, BCA, CAB) divided by the number of alternation opportunities (total arm entries minus two).

Behavioral testing - Active place avoidance (APA) test
The APA task is a test of hippocampus-dependent spatial learning. Mice (APP23 mice and non-transgenic littermate controls) were tested over five days in a rotating elevated arena (Bio-Signal group) that had a grid floor and a 32 cm high clear plastic circular fence enclosing a total diameter of grid of 77 cm. High-contrast visual cues were present on the walls of the testing room. The arena and floor was rotated at a speed of 0.75 rpm and a 500 ms, 60 Hz, 0.5 mA mild shock was delivered through the grid floor when the animal entered a 60 degree shock zone, and every 1,500 ms until the animal left the shock zone. The shock zone was maintained at a constant position in relation to the room. Recorded tracks were analyzed with Track Analysis software (Bio-Signal group). A habituation session was performed 24 h before the first training session in which animals were placed in the rotating arena for 5 min to explore but the mice did not receive any shocks during this period. After this initial testing APP23 mice were divided into two groups with mice matched so that the performance of the two groups of mice on day four of the task was the same. Four training sessions were held on consecutive days, one per day with a duration of 10 min.
Following a 7 week-period of treatment in which the mice were given SUS or sham treatment (in which the sham mice received all injections but ultrasound was not applied), they were retested in the task (reversal learning). For retesting the shock zone was switched to the opposite side of the arena and the visual cues were changed but mice were tested in the same room. The number of shocks delivered to the SUS-treated APP23 mice, sham-treated APP23 mice, and sham-treated wild-type mice were compared over the days of testing. As an additional measure we broke the 10 min interval into two 5 min intervals, the first a measure of long-term memory and the second of working/short-term memory. In the first 5 min from day 2 onwards mice are remembering the location of the shock zone from the previous day (hence long-term memory) whereas in the last 5 min mice have already received a shock in the first 5 min and so have more recent experience of the shock zone (hence short-term memory). Data was analyzed with a Two-Way ANOVA with day of testing as a within-subjects factor and simple effects of group tested with Fisher’s LSD post-hoc test.

**Behavioral testing - Novel object recognition (NOR) test**

Mice were also tested in the NOR test. A Y-shaped arena made of white Perspex (30 cm height x 16 cm length x 8 cm width) was used. Mice were placed in a start arm and the other arms contained the objects. A camera recorded the mice and Ethovision XT was used to analyze the time the mouse spent investigating less than 2 cm from the object with its nose pointed in the direction of the object. Between each trial the maze was thoroughly cleaned with paper towel and 70% ethanol. For two days prior to the test session mice underwent habituation to the Y-shaped maze for 8 min each without any objects in the arms. On the third day a sample phase was followed by a choice phase. In the sample phase two identical copies of an object were placed at the end of two arms. The mice explored the objects for 8 min. Following a 30 min delay in which the animal was placed back in its home cage, a choice phase was carried out in which the objects were replaced by a third identical copy of one of the objects and a novel object to which the mice had not been exposed. The choice phase was 4 min in duration. Times when an animal climbed on an object were not counted. Which objects were sample and novel objects and in which arm the novel object was placed in were counterbalanced within and across groups. We calculated the preference for the novel object as the total time spent exploring the novel object subtracted from the time spent exploring the familiar object (Fig. 4E). Differences between groups were assessed by Student’s t-test. We also
calculated a discrimination ratio, by dividing this measure by the total time spent exploring both objects (Fig. 4F).

**In vitro microglial uptake**

BV-2 microglia cells (kindly provided by Dr Trent Woodruff, University of Queensland) were maintained in DMEM containing 1% fetal bovine serum, non-essential amino acids and antibiotics. Cells were passaged when 80% confluent and a passage number of 10-15 was used for the experiments. For phagocytosis studies, cells were plated at a density of 30,000 cells per chamber in eight well-tissue culture chamber slides (Sarstedt). Aβ42 biotinylated at the N-terminus (JPET) was dissolved in DMSO and then diluted to a 1 mg/ml concentration in PBS and aggregated at 37 °C for 7 days to obtain fibrillar Aβ (fAβ42). Cells grown in the chamber wells were treated with 10 mg/ml human serum albumin (Sigma) in normal medium for 24 h or with normal cell medium alone (control). As a negative control for phagocytosis, the actin polymerization inhibitor cytochalasin D was added at a 5 µM concentration 1 h prior to the addition of fAβ42. Cells were then treated with 2.5 µg/ml fAβ42 for 60 min and then washed twice with normal medium and then fixed with 4% paraformaldehyde for 20 min. Cells were permeabilized by adding 0.1% Triton X-100 and blocked with 1% bovine serum albumin for 1 h, before being incubated with the anti-LAMP2 (lysosomal-associated membrane protein 2) antibody (1:500, Sigma) overnight at 4 °C. After washing with PBS, cells were incubated with Alexafluor 594-conjugated streptavidin to label biotinylated Aβ (1:1000, Invitrogen) for 1 h at room temperature and then stained with DAPI and cover-slipped with fluorescent mounting medium (Vectashield). Under confocal microscopy, three or four areas containing more than 100 cells in total were randomly chosen based on DAPI staining. Numbers of microglia containing phagocytosed fAβ42 were determined by counting the number of cells containing Alexafluor 594-labeled fAβ42 within LAMP2-positive areas and expressed as a percentage of the total number of cells counted. Data are representative of two experiments and expressed as mean ± standard error of the mean (SEM).
Supplementary Figure 1 - Absence of brain damage after either repeated or short-term scanning ultrasound (SUS) treatment. (A,B) The BBB is opened throughout the brain after SUS treatment, as evidenced by prevalent Evans Blue extravasation as early as 30 min after the treatment. (C-E) Absence of edemas, erythrocyte extravasation and 'dark' neurons revealed by Nissl staining (close-up: dentate gyrus) of cohort 1 (APP23 mice, 5 treatments over a period of six weeks) (C-D) and (E-H) hematoxylin and eosin staining, showing the cortex (E,F), and the hippocampus (G,H). (I,K) Absence of ischemic damage after SUS treatment of wild-type mice either 4 h (I) or 24 h after SUS treatment (J) using acid fuchsin staining. Scale bars: C=1mm, E,F=50 µm, D,G,H=200µm, I,K=50 µm
Supplementary Figure 2 - Skeleton analysis of microglia. 40 µm sections from the auditory cortex overlying the dorsal hippocampus (an area rich in plaques) stained with Iba1 using the nickel/DAB method (left) were converted to binary images and then skeletonized (right) using the Analyze Skeleton plugin by ImageJ. Sections from (A) Non-Tg, (B) sham-treated APP23, and (C) SUS-treated APP23. Scale bar: A-C=50 µm.
Supplementary Figure 3 - Increased Aβ uptake by microglial cells in the presence of albumin. Aβ42 uptake increases by 65% by the presence of albumin in BV-2 microglial cells (t-test, P=0.018). Cytochalasin D was included as control to inhibit phagocytosis. A BV-2 cell culture co-incubated with albumin is shown (green, LAMP2; red: Aβ). Scale bar: 25µm.
Supplementary Figure 4 - Analysis of IDE and tau phosphorylation after SUS treatment in AD mice. (A,B) Western blot showing concentration of the Aβ-degrading enzyme IDE (insulin-degrading enzyme) in the extracellular fraction obtained from SUS-treated and sham-treated APP23 mice. An age-matched wild-type (Non-Tg) mouse is included as control. (B) Quantification of IDE in the two groups in panel A (unpaired t-test, n.s., n=8 per group). (C, D) In AD, the protein tau is hyperphosphorylated at several residues including Ser202/Thr205, generating the AT8 epitope. Western blot showing AT8 immunoreactivity in the Triton-soluble fraction obtained from SUS-treated and sham-treated APP23 mice. An age-matched wild-type (Non-Tg) mouse was included as control. (D) Quantification of AT8 in the two groups in panel C (unpaired t-test, n.s., n=8 per group).
Supplementary Figure 5 - Analysis of SUS-treated mice for inflammatory markers. (A,B) Immunoreactivity (percentage immunoreactive area) for the astrocytic marker GFAP is increased in APP23 compared to Non-Tg mice, but there is no difference between sham-treated (A) and SUS-treated APP23 mice (B). (C,D) NFkB-positive nuclei as a marker of excessive, chronic inflammation are absent in wild-type mice. In APP23 mice NFkB-positive nuclei are low in numbers and are confined to plaques, with no obvious difference between SUS-treated (C) and sham-treated APP23 mice (D). (Blue, DAPI; green, Iba1; red, nuclear NFkB). Scale bars: 200μm.
Supplementary Figure 6 - Absence of astrogliosis but activation of microglia after acute ultrasound treatment in wild-type mice. Iba1 (A-C) and GFAP staining (D-F), 0 hours (A,D), 1 hour (B,E) and 24 hours (C,F) after SUS treatment. Scale bars: 100µm.
Supplementary Video

High-resolution 3D-reconstruction of a plaque imaged in a 40 µm section from a SUS-treated mouse.

Sec 1-5: A representative 3D projection of a plaque imaged in the cortex of a SUS-treated mouse. The microglia (green) can be seen surrounding the Aβ (red) containing plaque. Nuclei are labeled with DAPI (blue). Microglial cell bodies are not observed within the core of the plaque but they can be seen extending processes towards the core and wrapping around accumulations of Aβ.

Sec 6-13: Focusing on a selected region of microglia labeling close to the plaque, a 3D surface is generated. This surface highlights the branched morphology of the microglia. Looking beneath the surface, within the microglia, extensive accumulation of internalized Aβ can be observed.

Sec 14-24: Partial removal of the 3D surface of the microglia reveals the internal accumulation of Aβ. As the movie progresses, the microglial surface is further peeled back to fully reveal the internalized Aβ.

Sec 25-33: The microglial surface is restored. As the movie progresses, upon zooming out, the whole surface of the plaque again becomes visible.