Ultrasound Mediated Microbubbles Destruction Assists Dual Delivery of Beta-amyloid Antibody and NSCs to Restore Neural Function in Transgenic Mice of Alzheimer's Disease

qiong Zhu
Army Medical University
hai Cui
Army Medical University
yiyi Liao
Army Medical University
xue Guan
Army Medical University
ying He
Army Medical University
yani Rong
Army Medical University
yi Zhang
Army Medical University
chun Li
Army Medical University
zheng Liu
Army Medical University
yali xu (xuyali1976@163.com)
Army Medical University https://orcid.org/0000-0002-6311-8757

Research

Keywords: Alzheimer's disease, Ultrasound mediated microbubble destruction, Beta-amyloid, Neural stem cells, Blood-brain barrier

Posted Date: May 11th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-27241/v1

Ultrasound Mediated Microbubbles Destruction Assists Dual Delivery of Beta-amyloid Antibody and NSCs to Restore Neural Function in Transgenic Mice of Alzheimer's Disease

AUTHORS: Qiong Zhu[1], Hai Cui [1,2], Yiyi Liao [1], Xue Guan [1], Ying He [1], Yani Rong [1], Yi Zhang [1], Chun Li [1], Zheng Liu [1] and Yali Xu [1]

Affiliations

1.Department of Ultrasound, Xinqiao Hospital, Army Medical University (Third Military Medical University), Chongqing, China.

2.Department of Ultrasound, Armed Police Force Hospital, Sichuan, China.

Corresponding author

Xu Yali, M.D., Ph.D.

Department of Ultrasound, Xinqiao Hospital,

Army Medical University, Chongqing 400037, China.

Email: xuyali1976@163.com

Telephone: 86-23-68755681

Abstract

Background: To explore the feasibility, efficacy and safety of ultrasound mediated microbubbles destruction（UMMD） assisted dual delivery of β-amyloid antibody loaded by microbubbles ($MB_{A\beta}$) and neural stem cells (NSCs) on Alzheimer's disease（AD）.

Methods: 27 APP/PS1 double transgenic mice and 33 wild-type mice were used. The dual delivery of β-amyloid antibody and NSCs group (US+$MB_{A\beta}$+NSCs), single delivery of β-amyloid antibody group (US+$MB_{A\beta}$), US+MB group, Control group and Wild group, were involved in the experiment. $MB_{A\beta}$ or MB were injected via the tail vein, followed by NSCs or saline administration and exposed to ultrasound once a week for four times. The survival of NSCs was detected with the in vivo imaging method. Mice in each group were used for behavioral function evaluation and the pathology tests. Brain samples were used to detect β-amyloid deposition, BDNF and synaptophysin expression.

Results: BBB was opened by UMMD with an opening time about 10 h. The transplanted NSCs survived in AD brain for no more than 72 h. The learning and spatial memory function was significantly improved in the US+$MB_{A\beta}$+NSCs group, US+$MB_{A\beta}$ group came second. Immunochemistry results showed amyloid plaques reduction in the US+$MB_{A\beta}$+NSCs group at the cortex and hippocampus. Higher level of BDNF was demonstrated in the US+$MB_{A\beta}$+NSCs group than the US+$MB_{A\beta}$ group and the Control group with Western Blot and immunofluorescence examination, but synaptophysin remained no significant changes.

Conclusions: UMMD assisted combined delivery of β-amyloid antibody and NSCs to AD mice brain can help to clear the Aβ peptide, increase BDNF level and restore the impaired neural function, which was superior to β-amyloid antibody delivery group. Therefore, the combined

targeted delivery assisted by UMMD strategy may be a promising and safe method on treating AD.

Keywords: Alzheimer's disease; Ultrasound mediated microbubble destruction; Beta-amyloid; Neural stem cells; Blood-brain barrier

Background

Alzheimer ’s disease（AD） is the leading cause of dementia in the aged population and is defined as a progressive neurodegenerative disorder. The pathological hallmarks are the β-amyloid (Aβ) plaques and neurofibrillary tangles, which have become the most appropriate targets for the therapy of AD [1,2]. However, most of the therapeutants can hardly enter the brain owing to the blockage of blood brain barrier (BBB). Ultrasound (US) mediated microbubbles (MB) destruction (UMMD) induced BBB opening has provided a potential delivery approach for the therapeutic agents to enter the brain [3]. Several studies have focused on the reduction or clearance of Aβ plaque using US [4-7]. Nisbet and Xu have reported that both Aβ and Tau aggregates can be effectively cleared and the neuronal function has been restored with US [8-10]. Previous studies have proved that mesenchymal stem cells have been targeted and successfully homed into brain under UMMD induced BBB opening, which resulted in the improvement on neuronal loss and the cognitive and behavioral function in brain ischemic rats [11,12]. Other studies reported similar results following neuron stem cells (NSCs) implantation in AD animals [13,14]. Since Aβ peptide is released into the extracellular space whereas tau is distributed in the neuron and axon, Aβ may be more appropriate than Tau as a therapeutic target to be delivered into brain under UMMD in our research. Aiming to strengthen the recovery on the neuronal function and the clearance of Aβ deposits, a dual delivery of both the NSCs and Aβ antibody under UMMD was performed in APP/PS1 (+) transgenic AD mice in this study.

Materials and Methods

Experimental Animals

APP/PS1 double transgenic mice, B6/JNju-Tg（APPswe，PSEN1dE9）/Nju, were purchased from Animal Research Center of Nanjing University. Mice were housed in a specific pathogen free environment with a 12 h light-dark cycle at a constant temperature about 24 ℃. Twenty-seven 9~12-month-old APP/PS1 (+) mice and thirty-three age-matched wild-type mice, weighed 25-30 g, were used in this study.

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Army Medical University and were performed in accordance with the guidelines from the Chinese Animal Welfare Agency. The number of animals used was minimized. All mice were anesthetized intraperitoneally with 2, 2, 2-tribromoethanol (1.2%, 10 ml/Kg, Sigma-Aldrich, St. Louis, MO, USA), placed in a prone position with arms and legs immobilized. The hair of the mice skull was removed using depilatory creams to expose the parietal.

Diagnostic US parameters

A linear array probe (X4-12L) of a VINNO 70 diagnostic US scanner (Vinno Technology Co.Ltd, SuZhou, China) was used as MB trigger to open the BBB of mice in this study. For image-guided therapy, the system was modified, and the mode was called V-flash with adjustable therapeutic parameters and treatment area. In this study, mice were sonicated for 5 min with 3 MHz frequency, 50 Hz pulse repetition frequency and 26 cycles pulse length line by line at a density of 32/cm, and the transducer was positioned anterior to the connection line of ears to ensure the beams focused at the targeted area (Fig. 1).The MI value was around 0.8, the transmitting/intermittent time was 0.62/2 s, and the focal depth was set at 1 cm.

The preparation and characterizations of β-amyloid antibody-loaded and unloaded MB

The unloaded MB were prepared according to the protocols of the previous study [15]. And

the concentration of MB is (2-9) $\times 10^9$/ml [16]. The β-amyloid antibody loaded microbubbles ($MB_{A\beta}$) were prepared by an electrostatic absorption method with the purified anti-β-amyloid, 1-42 (mouse monoclonal, BioLegend, Inc. San Diego, CA) and MB. 10 μl of 1-42 anti-β-amyloid was added into the lyophilized lipid suspension of MB and incubated together for 1 h at room temperature, followed by the infusion of C_3F_8 in each vial. After agitation, MB labeled with β-amyloid antibody was formed. In this study, both MB and $MB_{A\beta}$ were diluted for 100 times with normal saline (NS).

The size and dispersion of the $MB_{A\beta}$ were measured by a Malvern Zetasizer Nano ZS90 detector (Malvern Instruments Inc., Worcestershire, UK). To further evaluate the property of $MB_{A\beta}$, the lipid membranes of $MB_{A\beta}$ were labeled with1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (1 μl, DiI, Yeasen Co. Ltd., Shanghai, China), and the β-amyloid antibody was labeled with FITC-labeled Goat Anti-Mouse Ig-G (1 μl, Beyotime Institute of Biotechnology, China). Both DiI and FITC were incubated for 45 min at room temperature. After incubation, the mixtures were washed for 3 times by centrifugation flotation at 300 rpm for 3 min to remove the extra DiI and FITC. The fluorescent microscopy of the labeled $MB_{A\beta}$ was observed by confocal laser scanning microscope (Leica TCS SP5, Wetzlar, Germany).

BBB Opening procedure and histological examination

To ensure the acoustic beam targeted to the hippocampus, the transducer was positioned anterior to the line of ears and at the rear of eyes. To confirm the BBB opening efficacy and opening time duration, Evens Blue (EB, 1%, Sigma-Aldrich, St. Louis, MO, USA) was injected intravenously after US exposure to assess the increased BBB permeability. During the 5 min

sonication, 300 μl MB or $MB_{A\beta}$ was administrated intravenously through the tail vein continuously to ensure there were enough cavitation nuclei in the mice brain. Both two-dimensional and contrast-enhanced US imaging modality were performed to confirm the targeted irradiation area. After US exposure, EB was injected at a dose of 50 mg/Kg via the tail vein at various time point (0 h, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h) (n=3 at each time point). One hour after EB injection, the mice were transcardially perfused (30 ml NS) and sacrificed. Then, the gross views of the harvested brains were observed to access the BBB opening capability and opening time duration.

To observe the safety of UMMD enhanced BBB opening, the mice (n=6) were transcardially perfused (30 ml NS followed by 10 ml 4% paraformaldehyde) and sacrificed immediately after the sonication. Then the brains were harvested and immersed in 4% paraformaldehyde for 24 h. Afterwards, the brains were made into 25 μm paraffin sections and hematoxylin-eosin staining was performed to assess the potential brain damages. Images were visualized with a light microscope (BX63, Olympus, Japan).

The preparation and characterization of Neural stem cells

Neural stem cells (NSCs) were harvested from the wild-type C57 embryos at embryonic day 12 to 14 (E12-E14) as described in the previous study [17]. NSCs were cultured in T25 culture flasks containing DMEF/F12 (Gibco, NY, USA) serum-free medium supplemented with 2% B27 supplement (50×,Gibco, NY, USA), 1% recombinant murine epidermal growth factor (EGF, 20ng/ml, PeproTech, NJ, USA), 1% recombinant murine basic fibroblast growth factor (bFGF, 20ng/ml, PeproTech, NJ, USA) and 1% penicillin–streptomycin solution (Hyclone, UT, USA) in a humidified atmosphere containing 5% CO_2 at 37 ℃. NSCs were grown as free-

floating neurospheres. The medium was half replaced every other day. In addition, neurospheres were dissociated to get single cell using StemPro Accutase cell dissociation reagent (Gibco, NY, USA) for passaging or transplantation. NSCs at the third to fifth passage were used in this study.

In vivo imaging of the luciferase labeled NSCs

To evaluate whether the NSCs could pass the pulmonary circulation and home to the brain or not, a whole-animal imaging was performed to detect bioluminescent signals of NSCs using the in vivo imaging Spectrum system (IVIS, Xenogen, Alameda, CA, USA). Images were acquired and analyzed by Living Image 4.5 (Xenogen, Alameda, CA, USA).

For the in vivo tracking, NSCs at the 3rd passage was labeled with firefly luciferase (fLuc) via lentivirus (GeneChem, Co. Ltd. Shanghai, China) transfection according to the manufacturer's protocol and seeded in 96-well plates at 5×10^4 cells/ml. Seventy-two hours after transfection, NSCs encoding fLuc were sorted by the puromycin at different concentration (Solarbio Science & Technology Co., Ltd., Beijing, China) and collected for culturing and passaging.

The Luciferase labeled NSCs (fLuc-NSCs, 200 μl，2×10^6 cells /ml) were injected into the APP/PS1 (+) mice (n=3) via the tail vein after BBB opening. The in vivo images of NSCs were captured 15 min after intraperitoneal injection of D-luciferin substrate (10 μL/g, Solarbio Science & Technology Co., Ltd., Beijing, China) to observe the distribution and survival of fLuc-NSCs in mice brain every 24 h until no bioluminescent signals were detected.

Treatment protocol

The treatment protocol was described in Table 1. Mice were randomly divided into five groups and the treatment protocol of each group was as follows: US+$MB_{A\beta}$+NSCs group

(UMMD assisted dual delivery group): during US exposure, the V-flash mode was switched on and the treatment center was focused at the hippocampus, 300 μl diluted $MB_{A\beta}$($2\text{-}9\times10^7$/ml) was administrated into the APP/PS1 (+) mice within 5 min, after the US exposure, 200 μl NSCs (2×10^6 cells /ml) were infused. US+$MB_{A\beta}$ group (single delivery of Aβ antibody): 300 μl diluted $MB_{A\beta}$ and 200 μl NS was given to the APP/PS1 (+) mice under US exposure for 5min. US+MB group: 300 μl MB and 200 μl NS infusion with US exposure on APP/PS1 (+) mice. Control group: 500 μl NS was administrated into the APP/PS1 (+) mice with a sham US exposure. Wild group: 500 μl NS was administrated into the APP/PS1 (-) mice with a sham US exposure.

The treatment schematic diagram was shown in Figure 1. The treatment was performed in each group once a week for four times. After the treatment, the mice were used for the behavioral test and the histological examinations after heart perfusion. Half of the brains were fixed with 4% paraformaldehyde, and the other hemispheres were used for western blot. The diagram and time sequences of the treatment procedure were illuminated in Figure 1.

Neural function evaluation with Morris Water Maze Test

The learning and memory function were assessed in a Morris water maze (MWM, Beijing Liuyi Co. Ltd., China) task, including the place-navigation test and spatial probe test. MWM was a white-colored circular water pool in 120 cm-diameter, 40 cm height and maintained at 25±2 ℃. The place-navigation was assessed for 5 consecutive days. A platform submerged 2 cm below the water surface was placed in the fourth quadrant. Mice were placed into the tank at the same point of each quadrant. If a mouse found the platform within 90 s and stayed on it for more than 10 s, it was considered successful. Otherwise, the mice were manually guided to the platform and remained on it for 10 s. The swimming paths and the time reaching the

platform were recorded by a computerized video imaging analysis system. The interval between each trial was 60 s.

After the final place-navigation test on the 5th day, the spatial probe test was carried on with the platform removed. The mice were placed at any one of the four quadrants. The numbers of crossings in the area where the platform originally located and the time spending in the target quadrant were recorded for 60 s.

Identification of NSCs with immunochemistry staining

Immunocytochemistry was performed on the neurospheres at the 3rd passage to identify NSCs. The neurospheres were cultured in the confocal dishes for 5 days and fixed with 4% paraformaldehyde for 5 min and treated with 0.1% TritonX-100 for 15 min. After washing with PBS, the neurospheres were sequentially blocked in 10% goat serum (Abcam, Cambridge, UK) for 10min followed by the incubation with the Anti-Nestin Antibody (Mouse monoclonal, 1:200, Millipore, MA, USA) overnight at 4 ℃. The next day, they were sequentially washed in PBS and incubated with Fluorescence-Conjugated affinity pure goat anti-mouse IgG (1:1000, ZSGB-Bio, Beijing, China) at 37 ℃ for 60 min, followed by a washing and incubating in Hoechst33342 (Sigma-Aldrich) for 5 min. Images were acquired by a laser scanning confocal microscope (TCS SP5, Leica, Germany).

Immunohistochemistry was performed by using specific antibody to identify senile plaques, brain-derived neurotrophic factor (BDNF) and synaptophysin (SYN) of the mice brain. After the fourth treatment, mice in each group were anesthetized and transcardially perfused with 30 ml NS. Then, one hemisphere of a mouse brain was extracted and fixed with 4% paraformaldehyde for 48 h and cut into 4μm-thick paraffin slide sagittally. After dewaxing,

hydration and antigen retrieval, the sections were sequentially treated with 0.25% TritonX-100 for 30 min and blocked with normal donkey serum (Abcam) for 30 min, followed by the incubation with specific antibody of anti-β-amyloid, 1-42 (1:100, BioLegend), BDNF (rabbit polyclonal, 1:100, Proteintech) and SYN (mouse monoclonal, 1:100, Millipore). Biotinylated antibody (SP9000, ZSBG-Bio, Beijing, China) or Alexa Fluor 647-labeled Goat Anti-Rabbit IgG (1:200, Beyotime) was used as the secondary antibody. Five slices of each sample were observed, and the images were captured with an Olympus BX51 microscope or a Leica laser scanning confocal microscope.

BDNF and SYN expression assessed with Western blotting

Brain samples at the targeted areas were homogenized in mortar with liquid nitrogen. The proteins were extracted in the protease and phosphatase inhibitors (Beyotime) on the ice for 30 min. Bicinchoninic acid protein determination assay (BCA protein assay reagent; Beyotime) was used to analyze protein concentration. Samples (10 μl) were electrophoresed in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. Then, the membranes were sequentially blocked in 5% nonfat dry-milk solution for 1 h, incubated with the primary antibodies of anti-BDNF (1:1000), anti-SYN (1:1000) and Tubulin (1:1000, Beyotime) overnight at 4 ℃. After washing the membranes with Tween-Tris-buffered saline buffer, the membranes were incubated in alkaline phosphatase-conjugated secondary antibody (1:5000, ZSGB-Bio) for 1 h. After the incubation, the membranes were washed three times and treated with enhanced chemiluminescence method (Millipore). Images were captured with a LAS-4000 mini (GE Healthcare, Wausaukee, WI). The bands were analyzed with the signal intensity ratio between the targeted protein and Tubulin using Image J.

Statistical analysis

All the data were presented as mean values ± standard deviation. To account for the multiple mean comparisons, one-way analysis of variance (ANOVA) with least significant difference (LSD) post hoc testing or nonparametric tests with Kruskal-Wallis were used. All the statistics were analyzed by SPSS 20.0 software. $P<0.05$ was considered as statistically significant.

Results

Characterization of $MB_{A\beta}$

The $MB_{A\beta}$ were in milky and stable at room temperature, and mainly ranging from 300 to 700 nm in diameter (Fig. 2D). $MB_{A\beta}$ can achieve the same imaging intensity as the MB with contrast-enhanced US. There were both red and green fluorescence on the membrane of MB with the fluorescence imaging, indicating FITC-labeled anti-Aβ-amyloid has been successfully loaded onto the DiI-labeled MB (Fig. 2A-C).

Characterization of NSCs

NSCs isolated from E12-14 C57 embryos were in free-floating neurospheres and capable of proliferation, self-renewal and differentiation. A remarkable expression of Nestin was revealed in the neurospheres with the immunofluorescence analysis (Fig. 2E, F).

In vivo tracking of the survival of luciferase labeled NSCs

In order to tracking the viability of fLuc-NSCs, in vivo imaging of mice was captured every day until no visible bioluminescence was recorded after transplantation. The results revealed the bioluminescence intensity at 48 h (Fig. 2H) was higher than that at 24 h (Fig. 2G), while none at 72 h (Fig. 2I). No bioluminescence was found in the other organs at any time. And the results indicated that fLuc-NSCs infusion via tail vein can go through the pulmonary circulation

and home to the brain, and fLuc-NSCs were able to proliferate for a limited time and survive for 48 h in AD mice brain.

Effect of UMMD on BBB opening

A patchy distribution of EB extravasation was observed at the US-exposed area in the gross brain samples, while no visible EB at the unexposed area (Fig. 3A-B). Moreover, EB extravasation was visible from the activation point to the 10^{th} h following UMMD. EB extravasation was evident in the previous 4 h and became weaker with time went on (Fig. 3C-J). At 10 h, only a trace of EB extravasation could be seen in the brain parenchyma and nearly none at 12 h. These results indicated that BBB can be opened by UMMD transiently and restorable with an opening window for no more than 12 h.

Histological findings were shown in Figure 3K-L. No erythrocyte leakages or other tissue damages were detected either in the cortex or hippocampus under the light microscope, which indicated the safety of BBB opening by this method.

Improved cognition performance with Morris water maze

In the place-navigation test, mice in the Wild group and the US+$MB_{A\beta}$+NSCs group displayed a similar latency. On day 3, 4 and 5, both the time spend of the Wild group and the US+$MB_{A\beta}$+NSCs group were shorter than the other groups and had significant difference with the Control group (P<0.05, Fig. 4F). US+$MB_{A\beta}$ group also had significant difference when compared with the control group on day 5 (P <0.05, Fig. 4F). The average time of the 5 days in the US+$MB_{A\beta}$+NSCs group had significant difference with Control and US+MB group but had no difference with the US+$MB_{A\beta}$ group (P <0.05, Fig. 4G). In the spatial probe test, the average time had no significant difference among each groups, but the average distance in the

target quadrant was much longer in the US+$MB_{A\beta}$+NSCs group than Control group (Fig. 4H, P <0.01), and the difference between the US+$MB_{A\beta}$ group and Control group was less significant(Fig. 4H, P <0.05). Significant difference was noted in the crosses number between the US+$MB_{A\beta}$+NSCs group and Control group (Fig. 4I, P<0.05).These data indicated an improvement on the impaired learning and spatial memory of the single Aβ antibody delivery and the combined implantation of Aβ antibody and NSCs group, especially the dual delivery group.

Aβ plaque reduction assessed by immunohistochemistry

Immunohistochemistry was performed with anti-β-amyloid to investigate whether the treatments prompt the amyloid plaques clearance in the brain of the APP/PS1 (+) mice (Fig. 5A-E). A significant difference was found in the number of plaques between the US+$MB_{A\beta}$+NSCs group and Control group both in the cortex and in the hippocampus (P <0.05, Fig. 5F,G). Although no significant difference was observed among the US+MB group, US+$MB_{A\beta}$ group and US+$MB_{A\beta}$+NSCs group, the number of plaques in US+$MB_{A\beta}$+NSCs group was fewer than the other two groups. No difference was found out between Wild group and US+$MB_{A\beta}$+NSCs group. These data showed that the NSCs transplantation combined with $MB_{A\beta}$ delivered by UMMD can efficiently help eliminate amyloid plaques in AD mice.

Elevated BDNF but not SYN expression with western blot and immunofluorescence (IF) method

To determine whether the combined delivery approach improves the expression of BDNF and SYN, IF and western blot were performed. IF result indicating an elevated BDNF expression in the US+$MB_{A\beta}$+NSCs group, second to that of Wild group both at the cortex and

hippocampus (Fig. 6 A-E). In contrast, much lower level of BDNF was detected in Control group. The expression of BDNF was improved in the US+MB group and the US+$MB_{A\beta}$ group when compared to Control group, but the degree was inferior to that in the US+$MB_{A\beta}$+NSCs group. Western blot analyses of BDNF were shown in Figure 6K. Much higher level of BDNF was demonstrated in the US+$MB_{A\beta}$+NSCs group than the US+$MB_{A\beta}$ group and Control group (P <0.05). Disappointingly, no obviously elevated expression of SYN was observed by IF (Fig. 6F-J) and western blot (Fig. 6L) in neither group. Thus, the treatment tried in the current study did not alter the level of SYN.

Taken together, these data confirmed that the combination of NSCs transplantation and $MB_{A\beta}$ mediated by US has recovered the expression of BDNF but not SYN in AD mice in the present study.

Discussion

AD affects millions of people; however, there is still no effective treatment. Therapeutic agent delivery to the brain is a potential approach for AD treatment. The great challenge of BBB blockage on these therapeutic agents delivered into brain has been resolved by the application of UMMD [18,19]. Stem cells such as NSCs or mesenchymal stem cells has been successfully and targeted transplanted to the AD animals, and consequently the neural function was improved and recovered [20,21]. Approaches involving the Aβ plaque clearance by Aβ antibody delivery in AD mice have also been reported [4-7]. But the failure of many clinical trials suggest inefficacy in the treatment of AD using only one target [22]. Aiming to further prompt the clearance of Aβ plaque and cognition improvement in AD mice, a dual delivery approach including both the NSCs and Aβ antibody under UMMD was tried in the present

study.

BBB opening window is important on the therapeutic targets delivered into brain. BBB permeability was increased safely, transiently and restorablely in mice brain before UMMD assisted delivery treatment in the study. EB extravasation was observed to evaluate the BBB opening and lasted for no more than 12 h in the wild type mice following UMMD. The amount of EB extravasation reached the peak at the first time of US exposure and remained a high level in the first 4 h, then decreased till 10 h and became invisible at 12 h. In order to get the optimal delivery efficiency, MB carried Aβ antibody was injected during US irradiation and NSCs was infused at the first time after finishing US exposure in the study. The optimal time for the brain targeted delivery of therapeutants was no more than 4 h following US exposure.

The survival and the proliferation of the transplanted NSCs were vital for AD recovery. The survival of the intravenous injected NSCs in AD mice were detected with fLuc-NSCs by in vivo imaging system. Luminescence was recorded at the calvaria at 24 h, 48 h and 72 h following fLuc-NSCs injection under UMMD assisted BBB opening. The luminescence intensity was higher at 48 h than at 24 h. At 72 h, no luminescence was observed. Few studies have emphasized on the survival time of NSCs in AD with the in vivo imaging method and it is difficult to quantify the viable cell number for the methodological problems [23,24]. Our results proved that the survival time of NSCs was limited for no more than 72 h in AD micro-environment. The possible reason might be that the US and MB parameters tried in this study were not the best for the living and survival of NSCs in AD mice brain and should be optimized in the future study. A repeated treatment strategy of once a week for four times has been preliminarily designed and carried out. According to the limited survival time of NSCs within

353 the AD brain, it is highly recommended to increase the transplantation of NSCs and therapeutic
354 frequency to twice a week rather than once a week for 4 times.
355 The combined delivery approach is superior to the Aβ antibody delivery assisted by UMMD
356 on the neural functional, pathological and molecular biological improvement in AD mice. As
357 demonstrated by the place-navigation test of MWM, the learning function was improved with
358 shorter training time in both the dual delivery group and the Aβ antibody delivery group. In
359 spatial probe test, the spatial memory function was greatly recovered with much longer distance
360 in the targeted quadrant in both the dual delivery and Aβ antibody delivery group, and with
361 much more crosses number in the dual delivery group compared to the control group. The
362 neural function recover was greater in the combined delivery group than the Aβ antibody
363 delivery group. All these results proved that the learning and memory function was repaired
364 greatly both in the dual delivery group and the Aβ antibody delivery group, especially in the
365 dual delivery group. Significant decrease on Aβ deposition was demonstrated in the
366 US+$MB_{A\beta}$+NSCs but not in Aβ antibody delivery group when compared to the control group
367 at the cortex and hippocampus, and the Aβ plaques were fewer in the dual delivery group than
368 Aβ antibody delivery group with no significant difference, which showed no superiority on Aβ
369 clearance with dual delivery approach to the single delivery method. Similarly, Aβ antibody
370 delivery approach could not greatly reduce the deposition of Aβ protein [24], combined with
371 the NSCs implantation could further help the reduction of Aβ protein. Blurton-Jones M has
372 reported that the cognitive and memory function was greatly improved without remarkable
373 pathological changes of Aβ deposition following NSCs transplantation [14]. From the similarity
374 and difference between their study and ours results, single target of NSCs or Aβ antibody could

not greatly prompt the Aβ clearance, however UMMD assisted dual delivery approach played an intensifying effect on the clearance of Aβ plaque in AD mice. BDNF is a neurotrophic molecule of structural and functional integrity of the hippocampus formation, as well as the consolidation of hippocampus-dependent memory [1,8]. Even single target of NSCs could result in BDNF elevation and cognition function recover in AD mice [13,14,21,24]. When combined with Aβ antibody and assisted with UMMD, BDNF was greatly improved at the cortex and hippocampus in both the dual delivery and single delivery group with immunofluorescence (IF) and western blot analysis, and the enhancement degree in the dual targets groups was greater than the single target group. This could explain the greater efficacy on neural function recovery in the combined delivery group.

SYN is correlated with the degree of cognitive decline and the progressing of AD. Give that increasing the expression of SYN is essential. An up-regulation of BDNF was demonstrated in our result, however, there were no significant improvement in SYN with the same methods between the combined delivery group and the single Aβ antibody delivery group, even though following a consecutive four times treatment. SYN was significantly reduced in relation to the deposition of Aβ plaques in the hippocampus and cortex in AD, leading to the loss of synapses and dysfunction of synaptic transmission [25-27]. According to our results, Aβ clearance has been improved in the dual delivery approach, while SYN remained nearly no recover. As we known, synapse formation and connection were a complex and long-term period [28]. Zhang W. et al have reported that after 10-weeks NSCs transplantation, an increased expression of SYN and post-synaptic protein-95 were demonstrated [20]. In the present study, SYN was detected 4 weeks post-treatment, and the results might be different if a longer time about 10

even more weeks was designed and performed. Besides, the in vivo imaging revealed that the NSCs survived no more than 72 h, so the effect of the implanted NSCs was weak and the effect on synaptic recover was limited. Furthermore, a non-invasive and repeatable method using intravenous infusion of NSCs was tried, and only part of NSCs was able to home to the AD brain through systemic circulation. So, the efficiency of NSCs on synaptic recover was restricted. Until now, no exact reasons are elaborated, and further study needs to be explored.

While this study still has some limitations. In the future study, a much longer observation time about 10 weeks should be designed due to the restricted recovery of synaptic function and the parameters optimization should be carried out to improve NSCs survival in AD micro-environment for the limited survival time.

Conclusions

A novel and dual delivery approach of UMMD assisted Aβ antibody and NSCs into AD brain could efficiently, safely increase the expression of BDNF, decrease the deposition of beta-amyloid protein and restore the impaired learning and the spatial memory function when compared to single Aβ antibody delivery approach, which may be a promising strategy on the treatment of AD.

Abbreviations

AD: alzheimer ’s disease; BBB: blood brain barrier; US: ultrasound; MB: microbubbles; UMMD: ultrasound mediated microbubbles destruction; NSCs: neuron stem cells; MBAβ: β-amyloid antibody loaded microbubbles; NS: normal saline; EB: evens blue; NSCs: neural stem cells; fLuc: firefly luciferase; fLuc-NSCs: Luciferase labeled NSCs; BDNF: brain-derived neurotrophic factor; SYN: synaptophysin.

Declarations

Ethics approval and consent to participate

The research has been approved by the Institutional Animal Care and Use Committee of Army Medical University.

Consent for publication

Not applicable.

Availability of data and materials

These datasets generated and/or analyzed in the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have declared that no competing interest exist.

Funding

The research was funded by grants of National Natural Science Foundation of China NSFC [81471795,81971774], the Army Cultivation Project in Medical Science and Technology [16QNP102] and the Chongqing Research Program of Basic Research and Frontier Technology [cstc2018jcyjAX0011].

Authors' contributions

Qiong Zhu was responsible for design, methodology, assemble and analysis the data, and manuscript writing. Hai Cui was responsible for design, assemble and analysis the data, and manuscript writing. Yiyi Liao, Chun Li, Xue Guan, Ying He, Yani Rong and Yi Zhang were responsible for methodology, assemble and analysis the data, and finance. Zheng Liu was responsible for supervision. Yali Xu was responsible for conceptualization, methodology,

manuscript writing, review and editing, and supervision.

Acknowledgements

Not applicable.

Author details

[1]Department of Ultrasound, Xinqiao Hospital, Army Medical University (Third Military Medical University), Shapingba District 480037, Chongqing, China. [2]Department of Ultrasound, Armed Police Force Hospital, Leshan 614300, Sichuan, China.

Reference

1. Bloom GS. Amyloid-beta and tau: the trigger and bullet in Alzheimer disease pathogenesis. Jama Neurol. 2014; 71: 505-8.
2. Blennow K, de Leon MJ, Zetterberg H. Alzheimer's disease. Lancet. 2006; 368: 387-403.
3. Burgess A, Dubey S, Yeung S, Hough O, Eterman N, Aubert I, et al. Alzheimer disease in a mouse model: MR imaging-guided focused ultrasound targeted to the hippocampus opens the blood-brain barrier and improves pathologic abnormalities and behavior. Radiology. 2014; 273: 736-45.
4. Jordao JF, Thevenot E, Markham-Coultes K, Scarcelli T, Weng YQ, Xhima K, et al. Amyloid-beta plaque reduction, endogenous antibody delivery and glial activation by brain-targeted, transcranial focused ultrasound. Exp Neurol. 2013; 248: 16-29.
5. Leinenga G, Gotz J. Scanning ultrasound removes amyloid-beta and restores memory in an Alzheimer's disease mouse model. Sci Transl Med. 2015; 7: 233r-278r.
6. Alecou T, Giannakou M, Damianou C. Amyloid beta Plaque Reduction With Antibodies Crossing the Blood-Brain Barrier, Which Was Opened in 3 Sessions of Focused Ultrasound in a Rabbit Model. J Ultrasound Med. 2017; 36: 2257-70.

7. Jordao JF, Ayala-Grosso CA, Markham K, Huang Y, Chopra R, McLaurin J, et al. Antibodies targeted to the brain with image-guided focused ultrasound reduces amyloid-beta plaque load in the TgCRND8 mouse model of Alzheimer's disease. Plos One. 2010; 5: e10549.
8. Nisbet RM, Gotz J. Amyloid-beta and Tau in Alzheimer's Disease: Novel Pathomechanisms and Non-Pharmacological Treatment Strategies. J Alzheimers Dis. 2018; 64: S517-27.
9. Nisbet RM, Van der Jeugd A, Leinenga G, Evans HT, Janowicz PW, Gotz J. Combined effects of scanning ultrasound and a tau-specific single chain antibody in a tau transgenic mouse model. Brain. 2017; 140: 1220-30.
10. Xu M, Zhou H, Liu Y, Sun J, Xie W, Zhao P, et al. Ultrasound-Excited Protoporphyrin IX-Modified Multifunctional Nanoparticles as a Strong Inhibitor of Tau Phosphorylation and beta-Amyloid Aggregation. ACS Appl Mater Interfaces. 2018; 10: 32965-80.
11. Cui H, Zhu Q, Xie Q, Liu Z, Gao Y, He Y, et al. Low intensity ultrasound targeted microbubble destruction assists MSCs delivery and improves neural function in brain ischaemic rats. J Drug Target. 2020; 28: 320-9.
12. Xu Y, Cui H, Zhu Q, Hua X, Xia H, Tan K, et al. Unilateral Opening of Rat Blood-Brain Barrier Assisted by Diagnostic Ultrasound Targeted Microbubbles Destruction. Biomed Res Int. 2016; 2016: 4759750.
13. Blurton-Jones M, Kitazawa M, Martinez-Coria H, Castello NA, Muller FJ, Loring JF, et al. Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. Proc Natl Acad Sci U S A. 2009; 106: 13594-9.
14. Zhang W, Gu GJ, Zhang Q, Liu JH, Zhang B, Guo Y, et al. NSCs promote hippocampal neurogenesis, metabolic changes and synaptogenesis in APP/PS1 transgenic mice. Hippocampus.

2017; 27: 1250-63.

15. Liu P, Wang X, Zhou S, Hua X, Liu Z, Gao Y. Effects of a novel ultrasound contrast agent with long persistence on right ventricular pressure: Comparison with SonoVue. Ultrasonics. 2011; 51: 210-4.

16. Gao S, Zhu Q, Dong X, Chen Z, Liu Z, Xie F. Guided longer pulses from a diagnostic ultrasound and intraclot microbubble enhanced catheter-directed thrombolysis in vivo. J Thromb Thrombolysis. 2017; 44: 48-56.

17. Zhu Q, Gao Y, Gao S, Chen Z, Liu Z, Xu Y. Cultivation of Neural Stem Cells and their Differentiation in different serum concentration. Chinese J of Tissue Engineering Research. 2017; 21: 2708-13.

18. Leinenga G, Langton C, Nisbet R, Gotz J. Ultrasound treatment of neurological diseases--current and emerging applications. Nat Rev Neurol. 2016; 12: 161-74.

19. Burgess A, Nhan T, Moffatt C, Klibanov AL, Hynynen K. Analysis of focused ultrasound-induced blood-brain barrier permeability in a mouse model of Alzheimer's disease using two-photon microscopy. J Control Release. 2014; 192: 243-8.

20. Zhang W, Wang GM, Wang PJ, Zhang Q, Sha SH. Effects of neural stem cells on synaptic proteins and memory in a mouse model of Alzheimer's disease. J Neurosci Res. 2014; 92: 185-94.

21. Gu G, Zhang W, Li M, Ni J, Wang P. Transplantation of NSC-derived cholinergic neuron-like cells improves cognitive function in APP/PS1 transgenic mice. Neuroscience. 2015; 291: 81-92.

22. Carpentier A, Canney M, Vignot A, Reina V, Beccaria K, Horodyckid C, et al. Clinical trial of blood-brain barrier disruption by pulsed ultrasound. Sci Transl Med. 2016; 8: 342r-343r.

23. Zheng Y, Huang J, Zhu T, Li R, Wang Z, Ma F, et al. Stem Cell Tracking Technologies for

Neurological Regenerative Medicine Purposes. Stem Cells Int. 2017; 2017: 2934149.

24. Hayashi Y, Lin HT, Lee CC, Tsai KJ. Effects of neural stem cell transplantation in Alzheimer's disease models. J Biomed Sci. 2020; 27: 29.

25. Li X, Zhu H, Sun X, Zuo F, Lei J, Wang Z, et al. Human Neural Stem Cell Transplantation Rescues Cognitive Defects in APP/PS1 Model of Alzheimer's Disease by Enhancing Neuronal Connectivity and Metabolic Activity. Front Aging Neurosci. 2016; 8: 282.

26. Morroni F, Sita G, Tarozzi A, Rimondini R, Hrelia P. Early effects of Abeta1-42 oligomers injection in mice: Involvement of PI3K/Akt/GSK3 and MAPK/ERK1/2 pathways. Behav Brain Res. 2016; 314: 106-15.

27. Tampellini D, Capetillo-Zarate E, Dumont M, Huang Z, Yu F, Lin MT, et al. Effects of synaptic modulation on beta-amyloid, synaptophysin, and memory performance in Alzheimer's disease transgenic mice. J Neurosci. 2010; 30: 14299-304.

28. Kim JA, Ha S, Shin KY, Kim S, Lee KJ, Chong YH, et al. Neural stem cell transplantation at critical period improves learning and memory through restoring synaptic impairment in Alzheimer's disease mouse model. Cell Death Dis. 2015; 6: e1789.

Figure 1 UMMD treatment diagram and timeframe in 5 groups of AD mice

The US probe was kept anterior to the line of ears of the mice brain. $MB_{A\beta}$, MB and/or NSCs were injected via tail vein per week for 4 times according to the groups. After the last treatment, cognitive function was performed with MWM.

Figure 2 Successful MB loaded with Aβ antibody, neurosphere and in vivo imaging of fLuc-labeled NSCs

Anti-β-amyloid antibody (Green) was successfully loaded on DiI-labeled MB (Red) (A-C, ×1600), and the size of $MB_{A\beta}$ was mainly distributed from 300nm to 700nm (D). NSCs were amassed as neurospheres at the 6th day of culture with expression of Nestin (Green, E-F, ×400). In vivo imaging, luminescence was observed at the calvaria following 24h and 48h with UMMD assisted BBB opening and fLuc-NSCs implantation. The luminescence intensity was higher at 48h (H) than at 24h (G) and disappeared at 72h (I).

Figure 3 EB extravasation at different time point and histological changes in wild mice

Strips of EB extravasation was observed in the gross views under US-exposed area (A, B). After US mediated BBB opening, the degree of EB extravasation was different at different time (0h, 1h, 2h, 4h, 6h, 8h, 10h and 12 h), and no EB was observed after 12h following UMMD (C-J). No capillary injuries and erythrocyte leakages were observed in cortex (K, ×200) and hippocampus (L, ×200) in HE stains.

Figure 4 The cognitive functions were analyzed with MWM after UMMD treatment

The search paths in the place-navigation test were shown (A-E). The latency to find the hidden platform was indicated with MWM analysis (F, * Wild vs. Control, % US+$MB_{A\beta}$ vs. Control, # US+$MB_{A\beta}$+NSCs vs. Control, P<0.05).The average time in the place-navigation test and their comparisons among 5 groups (G, * P<0.05, ** P<0.01). Spatial probe test of the distance in the target quadrant was shown in the histogram(H): Significant difference between US+$MB_{A\beta}$+NSCs vs. Control (** P<0.01), less significance between US+$MB_{A\beta}$ vs. Control (* P<0.05). The cross number in the target quadrant was significant different between US+$MB_{A\beta}$+NSCs vs. Control (I, * P<0.05).

Figure 5 The histological changes in 5 groups following UMMD treatment

Amyloid proteins were detected in the hippocampus (A1-E1, ×200) and cortex (A2-E2, ×200) by immunohistochemistry. The number of amyloid plaques was counted and statistically analyzed in the hippocampus (F) and cortex (G), respectively. (* P<0.05).

Figure 6 The expression of BDNF and SYN with IF and western blot analysis

Expression of BDNF (red) was shown in IF results (A-E, ×400). Obvious expression of BDNF (red) was demonstrated in the hippocampus (A1, E1) and cortex (A2, E2) in the Wild group and US+$MB_{A\beta}$+NSCs group, only faintly visible expression in the other three groups. Significant differences were noted between US+$MB_{A\beta}$+NSCs vs. Control, and US+$MB_{A\beta}$+NSCs vs. US+$MB_{A\beta}$ with western blot analysis (K, P<0.05). No obvious difference expression of SYN (red) was demonstrated by IF (F-J, ×400) and western blot analysis (L).

574

575 **Table1: Treatment protocols in different groups**

Group(n=6)	US	MB（300μl）	NSCs（200μl）	NS（200μl）
Wild	-	-	-	+
Control	-	-	-	+
US+MB	+	**MB**	-	+
US+$MB_{Aβ}$	+	**$MB_{Aβ}$**	-	+
US+$MB_{Aβ}$+NSCs	+	**$MB_{Aβ}$**	+	-

576

Figures

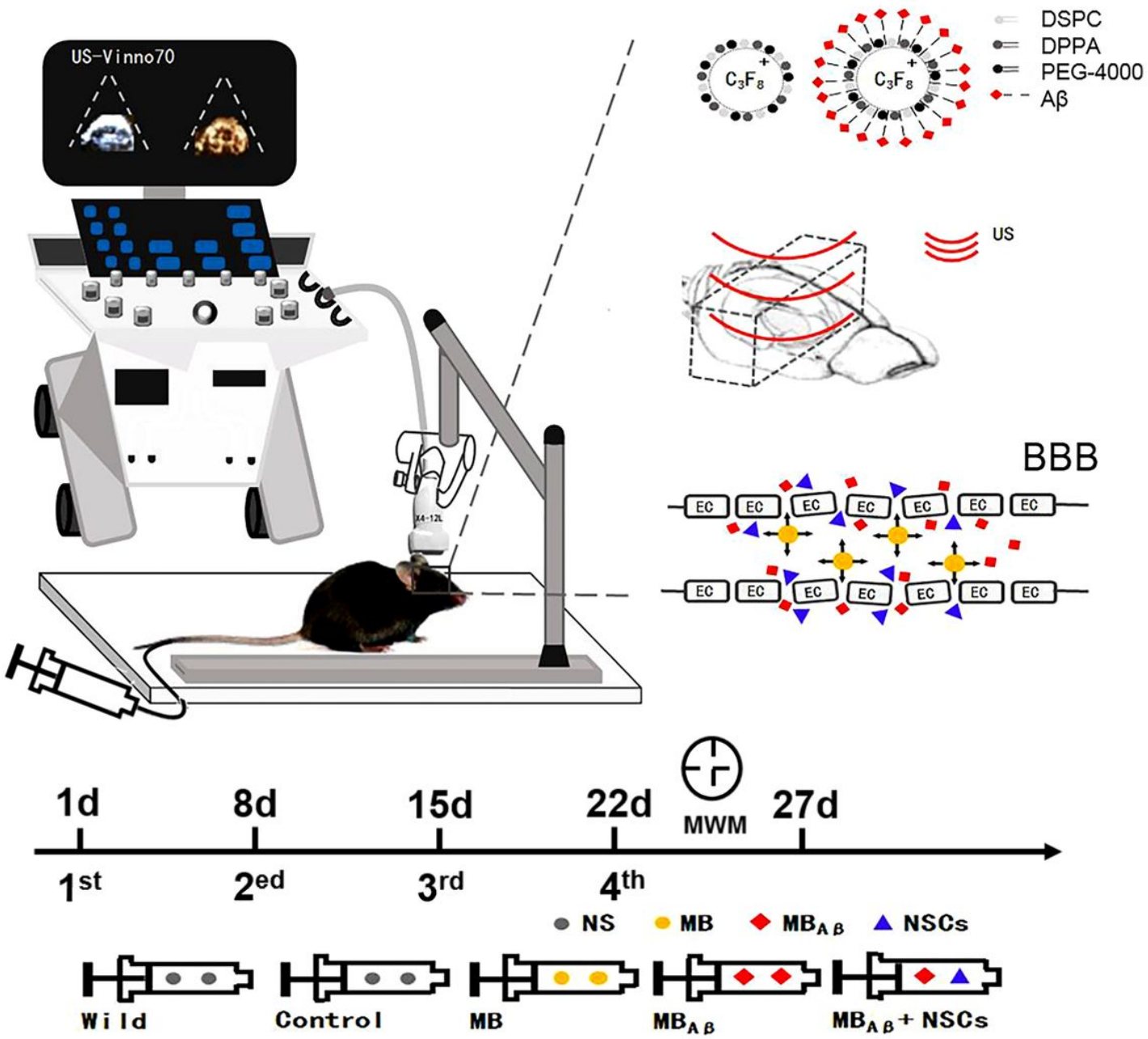

Figure 1

[See manuscript for figure legend.]

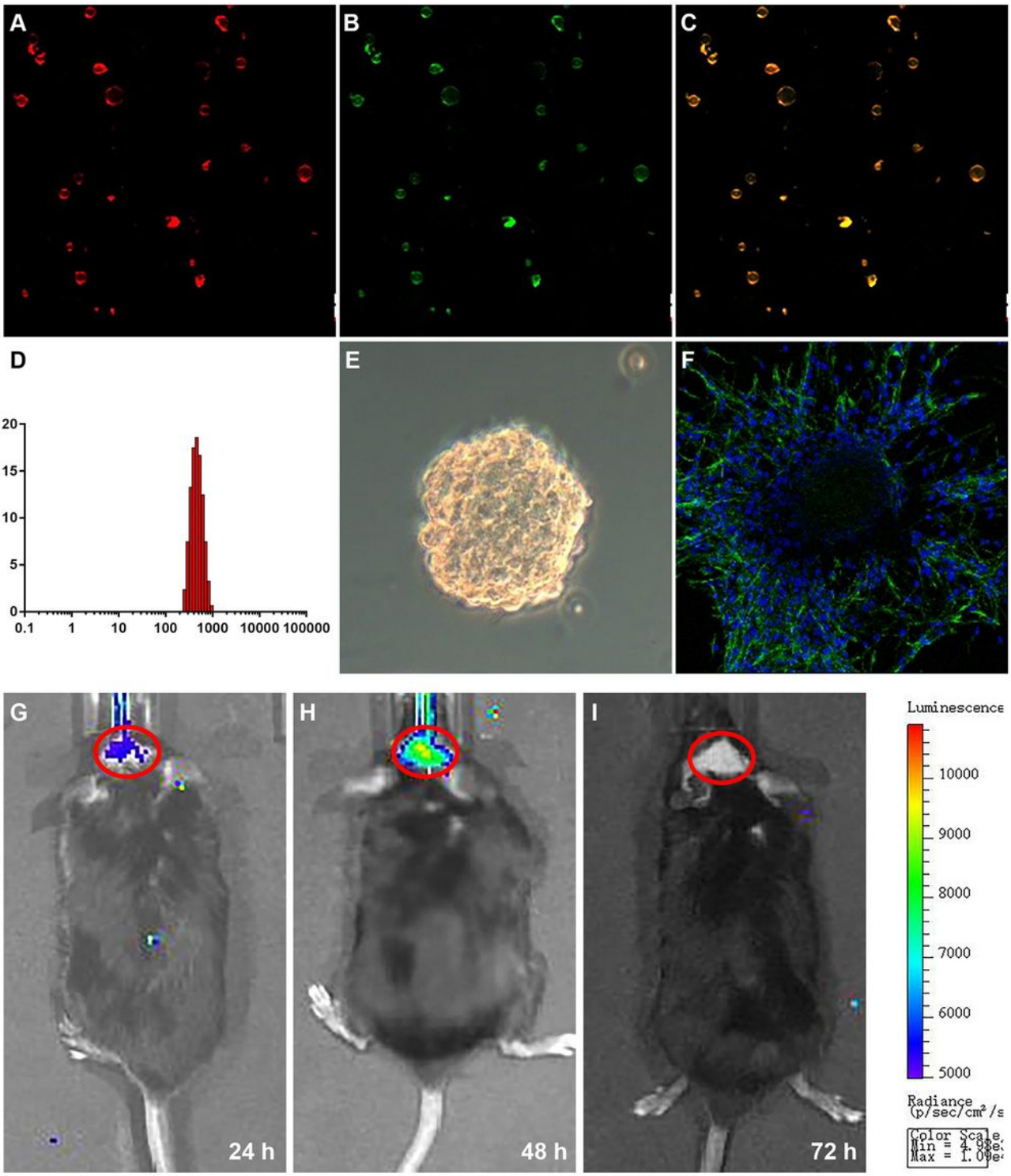

Figure 2

[See manuscript for figure legend.]

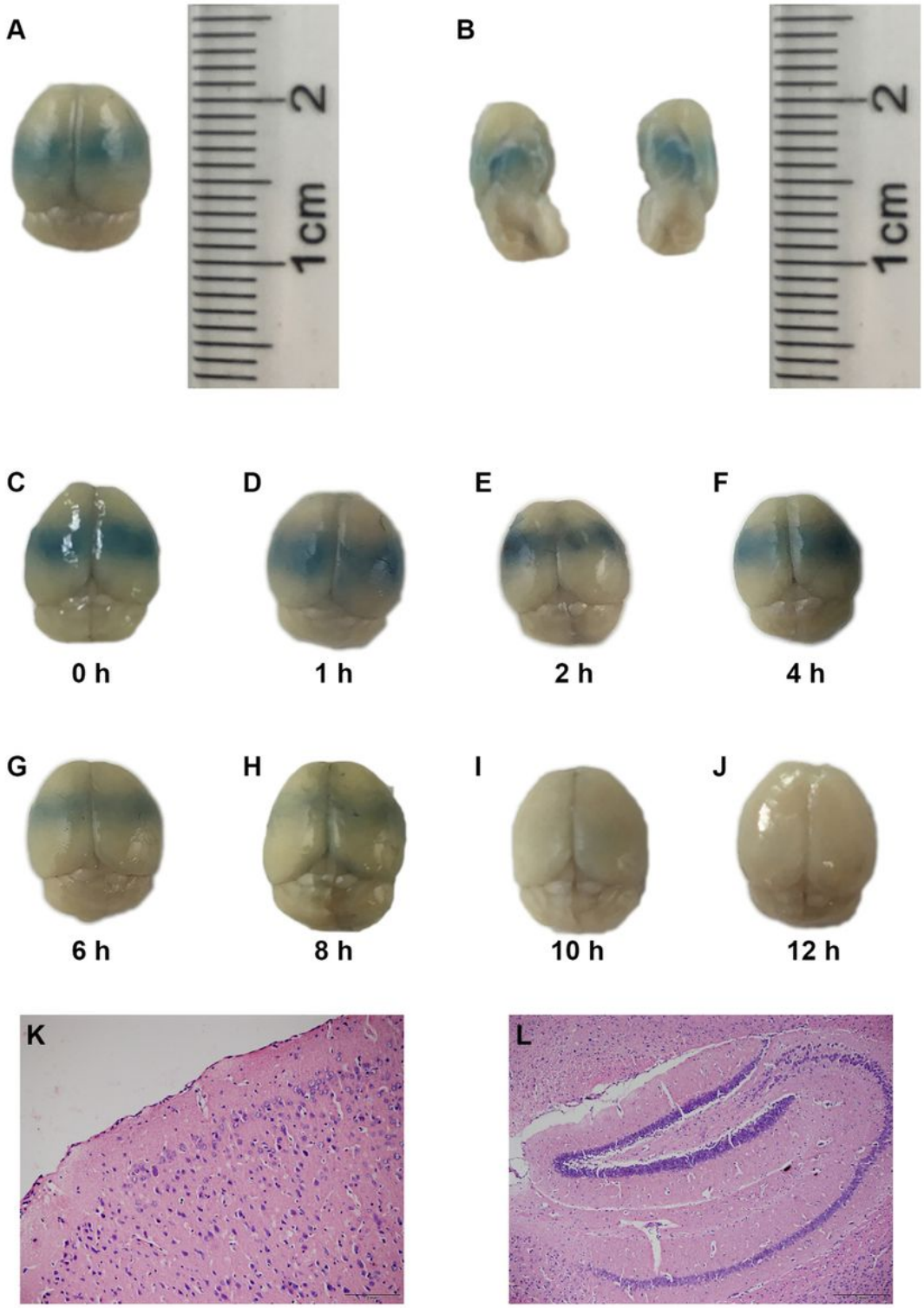

Figure 3

[See manuscript for figure legend.]

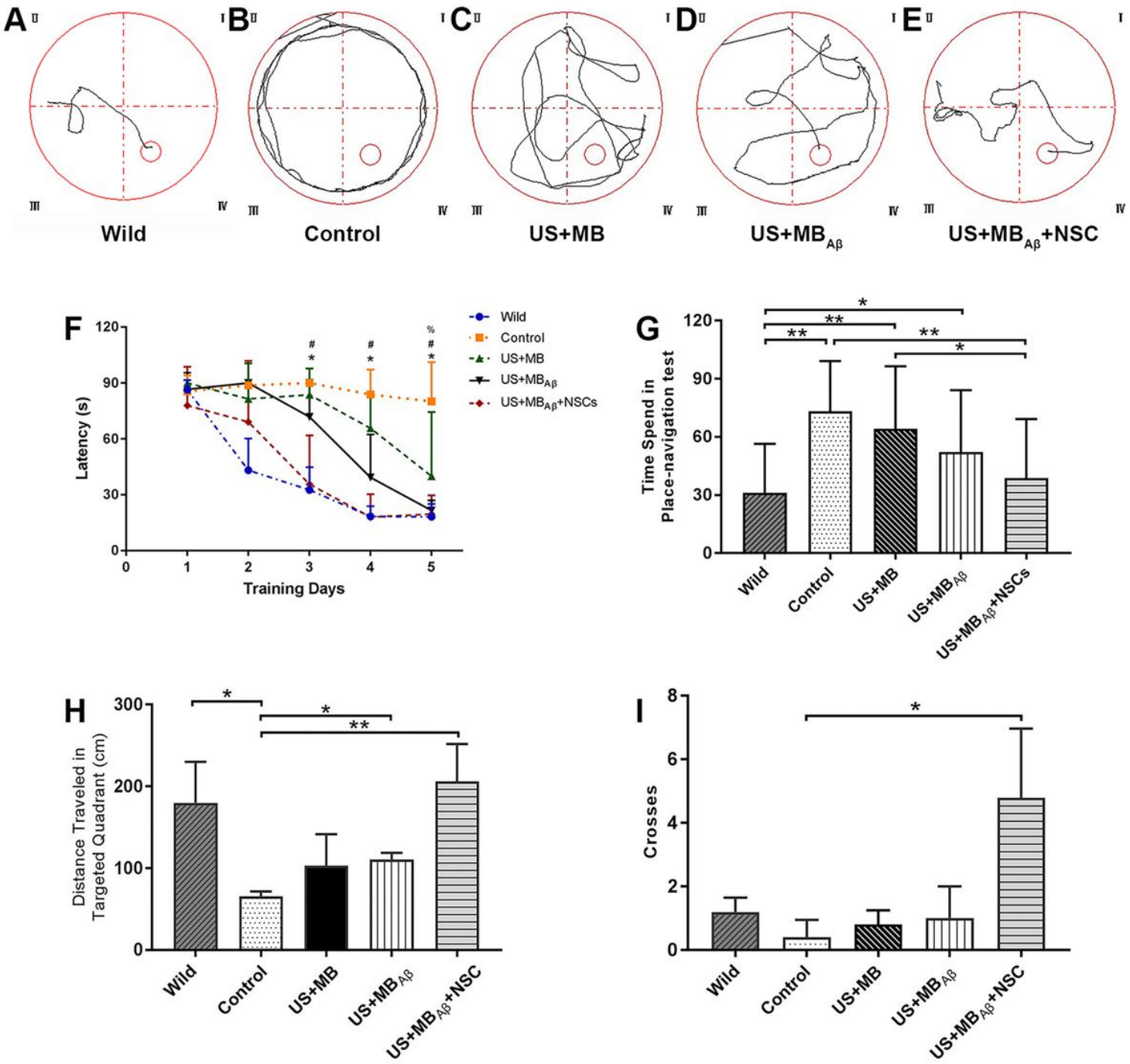

Figure 4

[See manuscript for figure legend.]

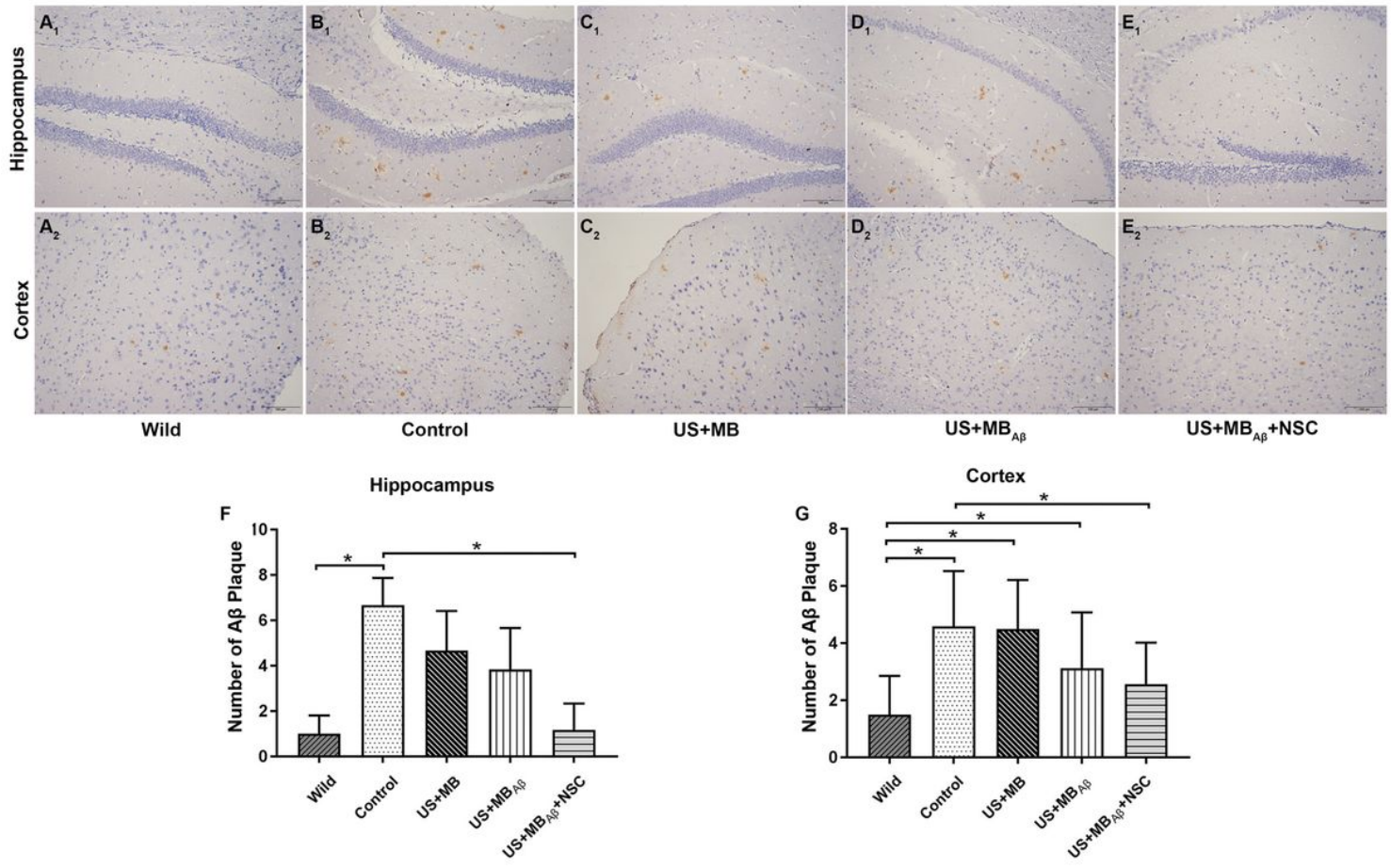

Figure 5

[See manuscript for figure legend.]

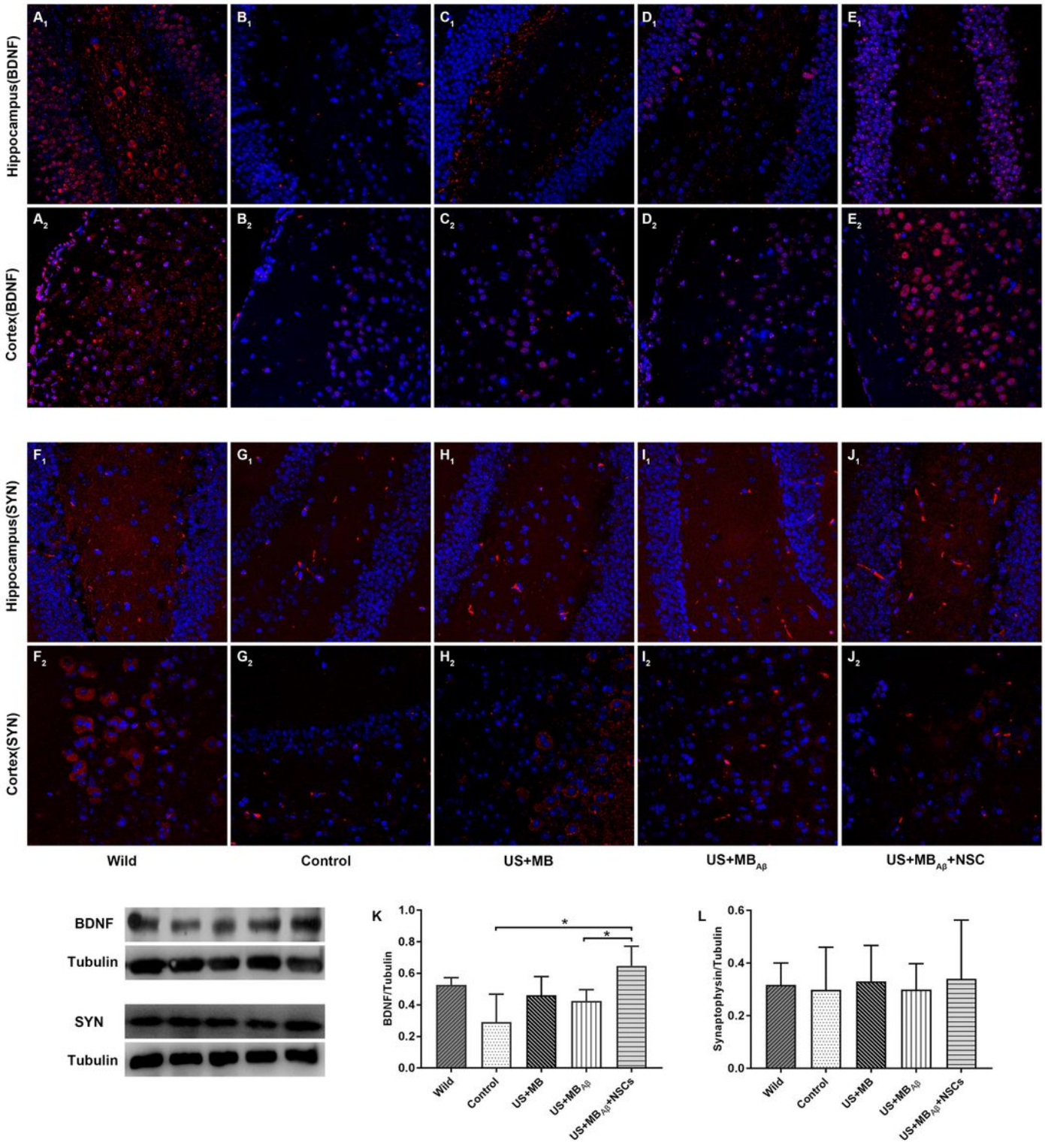

Figure 6

[See manuscript for figure legend.]

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table.pdf