Enhanced delivery of low-dose of aducanumab via FUS in 5xFAD mice, an AD model

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Research

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Abstract

Background

Aducanumab (Adu), which is a human IgG1 monoclonal antibody that targets oligomer and fibril forms of beta-amyloid (Aβ), has been reported to reduce amyloid pathology and improve impaired cognition after the administration of a high dose (10 mg/kg) of the drug in Alzheimer's disease (AD) clinical trials. The purpose of this study is to investigate the effects of a lower dose of Adu (3mg/kg) with enhanced delivery via focused ultrasound (FUS) in an AD mouse model.

Methods

The FUS with microbubbles opened the blood-brain barrier of the hippocampus for the delivery of Aducanumab. Combined therapy of FUS and Aducanumab was performed three times in total and each treatment was performed biweekly. Y-maze test, Brdu labeling, immunohistochemical experimental methods were employed in this study. In addition, RNA sequencing and ingenuity pathway analysis were employed to investigate gene expression profiles in the hippocampi of experimental animals.

Results

The combined treatment with FUS markedly increased the delivery of Adu into the brain by approximately 8.1 times in the brains. The combined treatment significantly restored cognitive impairment and decreased the level of amyloid plaques in the hippocampi of 5xFAD mice compared with Adu or FUS alone. The combined treatment with FUS and Adu increased reactive microglia and astrocytes associated with amyloid plaques in the hippocampi of 5xFAD mice. Furthermore, RNA sequencing identified 4 enriched canonical pathways such as phagosome formation, neuroinflammation signaling, CREB signaling and reelin signaling was altered in the hippocamis of 5xFAD given the combined treatment.

Conclusion

In conclusion, the enhanced delivery of a low dose of Aducanumab via FUS decreased amyloid deposits and restored cognitive function. This study provides better insight into establishing a solid therapeutic strategy for the treatment of AD as well as other neurodegenerative diseases.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease where cognitive functions, including memory, progressively deteriorate. Pathological features of AD include extracellular beta-amyloid (Aβ) plaques and intracellular neurofibrillary tangles, leading to neurodegeneration and neuronal cell death [1]. There have long been only four FDA-approved treatments for AD, such as donepezil, rivastagmine and galantamine, which alleviate symptoms by inhibiting acetylcholinesterase activity and memantine which is a partial NMDA antagonist [2]. According to the recently reported AD drug development pipeline and especially after the United States Food & Drug Administration (FDA) approved
aducanumab (Adu), the recent treatment development tends to be progressing more toward the fundamental treatment of the disease rather than merely focusing on symptom relief [3, 4].

Adu, a human IgG1 antibody designed for targeting aggregated forms of oligomer and fibril forms of Aβ, was approved by the FDA in 2021 [5] and is the first disease-modifying drug being used to slow the progression of AD and treat patients with mild cognitive impairment or the mild dementia stage of disease [1, 6]. In a clinical study, the accumulation of Aβ in the hippocampus was decreased by Adu in a dose-dependent manner (3–10 mg/kg) [2]. Consistent with the aforementioned outcome of a clinical trial, an animal study using an AD mouse model (Tg2576) showed that Aβ was significantly reduced in the group given a dose of 10 mg/kg or higher (30mg/kg) [6]. Acting as a double-edged sword, the BBB is essential for maintaining brain homeostasis. However, its function has long been a challenge in regard to applying potentially effective therapeutic agents, as BBB prevents approximately 98% of drug compounds with high molecular weight such as antibodies from penetrating the parenchyma [7].

Concentrating the acoustic pressure on the target area in the brain causes the cavitation effect of circulating microbubbles and temporarily opens the BBB [8]. FUS with microbubbles causes reversible opening of the BBB to both small and large molecules [9]. There are studies on the delivery of various therapeutic agents, such as chemotherapeutics [10–12] and cells [13, 14]. Furthermore, multiple pieces of evidence have reported that cerebral Aβ levels and plaque burden were reduced by opening the BBB only without delivering therapeutic agents in AD mouse models [15, 16]. Previously, we and other research groups reported that BBB opening by FUS increased adult hippocampal neurogenesis in rodents, which implies that FUS has therapeutic potential as an effective therapeutic strategy for AD [17–19].

Even though Adu has been approved by the FDA via an accelerated approval program, there may still be a challenging task in clinical phase IV. A previous study reported that only approximately 1.3% of Adu can reach the brain due to its large size by systemic administration [1]. In addition, a high concentration (10–60 mg/kg) of Adu could not be used because of its side effects such as amyloid-related imaging abnormalities including edema (ARIA-E) or microhemorrhage/superficial siderosis (ARIA-H) [6]. In this study, we investigated the effects of a lower dose of Adu with FUS in an AD mouse model.

Methods

Animals

The 5 familial AD mutations (5xFAD) mouse is a transgenic mouse with five familial mutations observed from early-onset AD families. This mouse expresses high levels of both mutant human amyloid precursor protein (APP695) with Swedish mutation (K670N, M671L), London mutation (V717I), Florida mutation (I716V), and human presenilin 1 (PS1) with two mutations (M146L and L286V). 5xFAD mice were purchased from Jackson Laboratory (Sacramento, CA) and maintained by crossing hemizygous transgenic mice with B6SJL F1 mice. The transgenic mice were identified by polymerase chain reaction (PCR), and non-transgenic littermates served as WT (wild type). The main characteristics of 5xFAD mice are extracellular amyloid deposition and gliosis beginning around 2 months [20]. Also, its phenotype can
be defined as spontaneous alternation in the Y maze in that the impairment in spatial working memory begins at approximately 4 to 5 months of age [20, 21]. Neuron loss has been observed in multiple brain regions in this model and begins at about 6 months of age [20, 22]. All mice were housed in groups of 2–5 per cage with *ad libitum* access to food and water, in a humidity- and temperature-controlled, specific pathogen-free environment (12 hours light cycle; lights on at 8 AM) in the Institute for Experimental Animals of Seoul National University. All experiments were approved by the Animal Care Committee of Seoul National University (Approval Number: SNU-201005-2-1).

**Focused ultrasound**

A 0.5 MHz single element focused transducer (H-107MR; SonicConcepts, Bothell, WA, USA) was used. The diameter of the transducer is 51.7 mm and the radius of curvature is 63.2 mm. The transducer was used with a conical container that could be filled with degassed water to efficiently transfer acoustic energy. A waveform generator (33220A, Agilent, Palo Alto, CA, USA) was connected to a 40 dB Radio Frequency Power Amplifier (210 L, ENI Inc., Rochester, NY, USA) to drive the FUS transducer, and a power meter (E4419B, Agilent) was used to measure the input electrical power (Fig. 1a).

**Magnetic resonance imaging**

Magnetic resonance imaging (MRI) was performed immediately following sonication with a Bruker 9.4 T 20 cm bore MRI system (Biospec 94/20 USR; Bruker, Ettlingen, Germany) and mouse head coil. A gadolinium-based MRI contrast agent, Gadobutrol (Gd; Gadovist; 0.2ml/kg), was injected intravenously. MRI sequence parameters are summarized in Table 1.

<table>
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<th>MRI sequences and parameters</th>
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**The preparation of Adu**
VH and VL sequences were identified in Biogen Idec's patent submission for WO2014089500A1 and were cloned into human IgG2a and kappa pcDNA3.1 vectors (GenScript, NJ, USA). Human Adu was produced using the Expi293 expression system and purified using protein A/G microbeads (Thermo Fisher Scientific, MA, USA).

**Confirmation of Adu delivery by FUS-mediated BBB opening**

First, FUS was sonicated unilaterally into the hippocampus of 5xFAD mice to confirm whether Adu was delivered into the brain of 5xFAD mice by FUS mediated BBB opening. Then, 24 hours later, the mice were sacrificed and compared with the contralateral hemisphere. We also examined quantitative measurement of Adu to determine whether the combined treatment with FUS enhanced the delivery of Adu into the brain. 5xFAD mice were divided into three groups: the 5xFAD + FUS, 5xFAD + Adu, and 5xFAD + FUS + Adu groups. The FUS was sonicated to the hippocampus bilaterally. Adu was injected intravenously immediately after FUS sonication, and the mice were sacrificed 24 hours later.

**Combined treatment with FUS and Adu**

All mice were divided into five groups: WT + Sham, 5xFAD + Sham, 5xFAD + Adu, 5xFAD + FUS and 5xFAD + FUS + Adu groups. For treatment, mice were anesthetized with 5% isoflurane in oxygen and animal heads were fixed on a stereotaxic frame (Narishige, Tokyo, Japan). Medical sterile ultrasound gel (ProGel-Dayo Medical Co, Seoul, South Korea) was used to fill the space between a coupling cone full of degassed water and skull for energy transfer efficiency. The FUS was targeted to four focal spots in the hippocampus bilaterally. DEFINITY microbubbles (0.04 ml/kg; Lantheus Medical Imaging, North Billerica, MA, USA) were injected intravenously 10 seconds before sonication. FUS (1-Hz burst repetition frequency, 10-ms bursts, 120 s in total, and average peak pressure 0.25 MPa) was started at the same time (Fig. 1a). Adu (3 mg/kg in saline) was injected intravenously at the end of FUS sonication. It was delivered three times in total and each treatment was performed every two weeks (Fig. 2a).

**Spontaneous alternation Y-maze test**

To investigate spatial working memory, spontaneous alternation in the Y-maze was investigated in experimental animals (Fig. 2b). Y maze test was performed three times 1 week after each treatment (Fig. 2a). The alternation performance was tested using asymmetrical Y-maze, consisting of 3 equal arms (40 × 15 × 9 cm), and constructed using black acrylic plastic. All mice were placed at the center of the Y-maze and allowed to explore freely for 8 minutes. All movements were recorded using a video camera and were analyzed to determine the alternation ratio by manually evaluating the number of triads containing entries into all three arms.

**5-Bromo-2'-deoxyuridine (BrdU) labeling**

BrdU was injected intraperitoneally twice a day for 4 days after treatment. Half of the group was injected after the 1st treatment and the rest of them was injected after the 3rd treatment (Fig. 2a).
**Immunohistochemistry**

Brains were fixed with 4% paraformaldehyde for 24 hours and transferred to 30% sucrose for 3 days and stored at -20 °C in cryoprotectant storage solution until use. Brains were cut into 30 µm coronal sections. Free-floating sections were washed in PBS and incubated in blocking solution (PBS, 5% normal goat serum, 0.2% Triton X-100) for 3 hours at room temperature. Sections were incubated with primary antibodies in blocking solution overnight at 4°C. The primary antibodies were used as follows: BrdU (Abcam, ab6326, 1:250), NeuN (Millipore, ABN78, 1:500), 6E10 (anti-human Aβ monoclonal antibody, Biolegend, SIG39320, 1:500), Iba-1 (Novus Biologicals, NB100-1028, 1:150, CO, USA or Wako, 19-19741, 1:150, VA, USA), CD68 (Bio-rad, MCA1957, 1:150, CA, USA), and GFAP (Abcam, ab53554, 1:150, MA, USA). After the primary immunoreaction, sections were incubated with Alexa 488 (Invitrogen, A11008, 1:500) and Alexa 594 (Abcam, A150156, 1:250) conjugated secondary antibodies. Immunostaining of the sections was visualized with an LSM 700 confocal microscope (Carl Zeiss, Jena, Germany) or an Axio Imager M2 (Carl Zeiss, Jena, Germany) light microscope. Images were analyzed using ImageJ software (Version 1.52a, NIH, USA).

**RNA sequencing and ingenuity pathway analysis (IPA)**

Three to four hippocampal samples per group were randomly selected for RNA sequencing analysis. Total RNA was extracted using a QIAGEN miRNeasy mini kit (Qiagen #217004). RNA (116 ng) was used to prepare RNA sequencing libraries using Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus. The libraries were sequenced by an Illumina HiSeq 3000 sequencer at Yonsei Genome Center (Seoul, South Korea). The data preprocessing included a quality check by FastQC and, where needed, trimming of the adapter sequences by Trimmomatic v0.32. The raw reads were aligned to the mouse reference genome (mm10) using the HISAT2 splice-aware aligner. The transcripts per million were quantified using StringTie. Significantly differentially expressed genes (DEGs) were defined by adjusted \( p \) values (\( q \) values) less than 0.05. For the canonical pathway, upstream regulatory networks, and molecular networks of DEGs, we used the commercial QIAGEN Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) software. For the canonical pathway analysis, \(-\log(q \text{ value}) > 1.3\) was taken as the threshold, a Z score > 1 was defined as the threshold of activation, and a Z score < -1 was defined as the threshold of inhibition. For upstream regulators, \(-\log(q \text{ value}) > 1.3\) was set as the threshold. The score was calculated by IPA for molecular networks, and significant changes in DEGs in the WT + Sham/5xFAD + Sham/5xFAD + FUS + Adu dataset were used for the comparison analysis.

**Statistical analysis**

All data are expressed as the mean ± standard error of the mean. Data were calculated using a one-way analysis of variance (ANOVA) and Kruskal-Wallis test followed by a least significant different (LSD) and Tukey's post hoc analysis. A value of \( p < 0.05\) was considered statistically significant for all measures. All statistical analyses were performed using SPSS (Version 25, SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA).
Results

FUS markedly improved the delivery of Adu into the targeted region of the brain, and Adu specifically bound to amyloid plaques in the hippocampi of 6 to 7 month-old 5xFAD mice

First, we investigated whether FUS with microbubbles can specifically open the BBB in targeted brain regions. After FUS sonication, we first acquired MR images without gadolinium and then performed MRI again with gadolinium to confirm whether the BBB is properly open. As shown in Fig. 1b, BBB opening in the hippocampus was confirmed through contrast-enhanced MRI. Then, to measure the increased delivery of Adu into the hippocampus after BBB opening by FUS treatment, we analyzed the hippocampal regions. FUS was given unilaterally (only to one hemisphere) to the hippocampus of experimental animals. A larger amount of intravenously administered Adu was detected in the ipsilateral region but not in the contralateral region (Fig. 1e). We also quantified the amount of Adu in the brains 24 hours after the treatment to investigate whether the combined treatment with FUS enhanced the delivery of Adu into the brains. The combined treatment markedly increased the delivery of Adu at 24 hours compared with Adu alone by approximately 8.1 times (Adu alone: 683.76 ± 259.16/40,000 µm², combined with FUS; 5541.24 ± 92 µm²/40,000 um², ###p < 0.001) (Fig. 1c and d). To confirm the specific binding of Adu with amyloid deposits in the hippocampus, co-staining with 6E10 and Adu targeted antibodies was assessed. The colocalization of amyloid plaques with Adu was observed in combined treatment group (Fig. 1c).

Combined treatment with FUS and low dose of Adu (3mg/kg) ameliorated cognitive impairments and significantly reduced the levels of amyloid plaques in the dentate gyrus of the hippocampus of 6 to 7 month-old 5xFAD mice

To investigate the effect of combined treatment with FUS and Adu on cognitive impairment in 5xFAD mice, the spatial learning memory was assessed using the Y-maze test. After the 1st treatment, the combined treatment group with FUS and Adu featured a significantly recovered alternation ratio compared with 5xFAD + Sham (Fig. 2c, WT + Sham: 69.74 ± 2.51%, 5xFAD + Sham: 46.74 ± 2.13%, 5xFAD + Adu: 55.93 ± 3.63%, 5xFAD + FUS: 58.14 ± 4.13%, 5xFAD + FUS + Adu: 59.17 ± 2.67%). After the 3rd treatment, the combined treatment group exhibited an even higher recovered alternation ratio than the 5xFAD + Sham mice (Fig. 2c, WT + Sham: 60.64 ± 2.17%, 5xFAD + Sham: 47.80 ± 4.52%, 5xFAD + Adu: 54.64 ± 3.66%, 5xFAD + FUS: 53.88 ± 3.88%, 5xFAD + FUS + Adu: 65.81 ± 2.07%). The combined treatment with FUS and Adu was more effective in alleviating the cognitive dysfunction assessed with the Y-maze test than the treatment with Adu or FUS alone. Thus, repeated combined treatment with FUS and Adu contributed to more significantly improved cognitive impairment in 5xFAD mice. To measure the therapeutic effects of combined treatment with FUS and Adu, we analyzed the accumulation of amyloid plaques in the hippocampus through immunostaining with 6E10 antibody. In the brains of WT-Sham mice, amyloid plaques were not observed at this age (7.5 months old, Fig. 2d).

All groups showed a significantly reduced number of amyloid plaques in the dentate gyrus of the hippocampus compared with 5xFAD + Sham; in particular, the decrease was remarkably reduced in the
5xFAD + FUS + Adu group (Fig. 2e, 5xFAD + Sham: 29.92 ± 2.92, 5xFAD + Adu: 20 ± 2.38, 5xFAD + FUS: 19.3 ± 1.66 and 5xFAD + FUS + Adu: 12.6 ± 1.92 /160,000 µm²). Additionally, areas of decreased amyloid beta were measured and compared. 5xFAD + FUS and 5xFAD + FUS + Adu groups showed a significantly reduced total area of amyloid plaques in the dentate gyrus of the hippocampus compared with 5xFAD + Sham group (Fig. 2f, 5xFAD + Sham: 3614.67 ± 498.24, 5xFAD + Adu: 2316.43 ± 331.8, 5xFAD + FUS: 1755.76 ± 218.31, 5xFAD + FUS + Adu: 1385.51 ± 374.3/160,000 µm²). Taken together, combined treatment of Adu with FUS ameliorated the amyloid plaques load in the hippocampi of 5xFAD mice.

**Combined treatment with FUS and Adu increased reactive microglia and astrocytes associated with amyloid plaques in the hippocampi of 5xFAD mice**

Microglia and astrocytes clear the pathological deposits of molecules such as Aβ through phagocytosis and degradation in the CNS [23]. To measure the localization of amyloid plaque-associated microglia, immunostaining of Iba-1, which is well known as a marker for reactive microglia, was performed for the four experimental groups, including 5xFAD + Sham, 5xFAD + Adu, 5xFAD + FUS, and 5xFAD + FUS + Adu (Fig. 3a). The number of reactive microglia within 20 µm of amyloid plaques larger than 500 µm² was significantly increased only in the 5xFAD + Adu group compared with the 5xFAD + Sham group (Fig. 3c, 5xFAD + Sham: 5.50 ± 0.60, 5xFAD + Adu: 6.89 ± 0.42, 5xFAD + FUS: 5.70 ± 0.38, 5xFAD + FUS + Adu: 6.67 ± 0.44/ amyloid plaques). No significant difference was observed in the number of microglia within 20 µm of amyloid plaques smaller than 500 µm² in all groups (Fig. 3c, 5xFAD + Sham: 3.88 ± 0.36, 5xFAD + Adu: 4.68 ± 0.47, 5xFAD + FUS: 3.50 ± 0.22 and 5xFAD + FUS + Adu: 3.86 ± 0.31 / amyloid plaques). Then, we examined the Iba-1⁺ microglial area and Iba-1⁺/CD68⁺ microglia to investigate whether the reactivation of microglia and potential phagocytic activity in microglia were altered in all groups (Fig. 3b). The Iba-1⁺ area in the dentate gyrus of the hippocampus was increased in the combined treatment group compared with the 5xFAD + Sham group (Fig. 3d, 5xFAD + Sham: 1600.03 ± 304.50 µm², 5xFAD + Adu: 2264.73 ± 760.19 µm², 5xFAD + FUS: 1716.90 ± 257.90 µm² and 5xFAD + FUS + Adu: 4568.93 ± 1976.34/26,500 µm²). The co-stained area with Iba-1 and CD68, which is a lysosome-associated membrane protein involved in phagocytosis being used as a marker for macrophages and other mononuclear phagocytes, in the dentate gyrus of the hippocampus was significantly increased in the combined treatment group (Fig. 3e, 5xFAD + Sham: 26.60 ± 4.73 µm², 5xFAD + Adu: 27.53 ± 10.19 µm², 5xFAD + FUS: 33.03 ± 6.65 µm², and 5xFAD + FUS + Adu: 48.42 ± 7.35 µm²).

Reactive astrocytes clustered around amyloid plaques, and the brain area occupied by these reactive astrocytes showed a significant increase in AD patients [24]. In addition, there are several lines of evidence that astrocytes can also eliminate amyloid plaques and neurons containing Aβ and enhance phagocytosis by microglia [25–27]. To investigate the number of amyloid plaque-associated astrocytes, we assessed the number of astrocytes within 20 µm of amyloid plaques. As shown Fig. 4a, confocal images showed that reactive astrocytes were recruited to amyloid plaques in all groups. The recruitment...
of astrocytes surrounding amyloid plaques smaller than 500 µm² was enhanced in the 5xFAD + Adu group, the 5xFAD + FUS group, and the combined treatment group (Fig. 4b, 5xFAD + Sham; 2.70 ± 0.21, 5xFAD + Adu; 4.20 ± 0.44, 5xFAD + FUS; 5.00 ± 0.62, 5xFAD + FUS + Adu; 4.10 ± 0.31/amyloid plaques). The recruitment of astrocytes was increased in 5xFAD mice treated with Adu alone and the combination treatment surrounding amyloid plaques larger than 500 µm² (Fig. 4b, 5xFAD + Sham: 5.52 ± 0.36, 5xFAD + Adu: 7.61 ± 0.47, 5xFAD + FUS: 6.94 ± 0.81, and 5xFAD + FUS + Adu: 8.27 ± 0.90 / amyloid plaques). These results strongly suggest that the activation of microglia and astrocytes associated with amyloid plaques was increased by the combined treatment with FUS and Adu.

**Combined treatment with FUS and Adu increased neurogenesis in the hippocampi of 5xFAD mice**

Adult hippocampal neurogenesis is normally active in neurologically normal subjects and decreases severely in patients with AD [28]. To examine whether combined treatment affects the neurogenesis in the hippocampus, we quantified the cells stained with BrdU, which is incorporated into dividing cells during the S-phase of the cell cycle and a marker of newborn cells [29] and NeuN, a neuronal marker in the subgranular zone and granular cell layer of the dentate gyrus (Fig. 5a). The number of BrdU⁺ and BrdU⁺/NeuN⁺ cells were compared after the 1st treatment and after the 3rd treatment. Only the combined treatment group (6.5 ± 0.81) showed a significantly increased number of BrdU⁺ cells in the hippocampus, compared with the 5xFAD + Sham group (3.14 ± 0.81) after the 1st and 3rd treatments (Figs. 5b and c). The combined treatment group (3.53 ± 0.5) also exhibited a highly increased number of BrdU⁺/NeuN⁺ cells compared with 5xFAD + Sham (1.6 ± 0.23) after the 1st and 3rd treatments (Figs. 5b and c). Overall, these results indicate that the combined treatment of FUS and Adu induced remarkable neurogenesis in the hippocampus.

**Differentially expressed genes (DEGs) associated with inflammation and synaptic plasticity pathways were revealed via transcriptome profiling in the hippocampi of 5xFAD mice treated with FUS and Adu**

To understand the underlying the action mechanisms related to the recovery of cognitive impairments and neuropathological characteristics by treatment with FUS combined with Adu in 5xFAD mice, RNA sequencing was performed in the hippocampi of the experimental animal groups. As shown Fig. 6a, a total of 21,833 genes DEGs were identified and displayed as a volcano plot. Among these genes, 146 DEGs were up-regulated and 128 DEGs were down-regulated significantly (q< 0.05). Among the 274 annotated DEGs, a functional prediction analysis was performed using IPA software (Fig. 6a). For canonical pathway analysis, a total of 32 enriched canonical pathways were identified by applying absolute z scores greater than 1. Among these pathways, a total of 12 pathways and 20 pathways were activated and inhibited, respectively (Fig. 6b). Furthermore, a total of 276 upstream regulators were
identified by applying the $p$ value of overlap $< 0.05$ threshold. Among them, 30 molecules were predicted to be activated and 43 molecules that were predicted to be inhibited (Fig. 6c). We also analyzed the network to show the interactions between molecules in the dataset (Fig. 6d). The highest ranked network which was sorted using the score values (score 49), was found to mainly affect 'neurological disease, hereditary disorder, organismal injury and abnormalities', involving 27 molecules. We found that 16 DEGs (ADAM22, C2CD5, CACNA2D1, CNOT1, CNOT2, CNOT4, CNOT6, CNOT6L, EHBP1, EXOC4, GABRA2, GABRA6, GABRB2, OTUD4, WNK3 and RN1) were up-regulated and 11 DEGs (ADPRH, Calm1, CNP, Dazap1, EPHX1, ESYT1, GGA1, MAP1S, PEX14, RHOT2, and SMIM12) were down-regulated. To investigate the similarity, difference and trend between the WT + Sham vs. 5xFAD + Sham and 5xFAD + Sham vs. 5xFAD + FUS + Adu datasets, a comparison analysis was performed using the comparison analysis function in IPA. As shown Fig. 6e, a total of 11 canonical pathways were found to be enriched in comparison analysis. In particular, four canonical pathways (phagosome formation, neuroinflammation signaling, CREB signaling in neurons and reelin signaling in neurons) ameliorated in 5xFAD + FUS + Adu mice compared with 5xFAD + FUS mice. Taken together, our data revealed that combined treatment with FUS and Adu altered pathways in the hippocampus that are related to neuroinflammation and neural activity.

Discussion

The BBB hinders therapeutic agents from penetrating into the brain and becomes an obstacle to CNS disease treatment [30]. Previously, we reported several studies regarding BBB opening by FUS [14, 19]. The safety of FUS has already been verified, and it has been currently used in clinical trials [11, 31, 32].

A significant correlation between cognitive decline and brain amyloid plaque levels in the living brain evaluated using PET-CT scan was reported [33]. Recently, the FDA approved Adu for the treatment of AD based on an evaluation of effects of the drug in clinical stages [6, 34]. However, the high dose of Adu (60 mg/kg) used in these studies induced ARIA-E in human clinical phase 1 [35]. According to the study, patients who were treated with 10 mg/kg of Adu experienced ARIA-E with headache, confusion, dizziness and nausea; and microhemorrhage; and superficial siderosis in clinical phase 3. Therefore, delivering an appropriate dose of Adu may be a key point for safety and effectiveness in AD.

In this study, we aimed to investigate if the combined treatment with FUS and Adu improves a very low BBB penetration ratio of Adu caused by its large molecular weight (approximately 150 kDa) in systemic administration. We first confirmed that FUS safely opened BBB and induced the increased penetration of Adu into the hippocampus of the animals (Fig. 1b).

Here, the impairments in cognitive function and the accumulation of amyloid plaques were ameliorated at a lower dose of Adu (3 mg/kg) with FUS in 5xFAD mice (Fig. 2). While the combined treatment group only showed significant restoration of cognitive impairment, spontaneous alternation Y-maze test did not show any significant difference between the FUS alone or Adu alone group and 5xFAD mice (Fig. 2c). Notably, the combined treatment resulted in a marked improvement in cognitive impairment after the 3rd
treatment (Fig. 2c). In addition, we also examined neuropathological changes, especially the amyloid plaque levels in the hippocampus, after treatment in 5xFAD mice. The 5xFAD + FUS + Adu mice showed the most significant reduction in amyloid plaques compared with the 5xFAD mice (Figs. 2d, E and f). A previous report by others focused on the effects of scanning ultrasound on the delivery of Adu into the brains and demonstrated that both Adu only and scanning ultrasound only groups reduced the total plaque area in the hippocampus with no additive effect observed with the combination treatment of scanning ultrasound and Adu using APP23 mice [15, 16, 36]. However, our results clearly showed that the combined treatment of FUS and Adu exerted beneficial effects on amyloid plaque reduction and cognitive function impairments in 5xFAD mice. Based on our results, it can be said that FUS enhanced the delivery of a low dose of Adu into the brain and attenuated the impairment of cognitive function by reducing the accumulation of amyloid plaques. In addition, FUS is considered to be very important in that the use of low dose drugs can minimize the side effects caused by Adu.

Even though the FDA approved the use of Adu via an accelerated approval program, the action mechanism underlying the treatment effects of Adu in the brain is still poorly understood. To understand the underlying the mechanisms of action, we investigated the changes in microglia, astrocytes and neurons after treatment with Adu in 5xFAD mice. Microglia are the only immune cells resident in the CNS, constitute 5–10% of total brain cells, and take up, phagocytose, and proteolyse both soluble and fibrillar forms of Aβ [37, 38]. Phagocytes such as microglia express Fc receptors (FcRs) on the cell surface and bind to the Fc region of antibodies. FcR activates phagocytosis, clearance of myelin debris and the inflammatory response [39]. The Fc portion of Adu can bind to FcRs expressed in microglia and opsonize Aβ for phagocytosis by microglia [40]. Early reports found that reactive microglia surround amyloid plaques in the brains of AD patients, and Aβ fibrils were found within the microglia [41]. In this study, the combined treatment did not affect the number of microglia surrounding amyloid plaques (Fig. 3c). This finding indicates that the recruitment of microglia around amyloid plaques was not changed by Adu. However, quantitative assessment of the CD68+/Iba-1+ area revealed a significant increase in the 5xFAD + FUS + Adu group (Fig. 3e). Furthermore, we identified that the phagosome formation pathway (PIPK1B, ROCK2, PIKFYVE, GPR137, AKT2, LIMK1, ADRA1D, GPR135, and RAC3) was activated in the combined treatment group using RNA sequencing and IPA (Fig. 6e). The activation of astrocytes, as demonstrated by increased GFAP expression, and amyloid deposition surrounded by activated astrocytes have a substantial impact on the AD state [42]. In the brains of AD patients and mouse models, there is a significant increase in GFAP immunoreactivities in plaque-associated astrocytes. Similar to the activation of microglia, reactive astrocytes phagocytose amyloid aggregates and dystrophic neurites and are involved in the inflammatory response to Aβ [27, 43]. Additionally, knockout of GFAP in an AD mouse model showed a 2-fold increase in amyloid plaque burden and twice the amount of dystrophic neurites [44]. Astrocytes were reported to be activated and uptake more Aβ in the brains of MRI-guided FUS treated mice [45]. Consistent with these results, we observed an increased number of plaque-associated astrocytes and a reduced number and size of amyloid plaques (Fig. 2, 4). Collectively, our data suggest that the combined treatment with FUS and Adu promotes glial phagocytosis and clearance of Aβ, which may induce a reduction in Aβ deposition in the brains of 5xFAD mice. To elucidate the precise molecular
mechanisms of phagocytosis associated with these pathways, more in-depth study is required both in *in vitro* and *in vivo* models.

Previous studies have reported that FUS-mediated BBB opening induces hippocampal neurogenesis [17, 18]. In this study, we investigated whether the combined treatment also induces neurogenesis and compared the effects of combined treatment with FUS or Adu alone. The combined treatment with FUS and Adu led to a higher number of BrdU+ and BrdU+/NeuN+ neurons compared with the FUS or Adu alone group after the 1st treatment but not after the 3rd treatment (Fig. 5). Future research is needed regarding the difference between the results of the 1st and 3rd treatments. As neurogenesis is induced only when the BBB is opened, it is assumed that changes in the intravascular microenvironment or the components of the tight junction may have played a role in promoting neurogenesis. In addition, brain-derived neurotrophic factor (BDNF) is reported to be one of the most important factors in inducing neurogenesis, and there is a report that FUS-mediated BBB opening increases the expression level of BDNF [14, 19, 46]. Therefore, these results show that the enhanced delivery of Adu could synergistically stimulate neurogenesis.

To understand the dynamic molecular processes induced by the combined treatment at the transcriptional level, transcriptome profiling was performed using RNA sequencing. We identified 32 canonical pathways based on significant DEGs and predicted 72 upstream regulators after combined treatment with FUS and Adu (Fig. 6a and c). In particular, the ‘neurological disease, hereditary disorder, organismal injury and abnormalities’ molecular network was identified, and increased activation of GABRB2, GABRA2 and GABRA6, which are related to the GABAergic pathway, was predicted in 5xFAD + FUS + Adu mice compared with 5xFAD mice (Fig. 6d). Several studies have reported the role of impaired function of GABA A receptors by modulating neuronal activity in AD [47, 48]. In addition, we found four promising target canonical pathways via a comparison analysis (WT vs. 5xFAD and 5xFAD vs. 5xFAD + FUS + Adu, Fig. 6e). Neuroinflammation signaling (GABRA2, GABRB2, BIRC6, MAPK9, AKT2, GABRA6, ATF4, and RAC3) and phagosome formation were activated by combined treatment with FUS and Adu. These results may explain why microglia and astrocytes were activated by the combined treatment. Both CREB signaling (TBP, GRIA4, CACNA2D1, PLCL2, GPR137, AKT2, ADRA1D, Calm1, GPR135, ATF4, and GNB2) and reelin signaling (DCX, ARHGEF7, MAPK9, AKT2, and LIMK1) in neurons were proposed to be activated genes in 5xFAD mice vs. 5xFAD + FUS + Adu mice (Fig. 6e). This suggests that cognitive impairment may be improved via these pathways. Therefore, these results indicate that gene sets related to proinflammation and inhibition of neuronal activity were reversed after combined treatment. In the future, we aim to investigate the detailed molecular mechanisms related to these datasets.

**Conclusion**

In conclusion, an effective treatment approach for AD is by improving cognitive function and reducing deposited amyloid plaques rather than alleviating symptoms and delaying progression. In this study, the enhanced delivery of a low dose of Adu via FUS reduced amyloid deposits and restored spatial memory. In addition, it was proven to be effective in neuropathological changes such as enhanced phagocytosis
and neurogenesis. Overall, this study provides insight into establishing a therapeutic strategy for the treatment of AD as well as other neurodegenerative diseases.

In this work, we demonstrate FUS with microbubbles induced BBB opening and increased the delivery of Adu into the brain. In *in vivo* experiment, the combined treatment alleviated the pathology and symptoms in AD mouse model. While achieving higher efficacy in the delivery and therapy, the combined treatment showed safety in mice. This study suggests that the optimized delivery technology using FUS can be applied to other drugs for CNS diseases.

**Abbreviations**

AD  
Alzheimer's disease  
FUS  
Focused ultrasound  
Aβ  
Amyloid-β  
Adu  
Aducanumab  
BBB  
Blood-brain barrier  
CNS  
Central nervous system  
MRI  
Magnetic resonance imaging  
ARIA-E  
Abnormalities including edema  
WT  
Wild type  
DG  
Dentate gyrus  
PBS  
Phosphate-buffered saline  
BrdU  
5-Bromo-2'-deoxyuridine  
NeuN  
Neuronal nuclei  
GFAP  
Glial fibrillary acidic protein  
RNA  
Ribonucleic acid
DEGs
Differentially expressed genes

IPA
Ingenuity pathway analysis

BDNF
Brain-derived neurotrophic factor

GABA
Gamma-aminobutyric acid

FcRs
Fc receptors

Declarations

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Availability of data and materials
The datasets used and/or analyzed in the current study are available from the corresponding authors on reasonable request.

Ethics approval and consent to participate
All works involving animals were approved by the Institute for Experimental Animals of Seoul National University. Also, all experiments were approved by the Animal Care Committee of Seoul National University (Approval Number: SNU-201005-2-1).

Competing interests
The authors declare that they have no competing interests.

Consent for publication
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Authors contributions
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Figures
Figure 1

FUS-mediated BBB opening significantly increased the delivery of the adu in the brain

a. A scheme of the FUS system set up for BBB opening in mice. b. Pre- and post-gadolinium T1-weighted images: FUS-mediated BBB opening was confirmed with MRI. c. Representative confocal images (20x) of human IgG (Aducanumab), Aβ stained with 6E10 antibody and DAPI in the dentate gyrus of the hippocampus. d. A bar graph showing the levels of Adu assessed with human IgG antibody in the hippocampus. e. FUS was treated unilaterally to the brains of experimental animals. A representative image of Western blotting with an antibody against human IgG after Adu injection and unilateral FUS treatment. Data are represented as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Tukey’s post hoc analysis. (**** p < 0.0001 compared with 5xFAD+FUS, ### p < 0.001 compared with 5xFAD+Adu, n = 5 mice for each group).
Figure 2

Treatment of FUS and Adu ameliorated cognitive impairment and levels of Aβ in the hippocampus

a. Timeline of FUS and Adu treatment in 5xFAD mice. b. Schematic illustration of the Y-maze spontaneous alternation test. c. Alternation ratio in the Y-maze test at one week after the 1st treatment and at one week after the 3rd treatment. d. Representative images (5X) of Aβ stained with 6E10 antibody and DAPI in the hippocampus. Bottom, representative images (20X) of Aβ stained with 6E10 antibody in the dentate gyrus of the hippocampus. e. A bar graph showing the number of amyloid plaques in the dentate gyrus of the hippocampus. f. A bar graph showing the total area of amyloid plaques in the dentate gyrus of the hippocampus. Data are expressed as the means ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Tukey’s post hoc analysis. (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 compared with 5xFAD-Sham mice, n = 11-15 mice for each group)

Figure 3

Treatment of FUS and Adu increased reactive microglia associated with amyloid plaques in the hippocampus

a. Representative images showing Iba-1-positive cells (green) surrounding Aβ plaques (red) in the dentate gyrus of the hippocampus b. Representative images of CD68(red) and Iba-1(green) contained in the dentate gyrus of the hippocampus. c. A bar graph showing the number of Iba1+ microglia within 20 µm from Aβ plaques that were larger than 500 µm² or smaller than 500 µm². d. A bar graph showing the total area of microglia. e. A bar graph showing the phagosome area (CD68+/Iba1+) in microglia. Data are expressed as the means ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Tukey’s post hoc analysis. (*p < 0.05 compared with 5xFAD-Sham mice, n = 3-4 mice for each group).
Figure 4

Treatment of FUS and Adu increased plaque-associated astrocytes in the hippocampus

a. Representative images showing GFAP-positive cells (red) surrounding Aβ plaques (green) in the dentate gyrus of the hippocampus. b. A bar graph showing the number of astrocytes within 20 μm from Aβ plaques that were larger than 500 μm² or smaller than 500 μm². Data are expressed as the means ± SEM. Statistical analyses were performed using the Kruskal–Wallis test. (*p < 0.05 and **p < 0.01 compared with 5xFAD-Sham mice, n = 3-4 mice for each group).

Figure 5

Treatment of FUS and Adu increased neurogenesis in the hippocampus
a. Representative images showing immunofluorescence of neuronal nuclear marker (NeuN, green) and 5-bromo-2’-deoxyuridine (BrdU, red) in the dentate gyrus of the hippocampus. Scale bars: 100 µm, SGZ: subgranular zone, GCL; granular cell layer.
b. Bar graphs showing the number of BrdU- and BrdU/NeuN-positive cells after the 1st treatment.
c. Bar graphs showing the number of BrdU- and BrdU/NeuN-positive cells after the 3rd treatment. Data are expressed as the means ± SEM. Statistical analyses were performed using one-way ANOVA followed by a least significant difference post hoc analysis. (*p < 0.05, **p < 0.01, and ***p < 0.001 compared with WT-Sham mice, #p < 0.05, ##p < 0.01, and ###p < 0.001 compared with 5xFAD-Sham mice, $p < 0.05 compared with 5xFAD+Adu mice, @p < 0.05 compared with 5xFAD+FUS mice, n = 5-10 for each group).

Figure 6

Treatment of FUS and Adu altered gene expression profiles in the hippocampus

a. Left figure is a volcano plot. The x-axis represents the log₂ conversion of the fold change (FC) values, and the y-axis represents the corrected significance level after base log₁₀ conversion (q value). Green dots in the volcano plot and right graph indicate all DEGs that were found to differ significantly (q value <0.05). The black bar in the right graph represents the number of genes with an absolute value of log₂ FC greater than 0.7, and the gray bar represents the number of genes with an absolute value of log₂ FC less than 0.7. b. Canonical pathway analysis. The upregulated DEGs (red bar) and downregulated DEGs (blue bar) were identified (Z score > 1 or < -1). c. A heatmap of upstream regulators (Z score > 1 or < -1). d. A gene interaction network map (upper) and related DEG FC values (lower). e. A heatmap of canonical pathways via comparison analysis (left) and four selected canonical pathways, including related molecules (right). Data are expressed as the means ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Tukey’s post hoc analysis. (*p < 0.05 compared with 5xFAD-Sham mice, n = 3-4 mice for each group).
Supplementary Files

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