Original Article
Effects of brain-derived neurotrophic factor-pretreated neuron stem cell transplantation on Alzheimer's disease model mice

Tong Li[1], Ying Yu[2], Hongliu Cai[2]

Departments of [1]Emergency, [2]Intensive Care Unit, The First Affiliated Hospital, Zhejiang University, Hangzhou 310003, Zhejiang, China

Abstract: Alzheimer's disease (AD) is a common case of dementia and its possible therapies, such as neuron stem cell (NSC) transplantation therapy, have been studied for years. In order to improve NSC transplantation effects, we were inspired to pretreat NSC using brain-derived neurotrophic factor (BDNF) before transplantation. The AD mouse model was constructed and effects of BDNF+NSC transplant group and traditional NSC transplant group were compared using the four indicators: conditions of learning and memory ability recovery tested by Morris Water Maze (MWM), number of basal forebrain cholinergic neurons, expression of synaptophysin, and number of acetylcholinesterase (ACHE)-positive fibers detected by chemical staining. Results showed all the four indicators were significantly lower in the AD model group than the control group ($P < 0.05$). Traditional NSC transplantation could improve these indicators to some extent but still possessed significant differences from the control group ($P < 0.05$). Especially, the BDNF+NSC transplant group showed significant improvements in the four indicators when compared with the AD model group ($P < 0.05$). Taken these data together, BDNF pretreatment improved the NSC transplantation effects, showing advantages over the traditional NSC transplantation. Our study could facilitate the application of stem cell transplantation therapy to AD treatment.

Keywords: Alzheimer's disease (AD), brain-derived neurotrophic factor (BDNF), neuron stem cell (NSC), transplantation

Introduction

Alzheimer's disease (AD) is a kind of chronic neurodegenerative disease accounting for a large proportion of dementia. It usually starts with a short-time memory loss [1] and gets worse over time, triggering various symptoms, including depression [2], aggressiveness, activity disturbances and psychosis [3]. AD patients are suffering from extremely inconvenience of life, shortened average life span and low society attention, which pose threats to them physically and mentally. Increased amyloid beta (Aβ) peptides are found depositing in the cerebral cortex of AD patients [4]. While recent studies indicate a cleaved amino-terminal fragment of Aβ precursor protein, N-APP, may be a major culprit in AD and Aβ plays a complementary role [5]. Besides the deposition of Aβ, AD patients are also characterized by their abnormal intracellular accumulation of tau protein [6], degen-

eration of basal forebrain cholinergic neuron (BFCN) [7], reduction of synaptophysin expression [8] and inhibition of acetylcholinesterase (ACHE) activity [9]. Deeper insight into AD and its neuropathology mechanisms has promoted research on AD treatment. New therapies, concerning traditional medicine [10], molecular targets [11] and neural stem cell (NSC) transplantation [12], have been attempted to treating AD.

NSC is a stem cell type exhibiting multi-potential ability to differentiate into glial cells or neurons [13] and is becoming a prospective cell therapy in treating various neurodegenerative diseases, including AD [14]. The grafted NSC has the capacity to migrate to the target regions in the brain and differentiate into the necessary type of cells [15], which enables related research on animal models. NSC transplantation is proved to be effective to alleviate the brain damage in AD models. For example, NSC

transplantation promotes the synaptic rebuilding and improves learning and memory abilities in AD rats [16]. It also improves memory [17], and rescues cognitive deficits by enhancing mitochondria biogenesis of AD model mice [18]. However, NSC transplantation for the treatment of AD is still at a preclinical stage, requiring long-term studies to testify the stable functional recovery. Pivotal issues, like safety and efficacy profiles of NSC therapy, should be emphasized [19]. Studies regarding these issues are indispensable to apply this promising treatment method to clinic.

In this study, we tried to improve the traditional NSC transplantation methods to achieve a better effect. The AD mouse model was constructed and the brain-derived neurotrophic factor (BDNF) was used to pretreat NSC before transplantation. To compare the effects of BDNF+NSC transplantation, we used Morris Water Maze (MWM) to test the learning and memory ability of different treated groups. Then changes in BFCN number indicated by nerve growth factor receptor (NGFR) p75, synaptophysin expression level and ACHE-positive fiber number were detected. These results allowed us to validate the advantages of BDNF-pretreated NSC transplantation, laying the foundation of its application for AD treatment.

Materials and methods

Isolation and cultivation of NSCs

The research was conducted in accordance with the guidelines of our institute and the Regulations for the Administration of Affairs Concerning Experimental Animals (approved by the State Council of China, 1988). Specific antigen-free (SPF) degree newborn C57BL/6 mice (Better Biotechnology, Nanjing, China) were fed *ad libitum* at 23°C. NSCs were isolated and cultured as follows. Mice were anesthetized with the hypodermic injection of xylazine (Sangong Biotech, Shanghai, China) (10 mg/kg) and ketamine (Sangong Biotech) (100 mg/kg). The subventricular zone and the hippocampus, the two regions with the highest neurogenic activity [13], were separated and minced. Then the tissues were treated with papain: ovomucoid (1:1) mixture at 37°C for 45 min. The papain mixture contained 30 U/μL papain, 240 μg/mL cysteine, 40 μg/mL DNase I in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) (Sigma-Aldrich, Shanghai, China). The ovomucoid mix-

ture contained L15 medium with 1.125 mg/mL trypsin inhibitor, 0.5 mg/mL bovine serum albumin (BSA) and 40 ng/mL DNase I (Sigma-Aldrich). The papain activity was blocked by adding additional one volume of ovomucoid mixture after digestion. Then the cell pellet was centrifuged at 80 g for 5 min, resuspended and cultured in the standard neurosphere medium DMEM/F-12 with 2 mm L-Glutamine, 20 ng/mL epidermal growth factor (EGF) and B27 supplement at 37°C for four days. Neurospheres were passaged with 0.05% trypsin followed by gentle mechanical trituration.

Construction of AD mouse model

The AD mouse model was constructed as previously described [20]. APP/PS1 transgenic mice (Better biotechnology) raised to 5 months old and 18 individuals (6 for each group) were anesthetized and then were placed in stereotaxic devices. In order to generate the AD mouse model, the specific cholinergic immunotoxin murine p75[NTR] saporin (mu p75-SAP, Advanced Targeting Systems, San Diego, USA) (1-1.2 μg/μL) diluted in phosphate buffered saline (PBS) was injected to the bilateral intracerebroventricular areas (anteroposterior (AP) -0.6 mm; mediolateral (ML) ± 1.2 mm; dorsoventral (DV) + 2 mm relative to the bregma). The injection was at a constant flow rate of 0.1 μL/min for 10 min, and a delay of 10 min was allowed before the needle was completely retracted in case of any aspiration of the toxin. Mice of the control group were injected following the same procedures with only PBS.

BDNF pretreatment and NSC transplantation

NSC was pretreated by BDNF (ProSpec-Tany, Ness-Ziona, Israel) before transplanted into the BNDF+NSC transplant group according to a former study [21]. Specifically, NSC single cell suspension was incubated in 100 ng/mL BDNF for 1 h. Then the pretreated and untreated cells were washed twice with PBS, and cell viability was examined with the trypan blue exclusion method. After trypsinization, wash and filter with 70 μm meshes, NSC were counted and suspended at the concentration of 1×10^6 cells/μL in 1× Hanks Balanced Salt Solution (HBSS) and 20 ng/mL EGF. Six mouse individuals of each group were anesthetized and injected with 5 μL of either BDNF-pretreated NSC (the BDNF+NSC transplant group), untreated NSC (the NSC transplant group) or PBS (the control
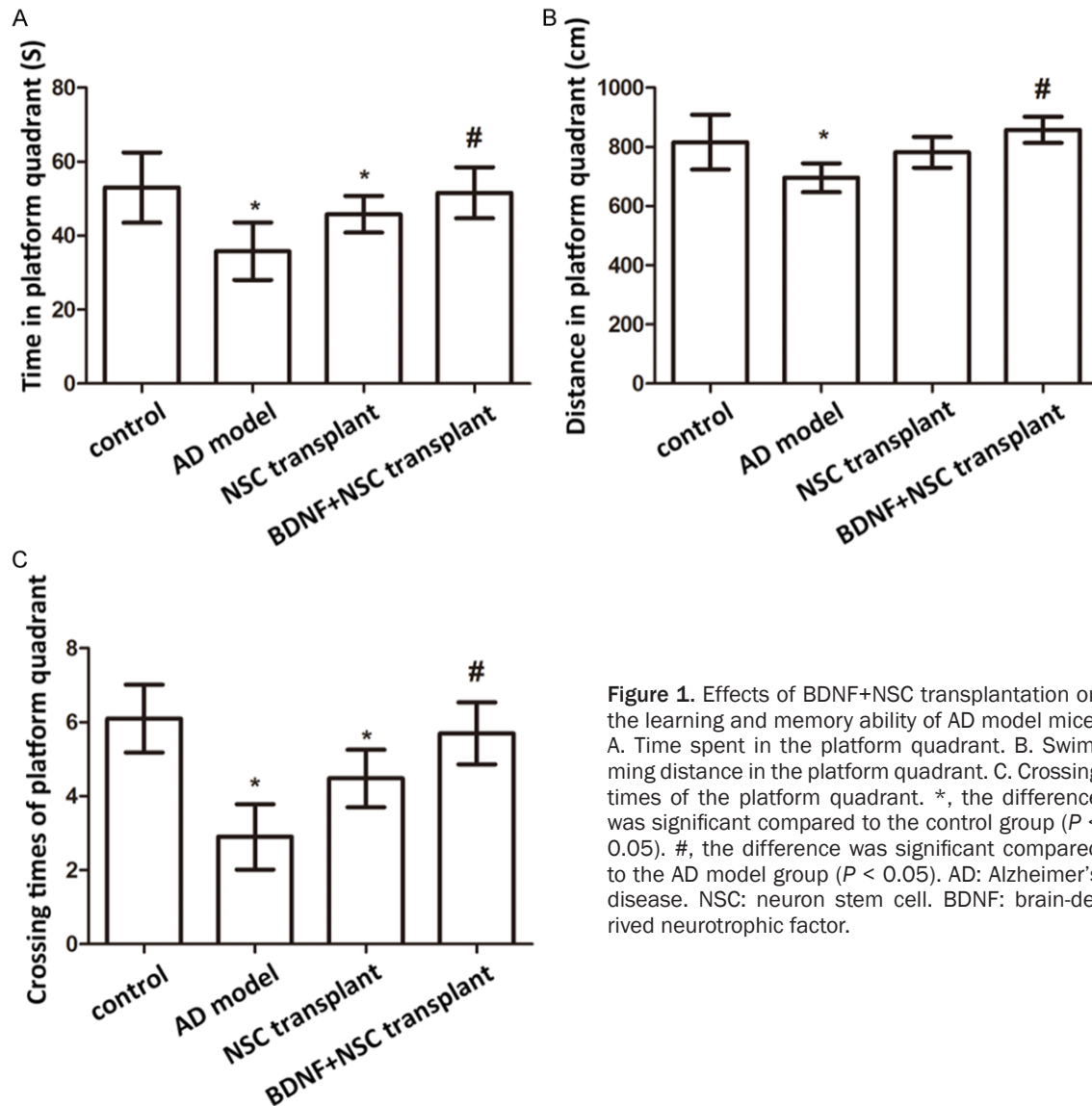
Figure 1. Effects of BDNF+NSC transplantation on the learning and memory ability of AD model mice. A. Time spent in the platform quadrant. B. Swimming distance in the platform quadrant. C. Crossing times of the platform quadrant. *, the difference was significant compared to the control group ($P < 0.05$). #, the difference was significant compared to the AD model group ($P < 0.05$). AD: Alzheimer's disease. NSC: neuron stem cell. BDNF: brain-derived neurotrophic factor.

group and the AD model group). Cells or vehicle were injected at a rate of 1 μL/min to bilateral hippocampi (AP -2.06, ML ± 1.85, DV -2.50). The needle was retracted after the sphere was completely diffused. All the transplanted mice were undergone behavioral tests at 5-6 weeks of post transplantation.

MWM analysis

Learning and memory abilities were further analyzed using the MWM test. All the groups under study, namely the control group, AD model group, NSC transplanted group and BDNF+NSC transplanted group, were tested. The maze consisted of a round tank of water (0.95 m in diameter) with 4 equal quadrants, and geometric cues were on the walls. An escape platform was placed 2-3 cm below the water surface. The water temperature was kept around 23°C. Mice were first trained 6 trials per day for 4 days. Each trial lasted for 10 min, with an interval of 30 min between two trials. The time limit was 60 s per trial. If an animal did not find the platform within the time limit, it was placed on it for another 10 s. The test began a day after the final trial. All the experiment procedures were videotaped and analyzed by image track software. Time spent in the platform quadrant (s), swimming distance in the platform quadrant (cm) and crossing times of the platform quadrant were recorded and compared.
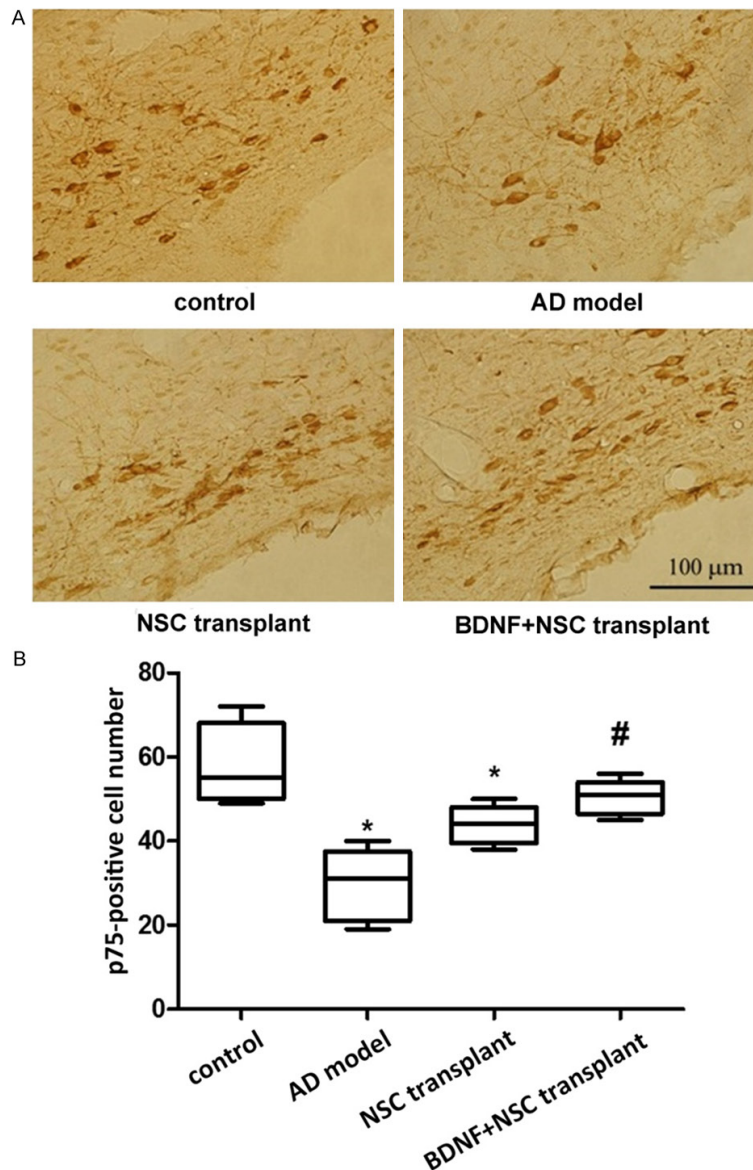
Figure 2. Effects of BDNF+NSC transplantation on increasing BFCN number indicated by p75-positive cell number. A. Immunohistochemistry results showing the signals of p75-posive cells. Scale bar = 100 μm. B. Histogram showing the number of p75-positive cells based on the counts in sections. *, the difference was significant compared to the control group ($P < 0.05$). #, the difference was significant compared to the AD model group ($P < 0.05$). AD: Alzheimer's disease. NSC: neuron stem cell. BDNF: brain-derived neurotrophic factor.

incubated with anti-NGFR p75 antibody or anti-synaptophysin antibody (Sigma-Aldrich) overnight at 4°C. Then they were incubated in the secondary antibody for 1 hour at 37°C. Diaminobenzidine solution was added for 5 min and then images were acquired using a microscope.

ACHE staining

ACHE staining was conducted following the procedures. The hippocampus sections of each group were washed three times in 0.1 M acetate buffer, each time for 1 min. Then the sections were incubated in buffer containing 0.05% acetycholine iodide, 4 M sodium citrate, 3 M copper sulfate, 0.1 M potassium ferricyanide and 0.065 M acetate buffer. After incubated for 30 min, the sections were washed by 1% ammonium sulfide solution for 3 min and 0.1 M sodium nitrate solution for 5 times. Then they were stained in silver nitrate solution for 2 min. After wash, dehydration and clearing, the sections were covered and then observed by a microscope. Five visual fields were randomly chosen to calculate the ACHE-positive fiber number in hippocampus CA1, which were indicated as the intersection number on the test line in the grid system.

Statistical analysis

Statistical analyses were performed using SPSS Statistics 19 software (IBM, New York, USA). Data were analyzed by one-way or two-way analysis of variance (ANOVA), followed by the Tukey HSD *post hoc* analysis if necessary. Results of MWM test were analyzed by three-way ANOVA with the trials as a repeated-measures factor. Differences were considered significant if $P < 0.05$.

Immunohistochemistry

Mice of each group were perfused with PBS followed by 4% paraformaldehyde (PFA). The brain tissues were fixed in PFA for 1-2 days, followed by a sequential 15% to 30% sucrose treatment. Then the samples were cryosectioned into 16 μm. After blocked in 5% BSA for 1 h, the sections were washed by PBS for 3 times, and
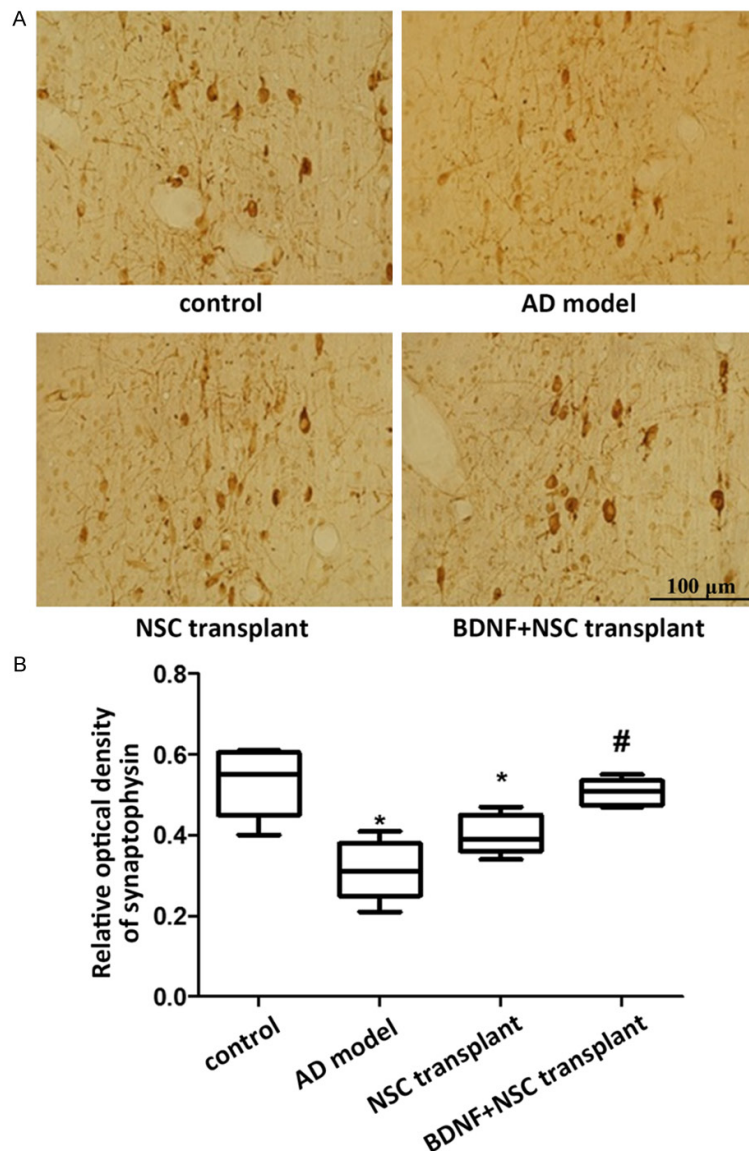
Figure 3. Effects of BDNF+NSC transplantation on promoting synaptophysin expression. A. Immunohistochemistry results showing the signals of synaptophysin. Scale bar = 100 μm. B. Histogram showing the signal density of synaptophysin gathering from the sections. *, the difference was significant compared to the control group ($P < 0.05$). #, the difference was significant compared to the AD model group ($P < 0.05$). AD: Alzheimer's disease. NSC: neuron stem cell. BDNF: brain-derived neurotrophic factor.

Results

BDNF+NSC transplantation improved better learning and memory ability

Time spent in the platform quadrant, swimming distance in the platform quadrant and the crossing times of platform quadrant were recorded and analyzed in the MWM test. Results reflected significant decreases of the above three parameters in the AD model group compared to the control group ($P < 0.05$) (**Figure 1A-C**). The performance of the NSC transplant group was a little better than the AD model group, with no significant difference. But the time spent and the crossing time of the platform quadrant were still significantly less than the control group ($P < 0.05$). The three parameters were significantly increased in the BDNF+NSC transplant group compared to the AD model group ($P < 0.05$). It can be inferred that the AD model mice were impaired in their learning and memory ability, while BDNF+NSC transplantation improved the learning and memory ability of AD model mice better than just transplanting untreated NSC.

BDNF+NSC transplantation increased BFCN number

Five visual fields were randomly chosen from each group after immunohistochemistry and then the numbers of BFCN were calculated. Results showed the BFCN number was significantly less in the AD model group than in the control group ($P < 0.05$) (**Figure 2A** and **2B**). Though NSC transplantation could increase the BFCN number to some extent, it still could be distinguished from the control group ($P < 0.05$). The BDNF+NSC transplant group showed a more significant increase compared to the AD model group ($P < 0.05$), indicating BDNF+NSC transplantation increased the BFCN number of AD model mice brain, which was more evident than traditional NSC transplantation.

BDNF+NSC transplantation promoted synaptophysin expression

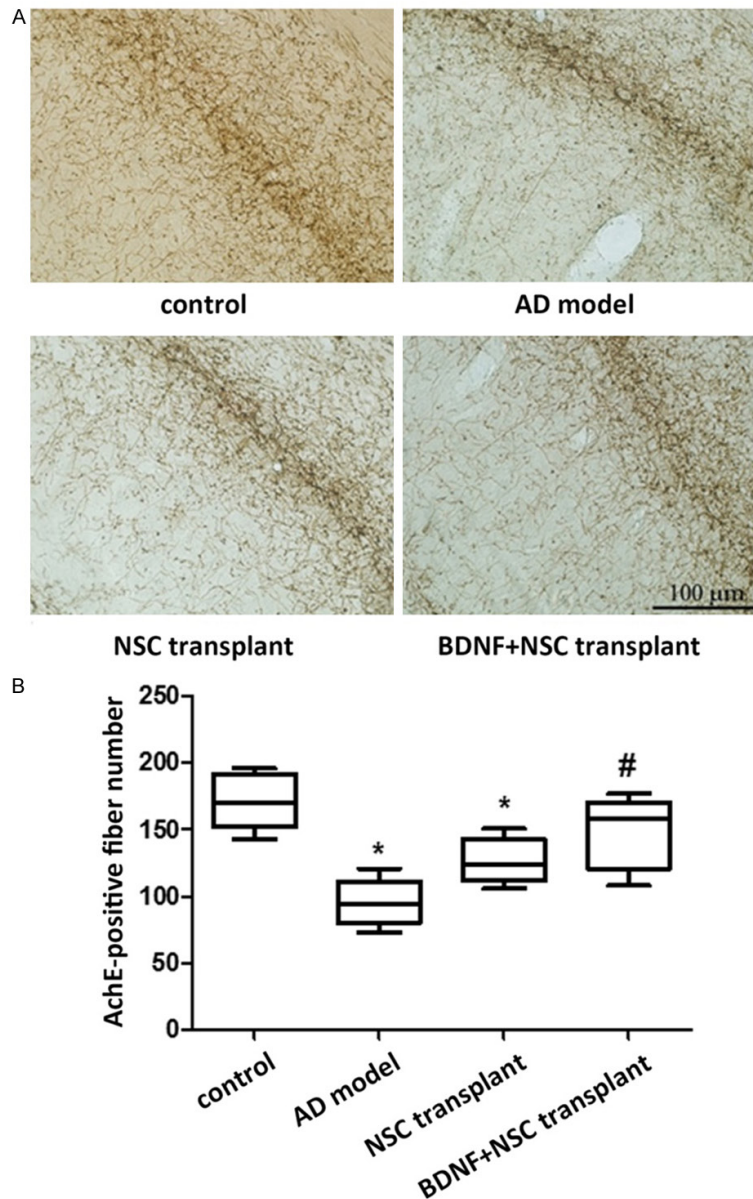Five visual fields were randomly chosen for synaptophysin detection. The AD model group

Figure 4. Effects of BDNF+NSC transplantation on increasing the ACHE-positive fiber number. A. Immunohistochemistry results showing the signals of AchE-posive fibers. Scale bar = 100 μm. B. Histogram showing the number of ACHE-positive fibers based on the counts in sections. *, the difference was significant compared to the control group ($P < 0.05$). #, the difference was significant compared to the AD model group ($P < 0.05$). ACHE: acetylcholinesterase. AD: Alzheimer's disease. NSC: neuron stem cell. BDNF: brain-derived neurotrophic factor.

tophysin at a much higher level, with significant difference from the AD model group ($P < 0.05$). These data indicated BDNF+NSC transplantation promoted synaptophysin expression greatly, having a better effect than the NSC transplant group.

BDNF+NSC transplantation increased AchE-positive fibers

Similar results were found in the number change of ACHE-positive fiber. The ACHE-positive fiber number in the AD model group was significantly decreased compared to the control group ($P < 0.05$) (**Figure 4A** and **4B**). After NSC transplantation, the number of ACHE-positive fiber increased, nonetheless, the difference still existed between the NSC transplant group and the control group. The ACHE-positive fiber number in the BDNF+NSC transplant group increased greatly compared to the AD model group ($P < 0.05$), indicating the better effect of BDNF+ NSC transplantation in increasing ACHE-positive fiber of AD model mice.

Discussion

This study introduces BDNF pretreatment in NSC transplantation to test the effects on AD treatment. The BDNF+NSC transplant group of AD mouse model and the traditional NSC transplant group were compared from four aspects, namely, learning and memory ability, the number of BFCN, the expression of synaptophysin and the number of ACHE-positive fibers. Results show the AD model mice after BDNF+NSC transplantation has better learning and memory ability, more BFCN number, higher synaptophysin expression and more ACHE-

showed significantly lower synaptophysin expression compared to the control group ($P < 0.05$) (**Figure 3A** and **3B**). After transplanted NSC, mice expressed synaptophysin a bit higher, but still possessed significant difference from the control group ($P < 0.05$). The BDNF+NSC transplant group expressed synap-

positive fibers. These results indicate BDNF pretreatment in NSC transplantation has better effects on AD treatment than simply transplanting NSC.

In addition to the MWM test, BFCN, synaptophysin and ACHE, whose quantity and expression are closely related to AD, were also detected to assess transplantation effects. BFCN has been demonstrated to play roles in memory function [22, 23]. Its perturbation may happen at very early stages of AD [24], thus it is an target for AD studies [25]. The synaptophysin knockout mice exhibit learning and memory impairments, like reduced object novelty recognition and reduced spatial learning [26]. Inhibition of ACHE leads to impeded neurotransmission and loss of its G4 form is found in the cortical and subcortical brain regions of some AD patients [27]. So the three factors were used in this study to reflect and compare the effects of different NSC transplantation methods. NSC transplantation with BDNF pretreatment showed more BFCN number, higher synaptophysin expression and more ACHE-positive fibers than traditional NSC transplantation, indicating its advantages over the latter.

Existed studies have utilized BDNF pretreatment to improve the effects of stem cell transplantation. When the human umbilical cord blood cells are transplanted into the cord injury site of rats together with BDNF, the Basso-Beattie-Bresnahan (BBB) scores, which reflect the functional recovery degree, are greatly improved [28]. Transplantation of human embryonic-derived NSC with BDNF pretreatment results in higher initial NSC engraftment and survival, increased neuroprotection and greater functional recovery compared to untreated NSC [21]. Especially, BDNF is an unspecific biomarker of many neurological and psychiatric diseases [29] and is related to the cognition improvement of NSC, implying its significant roles in AD pathology [30]. So in this study, we were inspired to try the BDNF pretreatment before NSC transplantation. As predicted, this strategy had advantages over NSC transplantation without any pretreatment. Consistent with the former results, this study indicated that BDNF pretreatment might be a candidate NSC transplantation approach, and could further facilitate the stem cell transplantation therapy of AD.

The important roles of BDNF have triggered studies on its functional mechanisms. BDNF prevents cell injuries induced by Aβ25-35 via binding to its receptor TrkB [31], and its up-regulation tends to play roles in the neuroprotective effect of NSC transplantation [32]. This study implied BDNF was a correlative factor in enhancing NSC transplantation effects, possibly due to its regulatory functions. Detailed mechanisms remain to be uncovered. It could not be denied based on our findings that NSC transplantation with BDNF pretreatment was more promising in AD treatment than traditional NSC transplantation.

In summary, this study introduces an improved NSC transplantation method with BDNF pretreatment, which shows better effects on AD model mice than traditional NSC transplantation. Increasing the efficacy of transplantation, NSC transplantation with BDNF pretreatment could be a promising method for AD treatment. Further fundamental mechanism studies and safety tests are still essential to clinically apply the stem cell transplantation therapy to AD treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hongliu Cai, Department of Intensive Care Unit, The First Affiliated Hospital, Zhejiang University, 79 qingchun Road, Hangzhou 310003, Zhejiang, China. E-mail: caihongliu456@126.com

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