Neural Stem Cells Improve Learning and Memory in Rats with Alzheimer's Disease

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Key Words

Alzheimer's disease · Neural stem cells · Nerve growth factor · Recombination adeno-associated virus

Abstract

Objective: We investigated whether neural stem cells (NSC) with transgenic expression of human nerve growth factor (*hNGF*) transplanted into the brain could offer a therapeutic option for the treatment of Alzheimer's disease (AD). **Methods:** We infused okadaic acid into rat lateral ventricles to establish a chronic AD animal model. In addition, NSC were stably transduced with *hNGF* and enhanced green fluorescent protein (*eGFP*) genes (*NSC-hNGF-eGFP*) by using a recombination adeno-associated virus serotype 2 (rAAV2) vector. These genetically modified stem cells were grafted into the cerebral cortex of AD rats. **Results:** AD model rats showed significant damage in learning and memory function, with the formation of senile plaques and neurofibrillary tangles in the cerebral cortex. The transferred *hNGF* gene conferred stable and high levels of protein expression in NSC in vitro. Moreover, the *NSC-hNGF-eGFP*, but not the NSC, survived, integrating into the host brain and enhancing cognitive performance after transplantation. **Conclusion:** The injection of okadaic acid into rat lateral ventricles constitutes a promis-

ing animal model for investigating selective aspects of AD. rAAV2-mediated *hNGF* delivery can render long-term and stable transduction of *hNGF* in NSC. NSC-*hNGF-eGFP* transplantation may offer a viable therapeutic approach for treatment of AD.

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Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative dementias, affecting more than 12 million people worldwide [1, 2]. The average survival from diagnosis is approximately 8 years. Symptoms of the disease include progressive impairments in learning and memory [3–5], and hallmark neuropathologic features of AD include depositions of extracellular senile plaques (SP), the formation of intracellular neurofibrillary tangles (NFT), and widespread neurodegeneration [6–9]. SP primarily comprise amyloid β -peptide (A β), which is generated from amyloid precursor protein (APP) [10, 11]. NFT are composed largely of hyperphosphorylated τ protein [12, 13]. Abnormal τ phosphorylation may cause NFT formation and cytoskeletal disruption, possibly leading to neuronal malfunction and death [14–16]. No

proven medical therapy is available to mitigate the devastating clinical manifestations of AD.

Neural stem cells (NSC) are undifferentiated cells capable of self-renewal or differentiation [17–19]. Recently, the transplantation of NSC has been proposed. This option might represent a plausible future therapy for AD, to provide unlimited stem cell numbers in vitro, stably express transgenes, release therapeutically active substances to maintain neuronal function, prevent the progressive loss of neurons, and induce regenerative responses in neurons [20–22]. Transplantation of NSC-*GDNF* (glial cell line-derived neurotrophic factor) into an animal model of Parkinson's disease prevented the degeneration of dopaminergic neurons in the substantia nigra, and resulted in limited functional recovery of the animals [23, 24].

This study examined (1) whether rAAV2 can maintain stable and high-level human nerve growth factor (*hNGF*) and enhanced green fluorescent protein (*eGFP*) transgene expression in NSC and (2) whether genetically modified NSC-*hNGF-eGFP* engraft well in the host brain and bring about functional effects, such as recovery from learning and memory impairments in AD rats. Our results indicated that rAAV2 vectors maintain high-level transgene expression in NSC, and that overexpression of *hNGF* in this way significantly improves learning and memory in AD rats.

Materials and Methods

Animals

Male Wistar rats (270–290 g body weight) and embryonic day 17 (E17) Wistar rat fetuses were used in this study. All rats were maintained in a temperature-controlled environment of $24 \pm 1^\circ\text{C}$ with a 12-hour dark/light cycle (dark cycle: 8:00 p.m. to 8:00 a.m.) with food and water supplied ad libitum throughout the experiments. All experimental protocols accorded with the Guidelines of Animal Use and Care of Hiroshima University.

Neural Stem Cell Culture

The forebrain cerebral cortex including the subventricular zones and the hippocampus was removed from E17 rat fetuses. After removal of the meninges, the tissue was minced and then digested in 0.25% trypsin solution with 0.1 mM EDTA at room temperature for 15 min. Following trituration through a plastic 5-ml pipette and a fire-polished Pasteur pipette, the dissociate was filtered through sterile gauze, washed twice in 5 ml of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (DMEM/F12; Invitrogen, Carlsbad, Calif., USA) to eliminate cell aggregates and debris. Cells were then counted with a hemocytometer. Finally, the cells were resuspended in DMEM/F12 containing 20 ng/ml epidermal growth factor (EGF; Sigma, St. Louis, Mo., USA), 20 ng/ml basic fibroblast

growth factor (Sigma), and B-27 growth medium (Sigma). The resulting cell suspension was plated onto poly-L-lysine (Sigma)-coated T-75 culture flasks (Iwaki, Tokyo, Japan) at a density of 5×10^4 cells/ml. Neurosphere cultures were maintained at 37°C in an incubator with 5% CO_2 and 95% humidity.

rAAV2 Vector Production

rAAV2 vectors were prepared as described previously [25]. The rAAV2 vector contains inverted terminal repeats flanking a transcription unit containing *hNGF* and *eGFP* cDNA driven by a cytomegalovirus promoter. The vector plasmid was packaged into the rAAV2 capsid using the AAVMax™ package system in a baby hamster kidney (BHK-21) cell line. Briefly, a recombinant herpes simplex virus type 1 (HSV1-rc/ Δ UL2), which is able to express adeno-associated virus Rep and Cap proteins, was used for rAAV2 replication and packaging. The rAAV2 proviral cell line (BHK/SG2) with *hNGF* and *eGFP* gene expression cassette inserted was established by transfecting BHK-21 cells with rAAV2 vector plasmid pSNAV-2-*hNGF-eGFP*. The BHK/SG2 cells were infected with HSV1-rc/ Δ UL2 to generate rAAV2 vectors. The cells were harvested, lysed by 3 freeze/thaw cycles, and the virions isolated by cesium chloride gradient centrifugation. The gradient fractions containing rAAV2 were dialyzed against sterile PBS, and stored at -80°C . Dot blot analysis demonstrated particle titers of 1×10^{12} virus genome/ml.

Transfection of rAAV2-hNGF-eGFP Viruses into NSC

For transfection experiments, 1×10^6 NSC were plated on 96-well plates in DMEM/F12 medium (as described above). Twenty-four hours after seeding, different multiplicity of infections (MOI) of the rAAV2-*hNGF-eGFP* virus (2×10^4 , 1×10^4 and 5×10^4) were added to the cell cultures. After 96 h, the culture supernatant was replaced with medium containing EGF and bFGF, and supplemented with B-27 growth medium.

Surgical Procedures

Rats were anesthetized with 50 mg/kg sodium pentobarbital, and placed in a stereotaxic apparatus (Narishige, Tokyo, Japan) according to the brain atlas of Paxinos and Watson [26]. Unilateral cannulas (model 2004; Alzet, Cupertino, Calif., USA) were implanted into the right lateral ventricle (coordinates: 0.8 mm posterior to the bregma, 1.3 mm right lateral to the midline, and 4.0 mm ventral to the skull surface). The cannulas were fixed to the skull with dental cement, and connected to the pump end with sterile, coiled polyethylene tubing. The pump was placed subcutaneously in the neck/shoulder area. Rats received an intramuscular injection of penicillin (20,000 U) into each thigh after the surgery and were given water with acetaminophen (0.35 mg/ml) for 3 days.

On day 0, 36 rats of the okadaic acid (OA)-treated group were intracerebroventricularly infused with OA (80 $\mu\text{g}/\text{day}$, Sigma), dissolved in 0.1 M phosphate-buffered artificial cerebrospinal fluid (CSF). Artificial CSF rats ($n = 36$) were intracerebroventricularly infused with artificial CSF. The sham operation rats ($n = 36$) received no infusion. The Alzet osmotic pumps (lifespan 28 days) were changed on day 28. On day 56, the pump and tubing were removed from the anesthetized rats. To confirm the OA dosage administered, the total infusion volume was determined for each animal. The rats were allowed to rest for 1 week, and the testing phases of learning, memory, and the exploratory task were performed as follows.

Morris Water Maze Test

The spatial learning and memory ability of rats was assessed in the Morris water maze [27]. The water maze (180 cm in diameter, 60 cm in height) was located in an experimental room rich in extra-maze cues and was filled with water (25°C) made opaque by white nontoxic tempera paint. An escape platform was hidden 1.5 cm below the water surface in a fixed location in one quadrant of the maze. Rats received 5 trials per day with 120-second intervals. On day 0, rats were allowed to swim freely for 120 s without a platform to acclimatize to the pool. On days 1–4, rats were trained to locate and escape onto the submerged platform from random release points around the pool. If an animal did not find the platform in 120 s, it was placed on the platform for 10 s and then returned to the home cage. The time to reach the platform (escape latency) was measured with a computerized tracking system (Etho Vision 2.0; Noldus Information Technology, Wageningen, The Netherlands). On day 5, the platform was removed, and a 120-second probe trial was run to examine retention of spatial memory. The percentage of time spent in each quadrant was recorded. All experiments were conducted in a blinded manner.

Open-Field Task

Exploratory locomotion activity was assessed by open-field testing. The apparatus consisted of a gray box open at the top and measuring 50 cm long $\times$ 30 cm wide $\times$ 20 cm high. The floor was divided into fifteen 10-cm squares. The testing took place under normal room illumination. Each rat was placed randomly in one corner of the box. The behavior was videotaped for 5 min. The following measurements were taken: number of squares entered (all 4 paws inside the square), time spent in the corner (the time spent in the 4 corner squares), and time spent rearing (an animal standing on its hind legs with forelegs in the air or against the wall). After each rat had been tested, the box was thoroughly cleaned to remove odor cues.

Histology

After deep anesthesia, animals were perfused intracardially with saline (0.5%), followed by 4% paraformaldehyde. The brains were then removed and fixed in paraformaldehyde for 24 h, and subsequently transferred to 30% sucrose at least 4 days before sectioning. Sections of 40–100 μ m in thickness were cut on a cryostat microtome and processed for thioflavin-S fluorescence [28] and Bielschowsky silver staining [29].

Transplantation

Transplanted cells were mildly trypsinized into single cells in 0.1 M phosphate-buffered saline (PBS). Cell viability was checked by trypan blue exclusion. The total number of cells was determined using a hemocytometer. The cells were centrifuged at 1,000 rpm for 5 min, and resuspended to produce a final concentration of 2×10^4 cells/ml. AD rats were anesthetized and placed under the stereotaxic instrument. The cells were loaded into a 5- μ l Hamilton syringe held in a micromanipulator (MP-85, Sutter Instruments, Novato, Calif., USA) for transplantation. The NSC-*hNGF-eGFP* rats ($n = 12$) were injected bilaterally with a 5- μ l NSC-*hNGF-eGFP* suspension into the hippocampus and cerebral cortex of AD rats (5 μ l/injection site/unilateral). NSC rats ($n = 12$) were injected with the same quantities of NSC suspension at the same sites. The other 12 rats were injected with the same quantities of culture medium at the same sites (medium group). Trans-

plantation coordinates for the hippocampus, relative to bregma (AP), the midline (ML), and the dura-ventral (DV) were 1.2 mm (ML), 1.7 mm (AP), and 3.5 mm (DV) for the hippocampus, and 2.0 mm ML, 2.0 mm AP, and 3.0 mm DV for the cerebral cortex. A 1- μ l cell suspension was injected over a period of 15–20 s into the transplantation site, and the needle was left in position for a further 5 s before being slowly withdrawn.

Immunofluorescence Staining

The cultured NSC were fixed with 4% formaldehyde for 10 min at room temperature. After 3 washes with PBS for 5 min each, the cells were incubated for 2 h at room temperature with blocking buffer (DMEM, 0.1% glycine, 0.1% lysine, 5% FBS, and 0.2% Triton X-100). Primary antibody against nestin (mouse monoclonal IgG, 1:100; Chemicon, El Segundo, Calif., USA) was diluted in blocking buffer and incubated overnight at 4°C. After 3 washes with PBS for 5 min each, the cells were incubated for 1 h at room temperature with goat anti-mouse IgG Alexa Fluor 488 conjugate (1:200; Invitrogen) diluted in blocking buffer without Triton X-100. Samples were then washed 3 times with PBS. Fluorescence was detected using an inverted fluorescence microscope (Eclipse 300E; Nikon, Düsseldorf, Germany), and digital images were captured with a 12-bit digital CCD camera (Visicam; Visitron, Munich, Germany).

Statistical Analysis

Results were presented as means $\pm$ SEM. The data were analyzed using 1-way or 2-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple range test. Repeated measures were also used when necessary. A statistical difference was accepted at a 5% level, unless indicated otherwise.

Results

Overexpression of NSC-hNGF-eGFP

NSC isolated from subventricular zones and the hippocampus of E17 rat fetuses could proliferate, self-renew and differentiate (fig. 1a–c). To select cells with the highest efficiency of *eGFP* expression, 3 different MOI of rAAV2-*hNGF-eGFP* virus were added in the cell suspension. MOI (5×10^5) of rAAV2-*hNGF-eGFP* virus showed the highest transduction efficiency, with approximately 87% of cells infected by day 4 (fig. 1d, e, 2).

Behavioral Analyses

To determine the effect of OA on learning and memory performance, all rat groups were tested in the Morris water maze and open-field task 63 days after surgery. The OA group spent a significantly longer time finding the hidden platform in the Morris water maze than the sham operation and artificial CSF groups ($p < 0.001$, fig. 3a). In the spatial probe trial, the OA group spent a significantly lower percentage of time in the target quadrant than rats in the sham operation and artificial CSF groups ($p <$

Fig. 1. NSC were isolated from the subventricular zone and hippocampus. **a** The NSC colonies formed neurospheres by self-renewal. **b** Primary NSC began to differentiate when bFGF and EGF were removed. **c** Nestin, a NSC marker, was expressed in neurospheres by immunocytochemistry. **d** NSC began to express NGF and eGFP after 24 h of infection. **e** NSC showed higher expressions of NGF and eGFP 72 h after the infection. Images are representative of 4 independent experiments. Scale bars: 50 μ m.

Fig. 2. The NSC were infected at different MOI of rAAV2-NGF-eGFP virus (2×10^4 , 1×10^5 and 5×10^5).

0.001, fig. 3b). The artificial CSF and sham operation groups showed similar behavior in the water maze task ($p > 0.05$). Swimming speed was not affected by the treatment throughout the testing (data not shown), indicating that the water-maze differences were not due to motor deficits. In the open-field task, OA-treated rats exhibited lower levels of locomotion activity and spent less time in

motion than the sham operation and artificial CSF groups ($p < 0.001$). The OA group also showed maximal habituation to the novel environment, at which time the other 2 groups still showed minimal habituation. There were no differences between artificial CSF and sham operation groups ($p > 0.05$, fig. 4a–c). These results suggest that spatial learning was significantly impaired in OA-treated rats.

The therapeutic effects of NSC-*hNGF-eGFP* on learning and memory in AD model rats were tested in the Morris water maze and open-field task on day 30 after transplantation. The NSC-*hNGF-eGFP* group exhibited significant improvements in performance in both the water maze and open-field testing. The escape latency ability of NSC-*hNGF-eGFP* rats was significantly different from that of the OA-, NSC-, and media-treated animals ($p < 0.001$); there were no differences among these 3 groups ($p > 0.05$, fig. 3a). NSC-*hNGF-eGFP* rats spent more time in the target quadrant than those in the OA, NSC, and media groups (fig. 3b), and showed normal exploratory behavior in the open-field task (fig. 4a–c). There was a significant difference between the NSC-*hNGF-eGFP* and OA groups in locomotion activity ($p < 0.001$), while the overall locomotion activities of the NSC-*hNGF-eGFP* and sham operation groups were not statistically different in the open-field task (fig. 4a–c). Differences were apparent between the NSC and OA groups in locomotion activity, but were not statistically significant ($p >$

Fig. 3. Spatial learning was assessed by the Morris water maze test. **a** Time taken by rats to find the hidden platform on day 5. **b** Time spent by rats in target quadrant during the spatial probe trial. Five trials were recorded following a 120-second intertrial interval. * $p < 0.001$ compared to OA-treated rats.

0.05). Analyses of the Morris water maze and open-field task results revealed that OA causes severe damage to the learning, memory, and exploratory activity of rats, and NSC-*hNGF-eGFP* transplantation induced a significant improvement in learning, memory, and exploratory behavior.

Histopathological Analyses

OA effects on SP deposition and NFT formation were evaluated by thioflavin-S fluorescence staining and Bielschowsky silver staining, respectively. SP deposits and NFT were observed in the cortex of OA-treated rats, but not in the artificial CSF control or sham operation groups (fig. 5a–d).

We used *eGFP* as a reporter gene to identify cellular transduction within the brain regions. We found that *hNGF* was expressed in different brain regions, and 30 days after transplantation, NSC-*hNGF-eGFP* were integrated into the surrounding host cells (fig. 6).

Fig. 4. Locomotion activity was assessed in the open-field task. **a** Number of squares entered within 5 min. **b** Number of times rats reared within 5 min. **c** Time spent in a corner within 5 min. * $p < 0.001$ compared to the OA-treated rats.

Discussion

This study demonstrated that chronic infusion of serine/threonine protein phosphatase (PP) inhibitor, OA, into rat lateral ventricles severely impairs learning, memory, and exploratory activity. In addition, SP deposition and NFT were formed in treated rat cortices. We also demonstrated that the OA-induced neurological dysfunction was improved through the transplantation of NSC-*hNGF-eGFP*. Our results suggest a role for NSC-*hNGF-eGFP* in the treatment of AD.

Fig. 5. SP deposition and NFT formation were detected by thioflavin-S fluorescence staining and Bielschowsky silver staining, respectively. **a** SP were detected in OA-treated rat cortex. **b** SP were not present in the cortex of control rats. **c** NFT accumulated in the cortex of OA-treated rats. **d** NFT were not detected in the control group. Scale bar: 50 μ m.

Fig. 6. rAAV2-modified transgene expression was observed in different regions of the rat brains after transplantation. **a** NGF were expressed in cortex after 15 days. **b** NGF were expressed in hippocampus and NSC were integrated into host cells 30 days after transplantation. Scale bar: 50 μ m.

Protein phosphorylation and dephosphorylation play an important role in the regulation of AD pathogenesis [30–32]. In vitro, OA causes hyperphosphorylation of τ , modification of synapse structure, and the destruction of stable microtubules. PP can restore the biological activity of abnormally phosphorylated τ in vitro [33], and a recent in vivo study found decreased PP2A activity and mRNA expression in AD brains [34], linking abnormalities in phosphatase activity and AD pathogenesis. Our current results strongly imply that OA acts by perturbing protein phosphorylation and dephosphorylation, which may in

turn affect several pathological processes such as the deposition of A β , inflammatory responses, oxidative injury, neurotransmitter deficits, and neuronal apoptosis. Our results also suggested that stereotaxic and chronic infusion of OA into lateral ventricles of rats might provide a useful AD animal model.

Neurotrophic factors have emerged recently as promising therapeutic tools for AD because they can prevent neuronal cell death [35–37]. A key to successful cell and gene therapy is the selection of appropriate therapeutic genes and the modes of molecular and cellular delivery

of that gene. NGF is the archetypical protein discovered in the 1960s for increasing both survival and functioning of cholinergic neurons. However, the blood-brain barrier does not allow penetration of this protein into the brain in vivo [38]. Using gene therapy, growth factors can be delivered directly to the brain [39–41], and genetically modified NSC expressing hNGF and eGFP offer long-term therapeutic efficacy as a delivery vehicle to treat neurodegenerative diseases [42, 43]. The transplantation of NSC-*hNGF-eGFP* into an experimental model of neural degenerative disease compensated for some deficits by releasing neurotrophic factors [36]. However, our knowledge of the mechanism of functional improvement in the AD model brain remains limited. The present study showed that NSC sustain their proliferation potential when cultured in DMEM/F12 (1:1) serum-free medium containing EGF and bFGF, which are both known to promote NSC proliferation [44, 45]. To understand the nature of differentiation, we induced NSC to differentiate into neurons and glial cells in vitro. Consistent with previous studies, our results confirmed that these cells isolated from the embryonic fetus have differentiation potential. To obtain genetically modified NSC clones, we selected recombinant adeno-associated virus (rAAV2) vector to infect NSC in vitro. Our data indicated that rAAV2 can efficiently and stably infect NSC. These findings support the hypothesis that NSC are ideal therapeutic-gene vehicles.

The formation of A β and the accumulation of NFT in the brain trigger the pathological cascade of AD, including the degeneration of neuronal elements [46, 47]. Intravenously delivered NSC were recently suggested to reduce progressive AD [48, 49], and transplantation studies using NSC have shown site-specific neuronal differentiation in neurogenesis sites of the adult brain [50]. NSC also show remarkable plasticity and an ability to respond to epigenetic signals in vivo by generating region-specific neurons [51–53]. In a different study, genetically modified NSC used for gene transfer by direct intracerebral injection of NGF promoted regeneration and reversed age-related atrophy of cholinergic neurons [54, 55]. In our study, NSC-*hNGF-eGFP* transplanted into rat brain secreted hNGF, and integrated into the surrounding host tissue as well as improving neurological function. These genetically modified cells survived in the long term, without perturbing the recipient brain and without becoming tumorigenic or immunogenic. However, transplantation of NSC did not achieve similar results. One explanation for this finding is that the modified NSC-*hNGF-eGFP*, but not unmodified NSC, survived and dif-

ferentiated into functional neurons and secreted hNGF over a long time. Secreted hNGF could act to increase the number of NSC acting directly on the NSC themselves or acting indirectly through augmentation of the cholinergic system, or a combination of both, although further characterization would be necessary to confirm this proposal. Both NSC and NGF rescue damaged cholinergic neurons, prevent the progressive loss of neurons, induce additional regenerative responses in neurons such as increased neurotransmitter turnover, provide neurotrophic and neuroprotective support, and maintain neuronal function [56].

In summary, our results demonstrated that chronic infusion of OA into rat brain ventricles can partly mimic the clinical pathology of AD, and that rAAV2 is a useful and efficient way to deliver *hNGF* directly into the NSC. Furthermore, genetically modified NSC-*hNGF-eGFP* show remarkable capacity to integrate into host tissue, secrete neurotrophic factor over the long term, and replace damaged or lost neural cells. Thus, using genetically modified NSC-*hNGF-eGFP* to replace damaged or lost neurons and secrete neurotrophic factor is a potential new approach toward restoring function in AD. Further investigations are needed to determine the specific therapeutic effects and explore the underlying mechanism.

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