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Human Adipose Tissue-Derived Mesenchymal Stem Cells Improve Cognitive Function and Physical Activity in Ageing Mice

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Brain ageing leads to atrophy and degeneration of the cholinergic nervous system, resulting in profound neurobehavioral and cognitive dysfunction from decreased acetylcholine biosynthesis and reduced secretion of growth and neurotrophic factors. Human adipose tissue-derived mesenchymal stem cells (ADMSCs) were intravenously (1×10^6 cells) or intracerebroventricularly (4×10^5 cells) transplanted into the brains of 18-month-old mice once or four times at 2-week intervals. Transplantation of ADMSCs improved both locomotor activity and cognitive function in the aged animals, in parallel with recovery of acetylcholine levels in brain tissues. Transplanted cells differentiated into neurons and, in part, into astrocytes and produced choline acetyltransferase proteins. Transplantation of ADMSCs restored microtubule-associated protein 2 in brain tissue and enhanced Trk B expression and the concentrations of brain-derived neurotrophic factor and nerve growth factor. These results indicate that human ADMSCs differentiate into neural cells in the brain microenvironment and can restore physical and cognitive functions of aged mice not only by increasing acetylcholine synthesis but also by restoring neuronal integrity that may be mediated by growth/neurotrophic factors.

Key words: ageing; cognitive function; physical activity; human adipose-derived mesenchymal stem cell; brain-derived neurotrophic factor; nerve growth factor

Ageing is associated with progressive functional and structural deterioration of neural systems, affecting both cognitive and motor functions (Kluger et al., 1997; Volkow et al., 1998). Cholinergic nerve cells in the basal forebrain undergo neurodegenerative changes during normal ageing as well as in Alzheimer's disease (AD; Bartus et al., 1982; Grothe et al., 2012). Progressive loss of cholinergic neurons, marked by reduced choline acetyltransferase (ChAT) activity leading to decreased acetylcholine (ACh) release and p75 neurotrophin receptor expression, occurs during ageing and AD (Whitehouse et al., 1982; Bierer et al., 1995; Mufson et al., 2002; Gil-Bea et al., 2005; Roman and Kalaria, 2006; Contestabile et al., 2008). The degree of cholinergic neuron loss is closely associated with the severity of the cognitive deficits (Pizzo et al., 2002).

In addition to cognitive function, physical activity is also associated with ACh release (Dudar et al., 1979). As ACh receptors regulate the balance of muscle excitation and inhibition (Jospin et al., 2009; Barbagallo et al., 2010), and ACh impacts stamina and action potential of muscles (Lund et al., 2010), ACh receptor mutations lead to motor neuron degeneration. Indeed, ageing reduces ACh release and diminishes motor performance (Freeman and Gibson, 1988). These results suggest that activating the cholinergic system in aged animals may enhance cognitive function and physical activity.

Additional Supporting Information may be found in the online version of this article.

D. Park and G. Yang contributed equally to this work.

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Ageing leads to decreased release of growth and neurotrophic factors, including brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF; Diogenes et al., 2011; Niewiadomska et al., 2011). These alterations during ageing inhibit neurogenesis and thereby accelerate shrinkage of the hippocampus (Karege et al., 2002), which is associated with behavioral and functional deficits in hippocampus-dependent learning and memory tasks (Rosenzweig and Barnes, 2003). Young $BDNF^{-/-}$ mice have reduced cholinergic innervation (Ward and Hagg, 2000) and a reduced number of cholinergic cells in the medial septum, which is accompanied by a decrease in ChAT activity in the hippocampus (Grosse et al., 2005). In contrast, BDNF and NGF promote survival of developing cholinergic forebrain neurons and attenuate the loss of neurons following excitotoxic insults (Burke et al., 1994; Sofroniew et al., 2001). In addition, NGF upregulates several cholinergic markers (Pongrac and Rylett, 1998; Oosawa et al., 1999; Auld et al., 2001), and BDNF is required for maturation of cholinergic nerves and plays a critical role in long-term potentiation required for memory consolidation (Ward and Hagg, 2000; Diogenes et al., 2011).

Interestingly, neurotrophins, including BDNF and NGF, play roles in skeletal muscle adaptation (Sakuma and Yamaguchi, 2011). Notably, BDNF is also involved in the development of motor coordination (Strand et al., 2007). Several studies have demonstrated that age- and AD-related dysfunctions of the cholinergic system related to physical activity as well as cognitive function are ameliorated by NGF and BDNF treatment (Williams et al., 1986; Casamenti et al., 1994; Kordower et al., 1994; Murray et al., 1994).

Stem cell therapy has recently been noted as a novel strategy to treat neurological disorders such as AD, Parkinson's disease (PD), stroke, and spinal cord injury (SCI; Lindvall and Kokaia, 2006; Kim and de Vellis, 2009; Park et al., 2012a,b). In contrast to a transient improvement in body function by pharmaceuticals, stem cells may prevent or delay host cell death and restore injured tissues (Lindvall and Kokaia, 2006; Blurton-Jones et al., 2009; Kim and de Vellis, 2009). Mesenchymal stem cells (MSCs) have been isolated from several tissues, such as bone marrow, adipose tissue, umbilical cord blood, and the amniotic membrane (Pittenger et al., 1999; Díaz-Prado et al., 2011). Adipose tissue-derived MSCs (ADMSCs) have recently received attention as a promising source of cells for cell therapy. ADMSCs can be easily harvested from patients by a simple and minimally invasive method and are more abundant than other sources such as bone marrow-derived MSCs (BMMSCs; Fraser et al., 2006; Parker and Katz, 2006). Adipose tissue contains hundreds of thousands of MSCs in each gram of fat (Sen et al., 2001), whereas BMMSCs in the bone marrow fraction constitute a mere 0.0001–0.01% of all nucleated cells (Pittenger et al., 1999). In particular, ADMSCs differentiate into several cell types (Constantin et al., 2009), and, unlike embryonic stem cells, are an ethically uncontroversial source for stem cell therapy (Díaz-Prado et al., 2011).

Accordingly, ADMSCs have been tested in diverse conditions, including PD, cerebral palsy, angina, stroke, and SCI (Kim et al., 2010; Ra et al., 2011). ADMSCs secrete various growth/neutrophic factors such as NGF, BDNF, and vascular endothelial growth factor, so it is expected that these cells might exert neuroprotective activity and thereby improve body functions in aged animals (Kim et al., 2010; Lopatina et al., 2011). In the present study, we investigated the recovery effects of ADMSCs on cognitive and neurobehavioral dysfunctions in aged mice.

MATERIALS AND METHODS

Animals

Young (8-week-old) and old (18-month-old) male ICR mice (n = 10/group) were obtained from Daehan-Biolink (Eumseong, Korea). The animals were housed in a room with a constant temperature (22°C ± 2°C), relative humidity of 55% ± 10%, and a 12-hr light/dark cycle. The animals were fed standard rodent chow and purified water ad libitum. All experimental procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Laboratory Animal Research Center at Chungbuk National University, Korea.

Preparation and Transplantation of ADMSCs

Human ADMSCs were prepared under good-manufacturing-practice conditions (RNL BIO, Seoul, Korea). In brief, human abdominal subcutaneous fat tissue was obtained by simple liposuction with informed consent from a 53-year-old female donor (Choi et al., 2011; Ra et al., 2011). The adipose tissues were digested with collagenase I (1 mg/ml) under gentle agitation for 60 min at 37°C, filtered through a 100-mm nylon sieve to remove cellular debris, and centrifuged at 470*g* for 5 min to obtain the pellet. The pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY) containing 0.2 mM ascorbic acid and 10% fetal bovine serum (FBS) obtained from a bovine spongiform encephalopathy-free herd. The cell suspension was recentrifuged at 470*g* for 5 min, and the cell pellet was collected. The cell fraction was cultured overnight at 37°C/5% CO^2 in DMEM. Cell adhesion was checked 24 hr later under an inverted microscope, and nonadherent cells were removed by washing with phosphate-buffered saline (PBS). The cell medium was changed to Keratinocyte-SFM (Invitrogen) medium containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/ml recombinant EGF, and 5% FBS. The cells were maintained for 4–5 days until confluent (passage 0). When the cells had reached 90% confluence, they were subculture expanded in the Keratinocyte-SFM medium until passage 3. FBS from cultured MSCs was completely removed by several washes with PBS and was verified by testing the albumin level below the measurement limit using a bovine albumin enzyme-linked immunosorbent assay quantification kit (Bethyl Laboratories, Montgomery, TX).

ADMSCs were dissolved in appropriate volumes of saline: 1×10^6 cells/100 μl/mouse and 4×10^5 cells/2 μl/mouse for intravenous (IV) and intracerebroventricular (ICV)

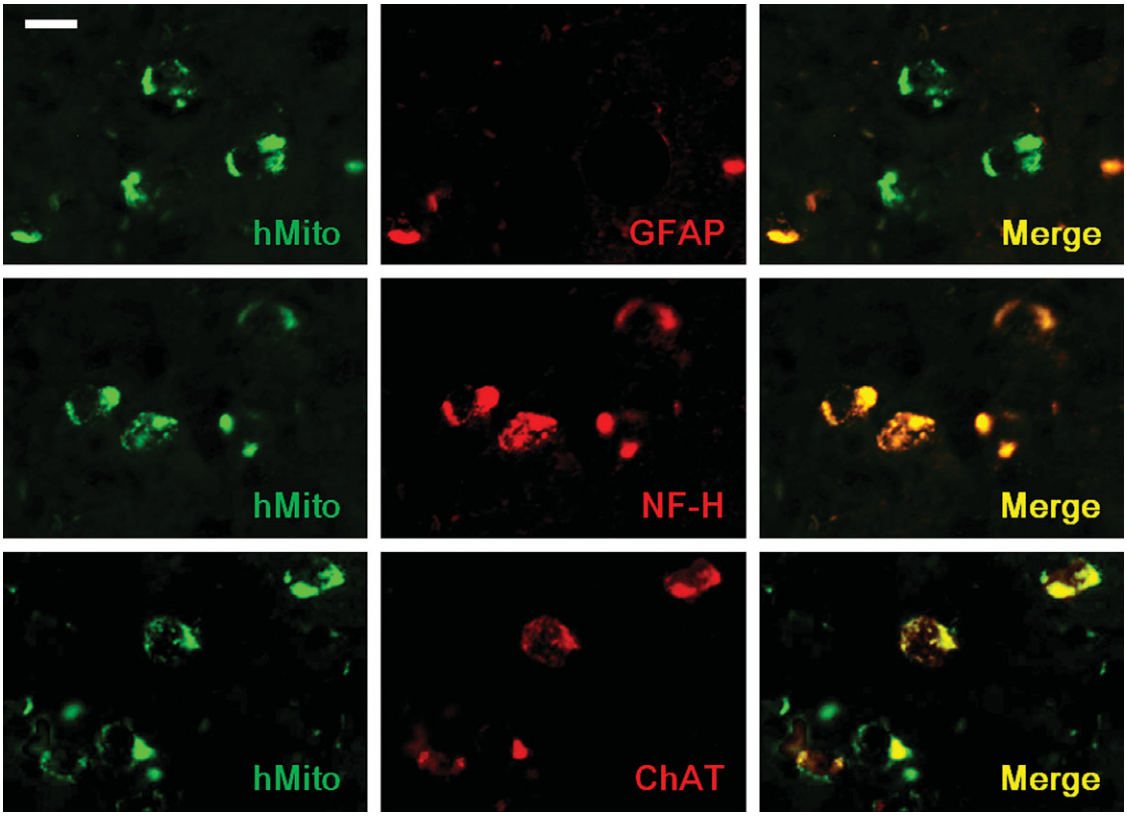

Fig. 1. Differentiation of human adipose tissue-derived mesenchymal stem cells (ADMSCs; hMito-stained) into astrocytes (GFAP-stained) or neurons (NF-H-stained) and expression of choline acetyltransferase (ChAT) 2 weeks after repeated intravenous transplantation (1×10^6 cells) into 18-month-old mice four times at 2-week intervals. Scale bar = 10 μm.

transplantations, respectively. Mice were anesthetized with enflurane and positioned in a stereotaxic frame. After incision of the skin and drilling of a hole, ADMSCs were transplanted ICV at the following coordinates: posterior, 1.0 mm; lateral, 2.0 mm; and ventral, 3.0 mm from bregma or transplanted IV via the tail veins once or four times at 2-week intervals.

Measurement of Locomotor Activity

Spontaneous activities and exploratory behaviors were evaluated using a video tracking system (Smart v2.5; Panlab, Barcelona, Spain) connected to a CCTV monitor (Samsung, Changwon, Korea) 4 weeks after single transplantation or 1 week after final transplantation in the repeated-dose groups (Gomez et al., 2012). Mice were placed in a quiet chamber with dim light, and the types of movements, i.e., resting, slow-moving, and fast-moving times were recorded for 5 min, and the ratio was analyzed.

Measurement of Learning and Memory Functions

Passive avoidance performance was assessed by nine consecutive trials at 5-min intervals to evaluate memory acquisition and retention. The latency time of remaining in a room with the light on was recorded following electric shock (1 mA for 2 sec) in a dark compartment. The endpoint was set to 300 sec, denoting full acquisition of memory (Park et al., 2012a,b).

Water maze trials were performed in a circular bath filled with water maintained at 22°C ± 2°C to evaluate spatial memory. The bath was divided into four quadrants, and a hidden escape platform (10 cm in diameter) was submerged in the center of one quadrant. The rats were trained to learn to find the hidden platform, based on several cues external to the maze. Three trials were conducted on each day with 5-min intervals for 7 consecutive days. The mean time spent to escape onto the platform was recorded (Park et al., 2012b).

Brain Tissue ACh Analysis

The rats were sacrificed at the end of learning/memory testing, and ACh concentration in the brain was evaluated with a modified hydroxylamine reactive assay (Hestrin, 1949). In brief, the brain was removed after intracardial perfusion with cold saline, homogenized in 20 volumes of 0.1 M sodium phosphate buffer, and centrifuged at 13,500 rpm for 6 min at 4°C to obtain the supernatant. An aliquot (50 μl) of the supernatant was mixed with 1 ml hydroxylamine chloride (2 M) and 1 ml sodium hydroxide (3.5 N) to stop additional enzymatic reactions. After an 1-min incubation, 1 ml hydrochloric acid (3.9 N) and 0.5 ml ferric chloride (0.37 M) were

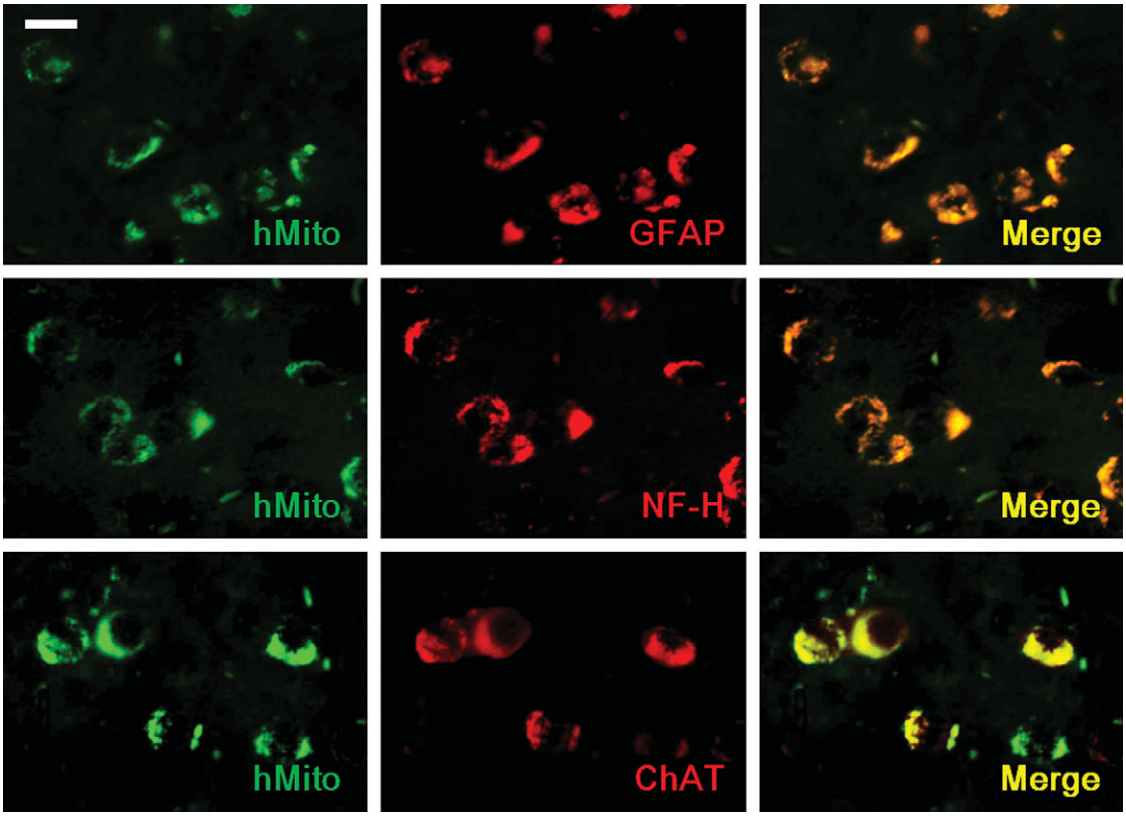

Fig. 2. Differentiation of human adipose tissue-derived mesenchymal stem cells (ADMSCs; hMito-stained) into astrocytes (GFAP-stained) or neurons (NF-H-stained) and expression of choline acetyltransferase (ChAT) 2 weeks after repeated intracerebroventricular transplantation (4×10^5 cells) into 18-month-old mice four times at 2-week intervals. Scale bar = 10 μm.

added gradually to the mixture. The upper layer was measured at 540 nm following centrifugation at 1,500 rpm for 15 min.

Brain Section Immunohistochemistry

The rat brains were perfusion fixed with 10% paraformaldehyde solution and postfixed in the same solution for 48 hr, followed by cryoprotection in 30% sucrose for 72 hr to confirm the distribution and survival of transplanted ADMSCs. Coronal cryosections of 30-μm thickness were prepared and processed for double immunostaining for human mitochondria (hMito), ChAT, NF-H (for neurons), or glial fibrillary acidic protein (GFAP; for astrocytes) using antibodies specific to hMito (1:200; mouse monoclonal; Chemicon, Temecula, CA), ChAT (1:200; rabbit polyclonal; Chemicon), NF-H (1:200; rabbit polyclonal; Chemicon), or GFAP (1:200; rabbit polyclonal; Chemicon). Brain sections were incubated with primary antibodies overnight at 4°C and with secondary antibodies conjugated with Alexa Fluor-488 or -594 (1:500; rabbit polyclonal; Molecular Probes, Eugene, OR) for 2 hr at room temperature. All samples were examined immediately after staining and photographed with a laser-scanning confocal microscope (LSM710; Zeiss, Oberkochen, Germany).

Western Blot Analysis

Whole brains of mice were homogenized in RIPA buffer (Sigma, St. Louis, MO) with protease inhibitors. Proteins were obtained by centrifugation at 15,000 rpm and 4°C for 15 min and quantified by using the BCA Protein Assay kit (Pierce, Rockford, IL). Proteins were denatured by boiling for 5 min at 95°C in 0.5 M Tris-HCl buffer (pH 6.8) containing 10% sodium dodecyl sulfate (SDS) and 10% ammonium persulfate, separated by electrophoresis on 7.5% or 15% SDS-polyacrylamide gels (SDS-PAGE), depending on protein size, and transferred to a polyvinylidene difluoride membrane in 25 mM Tris buffer containing 15% methanol, 1% SDS, and 192 mM glycine. After blocking for 2 hr with 5% skim milk in Tris-buffered saline-Tween (TBS-T; 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20), the membrane was incubated with antibodies specific for microtubule-associated protein 2 (MAP2; 1:500; rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) for 3 hr at room temperature and NGF (1:500; rabbit polyclonal; Santa Cruz Biotechnology), BDNF (1:500; rabbit polyclonal; Santa Cruz Biotechnology), or Trk B (1:500; rabbit polyclonal; Santa Cruz Biotechnology) overnight at 4°C. After washing with TBS-T, the membrane was incubated with a secondary goat

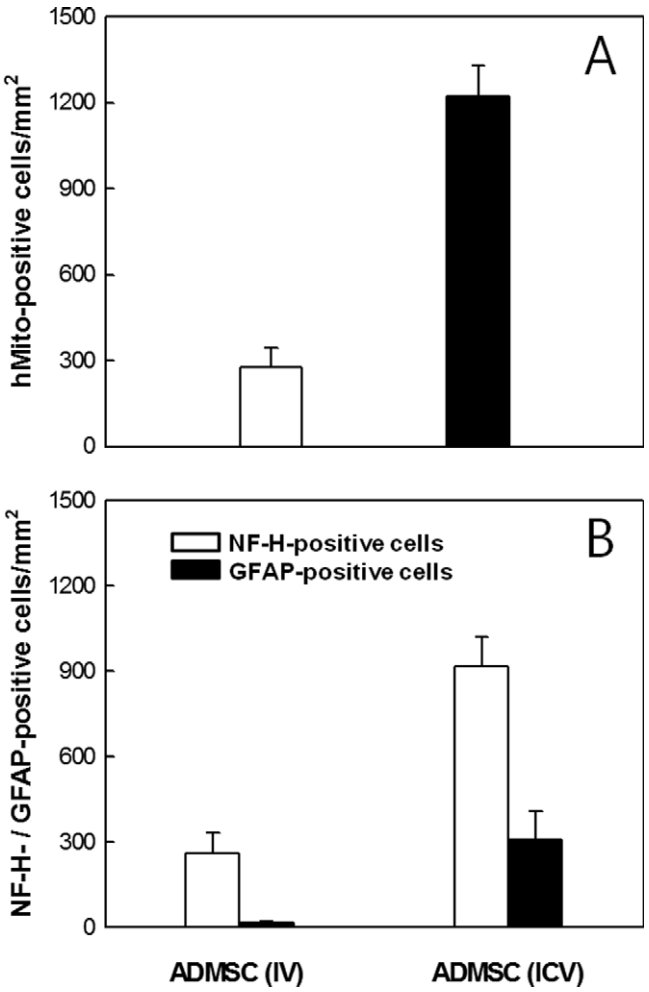

Fig. 3. Cell counts in the hippocampal region 2 weeks after repeated intravenous [IV; 1×10^6 adipose tissue-derived mesenchymal stem cells (ADMSCs)] or intracerebroventricular (ICV; 4×10^5 ADMSCs) transplantation into 18-month-old mice four times at 2-week intervals (**A**) and differentiation into neurons (NF-H positive) or astrocytes (GFAP positive; **B**).

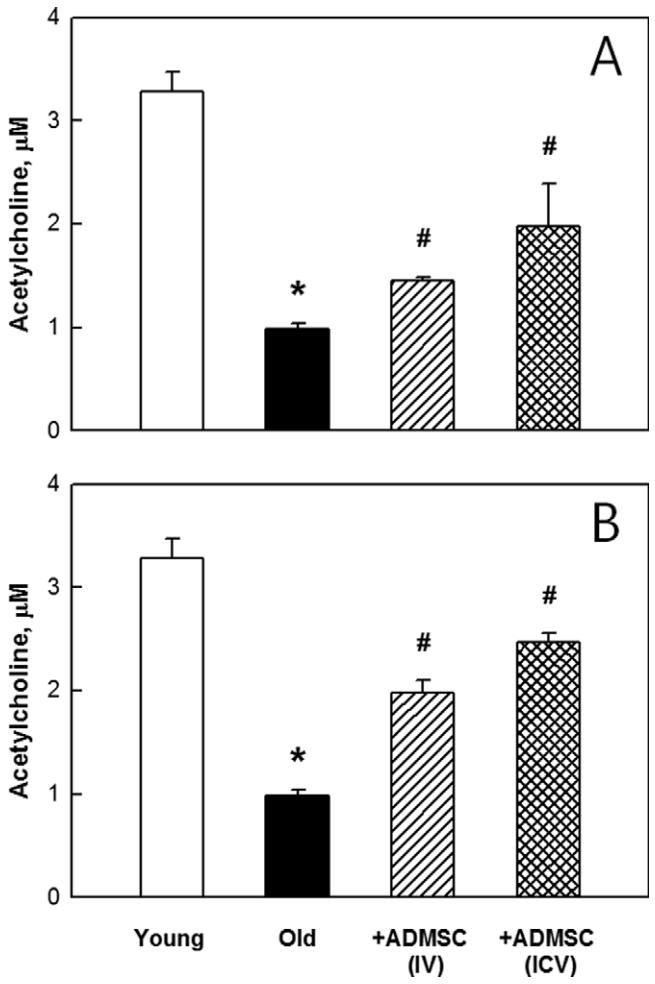

Fig. 4. Acetylcholine (ACh) concentrations in brain tissue 5 weeks after single intravenous (IV; 1×10^6 cells) or intracerebroventricular (ICV; 4×10^5 cells) transplantation of adipose tissue-derived mesenchymal stem cells (ADMSCs) into 18-month-old mice (**A**) and 2 weeks after repeated IV (1×10^6 cells) or ICV (4×10^5 cells) transplantation four times at 2-week intervals (**B**). *Significantly different from young mice ($P < 0$ .05). #Significantly different from old mice ($P < 0.05$).

anti-rabbit IgG conjugated with horseradish peroxidase (1:2,000; Santa Cruz Biotechnology) for 2 hr at room temperature. The membrane was then developed using an enhanced chemiluminescence solution (Pierce).

Statistical Analysis

Data are presented as mean ± standard error. Statistical significance between groups for the behavioral data was determined by one-way analysis of variance followed by post hoc Tukey's multiple-comparison tests. $P < 0.05$ was considered statistically significant.

RESULTS

hMito immunoreactivity was detected in the brain tissue 5 weeks after single IV transplantation of ADMSCs (1×10^6 cells/mouse) into 18-month-old mice (Supp. Info. Fig. 1). These cells differentiated into (NF-H-positive) neurons but not (GFAP-positive) astrocytes. Interestingly, ADMSCs that differentiated into neurons did not express ChAT, suggesting that the cells did not differentiate into cholinergic nerves or motor neurons. Additionally, single ICV transplanted ADMSCs (4×10^5 cells/mouse) differentiated into neurons, but not astrocytes, 5 weeks posttransplantation (Supp. Info. Fig. 2).

Transplanted ADMSCs were detected in brain tissue 2 weeks after the final IV dose of ADMSCs (1×10^6 cells/mouse) following repeated (four times at 2-week intervals) transplantations and were confirmed to differentiate into neurons, and, in part, into astrocytes (Fig. 1). Notably, some of the transplanted cells

expressed ChAT, a marker of cholinergic and motor neurons. In addition, ICV transplanted cells (4×10^5 cells/mouse) differentiated into neurons and astrocytes 2 weeks after the final transplantation (Fig. 2) and expressed ChAT, suggesting that these cells had started to differentiate into cholinergic nerves or motor neurons.

The numbers of ADMSCs in the hippocampal area following repeated (four times) IV (1×10^6 cells/mouse) or ICV (4×10^5 cells/mouse) transplantation were 274.3 cells/mm^2 and 1,223.8 cells/mm^2, respectively (Fig. 3A). Most of the cells detected in the brain after IV transplantation had differentiated into neurons (94% for neurons; 6% for astrocytes; Fig. 3B). However, a higher portion of the cells differentiated into astrocytes after ICV transplantation (74.7% for neurons; 15.3% for astrocytes).

ACh concentration (0.97 μmole/g tissue) in the brain of aged (18-month-old) mice was much lower (30%) than that (3.28 μmole/g tissue) in young (8-week-old) animals (Fig. 4A). However, a single IV transplantation of ADMSCs significantly restored the ACh level to 1.40 μmole/g tissue, and ICV transplantation further increased it to 2.13 μmole/g tissue, indicating a higher efficacy than for IV transplantation. In comparison with a single dose, repeated transplantation led to higher ACh levels, showing 2.00 and 2.33 μmole/g tissue following IV and ICV transplantations, respectively (Fig. 4B).

The moving times of old mice decreased significantly to 38%, whereas the resting time (62%) increased significantly in comparison with young animals exhibiting 75% moving (slow- and fast-moving) activity (Fig. 5A). Notably, a single IV transplantation with ADMSCs improved the activity of old mice almost completely to the level of young animals. A similar activity-enhancing effect was achieved by a single ICV transplantation. Repeated IV and ICV transplantation of ADMSCs also substantially recovered the activity of aged mice (Fig. 5B), regardless of transplantation route.

Old animals displayed severely impaired learning and memory functions as assessed by both passive avoidance (Fig. 6A,C) and the Morris water maze (Fig. 6B,D) performances. The aged mice showed a delayed increase in retention time and long latency time during repeated trials in the passive avoidance and Morris water maze performances, respectively, in contrast to full memory acquisition at the seventh trial in young animals. Notably, single IV or ICV transplantation of ADMSCs improved cognitive function in mice, and ICV treatment was superior to IV injection (Fig. 6A,B). Greater degrees of passive avoidance and water maze performance restoration were attained after repeated IV and ICV transplantations of ADMSCs, and the ICV injection was superior to the IV route (Fig. 6C,D).

The content of MAP2, a neuronal skeletal protein, decreased markedly in 18-month-old animals compared with young animals (Fig. 7). In addition, BDNF and NGF, growth/neurotrophic factors associated with cholinergic function, and the BDNF receptor Trk B decreased greatly. The single IV and ICV transplantations of ADMSCs remarkably restored MAP2 expression to levels similar to those in young animals. BDNF was also restored by single IV or ICV ADMSC transplantation in parallel with the recovery of Trk B, although NGF did not increase. Interestingly, repeated treatment with ADMSCs fully restored MAP2, BDNF, and Trk B, in addition to recovering NGF.

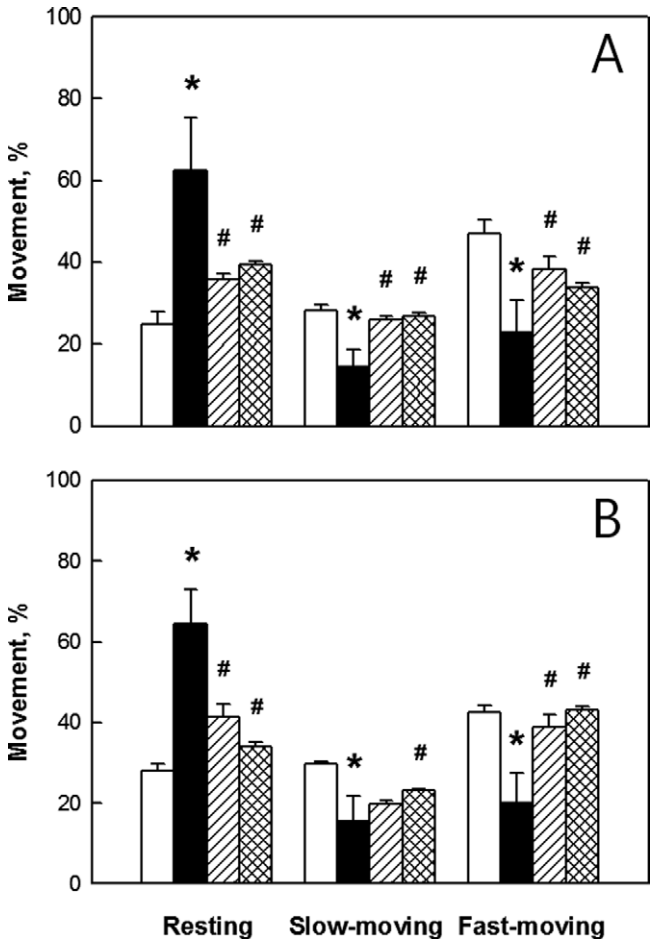

Fig. 5. Locomotor activity (resting, slow-moving, and fast-moving times) of mice measured 4 weeks after single intravenous (IV) or intracerebroventricular (ICV) transplantation of adipose tissue-derived mesenchymal stem cells (ADMSCs) into 18-month-old mice (**A**) and 1 week after repeated IV or ICV transplantation of ADMSCs four times at 2-week intervals (**B**). Open bars, young mice (8 weeks old); solid bars, old mice (18 months old); hatched bars, old mice transplanted IV with ADMSCs (1×10^6 cells); cross-hatched bars, old mice transplanted ICV with ADMSCs (4×10^5 cells). *Significantly different from young mice ($P < 0.05$). #Significantly different from old mice ($P < 0.05$).

DISCUSSION

Ageing and AD induce cognitive deficits via cholinergic dysfunction (Terry and Buccafusco, 2003; Miller and O'Callaghan, 2005). Additionally, synaptic loss fol-

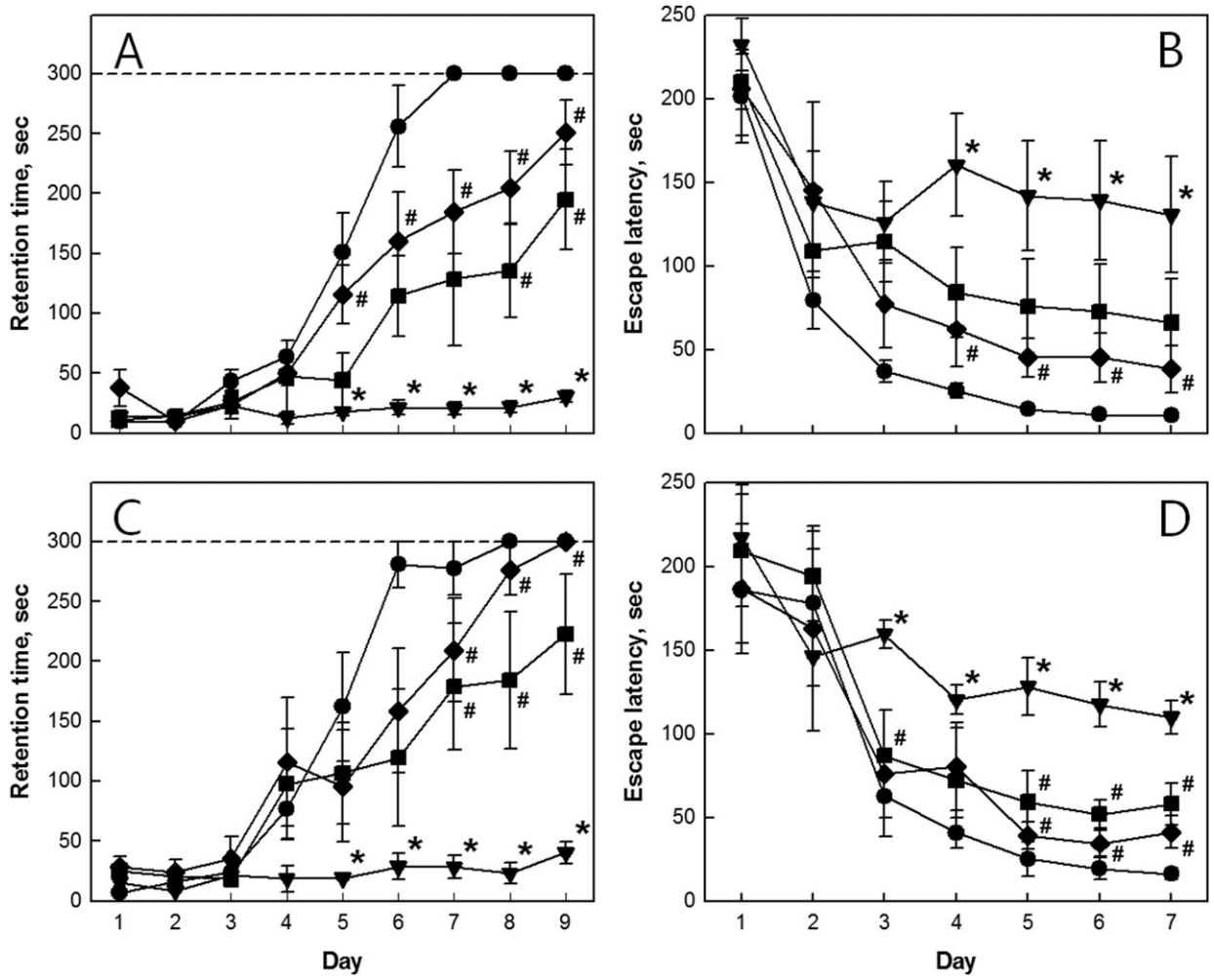

Fig. 6. Passive avoidance (**A,C**) and Morris water maze (**B,D**) performances of mice measured 4 weeks after single intravenous (IV) or intracerebroventricular (ICV) transplantation of adipose tissue-derived mesenchymal stem cells (ADMSCs; A,B) into 18-month-old mice and 1 week after repeated IV or ICV transplantation of ADMSCs four times at 2-week intervals (C,D). Circles, young mice (8 weeks old); triangles, old mice (18 months old); squares, old mice transplanted IV with ADMSCs (1×10^6 cells); lozenges, old mice transplanted ICV with ADMSCs (4×10^5 cells). *Significantly different from young mice ($P < 0.05$). #Significantly different from old mice ($P < 0.05$).

lowed by neuronal death, particularly in discrete brain regions related to memory and cognition, is observed in the ageing brain. The hippocampus and subcortical areas show decreased ChAT activity and ACh levels in patients with AD (Auld et al., 2001). In particular, the hippocampus is the brain region most vulnerable to the ageing process (Eichenbaum, 2001; Miller and O'Callaghan, 2005). The hippocampal cholinergic system has long been implicated in several functions such as arousal, attention, and behavior as well as in certain aspects of learning and memory (Bartus et al., 1982; Inglis and Fibiger, 1995; Karczmar, 1995). Therefore, ageing animals show memory deficits and behavioral impairments (Altun et al., 2007; Barquer et al., 2009) in parallel with decreased brain ACh levels (Freeman and Gibson, 1988; Terry and Buccafusco, 2003).

In the present study, ADMSCs were detected in hippocampal regions of 18-month-old mice after IV or ICV transplantation. The lesion tropism of ADMSCs might be triggered by growth factors such as stromal cell-derived factor-1 and chemokines including tumor necrosis factor-α (Baek et al., 2011). In our previous studies, F3 neural stem cells (NSCs) also showed lesion tropism in both kainic acid-induced hippocampal injury and AF64A-induced cholinergic nerve injury models (Park et al., 2012a,b). Notably, leakage and increased permeability of the blood–brain barrier with ageing have been confirmed in healthy animals and humans, which might be mediated by oxidative damage following microglial activation, iron accumulation in astrocytes, and declining estrogen levels (Popescu et al., 2009; Simpson et al., 2010). Accordingly, IV-injected ADMSCs migrated into the aged brain, as confirmed in the present study.

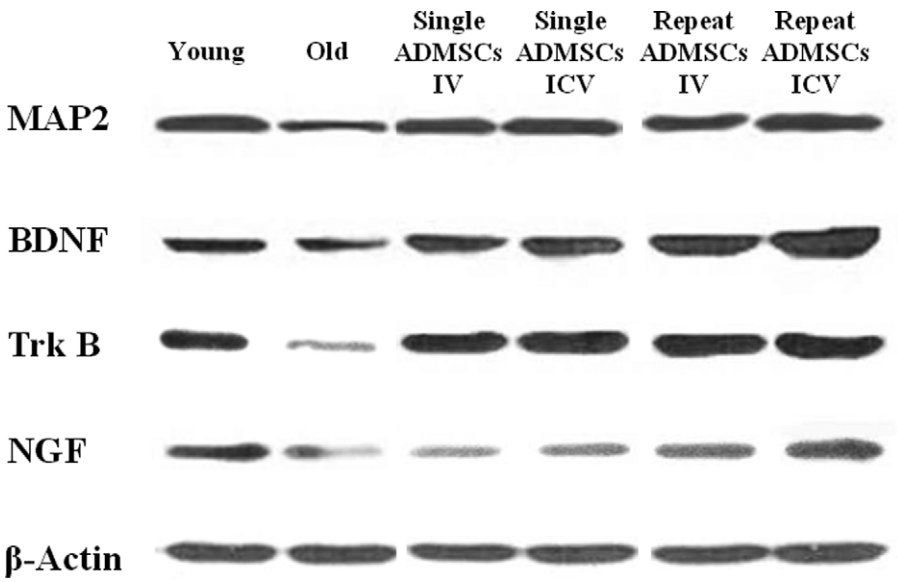

Fig. 7. Expressions of microtubule-associated protein 2 (MAP2), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and Trk B 5 weeks after single intravenous (IV) or intracerebroventricular (ICV) transplantation of adipose tissue-derived mesenchymal stem cells (ADMSCs) into 18-month-old mice and 2 weeks after repeated IV or ICV transplantation of ADMSCs four times at 2-week intervals. Young, 8 weeks old; old, 18 months old; single ADMSC IV, old mice transplanted IV with ADMSCs (1×10^6 cells); single ADMSC ICV, old mice transplanted ICV with ADMSCs (4×10^5 cells); repeat ADMSC IV, old mice transplanted IV with ADMSCs four times; repeat ADMSC ICV, old mice transplanted ICV with ADMSCs four times.

However, the number of cells that migrated to the hippocampal region following ICV (4×10^5 cells/mouse) transplantation was much greater than that after IV (1×10^6 cells/mouse) injection and was also greater after repeated treatment than after single administration. Notably, the cells differentiated into neurons in the brain and into astrocytes in the case of repeated transplantation. More interestingly, some ADMSCs displayed ChAT expression 8 weeks after initial transplantation, indicating further differentiation into cholinergic neurons. Growth/neurotrophic factors such as NGF, ciliary neurotrophic factor, and neurotrophin-3 promote cholinergic differentiation in human embryonic stem cells (Nilbratt et al., 2010), suggesting that peripheral stem cells can differentiate into neural cells in the central nervous system microenvironment. Despite the lack of ChAT expression 5 weeks after single transplantation of ADMSCs, the brain ACh concentration recovered significantly, which might have been due to activation or restoration of the host cholinergic neurons as confirmed by increased MAP2 levels. Therefore, the higher ACh concentration following repeated ICV transplantation may originate from both stem cells expressing ChAT and preserved host cholinergic neurons. It is thought that ADMSCs can differentiate into neurons, particularly cholinergic and motor neurons, and into glial cells, including astrocytes, according to the exposure time to the brain microenvironment.

Ageing diminishes motor performance (Freeman and Gibson, 1988), and body movement declines gradually with increasing age in studies using *Caenorhabditis elegans* (Herndon et al., 2002). In the present study, the resting time in old animals was much longer than that of young animals, indicating decreased physical activity. However, transplantation of stem cells into aged animals markedly restored locomotor activity. It is believed that transplantation of ADMSCs affected both central and peripheral cholinergic and motor neurons, because an increase in ACh release is related to behavioral activation (Mizuno et al., 1991), and muscarinic agonists improve locomotor behavior in aged animals (Glenn et al., 2004). Moreover, restoration of activity was contributed not only by BDNF involved in dopaminergic function and motor coordination (Strand et al., 2007; Boger et al., 2011) but also by NGF upregulating cholinergic activity (Pongrac and Rylett, 1998; Oosawa et al., 1999; Auld et al., 2001), because neurotrophins, including BDNF and NGF, play roles in skeletal muscle adaptation (Sakuma and Yamaguchi, 2011). The degree of recovery of physical activity was similar among the groups, regardless of injection route and dosage of ADMSCs, suggesting a major role for peripheral effects on neuromuscular transmission.

The MAP2 content in aged animals was markedly reduced, and decreased immunoreactivity was also observed in the hippocampal region (Himeda et al., 2005). Brain MAP2 levels recovered fully following transplantation of ADMSCs, which might have been due to the activated and recovered host neurons, in addition to expression in transplanted ADMSCs. It is well known that NGF and BDNF are responsible for the development, differentiation, maintenance, and

repair of neurons (Schabitz et al., 2000; Hsu et al., 2007). Transplantation of murine BMMSCs into the ischemic brain enhances neurogenesis and oligodendrogenesis (van Velthoven et al., 2010a,b), suggesting that the cytoskeleton is preserved and/or regenerated, which might be mediated by growth/neutrophic factors. Repeated transplantation of BMMSCs after hypoxia-ischemia-induced injury increases the expression of diverse growth/neurotrophic factors and cytokines responsible for cellular growth and proliferation (van Velthoven et al., 2010b, 2011). In the present study, it was confirmed that the recovered MAP2 level originated from neurogenesis and/or restoration of cellular integrity, which had been lost in the aged brain before ADMSCs transplantation.

Transplantation of stem cells improves cognitive function in AD model animals by enhancing hippocampal synaptic density mediated by BDNF (Xuan et al., 2008; Blurton-Jones et al., 2009). In addition, exogenous NGF rescues cholinergic neurons in the basal forebrain and improves cognitive function in aged animals (Markowska et al., 1994), and NSCs overexpressing NGF restore memory deficits in AD model animals (Lee et al., 2012). Indeed, ADMSCs secrete various growth/neurotrophic factors, including BDNF and NGF (Kim et al., 2010; Lopatina et al., 2011). BDNF and NGF stimulate cholinergic phenotypes by increasing ChAT activity in cultures enriched with embryonic rat motor neurons (Wong et al., 1993). Because BDNF exert its action via Trk B receptors (Conover et al., 1995), the full recovery of Trk B expression directly affected BDNF activity to restore cognitive and physical functions. Actually, cognitive function of aged mice recovered in an ACh concentration-dependent manner and not in parallel with BDNF and NGF. Therefore, it is believed that the number of ADMSCs, particularly ChAT-expressing cholinergic neurons in the brain, may play a critical role improving cognitive function.

In conclusion, we have demonstrated that ADMSCs migrated to the ageing brain, differentiated into neural cells, and improved cognitive function and physical activity not only by restoring ACh levels but also by protecting and activating host neurons by secreting growth and neurotrophic factors such as BDNF and NGF.

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