

Title: Transplantation of Primed or Unprimed Mouse Embryonic Stem Cell-derived Neural Precursor cells Improves Cognitive Function in Alzheimerian Rats

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Running title: ESC-derived NPCs function in Alzheimerian rat

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized by progressive and irreversible decline of memory. Neuropathological features include the progressive degeneration of cholinergic neurons in the forebrain cholinergic projection system especially nucleus basalis of Meynert (nbM). New cell therapeutic approaches for the replacement of degenerated cells are being researched. The aim of this study was to investigate the production of cholinergic neurons from mouse embryonic stem cells (ESCs) and potential for utilizing ESC-derived neuronal precursor cells (NPCs) and primed NPCs (PNPCs) for cell restorative therapy in a rodent model of AD. NPCs were produced by growth factor-mediated selection under serum free conditions and differentiated better into cholinergic neurons when NPCs primed with Shh (~22%) in comparison with different cholinergic promoting factors. Behavioral assessment of unilateral nbM ibotenic acid-lesioned rats by Morris water maze and spatial probe test revealed a significant behavioral improvement in memory deficits following transplantation with NPCs and/or PNPcs. Immunohistochemical analysis revealed that the majority (~70%) of the NPCs and/or PNPcs retained neuronal phenotype and ~40% of them had a cholinergic cell phenotype following transplantation with no tumor formation, indicating that these may be safe for transplantation. This experimental study has important implications as it suggests that the transplantation of mouse ESC-derived NPCs and/or following commitment to a cholinergic cell phenotype can promote behavioral recovery in a rodent model of AD.

Key words: Alzheimer's disease; Behavior; Cholinergic neurons; Differentiation; Neuronal precursor cells; Transplantation.

Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders affecting elderly people (Oliveira and Hodges, 2005; Power et al., 2003). In AD there is an established correlation between the progressive and irreversible decline of memory and the loss of cholinergic neurons in the forebrain cholinergic projection system (FCPS) (Isomae et al., 2003). The diffuse projection of the FCPS, more specifically the nucleus basalis of Meynert (nbM) and medial septal area (MSA), reaches the hippocampal formation, the frontal and parietal cortices, the olfactory bulb, and the basolateral nucleus of the amygdala (Bartus, 1985). As the loss of cholinergic neurons in the nbM of AD patients is one of the prominent neuromorphological changes in these patients, one experimental model of AD is based on lesioning of the nbM in animals (Cole and Frauandtschy, 1997). Because drug therapies could not improve symptoms in this model, neuronal grafts were considered as an alternative treatment (Oliveira and Hodges, 2005). In the first attempts, neuronal tissue from embryonic hippocampus was used for transplantation into the basal forebrain of injured animals. This treatment improved memory function, but because of ethical considerations surrounding the use of fetal tissue and its general lack of availability, alternatives have been sought (Fine et al., 1985). Identification of an expandable population of cells that can differentiate into neurons, embryonic stem cells (ESCs), has given rise to hopes for clinical application of these in vitro produced neuronal cells in the cell replacement procedures for neurodegenerative disorders such as AD (Harrower et al., 2006; Gao et al., 2006), Parkinson disease (Kim et al., 2002; Roy et al., 2006; Yang et al., 2008), and traumatic brain injury (Gao et al., 2006).

Recent reports describe the derivation of neuronal cells such as GABAergic, glutamatergic, dopaminergic, serotonergic and motor neurons from ESCs using a number of different strategies (for review see (Cai and Grabel, 2007)). However, there are few reports on the production of

cholinergic neurons from ESCs. In one study it was reported that treatment of mouse ESCs with noggin to induce the production of neuronal progenitor cells (NPCs) followed by treatment of these NPCs with sonic hedgehog (Shh) increased the percentage of cholinergic neurons (Shindo et al., 2006). Knowledge of the factors controlling development of NPCs and the factors controlling differentiation of these precursors into mature cholinergic neurons is essential before clinical production of cholinergic neurons can be realistic.

Here, we describe the use of essential factors to differentiate cholinergic neurons after selection of nestin-positive cells from mouse ESCs. These factors include: Shh, retinoic acid (RA), leukemia inhibitory factor (LIF), nerve growth factor (NGF), and interleukin-6 (IL6). These factors were selected because of their known effects on the cholinergic system including: (i) culture of forebrain cholinergic neurons (Yuhara et al., 2003), (ii) fetal and adult NPC cultures (Wu et al., 2002; Reilly et al., 2002), (iii) sympathetic neuronal culture and study of transdifferentiation of adrenergic sympathetic neurons to cholinergic neurons (Brodski et al., 2000; Lewis et al., 1994; Fukada, 1985), and (iv) differentiation of ESCs toward motor neurons and cholinergic neurons (Wichterle et al., 2002; Okada et al., 2004). We also assessed the effect of ESC-derived NPCs, with or without pretreatment by cholinergic promoting factors, on spatial memory and histopathology of the cholinergic system after transplantation into the brain of nbM-lesioned rats. Our data demonstrate that mouse ESC-derived NPCs can differentiate more efficiently into cholinergic neurons during in vitro culture after nestin-positive selection accompanied by some exogenous factors and that grafting of in vitro produced unprimed or primed ESC-derived NPCs can improve memory function in the rat model of AD, probably by generating new synapses as well as by secretion of acetylcholine.

Materials and methods

Mouse ESC culture

Mouse ESCs of line Royan B1 (Baharvand and Matthaiei, 2004) that were derived from the C57BL/6 strain were grown on a feeder layer of primary mouse embryonic fibroblasts (MEF) in tissue culture flasks. Cells were maintained in ESC medium that consisted of DMEM (Gibco, 10829-018) containing 15% fetal bovine serum (Gibco, 16141-079), 0.1 mM β -mercaptoethanol (Sigma, M7522), 2mM glutamine (Gibco, 15039-027), 0.1mM non-essential amino acids (Sigma, M7145) and 1000 IU/ml leukemia inhibitory factor (LIF, Chemicon, ESG 1107).

Induction of differentiation

For differentiation into neuronal phenotypes, 10^5 ESCs/ml were cultivated for 4 days in 5 ml ESC medium without LIF in non-adhesive bacterial dish (6 cm) supplemented with 15% FBS, to form aggregates or embryoid bodies (EBs). About 20-30 EBs with diameters of 100-200 μ m were plated in each cm^2 of standard tissue culture dishes at day 4 (4d) and cultivated in DMEM+15% FBS for one day (4+1d). The subsequent selection of neuronal precursor cells was carried out as previously described (Okabe et al., 1996), with some minor modifications. After attachment of EBs, the medium was replaced with DMEM/F12 medium (Gibco, 12456018) supplemented with 5 μ g/ml insulin (Sigma, 16634), 30 nM sodium selenite (Sigma, S5261), 50 μ g/ml transferrin (Sigma, T5391), 2mM glutamine, 0.1mM non-essential amino acids, and 5 μ g/ml fibronectin (Invitrogen, 12173-091). The culture medium was completely replaced with fresh medium every 2 days. NPCs were selected after cultivation for 7 days (day 4+8d). At day 4+8d, EBs were dissociated with 0.05% trypsin/0.53 mM EDTA (Gibco, 15305-014) in Hank's balanced salt solution (HBSS, Gibco, 14185-052), collected by centrifugation, and replated onto poly-L-ornithine (Sigma, P3685)/laminin (Sigma, L2020)-coated tissue culture dishes in DMEM/F12 containing 20 nM progesterone (Sigma, P7556), 100 μ M putrescine (Sigma, P5780), 1 μ g/ml

laminin, N2 supplement (Gibco, 17502-048), 2mM glutamine, 0.1mM non-essential amino acids, basic fibroblast growth factor (bFGF, 10 ng/ml; Sigma, F0291), and epidermal growth factor (EGF, 20 ng/ml; Sigma, E4127) for 4 days until day 4+12d. The medium was replaced every 2 days. At day 4+12d, some of the cells were primed (primed neuronal precursor cells; PNPCs) with cholinergic promoting factors [LIF (Chemicon, ESG 1107), IL6 (Chemicon, IL006), (Sigma, S0191), RA (Sigma: R2625), and NGF (Chemicon, NC010)] for another 6 days (4+12d to 4+18d) in DMEM/F12 medium with 5% FCS, 2mM glutamine, 0.1mM non-essential amino acids and 1% N2 supplement. The optimum concentration of each cholinergic promoting factor was determined based on prior studies (reference concentration). Three doses were used for each one (reference concentration, and a half and twice of reference concentration, Table-1). The medium was replaced every 2 days. At day 4+18d, the differentiation of mature neurons was induced by ‘Neurobasal’ medium (Gibco, 211030-049) plus 2% B27 (Gibco, 17504-044), 2 mM glutamine, 0.1 mM non-essential amino acids, and 10% FCS. The cells were then cultured for one week (day 4+25). One-fourth of the medium was replaced every 3 days.

Prior to transplantation, cultured cells (NPCs, or PNPCs) were labeled with bromodeoxyuridine (BrdU, 5 μ M, Sigma, 858811) for 4 days in the proliferation phase (day 4+8 up to 4+12). ESCs also were incubated with 5 μ M BrdU for 2-3 days but in the presence of LIF in their routine culture medium, as described above. After labeling, the cells were harvested with trypsin, extensively washed with phosphate-buffered saline (PBS), suspended at a density of 10⁵ cells/ μ l in PBS containing 15% rat serum, and maintained on ice until transplantation.

Animals and Experimental design

All animal manipulations were approved by the Institutional Animal Care and Use Committee of the Royan Institute. The animals were behaviorally assessed using the Morris water maze (MWM) test for 2 days in 4 repeated trials per day, and those animals that did not meet criteria

were not used for further study. A total of thirty-six male Sprague–Dawley rats, weighing about 300g, with normal MWM behavior were used in the study and randomly divided into six equal groups: control with no treatment, sham-lesioned which received only vehicle injection, nbM lesion plus vehicle injection, and nbM lesion plus NPC, PNPC, or ESC transplantation.

nbM lesioning

The experimental model of AD animals was anesthetized using an intraperitoneal (ip) injection of fresh chloral hydrate (175 mg/kg, in saline) (Cole and Frauandtschy, 1997). After being fixed carefully in the stereotaxic apparatus (Model SR - 6N, Narishige, Japan), the rat's scalp was cut, a small craniotomy was drilled, and 10 µg ibotenic acid (Ibo) in 1 µl of 0.1 mM PBS was infused through a glass capillary (inner diameter ~50 µm) into the right nbM (coordinates from bregma: AP=-0.9, L=2.8, V=6.8) using a 25 µl Hamilton syringe. The injection speed was maintained by a microinjection unit (David Kopf Instruments) at 0.2 µl/min. The infusion was made over 5 minutes, and the capillary tube was left in place for 3 minutes before being withdrawn over an additional 3 minutes. Animals were given 65,000 units of sodium penicillin (i.m.) and 2 ml lactated Ringer's solution (i.p.) and kept on a thermo pad until awake.

Transplantation of cells

Four weeks after sham surgery or nbM-lesioning, cells were transplanted at the same coordinates listed above. 2×10^5 cells in 2 µl were injected into the right nbM of anesthetized rats using a 5 µl Hamilton syringe with a 26-gauge needle. The injection speed was maintained at 0.2 µl/min. The needle was held in place for 5 min after injection before gradually withdrawing it. Animals were given 65,000 units of sodium penicillin (i.m.) and 2 ml lactated Ringer's solution (i.p.) and kept on a thermo pad until awake. To minimize possible immune rejection, all animals (vehicle and cell groups) were treated with the immunosuppressor NEORAL cyclosporine (Novartis

Pharmaceuticals Corp., East Hanover, New Jersey) at 10 mg/kg in saline, S.C., from 1 to 2 days before grafting and throughout the course of the experiment.

Morris water maze test

The water maze was a circular pool, 150 cm in diameter, 60 cm high, painted black. The pool was filled with 40 cm of water that was maintained at a temperature of 22-27 °C. The pool was divided into four quadrants, with four starting locations, north (N), east (E), south (S), and west (W), at equal distances from the rim. During testing, a black escape platform, 12 cm in diameter and located 2 cm below the water, was placed at the center of the north-east (NE) quadrant in the pool, approximately 37.5 cm from the near sidewall (this platform blends into the black background of pool when submerged). This escape platform was always in the same quadrant. The pool was surrounded by many cues external to the maze; these were visible from the pool and could be used by the rats for spatial orientation. The position of the cues remained unchanged throughout training. Four weeks post-surgery rats were trained for 4 days, with 4 trials per day, to find and climb onto the hidden escape platform. This program of tests was repeated two times for each experimental surgery group. A spatial probe trial (see below) also was performed at the end of each phase. At the start of each trial, the rat was placed in the water randomly at one of four starting locations (N, S, E, and W), facing the wall of the pool, and allowed to swim to the platform. A trial was terminated as soon as the rat had climbed onto the escape platform or when 90 s had elapsed. Rats not finding the platform within 90s were guided to it and allowed to remain on the platform for 20 s. At the end of each trial the rat was removed from the tank and placed in a holding box for 1 min.

Spatial Probe trial

One day after the fourth trial of the fourth day, an additional trial was given as a probe trial; the platform was removed, and the time the rat spent in the four quadrants was measured for 60 s. In

the probe trial, all rats started from the same start position, opposite to the quadrant where the escape platform had been positioned during training.

Swim path lengths, escape latency, and swim speed of each trial were recorded using a video camera mounted above the center of the pool. The video signal was sent to a video tracking device that digitizes the location of the rat and represents this location in *x*- and *y*-coordinates. This signal is sent to a computer where it is analyzed using RADIAB-1 software (produced by Nomirei, Iran).

Immunofluorescence staining

Tissue processing for staining of brain sections was performed after the final MWM test. All rats were deeply euthanized around 5 weeks post-transplantation with 300 mg/kg Choral hydrate (i.p.). They were then intracardially perfused with 65 ml 0.1 M ice-cold PBS and then 500–800 ml of ice-cold 4% paraformaldehyde in PBS, pH 7.4. The brains were postfixed overnight and then infiltrated with 30% sucrose until they sank (usually 2 days). Serial coronal sections at a thickness of 30 μ m were cut with a cryostat (Leica CM1900, Meyer Instruments Inc., Houston, Texas). All of the brains were processed for staining, some brain sections from all animals transplanted with NPCs, PNPCs or ESCs were stained by H&E to assess for possible tumor formation and three brains randomly selected from each group for immunohistochemical studies (ten section from each one). Cultured cells on slides or coverslips were fixed with ice-cold 4% paraformaldehyde for 20 min and rinsed with PBS for three times. Sections (10 per rat separated by 30 μ m) or cells were incubated for 30 min in 5% normal serum produced from the same species as secondary antibody (goat or rabbit) plus 0.3% bovine serum albumin+0.25% Triton X-100 in Tris-buffered saline (TBS). This blockade of nonspecific binding and permeabilization was followed by an overnight incubation with primary antibodies at optimal concentrations at 4°C. After five 20-min PBS rinses for sections, or five 5-min PBS rinses for cells, samples were

incubated with species-specific secondary antibodies for 30 or 120 min at room temperature, in the dark. Secondary antibodies included: FITC conjugated goat anti-mouse (Chemicon, AP124F, 1:100), FITC conjugated rabbit anti-goat (Chemicon, AP106F, 1:200), AMCA conjugated goat anti-mouse (Chemicon, AP124M, 1:100), or Cy5 conjugated goat anti-rat (Chemicon, AP136S, 1:200). After three 20-min rinses with PBS, sections were mounted onto slides and coverslipped. After three 10-min rinses with TBS, cells were counterstained with 1 μ g/ml propidium iodide (PI, Sigma, P4864) for 3 min at room temperature. Omission of primary antibodies was used as a control for all markers. Primary antibodies included: neuronal cell adhesion molecule (NCAM) rat monoclonal antibody (Chemicon MAB310, 1:200), BrdU mouse monoclonal antibody (Sigma B2531, 1:750), choline acetyltransferase (ChAT) goat polyclonal antibody (Chemicon AB144P, 1:200), glial fibrillary acidic protein (GFAP) mouse monoclonal antibody (Chemicon MAB3402, 1:250), microtubule-associated protein 2 (MAP2) mouse monoclonal antibody (Sigma M2320, 1:250), nestin mouse monoclonal antibody (Chemicon MAB353, 1:200), and β -tubulin III mouse monoclonal antibody (Sigma T8660, 1:200). Labeled cells and sections were examined with a fluorescence microscope (Olympus, BX51, Japan) and images were acquired with an Olympus D70 camera.

Effect of Ibo on cholinergic neuron depletion in nbM-injured animals was assessed by DAB staining of ChAT antibody in some sections of normal, nbM-lesioned and sham-lesioned groups. In this protocol, the secondary antibody was peroxidase-conjugated rabbit anti-goat and the sections were incubated with 0.05% DAB - 0.015% H₂O₂ in 0.01 M PBS, pH 7.2 solution at the end of incubation with the secondary antibody.

Reverse transcription-polymerase chain reaction analysis

Total RNA was isolated as described using the RNeasy Mini Kit (Qiagen, 74104). Prior to reverse transcription (RT), a sample of the isolated RNA was treated with 1 U/ μ l of RNase-free

DNaseI (EN0521, Fermentas) per 1 µg of RNA (in order to eliminate residual DNA) in the presence of 40 U/µl of ribonuclease inhibitor (E00311, Fermentas) and 1x reaction buffer with MgCl₂ for 30 min at 37 °C. Standard reverse-transcription reactions were performed with 2 µg total RNA using Oligo (dT)18 as a primer and Superscript II RNase H⁻ reverse transcriptase kit (InvitrogenTM, 11917-010), according to the manufacturer's instructions. For every reaction set, one RNA sample was prepared without Reverse Transcriptase (RT⁻ reaction) to provide a negative control in the subsequent PCR. The sequences of primers were: ChAT (Choline Acetyltransferase), forward: 5'-GTGAACTCCCTGCTCCCAGA-3' and reverse: 5'-CTCAGTGCCAGAAGATGGTTGT-3'; VACHT (Vesicular Acetylcholine Transporter), forward: 5'-CCCACCTCCTCTAATGAGTACC-3' and reverse: 5'-GCAGGAAGGACAAACAGATGC-3'; HB9 (motoneuron marker), forward: 5'-GGCGCTTTCCTACTCATAACC-3' and reverse: 5'-TCCTCTTCCGTCTTCTTCTCAC-3'; OCT-4, forward: 5'-GGCGTTCTCTTTGGAAAGGTGTTC-3' and reverse: 5'-CATACTCGAACCACATCCTTCTCTA-3'; and β-tubulin, forward: 5'-TCACTGTGCCTGAACTTACC-3' and reverse: 5'-GGAACATAGCCGTAAACTGC-3'.

The PCR reactions were performed on a Mastercycler gradient machine (Eppendorf, Germany) for 30 cycles. Products were electrophoresed on 1.7 % agarose gel. The gels were stained with ethidium bromide (0.5 µg/ml) and photographed on a UV transilluminator (Uvidoc, UK). Gel images were analyzed using the UVI bandmap program (Uvitec, Cambridge, UK).

Cell counting and histological evaluation

Two slides from each group of treatments from three different in vitro experiments were stained (total of 6 slides from each group). For cell counting 40 random images prepared from different sites of each slide and fluorescent stained cells were counted in each image and examiner was blinded to the groups. ChAT-positive cells and β-tubulin III-positive cells that were stained with

specific fluorescent dye as obvious in images were counted to determine the number of cholinergic cells and neurons per total number of cells (stained with PI).

As described above all of the brains were processed and some sections from each brain randomly selected and stained with H&E for analysis of tumor generation. For counting of immunohistochemically stained cells, 10 sections from each one of three randomly selected brains of each group of rats: transplanted with NPCs, PNPCs or ESCs were selected. Ten images with 100X magnification were prepared from each selected brain. Evaluation of β -tubulin III- and ChAT-positive cells per BrdU- and NCAM-positive cells that were stained with just one or both of fluorescent dyes as they were obvious in images was performed.

Statistical analysis

The data were expressed as mean \pm SEM (standard error of mean). Measures (time latency, speed, and distance) of MWM were averaged per rat within each session. Lesioning and treatment effects on the acquisition of the water escape task were assessed with repeated measures analysis of variance (ANOVA) to find differences between groups. One-way ANOVA followed by the Tukey post *hoc* test multiple group comparison was used to analyze group differences of the data collected from each day of MWM, probe trials, and image analysis. A difference between groups was considered as statistically reliable if the $P < 0.05$.

Results

Neuronal Differentiation

In vitro differentiation of cholinergic neurons from ESCs involves 5 steps: (i) formation of cells of all three primary germ layers in EBs, (0-4 days) (ii) selective differentiation of neuroectodermal cells by growth factor removal (serum depletion) (4+1d to 4+8d), (iii) proliferation and maintenance of neuronal precursor cells in the presence of bFGF and EGF (4+8d to 4+12d), (iv) cholinergic differentiation induction by withdrawal of bFGF/EGF (Fig. 1A) and the addition of cholinergic promoting factors (4+12d to 4+18d), and (v) the maturation and maintenance of functional neurons and glial cells by withdrawal of the growth factors and inducers and the addition of neuronal medium and B27 (4+18d to 4+25d, Fig. 1C).

RT-PCR analysis showed the expression of cholinergic specific genes (ChAT and VACHT) and motor neuron marker (Hb9) in different groups in step 5 (Fig. 1G). Oct-4, a marker of pluripotent stem cells, was only expressed in ESCs and early NPCs (Fig. 1G). Immunofluorescence analysis of the cells at step 3 showed that $91.53 \pm 2.08\%$ of the cells expressed nestin, a NPC marker (Fig. 1B) and that, at the final stage (step 5), the majority of differentiating cells expressed neuron specific β -tubulin III (Fig. 1D, $72.47 \pm 2.70\%$), or MAP2 (Fig. 1E, $67.11 \pm 1.23\%$), while some expressed GFAP (Fig. 1F, $15.79 \pm 2.38\%$). The percentage of cholinergic neurons (Fig. 1H) was in a concentration-dependent manner showing significant increase in the percentage of cholinergic neurons with 100 and 200 ng/ml Shh, 1 and 2 μ M RA, and 500 iu/ml LIF (at least $P < 0.05$, Table-1). The percents were significantly increased (Fig. 1I) in comparison with control ($11.67 \pm 1.09\%$), when the cells treated with optimum concentration of Shh (100 ng/ml, $22.45 \pm 1.52\%$), RA (1 μ M, $17.05 \pm 1.25\%$), or LIF (500 iu/ml, $17.78 \pm 0.91\%$), but not IL6 ($14.08 \pm 1.36\%$). No synergistic effect was observed with combination treatments of Shh+RA ($18.53 \pm 1.75\%$), and/or RA+LIF ($18.26 \pm 1.70\%$). In contrast, IL6+LIF and/or NGF decreased

cholinergic differentiation significantly ($8.25\pm 0.98\%$ and $7.64\pm 0.50\%$, $P<0.05$) and the combination of NGF and Shh or LIF and or IL6 did not increase the number of cholinergic neurons over NGF alone.

Based on these data, Shh (100ng/ml)-treated NPCs were selected as the PNPCs for transplantation experiments.

Grafted NPCs and PNPCs in cognitive function of nbM-lesioned rats

Cognitive function, particularly spatial learning, and memory, which are known to be affected by nbM damage, were assessed with the reference memory task of the MWM test. Rats from all six groups were tested starting at 4 weeks post-surgery. The test spanned 4 consecutive days and included 4 trials per day for each animal, to enhance sensitivity. The time needed for rats to find the hidden platform (latency), the path length (distance) and the speed (velocity) they swam were recorded, averaged over 4 trials per rat, and then over 6 animals for each day for each group (Fig. 2).

There was no significant difference in speed of swimming (velocity) between groups. The path lengths (distance) and time (latencies) were similar so only the time (latency) data are presented. Differences in MWM performance across all days within groups and between groups were analyzed by repeated measures ANOVA. One way ANOVA followed by the Tukey post *hoc* test was used to find differences between groups at each day and for the spatial probe test. All animals were able to locate the hidden platform during acquisition, and performance improved across trials ($P<0.05$) except in ESC and vehicle transplanted groups. We established that nbM-lesioned animals have impaired memory function in MWM test in different sessions and also in spatial probe test before any cell transplantation. As shown in Fig 2A, lesioned rats required more time to find the platform compared to sham-lesioned and normal control groups in second, third and fourth training days: day2 ($P=0.013$; $P=0.02$), day3 ($P=0.029$; $P=0.003$) and day4 ($P=0.025$;

$P=0.007$). nbM-lesioned rats also spent less time in the goal quadrant in spatial probe test (Fig. 2B) compared with control ($P=0.006$) and sham-lesioned rats ($P<0.003$).

NPC and PNPC transplanted rats performed similarly in the MWM test (Fig. 2C) and the spatial probe test (Fig. 2D); there were no significant differences between them. During the first and second days in MWM test, the time latencies to find the platform was similar in neuronal-(NPC and PNPC) and vehicle-transplanted groups, but during the third and fourth days the latency decreased significantly more in the neuronal-transplanted rats (Fig. 2C, $P<0.05$). In comparisons of the vehicle and neuronal (NPC and PNPC)-transplanted rats with control and sham-lesioned rats, we found that the mean latencies to find the platform were significantly different among them ($P<0.05$, Fig. 2C). ESC-transplanted rats required significantly more time than neuronal transplanted rats (at least $P<0.05$) or than sham and normal control rats ($P<0.004$) at all days.

The results of spatial probe test were significantly different between treatment groups: ESC vs. sham-lesioned ($P<0.0001$), ESC vs. normal control ($P<0.0001$), ESC vs. NPC ($P=0.002$) and ESC vs. PNPC ($P=0.001$) (Fig. 2D). When vehicle-transplanted rats were compared with both normal and sham-lesioned rats, we found significant differences between them as well ($P<0.001$, Fig. 2D). Our results from MWM task indicate that the memory function of neuronal transplanted rats did not reach the level of normal rats, but when the results of spatial probe test were compared, there was a significant difference between neuronal cell (NPCs and PNPCs)-transplanted and vehicle transplanted rats (Fig. 2D, NPC vs. vehicle, $P=0.014$; PNPC vs. vehicle, $P=0.003$).

Histological analysis of lesions and grafts

Analysis of coronal sections of Ibo-injected brains showed a marked cholinergic depletion in nbM-lesioned rats (Figs. 3A–C). To characterize survival and differentiation of grafted cells after completion of the MWM test (5 weeks post-transplantation), the brains of all rats were removed

after perfusion fixation with paraformaldehyde. Transplanted mouse cells were identified in rat brain tissue using a mouse specific NCAM monoclonal antibody. Using immunofluorescent staining, we detected grafted cells primarily near the injection site (Fig. 3D–F). Grafted mouse cells (NPCs, PNPCs, and ESCs) were also found in some sections of host brain tissue by staining with anti-BrdU antibody. Double staining for BrdU and NCAM was used to determine those transplanted cells that had differentiated into neuronal cells (Figs. 3G–I). To determine whether grafted cells differentiate into neurons, cholinergic neurons, and/or glial cells in the Ibo-injured nbM, cryostat sections were double stained with monoclonal antibodies against β -tubulin III and NCAM (Figs. 3J–L), ChAT and BrdU (Figs. 3M–O), and GFAP and BrdU, respectively. We found that the majority of grafted NPCs ($69.44 \pm 2.67\%$) and PNPCs ($73.15 \pm 2.71\%$) were β -tubulin III positive in the nbM, 5 weeks post grafting, and that $22.09 \pm 2.53\%$ of NPCs and $19.86 \pm 1.46\%$ of PNPCs were GFAP-positive (astrocytic phenotype). The counts of cholinergic cells indicated that most of the differentiated neurons, from NPCs ($43.67 \pm 1.35\%$) and PNPCs ($41.73 \pm 2.61\%$), were cholinergic. These data indicated that: (i) there was no significant difference between the NPC and PNPC transplanted groups with respect to cholinergic differentiation and (ii) there was a marked increase in cholinergic differentiation in vivo compared with in vitro suggesting that local inducing factors in the nbM may have a potent effect on induction of cholinergic differentiation. No neuronal differentiation was observed in the ESC transplanted group (Figs. 3I, L, and O). Further, H&E staining revealed that ESCs (Fig. 3R), but not NPCs or PNPCs (Figs. 3P and Q), formed large tumors with hypertrophic and dividing nuclei in all transplanted rats.

Discussion

One of the major findings of this study indicated that ESC-derived NPCs show a different profile of neuronal differentiation *in vitro* compared to that seen *in vivo* after transplantation into the brain of nbM-lesioned rats. We also demonstrated that when mouse ESC-derived NPCs were grafted into the injured basal forebrain, they induced improvements in spatial learning and memory of nbM-lesioned rats.

In this *in vitro* study, we investigated the effects of some cholinergic promoting factors on cholinergic differentiation of ESC-derived neuronal cells. Our findings indicated that Shh, RA, and LIF increased cholinergic differentiation in a concentration-dependent manner but NGF and IL6 did not. Among these factors, Shh had the more potent effect and combination treatments using Shh+RA or RA+LIF showed no synergistic effect. In support of the presumed role of Shh in cholinergic survival and differentiation, it was reported that expression of Shh gene exist in different regions of adult CNS, such as the basal forebrain and spinal cord, that are replete with cholinergic neurons (Furusho et al., 2006). In studies of the development of motor neurons, it has been reported that several signaling molecules, including Shh and RA, are important (Wichterle et al., 2002; Anderson, 2001). RA induces differentiation of neuronal progenitors along the rostrocaudal axis (Okada et al., 2004), while Shh induces differentiation along ventrodorsal axis (Anderson, 2001). While differentiation of ESCs into spinal cord motor neurons may be successfully achieved by treatments with RA and Shh (Wichterle et al., 2002), the mechanism of differentiation of cholinergic neurons and interneurons is far from clear. Similar to other studies, we found that RA could direct differentiation of ESCs toward a generalized cholinergic fate (Stavridis and Smith, 2003; Okada et al., 2004; Bain et al., 2000; Fraichard et al., 1995). Our study further shows that treatment with RA+Shh does not have a synergistic effect in increasing the number of all types of cholinergic neurons compared with treatment with RA alone. Thus,

RA alone could increase the differentiation to a cholinergic fate as did Shh, but it is possible that when they act together, they just increase the proportion of cholinergic motor neurons without affecting the total numbers of generalized cholinergic neurons; further studies are necessary to clarify this issue. LIF has not previously been shown to effect cholinergic differentiation of ESC-derived neuronal cells but, in other studies of sympathetic ganglion neurons and LIF gene knockout mice, the effect of this factor on cholinergic differentiation has been reported (Fukada, 1985; Patterson, 1994). It was also demonstrated that LIF mRNA is expressed in cholinergic and GABAergic basal forebrain neurons (Lemke et al., 1999). We found that effect of LIF on cholinergic differentiation is specific for LIF, as treatments with IL6, another cytokine related to LIF family, had no effect on cholinergic differentiation, despite the fact that they both belong to same family of cytokines. This difference could be related to their different receptors: LIF exerts its effect through heterodimerization of LIFR and gp130, whereas IL6 binds to IL6R and gp130 (Bartoe and Nathanson, 2000). The specific activation of LIFR may play an important role in cholinergic differentiation, but further studies are needed to identify the mechanism of LIF in cholinergic differentiation of ESC-derived neuronal cells. Some studies have also suggested a role for NGF in survival and, perhaps also, the development of forebrain cholinergic neurons (Nonner et al., 1996) or cholinergic differentiation of sympathetic neurons (Brodski et al., 2000; Madziar et al., 2005) or neuroblastoma cells (Szutowicz et al., 2004). In this study, we did not find any increase in number of cholinergic neurons after treatment with NGF but further studies will need to ascertain whether NGF is only a cholinergic survival factor or whether it also contributes to differentiation of cholinergic neurons.

Moreover, we compared and contrasted the effects of transplantation of primed (Shh treated) and/or unprimed mouse ESC-derived NPCs in nbM-lesioned rats using a spatial reference memory test. Behavioral tests after transplantation indicate that grafted NPCs and/or PNPCs

integrated into the lesion site and improved memory function in spatial probe test of the MWM, when compared with vehicle-transplanted and ESC-transplanted rats. A significant effect was seen between vehicle-transplanted and NPC- or PNPC-transplanted rats in the MWM during last training two days and in the spatial probe test, indicating that cell transplantation did have some beneficial effect in reference memory function. The beneficial effect of the grafted NPCs or PNPCs could be attributed to one or both of two functions of the cells in vivo: (i) simple secretion of acetylcholine from transplanted cells or (ii) actual functional integration into the host tissue (Wernig et al., 2004). Previous studies of genetically engineered acetylcholine-producing fibroblasts transplanted into the hippocampus of septohippocampal-lesioned rats proposed that simple secretion of acetylcholine from these cells could improve memory function (Dickinson-Anson et al., 1998), in the absence of integration. We found more cholinergic differentiation (~40%) after transplantation of NPCs and/or PNPCs in the brain of nbM-lesioned animals in comparison with in vitro Shh-treated NPCs (~22%). This is similar to the findings of Wu et al, which indicated that there is a regional specificity for neuronal differentiation and that it was possible that additional signals from astrocytes or the three dimensional milieu of the host brain play a role (Wu et al., 2002). In another study (Yang et al., 2002) of grafting of ESC-derived NPCs into the brain of striatum-lesioned rats, it was shown that these cells differentiated into dopamine releasing neurons. These results show the utility of recreating the cellular environment of the developing brain while driving specific neurogenesis from ESC-derived NPCs. Although other studies have suggested that priming before transplantation may improve the differentiation of the graft in spinal cord and hippocampus of injured animals (Gao et al., 2005; Gao et al., 2006), we found that unprimed NPCs differentiate to regional specific neurons in nbM. It is possible, therefore, that the local milieu plays a very important role (for review see (Lathia et al., 2007; Jones and Wagers, 2008). Further studies are required to determine whether newly

differentiated neurons are functionally integrated into the injured nbM and to characterize the mechanisms underlying stem cell differentiation in the damaged CNS. Moreover, we tested the reference memory function of neuronal transplanted animals. Consolidation of this kind of memory requires generation of new synapses between grafted cells and host neurons, in addition to secretion of acetylcholine. Thus, we propose that transplanted cells contributed to improvement of memory function by both making new synapses and also acetylcholine secretion. Transplanted NPCs which were isolated from fetal human brain integrated into the hippocampus of traumatic brain-injured animals and improved their memory function in the MWM test (Gao et al., 2006). There are some reports of transplantation using embryonic ventral forebrain in rats with damage to the FCPS. Transplantation of these cells to neocortex improved the performance in passive avoidance and elevated plus maze (Shoham and Emson, 1997), the radial arm maze (Allen et al., 1990; Hodges et al., 1991), multi-choice reaction time task (Muir et al., 1992), and in the MWM (Gage and Gage, 1986). To date, there are few studies of transplantation of ESC-derived neurons in nbM-lesioned animals and effect of these cells on cognitive function. One recent study reported that mouse ESC-derived NPCs that were primed with Shh, heparin and bFGF, improved memory function in eight-arm radial maze test in nbM-lesioned mice (Shindo et al., 2006).

We also examined the effect of undifferentiated ESC transplantation in nbM-lesioned rats using a spatial reference memory test. ESC-transplanted animals in our study showed a marked memory dysfunction, presumably due to the generation of tumors from ESCs, which is consistent with (Shindo et al., 2006) whereas there were not found any tumor cells in NPC or PNPC transplanted animals. Roy et al., have transplanted human ESCs-derived dopaminergic neurons into the parkinsonian rats; the dopaminergic implants yielded a significant, substantial, and long-lasting restitution of motor function (Roy et al., 2006). However, although rich in donor-derived tyrosine

hydroxylase-expressing neurons, the grafts exhibited expanding cores of undifferentiated mitotic neuroepithelial cells, which can be tumorigenic. Moreover, Yang et al., have demonstrated that human ESC-derived dopaminergic neurons and their progenitors survive for 5 months in the brain of an experimental rat model of Parkinson's disease and contribute to locomotor functional recovery. However, in their extensive analyses show no obvious tumor formation after 5 months of survival (Yang et al., 2008). Therefore, this leaves open the questions of whether ESC-produced neuronal cells can functionally engraft in a safe manner and how to accomplish this.

Conclusions

We have demonstrated that mouse ESC-derived NPCs and PNPCs survive and differentiate into neurons, especially cholinergic neurons for five weeks after grafting in the brain of an experimental rat model of AD and contribute to cognitive functional recovery. Although we transplanted NPCs and/or PNPCs, the neurons in the graft exhibited a cholinergic phenotype, indicating that regional cues in the nbM direct cell differentiation toward the cholinergic lineage. The improvement in reference memory function in grafted animals suggests that transplanted cells exert their effect by generating new synapses as well as acetylcholine secretion. The ESC-derived NPCs and PNPCs display greatly diminished proliferative activity and do not result in obvious tumor formation in the long term graft, suggesting their potential therapeutic application in Alzheimer's treatment.

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Figure legends:

Figure 1. Characterization of mouse ESC-derived neuronal progenitors and neurons. Phase contrast microscopy (A) and immunofluorescence staining of progenitors at step 4 with anti-nestin (green, B). Phase contrast microscopy (C) and immunofluorescence staining of mature neurons at step 5 with anti- β -tubulin III (green, D), microtubule associate protein-2 (MAP-2, green, E), and glial fibrillary acidic protein (GFAP, green, F) in Shh-PNPCs. RT-PCR analysis of undifferentiated ESCs, and differentiated neurons at step 4 (NPCs) and step 5 (all other lanes, PNPCs) (G). The PNPCs expressed cholinergic markers (ChAT and VChAT) and motoneuron marker (HB9). (H) Immunofluorescence staining of mature neurons at step 5 with (D) anti-choline acetyltransferase (ChAT). (I) Mean percent of cholinergic neurons (ChAT-positive cells) per total number of cells (determined by propidium iodide staining) in different treated and non-treated cell cultures. Shh, LIF, RA, Shh+RA, or LIF+RA treatment significantly increased the proportion of cholinergic neurons in comparison with non-treated cell cultures. In contrast, NGF or IL6+LIF treatment significantly decreased the proportion of cholinergic neurons in comparison with non-treated cell cultures. The red color in B, D, E, F, and H represents nuclei counterstained with propidium iodide. ** $P < 0.01$ and * $P < 0.05$. Scale bars: 100 μ m.

Figure 2. Results of the MWM reference memory test (A, C) and spatial probe test (B, D) in normal animals and animals after nbM lesioning, before and after cell transplantation (NPCs and/or Shh-primed NPCs). (A) There are significant differences in mean latencies of lesioned animals compared with both control animals (\ddagger : $P < 0.02$) and sham-lesioned animals (\dagger : $P < 0.029$). (B) The mean time that nbM-lesioned animals spent in the goal quadrant (the quadrant which the escape platform was in during the previous sessions) is significantly lower than that of the normal (*: $P = 0.006$) and sham-lesioned animals (#: $P < 0.003$). (C) There are significant

differences in mean latencies of the normal and sham-lesioned animals, (* $P < 0.05$, * $P < 0.004$, † $P < 0.028$) compared with both groups of neuronal transplanted animals (NPC and PNPC).

During the first and second days, the time latencies to find the platform was similar in neuronal- (NPC and PNPC) and vehicle-transplanted groups, but during the third and fourth days the latency decreased significantly more in the neuronal-transplanted rats ($P < 0.05$). As shown in histological studies, most NPCs and PNPCs differentiated into neurons and cholinergic neurons after transplantation whereas generation of tumor cells in brain of ESC-transplanted rats was related to a marked disruption in learning and memory function. Note, the rats in fig. 2A were in first MWM trainings and they spent much more time to find the hidden platform compared with fig. 2C, that was taken after transplantation when rats had been well trained on MWM tank, but consider that in the ESC-transplanted group the scores partially remained the same. (D) The mean time that the animals spent in the goal quadrant was significantly longer in the NPC- and PNPC-transplanted animals compared with the vehicle-transplanted group ($\times P < 0.014$, ## $P < 0.0001$, and * $P < 0.0001$ compared with both normal and sham-lesioned rats, # $P < 0.002$ compared with both NPC and PNPC transplanted groups).

Figure 3. Staining of brain sections by immunohistochemistry (A-C) and light microscopy (P-R), or immunofluorescence microscopy (D-R). DAB staining of ChAT-positive cells in normal (A), sham-lesioned (B), and nbM-lesioned animals (C) shows marked cholinergic depletion in nbM-lesioned brain section. Neuronal and glial differentiation from mouse ESC-derived NPCs, PNPCs, and ESCs, one month post-transplantation in nbM of lesioned adult rats (D-R). Mouse neuronal cells in nbM of injured rats stained with mouse specific NCAM (D-F). Double staining with BrdU (red) and NCAM (blue) was used to determine the extent of differentiation to neuronal cells from the grafted cells (G-I). Double stained BrdU and NCAM cells were not detectable in

the ESC-transplanted group (I). Double staining for β -tubulin III and NCAM indicated that some grafted cells had differentiated into neurons in the NPC- and PNPC-transplanted animals (J and K) but not in the ESC-transplanted animals (L). ChAT- and BrdU-positive cells are seen in the NPC- and PNPC-transplanted rats (M and N) but no ChAT-positive cells is seen in the ESC-transplanted group (O). Double staining for GFAP and BrdU indicated that some grafted cells had differentiated into glial cells (white arrows) in the NPC- and PNPC-transplanted animals (P and Q) but in the ESC-transplanted animals there were just Brdu stained nuclei of transplanted cells (R). H&E staining indicates that ESCs grew into large tumors containing hyperdense cells with dividing nuclei (U), while there was only a normal distribution and morphology in the NPC and PNPC grafts (S and T). Scale bars: 100 μ m. White arrows in P and Q indicate cells double stained with BrdU and GFAP, in S, T, and U indicate the injection site.

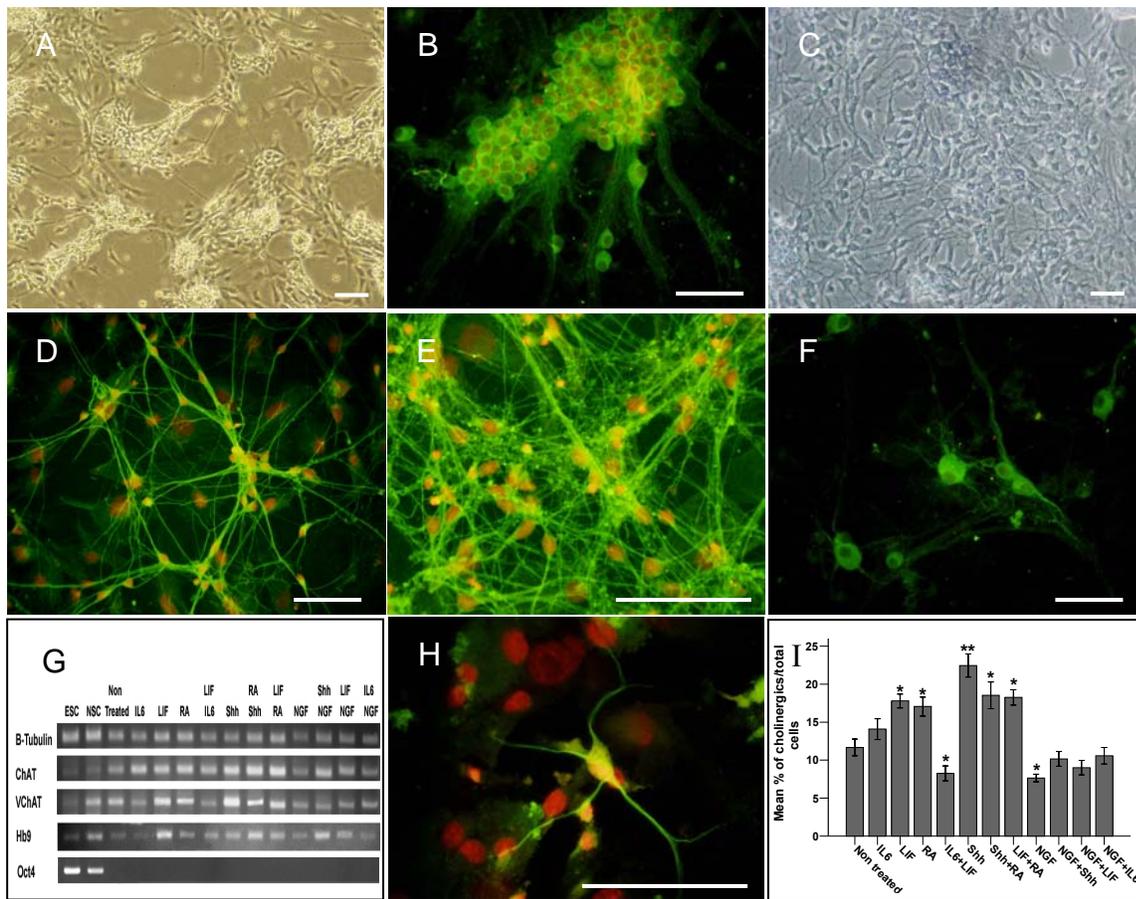


Figure 1.

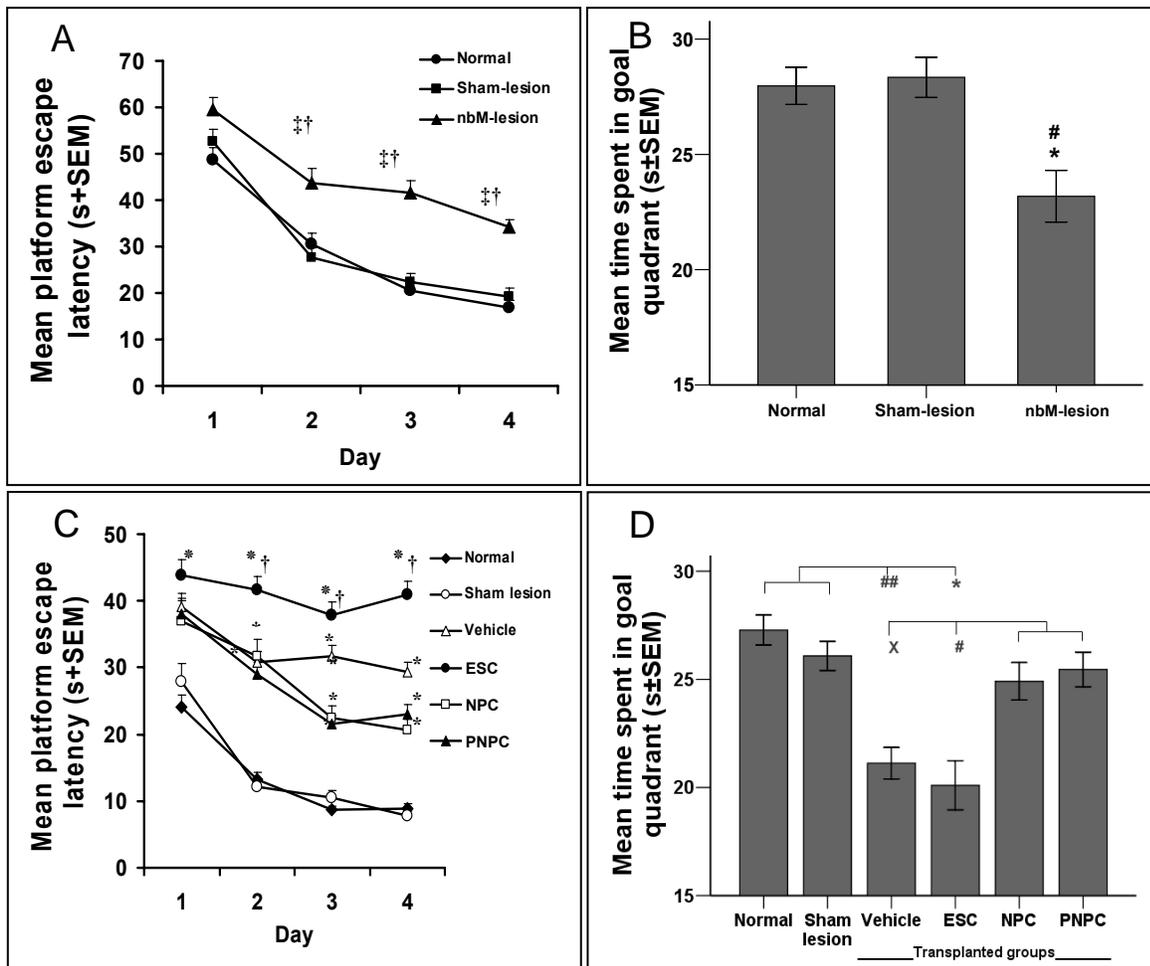


Figure 2.

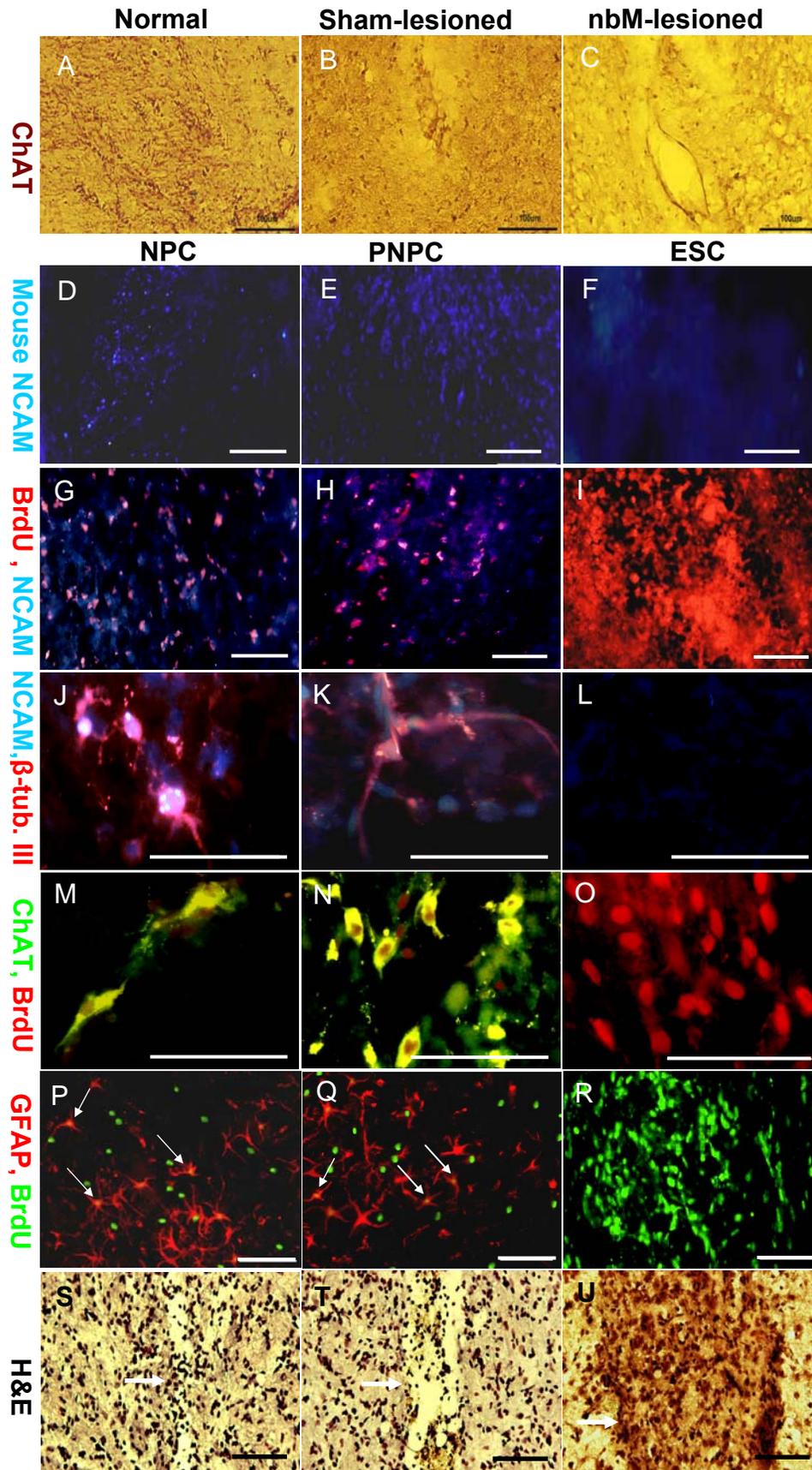


Figure 3.

Table 1. The effect of growth factor concentration on cholinergic neuron differentiation.

Component	Concentration	Cholinergic neurons (%)*
Shh (ng/ml)	50	17.39±1.32
	100	22.45±1.52 a
	200	22.37±0.97 a
RA (µM)	1	17.05±1.25 a
	2	15.73±1.04 b
	5	9.19±1.75
LIF (iu/ml)	250	15.28±1.35
	500	17.78±0.91 a
	1000	12.91±1.42
IL6 (ng/ml)	5	14.08±1.36
	10	13.81±1.62
	20	10.46±1.18
NGF (ng/ml)	25	6.47±1.29
	50	6.81±0.74
	100	7.6±0.50
Control (no growth factor)		11.67±1.09

*: The data were presented as mean ± SEM. a: P<0.05, b: P<0.01 in comparison with other concentration with same component.