Research paper

Migration of neural precursor cells derived from olfactory bulb in cochlear nucleus exposed to an augmented acoustic environment

Yang Chen, Jianhua Qiu *, Fuquan Chen, Shunli Liu

Department of Otolaryngology, Xijing Hospital, Fourth Military Medical University, Xi’an 710032, PR China

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Abstract

The regeneration of the auditory neural system remains a challenge in hearing restoration. Acoustic signals may induce a site-specific cell replacement in the auditory system. This hypothesis was tested with grafted implantation of neural precursor cells (NPCs) along the cochlear nucleus in the adult host followed by an augmented acoustic stimulation. NPCs were obtained from the olfactory bulbs at embryonic day 14–16 and were transplanted into the inside border of cochlear nucleus. The labeled cells survived at least 2 weeks, verified by Hoechst 33342 fluorescence, and by immunostaining for a neuronal marker. In some cases NPCs had migrated directionally to the root of the auditory nerve. This observation demonstrates the survival and migration of NPCs from the olfactory bulb (OB) along the adult auditory nerve in an augmented acoustic environment following implantation.

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1. Introduction

Impairment to the sensory epithelium, the organ of Corti, in the mammalian cochlea results in a series of pathological changes of the auditory system. The primary damage is often selective to the sensory epithelium. As in other afferent systems, degeneration of the spiral ganglion neurons and the cochlear nucleus neurons happens secondary to the loss of cochlear sensory epithelium (Leake and Hradek, 1988; Ryals et al., 1989). Moreover, the antero-ventral cochlear nucleus (AVCN) of D2 (DBA/2) mice show a progressive loss of tissue volume of about 15–20%, the number of neurons of about 25–35%, and a small decrease in the neuronal size (Willott and Bross, 1996). It is of interest to explore novel approaches for restoration of auditory function. One strategy would be the replacement of degenerated or absent spiral ganglion neurons and cochlear nucleus neurons using cell replacement therapy. Stem cells are interesting candidates for cell replacement therapy. Embryonic dorsal root ganglion neurons and embryonic stem cells have been transplanted into the modiolus at the first cochlear turn (Hu et al., 2004). At 9 weeks following implantation, the implanted DRG neurons were found to have migrated along the auditory nerve in the internal meatus. At the same postoperative time, the ES cells had migrated into the brain stem close to the ventral cochlear nucleus.

Progenitor cells in the subventricular zone (SVZ) proliferate throughout life in rodents. These proliferating progenitor cells migrate into the olfactory bulb (OB) via the rostral migratory stream (RMS) and differentiate into the interneurons found in the OB granule cell layer and glomerular layer (Garcia-Verdugo et al., 1998).

Progenitor cells isolated directly from the SVZ of neonatal animals were grafted into multiple brain regions in the
neonate or the adult. These grafted cells migrated and differentiated into neurons within multiple regions including the OB, the cortex and the striatum (Doetsch and Alvarez-Buylla, 1996; Lim et al., 1997; Lois and Alvarez-Buylla, 1994; Rousselot et al., 1995). Multipotential precursors with stem cell features can be isolated from the SVZ but also from the entire rostral extension, including the distal portion within the OB (Gritti et al., 2002). SVZ/OB progenitor cells could serve as a useful source of neurons for cell transplantation. Neural stem cells have been tested in the treatment of nervous system disease, as Parkinson’s disease, Huntington’s disease, Alzheimer’s disease and spinal cord injury (Björklund et al., 2003; Blesch et al., 2002; Storch and Schwarz, 2002; Sugaya and Brannen, 2001; Wagner et al., 1999).

It was found that enriched odor environment could influence proliferation and survival of newborn olfactory interneurons in adult mice (Rochefort et al., 2002). This demonstrates that cell proliferation and survival of OB neurons might be regulated independently. Meanwhile, the genetic, progressive, sensorineural hearing loss in D2 mice and several other inbred strains is ameliorated by treatment with an augmented acoustic environment (AAE) (Turner and Willott, 1998; Willott and Turner, 1999; Willott et al., 2000, 2005; Willott and Bross, 2004). In this study, we investigate the possibility for NPCs derived from rat OB to migrate and survive in the cochlear nucleus in an augmented acoustic environment.

2. Methods

2.1. NPC isolation and culture (Vicario-Abejon et al., 2003)

Reagents for tissue culture were purchased from HyClone (USA), Sigma (St. Louis, MO). Fibroblast growth factor-basic, bFGF and EGF were purchased from PeproTech (Rocky Hill, NJ) and B27 purchased from Invitrogen (Carlsbad, CA).

Neural stem cells were prepared from Wistar rat embryonic olfactory bulb on the gestational days (E) 14.5, E15.5, and E16.5 considering the day on which a vaginal plug as E0.5. Upon the removal of brain, the olfactory bulbs were dissected at the level marked in Fig. 1A of an E15.5 rat brain. Cells were obtained by mechanical dissociation of dissected and pooled olfactory bulbs, followed by mild trypsinization. After trypsin inhibition with heat inactivated 10% FBS, the cells were resuspended in DMEM/nutrient mixture F12 (F12)/ and B27, plated on uncoated tissue culture dishes at a density of 35,000 cells per square centimeter and incubated at 37°C in a 5% CO2 atmosphere. FGF-2 and EGF (20 ng/ml each) were added daily to expand the proliferative precursor cell population. Cells growing as floating aggregates or “spheres” (Reynolds and Weiss, 1996) were passaged every 4–7 d by mechanical procedures and plated at 5000 cells per square centimeter at each passage. Cells were pulsed with 10 µM 5-bromo-2-

![Fig. 1. Cells that respond to FGF-2 plus EGF by proliferating and forming spheres can be isolated from the embryonic olfactory bulb.](image)
deoxyuridine (BrdU) (Sigma, St. Louis, MO) for 24 h before fixation. Hoechst 33342 (10 μg/ml) was added to the culture medium 1 h prior to transplantation.

To initiate olfactory bulb stem cell differentiation, after FGF-2 and EGF were removed the corresponding passage and cells were plated for 10 d on glass coverslips precoated with 15 μg/ml poly-L-lysine in DMEM/F12/B27 at a density of 100,000 cells per square centimeter. The cells were then fixed with 4% paraformaldehyde to determine whether dividing cells could differentiate to neurons, astrocytes, and oligodendrocytes.

2.2. NPC transplantation

Following in vitro expansion for 10 days, Hoechst 33342 (10 μg/ml) was added to the culture medium 1 h prior to transplantation. These Hoechst-33342-labeled OB cells were resuspended by trypsinization and collected by centrifugation (1000 rpm, 5 min). Single cell suspensions were prepared in PBS. Nucleated OB cells were counted using a cytometer to ensure an adequate cell number for transplantation of approximately 2.0 × 10⁴ NSCs in 1 μl DMEM/F12 media. Cells were implanted into the medial side of right cochlear nucleus of adult Wistar rats (250–300 g, n = 40) at the following coordinates calculated from bregma: anterior–posterior = −10.3 mm; medial–lateral = −3.85 mm; depth = −6.1 mm. The cells were delivered by a glass capillary with an inner diameter of approximately 100 μm, attached to a 10 μl Hamilton syringe, to deliver 4.0 × 10⁴ cells per graft site. To avoid backflow along the needle track, the needle was left at the appropriate depth for 5 min after cell infusion. The animals were divided into two groups. Group A of 20 rats were placed in the augmented acoustic environment after transplantation and the control group of 20 rats remained in the normal environment. On a given surgery day, an equal number of animals from each group were transplanted when possible.

2.3. Exposure to the augmented acoustic environment

Animals of group A were placed in steel cages (40 × 30 × 30 cm) with a wire lid through for access to food and water. They received consecutive 12-h nights of AAE beginning at the day of transplantation. To produce the AAE, a RadioShack Supertweeter was mounted above the cages. The signal was a broadband noise shaped, timed, and gated by Coulbourn Instruments modules (rise/fall = 10 ms, duration = 200 ms, rate = 2/s), then passed through an amplifier (Yamaha AX-500U) before being sent to the speakers. Acoustic calibration was performed using a Bruel and Kjær Type 2606 measuring amplifier connected to an external band-pass filter and 1/8-in. microphone. The calibration microphone was positioned at various points within the cages, and the signal was set at the level producing a mean SPL of 70 dB. The signal was timed to be on between 8.30 p.m. and 8.30 a.m. The parameters of the AAE signal were chosen so that maximum acoustic stimulation could be provided without producing hearing loss or other adverse effects. Broadband noise was used so as to produce maximal stimulation of the baso-apical middle segment of the basilar membrane. The 70 dB SPL intensity was used because previous work had shown that chronic exposure to sounds below 75–80 dB does not produce hearing loss (NIH Consensus Conference, 1990). The AAE occurred at night so as to provide the maximum amount of stimulation to the waking animal, since rats are nocturnal, without significantly altering the sleep cycle.

2.4. Immunofluorescent staining and phenotypic analysis

2.4.1. In vitro

Plates were fixed for 30 min in 4% paraformaldehyde and washed three times for 5 min with PBS. Fixed cells were blocked for 30 min in PBS containing 5% normal goat serum and 0.25% Triton X-100, and then incubated overnight at 4 °C with primary antibodies diluted in the PBS. Cells were washed three times and incubated with secondary antibodies diluted in the PBS, for 3 h at room temperature. Finally, cells were washed three times with PBS including 30 min incubation with Hoechst 33342. For BrdU staining, fixed cells were pretreated with 2 M HCl at 37 °C for 30 min followed by six washes with PBS prior to blocking. The primary antibodies used were: mouse anti-BrdU (1:1000, Sigma), rabbit anti-GFAP (1:200; Chemicon), mouse anti-nestin (1:250; Chemicon), mouse anti-NeuN (1:300; Chemicon), rabbit anti-Musashi (1:200; Chemicon), and rabbit anti-NSE (1:200; Chemicon). The secondary antibodies used were goat anti-species conjugates of FITC, Cy3 or Rhodamine (1:100; Boster Biological Technology Company, Wuhan, China). The exclusion of the primary or secondary antibodies in control sections was used to demonstrate antibody specificity.

2.4.2. In vivo

At 2 weeks post-grafting, animals were perfused with 100 ml cold PBS followed by 300 ml cold 4% paraformaldehyde (pH 7.4). Coronal sections were made through the cochlear nucleus from frozen brains using a sliding microtome set at 16 μm. Eight sets of sections were collected from each brain. Tissue sections were blocked for 10 min at 37 °C in PBS containing 5% normal goat serum and then incubated for 24 h at 4 °C with primary antibodies diluted in the blocking solution. Sections were washed three times for 5 min each with PBS, followed by a 3 h, room temperature incubation with secondary antibodies diluted in blocking solution. Primary antibodies used were rabbit anti-NSE (1:200; Chemicon), mouse anti-GFAP (1:200; Chemicon), mouse anti-NeuN (1:300; Chemicon) and rabbit anti-Musashi (1:200; Chemicon). Secondary antibodies used were goat antispecies conjugates of FITC, Cy3 or Rhodamine (1:100; Boster). Each grafted Hoechst
positive cell observed within right cochlear nucleus was evaluated.

3. Results

3.1. Isolation and characterization of stem cells from the olfactory bulb

To isolate local olfactory bulb stem cells, a cell suspension was prepared from rat E14.5 OB (Fig. 1A) and plated under tissue culture conditions in the presence of FGF-2 and EGF. One day after plating, some rounded cells started to divide (Fig. 1B, arrowheads). A smaller proportion of the cells differentiated into neuron like cells (Fig. 1C, arrow). Daily inspection of the cultures showed that the rounded cells divided, forming cell aggregates or spheres (Reynolds and Weiss, 1996) (Fig. 1C) which reach confluence in the presence of the mitogens by day 6 (Fig. 1D). Cell suspensions were also prepared from the E15.5 and E16.5 OB. After plating, the E15.5- and E16.5-derived cells proliferated and formed spheres in response to FGF-2 plus EGF, with the same temporal pattern as the E14.5 OB-derived cultures.

Under these standard growth conditions, cells could be passed every 4–7 d for at least 3 months for a total of 20 passages. The average doubling time of these cells was approximately 26 h. The increase in cell number varied between passages, but a tendency toward lower proliferation was observed in the later passages. Cell proliferation required the presence of FGF-2 and EGF even after long periods in culture, indicating that the cells had not been transformed into growth factor independent cells (data not shown). About 97% of cells expressed nestin (Fig. 2A,C), and about 91% of cells expressed Musashi (Fig. 2A,D), another marker of neural stem cells, in cultures grown in the presence of FGF-2 and EGF for up to 2 months (passage 10). On average, 99% of cells were labeled by Hoechst 33342 and only 65% of the cells incorporated BrdU (Fig. 2B,D). Similarly, the E15.5 and E16.5 cultures proliferated extensively and were composed of 97% nestin-positive cells. Together, these results indicate that OB cultures were composed predominantly of highly proliferative neural precursor cells that could be expanded for long periods of time.

To test whether these proliferative precursor cells possessed stem cell features such as multipotentiality and self-renewal capacity, twelve clonally derived secondary spheres and twelve tertiary spheres were analyzed by triple immunostaining. About 56% of the spheres generated neurons, astrocytes, and oligodendrocytes after differentiation, indicating that the founder cells were multipotent (Fig. 2E–G). Other clonally derived spheres gave rise to neurons and astrocytes (25%), or astrocytes and oligodendrocytes (5%), or only astrocytes (14%). Subclonal analysis of clonally derived secondary spheres confirmed the presence of cells that were multipotent with self-renewal capacity.

3.2. Migration and neuronal differentiation of grafted NPCs

The ratio of cells that incorporated BrdU was much lower than Hoechst 33342 in vitro, the latter label was used to identify grafted cells in tissue sections. The reliability of this method was demonstrated by other investigators, whose results showed no transfer of BrdU from live or dead cells to the host cells (Muraoka et al., 2006), surviving grafted cells were identified in all animals by Hoechst 33342-positive, in intact nuclei. NPCs identified by Hoechst 33342-positive, intact nuclei, were injected accurately within the inside border of the cochlear nucleus in 9 rats of group A and 8 rats of control group. These grafted cells were assayed by immuno-fluorescence for the detection of NSE or NeuN, markers for neuronal differentiation (Weyer and Schilling, 2003). The other species were abandoned when undershoot happened. This was done to determine if neural precursor cells derived from rat OB would migrate and differentiate into neurons in the cochlear nucleus in an augmented acoustic environment. Directional migration from the grafted cells clearly occurred in some animals exposed to the augmented acoustic environment. Part of the grafted cells migrated from the graft core toward the root of the 8cn and tz, but migration was not apparent in the control group at 2 weeks survival (Fig. 3A,B). The average migration distance is 530 ± 126 μm (n = 9) in group A and 152 ± 48 μm (n = 8) in control group.

Neurite outgrowth from the differentiated grafted NPCs was observed based on the presence of Hoechst 33342 labeled nuclei and NSE antibody staining (Fig. 3C). The number of GFAP positive double-labeled cells was greater than NSE positive grafted cells (Fig. 3D). Grafted cell (blue nuclei) expressed neurotransmitter of cochlear nucleus was observed by triple immunostaining of NeuN (red nuclei) and glutamate (green) (Fig. 3E). Newly generated neurons, verified by co-expressing NSE and NeuN, were approximately 10% of the grafted cells labeled by blue nuclei.

4. Discussion

The present study was designed to determine if NPCs from the OB, which can be differentiated into presumptive neurons in vitro, exhibit neuronal characteristics in the cochlear nucleus in vivo. In vitro analysis confirmed that the isolated and cultured NPCs from OB used in these studies, exhibited the ability to differentiate into neurons and astrocytes, indicating the multipotentiality of the starting cell population. This represents the first demonstration that NPCs from OB engraft, migrate, differentiate, and survive in the cochlear nucleus in vivo. Exposure to an augmented acoustic environment after transplantation induced the grafted cells migration towards the root of the cochlear nerve and across auditory fiber of the tz. These cells underwent large-scale differentiation with approximately 10% of newly generated neurons co-expressing NSE and NeuN, specific markers for neurons. Furthermore, some differentiated neurons expressed glutamate an
excitatory, afferent neurotransmitter. These results indicate that adult neural progenitor cells may provide an alternative cell source useful for transplantation therapy in the treatment of auditory neural path disease.

Previous studies investigated the effect of transplanting adult neural progenitor cells into either neurogenic or non-neurogenic regions of the normal adult brain. The majority of these studies showed that transplantation of
either adult hippocampal-derived or SVZ-derived neural progenitor cells into homotrophic or heterotrophic neurogenic regions resulted in site-specific neuronal differentiation (Gage et al., 1995; Suhonen et al., 1996; Herrera et al., 1999; Richardson et al., 2005b). However, transplantation into non-neurogenic sites such as the striatum or cerebellum resulted in the majority of transplanted cells differentiating into glia (Suhonen et al., 1996; Herrera et al., 1999; Dziewczapolski et al., 2003; Richardson et al., 2005a). This suggests that the in vivo differentiation pattern in non-neurogenic regions such as the striatum may not reflect the entire lineage potential of transplanted adult neural progenitor cells. This could either be because environmental cues conducive for neuronal differentiation are absent in these regions or because of the presence of cues inhibitory for differentiation to a neuronal lineage. In con-
trast to these observations, Zhang et al. (2003) reported that transplantation of adult neural progenitor cells derived from the SVZ into the normal adult striatum resulted in extensive neuronal differentiation 4 weeks post-transplantation. The apparent disparity observed regarding neuronal differentiation of SVZ-derived adult neural progenitor cells transplanted into the normal adult striatum (Herrera et al., 1999; Zhang et al., 2003; Richardson et al., 2005a) may reflect inconsistencies in the state of neuronal differentiation of the transplanted cultures as well as methodological differences between experiments. However, the extent of neuronal differentiation reported by Zhang et al. (2003) raises the possibility that appropriate environmental cues for the induction of neuronal differentiation are present in the adult striatum.

An essential issue in a cell replacement strategy for the auditory system is that the implant, apart from the long time survival and high survival rate, has a potential to migrate centrally towards the auditory neurons and to form neuronal connections further centrally within the auditory system. In the present study, the first step was taken by showing that NPCs from OB can migrate directionally to the root of auditory nerve and differentiate into glutamate positive neurons when the animals were subjected to augmented acoustic environment. This suggests the possibility for a biological implant to replace degenerated or absent cochlear nucleus neurons. We speculate that acoustic cues may play an important role in cell migration. Neural progenitors implanted into the brain traveled along the rostral migratory pathway to differentiate into neurons in the olfactory bulb (Fricker et al., 1999; Suhonen et al., 1996). A recent study shows that region specific cues are important in the neuronal differentiation and migration of naive embryo stem cells (Harkany et al., 2004). It appears that certain factors could “guide” foreign cell migration. In this study, the NPCs from OB could have been “directed” by afferent information in the augmented acoustic environment, representing an auditory-specific migration. We also found that the NPCs from OB in non-augmented acoustic environment migrated diffusely around the graft core.

In summary, the present findings demonstrate that allogeneic grafted NPCs from OB survive 2 weeks following transplantation in the adult VCA. The implanted Hoechst 33342 labeled cells also migrate directionally towards the root of auditory nerve in the augmented acoustic environment. This demonstrates not only the survival of differentiated neuronal tissues and pluripotent potential of NPCs from OB in the adult auditory system but also the potential ability of these implanted cells to migrate in the augmented acoustic environment. The present findings may help to determine if biologically active neuronal tissue and pluripotent NPCs could be used to restore the degenerated adult auditory system. However, in order to restore hearing, the establishment of essential synaptic contacts between the implanted cells and the host auditory neurons needs to be demonstrated. This will be an exiting topic for future research.

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References


