Original Article

Intracerebral neural stem cell transplantation improved the auditory of mice with presbycusis

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Abstract: Stem cell-based regenerative therapy is a potential cellular therapeutic strategy for patients with incurable brain diseases. Embryonic neural stem cells (NSCs) represent an attractive cell source in regenerative medicine strategies in the treatment of diseased brains. Here, we assess the capability of intracerebral embryonic NSCs transplantation for C57BL/6J mice with presbycusis in vivo. Morphology analyses revealed that the neuronal rate of apoptosis was lower in the aged group (10 months of age) but not in the young group (2 months of age) after NSCs transplantation, while the electrophysiological data suggest that the Auditory Brain Stem Response (ABR) threshold was significantly decreased in the aged group at 2 weeks and 3 weeks after transplantation. By contrast, there was no difference in the aged group at 4 weeks post-transplantation or in the young group at any time post-transplantation. Furthermore, immunofluorescence experiments showed that NSCs differentiated into neurons that engrafted and migrated to the brain, even to sites of lesions. Together, our results demonstrate that NSCs transplantation improve the auditory of C57BL/6J mice with presbycusis.

Keywords: Presbycusis, neural stem cells, transplantation, C57BL/6J mice, primary auditory cortex, apoptosis, auditory brain stem response (ABR)

Introduction

Presbycusis, which is associated with difficulty in speech discrimination and sound detection and localization, influences an individual’s well being and productivity and causes communication problems, isolation, and social withdrawal. The prevalence of presbycusis increases proportionally with age. Although the potential mechanisms underlying presbycusis are not completely understood, the age-related decline in acuity is generally attributed to at least two factors: progressive peripheral degeneration [1] involving the degeneration of the stria vascularis [2], hair cells and spiral ganglions [3-5] and a central degeneration involving neuron degeneration in the primary auditory cortex (AI) of the central nervous system (CNS) [6-8]. Central auditory dysfunction is a prominent component of presbycusis [9]. Among the aforementioned factors, apoptosis of neurons in the AI plays a major role in central presbycusis [10-14].

The NSCs transplantation strategy has received significant attention as an alternative therapy for neurodegenerative diseases in preclinical studies. However, few reports have examined the application of NSCs to the treatment of central presbycusis. Based on pathogenetic findings in presbycusis and successful examples of NSCs-based therapies for stroke [12, 15, 16], Parkinson’s disease [17, 18] and spinal cord injuries [19, 20], the transplantation of embryonic NSCs into the AI provides insights into progressive therapeutic strategies for presbycusis. NSCs are defined as undifferentiated cells that are capable of self-renewal and give rise to three brain-derived major cell types: neurons, astrocytes and oligodendrocytes. They can be

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transplanted into patients as autografts or as allografts with unique immunological properties. NSCs are thought to replace apoptotic neurons and support existing neurons by inhibiting apoptosis and promoting better recovery of function. The goals of this study were to characterize the survival, migration and differentiation of embryonic NSCs that are transplanted into the AI and to determine the increase in auditory function of C57BL/6J mice with presbycusis. Ultimately, we aimed to document the role of apoptosis in central presbycusis [10].

Materials and methods

Animals and tissue preparation

All investigations were approved by the ethical committee of The Third Military Medical University for animal care. C57BL/6J mice, which are known for their rapid development of hearing loss, particularly in the high-frequency range, by one year of age [21, 22], that were aged 2 months and 10 months were chosen for this experiment. These age groups were termed the young group and aged group, respectively. Each group contained 20 C57BL/6J mice. Simultaneously, a pregnant C57BL/6J mice (E14 days) was prepared to culture the NSCs. The C57BL/6J mice were housed 2 or 3 per cage, had free access to food and water and were housed under suitable temperature and humidity conditions and a normal 12/12 h light/dark cycle.

Experimental design

Two days before transplantation, the cultured NSCs were passaged for the third time, and fluorescence immunocytochemistry was carried out to examine the expression of NSCs markers and differentiated markers. The transplantation took place just before the NSCs were co-cultured with DAPI. Two groups were assessed: the young group (n=15) and the aged group (n=15) at 2 weeks, 3 weeks and 4 weeks after transplantation. From the 2nd week and every week thereafter, 5 mice from each of the 2 groups after the operation underwent ABR tests and were sacrificed to evaluate neuronal apoptosis and the survival, migration and differentiation of the transplanted cells in the AI by the TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling)-POD (peroxidase) assay and fluorescence immunohistochemistry method, respectively. The entire procedure is shown in Figure 1.

Embryonic neural stem cell preparation and transplantation

The isolation and expansion of embryonic NSCs from embryonic day 14 cerebral cortex followed the protocol described by Andrew Chojnacki [23]. In short, using sterile technique, embryonic NSCs were dissociated from the cerebral cortex of C57BL/6J mice embryos at 14 days of gestation. Cells were suspended in NEUROBASAL™ (Gibco, New York, NY, US) medium followed by centrifugation. After the cells were separated into a single-cell suspension, primary cultures are plated at a density of 10^5-10^6 cells mL^-1 in NEUROBASAL™ medium supplemented with basic fibroblast growth factor (b-FGF, 20 ng/mL; Gibco) and epidermal growth factor (EGF, 20 ng/mL; Sigma, USA). Two to three days were required for neurosphere formation. The cultures were then passaged every 7–10 days by mechanical dissociation. Embryonic NSCs were passaged 2–3 times before transplantation. Two days before transplantation, fluorescence immunocytochemistry was carried out to examine the expression of an NSCs marker (anti-nestin antibody; Sigma, 1:600), self-proliferation marker (anti-BrdU, Sigma, 1:1,000) and differentiation markers, including anti-β-tubulin III antibody (Sigma, 1:600) and anti-GFAP antibody (Sigma, 1:400) according to previous studies [23, 24] (Figure 2). On the day of transplantation, neurospheres were dissociated mechanically into a single cell suspension and cultured with 4'6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) in fresh neurosphere media for 20 minutes. Then, the cells were harvested, washed and resuspended in culture media to a final concentration of 1×10^5 mL^-1 and were kept on ice before transplantation. To ensure that the cells were labeled before transplantation, a portion of the single cell suspension that was labeled with DAPI was viewed using laser confocal microscopy (TCS Sp2: Leica, Wetzlar, Germany). After pentobarbital anesthesia, the C57BL/6J mice were placed in a digital stereotactic frame (Stoelting, Wood Dale, IL, USA) and received a total of 1×10^5 mL^-1 suspended cells (n=15 each group) in a volume of 3 μL injected into the AI using a Hamilton 701N syringe (Sigma-Aldrich) at the following coordinates: 3.0 mm
posterior to the bregma and 3.0 mm lateral right to the midline. After the injections were completed, the needle was left in place for 5 minutes to allow the deposits to diffuse off of the needle tip before slowly retracting the syringe. The exact anatomy of the AI was identified according to George Paxinos [25].

Auditory Brainstem Response (ABR)

For the ABR recordings, all of the C57BL/6J mice, both pre-transplantation (n=15 from the young group and n=15 from the aged group) and post-transplantation at 14 days (n=5 from each group), 21 days (n=5 from each group) and 28 days (n=5 from each group) were anaesthetized by intramuscular injection with a mixture of ketamine (100 mg/kg) and xylazine (25 mg/kg) and placed on a thermostatic heating pad to ensure their body temperatures remained at 38.5°C. ABR testing was conducted in a sound-attenuating chamber. The stimulus presentation, ABR acquisition, equipment control and data management were coordinated using the computerized Intelligent Hearing System (IHS, US), and Smart-EP (version 10) software was used to acquire the ABR acoustic stimuli. We chose a pair of high-frequency transducers (Mike Ravicz, Somerville, MA) to generate specific acoustic stimuli. The active recording electrode and the reference were placed at the vertex of the skull and right mastoid prominence, respectively. The ground was inserted in the nasal tip. The ABR threshold testing facility and technique have been described in detail [5, 26]. Briefly, the stimuli were rarefaction clicks and pure tones at click, 8 kHz, 16 kHz, presented at 90 dB SPL at a rate of 11.1 s. Auditory thresholds were obtained for each stimulus by reducing the SPL from 90 dB in 10-dB steps and finally in 5-dB steps up and down to identify the lowest level at which an ABR could be recognized. If no waveform was identified at the highest presentation level (90 dB SPL) for a particular frequency, the threshold was then documented as 90 dB SPL. The waveform was filtered from 100 Hz to 1,500 Hz, with a gain of 100 and window of 10 ms. A total of 1,024 sweeps were obtained for each waveform. The Threshold was determined as the minimal SPL to which a repeatable and just-detectable response of peaks III was visible.

Observation of the auditory cortex morphology

Transmission Electron Microscopy (TEM): Before transplantation, 25% of two groups of mice aged 2 months (n=2) and 10 months (n=2) were perfused intracardially with 0.9% phosphate-buffered saline (PBS) followed by 2% glutaraldehyde. For each mice, the brain was removed, and the AI (as described above) was dissected out entirely and immersed in 2% glutaraldehyde for 6 h at 4°C. The immersed tissues were washed in PBS for 10 minutes at 4°C and then post-fixed in 1% osmic acid in 0.2 M phosphate buffer (pH 7.4) followed by another 10 minutes of washing in PBS at 4°C. The tissues were dehydrated in a graded ethanol series and then treated with propylene oxide for approximately 30 minutes before being infiltrated with a mixture of epoxy resin 618 and propylene oxide (1:1 for 2 h, then 2:1 overnight) and then pure epoxy resin 618 for 6 h. After drying at 60°C for 48 h, the tissues were cut on an LKB V ultramicrotome. Serial ultrathin sections were distributed on copper grids and stained with uranyl acetate followed by lead citrate and visualized using a CM-120 transmission electron microscope (Philips, The Netherlands).

Tissue morphology and apoptosis: Following the ABR, 6 mice both pre-transplantation (n=3 from each group) and post-transplantation at 14 days (n=3 for each group), 21 days (n=3 for
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each group) and 28 days (n=3 for each group) were transcardially perfused with PBS followed by a 4% paraformaldehyde solution. The brains were extracted and postfixed in 4% paraformaldehyde for at least 24 hours at 4°C. The entire AI of each mice was embedded in paraffin wax. The tissues were sectioned on a cryostat and stained with hematoxylin and eosin prior to the TUNEL–POD assay.

Neuronal apoptosis in the AI was detected using an In Situ Cell Death Detection Kit with Peroxidase (POD) (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Briefly, paraffin-embedded AI was cut into sections, which were dewaxed in xylene and hydrated in a graded alcohol series and then washed 3 times with PBS. Tissue sections were incubated for 15 minutes at room temperature with Proteinase K working solution, and the slides were rinsed twice with PBS. A labeling protocol was carried out on these slides by immersing them in the TUNEL reaction mixture for 60 minutes at 37°C in a humidified atmosphere in the dark. Samples were analyzed in a drop of PBS under a fluorescence microscope at this state. Then, the samples were reacted with Converter-POD in a humidified chamber for 30 minutes at 37°C. Apoptotic nuclei were obtained using the peroxidase-DAB reaction, after which the sections were counterstained with hematoxylin. TUNEL-labeled cells were counted in five representative sections per animal.

Immunohistofluorescence

The immunohistofluorescence methods that were used to determine the survival and migration of grafted NSCs cells in vivo are similar to those reported previously [27]. After the final ABR test, the mice (n=2 from each group) were deeply anesthetized with pentobarbital (left ventricle perfusion), and their AI tissues were processed similarly to treatment described above with hematoxylin and eosin staining. The site of the needle track inside the AI was examined. Then, the tissues were cut into 20-mm-thick coronal sections on a cryostat microtome. Grafted NSCs were detected using fluorescent methods. They were pre-labeled with DAPI (blue) and had blue nuclei when examined under a fluorescence microscope. Following 3 rinses in PBS, these sections were made transparent by incubation in 0.3% H2O2 and then immersed in 0.3% Triton X-100. The performance was blocked with 10% normal goat serum for 30 minutes at room temperature. Those sections were incubated overnight at 4°C in primary antibody (monoclonal anti-β-tubulin III (neuronal) antibody, Sigma, 1:600), rinsed and then incubated with fluorescein isothiocyanate(FITC)-conjugated IgG for 60 minutes at room temperature. Fluorescence signals were detected with a laser confocal microscope (TSC SP2, Leica).

Statistics

Data were analyzed using SPSS v. 13.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons between pre- and post-transplantation were performed with a two-tailed analysis of variance with a paired-samples T-test. The results were considered significant at P<0.05. All results are expressed as mean±s.e.m.

Results

Presbycusis pathology

The TEM method, hematoxylin and eosin staining and the TUNEL-POD assay were used to detect the pathology of presbycusis. Using TEM, neurons of the AI in the young group displayed typical ultrastructural features. Generally speaking, neurons (Figure 2A) looked polygonal or oval with round nuclei. The nuclear membrane was intact and smooth. The chromatin was uniformly but lightly dispersed throughout the nucleus, which remained translucent. The cytoplasm contained abundant organelles, including mitochondria and rough endoplasmic reticulum. The apoptotic neurons (Figure 2B) and characteristic ultrastructural features of the AI in the aged group were also observed using TEM. The neurons were shrunken, which was accompanied by highly condensed heterochromatin. Simultaneously, heterochromatin accumulated around the edges of cell membrane. The nuclei were fragmented. In addition to nuclear changes, there were significant differences in the cytoplasmic organelles, including the dissolution of the rough endoplasmic reticulum, ribosomes and mitochondria. Additionally, there were apoptotic bodies within the concentrated cytoplasm. After hematoxylin and eosin staining, the neurons (Figure 2C) in the auditory cortex were intact with a tight and regular arrangement in
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Figure 2. Presbycusis Pathology. The ultrastructure of neurons in the auditory cortex was measured by transmission electron microscopy (A-B): A. Neurons appear polygonal or oval with round nuclei. The nuclear membranes were intact and smooth. The chromatin was uniformly but lightly dispersed throughout the nuclei, which remained translucent. The cytoplasm contained abundant mitochondria and rough endoplasmic reticulum (scale bar=2 μm); B. The neurons are shrunken, which is accompanied by highly condensed heterochromatin that accumulated around the edges of cell membrane. The nuclei are fragmented, and the rough endoplasmic reticulum, ribosomes and mitochondria are dispersed. Histopathological changes in the AI were examined by hematoxylin and eosin staining (C-D): C. in the young group, the neurons in the auditory cortex were intact, with a tight and regular arrangement; D. the neurons in the aged group were shrunken and had membrane blebbing and pathological capillary changes, including tortuosity and looping. Apoptosis in the AI (E-F): E. apoptotic neurons in the young group; F. apoptotic neurons in the aged group. ABR tests, *P<0.05, n=15 for the young group; n=15 for the aged group (G): the average auditory threshold of the young group was maintained at 40 dB SPL or so, while the auditory threshold of the aged group was approximately 80 dB SPL and showed significant hearing loss at high frequencies.

the young group. By contrast, the neurons (Figure 2D) in the aged group were shrunken and exhibited membrane blebbing. There were also pathological capillary changes, including tortuosity and looping. To evaluate the rate of apoptosis in the AC, we examined AI tissues using the TUNEL-POD assay. The DAB method was performed to assess the apoptotic rate pre-transplantation in the AI in the young and aged groups. Only rare apoptotic cells (Figure 2E) were observed in young group, while the neuronal apoptosis index (Figure 2F) was sig-
Significantly increased in the AI of the aged group. Accompanied by the pathology of presbycusis described above, the ABR thresholds prior to NSCs transplantation from each group are shown in Figure 2G. The average auditory threshold of the young group was maintained at 40 dB SPL or so, while the aged group’s auditory threshold remained at approximately 80 dB SPL and showed significant hearing loss at high frequencies.

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The formation of neurospheres began 2 days later than the dissociation of single cells. Neurospheres and their differentiations were both examined by the way of immunofluorescence (Figure 3). Neurospheres (Figure 3A) are phase-bright and have a round morphology. They are also highly cellular. A large number of neurospheres were positively stained by anti-Nestin antibody (Sigma, US, 1:600) (Figure 3D) and anti-BrdU antibody (Sigma, US, 1:1000) (Figure 3E), showing the potential of NSCs for self-proliferation. They can also be differentiated (Figure 3B) into β-tubulin III-positive neurons (Figure 3H) and GFAP-positive astrocytes (Figure 3I), which demonstrates the multipotent properties of differentiation of these cells. All of the nuclei are stained blue (Figure 3C and 3G). The cultured cells were prepared and
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Figure 4. The auditory thresholds of C57BL/6J mice were examined both before and after the operations. A. In the young group, there was no significant difference in the ABR threshold before and 2 weeks, 3 weeks and 4 weeks post-transplantation (P>0.05, n=5). B. The average threshold of the aged group was apparently enhanced at both 2 and 3 weeks post-transplantation (P<0.05, n=5) compared to the thresholds before embryonic NSCs transplantation; this enhancement was not detected at 4 weeks post-transplantation (P>0.05, n=5).

transplanted. 2 weeks, 3 weeks and 4 weeks later, the mice underwent the ABR test. The ABR thresholds prior to NSCs transplantation from each group are shown in Figure 3. In the young group, there was no significant difference in the ABR threshold before and 2 weeks, 3 weeks and 4 weeks post-transplantation (P>0.05, Figure 4A). The average threshold of the aged group was apparently decreased at 2 and 3 weeks post-transplantation (P<0.05, Figure 4B) compared to the thresholds before embryonic NSC transplantation, but no such change was observed at week 4 post-transplantation.

Survival and migration of grafted embryonic neural stem cells in the mice brain

The survival and migration of grafted NSCs in vivo, which appeared around the site of the needle track, were evaluated by immunohistochemistry. Owing to pre-labeling with DAPI, the grafted NSCs were detected easily by visualizing the distribution of DAPI-stained nuclei in the AI area (Figure 5A). The grafted cells differentiated around the needle track and entered the pinhole to restore the injury (Figure 5B and 5C).

Decreased levels of apoptosis in the auditory cortex following embryonic NSCs transplantation

Because apoptosis is associated with nuclear DNA fragmentation, we examined AI with the TUNEL-POD assay, which detects apoptotic cells in situ. A fluorescence method was used to determine the apoptotic rate pre-transplantation in the AI in the young and aged groups. Only rare apoptotic cells were observed in young group (Figure 6A-C), while the neuronal apoptotic index was significantly increased in the AI of the aged group (Figure 6D-F). The young group showed no differences before and after transplantation for all tissues examined (Figure 6G-I), while there were significantly fewer TUNEL-positive cells after transplantation in the aged group (Figure 6J-L). The apoptotic rate in the AI of aged group postoperation was significantly lower compared to the apoptotic rates preoperation. The apoptotic rate decreased approximately 10% in the aged group (P<0.05) compared to the young group, which showed no difference both before and after transplantation (Figure 5M) (P>0.05).

Discussion

In the current study, we present a novel approach to treat an animal model of presbycusis. In this study, we show that auditory function can be partially restored by intracerebral embryonic NSCs transplantation. This experimental therapy was chosen because of the lack of restorative medicines and effective therapeutics currently available for the treatment of presbycusis and because of the accumulated evidence of the benefit of stem cell
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transplantation for other brain diseases [28-31].

Previous studies about the development of effective therapeutics for presbycusis have shown that treatment is a slow and difficult process. Moreover, some therapeutic advances mainly focus on peripheral degeneration, such as the regeneration and remodeling of hair cells and spiral ganglia. However, degeneration of the CNS, especially apoptosis of the neurons in the AI, plays a major role in presbycusis. Although a series of studies have shown that the replacement and restoration of functional neurons using intracerebral stem cell grafts contributes to behavioral improvements in Parkinson’s disease and stroke, and some approaches have even presented regeneration of peripheral systems [32-34], there are few studies of central presbycusis. In our study, we adapted intracerebral NSCs transplantation as an in vivo bioassay that provides opportunities to study NSCs survival and differentiation and, importantly, allows the evaluation of potential degenerative changes in C57BL/6J mice, which represent an animal model of presbycusis.

We chose the C57BL/6J mice as an ideal model for exploring age-related changes in the central auditory system because they exhibit rapid, high-frequency hearing loss during the first year of life, unlike CBA mice that exhibit minimal hearing loss at 18 months [35]. For a successful transplantation therapy, we sought to utilize embryonic NSCs because they are more readily cultured and passaged, better characterized and not tumorigenic compared to other types of stem cells. Most importantly, previous experience has indicated the survival and migration of NSC grafts and the beneficial effects of these cells in various models of neurodegenerative disease.

We performed TEM and hematoxylin and eosin staining to assess the morphology of the AI prior to transplantation, especially with regard to apoptotic neurons, which were easily detected in the aged group but were rare or not detected in the young group. These observations clarify that the pivotal pathology in C57BL/6J mice over 10 months of age is neuronal apoptosis. Auditory function restoration was assessed by the ABR, with results confirming our hypothesis that embryonic NSCs can significantly decrease the ABR threshold in the aged group 2 and 3 weeks post-transplantation. By contrast, there was no difference in the ABR threshold in the aged group 4 weeks post-transplantation and in the young group at any time post-transplantation. Therefore, embryonic NSCs transplantation can act as an effective approach to ameliorate the auditory ability of C57BL/6J mice with presbycusis. A qualitative immunofluorescence analysis illustrates the graft survival, migration and differentiation into neurons in the AI to replace apoptotic neurons and repair lesioned sites 2 and 3 weeks post-transplantation. Nevertheless, the grafted cells gradually disappeared by the 4th week post-transplantation. This disappearance is possibly due to the long maturation process that embryonic NSCs or their differentiated cells must undergo in vivo, as reported in studies on implanted human NSCs that show a significant

**Figure 5.** Survival and migration of grafted NSCs in vivo. A. Nuclei of grafted NSCs, pre-labeled with DAPI. B. differentiated neurons of the grafted cells not only surround the needle track but also enter the pinhole to restore the injury (stained green with β-III-tubulin).
reduction in the 4th week similar to the results found in previous studies [24, 36, 37]. Meanwhile, the deceased apoptotic level of the aged group after transplantation but not in the young group supports the cause of the variation in auditory function. However, cell counts and apoptotic rates were estimated mainly according to the following principles [38-40]: 1) condensation of chromatin and cytoplasm (apoptotic cells); 2) cytoplasmic fragments with or without condensed chromatin (apoptotic bodies); and 3) chromatin fragments (micronuclei). On the other hand, none of the apoptotic neurons had excellent staining for hematoxylin.

The values represent percentages relating to a minimum of 1,000 counted cells. Considering these results, we proposed that embryonic NSCs transplantation elevated the auditory function of the aged group after the transplantation for 2 and 3 weeks by replacing and restoring the apoptotic neurons in the AI, while there is likely no apparent change post-transplantation by the 4th week due to the reductions in the differentiated grafted cells.

Recently, several studies have reported that engraftment of stem cells or their derivatives, such as dopamine (DA) neurons, might replace

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**Figure 6.** Apoptosis levels are determined by the TUNEL-POD method; Nuclei were stained with DAPI (blue); B. only rare apoptotic cells were observed in the young group; E. Of all tissues examined, there is no distinct change in the tissues of the young group post-transplantation; H. the neuronal apoptosis index was significantly increased in the AI of the aged group; K. The aged group showed significantly fewer TUNEL-positive cells post-transplantation; L. Neuronal apoptosis index in the AI; M. Statistical analysis of the apoptotic rate in the AI between the young group and the aged group, all of the apoptotic neurons were labeled with green fluorescence. All values are expressed as mean±SEM. *P<0.05 versus pre-operation. n=9 for the young group; n=9 for the aged group.
lost neurons and induce functional improvement by releasing therapeutic molecules that are neuroprotective or modulate inflammation [17, 41, 42]. In a recent study of Parkinson’s disease, pluripotent stem cells (PSC)-derived DA neurons that efficiently engraft in three host species, including NOD-SCID IL2Rg-null mice, Sprague Dawley rats and rhesus monkeys, demonstrate excellent graft survival and subsequent improvement of behavioral outcomes as detected by electrophysiological data [17]. Another case of stem cell-based therapy for Parkinson’s disease also showed that DA neurons that were differentiated from induced pluripotent stem (iPS) cells transplanted in vivo displayed cell development, neuronal maturation and neuronal cell survival and allowed the evaluation of potential degenerative changes [28]. In addition, there are NSCs in the brain that generate new neurons and glial cells in response to neurodegeneration [16, 42]. In our view, despite the achievements in the treatment of the degenerative diseases described above, presbycusis is more suitable for stem cell therapy because of its relatively better environment for graft survival without inflammation compared to stoke and rare complications concerning of Parkinson’s disease after regeneration therapy.

To our knowledge, our research is a pioneer study in central presbycusis. Future studies must face the challenge of finding the mechanisms underlying the observed improvements [16]: (1) immunomodulation, trophic actions and neuroprotection; (2) production of cells that are mechanically and structurally compliant with native tissues and integration of the transplanted tissue with the native tissue without immunological rejection; and (3) the correlation between the grafted cells and the native cells and how responses to downstream networks affect the computations of behavioral functions [43]. Clinical studies with these cells will not be possible until more solid preclinical experiments have been conducted.

In conclusion, presbycusis is an ideal model for NSCs regeneration therapy because of the lack of inflammation in its environment and its rare complications. Furthermore, an analysis of the AI demonstrates high levels of neuronal apoptosis in the aged group that decrease after the implantation of embryonic NSCs. We also determined the survival, migration and differentiation of the NSCs grafts. An electrophysiology data analysis demonstrates an enhancement of auditory function. Taken together, our results suggest that auditory function can be recovered by the replacement and restoration of the apoptotic neurons using regeneration therapy.

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Disclosure statement

All of the authors declare that there are no conflicts of interests during the process of research and publication.

All animal work was approved by our Institutional Animal Care and Research Advisory Committee and studied according to institutional guidelines.

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