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Original Article

Different characteristics of auditory neuronal stem cells derived from different regions of embryonic rats in vitro

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Abstract: In severe cases of sensorineural hearing loss where the numbers of auditory neurons are significantly depleted, stem cell-derived neurons may provide a potential source of replacement cells. In this manuscript, we examined the distinct differentiation of NSCs from different regions of embryonic brain respectively. Two types of NSCs were isolated from E13-15 auditory cortex and otocyst of rats. The neuronal differentiation of NSCs and the electrical function of differentiated neurons were examined in two groups. There were more neuronal marker β-tublin III positive cells in NSCs from auditory cortex than those from otocyst. However the differentiated neurons whether differentiated from auditory cortex or otocyst both expressed sensory neurons specific marker Brn3a protein and exhibited biological electrical reactivity after stimulated by substance P. Therefore, these findings indicated that two different source of NSCs could differentiate into functional mature sensory neurons, among which NSCs from auditory cortex have the tendency to differentiate into more neurons than those from otocyst finally.

Keywords: Auditory cortex, otocyst, neural stem cells, differentiation

Introduction

Deafness is the most common disability especially in elder population over 65 years. As a consequence of deafferentation due to the sensory cell loss or degeneration, death of auditory nerve cells or spiral ganglion cells (SGC) may occur over time. Sensory cell replacement has been found to occur spontaneously in both the vestibular and auditory sensory epithelium, but the injured auditory neurons were difficult to regenerate [1, 2].

Numerous experiments have reported the rescue of auditory neurons from sensori-neural hearing loss-induced degeneration by exogenous infusion of neurotrophins. Although significant survival is observed during the delivery of neurotrophins into the cochlea, this promoting effect is lost immediately following the cessation of treatment. In addition, the long-term application of exogenous neurotrophins to the cochlea using a pump-based delivery system is problematic, due to the increased risk of infection, which is currently used for delivery in experimental animals [3-5].

Given these difficulties, cell-based therapies are presently being investigated as a long-term solution to auditory neuron degeneration in the deafened mammalian cochlea [6, 7].

Neural stem cells (NSCs), located at various sites of the CNS, are able to proliferate in vitro and form neurospheres. NSCs are multipotent cells capable of differentiating into neurons, astrocytes and oligodendrocytes. So NSCs are promising candidates for cochlear cell-based therapy as they have the potential to provide large numbers of replacement neurons to the degenerating auditory nerve [8, 9]. But the characteristic of NSCs derived from different auditory related regions is not clear. In this experiment, the growth, differentiation and function of NSCs from embryonic otocyst and auditory cortex were examined respectively.

Materials and methods

Cell preparation

All experiments were approved by the animal care and experimental committee of the Third

Medical Military University. Primary culture of auditory NSCs was prepared from embryos (E13-15) of Wistar rats as described previously [8]. Briefly, the tissue was rapidly dissected from otocyst and auditory cortex respectively and placed into 3 ml tubes containing 0.25% trypsin. The tissues were then mechanically dissociated into single-cell suspension. Cell number and viability were assessed by staining with 0.4% trypan blue. Single-cell suspensions were then transferred to growth medium consisting of NB + 2% B27 supplemented with 20 ng/ml human recombinant basic fibroblast growth factor (bFGF, Gibico Invitrogen, USA), 20 ng/ml of epidermal growth factor (EGF, Gibico Invitrogen, USA) at 1×10^6 cells/ml. The cells were plated into culture flasks and maintained under a humidified atmosphere of 5% CO_2 in air at 37°C. After 5 days *in vitro*, the neurospheres were dissociated into single-cell suspension and seeded onto 96-well plates at 1-2 cells per well. The neurosphere subcultures were digested and another passage was performed as before. The cell passage protocol was performed every 6 days to obtain neurospheres originating from a single primary cell. Secondary or tertiary neurospheres were used for subsequent identification experiments through 1:400 mouse anti rat nestin antibody [9]. For the differentiating experiments, cells from different regions were plated at a density of 5×10^4 cells/cm^2 in NB + 2% B27 + 2% FBS medium. After 24 h, media were replaced with serum -free control media (NB + 2% B27, Hyclone). Media were replaced every other day and cells were allowed to differentiation for 7-10 days [10].

Immunocytochemistry identification of differentiated cells

The differentiated cells from NSCs were characterized by certain antibodies, including rabbit anti-rat glial fibrillary acidic protein (GFAP; IgG, 1:400; Sigma-Aldrich, USA) which is a specific marker for astrocyte, and the specific neuronal cell marker: mouse anti-rat β-tubulin III antibodies (1:800; IgG, Sigma-Aldrich, USA). Rabbit anti-rat Brn3a (IgG, 1:800, Abcam) was applied for identification of sensory neurons. Typical immunocytochemical procedures were applied for identification the differentiated cells. Briefly Cells were cultured on coverslips and fixed for 20 min in 4% PFA in PBS at room temperature for 20 min. For labeling, coverslips were briefly washed with PBS and incubated 20 min in blocking buffer (0.1% Triton + 5% normal goat serum in PBS) at room temperature. Subsequently, the cells were incubated with the above primary antibodies overnight at 4°C. Signals were visualized using either FITC- or Rhodamine-conjugated secondary antibodies (Jacks on Immunoresearch Labs) for 1 h at 37°C. Nuclear counterstaining was done with 300 nM of 4-6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) for 3 min. Positive cells were scanned with a Leica confocal microscope (SP-2, Leica, Germany). For negative controls, the cells were processed by the same immunofluorescent staining technique, but with omission of the primary antibodies [11, 12].

Western blotting analysis

Total cell lysates were prepared by re-suspending 1×10^6 cells in 100 μl Gel Sample Buffer (62.5 mM Tris-HCl, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS and 0.025% bromophenol-blue) and boiled for 5 min. Protein samples were run on polyacrylamide resolving gel (10%) and transferred onto a nitrocellulose membrane by electro-blotting at a constant current of 250 mA. Immunoblotting was performed by incubating the membrane overnight (4·C) with the primary antibodies: mouse anti-rat β-tubulin III (1:800; Sigma-Aldrich), rabbit anti-rat GFAP (1:600; Sigma-Aldrich) respectively and detected using HRP-conjugated secondary antibody. Protein bands were visualized by an enhanced chemical luminescent detection system (Millipore) [10]. The data were normalized by running parallel Western blots with β-actin as an internal control. The optical density was quantified by the Image-Pro Plus 6.0 software. Separate experiments were conducted three times.

Calcium activity in differentiated neurons

The differentiated neuronal cells from embryonic different regions were incubated with 10 nM Fura-3-AM in Ca^{2+}-free D-Hanks medium for 20 min. After washed with 0.1 M PBS for three times, the medium was replaced with NB + 2% B27 medium again. Then the differentiated neuronal cells were observed timely with laser con-focal scanning microscope at xyt model to examine the changes of free Ca^{2+} concentration in differentiated neurons stimulated by 500 nM substance P [12].

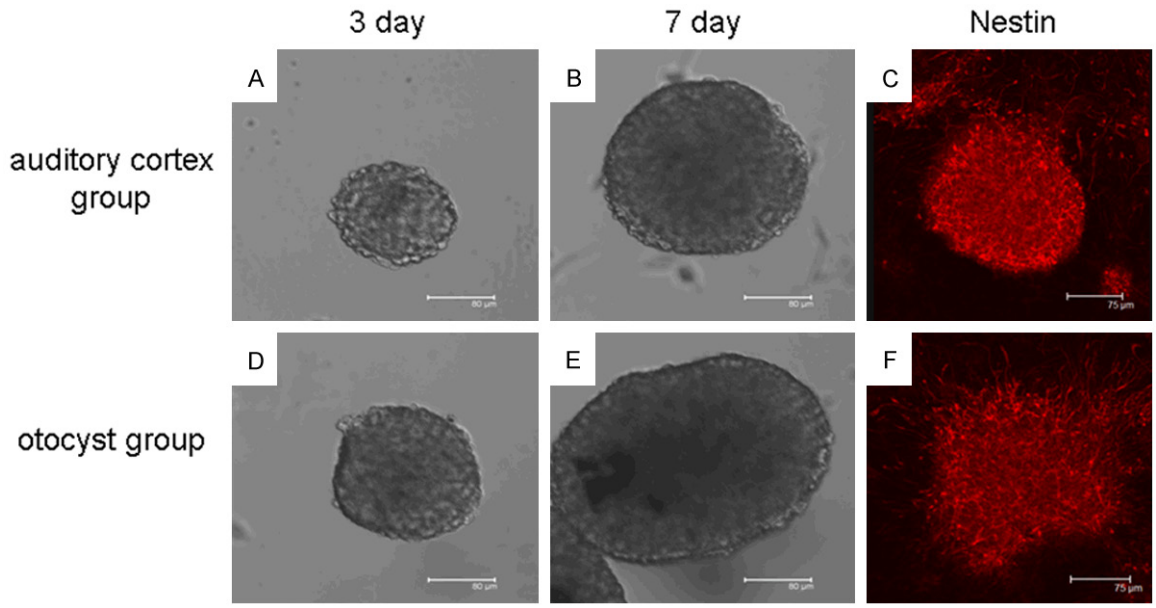

Figure 1. The growth and identification of NSCs from different regions *in vitro*. A, B. Growth of NSCs from auditory cortex at 3 and 7 days. D, E. Growth of NSCs from otocyst at 3 and 7 days. (Scale bar = 80 μm); C, F. Immunocytochemical staining of NSCs with Nestin in two groups (Scale bar = 75 μm).

Statistical analysis

The percentage of positive cells in relation to the total cells was determined in 5 random fields under a 40× objective for each group in five independent experiments. All data were presented as mean ± SD. Statistical analysis of data was performed by a one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

Results

NSCs from different regions have the ability to proliferate and differentiate into different types of neural cells

On the second day after primary cultures, the cells growing as spheres in suspension are NSCs and express the neural stem cell specific marker Nestin. But the diameter of neurospheres from auditory cortex were bigger than those from otocyst at 3 and 7 days after culture (**Figure 1**). At the same time, the NSCs have a multi-differentiation potential which can differentiate into neurons and astrocytes after withdraw of bFGF (**Figure 2A**).

Differentiation of NSCs derived from different regions

To confirm differentiated cells from NSCs in the various culture conditions, specific markers for astrocytes and neurons were used for immunofluorescent staining. It was demonstrated that 7 days after differentiation 67% ± 5.2 of differentiated cells with long and thin dendrites derived from auditory cortex media expressed the neuronal specific marker β-tubulin III, which was significantly greater than those from otocyst (45% ± 4.8 β-tubulin III positive cells). On the other hand, the differentiated cells with short and thick processes expressed the astrocyte specific marker GFAP was less than those from otocyst group (**Figure 2A** and **2B**).

Moreover, western blot analysis confirmed β-tubulin III expression in differentiated neuron of the two media conditions. During the differentiating process, the level of β-tubulin III expression in auditory cortex group was higher than those in the otocyst at 7 days after differentiation. On the contrary, the protein of GFAP in auditory cortex group was apparently less than that in otocyst group at 7 days after differentiation (**Figure 3**).

Brn3a protein expression in differentiated neurons of two groups

The differentiated auditory neurons from NSCs derived from different regions both expressed Brn3a protein, which was the specific marker of sensory neurons. There is no difference in two groups, which indicated the neurons differenti-

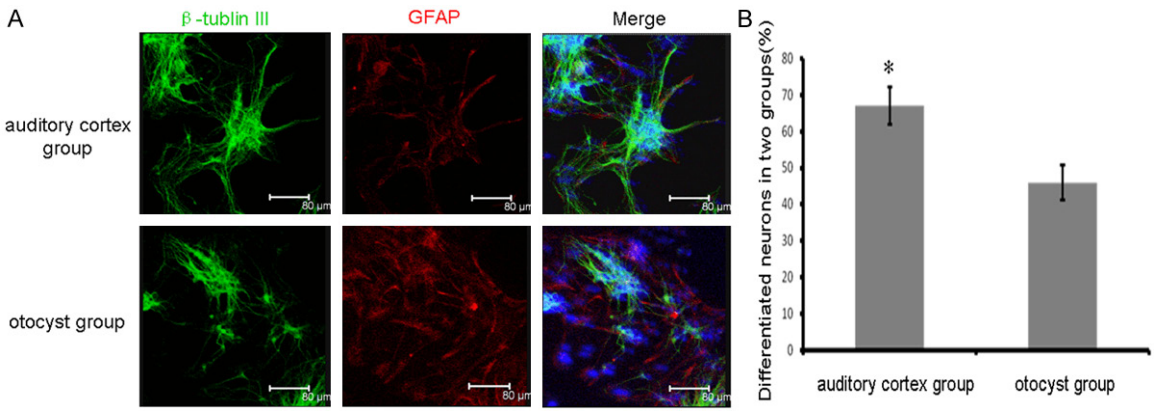

Figure 2. Identification and quantification of differentiated neural cells from different regions *in vitro*. A. Immunocytochemical staining of differentiated neural cells in two culture media. β-tubulin III staining (green) indicates neurons; GFAP staining (red) indicates astrocytes. The nuclei were counterstained with DAPI (blue). (Scale bar = 40 μm). B. Quantification of differentiated neurons in two group. $^{*}P < 0.05$ indicates statistical significance compared with the otocyst group.

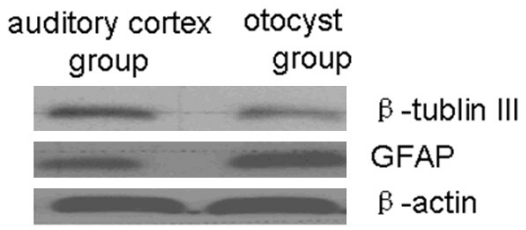

Figure 3. Western blotting analysis of β-tubulin III and GFAP protein in differentiated cells from two different regions of NSCs *in vitro*.

ated from NSCs derived from different regions were of the characteristics of sensory neurons (**Figure 4**).

Calcium activity in differentiated neurons from auditory NSCs derived in different regions

After adding 500 nM substance P into the differentiated auditory neurons from different group, the Ca^{2+} inside the differentiated cells effluxed quickly out of the cytoplasm. The concentration of free calcium which indicated as fluorescence density in cytoplasm significantly decreased and restored the normal level with several seconds as shown in **Figure 5**. However, the concentration of free calcium inside the differentiated neurons from NSCs had no difference between the two groups.

Discussion

Because NSCs have the potential of self-renewal and are capable of multi-lineage differentiation, NSCs has been considered to be a promising ideal candidate to provide large numbers of replacement neurons to the degenerating auditory nerve. However, some studies have shown that the proliferation and differentiation of NSCs located in various regions is entirely different [10, 13]. Therefore, investigating the self-renewal capacity of NSCs derived from different auditory regions of CNS and inducing their directed differentiation into specific neural cell types becomes a major challenge for the treatment of sensori-neural hearing loss [12, 13].

In this experiment, it was indicated that the cells whether from embryonic auditory cortex or otocyst were capable of self-renew and forming spheres that express NSCs specific marker Nestin. Among which the neurospheres from auditory cortex were larger than those from otocyst at the same time point in culture, which indentified that the ability of proliferation in NSCs from auditory cortex is stronger than those from otocyst. But after withdraw of bFGF, the spheres in two groups can both differentiation into neurons and astrocytes respectively, which indicated the cells in two groups are of pluripotent [9, 14].

But as exhibited by immunocytochemistry staining and Western Blot examination, it was indicated the after 7 days differentiation, the number of NSCs distinct differentiating into sensory neurons was greater in auditory cortex group than those in otocyst group. On the other

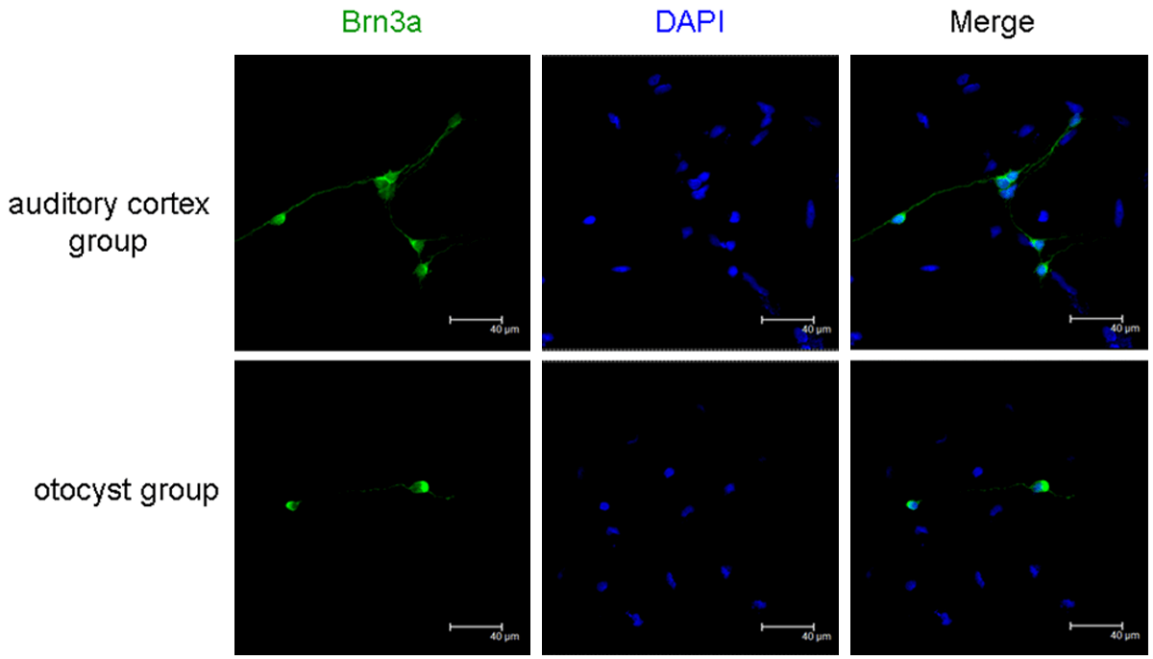

Figure 4. Immunocytochemical staining of differentiated neurons from two regions of NSCs with Brn3a. The nuclei were counterstained with DAPI (blue) (bar = 40 μm).

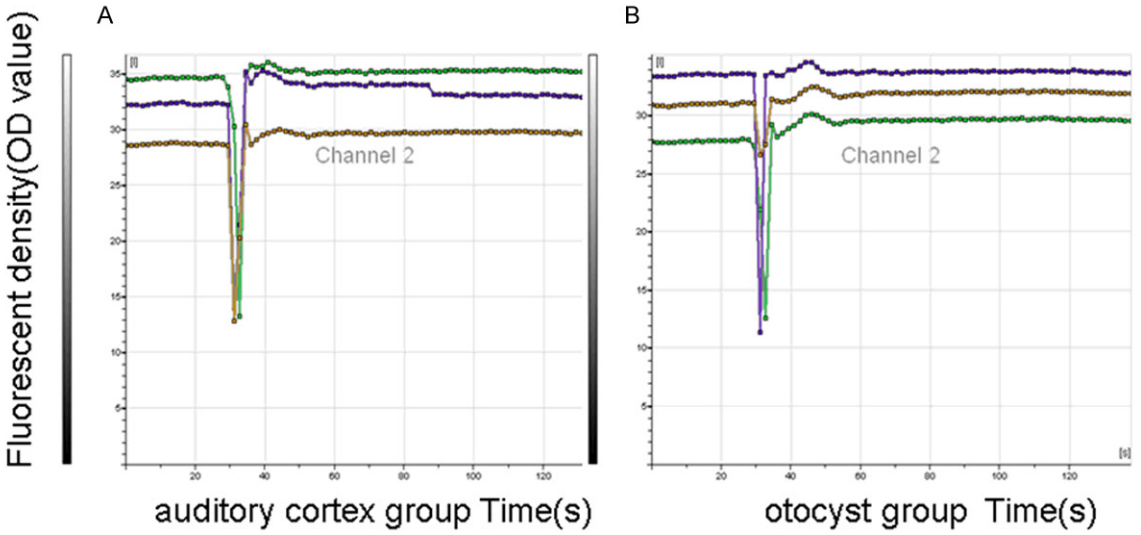

Figure 5. The changes of free calcium ions in cytoplasm of differentiated neurons after stimulated by substance P. A. The curve of free calcium concentration in neurons differentiated from auditory cortex NSCs. B. The curve of free calcium concentration in neurons differentiated from otocyst NSCs. Note: The line represents continuous change of free calcium ions concentration in cytoplasm of one neurons.

hand, the astrocytes differentiated from NSCs in auditory cortex group were less than those in otocyst group. It was shown that the NSCs from auditory cortex have stronger tendency to distinct differentiate into sensory neurons than those from otocyst. So NSCs from auditory cortex may be a candidate for the treatment of auditory neuron degeneration in the deafened in the future.

Further, on the membrane and synapses of the differentiated neurons from NSCs in different

regions can both express Brn3a, which is one of the most important transcript factors and specific marker in sensory neurons development. So the expression of Brn3a in differentiated neurons indicated that the NSCs whether form auditory cortex or otocyst can both differentiated into mature sensory neurons [15, 16].

Furthermore, the free Ca^{2+} inside the cytoplasm of differentiated neurons from two regions conditions can efflux quickly out of cytoplasm and restored at normal level after stimulated by substance P. It was indicated differentiated sensory neurons could receive outside stimulation and produce electric reactivity. As it has been identified, the calcium in neural cells took part in a great of metabolism and biological functional activities. So the sensory neurons differentiated from two regions have basic biological functions [17, 18].

In all, the NSCs whether from embryonic auditory cortex or otocyst can both differentiated into mature sensory neurons, among which NSCs from auditory cortex may have more potent proliferation ability and tendency to differentiate into more neurons. It is suggested NSCs from auditory cortex can be an ideal cell for the treatment of sensori-neural hearing loss. Furthermore, the mechanism and signaling pathway of the difference in distinct differentiation between the two types of NSCs are still to be further investigated [19-21].

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Disclosure of conflict of interest

None.

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