Cell Mol Neurobiol (2015) 35:861–869
DOI 10.1007/s10571-015-0180-z

ORIGINAL RESEARCH

Ginkgo Biloba Extract Enhances Differentiation and Performance of Neural Stem Cells in Mouse Cochlea

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Received: 15 February 2015 / Accepted: 11 March 2015 / Published online: 31 March 2015

Abstract *Ginkgo biloba* extract (GBE) has been widely used for treatment of neural damage and disorders. Neural stem cells (NSCs) hold promise as a treatment of hearing loss caused by neural damage. However, the biological functions of GBE in modulating NSC behaviors in the cochlea are still largely elusive. In this study, we sought to explore the effects of GBE on the differentiation and performance of NSCs from mouse cochlea. Our data showed that GBE treatment promotes cell survival and NSC proliferation. In addition, GBE treatment also increases NSC differentiation to neurons and enhances the performance of mature neural networks evident by the increased frequency of calcium oscillation. Moreover, neurite outgrowth is also dramatically increased upon GBE treatment. Overall, our study demonstrates the positive regulatory role of GBE in NSC proliferation and differentiation into functional neurons in vitro, supporting the potential therapeutic use of GBE in hearing loss recovery.

Keywords *Ginkgo biloba* extract · Neural stem cells · Differentiation · Cochlea

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Introduction

The *Ginkgo biloba* leaf is one of the best explored Chinese herbal medicines. It contains 22–24 % Ginkgo flavonoids and 5–7 % terpene trilactones (gingolides and bilobalide). The main ingredients of *Ginkgo biloba* extract (GBE) EGb 761 are ginkgolid A, B, C and bilobalide (EGb 761: *Ginkgo biloba* extract, Ginkor 2003). The majority of the studies on the biological activity of GBE were focused on neural modulatory activities. Clinical trials indicated the efficacy of GBE in treating cognitively impaired patients (Itil et al. 1996). Patients receiving GBE showed improvements in cognitive functions, such as memory retention, learning rate, speed of response and attention (Hofferberth 1994; Semlitsch et al. 1995). Moreover, GBE was reported to have anti-tumor, anti-aging, hepato-protective and cardio-protective properties (Tunali-Akbay et al. 2007; Zhang et al. 2008). Recently, GBE was demonstrated to regulate different neurotransmitter systems, where it can strongly inhibit monoamine oxidase A and synaptosomal uptake of DA, 5-HT and norepinephrine (Fehske et al. 2009; Ramassamy et al. 1992). GBE also displays free radical scavenger activity and has neuroprotective and anti-apoptotic properties, such as inhibition of amyloid-β neurotoxicity and protection against hypoxic challenges and increased oxidative stress (Kampkotter et al. 2007; Wu et al. 2006). In addition, GBE was shown to act through heme oxygenase 1 to exhibit neuroprotective functions in ischemic reperfusion brain injury in mouse (Saleem et al. 2008; Nada and Shah 2012; Nada et al. 2014). These findings suggested that apart from antioxidant, neuritogenic and angiogenic properties, GBE also exerts protective functions following stroke.

Neural stem cells (NSCs) are multipotent cells that can self-renew, proliferate and in turn differentiate into neurons and

glial cells (Gage 2000). Recent studies indicate that NSCs can be activated after neuronal injury and can migrate to the injured sites to replace the lost neurons, suggesting its potential therapeutic use in the treatment of neural damage and neurodegenerative diseases (Nakatomi et al. 2002; Russo et al. 2011). However, the mechanisms controlling NSC self-renewal and differentiation are still largely unknown. Primary NSC cultures provide an in vitro model to study the development of the nervous system. Protocols to isolate and expand these cells in vitro have recently been developed in replacement cell therapies (Gage 2000; Chaddah et al. 2012). Early NSCs can be derived from embryonic stem cells (ESCs) and pluripotent stem cells, and the derived NSCs proliferate in response to the mitogens, forming small clusters of cells called neurospheres, which in turn further develop into an in vitro neural network (Chaddah et al. 2012).

In the vertebrate inner ear, hair cells are the sensory receptors of both the auditory and vestibular system. In mammals, the auditory hair cells are located in the cochlea. Neurons of the auditory or vestibulocochlear nerve innervate cochlear and vestibular hair cells. Damaged hair cells result in decreased hearing sensitivity and, in severe cases, complete hearing loss (Yuan et al. 2014). Hearing loss can also be caused by primary degeneration of spiral ganglion neurons (SGNs) or secondary degeneration of neurons after hair cell loss. Therefore, replacement of these neurons is the first step to restore sensory neuronal hearing loss. Inner ear stem cells are capable of forming neurospheres in vitro and differentiate into hair cells and neurons, and a successful cochlea transplant derived from inner ear stem cells has been reported recently (Hu et al. 2005). Several stem cells are available as the source: neural stem cells (NSCs), dorsal ganglion neurons, bone marrow stromal cells and ESCs. In vitro studies using co-culture of ESC-derived neural progenitors with cochlear or vestibular sensory epithelial cells have demonstrated they are able to further differentiate into bipolar neurons and promote neurite outgrowth attaching to sensory hair cells (Coleman et al. 2007; Matsumoto et al. 2005). Further studies are required to understand the cell biology of NSC differentiation in order to advance the technique using inner ear stem cell therapy to treat hearing loss.

In this study, we aimed to investigate the role of GBE in the cochlear NSC self-renewal and differentiation process. Our results showed that GBE significantly increases NSC cell survival, proliferation and neurosphere formation in vitro. Furthermore, we found that GBE also improved differentiation of NSCs to neurons and promoted the formation of the neural network as well as its functions, evident by more complex neurite outgrowth and frequent calcium oscillations. Our results reveal the importance of GBE in the differentiation of cochlear NSC to neurons, thus supporting the role of GBE as a potential therapeutic agent in the treatment of hearing loss.

Materials and Methods

Mouse Cochlea NSC Isolation and Culturing

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Eye and ENT Hospital of Fudan University. The IACUC committee members at the Eye and ENT Hospital of Fudan University approved this study. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Early postnatal (P1–3) Balb/c mice were decapitated, and the temporal bone was dissected out after removal of the brain and transferred into ice-cold Hank's balanced salt solution (Invitrogen). The otic capsule was freed from the otic bulla, opened and removed to visualize the membranous labyrinth of the cochlea. The cochlear duct (organ of Corti, spiral ligament and stria vascularis) was microdissected from the modiolus where the spiral ganglion resides. For isolation of sphere-forming stem cells, we used a modified neurosphere assay protocol that was established previously (Diensthuber et al. 2009). In brief, spiral ganglia were enzymatically digested in 0.125 % trypsin/EDTA in phosphate-buffered saline (PBS) for 5 min at 37 °C. This process was terminated by a cocktail of 10 mg/ml soybean trypsin inhibitor (Worthington, Lakewood, NJ) and 1 mg/ml DNase I (Worthington) in Dulbecco's modified Eagle's medium and Nutrient Mixture F-12 (DMEM/F12; Sigma) followed by trituration with pipette tips to achieve mechanical dissociation and the use of a 70-μm cell strainer (BD Falcon) to obtain a homogeneous single-cell suspension. The cells were then cultured in poly-HEMA-coated suspension culture six-well plates (Sigma) in a volume of 2 ml of DMEM/F12 supplemented with N2 (Invitrogen), B27 (Invitrogen), 20 ng/ml epidermal growth factor (EGF), 10 ng/ml basic fibroblast growth factor (bFGF), 50 ng/ml insulin-like growth factor-1 (IGF-1), 50 ng/ml heparan sulfate (all growth factors from Sigma-Aldrich) and 50 μg/ml ampicillin. Analysis of sphere size was determined by measuring the maximum sphere diameter.

NSC Differentiation

Neurospheres were harvested after 3–5 days and mechanically dissociated following enzymatic digestion by treatment with Accumax (PAA Laboratories) to achieve a

20,000 cell/ml suspension. Cells were then cultured in differentiation conditions by transferring them to plastic 4-well tissue culture plates with a 0.1 % gelatin-coated surface containing 100 µl of DMEM/F12 per well supplemented with N2, B27, 50 ng/ml brain-derived neurotrophic factor (BDNF), 50 ng/ml NT-3 (both from R&D Systems) and 50 µg/ml ampicillin. Three quarters of the medium was replaced by fresh, prewarmed medium every other day. Analysis of marker expression (MAP-2, Nestin) in cells was done after attachment of the single cells to tissue culture plates. Immunofluorescence and Western blot analysis of differentiated cultures were performed after a period of 8–10 days in vitro.

GBE Treatment

GBE was purchased from Fuwei Ginkgo Co., Ltd. (Pizhou City, China). Culturing and differentiation media used in GBE-treated experimental groups were supplemented with GBE at assay-specific concentrations, and the same media without GBE were used as control treatment. All media were exchanged every 5–7 days to ensure the availability of growth factors and GBE components in the culture.

Immunofluorescence

Cells were fixed with 4 % paraformaldehyde for 30 min at room temperature. After permeabilization with 0.1 % Triton X-100 for 15 min and blocking with 5 % BSA/PBS for 20 min, cells were stained with antibodies against nestin or MAP-2 (Abcam) in 5 % BSA/PBS at room temperature for 2 h. After washing, cells were incubated with FITC- or rhodamine-conjugated secondary Abs (Santa Cruz) for 1 h. Nuclei were visualized with DAPI. Cells were then imaged by Carl Zeiss scanning confocal microscope.

Western Blot

Cells were washed twice with ice-cold PBS and lysed in a RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 1 % Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/ml each of aprotinin and leupeptin, and 1 mM Na_3VO_4. Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were immunoblotted with anti-MAP-2 or anti-GAP-43 antibodies (Sigma) for 2 h, followed by HRP-conjugated secondary antibody (Santa Cruz) for 1 h. After washing, specific bands were visualized using ECL detection reagents.

MTT Assay

Cells grown in 96-well plates were treated with different concentrations of GBE (25–100 mg/l) for 24 h. Then 10 µl of MTT (5 mg/ml) was added to each well, and the plate was incubated at 37 °C in the dark for 4 h. Then the media along with MTT were removed, and the formazan crystals were solubilized by adding DMSO (100 µl/well). Finally, the reduction of MTT was quantified by reading the absorbance at 570 nm by a microplate reader. Effects of the GBE on cell viability were calculated using cells treated with PBS as control.

BrdU Incorporation Assay

NSC proliferation was assessed by bromodeoxyuridine (BrdU) incorporation assay using the Proliferation Assay Kit (Millipore). Cells were seeded in 96-well plates (5000 cells/well) and treated with the indicated concentrations of taurine for 48 h. BrdU was added during the last 2 h of the incubation period. The assay was performed according to the manufacturer's instructions.

Calcium Imaging

Cells were washed twice with standard solutions (contain 150 mM NaCl, 5 mM KCl, 2 mM CaCl, 1 $MgCl_2$, 10 mM HEPES, 10 mM D-glucose, pH 7.3) and then loaded with 2.5 µM Fluo-4-AM (Dojindo Laboratories) and pluronic F-127 (Sigma). After 45-min incubation at 37 °C, Fluo-4-AM was removed, and then cells were incubated in standard solution for another 30 min. Cells were then imaged by a Carl Zeiss scanning confocal microscope. The excitation and emission wavelengths for Fluo-4-AM were 488 and 510 nm, respectively. The frequency of the oscillations was calculated as the number of spikes per min. The relative fluorescence amplitudes of the calcium spikes $\Delta F/F$ were measured by normalizing the fluorescence for each cell to the average fluorescence intensity.

Statistical Analysis

Data were presented as the mean ± SEM from three independent experiments. Statistical analysis between groups was performed by unpaired Student's t test, and p values <0.05 were considered significant.

Results

GBE Improved Viability of Mouse Cochlear NSCs In Vitro

In our study, we used EGb 761, a standardized and well-defined product of GBE, to treat the cells and determine the effect on NSC differentiation. Cochlear NSCs were isolated as described in the "Materials and Methods" section.

To confirm the stemness of the cultured cells, immunofluorescence was performed by using an antibody against the neuron progenitor marker nestin (Rietze et al. 2001), and the results showed that the majority of the cells were nestin positive (Fig. 1a), which indicated these cells had the neural progenitor status. Next, the effect of GBE on cell viability was determined by treating the cells with different concentrations of GBE for 24 h. Anti-tubulin antibodies were used to visualize the cell morphology, and an MTT assay was conducted to determine the cell viability. Both the morphological and MTT results showed that the NSC cell viability was increased from 0 to 50 mg/l GBE treatment, while higher concentrations of GBE treatment from 75 to 100 mg/l significantly decreased the cell viability (Fig. 1b–f). The optimal dosage of GBE was 50 mg/l, which exhibited the highest viability increase compared to control treatment (Fig. 1g). With the BrdU incorporation assay (Fig. 1h), we confirmed that the proliferation of cultured NSCs is also significantly improved in response to various concentrations of GBE treatment. Moreover, 50 mg/l of GBE again exerted a better proliferation effect on cultured NSCs. Therefore, this optimal dosage was used in all of the following experiments.

GBE Treatment Improved the Capability of Neurosphere Formation

Next, we investigated the role of GBE in NSC neurosphere formation. Neurospheres in an in vitro culture system are composed of free-floating clusters of NSCs, and the number of neurospheres and their size can be used as a gage to assess the growth of NSCs in vitro. For the neurosphere formation assay, growth factors were added to the neural stem cell basal media, supplemented with or without 50 mg/l GBE in GBE or control-treated experimental groups (see "Materials and Methods" section). Our results indicated that 3 days of GBE treatment significantly increased the size of neurospheres (Fig. 2a). This gap in neurosphere diameter in the presence or absence of GBE continued to increase with prolonged exposure to GBE (Fig. 2b). The neurosphere number was also increased significantly in the presence of GBE, especially after longer exposure (Fig. 2c). These results suggested that GBE treatment significantly enhanced the capability of neurosphere growth and formation.

GBE Enhances the Capability of NSCs to Differentiate into Neurons

NSCs have the potential to differentiate into neurons and form a functional neural network, and a successful stem cell-derived cochlear transplant has been reported. To investigate the effects of GBE on NSC differentiation into neurons, we performed immunofluorescence against microtubule-associated protein-2 (MAP-2), a neuron-specific cytoskeletal protein, in a 2-week NSC culture in the presence and absence of GBE. As a result, the number of MAP-2

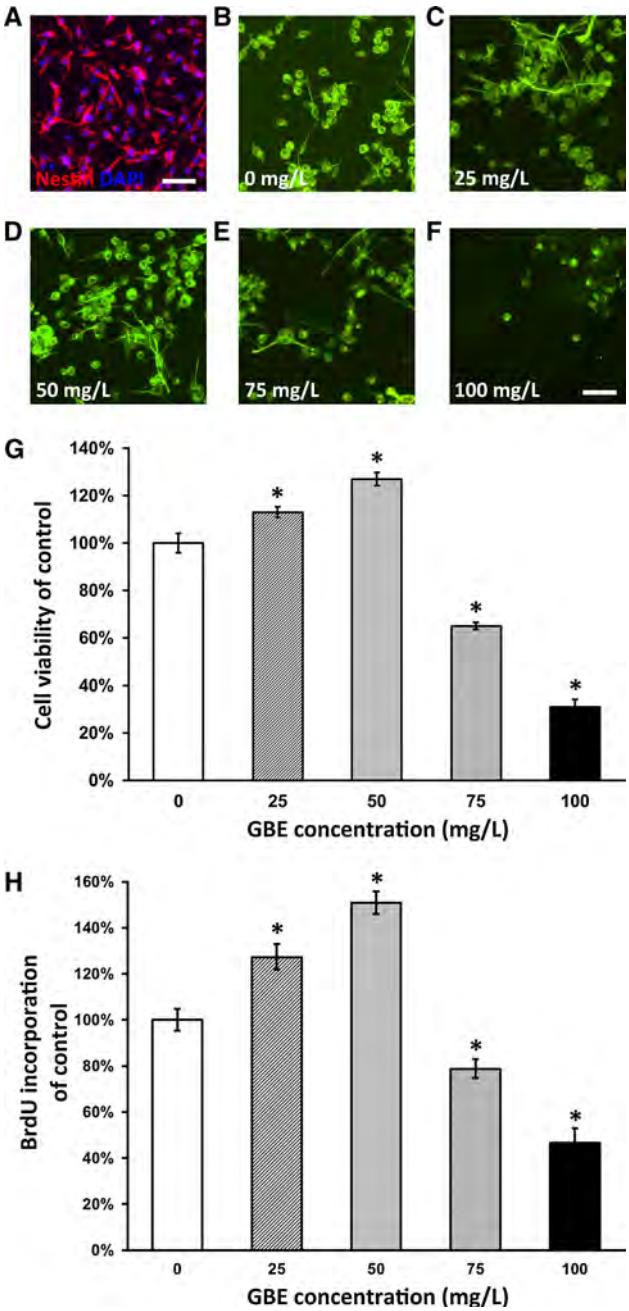

Fig. 1 GBE improved the viability and proliferation of mouse cochlea NSCs in vitro. **a** The identification of NSCs, revealed by immunostaining against nestin (*red*), and nuclei were stained by DAPI (*blue*). **b–f** Representative photomicrographs of NSCs exposed to different concentrations of GBE treatment (0–100 mg/l); cells were stained by tubulin. Relative cell viability and proliferation of the NSCs to the control (0 mg/l) following GBE treatment, measured by MTT assay (**g**) and BrdU incorporation assay (**h**). *Scale bar* 100 μm. Data are presented as the mean ± SEM. $*p < 0.05$ versus control (Color figure online)

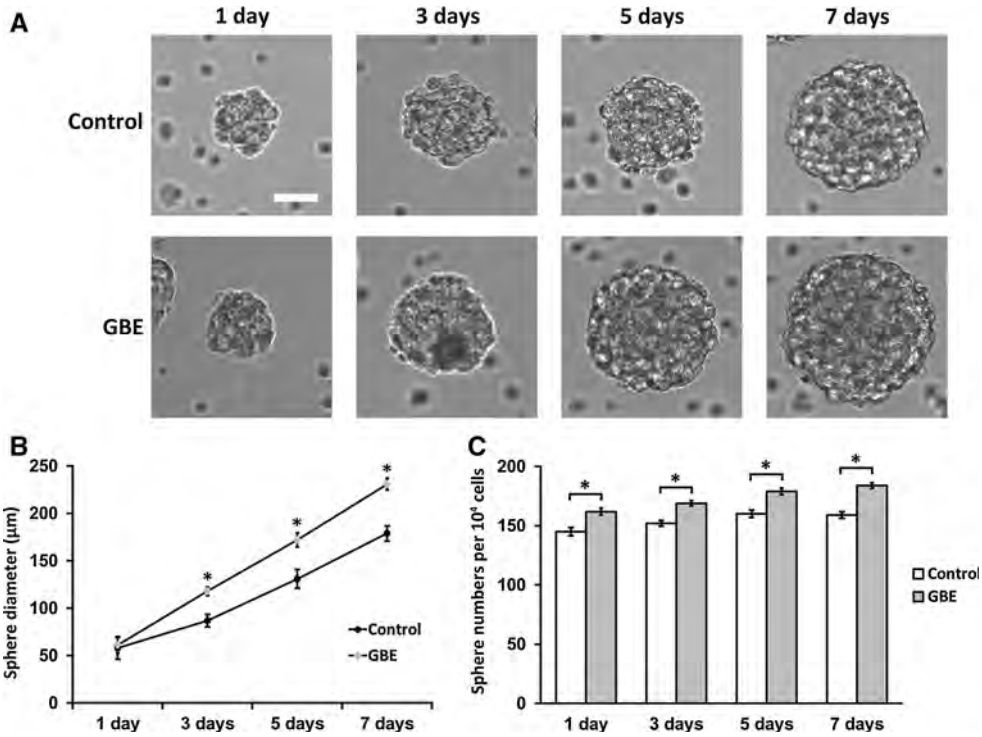

Fig. 2 GBE treatment improved the capability of neurosphere formation. **a** Representative images of neurosphere formation in the cultures at different time points after GBE treatment compared to control. **b** The average sphere diameter (μm) was measured at the indicated time points after GBE treatment. **c** The number of neurospheres generated per 10^4 NSCs was measured at the indicated time points after GBE treatment. *Scale bar* 50 μm. Data are presented as mean ± SEM. $*p < 0.05$ versus control

positive cells was significantly increased by 2-week GBE treatment (Fig. 3a, b). Western blotting results also indicated a prominent increase of the MAP-2 protein level with GBE treatment (Fig. 3c).

Neuron cells have characteristic treelike surface protrusions called dendrites, which physically contact other neurons to form a neural network and propagate electrical stimulations throughout the dendritic tree. Dendrites play a critical role in integrating synaptic inputs and determining the extent to which action potentials are produced by the neuron (Urbanska et al. 2008); therefore, we next examined dendritic tree formation to assess whether GBE also affects neurite outgrowth. As indicated by MAP-2 immunostaining, we found that although GBE has no effect on the number of primary dendrites of the differentiated neurons (Fig. 4a, b), the number of dendritic end tips was nevertheless significantly increased by GBE treatment (Fig. 4c). Moreover, the average length of the neurite was also significantly increased in GBE-treated NSC culture (Fig. 4d). We further examined the expression of growth-associated protein-43 (GAP-43) in either control- or GBE-treated NSC-differentiated neuron culture. GAP-43 is a neuronal protein associated with neurite outgrowth and has also been shown to be an efficient marker for the presence of neuronal growth cones (Meiri et al. 1986). We found that the GAP-43 protein level was greatly increased in GBE-treated culture compared to controls, confirming our initial conclusion that GBE treatment promoted neurite outgrowth (Fig. 4e). Taken together, these results demonstrated that GBE treatment significantly promotes NSC differentiation to neurons and the neurite outgrowth in the in vitro cochlear NSC culture.

GBE Enhanced the Spontaneous Ca^{2+} Oscillations in NSC-Differentiated Neural Networks

Calcium plays a major role in controlling neural excitability, in that calcium oscillations reflect the transduction activity of the neurons, which can be seen as an indicator of overall neural excitability in the neuronal network. To assess the effect of GBE on calcium signaling in the NSC-differentiated neural network, we traced the spontaneous calcium oscillations in both GBE- and control-treated cultures. Our results indicated that GBE treatment remarkably increased the number of neurons generating spontaneous calcium oscillation in the NSC-differentiated neural network (Fig. 5a, b). Furthermore, we found that the frequency of the dendritic calcium spikes was also significantly increased by GBE treatment (Fig. 5c). These results indicated that GBE treatment profoundly elevated the calcium signaling in the culture, strongly suggesting that GBE is able to escalate the excitability of the cochlear NSC-derived neural network.

Discussion

In this study, we examined the effect of GBE on the proliferation, differentiation and neurite outgrowth of NSC-differentiated neurons. We found the optimal dosage of GBE to enhance NSC viability and proliferation. Our

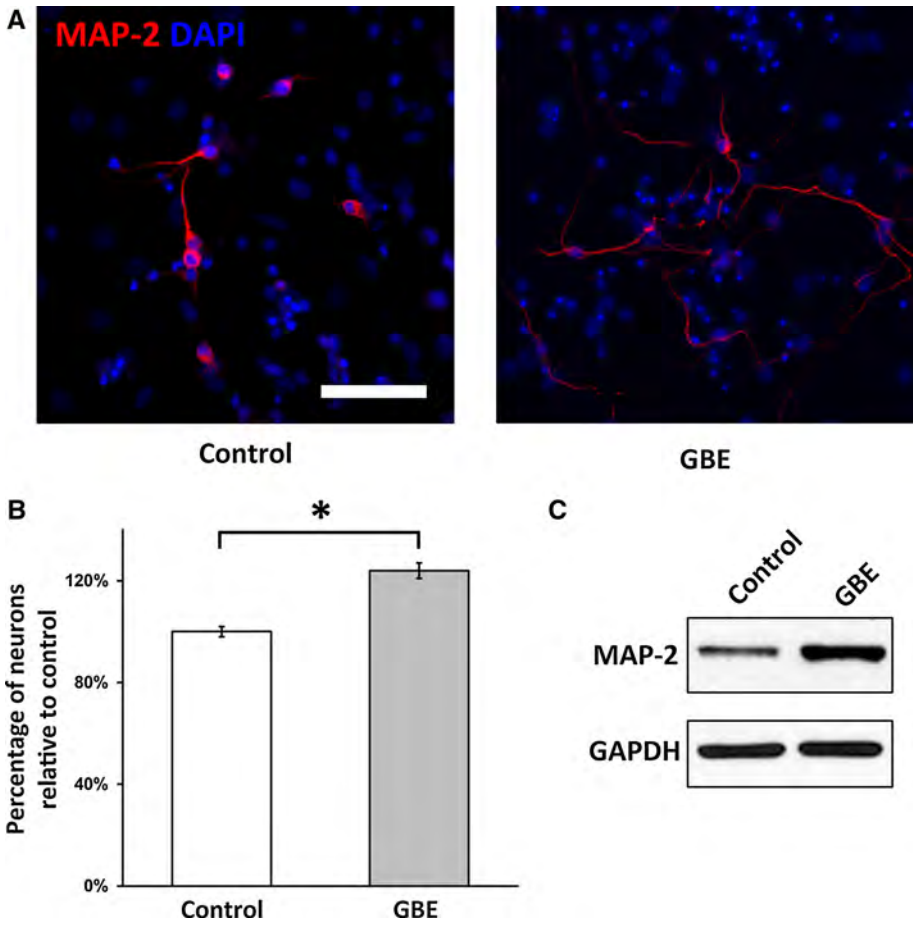

Fig. 3 Differentiation of NSCs to neurons after 2 weeks' culture. **a** Control- or GBE-treated cultures in vitro. Neurons were stained by MAP-2 (*red*), and nuclei were stained DAPI (*blue*). **b** Density of neurons in culture relative to the control after GBE treatment. **c** Western blot analysis of MAP-2 expression in control- or GBE-treated cultures. *Scale bar* 100 μm. Data are presented as the mean ± SEM. $*p < 0.05$ versus control (Color figure online)

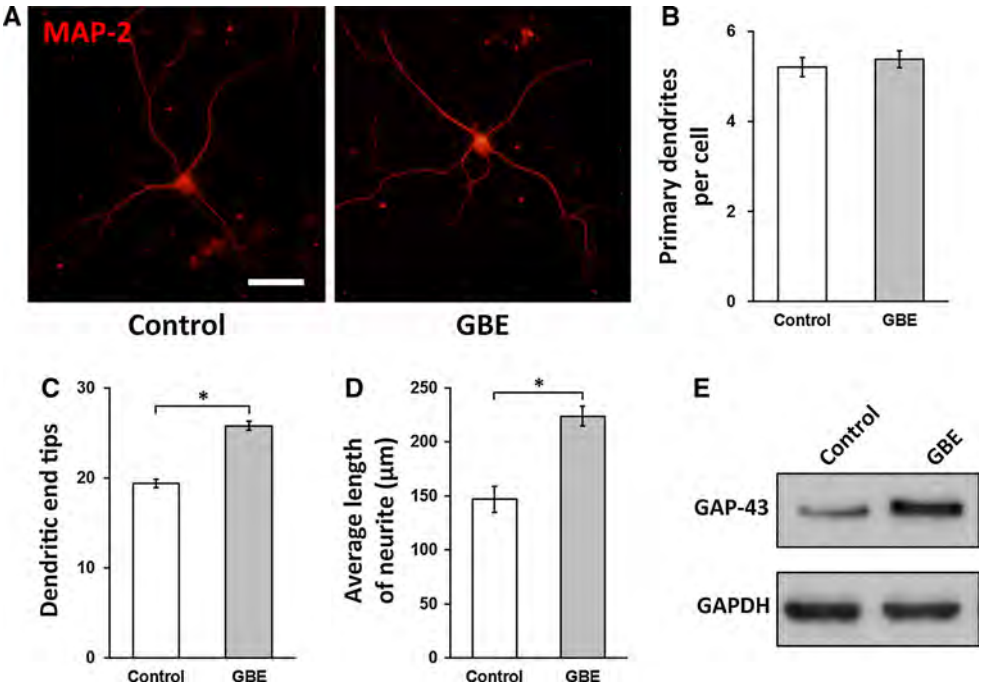

Fig. 4 GBE treatment promoted neurite outgrowth. **a** Representative images of NSC-differentiated neurons after culturing for 14 days in control- and GBE-treated cultures. Cells were immunostained by MAP-2 (*red*). Statistical analysis of the number of primary dendrites per cell (**b**), number of dendritic end tips (**c**) and average length of neurites (**d**) in the experimental groups. *Scale bar* 100 μm. Data are presented as the mean ± SEM. $*p < 0.05$ versus control. **e** Western blot analysis of GAP-43 expression in control- or GBE-treated cultures (Color figure online)

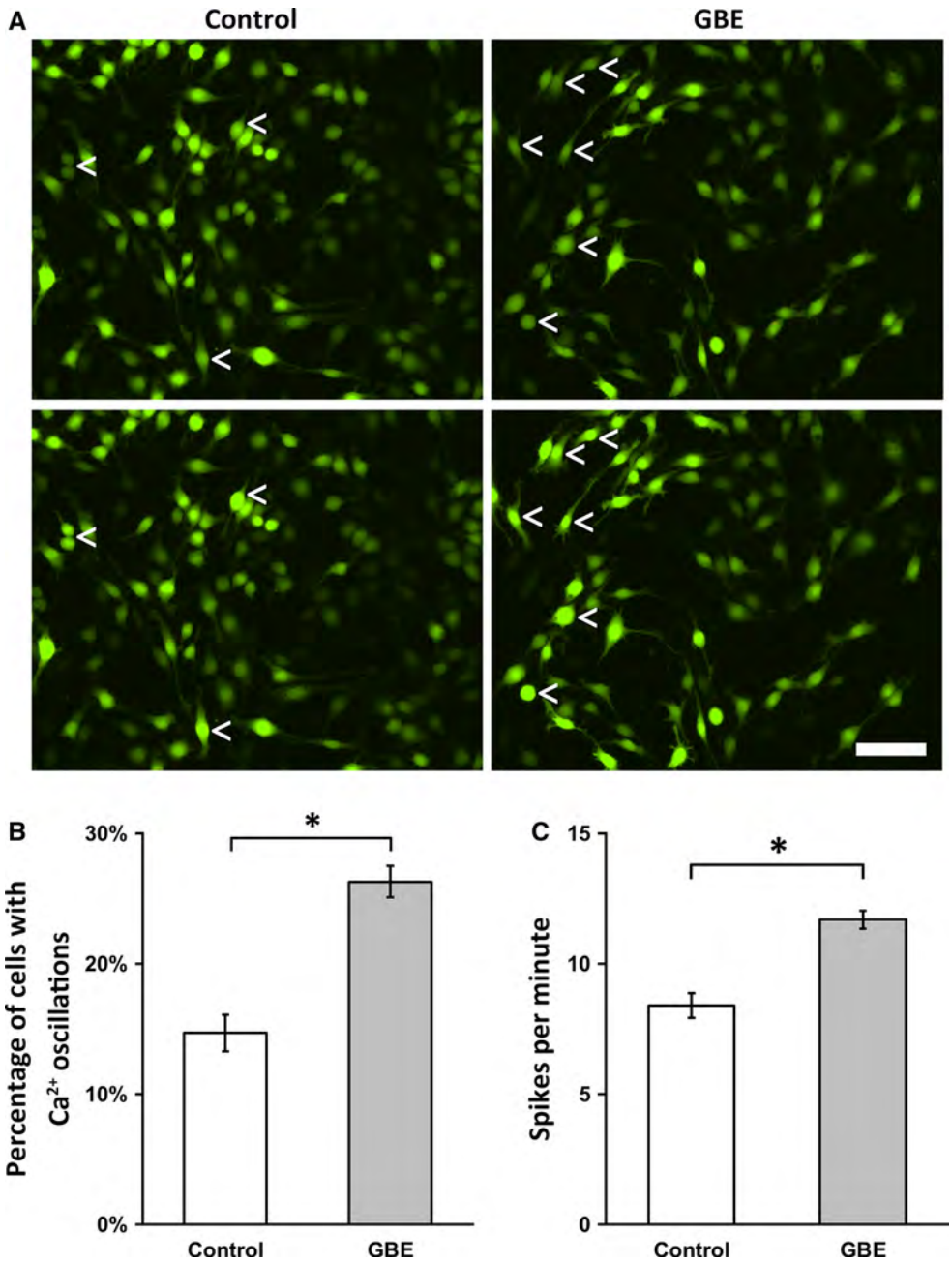

Fig. 5 Spontaneous Ca^{2+} oscillations in NSC-differentiated neural networks under GBE treatment. **a** Confocal images of both control- and GBE-treated cultures loaded with fluo-4-AM, with *arrowheads* pointing to neurons showing Ca^{2+} spikes in the adjacent frames. *Scale bar* 100 μm. **b** Percentage of cells with Ca^{2+} oscillations in control- and GBE-treated cultures. **c** Frequency of the oscillations was measured in control- and GBE-treated cultures. Data are presented as the mean ± SEM. $*p < 0.05$ versus control

following results showed that GBE treatment significantly increased neurosphere formation and growth. The capability of NSC differentiating into mature neurons was also remarkably enhanced upon GBE treatment, as indicated by the increased level of neuron-specific cytoskeleton protein MAP-2. Moreover, GBE treatment also promotes neurite outgrowth by increasing the average length of neurites as well as the complexity of the dendritic tree. More importantly, we demonstrated that the overall excitability of the in vitro neural network was escalated, indicated by elevated levels of spontaneous calcium oscillation. Taken together, these results established that GBE treatment was capable of enhancing the cochlear NSC proliferation and differentiation into neurons, which provides novel insights for the application of GBE in NSC regeneration therapy to treat hearing loss and other neurodegenerative diseases. The involvement of GBE in alleviating hearing problems is intriguing. GBE, when administered together with infusion therapy, was shown to be effective in treating tinnitus aurium, a perception of "ringing" in the ears (Morgenstern and Biermann 2002). This suggested GBE exerts beneficial effects on hearing problems; however, the underlying mechanism remains elusive.

It has been demonstrated that GBE protects cells against damage induced by a variety of extrinsic stimuli. GBE was shown to exert a protective effect on PC12 cells injured by the neurotoxin paraquat (Kang et al. 2007) on human neuroblastoma SH-SY5Y cells under hydrogen peroxide-induced oxidative stress (Shi et al. 2009) and against neurotoxicity in mouse brain in an animal model of Parkinson's disease (Rojas et al. 2009) by suppressing apoptosis. However, some studies also demonstrate that GBE may induce apoptosis and inhibit cellular proliferation in ESCs (Chan 2006), effector T cells (Kotakadi et al. 2008) and tumor cells (Kim et al. 2005). In our study, we also found GBE promoted cochlear NSC viability at moderate concentrations, whereas it greatly reduced cell viability at higher concentrations (Fig. 1). Similar to our study results, GBE also exhibited a biphasic effect on apoptosis in bone marrow mesenchymal stem cells (BMSCs) (Li et al. 2011). Low concentrations of GBE were found to aggravate hypoxia/serum deprivation-induced apoptosis, whereas high concentrations significantly prevented BMSC apoptosis. Taken together, these and our current report indicate possible selective and divergent effects of GBE on different types of cells at different dosages.

Hair cells are the sensory receptors of both the auditory and vestibular systems in all vertebrates, and they cannot be regenerated in vivo. Loss of the sensory hair cells from the inner ear causes sensorineural deafness; hence, restoring the inner ear hair cells has always been a major challenge for treating hearing loss. A cochlear implantation remains the only way to treat hair cell loss-induced hearing loss (Zong et al. 2014). Several groups have also reported the ability of cultured cochlear progenitor cells to differentiate into neurons (Parker et al. 2007; Wei et al. 2008). There has been significant progress in finding the mechanisms for hair cell regeneration, and many biotechnological approaches have led to new methods of regenerating hair cells (Parker 2011). Recently, a human stem cell-derived transplant was reported to successfully restore hearing loss (Chen et al. 2012). In our present study, we demonstrated that GBE treatment greatly enhanced the proliferation and differentiation of in vitro cultured cochlear NSCs, and most importantly the performance of these differentiated neurons, indicating the potential of using GBE in vivo to enhance the transplant efficacy.

Conclusion

In summary, our study reveals that GBE can significantly enhance the proliferation of mouse cochlear NSCs and more importantly their differentiation into functional neurons in vitro. Moreover, the NSC-derived neurons exhibit more complex neurite outgrowth and better neural network performance in the presence of GBE. Our findings strongly support the use of GBE as a potential therapeutic agent in the treatment of hearing loss.

Acknowledgments This work was funded by the National Natural Science Foundation of China, General Program (no. 81371093).

Conflict of interest Congpin Wang and Zhao Han declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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