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REGULAR ARTICLE

Taurine enhances mouse cochlear neural stem cell transplantation via the cochlear lateral wall for replacement of degenerated spiral ganglion neurons via sonic hedgehog signaling pathway

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Abstract

The aim of this paper is to investigate the potential beneficial effects of taurine in cochlear neural stem cell (NSC) transplantation and elucidate the underlying molecular mechanism. The NSC cells were isolated from neonatal Balb/c mice and an auditory neuropathy gerbil model was established by microinjection of ouabain. The spiral ganglion neurons (SGN) were characterized with immunofluorescence stained with Tuj1 antibody. Cell proliferation was determined by BrdU incorporation assay and the morphologic index was measured under the light microscope. The relative protein level was determined by immunoblotting. The hearing of the animal model was scored by click- and tone burst-evoked auditory brainstem response (ABR). Here we consolidated our previous finding that taurine stimulated SGN density and the proliferation index, which were completely abolished by Shh inhibitor, cyclopamine. Transplantation of cochlear NSCs combined with taurine significantly improved ouabain-induced auditory neuropathy in gerbils. In addition, cyclopamine antagonized taurine's effect on glutamatergic and GABAergic neuron population via suppression of VGLUT1 and GAT1 expression. Mechanistically, taurine evidently activated the Sonic HedgeHog pathway and upregulated Shh, Ptc-1, Smo and Gli-1 proteins, which were specifically blockaded by cyclopamine. Here, for the first time demonstrated we that co-administration with taurine significantly improved NSC transplantation and the Shh pathway was identified in this beneficial effect.

Keywords Taurine · Spiral ganglion neuron · Sonic hedgehog · Cyclopamine · Neural stem cell (NSC)

Introduction

Taurine is one of the most common amino acids that widely exists in a diversity of tissues with fundamental physiological roles (Ripps and Shen 2012). The pancreas is a major source of endogenously synthesized taurine via the cysteine sulfinic acid pathway (Chang et al. 2013), which is also supplemented from a regular diet such as egg, meat and seafood (Bouckenooghe et al. 2006). The critical physiological actions of taurine have been recognized in the functioning and development of cardiovascular, skeletal muscle, retina and the central nervous system (CNS), which are consistent with a localized high concentration of endogenous taurine. Assembling investigations indicate the protective roles of dietary taurine on the neural system and the intracerebral taurine concentration in rodents was shown to be significantly provoked in the neurons exposed to hypoxic condition (Maia et al. 2014). Taurine administration has also been demonstrated to ameliorate the impairment of differentiation, proliferation and cell viability of neural progenitor cells in response to short of oxygen and glucose supply (Wang et al. 2015a). In vitro treatment of mouse embryonic mesencephalon-, dentate gyrus- and human embryonic brain-derived neural progenitor cells with taurine significantly promoted cell proliferation (Hernandez-Benitez et al. 2010; Hernandez-Benitez et al.

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2013; Shivaraj et al. 2012). Consistent with the known neural protective actions, we previously demonstrated that taurine treatment remarkably stimulated proliferation, differentiation and neurite outgrowth of cochlea spiral ganglion progenitor cells as well (Wang et al. 2015b). However, the in vivo actions and molecular mechanism are still to be defined.

Auditory neuropathy is a hearing loss disorder with an intact sound response but a defect in signal transmission, which plays a substantial role in hearing impairment and deafness in the population of all ages (Starr et al. 1996). Effective clinical treatments are still being sought with available options that include hearing aids, cochlear implants and other technologies (Chen and Oghalai 2016). With advances in stem cell research, more intensive investigations have been conducted to explore the potential application of auditory stem cells, which might represent a promising direction to conquer this complication (Lang et al. 2015).

Etiologic studies have increasingly uncovered the critical role of the Hedgehog signaling pathway in incidence and progression of auditory neuropathy, which is canonical in transmitting information to embryonic cells for appropriate differentiation and a key master in animal development in all bilaterians (Ingham 2001). Notably, Hedgehog signaling is increasingly recognized as being critical in adult stem cells in diverse tissues including hematopoietic cells, mammary and neural stem cells (Parisi and Lin 1998). For instance, the titration of GLI3 repressor activity by sonic hedgehog signaling is critical for maintaining multiple adult neural stem cells and astrocyte functions (Petrova et al. 2013). Palma et al. demonstrated that sonic hedgehog controlled stem cell behavior in both the postnatal and adult brain (Palma et al. 2005). Machold et al. proposed that sonic hedgehog was required for progenitor cell maintenance in telencephalic stem cell niches (Machold et al. 2003). The critical role of sonic hedgehog signaling in auditory neural progenitor cells has been highlighted by several investigations, where an approximate 1.5- to 4-fold increase of sonic hedgehog transcripts in response to taurine treatment definitely indicates that the Shh pathway is activated by taurine and in turn contributes to its protective effect (Ramos-Mandujano et al. 2014). Therefore, here we investigate this possibility both in cell culture and in animal disease model.

Methods

Isolation and differentiation of cochlear NSCs

The animal study was approved by the Institutional Committee of Ethics on Experimental Animals in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of The Second Xiangya Hospital, Central South University. The mice were anesthetized with sodium pentobarbital for surgical operation purpose. The cochlear NSCs were isolated following the previously described protocol (Xiao et al. 2015). In brief, the 1-day postnatal Balb/C mice (three mice for a batch of NSCs) were sacrificed by decapitation and temporal bone was extracted and transferred into ice cold Hank's balanced salt solution (Invitrogen, MO, USA). The otic capsule was subsequently isolated from the otic bulla and opened to expose the membranous labyrinth of the cochlea. The spiral ganglia were obtained from the modiolus via complete removal of the cochlear duct including Corti, spiral ligament and stria vascularis, which was in turn enzymatically digested with 0.125% trypsin/EDTA in phosphate-buffered saline (PBS) at 37 °C for 5 min. The excess enzymatic activity was quenched by a cocktail of 10-mg/ml soybean trypsin inhibitor (Worthington, USA) and 1 mg/ml DNase I (Worthington) dissolved in DMEM/F12. Homogenous single-cell suspension was achieved via repeated pipetting and passing through a 70-μm cell strainer (BD Falcon, USA). Cells were sub-cultured every 7 days. For proliferation assay, NSCs were maintained in DMEM-F12 medium supplemented with 2% B-27, 20 ng/ml EGF and 20 ng/ml FGF-2 (R&D Systems, USA). For differentiation assay, NSCs were firstly seeded in proliferation medium and allowed for attachment overnight. Replacement with growth factor-free medium supplemented with 2% B-27, 10% fetal bovine serum (FBS, Gibco, USA) and 1 mM retinoic acid (Sigma, MO, USA) suppressed cell division and promoted cell differentiation into a mixed lineage of neurons, astrocytes and oligodendrocytes. Cells were harvested after 7 days of consecutive culture with regular fresh medium changes and subjected to proliferation and differentiation analysis. For treatment, taurine was purchased from Sigma and added into the culture medium at indicated concentrations.

SGN morphology

The SGN count was performed at 30 days post-NSC transplantation in presence of taurine or cyclopamine. Animals were sacrificed by decapitation and both cochleae were dissected and perfused with a fixative solution containing 4% paraformaldehyde in PBS (pH 7.2–7.4) for 1 h at 4 °C. The apical portion of the bony cochlea was gently opened to allow the fixative to be perfused through the cochlea. After rinsing (2 × 5 min) in PBS, the cochleae were decalcified overnight in EDTA solution at room temperature and then soaked in 15% and 30% sucrose solution for 8 h. The cochleae were placed in a Tissue-Tek OCT compound for cryostat sections. The cochleae were oriented to obtain cross-sections of the Rosenthal canal. The sections were cut to 14-μm thickness by cryostat and mounted on Premiere® slides. SGN density was tested with immunofluorescence staining by using an anti-Tuj1 antibody for detecting neurons and DAPI for detecting nucleus at three different locations across the basilar membrane from the apex to the apical, middle and basal parts of the cochlea,

respectively. The cell proliferation of SGN was tested with BrdU incorporation assay and nucleus labeled by DAPI. Confocal images were acquired using a confocal laser-scanning microscope (710 META; Zeiss) with 10× objectives and the image stacks were ported to image-processing software.

BrdU incorporation

Briefly, two doses of BrdU (50 μg/g body weight each dose) were injected to animals every 6 h. Four hours after the second injection, animals were sacrificed to dissect the cochleae, followed by sectioning as described above. BrdU staining was then performed following a previously established method (An and Kang 2013).

Western blotting

The cells were lysed in ice-cold RIPA buffer and the cell debris was discarded by centrifugation. The protein concentration in the resultant supernatant was measured using the BCA Protein Assay Kit (ThermoFisher, MO, USA) following the manufacturer's instruction. The samples were resolved by SDS-PAGE and transferred onto a PVDF membrane on ice (320 mV, 2 h). The membrane was blocked with 5% BSA in TBST buffer and incubated with primary antibodies at 4 °C overnight. After a rigorous wash with TBST for 6 × 5 min, the protein blots were detected with species-specific secondary antibodies at room temperature for 1 hand visualized using the enhanced chemiluminescence method (ECL, Millipore, CA, USA).

Ouabain-induced auditory neuropathy gerbil model

The ouabain-induced auditory neuropathy model was established following the previously described protocol (Qu et al. 2012). The Mongolian gerbils were purchased from the Institutional Experimental Animal Center and maintained in a pathogen-free environment. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the Second Xiangya Hospital, Central South University. The gerbil was subjected to anesthesia with sodium pentobarbital (40 mg/kg) immediately before surgery. A heating pad (39 °C) was applied to maintain the body temperature. In each animal, the right ear was denervated by ouabain application to the round window (RW) niche under sterile conditions. The underlying muscles and facial nerve were identified and dissected using an iris scissor to expose the lateral wall of the bulla and the RW niche was exposed after drilling the bone for a 200-μm opening. Ten microliters of a 1 mM ouabain solution in normal saline was placed in the RW niche for 1 h. Then, the ouabain was removed with a small piece of filter paper. Finally, the surface of the bulla was fully closed with dental cement and the incisions were closed with sutures. Histological examination was performed by H&E staining to confirm the success in induction of auditory neuropathy. For NSCs injections, the RW niche was re-exposed as described above and a microdissection needle was used to expose the cochlear nerve trunk via a 200-μM opening in the bone separating the nerve from the floor of the RW niche. A 10-μl Hamilton syringe coupled to a micropipette (tip diameter: 100 μM) was placed in a micromanipulator and inserted 1.5 mm into the opening. A volume of 5-μl cells was injected: half at the 1.5-mm depth and the rest after retracting the electrode by 1 mm. The incision was closed as above and the animal transferred to a homeothermic blanket. To prevent cell rejection, cyclosporine A (10 mg/kg subcutaneous) was given daily from the day of transplantation until the end of the experiment. Animals were divided into four groups: (1) control group (animals denervated by ouabain only application to the RW), (2) control + NSCs (ouabain-treated animals inoculated with cultured cochlea NSCs), (3) control + NSCs + taurine (ouabain-treated animals inoculated with cultured cochlea NSCs and taurine) and (4) control + NSCs + taurine + cyclopamine (ouabain-treated animals inoculated with cultured cochlea NSCs and taurine and cyclopamine).

Auditory brainstem response

The hearing capacity of gerbils was scored at baseline just before and 8 days after ouabain treatment following the previously described auditory brainstem response (ABR) method. Three electrodes were implanted subcutaneously beneath the pinna of the target ear (reference electrode), the apex of the nose (ground) and at the vertex (active electrode). The stimulus signal was generated with an Intelligent Hearing Systems device (Bio-Logic Systems, USA). The test environment is a quiet shielding room, in which the ambient noise is less than 20 dB sound pressure levels (SPL). Click sounds were produced at a rate of 57.7/s to evoke the ABRs. Tone burst sounds at 4, 8 and 16 kHz (0.2-ms rise/fall time and 1-ms flat segment) were generated to estimate the frequency-specific thresholds. The responses of 1024 sweeps were averaged at each intensity level step. The intensity of the stimulus was varied at stepwise increments of 5-dB SPL.

Statistical analysis

All data expressed in this study were acquired from at least three independent experiments. Statistical analysis was conducted with SPSS 23.0 software. One-way ANOVA followed by Turkey's test were used for multiple

group comparison. Significance was calculated as P value and $P < 0.05$ was considered as significantly different.

Results

Taurine stimulated SGN density and relative cell proliferation

Our previous data implicated a significant beneficial effect of taurine on both proliferation and differentiation of SGNs in vitro; therefore, we sought to investigate this potential further in vivo. Here we employed an ouabain-induced auditory neuropathy gerbil model and the hearing status was determined by ABR threshold evaluation before ouabain administration and 3, 7, 14, 30 and 60 days post-transplantation of SGNs. The scheme to establish the auditory neuropathy in gerbils and NSC implantation is illustrated in supplementary Fig. S1A. The success in establishment of auditory neuropathy was confirmed pathologically by H&E staining as shown in supplementary Fig. S1B. The inoculation site of NSCs is clearly shown in supplementary Fig. S1C. The SGNs were further characterized by immunostaining with Tuj1 antibody (Fig. 1a, a‴, untreated control was supplemented in Fig. S2A), which demonstrated a remarkable induction of SGN increase post-transplantation of NSCs. Co-administration with taurine significantly improved SGN population (Fig. 1b), while application of Shh-specific inhibitor, cyclopamine, oppositely inhibited the beneficial effect of NSC transplantation. To further understand the beneficial actions of NSCs, the cell proliferative index was measured in response to taurine and cyclopamine treatment either alone or in combination. The BrdU incorporation results presented in (Fig. 1c, c‴, d) indicate the evident promotion of DNA synthesis upon taurine treatment in vivo (untreated control was supplemented in Fig. S2B), which was partially abolished by co-treatment with cyclopamine. Our results suggest the significant benefits of

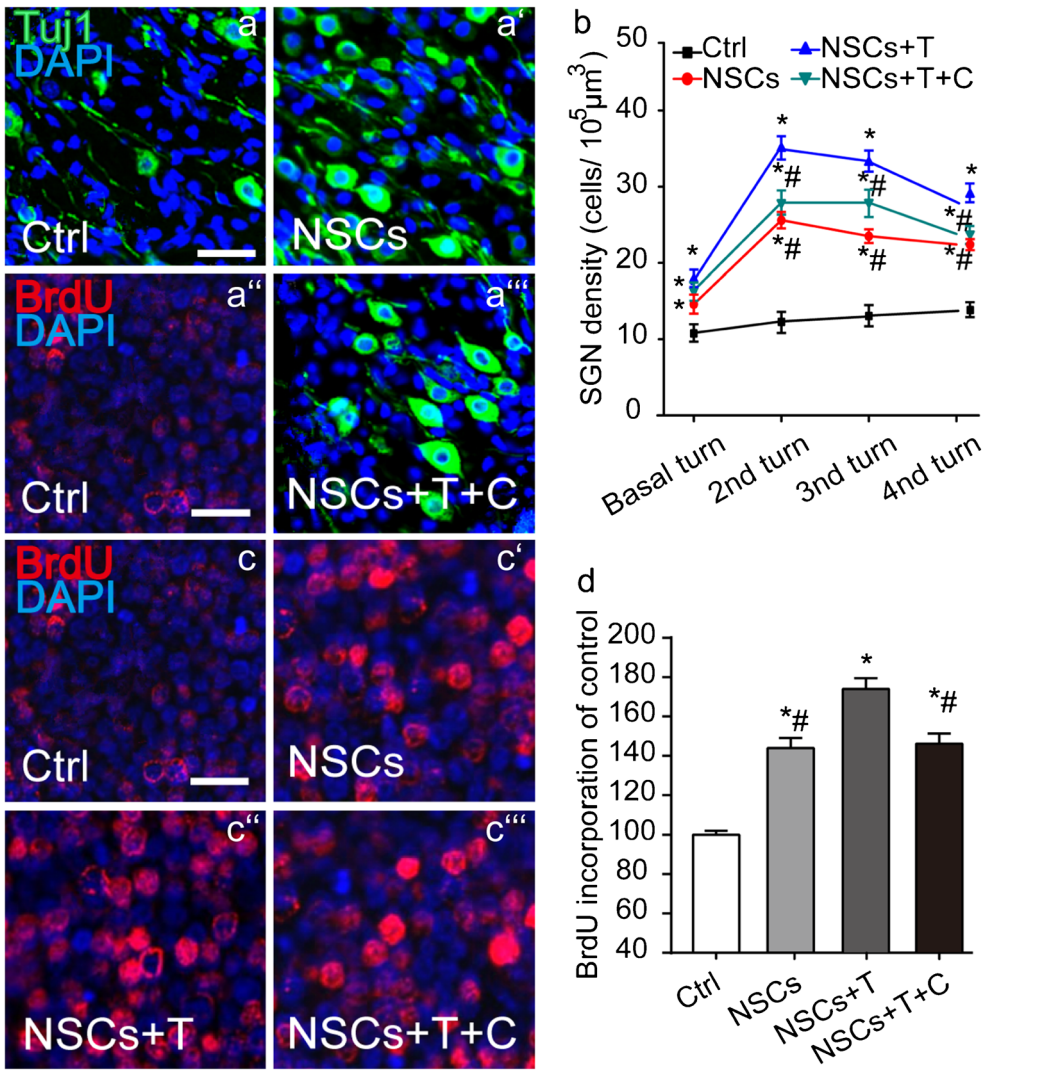

Fig. 1 SGN density and relative cell proliferation at 30 days after NSC transplantation in auditory neuropathy gerbils by ouabain injury. **a**, **a'''** Representative images of SGNs labeled with a neuronal marker (Tuj1, green) and a nucleus marker (DAPI, blue) in Rosenthal's canal at the basal turn. **b** Average neuron counts in each turn of the spiral ganglion 30 days after transplantation. **c**, **c'''** Representative photomicrographs of BrdU-labeled cells (nucleus labeled with DAPI) in four experimental groups, measured by BrdU incorporation assay. **d** Quantitative changes of SGN density at four locations 30 days of the transplantation of cochlear NSCs. Scale bar = 50 μm. Histogram bars represent mean ± SEM. $^{*}P < 0.05$ versus Ctrl, $^{\#}P < 0.05$ versus NSCs + T. $n = 8$ each group

NSCs in improvement of auditory neuropathy in vivo. Notably, our data further highlighted the potential critical role of the Shh pathway in this effect.

Taurine improved ABR

Next, the hearing capacity in auditory neuropathy gerbils in response to taurine and cyclopamine treatments was evaluated by measurement of click- and tone burst-ABR (Fig. 2a, b). Consistent with the pathological characteristics, ouabain induced severe hearing capacity impairment as indicated by the remarkable increase of ABR thresholds. In-site transplantation of NSCs cell markedly lowered both the click- and tone burst-evoked ABR threshold in the ouabain-induced animals, which was further decreased by the taurine-imposed synergistic effect. However, while we co-treated the transplantation mixture with cyclopamine, this beneficial effect on auditory restoration was greatly compromised, which highlighted the predominate role of the Shh pathway underlying this phenotype.

Cyclopamine antagonized taurine's effect on glutamatergic and GABAergic neuron population

Excitatory and inhibitory neurotransmitters were a pair of counterparts and orchestrated the regulation of cochlea neural impulses. We sought to investigate the differentiation of glutamatergic and GABAergic neurons, which secreted excitatory and inhibitory neurotransmitters, respectively, in NSCs in response to taurine treatment. The immunoblotting against the glutamatergic neuron-specific marker, vesicular glutamate transporter 1 (VGLUT1), displayed a significant increase of this population by taurine treatment, whereas co-treatment with cyclopamine remarkably compromised this effect (Fig. 3a). Again, the influence of taurine on the GAT1 protein (GABAergic neuron) was further characterized using immunoblotting (Fig. 3b). Consistent with previous reports that taurine induced glutamatergic and inhibited GABAergic neuron differentiation, our results further demonstrated that these effects greatly depended on the downstream Shh pathway, which was effectively blocked by cyclopamine.

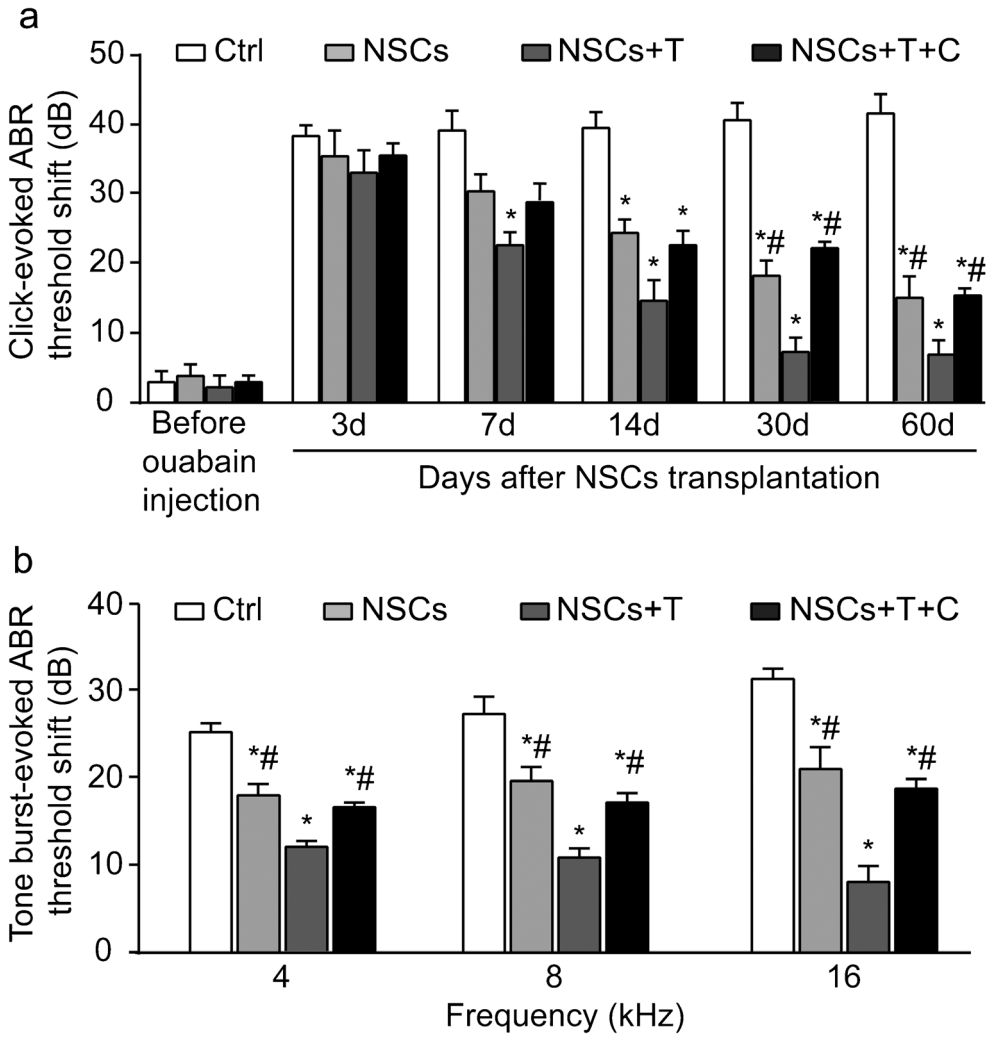

Fig. 2 Transplantation of cochlear NSCs combined with taurine (NSCs + T) attenuated ouabain-induced click-evoked and tone burst-evoked auditory brainstem response (ABR) threshold shift in the auditory neuropathy gerbils by ouabain injury. In contrast, cyclopamine injection (NSCs + T + C) inhibited the effect of taurine. **a** The click-evoked ABR threshold was measured at baseline just before ouabain administration and again 3 days 7 days, 14 days, 30 days, and 60 days after cochlear NSC transplantation. **b** The tone burst-evoked ABR threshold was measured at 30 days after cochlear NSC transplantation. Histogram bars represent mean ± SEM. $^{*}P < 0.05$ versus Ctrl, $^{\#}P < 0.05$ versus NSCs + T. $n = 7$ each group

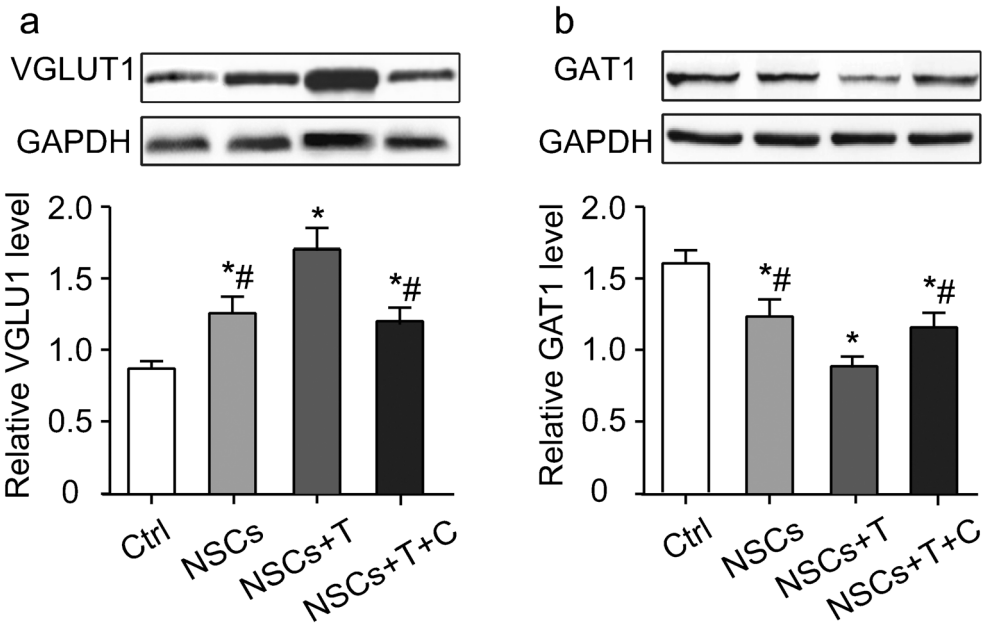

Fig. 3 Cyclopamine injection inhibited the effect of taurine on VGLUT1 **(a)** and GAT1 **(b)** protein expression in the auditory neuropathy gerbils by ouabain injury. VGLUT1 and GAT1 protein levels, as detected by western blotting. GAPDH was used as a loading control. Histogram bars represent mean ± SEM. $^{*}P < 0.05$ versus Ctrl, $^{\#}P < 0.05$ versus NSCs + T. $n = 8$ each group

Cyclopamine suppressed Ptc-1, Smo and Gli-1 expression induced by taurine in NSCs

Our previous investigations suggested that the Shh pathway was mechanistically involved in taurine-induced pro-proliferation and -differentiation, which was readily suppressed with a specific inhibitor. We sought to characterize the detailed molecular profile along the Shh pathway in response to taurine and cyclopamine. Four critical factors including Shh, Patched-1 (Ptc-1), Smoothened (Smo) and Gli-1 were quantitatively interrogated in NSCs cell treated with either taurine alone or combined with cyclopamine. As shown in (Fig. 4a), taurine treatment greatly stimulated upregulation of Shh (Fig. 4b), Ptc-1 (Fig. 4c), Smo (Fig. 4d) and Gli-1 (Fig. 4e), which was abolished by addition of cyclopamine. Our data disclosed that the taurine activated Shh pathway via upregulation of Ptc-1, Smo and Gli-1 protein, and cyclopamine significantly reversed this effect.

Discussion

Accumulative evidences suggested the potential protective actions of taurine in auditory neuropathy. Our previous study indicated that taurine treatment promoted proliferation, differentiation and neurite outgrowth of spiral ganglion progenitor cells in vitro as well and elucidated the critical involvement of the Shh pathway (unpublished data). In this study, we further investigated the beneficial effects of taurine in vivo via employment of an auditory neuropathy gerbil model and clarified the important role of Shh signaling pathway in mediating this effect under physiological conditions. Following a previously described protocol, we successfully established an auditory neuropathy animal model via injection of ouabain, which was pathologically confirmed. Immunostaining detected significant a increase of SGNs in response to NSC transplantation, which was further enhanced by co-administration with taurine and antagonized by cyclopamine. The proliferative index of SGNs interrogated by BrdU incorporation was greatly stimulated by NSCs, taurine manifested synergistic actions while blockaded by cyclopamine. The hearing capacity was significantly restored in ouabain-induced auditory neuropathy gerbils by NSC transplantation as indicated by the decrease of both click- and tone burst-evoked ABR thresholds. Taurine synergistically improved this effect, which was antagonized by cyclopamine. Moreover, the differentiation potential of SGNs into glutamatergic neurons was remarkably induced while the direction into GABAergic neurons was conversely suppressed in response to taurine treatment, which was in agreement with the physiological function of glutamatergic neurons in producing the excitatory neurotransmitters in this setting. Consistent with the results from a cDNA profiling investigation into taurine-treated neural progenitor cells (Ramos-Mandujano, Hernandez-Benitez and Pasantes-Morales 2014), we further confirmed that several key factors in sonic hedgehog pathway including Shh, Ptc-1, Smo and Gli-1 were significantly upregulated in SGNs upon taurine treatment, which unambiguously indicated that the Shh pathway was activated by taurine. Notably, via employment of Shh-specific inhibitor, cyclopamine, our study highlighted the importance of the Shh pathway in mediating taurine-elicited protective effects on SGNs. Cyclopamine is a highly potent Shh inhibitor by influencing the balance between the active and inactive form of the smoothened protein (Chen et al. 2002). With co-administration with taurine, the beneficial effects in synergistic restoration of the hearing capacity

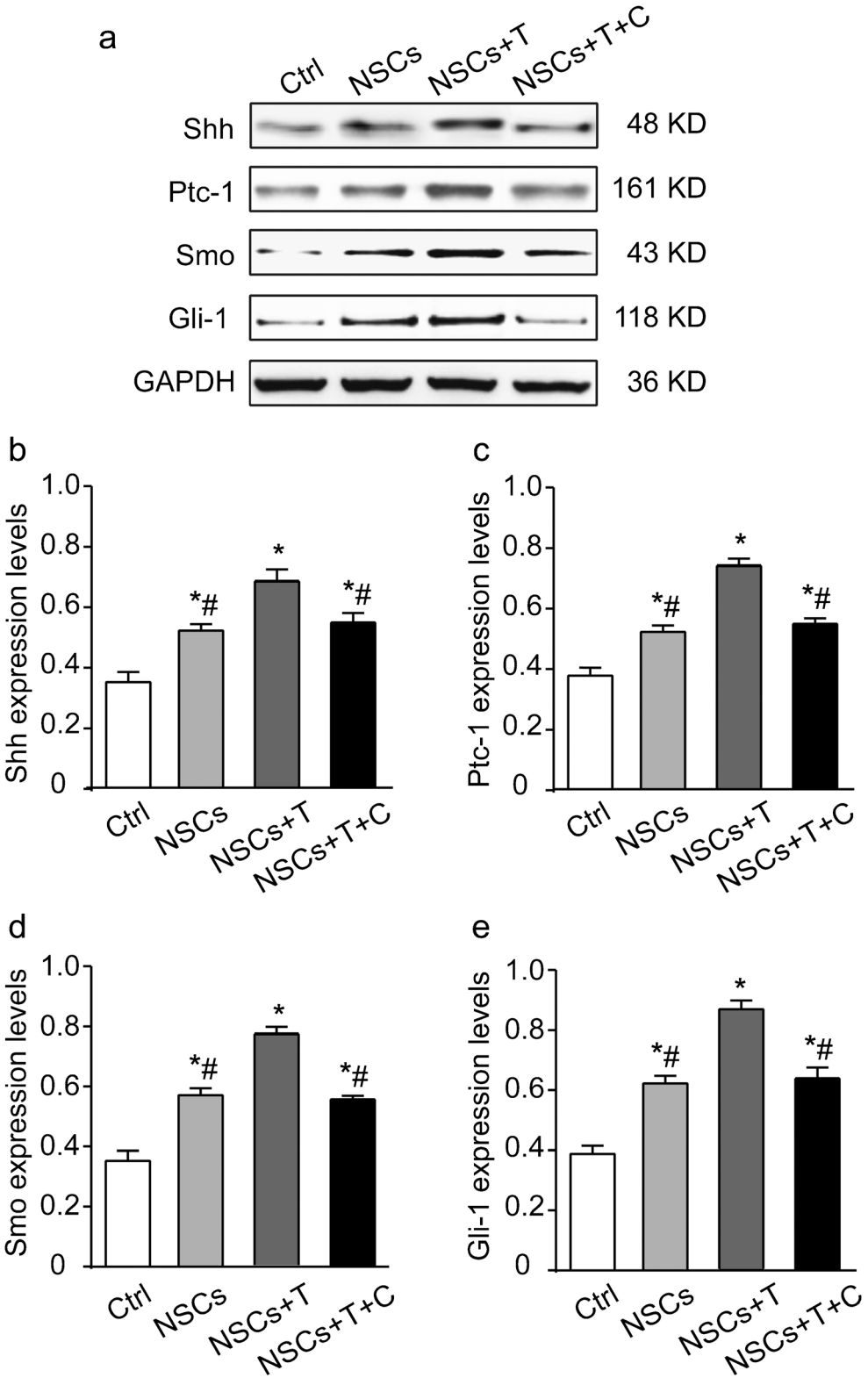

Fig. 4 Transplantation of cochlear NSCs combined with taurine (NSCs + T) upregulated the levels of Shh, Ptc-1, Smo and Gli-1 protein expressions in auditory neuropathy gerbils by ouabain injury. In contrast, cyclopamine injection (NSCs + T + C) inhibited the effect of taurine. **a** Shh, Ptc-1, Smo and Gli-1 protein levels, as detected by western blotting. **b**–**e** Quantitative analyses of Shh, Ptc-1, Smo and Gli-1 proteins levels. All four proteins were upregulated in the two groups of transplantation of NSCs alone (NSCs) or combined with taurine (NSCs + T) relative to the control (Ctrl) group. Histogram bars represent mean ± SEM (*$P < 0.05$ versus Ctrl,$^{\#}P < 0.05$ versus NSCs + T). $n = 6$ each group

imposed by taurine was readily abrogated by cyclopamine, which emphasized the predominate role of the Shh pathway underlying this phenotype. To the best of our knowledge, for the first time we demonstrated that taurine co-administration significantly improved the therapeutic actions of NSC transplantation in a gerbil disease model and clarified the involvement of the Shh pathway, which highlighted the potential for future clinical exploitation aiming to activate the Shh pathway

for auditory impairment. However, the signaling pathway during taurine-activated Shh is still to be defined. In addition to sonic hedgehog signaling, the gene expression profile demonstrated that taurine also regulated genes involved in the Wnt pathway, cellular adhesion, cell survival and mitochondrial functioning (Ramos-Mandujano, Hernandez-Benitez and Pasantes-Morales 2014), which suggests multiple candidate targets of taurine and requires further comprehensive investigation.

Taurine has been increasingly recognized as having beneficial effects on hearing and potential therapeutic application for this complication. For instance, Wang et al. demonstrated that taurine enhanced excitability of mouse cochlear neural stem cells by selectively promoting differentiation of glutamatergic neurons over GABAergic neurons (Wang et al. 2015b). Ye et al. reported that taurine attenuated bilirubin-induced neurotoxicity in the auditory system in neonatal guinea pigs (Ye et al. 2013). Liu et al. demonstrated that taurine attenuated aminoglycoside ototoxicity by inhibiting inducible nitric oxide synthase expression in the cochlea (Liu et al. 2008). In addition, taurine has also been shown to modulate calcium influx through L-type voltage-gated calcium channels in isolated cochlea outer hair cells in guinea pigs (Liu et al. 2006). Brozoski et al. reported that supplemental dietary taurine significantly attenuated tinnitus and improved auditory discrimination by increasing inhibitory tone and decreasing noise in the auditory pathway (Brozoski et al. 2010). In line with these elegant studies, our data acquired in the disease animal model demonstrated that taurine significantly enhanced the restoration of hearing capacity via selectively promoting differentiation of glutamatergic neurons over GABAergic neurons. And the molecular mechanism underlying this effect was further elucidated in this study as being involved with Shh pathway activation.

Hearing loss imposes severe threats to the quality of life and health conditions of patients worldwide with limited clinical options for therapeutic purposes (Lasak et al. 2014). Neural stem cell transplantation is the sole option for hearing rehabilitation despite the wide usage of hearing aids to ameliorate hearing disorder (Muller and Barr-Gillespie 2015). However, limited success has been achieved in respect with efficient differentiation into functional neurons, glia, hair cells and supporting cells (Kesser and Lalwani 2009). Here we offered an alternate way to improve the efficiency of NSC transplantation via co-treatment with taurine, which potentiated both proliferation and differentiation in vivo.

In summary, here we showed Shh blockage greatly abrogated the cell proliferation and differentiation of NSCs stimulated by, which highlighted Shh signaling in the taurine-elicited protective effect on neural progenitor cells and potentially could serve as activating targets for therapeutic purposes.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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