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7,8,3′-Trihydroxyflavone, a potent small molecule TrkB receptor agonist, protects spiral ganglion neurons from degeneration both *in vitro* and *in vivo*

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ABSTRACT

Most sensorineural hearing loss cases occur as a result of hair cell loss, which results in secondary degeneration of spiral ganglion neurons (SGNs). Substantial loss of SGNs reduces the benefit of cochlear implants, which rely on SGNs for transmitting signals to the central auditory centers. Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) play essential roles in cochlear development and are required for SGN survival. Here we report that 7,8,3′-trihydroxyflavone (7,8,3′-THF), which is a small molecule agonist of tyrosine receptor kinase B (TrkB), promoted SGN survival with high potency both *in vitro* and *in vivo*. The compound protected the SGNs in a TrkB-dependent manner, as its effects on SGNs disappeared when the TrkB was blocked. Application of 7,8,3′-THF in the bulla of conditional connexin26 (cCx26)-null mice dramatically rescued SGNs in the applied ear compared to untreated control cochlea in the same animal. Our findings suggest that 7,8,3′-THF is a promising therapeutic agent protecting the SGNs from degeneration both *in vitro* and *in vivo*.

1. Introduction

Both BDNF and the TrkB receptor are expressed in the cochlea [1,2]. In addition to its prominent roles in the cochlear development [3], exogenous applications of BDNF directly into the cochlear labyrinth rescue SG neurons from degeneration that caused by loss of the hair cells (HCs) in adult animals [4,5]. However, the short *in vivo* half-life and the poor pharmacokinetics of BDNF severely limit its efficacy and makes its direct clinical applications difficulty [6].

The use of small molecule agonist of BDNF is a promising alternative to the success applications of BDNF. 7,8-Dihydroxyflavone (7,8-DHF) has recently been identified as a small molecular TrkB receptor agonist that selectively binds and activates TrkB receptors in neurons [7]. Studies show that 7,8-DHF fully mimics BDNF and exhibits promising therapeutic effects in animal models of depression [8], Alzheimer's disease (AD) [9] and Rett Syndrome [10]. In searching for a more potent 7,8-DHF analog for triggering TrkB activation with better *in vivo* pharmacokinetics, Ye's group has conducted an extensive structural–activity relationship (SAR) study which suggested that 7,8,3′-trihydroxyflavone (7,8,3′-THF) is a more robust compound for stimulating TrkB phosphorylation than the parent 7,8-DHF, with 2–3 times higher potency [11]. However, its *in vitro* and *in vivo* neuroprotective effects have not been tested yet. In this study, we examined the potency of this newly-identified small molecule TrkB agonist using both organotypic cultures and a genetic animal model of degeneration of the spiral ganglion neurons (SGNs).

2. Materials and methods

2.1. Organotypic cultures

Cochlear tissues used were obtained from C57/BL6 (Charles River Inc., Wilmington, MA) or $TrkB^{F616A}$ [7] mice. Only the middle cochlear turn were used in the cultures to ensure consistency. All animal use protocols in this study were approved by the Emory Institutional Animal Care and Use Committee. Cochleae from postnatal day 1 (P1) or P2 were dissected in ice-cold Hanks' balanced salt solutions (HBSS, Invitrogen, Carlsbad, CA). Tissue dissection procedures were the same as our published protocol [12]. The explants were placed onto glass coverslips coated with poly-D-lysine

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(Invitrogen, 500 μg/ml, for 1 h at 20 °C) and laminin (Invitrogen, 50 μg/ml in HBSS, for 2 h at 37 °C). Cultures were incubated in a defined serum-free culture medium, which contained Dulbecco's modified Eagle's medium (DMED, Sigma–Aldrich, St. Louis, MO), 10 μl/ml N2 supplement (Invitrogen Inc.), 25 mM HEPES (Sigma–Aldrich Inc.), 6 mg/ml glucose, and 1 mM gentamicin (Sigma–Aldrich Inc.), at 37 °C with 6.5% CO_2 for 72 h. Culture medium was replaced every 24 h. In some experiments, 100 ng/ml BDNF (Invitrogen, CAT# PHC7074) or 300 nM 7,8,3′-THF (Indofine chemical company, Hillsborough, NJ, CAT# T-411) was added as indicated in the text.

2.2. Immunolabeling assays and cochlear resin sectioning

Cultured segments of the cochlea or cryo-cochlear sections (8 μm in thickness, cut with a Leica CM1850, Germany) were used in immunolabeling. Details of immunolabeling protocol can be found in our published paper [13]. Samples were first incubated in primary antibody (1:400) at 4 °C overnight. A mouse monoclonal antibody against class III β-tubulin (TUJ1, Cat# MMS-435P, Covance, Princeton, NJ) was used to label SGNs. Processed samples were mounted in an antifade solution (Fluoromount-G, Southern Biotech, Birmingham, AL), which also contained DAPI to reveal the location of cell nuclei. Images were acquired by confocal microscopy (Zeiss LSM, Carl Zeiss, Germany) and analyzed by Image J software package (NIH, http://rsbweb.nih.gov/ij/features.html).

The survival of SGN *in vivo* was determined at P60 from consecutive midmodiolar resin sections (5 μm in thickness, cut with a Microm HM 335E microtome, Germany). Tissues were fixed by transcardiac perfusion of 4% paraformaldehyde (PFA) in PBS. Cochleae were dissected out and subsequently perfused via the oval window with 2% PFA and 2.5% glutaraldehyde in PBS and post-fixed at 4 °C overnight. Decalcification in 0.35 M EDTA in PBS (pH 7.4) at 4 °C was carried out for 72 h. The samples were then immersed in 2% osmium tetroxide for 2 h, followed by gradual dehydration in alcohol of increasing grades, infiltrated, and embedded in epoxy resin. Cochlear sections were stained with toluidine blue and examined with a light microscope.

2.3. Electrophysiological recordings

Borosilicate glass capillary pipettes (Sutter Instrument Co., Novato, CA) were pulled on a micropipette Puller (P-2000, Sutter Instrument Inc.) and the electrodes (with a resistance of 1–3 MΩ) were fire-polished (MF-830, Narishige, Japan) before uses. HEPES buffered HBSS solution was used as the external recording solution, which contained (in mM): NaCl 137, Na_2HPO_4 0.2, KCl 5.4, KH_2PO_4 0.4, $MgCl_2$ 1, $CaCl_2$ 1.2, and HEPES 10. The pipette internal solution contained (in mM): KCl 140, $MgCl_2{\cdot}2H_2O$ 2, $CaCl_2$ 1.2, EGTA 10 and HEPES 10. pH was buffered to 7.3 with KOH. Coverslips with cultured SGNs were placed in a perfusion chamber mounted on a Zeiss upright fixed-stage microscope (AxioSkop II, Zeiss). Experiments were controlled by Clampex software (version 9.1). Signals from a patch-clamp amplifier (Axon Axopatch 200B, Molecular Devices, Sunnyvale, CA) were digitized via a Digidata 1322A interface (Axon Instruments Inc., Foster City, CA). The series capacitance was compensated and a tight seal (>1 GΩ) was maintained during recordings. Data were analyzed offline using pClamp (version 9.2, Axon) and Origin 7.0 (OriginLab, Northampton, MA) software.

2.4. In vivo delivery of 7,8,3′-THF

cCx26 null mice young than P2 were cryo-anesthetized by placing the pups on ice for 3 min. A retro-auricular approach was used to expose the tympanic membrane and tympanic ring. The tympanic ring with tympanic membrane was detached from posterior adjacent tissue to allow exposure of stapedial artery and round window niche. A small cube (2–3 mm^3) of absorbable gelatin sponge (Gelfoam, Pfizer Inc., New York, CAT# 59-9863) soaked with 15 μL of 7,8,3′-THF (200 μM) in PBS containing 0.1% DMSO was placed on the round window membrane. The above surgery was performed in the left ears of cCx26 null mice. The right cochleae were not treated and used as controls. The same procedure was repeated at P30 to give the second application of 7,8,3′-THF. At P60, the animals were sacrificed after auditory brainstem response (ABR) thresholds were tested from both ears. Details of the testing methods for ABRs are given in our published papers [14]. Both cochleae were processed for histological analysis for evaluating effects of 7,8,3′-THF.

2.5. Quantitative analysis

The spiral ganglia were imaged using a confocal microscope using a 40× lens (N.A. 1.2 oil lens, Zeiss LSM, Carl Zeiss, Germany), with 10 μm distance between each optical section. NIH Image J software (Version 1.63, NIH, Bethesda, Maryland) was used for quantitative analysis. β-Tubulin provided good perinuclear immunolabeling of SGNs and thus permitted distinction of the nuclei in each cell. Cells were counted in each optical section when the nucleus comprised more than ≈40% of the cell area, smaller nuclei were disregarded. As SGNs are approximately 10 μm in diameter and optical sections were taken every 10 μm, therefore nucleus from the same SGN would not be counted twice. Neuronal counts from each optical section were summed for each image stack to give the neuron count for each explants of cochlea culture. Neuron density per each 100 μm^3 was subsequently calculated.

In vivo SGN density was quantified from midmodiolar section of P60 mouse cochleae. The resin cochleae were cut into 5 μm thickness sections. Every other section was collected to assure that neurons were counted only once. To determine the in vivo SGN densities, digitized light microscopic images (at 40× magnification, Zeiss Axiovert 135TV, Carl Zeiss, Germany) of the Rosenthal's canal at basal, middle and apical cochlear turns were analyzed using Image J software. Both type-I and type-II SGNs were counted. SGN densities were calculated by dividing the number of SGNs by the cross-sectional area of Rosenthal's canal at each cochlear location.

All quantitative analyses were performed in a blind fashion by two investigators, independently of one another, and both sets of data were subsequently averaged. GraphPad Prism 5 (GraphPad Software Inc., USA) and Origin 7.0 (OriginLab, Northampton, MA) software were used. Statistical comparisons were made using one-way ANOVA's with post hoc Turkey's tests, or Students two-tailed unpaired *t* tests. Data were presented as mean ± standard error of the mean (SEM). The level of significance was set at $p < 0.05$.

3. Results

We first evaluated the neuroprotective effects of 7,8,3′-THF using cochlear organotypic cultures in which the HCs were killed by applying gentamicin [15] since HCs are known to be the major source of endogenous neurotrophins for SGNs. Cultured in a defined media and without exogenous activation of TrkB, we found that SGN soma density was 19 ± 4 neurons/100 μm^3 ($n = 8$) after three days in culture (Fig. 1a). In contrast, addition of 7,8,3′-THF in the culture medium dramatically rescued SGNs in a dose dependent manner and the saturating concentration was found at 300 nM (data now shown). After three days of culture with defined media supplemented with 7,8,3′-THF (300 nM), we found the density of SGNs was 109 ± 19 neurons/100 μm^3 ($n = 8$, $p < 0.001$)

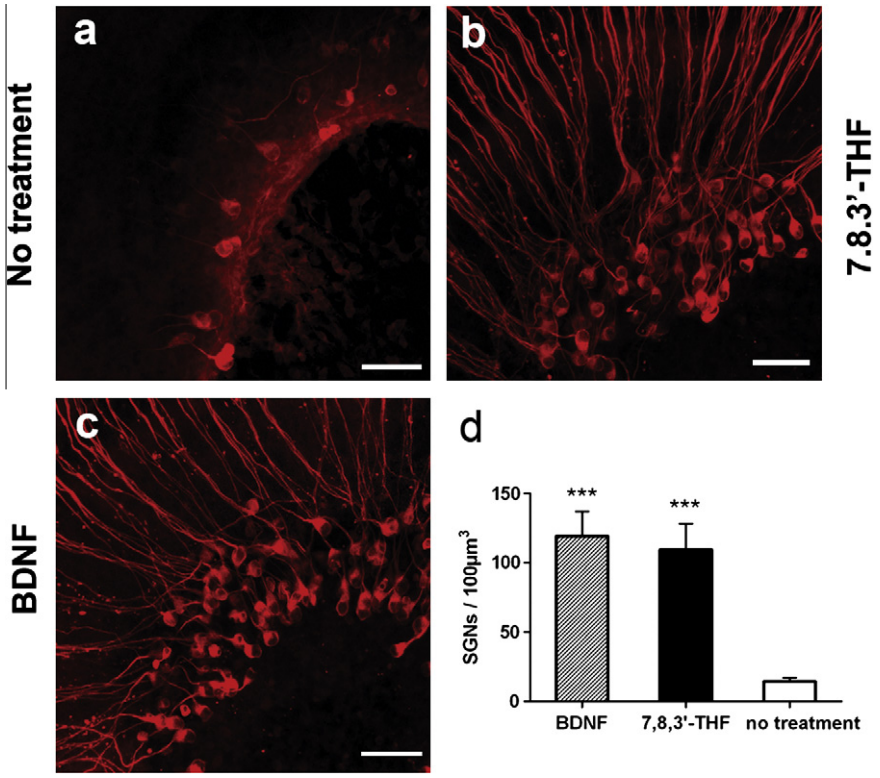

Fig. 1. Neuronal protective effect of 7,8,3′-THF on SGNs tested in organotypic cochlear cultures. Confocal images of β-tubulin immunolabeled SGNs maintained in organotypic cochlear cultures for 3 days with no supplement (a), treated with 7,8,3′-THF 300 nM, (b), or BDNF 100 ng/ml, (c). Scale bars represent approximately 50 μm in all panels. Results are quantified for SGN soma densities in (d) for BDNF, 7,8,3′-THF and control groups. $^{***}p < 0.001$, tested by the student's *t* test by comparing to corresponding controls (no treatment group). Data are presented as mean ± SEM.

(Fig. 1b), which was similar to that obtained when 100 ng/ml BDNF was added in the culture media (119 ± 18 neurons/100 μm^3, $n = 8$) (Fig. 1c). These survival promotional effects of 7,8,3′-THF were consistent with previously reported BDNF effects obtained in organotypic cochlear cultures [16].

We next tested the electrical excitability of survived SGNs in order to assess their functions *in vitro*. Transient inward sodium currents were reliably elicited by depolarizing voltages from SGNs cultured with 7,8,3′-THF for three days ($n = 8$). Under the current clamp, a single action potential (AP) was evoked by suprathreshold current stimuli from both fresh-dissected SGNs and those cultured with 7,8,3′-THF (Fig. 2). The threshold currents needed to elicit APs were similar in fresh-dissected and cultured SGNs (90 ± 12 pA ($n = 10$) vs. 93 ± 14 pA ($n = 10$), respectively). These data indicated that SGNs survived with the help of 7,8,3′-THF after 3 days were electrically excitable.

It has been shown that a TrkB point mutation (F616A) renders the receptor sensitive to the functional blockage by 1NMPP1 [17], which has been used to effectively null the TrkB functions in a controlled manner [18]. To further test whether the neuroprotective effect of 7,8,3′-THF is dependent on the TrkB activation, we used cochleae isolated from TrkB^{F616A} knock-in mice. Contrary to the dramatic increase of SGN density by the applications of 7,8,3′-THF in cochlear cultures of WT mice (Fig. 1), the protective

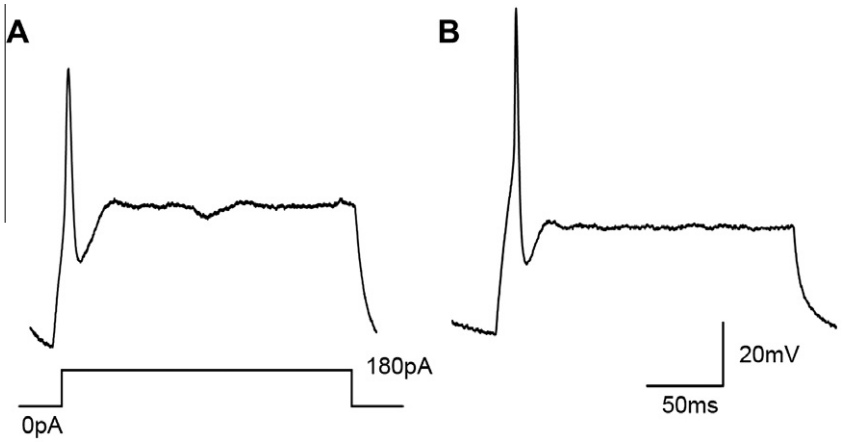

Fig. 2. SGNs rescued by 7,8,3′-THF are electrically excitable. APs recorded under current clamp from SGNs either freshly dissected (A) or cultured for 3 days in the presence of 7,8,3′-THF (B).

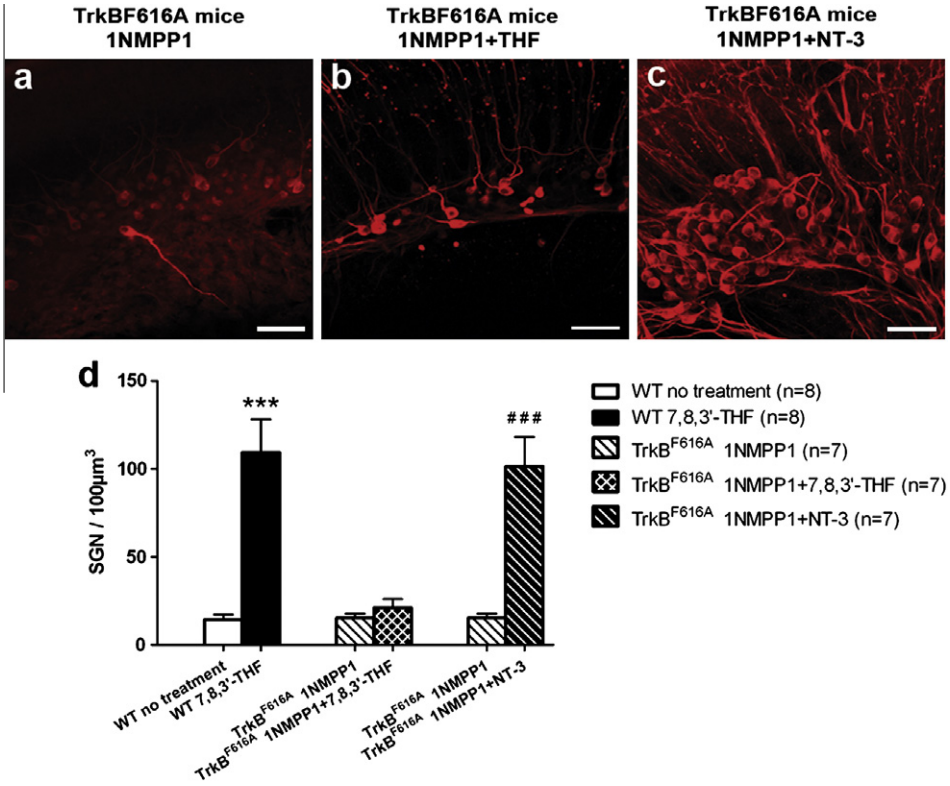

Fig. 3. Neuronal protective effect of 7,8,3′-THF is TrkB dependent. Organotypic cultures of the cochleae obtained from $TrkB^{F616A}$ knock-in mice were treated with 1NMPP1 (100 nM) to selectively block TrkB. While TrkB was inactivated, supplement of 7,8,3′-THF showed little neuroprotective effect (comparing (a) and (b)). In control experiments, NT-3 enhanced the SGN survival (c). Scale bars represent approximately 50 μm in all panels. Results are quantified in (d) by counting SGN soma density. $^{***}p < 0.001$, compared to WT with no exogenous activation of TrkB. Statistical analyses were performed by student's *t* tests. Data are presented as mean ± SEM.

effects of 7,8,3′-THF were abolished by blockage of TrkB with 100 nM 1NMPP1 (Fig. 3a and b). As a control test, we found that the survival promoting effect exerted by the NT-3 through the TrkC receptor was preserved when the TrkB was blocked by 1NMPP1 (Fig. 3c). These results further supported that 7,8,3′-THF selectively activated TrkB in exerting its neuroprotective actions of SGNs.

Our previous studies demonstrated that hair cells in the middle and basal turns of cCx26 null mice degenerate rapidly during postnatal day 14 (P14) to P21. Subsequently most SGNs in the Rosenthal's canal in these locations die by P60, only those in the apical turn survived [19,20]. We therefore used cCx26 null mice to test the *in vivo* efficacy of 7,8,3′-THF in protecting the SGNs. We applied the 7,8,3′-THF only to the left bulla and the right ear was used as an untreated control in order to compare the protective effects in the same animal. Both cochleae were examined 2 months after birth.

Morphology rescue effects were observed from cochlear sections. As expected, data obtained at the same developmental stage in the Rosenthal's canal of the cochlea obtained from WT animals demonstrated densely packed SGNs and nerve fibers (Fig. 4C). This is in sharp contrast to the results obtained from cCx26 null mice, in which a substantial loss of SGNs was observed on the untreated side of the cochlea in both the middle (indicated by a small arrow in Fig. 4A) and basal (indicated by a larger arrow in Fig. 4A) turns. SGNs in the 7,8,3′-THF treated side of the cochlea in the same animal were significantly preserved (Fig. 4, compare A and B). At the basal and middle turns ($n = 6$), the SGN densities were 1768 ± 168 neurons/100 μm^3 and 2506 ± 202 neurons/100 μm^3, respectively. These were significantly higher than densities in the untreated contralateral cochleae obtained from the same locations (548 ± 67 neurons/100 μm^3 and 826 ± 89 neurons/100 μm^3, respectively, $p < 0.001$). The SGN degeneration was not significant at the apical turn in cCx26 mice [19]. The differences in SGN density we observed at the basal turn were not statistically significant as well (Fig. 4D).

4. Discussion

BDNF exerts strong survival and neuroprotective effects on many types of CNS and PNS neurons [21], including the SGNs [22,23]. The preclinical evidence strongly supports the idea that BDNF might be useful as a therapeutic agent for a variety of neurological disorders. However, the outcomes of several clinical trials using recombinant BDNF are disappointing [24]. Presumably, this is due to poor delivery and the short *in vivo* half-life of BDNF [6]. 7,8-DHF is a recently identified small molecule TrkB receptor agonist that exhibits promising therapeutic effects for numerous neurological diseases [9–11,25]. Since our previous studies suggested that 7,8,3′-THF may possess a more robust TrkB stimulatory effect [11], this derivative compound would presumably exhibit potent efficacy in treating BDNF-related neurological diseases. The work presented here gives the first experimental confirmation for the neuroprotective effects of this new compound both *in vitro* and *in vivo*.

The cochlear implant is used by more than 100,000 people worldwide and is currently the only effective therapeutic treatment for patients suffering from severe sensorineural hearing loss. This device provides auditory cues by directly stimulating SG neurons, thus bypassing the damaged or missing hair cells. The survival of SGNs in the cochlea is critical for the optimal performance of cochlear implants [26,27]. Loss of significant amounts of SGNs also diminishes the hope for developing future treatments that are based on the regeneration of hair cells [28]. Exogenous applications of BDNF directly into the cochlea by either osmotic pump infusion or transplanting engineered cells that overexpressing BDNF to prevent the degeneration. Approximately 80–90% of SG neurons survived in the treated ear, compared to <30% survival rate in contralateral untreated cochlea of the same animal [4,5,29,30]. However, the use of BDNF and complicated delivery

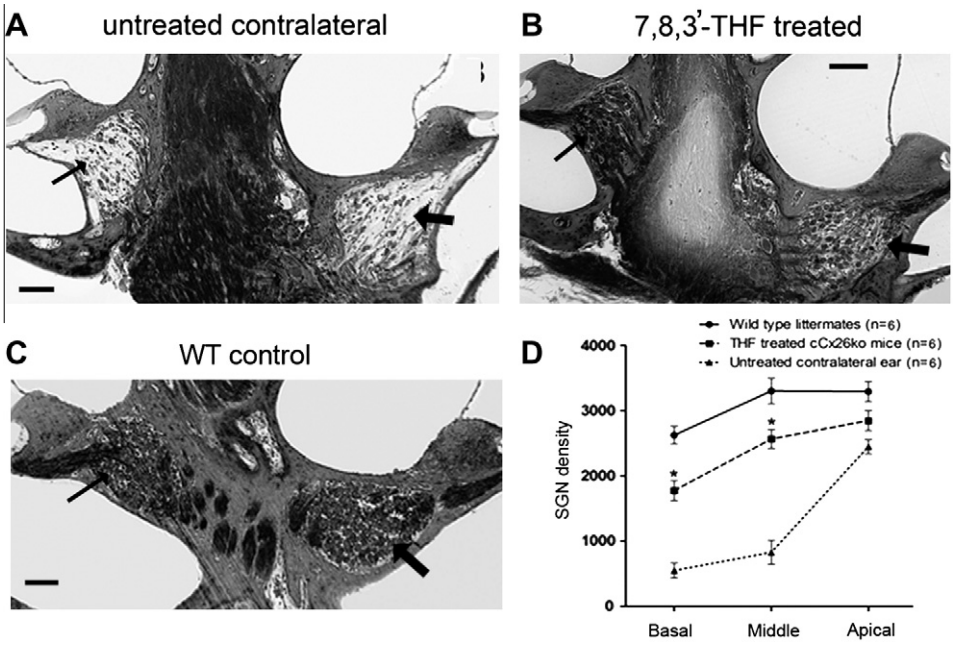

Fig. 4. 7,8,3′-THF protected SGNs in the Rosenthal's canal of cCx26 null mice from degeneration. Cochlear sections showing Rosenthal's canal at basal (indicated by larger arrows in panels) and middle (indicated by small arrows in panels) turns in untreated (A) and contralateral treated (B) cochlea in the same animal. Cochlear sections obtained from wild type mice from the same littermate are presented as a control (C). Scale bars represent approximately 500 μm in all panels.

methods make the previous approaches unlikely to be successful for translational uses in medical care. We believe the identification of 7,8,3′-THF reported here has brought us one step closer to the goal of translating the basic research of neuroprotection of SGNs into clinical practice.

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