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Netrin 1 mediates protective effects exerted by insulin-like growth factor 1 on cochlear hair cells

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ABSTRACT

Sensorineural hearing loss (SNHL) is mainly caused by the damage of cochlear hair cells (HCs). As HCs and supporting cells (SCs) do not proliferate in postnatal mammals, the loss of HCs and SCs is irreversible, emphasizing the importance of preserving their numbers to prevent SNHL. It is known that insulin-like growth factor 1 (IGF1) is instrumental in the treatment of SNHL. Our previous study indicates that IGF1 protects HCs against aminoglycoside by activating IGF1 receptor and its two major downstream pathways, PI3K/AKT and MEK/ERK, in SCs, which results in the upregulation of the expression of the Netrin1-encoding gene (*Ntn1*). However, the mechanisms underlying IGF1-induced protection of HCs via SC activation as well as the role of NTN1 in this process have not been elucidated. Here, we demonstrated that NTN1, similar to IGF1, promoted HC survival. NTN1 blocking antibody attenuated IGF1-induced HC protection from aminoglycoside, indicating that NTN1 is the effector molecule of IGF1 signaling during HC protection. *In situ* hybridization demonstrated that IGF1 potently induced *Ntn1* expression in SCs. NTN1 receptors were abundantly expressed in the cochlea; among them, UNC5B mediated IGF1 protective effects on HCs, as NTN1 binding to UNC5B inhibited HC apoptosis. These results provide new insights into the mechanisms underlying IGF1 protection of cochlear HCs, suggesting a possibility of using NTN1 as a new treatment for SNHL.

1. Introduction

The cochlea is the primary auditory organ in the inner ear that transduces sound signals to the auditory neurons, which in turn deliver them to brainstem structures for processing. Cochlear hair cells (HCs) responsible for this mechano-electrical transduction work as sound detectors and amplifiers. The mammalian cochlear HCs and surrounding supporting cells (SCs) lose their proliferation ability during embryonic stages and cannot regenerate if damaged after birth. HC destruction is the main cause of sensorineural hearing loss (SNHL), which is intractable because of HC inability to regenerate. Therefore, the maintenance of HC and SC numbers after cochlear injury is one of the most important strategies to treat SNHL.

Insulin-like growth factor 1 (IGF1) is a member of the insulin family of proteins that control cell proliferation, differentiation, and apoptosis in various tissues and organs (Varela-Nieto et al., 2007). IGF1 signaling through the corresponding receptor activates downstream pathways important for the development of the cochlear structure (Camarero et al., 2001; Okano et al., 2011) and maintenance of its function (Cediel et al., 2006; Woods et al., 1996), as evidenced by IGF1 protective effects exerted on damaged cochlear HCs *in vivo* and *in vitro* (Fujiwara et al., 2008; Hayashi et al., 2013; Iwai et al., 2006; Lee et al., 2007b). Furthermore, clinical trials have shown that IGF1 is effective in treating idiopathic sudden SNHL, which is one of the most common SNHL types in humans (Nakagawa et al., 2014, 2012, 2010).

IGF1 binds to its tyrosine kinase receptor (IGF1R) and activates two main signaling pathways of phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase kinase (MEK)/

Abbreviations: IGF1, insulin-like growth factor 1; IHCs, inner hair cells; NTN1, Netrin 1; OHCs, outer hair cells; SCs, supporting cells; SNHL, sensorineural hearing loss.

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extracellular signal-regulated kinase (ERK) (Varela-Nieto et al., 2007); both pathways regulate cell survival by inhibiting apoptosis and promoting cell cycle (Holmstrom et al., 1999; Laviola et al., 2007; Pages et al., 1993; Shepherd et al., 1998). In a previous study, we revealed that IGF1 induced SC proliferation and inhibited HC apoptosis by activating PI3K/AKT and MEK/ERK signaling in SCs (Hayashi et al., 2013), suggesting that IGF1 acts on SCs, which in turn mediate protective effects on HCs via surface-expressed or secreted factors. To identify HC effectors upregulated by IGF1, we performed comprehensive gene expression profiling to compare IGF1-treated and untreated cochlear explant cultures exposed to neomycin, an antibiotic of the aminoglycoside group known to cause irreversible hearing loss by damaging the cochlea. As a result, we identified two genes upregulated in IGF1-treated cultures, including *netrin 1* (*Ntn1*) (Hayashi et al., 2014).

Netrin 1 (NTN1) belongs to a family of laminin-related secretory proteins and is involved in axon guidance and cell migration via activation of six canonical receptors, UNC5A, UNC5B, UNC5C, UNC5D, NEOGENIN, and DCC (Serafini et al., 1994). In rodents, the *Ntn1* gene is expressed in many tissues other than the central nervous system, including the inner ear (Matilainen et al., 2007). Recent studies have revealed that NTN1 is also involved in tissue morphogenesis and survival as an anti-apoptotic factor (Hebrok and Reichardt, 2004; Liu et al., 2004; Nguyen and Cai, 2006; Rajasundari et al., 2011).

2. Materials and methods

2.1. Animals

ICR mice were purchased from Japan SLC (Hamamatsu, Japan). All animal procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Research Committee of the Graduate School of Medicine, Kyoto University (No. 11179). Animals were maintained under the supervision of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

2.2. Preparation and treatment of cochlear sensory epithelium explant cultures

Cochlear explant cultures were established as previously described (Yamamoto et al., 2006). Briefly, P2 ICR mice were euthanized with carbon dioxide and the cochleae were dissected out. Then, surrounding tissues were removed to obtain the organs of Corti, which were placed onto cell culture inserts (Becton Dickinson, Franklin Lake, NJ, USA) and incubated in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Inc., St. Louis, MO, USA) supplemented with D-glucose (6 g/l) and penicillin G at 37 °C in a humidified 5% CO_2 atmosphere. To exclude the possibility of effects caused by serum growth factors or hormones, serum-free medium was used in all experiments. Treatments started 24 h after dissection and incubation *in vitro* to stabilize the explants.

To determine NTN1 protective effects against aminoglycoside antibiotics, cochlear explant cultures grown in the medium containing 0.9 mg/ml neomycin sulfate (N6386, Sigma-Aldrich) or 100 μg/ml gentamicin sulfate (1405-41-0, Sigma-Aldrich) were treated for 24 h with various concentrations of NTN1 (1109-N1, R&D Systems, Minneapolis, MN, USA) or with only vehicle (n = 6–9). Explants cultured for 48 h without any treatment were used as control.

To detect NTN1 in the culture medium by western blotting, four cochlear explants were treated with only 1 μg/ml IGF1 (Orphan-Pacific Pharma Inc., Tokyo, Japan) or with 0.9 mg/ml neomycin ±1 μg/ml IGF1 (n = 5–9 for each condition) for 24 h before collecting culture supernatants. The supernatant from untreated explants was used as control.

To determine whether IGF1 protected HCs from neomycin through NTN1, an anti-NTN1 blocking antibody (Cayre et al., 2013) (AF1109, R&D Systems) or non-specific IgG (AB108C, R&D Systems) were added at 2 μg/ml to cochlear explant cultures treated for 24 h with 0.9 mg/ml neomycin and 1 μg/ml of recombinant human IGF1 (n = 6–8).

Cochlear explants treated for 24 h with 0.9 mg/ml neomycin with or without 1 μg/ml IGF1 or 1 μg/ml NTN1 were used to examine the expression of *Ntn1* mRNA, the effects of UNC5B blockade, the involvement of the MEK/ERK and PI3K/AKT signaling pathways, and cell cycle progression.

To determine UNC5B involvement in HC protection, an anti-UNC5B antibody (AF1006, R&D Systems) or non-specific IgG (AB108C, R&D Systems) were added at 2 μg/ml to the cultures for 24 h (n = 6–7). To test the activation of MEK/ERK and PI3K/AKT signaling, MEK inhibitor U0126 (19–147, EMD Chemicals, Gibbstown, NJ, USA) and PI3K inhibitor LY294002 (440202, EMD Chemicals) were used at 10 μM (n = 4–6). The effects on the cell cycle were examined by adding 3 μg/ml BrdU (Sigma-Aldrich) (n = 4–5). To test the involvement of cell proliferation in HC protection, explants were cultured for 24 h with 0.9 mg/ml neomycin and 2.8 mM aphidicolin (A0781, Sigma-Aldrich) (n = 5); the specificity and toxicity of aphidicolin were examined as described previously (Hayashi et al., 2013).

Apoptosis in cochlear epithelium was determined by treating the explants with 0.9 mg/ml neomycin with or without NTN1 for 24 h (n = 7) and immunostaining with anti-cleaved caspase 3 antibodies.

2.3. Immunohistochemistry

The samples were fixed at room temperature (RT) for 15 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), rinsed with phosphate-buffered saline (PBS), and incubated in blocking solution (10% goat serum with 0.2% TritonX-100) for 30 min at RT. The samples were then incubated with the following primary antibodies: rabbit polyclonal anti-myosin7a (1:500; Proteus Bioscience Inc., Ramona, CA, USA), rabbit polyclonal anti-cleaved caspase 3 (1:100; Cell Signaling Technology, Danvers, MA, USA), and mouse monoclonal anti-BrdU (1:50; BD Biosciences, Franklin Lakes, NJ, USA) overnight at 4 °C and then with Alexa-488- or -546-conjugated anti-rabbit or anti-mouse goat IgG (1:500; Invitrogen, Grand island, NY, USA) for 1 h at RT. F-actin (actin filaments) were visualized by incubation with Alexa-488- or Alexa-546-labeled phalloidin (1:100; Invitrogen) for 1 h at RT. The samples were then counter-stained with 2 mg/ml DAPI (Invitrogen) in PBS to visualize nuclei.

2.4. Western blotting

Culture medium of explants incubated at different conditions (250 μl) was concentrated using an ultra centrifugal filter (Merck Millipore, Germany). Then, total proteins in the medium were separated by SDS-PAGE on 5–20% gradient gel (Wako, Japan) and transferred onto polyvinylidene fluoride membranes (Merck Millipore). The membranes were blocked with 5% BSA in PBS with 0.1% Tween 20 for overnight, followed by incubation with primary rabbit monoclonal anti-NTN1 antibodies (1:3000; ab126729, Abcam, Cambridge, UK) for 1 h at RT. The membranes were then treated with secondary antibody, biotinylated anti-rabbit IgGs (1:1000; GE Healthcare Life Sciences, Marlborough, MA, USA) for 1 h at RT, and then, with an appropriate horseradish peroxidase-conjugate (GE Healthcare Life Sciences) for 1 h at RT. Immunoreactivity was

detected using ECL detection reagents (Nacalai Tesque, Japan).

2.5. In situ hybridization

Digoxigenin (DIG)-labeled sense and antisense RNA probes were prepared from plasmids containing partial cDNA sequences of mouse *Unc5a* (Matilainen et al., 2007), *Unc5b* (Matilainen et al., 2007), *Unc5c* (Matilainen et al., 2007), *Unc5d* (Matilainen et al., 2007), *Dcc* (Matilainen et al., 2007), *Neo1* (neogenin) (Keeling et al., 1997), and *Ntn1* (Noda et al., 2012) as previously described (Wu and Oh, 1996). Corresponding sense probes were used as negative controls.

Frozen sections from the P2, P7 or P28 cochleae were processed as previously described (Wu and Oh, 1996). Briefly, the cochleae were fixed overnight with 4% paraformaldehyde in PBS, dehydrated in 30% sucrose, and embedded in Tissue-Tek® O.C.T.™ compound (Sakura, Japan). The samples were sectioned at 12-μm thickness, mounted on MAS-coated glass slides (Matsunami, Japan), and stored at −80 °C until analysis. The slides were rehydrated, post-fixed, permeabilized with 10 μg/ml proteinase K for 4 min, and prehybridized for 1 h at RT in hybridization buffer containing 50% formamide, 5× SSC (both from Nacalai Tesque, Kyoto, Japan), 1% SDS, 50 μg/ml yeast RNA, and 50 μg/ml heparin (all from Sigma-Aldrich). Hybridization was performed at 70 °C overnight in sealed plastic bags containing up to 4 glass slides in 5 ml of hybridization solution with 0.2 μg/ml probe. On the following day, slides were washed first in 50% formamide with 6× SSC and 1% SDS at 70 °C, then in 50% formamide with 2.4× SSC at 65 °C, and finally in 1× Tris-buffered saline, pH 7.4, with 0.1% Tween 20 (TBST; Sigma-Aldrich) at RT. Slides were then blocked in 5% sheep serum (Sigma-Aldrich) at RT for 1 h, and DIG-labeled RNA hybrids were detected with alkaline phosphatase-conjugated anti-DIG Fab fragments (1:4000 in TBST; Roche, Basel, Switzerland) diluted overnight at 4 °C. Slides were washed in TBST and NTMT containing 100 mM NaCl (Nacalai Tesque), 100 mM Tris-HCl pH 9.5, 10 mM $MgCl_2$ (Wako), 0.1% Tween-20, and 480 μg/ml levamisole (Sigma-Aldrich). The reaction was developed by adding NBT/BCIP substrate (Roche) for 12 h at RT.

2.6. Whole-mount in situ hybridization

The cochlear explants treated with 0.9 mg/ml neomycin, 1 μg/ml IGF1, or both were fixed overnight with 4% paraformaldehyde in PBS, dehydrated through a graded methanol series (25% methanol/PBS-Tween 20 (PBST), 50% methanol/PBST, 75% methanol/PBST, 100% methanol), and stored in 100% methanol at −20 °C. Then, the explants were first rehydrated through a graded methanol series, post-fixed, permeabilized with 10 μg/ml proteinase K for 17 min, and prehybridized for 1 h at 70 °C in the hybridization buffer (50% formamide, 5× SSC, 1% SDS, 50 μg/ml yeast RNA, and 50 μg/ml heparin). Hybridization was carried out at 70 °C overnight in 1.5 ml tubes containing up to 6 explants in 400 μl of hybridization solution with 0.5 μg/ml probe. On the following day, the explants were sequentially washed in 50% formamide with 5× SSC and 1% SDS at 70 °C, in 500 mM NaCl, 10 mM Tris-HCl pH 7.5, and 0.1% Tween-20 at RT, in 50% formamide with 2× SSC at 65 °C, and finally in TBST at RT. Explants were then blocked in 10% sheep serum at RT for 1 h. DIG-labeled RNA hybrids were detected as described for frozen sections. The following day, the explants were washed in TBST with 480 μg/ml levimasole and then in NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, and 0.1% Tween-20). The reaction was developed by adding NBT/BCIP substrate for several hours or overnight at RT.

2.7. Image analysis

Images were obtained with a Spot digital camera mounted on a BX50 microscope (Olympus, Tokyo, Japan) for *in situ* hybridization, and Leica TCS-SPE or SP8 confocal microscope (Leica Microsystems Inc., Wetzlar, Germany) for immunohistochemistry. The images were processed using Adobe Photoshop CC and Adobe Illustrator CC. Image manipulations were limited to adjustments of level, hue, and saturation.

2.8. Cell counts

The total length of each organ of Corti was measured using ImageJ software (NIH). Briefly, after IHCs and OHCs were identified by immunohistochemistry of myosin7a, lines were drawn between IHCs and the first row of OHCs through the whole cochlear duct and the total length of the organ of Corti was calculated. Only the basal portion of each explant (60%–80% from the apex) was selected for analysis because HCs in the apical portion are resistant to aminoglycosides and 80%–100% cells from the apex are often damaged by preparation. To avoid the bias in selecting regions for cell counting, the numbers of all inner HCs (IHCs) and outer HCs (OHCs) or SCs in the basal portion were counted for each sample to calculate average numbers of IHCs and OHCs or SCs per 200 μm. HCs with remaining stereocilia labeled with phalloidin were considered alive. Cleaved caspase 3-positive HCs were counted if they were co-stained with DAPI and their hair bundles were stained with phalloidin. BrdU-positive cells co-stained with DAPI were counted in the epithelial layer of the basal turn.

2.9. Statistical analysis

Student's t-test and one-way ANOVA with post-hoc test (Tukey's method) were used for comparison between two groups and among multiple groups, respectively. The data were expressed as the mean ± standard error (SE); p values below 0.05 were considered statistically significant.

3. Theory

We hypothesized that NTN1 plays a critical role in mediating HC protection against aminoglycosides by IGF1. To test it, we determined whether the release of NTN1 protein into culture medium of cochlear sensory epithelium explant was induced by IGF1 and whether NTN1 itself was effective in preventing the damage of cochlear HCs by aminoglycoside. In addition, we gained mechanistic insights into NTN1 function in the cochlea by examining the expression of *Ntn1* mRNA within the cochlea after IGF1 treatment, identifying the NTN1 receptors responsible for the protective effect, and determining whether NTN1 mediates the whole spectrum of IGF1 activity towards HCs.

4. Results

4.1. NTN1 protects cochlear hair cells against neomycin

In our previous study, we used cochlear explants established from postnatal day (P) 2 mice to demonstrate that IGF1 protected cochlear HCs against neomycin (Hayashi et al., 2013). In this study, we employed the same system to determine whether NTN1, a candidate effector of IGF1 signaling, had similar effects on damaged HCs. Our results indicate that the administration of 1 μg/ml NTN1 markedly protected both the IHCs and OHCs from damage by neomycin (Fig. 1), which was similar to the effect produced by IGF1 (Hayashi et al., 2013). NTN1 protection against neomycin was

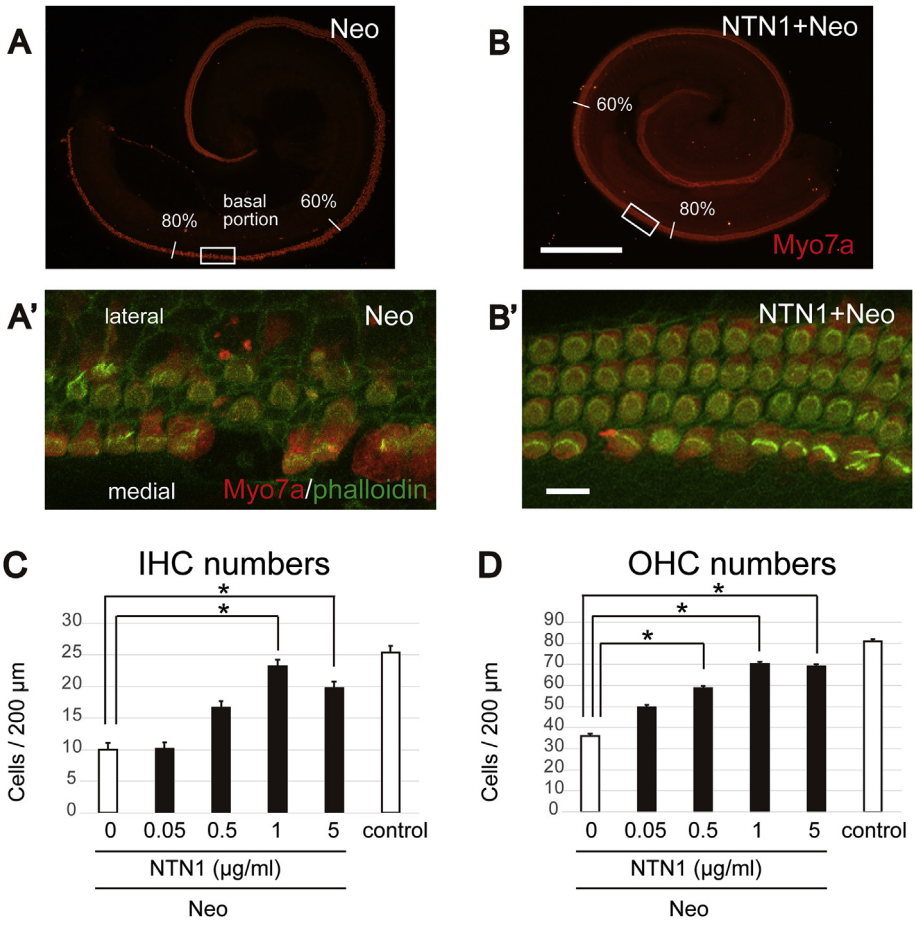

Fig. 1. NTN1 protects cochlear hair cells against neomycin damage. **A**–**B**′. Representative images of the cochlear explants treated with 0.9 mg/ml neomycin (Neo; **A** and **A**′) or Neo and 1 μg/ml NTN1 (**B** and **B**′) and stained for myosin 7a (Myo7a, red) and actin filaments (phalloidin, green). **A**′ and **B**′ present magnified images of the boxed regions in **A** and **B**, respectively. **C** and **D**. Survival of Neo-treated hair cells incubated with different concentrations of NTN1. NTN1 protected both the inner hair cells (IHCs) (**C**; $p < 0.0001$) and outer hair cells (OHCs) (**D**; $p < 0.0001$). Scale bar, 10 μm. The data are expressed as the mean ± SE; *$p < 0.05$ by Tukey's method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

statistically significant for both IHCs and OHCs ($p < 0.0001$, by one-way ANOVA) and was comparable to that of IGF1 (Fig. 1C and D). We confirmed the effect of the IGF1-NTN1 axis in HC protection against another aminoglycoside, gentamicin (Supplementary Fig. 1). Both IGF1 and NTN1 protected cochlear OHCs against gentamicin and the maximal effect of NTN1 was comparable to that of IGF1. These results indicate that NTN1, similar to IGF1, protected cochlear HCs against aminoglycosides.

4.2. NTN1 blockade attenuates IGF1-induced protection of neomycin-treated HCs

The observed activity of NTN1 suggests that it plays a role in the protection of HCs by IGF1. To further investigate this effect, we examined if IGF1 treatment induced NTN1 protein release into culture medium using western blotting analysis (Fig. 2A and B) (Jarjour et al., 2011; Shimizu et al., 2013). The results indicated that the conditioned medium of the explants treated with neomycin and IGF1 contained significantly higher amounts of NTN1 protein than that of explants treated with neomycin only although IGF1 only, neomycin only, or IGF1 and neomycin treatment induced significantly higher amount of NTN1 compared with untreated samples ($p < 0.0001$ by one-way ANOVA and $p < 0.05$ by Tukey's method). As the next step to confirm the involvement of NTN1 in the protection of HCs by IGF1, we added a blocking antibody (Ab) against NTN1 (Cayre et al., 2013) to cochlear explant cultures treated with neomycin and IGF1. IGF1-induced protection of both IHCs and OHCs was markedly attenuated by NTN1 Ab (Fig. 2C) but not by non-specific IgG (Fig. 2D). This effect of NTN1 Ab was statistically significant for both IHCs and OHCs ($p < 0.05$; Fig. 2E and F), indicating that secreted NTN1 is involved in IGF1 protection of cochlear HCs against aminoglycoside. Considering that IGF1 acts mainly on SCs, we hypothesized that NTN1 produced by IGF1-stimulated SCs played a role in protecting HCs treated with aminoglycoside.

4.3. Ntn1 is highly expressed in SCs after treated with IGF1 and neomycin

Our previous study has demonstrated that *Ntn1* expression is significantly upregulated by IGF1 in neomycin-treated cochlear cells (Hayashi et al., 2014). We did not detect *Ntn1* expression in the sensory epithelium or spiral ganglion cells of the cochlea in physiological conditions by *in situ* hybridization (data not shown). To identify cochlear cell populations expressing *Ntn1* after IGF1 stimulation, we performed whole-mount *in situ* hybridization (WISH) in cochlear explants treated with neomycin (Fig. 3A and B), IGF1 (Fig. 3C and D), and neomycin with IGF1 (Fig. 3E–H). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis showed that *Ntn1* expression was transiently upregulated

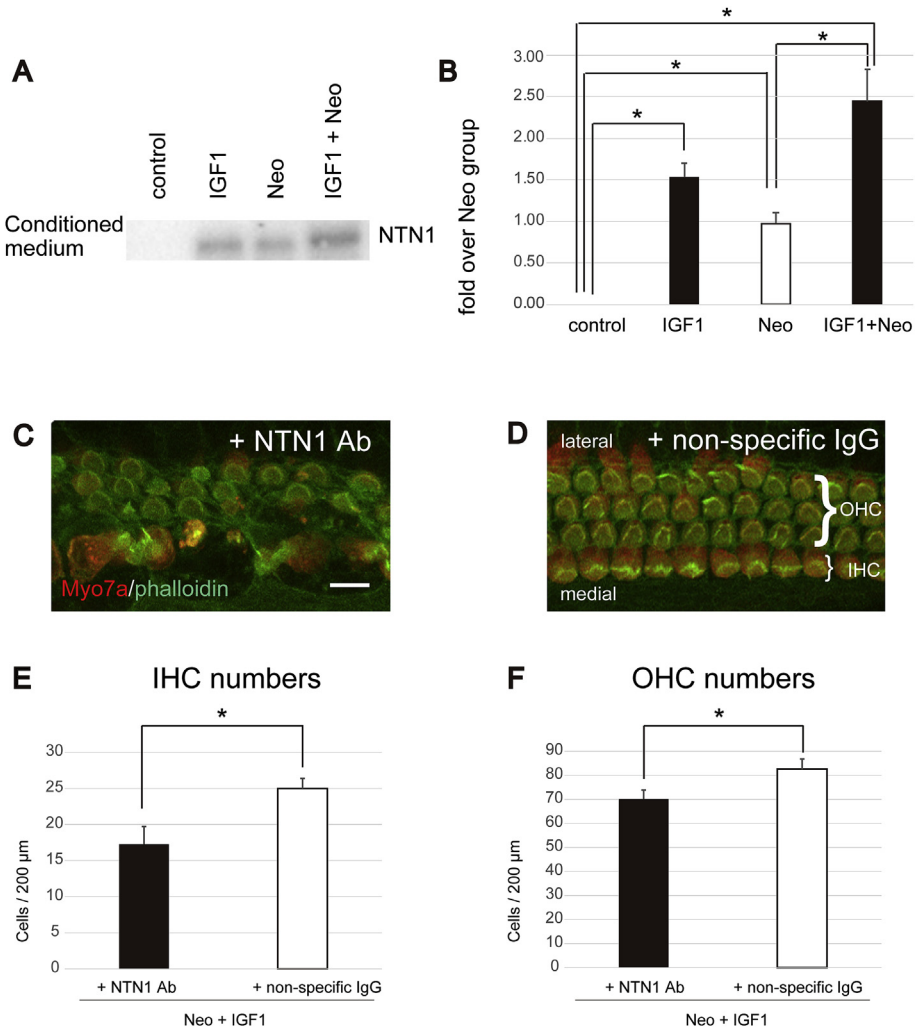

Fig. 2. IGF1 induces NTN1 release into culture medium and NTN1 blocking attenuates IGF1-induced protection of neomycin-treated hair cells. A, B. The expression of the NTN1 protein was evaluated in the conditioned medium from untreated (control), IGF1-treated, neomycin (Neo)-treated, or IGF1 + Neo-treated samples. A representative image of western blotting (A) shows that conditioned medium from IGF1-, Neo- and IGF1 + Neo-treated samples contained NTN1, and that IGF1 + Neo induced the release of significantly higher amounts of NTN1 than only Neo (B). C–F. Cochlear explant cultures were treated with Neo and IGF1 in the presence of the NTN1 antibody or non-specific IgG. C, D. Representative images of the cultures incubated in the presence of the NTN1 antibody (C) or non-specific IgG (D). The specimens were stained for myosin7a (Myo7a, red) and actin filaments (phalloidin, green). E, F. Survival of the inner hair cells (IHCs) and outer hair cells (OHCs). The addition of NTN1 Ab caused significant attenuation of IGF1 protective effects on the IHCs (E) and OHCs (F). Scale bar, 10 μm. The data are expressed as the mean ± SE; *$p < 0.05$ by Tukey' s method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3 h after IGF1 administration returning to control levels after 12 h (Hayashi et al., 2014). Therefore, we analyzed *Ntn1* mRNA levels in the cochlear explants 3 h after IGF1 treatment.

Weak *Ntn1* expression was detected in spiral ganglion cells but not in the sensory epithelia of the neomycin-treated cochlear cultures (Fig. 3A and B), while in IGF1-treated explants, high levels of *Ntn1* transcripts were detected in spiral ganglion cells but not in the sensory epithelium (Fig. 3C and D). In contrast, the combined treatment with neomycin and IGF1 stimulated strong *Ntn1* expression in both spiral ganglion cells and the sensory epithelium (Fig. 3E and F). Close observation of the cochlear basal turn revealed that IGF1 with neomycin induced *Ntn1* expression in several types of SCs, including Hensen's and Claudius' cells (black arrowhead in Fig. 3F) and inner sulcus cells (white arrowhead in Fig. 3F), as well as in pillar cells located between the IHCs and OHCs (black arrow in Fig. 3F). Compared with SCs, *Ntn1* expression in both IHCs and OHCs was weak (Fig. 3F). Sectioning of the WISH samples (Fig. 3G and H) also showed that almost all SCs including Deiters' cells (white arrows in Fig. 3G and H) and Hensen's and Claudius' cells (black arrowheads in Fig. 3G and H) expressed *Ntn1*. However, we could observe only weak *Ntn1* signals in the IHCs and OHCs (Fig. 3G and H). These results indicate that IGF1 induces *Ntn1* expression in spiral ganglion cells and SCs only when the sensory epithelium is damaged.

4.4. *UNC5B is the only receptor expressed in the sensory epithelium through the whole cochlear duct*

NTN1 exerts its effect through several receptors, including six canonical receptors, DCC, NEOGENIN, UNC5A, UNC5B, UNC5C, and UNC5D. In contrast to the embryonic cochlea, the expression patterns of NTN1 and its receptors in the postnatal cochlea are not well investigated (Matilainen et al., 2007). To determine cell populations responding to NTN1, we examined the expression of its canonical receptors in the postnatal cochlea under physiological conditions by *in situ* hybridization (Fig. 4). In spiral ganglion cells, all receptors except *Unc5a* were expressed (black arrows in Fig. 4A, C, H, K and N), while in the cochlear sensory epithelium, *Unc5a*, *Unc5b*, and

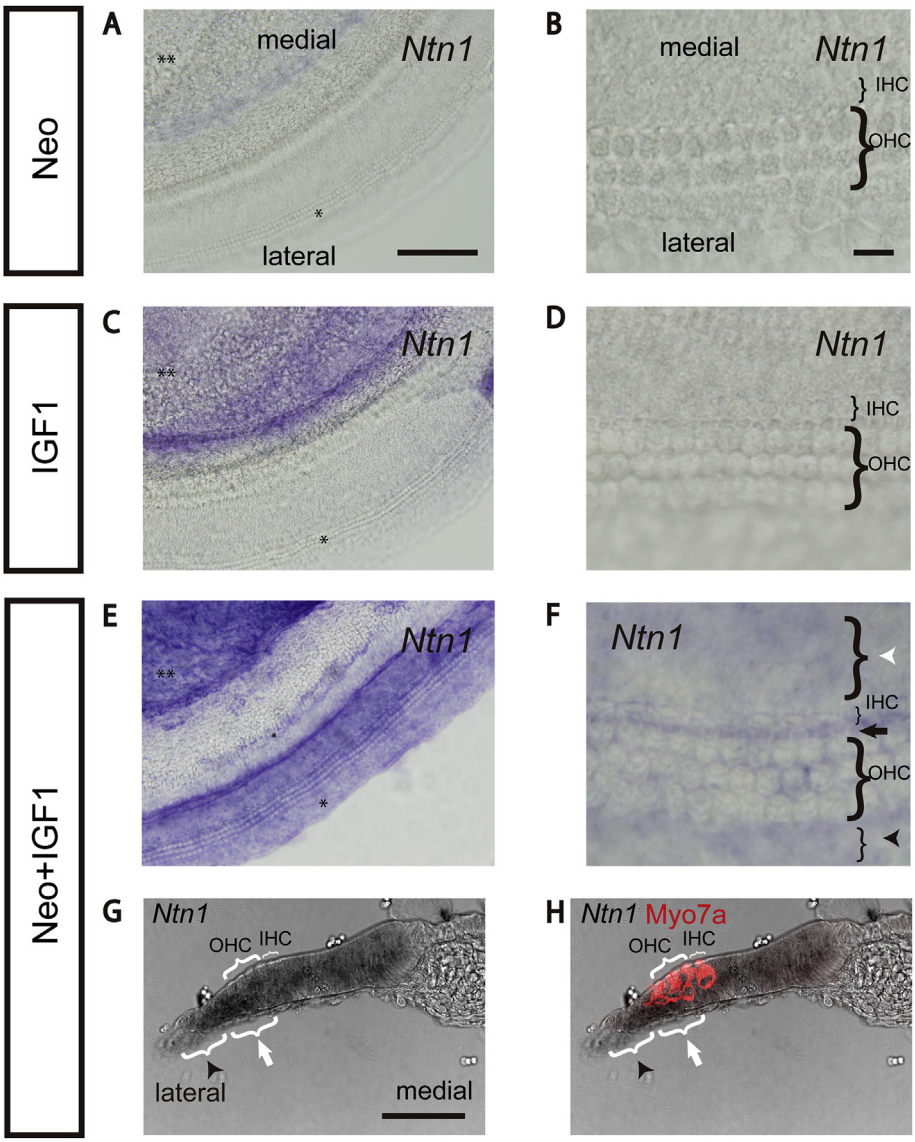

Fig. 3. IGF1 effects on *Ntn1* expression in neomycin-treated supporting cells. Cochlear explant cultures were treated with neomycin (Neo; **A, B**), IGF1 (**C, D**), or Neo with IGF1 (**E**–**H**) and analyzed for *Ntn1* expression by whole-mount *in situ* hybridization (WISH). Neo + IGF1 treated WISH samples were sectioned to determine the area of *Ntn1* expression more precisely (**G**, **H**). Neo did not induce *Ntn1* expression in the cochlear sensory epithelium (**A**, single asterisk; **B**), although very weak expression was observed in spiral ganglion cells (**A**, double asterisks). IGF1 did not influence *Ntn1* expression in the sensory epithelium (**C**, single asterisk; **D**), but significantly induced it in spiral ganglion cells (**C**, double asterisks). IGF1 + Neo induced *Ntn1* expression in the sensory epithelium (**E**, single asterisk; **F**) and spiral ganglion cells (**E**, double asterisks) of the Neo-treated cochlea. Stronger *Ntn1* expression was observed in supporting cells (Deiters' cells, pillar cells, inner sulcus cells, and Hensen's and Claudius' cells marked by white arrows, black arrows, white arrowheads, and black arrowheads, respectively) than in hair cells (**F**, **G**, **H**). The explants were stained for myosin7a (Myo7a) (**H**). Scale bars: 100 μm (**A**, **C**, **E**), 10 μm (**B**, **D**, **F**), and 50 μm (**G**, **H**). IHC: inner hair cell, OHC: outer hair cell.

Unc5c transcripts were detected (white arrows in Fig. 4E, H, and K, respectively, and Fig. 4F, G, I, J, L and M). *Unc5a* was expressed in the IHCs and OHCs (black and white arrowheads, respectively; Fig. 4F and G) of the cochlear apical turn. In contrast, *Unc5c* transcripts were detected in SCs, inner sulcus cells (white asterisks) and Hensen's and Claudius' cells (double white asterisks) of the cochlear apical turn (Fig. 4L and M). However, a clear basal-to-apical gradient in *Unc5a* and *Unc5c* expression was observed. *Unc5a* and *Unc5c* transcripts were detected only in the apical turn, but not in the basal turn (Fig. 4E–G and K–M). In contrast, *Unc5b* was the only receptor expressed through the whole cochlear duct (white arrows, Fig. 4H) including the basal turn where NTN1 protection of HCs was observed; *Unc5b* transcript was observed in the IHCs, OHCs, and several types of SCs in both the apical and basal turns of the cochlea (Fig. 4H–J). *Unc5b* expression disappeared in the cochlear at a later stage, P28, although it was detected at P7 (Supplementary Fig. 2).

4.5. HC protection by IGF1 is mediated via the UNC5B receptor

Considering that UNC5B is the only receptor expressed in HCs of the cochlear basal turn, we hypothesized that NTN1 induced by IGF1 exerted its effect through the UNC5B receptor. Before testing this hypothesis, we first confirmed that Unc5b was expressed in the cochlear explants treated with neomycin (Fig. 5A–C) with similar pattern with the physiological condition (Fig. 4I and J). Secondly, to test our hypothesis, we investigated whether UNC5B inhibition would result in the attenuation of NTN1 protection for neomycin-damaged HCs by blocking the UNC5B receptor with a specific antibody (UNC5B Ab) (Kohler et al., 2013; Ly et al., 2005). After

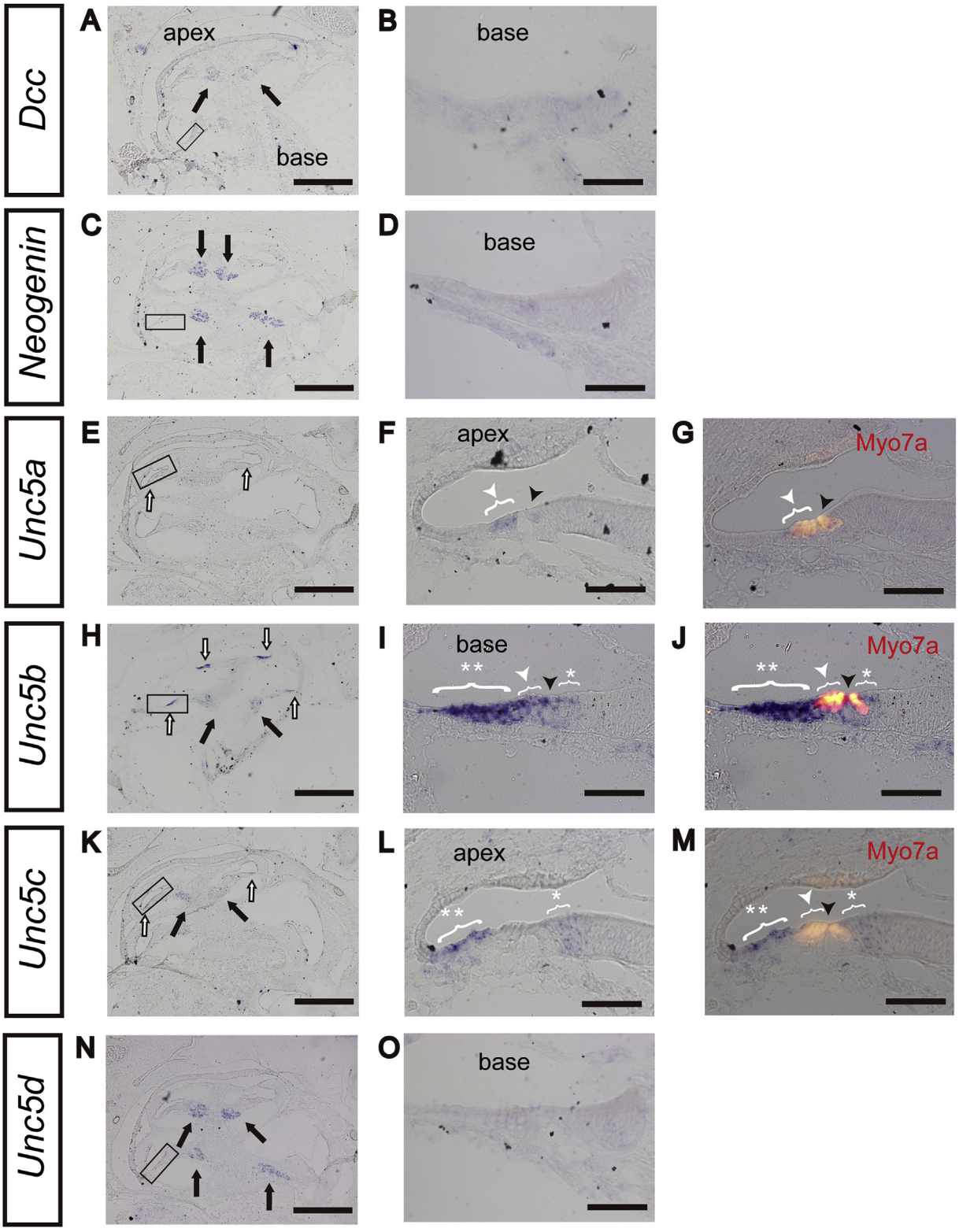

Fig. 4. Expression of NTN1 receptors in the P2 cochlea. Expression patterns of six canonical NTN1 receptors were examined by *in situ* hybridization (ISH). **A**, **B.** Representative images of *Dcc* expression in the cochlea shown at low (**A**) and high (**B**, basal turn) magnification. *Dcc* mRNA was not detected in the sensory epithelium, but was observed in spiral ganglion cells (black arrows). **C**, **D**. Representative images of *Neo1* (*Neogenin*) expression in the cochlea shown at low (**C**) and high (**D**, basal turn) magnification. *Neo1* transcripts were detected in spiral ganglion cells (black arrows) but not in the sensory epithelium. **E**–**G.** Representative images of *Unc5a* expression in the cochlea shown at low (**E**) and high (**F** and **G**, apical turn) magnification. *Unc5a* mRNA was not detected in spiral ganglion cells or the sensory epithelium of the basal turn, but expressed in the sensory epithelium of the apical turn (white arrows, **E**). Immunostaining for myosin 7a (Myo7a, red in **G**) indicated that *Unc5a* expression was restricted to the inner and outer hair cells (black and white arrowheads, respectively; **F**, **G**) in the apical turn. **H**–**J.** Representative images of *Unc5b* expression in the cochlea shown at low (**H**) and high (**I** and **J**, basal turn) magnification. *Unc5b* mRNA was detected in spiral ganglion cells (black arrows) and the sensory epithelium of both the apical and basal turns (white arrows; **H**). In the sensory epithelium, immunostaining for myosin 7a (**J**) indicated that *Unc5b* mRNA was expressed both in the inner and outer hair cells of the basal turn (black and white arrowheads, respectively; **I** and **J**) and in supporting cells (single and double asterisks; **I** and **J**). **K**–**M.** Representative images of *Unc5c* expression in the cochlea shown at low (**K**) and high (**L** and **M**, apical turn) magnification. *Unc5c* mRNA was detected in spiral ganglion cells and the sensory epithelium of the apical turn (black and white arrows, respectively; **K**). In the sensory epithelium, immunostaining for myosin 7a (**M**) indicated that *Unc5c* mRNA was expressed in supporting cells (inner sulcus cells, and Hensen's and Claudius' cells indicated by single and double white asterisks, respectively; **L**, **M**) of the apical turn, but not in the inner or outer hair cells (black and white arrowheads, respectively; **L**, **M**). **N**, **O**. Representative images of *Unc5d* expression in the cochlea shown at low (**N**) and high (**O,** basal turn) magnification. *Unc5d* mRNA was detected in spiral ganglion cells (black arrows), but not in the sensory epithelium (**O**). Scale bars: 500 μm (**A**, **C**, **E**, **H**, **K**, **N**), 100 μm (**B**, **D**, **O**), and 50 μm (**F**, **G**, **I**, **J**, **L**, **M**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

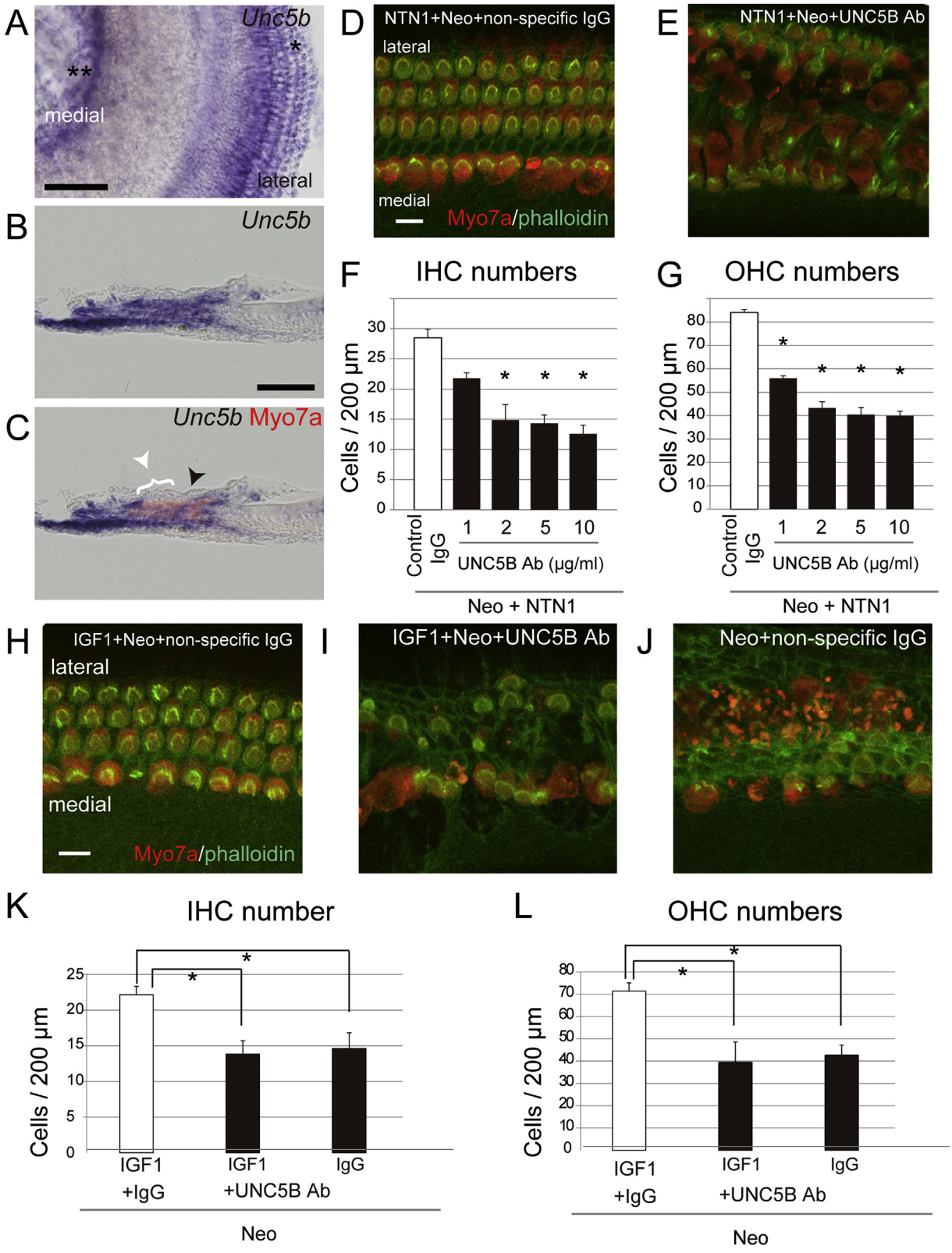

Fig. 5. IGF1 and NTN1 protects neomycin-treated hair cells of the cochlear basal turn via the UNC5B receptor. A—C. Cochlear explant cultures were treated with neomycin and analyzed for *Unc5b* expression by whole-mount *in situ* hybridization (WISH) (**A**). Neo-treated WISH samples were sectioned to determine the area of *Unc5b* expression more precisely (**B**, **C**). The explants were stained for myosin7a (Myo7a) (**C**) to indicate the position of hair cells. *Unc5b* is expressed in both spiral ganglion cells and the sensory epithelium (A, double and single asterisks, respectively). Sectioning of the WISH samples showed that *Unc5b* is expressed in inner hair cells (C, black arrowhead), outer hair cells (C, white arrowhead), and several types of supporting cells. **D—G.** Cochlear explant cultures were treated with neomycin (Neo) and NTN1 in the presence of 2 µg/ml non-specific IgG (**D**) or 2 µg/ml UNC5B antibody (Ab) (**E**) and stained for myosin7a (Myo7a, red) and actin filaments (phalloidin, green). UNC5B Ab significantly attenuated NTN1 protective effects on the inner hair cells (IHCs; **F**, $p < 0.0001$) and outer hair cells (OHCs; **G**, $p < 0.0001$). **H—L.** Cochlear explants were treated with Neo + IGF1 (**K**, **I**) or Neo alone (**J**) and incubated with 2 µg/ml non-specific IgG (**H**, **J**) or 2 µg/ml anti-UNC5B Ab (**I**) and stained for myosin7a (Myo7a, red) and actin filaments (phalloidin, green). **K**, **L.** Numbers of surviving IHCs (**K**) and OHCs (**L**) treated as above were counted. IGF1-induced protection of the IHCs and OHCs was significantly attenuated by blocking the UNC5B receptor ($p < 0.05$ for IHCs and OHCs). No significant difference in IHC and OHC numbers was observed between the UNC5B Ab + neomycin + IGF1 group and the non-specific IgG + neomycin group. Scale bars, 50 µm (A—C) and 10 µm (D, H). The data are expressed as the mean ± SE; *$p < 0.05$ by Tukey's method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

confirming that UNC5B Ab in the wide concentration range was not toxic to the cochlear explant cultures (data not shown), we added UNC5B Ab or non-specific IgG to the cochlear explants treated with NTN1 and neomycin. UNC5B Ab, but not non-specific IgG, significantly attenuated the protective activity of NTN1 on neomycin-damaged HCs ($p < 0.0001$ for the IHCs and OHCs, one-way ANOVA; Fig. 5D–G), suggesting that UNC5B is specifically required for NTN1 activity on HCs.

Next, to examine whether the pro-survival effects of IGF1 towards HCs were mediated by UNC5B, we used UNC5B Ab (Fig. 5H–L). Neomycin treatment damaged the structure of the cochlea, which was rescued by IGF1 administration (Fig. 5H,K and L); however, UNC5B Ab reversed the protective effect of IGF1 on both the IHCs and OHCs (Fig. 5I,K,and L, $p = 0.00565$ and $p = 0.0041$ by one-way ANOVA, respectively; $p < 0.05$ by Tukey's method). It should be noted that there was no significant difference in HC survival between the explants treated with neomycin + IGF1 + UNC5B Ab and neomycin + non-specific IgG (Fig. 5J–L), indicating that the blockade of the UNC5B receptor completely abolished HC protection rendered by IGF1. These results strongly suggest that IGF1 pro-survival activity is mediated via the UNC5B receptor and that NTN1 is an effector of IGF1 protection of cochlear HCs against aminoglycoside, neomycin.

4.6. HC protection by NTN1 is independent of PI3K/AKT or MEK/ERK signaling

Several signaling pathways are activated in response to NTN1 binding to its receptors in various cells and tissues (Barallobre et al., 2005; Forcet et al., 2002); however, little information is available on signaling mechanisms downstream of the UNC5B receptor. Thus, in UNC5B-expressing renal proximal tubular epithelial cells, NTN1

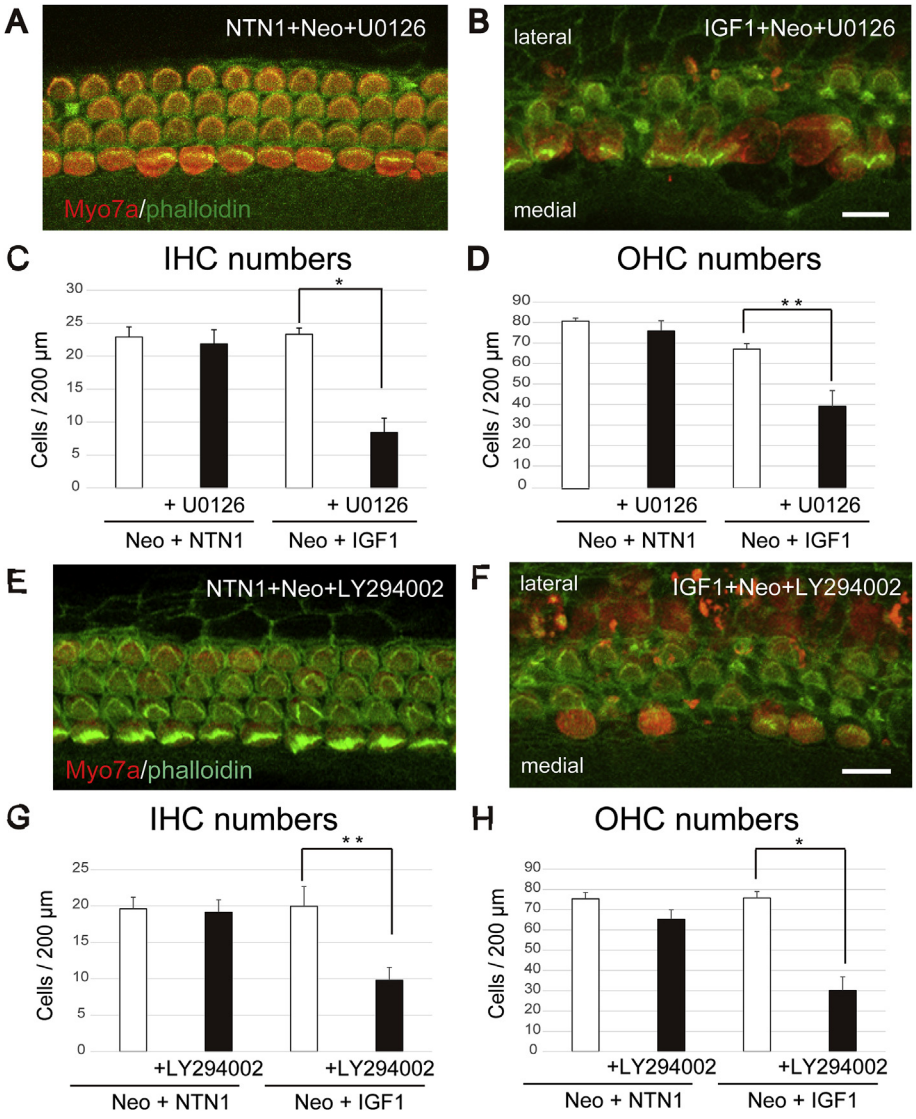

Fig. 6. Inhibition of PI3K/AKT or MEK/ERK signaling pathway does not attenuate NTN1-induced hair cell protection. **A**, **B**. Cochlear explant cultures treated with neomycin (Neo) and NTN1 (**A**) or IGF1 (**B**) were incubated with U0126 (10 μM) and stained for myosin7a (Myo7a, red) and actin filament (phalloidin, green). **C**, **D**. Cochlear explant cultures were treated as above and surviving inner hair cells (IHCs; **C**) and outer hair cells (OHCs; **D**) were counted. U0126 treatment did not attenuate the protection of the IHCs or OHCs by NTN1, but significantly decreased that by IGF1 ($p < 0.05$). **E**, **F**. Cochlear explant cultures treated with Neo and NTN1 (**E**) or Neo and IGF1 (**F**) were incubated with LY294002 (10 μM) and stained for myosin7a (Myo7a, red) and actin filaments (phalloidin, green). **G**, **H**. Explant cultures were treated as above and surviving IHCs (**G**) and OHCs (**H**) were counted. LY294002 did not attenuate the protection of the IHCs or OHCs by NTN1, but significantly reduced that by IGF1. Scale bars, 10 μm. The data are expressed as the mean ± SE; $^{*}p < 0.01$ and $^{**}p < 0.05$ by Student's *t*-test.

rapidly activated ERK and AKT (Wang et al., 2009). Other studies have revealed that the PI3K/AKT and MEK/ERK pathways are involved in the protection of HCs by IGF1 (Battaglia et al., 2003; Hayashi et al., 2013; Jiang et al., 2006). Therefore, we tested if HC protection by NTN1 was mediated via PI3K/AKT or MEK/ERK signaling by using appropriate pharmacologic inhibitors.

Cochlear explant cultures treated with neomycin together with NTN1 or IGF1 were incubated with MEK inhibitor U0126 or PI3K inhibitor LY294002 and analyzed for HC survival (Fig. 6). The results indicated that neither U0126 nor LY294002 attenuated the protective effect of NTN1 on HCs (Fig. 6A,C,D and E,G,H, respectively), while significantly reducing that of IGF1 (Fig. 6B,C,D and F,G,H, respectively) for both IHCs ($p = 0.0006$ and $p = 0.00326$, respectively, by one-way ANOVA; $p < 0.05$ by Tukey's method) and OHCs ($p = 0.0002$ and $p < 0.0001$, respectively, by one-way ANOVA; $p < 0.05$ by Tukey's method).

These results demonstrate that HC protection by NTN1 is not mediated via MEK/ERK or PI3K/AKT signaling.

4.7. NTN1 suppresses caspase 3 activation in HCs

IGF1 protects HCs from aminoglycosides by inhibiting apoptosis and promoting the cell cycle (Hayashi et al., 2013). Among the six canonical NTN1 receptors, DCC and UNC5B act as so-called "dependence receptors," because they induce apoptosis in the absence of its cognate NTN1 ligand, but inhibit apoptosis upon NTN1 binding (Llambi et al., 2001; Mehlen et al., 1998). However, little is known about the downstream signaling mechanisms, which are activated by NTN1 ligation of these dependence receptors and are responsible for anti-apoptotic activity.

Here, we examined anti-apoptotic effects caused by NTN1 by assessing the activation status of caspase 3, a cysteine-aspartate protease with a central role in the execution of cell apoptosis (Fig. 7). Immunohistochemistry analysis showed that neomycin induced caspase 3 cleavage in cochlear explants, which indicated caspase 3 activation and apoptosis, while NTN1 completely reversed this effect in both the IHCs and OHCs (Fig. 7; $p < 0.01$).

These results demonstrated that NTN1, similar to IGF1 (Hayashi et al., 2013), inhibited neomycin-induced apoptosis in the IHCs and OHCs, suggesting the role of NTN1 as the effector of IGF1 during HC protection.

4.8. NTN1 does not promote the cell cycle in HCs or SCs

IGF1 has also been shown to protect cochlear HCs by promoting the cell cycle in Hensen's and Claudius' cells (Hayashi et al., 2013). Moreover, NTN1 and UNC5B are known to increase the proliferation of several cell types including Schwann cells (Lee et al., 2007a) and renal tubular epithelial cells (Wang et al., 2009). To investigate whether NTN1, similar to IGF1, induced cell proliferation in the sensory epithelium of the cochlea, we analyzed 5-bromo-2′-deoxyuridine (BrdU) incorporation indicative of the S phase of mitosis and cell cycle progression (Fig. 8).

In neomycin-treated cochlear explants, no BrdU-positive HCs and very few BrdU-positive SCs were observed (Fig. 8A). However, the addition of IGF1 significantly increased the number of BrdU-

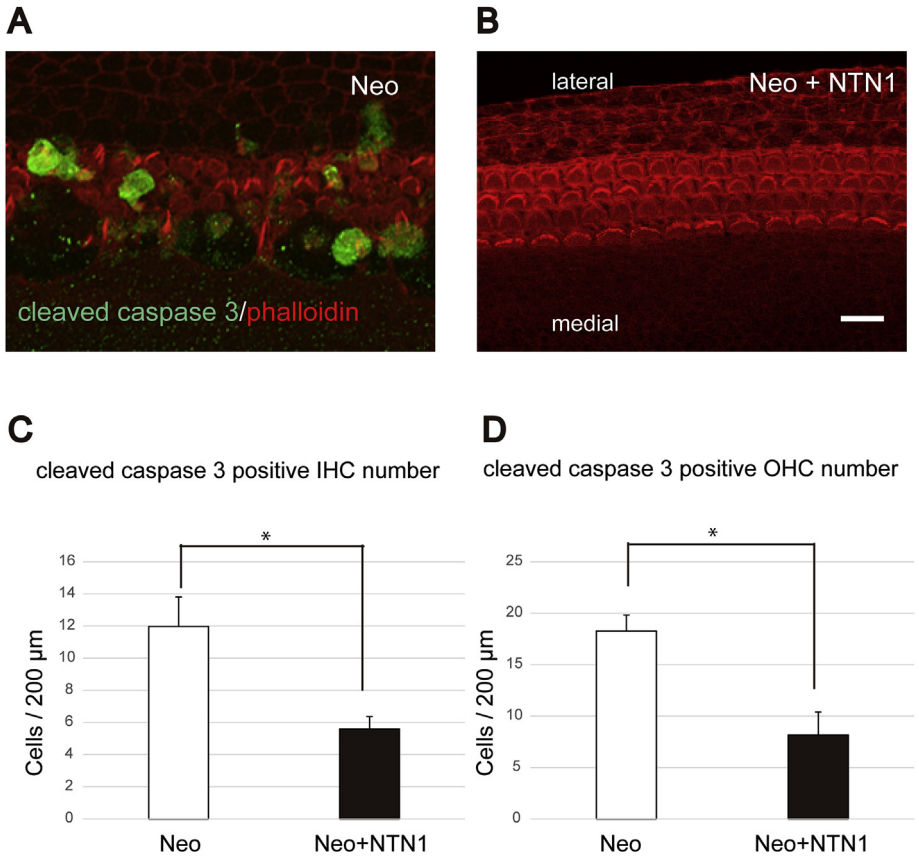

Fig. 7. NTN1 inhibits caspase 3 activation in hair cells. A, B. Cochlear explant cultures treated with neomycin (Neo) alone (**A**) or together with NTN1 (**B**) were stained for cleaved caspase 3 (green) and actin filaments (phalloidin, red). **C, D.** Apoptotic inner hair cells (IHCs, **C**) and outer hair cells (OHCs, **D**) were counted. NTN1 administration significantly decreased the numbers of cleaved caspase 3-positive IHCs and OHCs. Scale bar, 10 µm. The data are expressed as the mean ± SE; *$p < 0.05$ by Student's *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

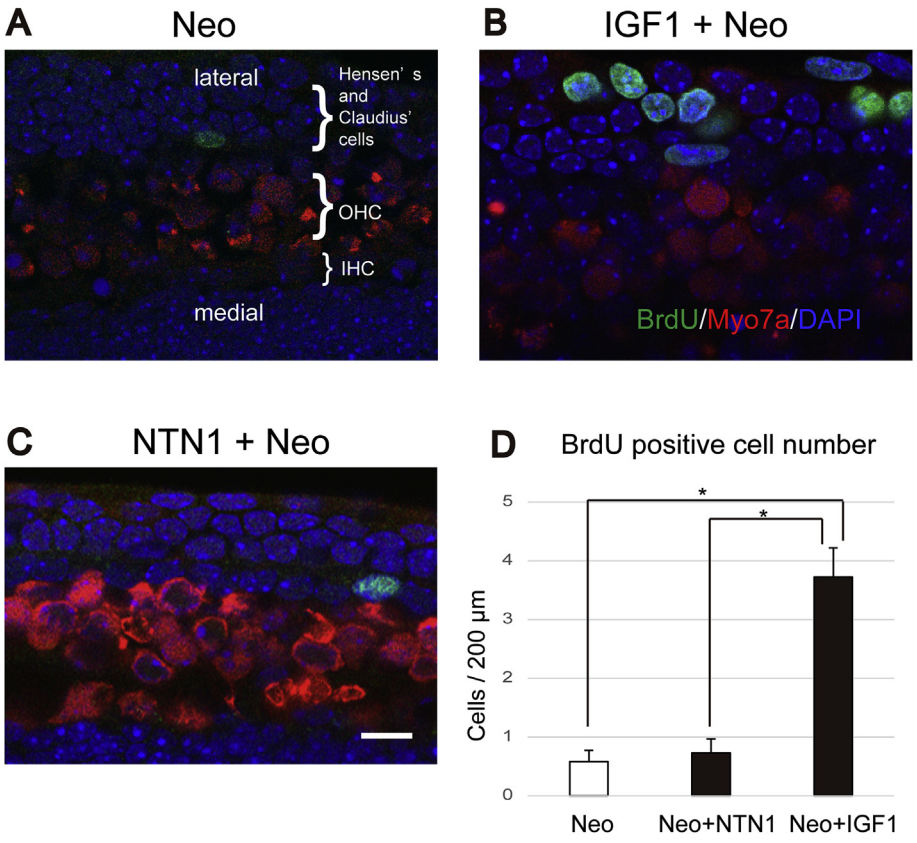

Fig. 8. NTN1 treatment does not promote the cell cycle in hair cells or supporting cells. **A**–**C.** BrdU uptake in the cochlear explants treated with neomycin (Neo; **A**), Neo and IGF1 (**B**), or Neo and NTN1 (**C**). Explants were immunostained with the anti-BrdU (BrdU, green) and anti-myosin7a (Myo7a, red) antibodies and counter-stained with DAPI (blue). **D**. Numbers of BrdU-positive cells. No hair cells and few supporting cells (Hensen's cells and Claudius' cells) were BrdU-positive in cultures treated with Neo alone or together with NTN1. Treatment with Neo and IGF1 significantly increased the number of BrdU-positive Hensen's and Claudius' cells ($p = 0.0002$ by one-way ANOVA). Scale bar, 10 μm. The data are expressed as the mean ± SE; $^{*}p < 0.05$ by Tukey's method. IHC: inner hair cell, OHC: outer hair cell. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

positive Hensen's and Claudius' cells (Fig. 8B and D; $p = 0.0002$ by one-way ANOVA and $p < 0.05$ by Tukey's method), which is consistent with a previous report (Hayashi et al., 2013). In contrast, the addition of NTN1 did not result in significant increase of BrdU incorporation either in HCs or SCs (Fig. 8C and D), indicating that NTN1 protective effects against aminoglycoside toxicity were not associated with cell cycle promotion in HCs or SCs.

To confirm this observation, we examined NTN1 protection of HCs treated with aphidicolin, a specific inhibitor of DNA polymerase α and δ, which blocks the cell cycle at the early S phase (Fig. 9). Consistent with our previous results (Hayashi et al., 2013), the protection of the OHCs by IGF1 was significantly attenuated by aphidicolin (Fig. 9A and E; $p < 0.05$), indicating that SC proliferation is associated with OHC protection by IGF1. The addition of aphidicolin to the explants treated with neomycin and NTN1 did not attenuate NTN1 protection of both the IHCs and OHCs (Fig. 9B, D, and F). This result indicated that HC survival supported by NTN1 was independent of SC proliferation, suggesting that NTN1 and IGF1 may exert beneficial effects on cochlear HCs via distinct mechanisms.

5. Discussion

Our previous study has demonstrated that *Ntn1* is one of genes upregulated by IGF1 via the PI3K/AKT and MEK/ERK pathways (Hayashi et al., 2014). However, the role of NTN1 in the protection of cochlear HCs by IGF1 remained unknown. In this study, we showed that NTN1 binding to the UNC5B receptor may mediate HC protection by IGF1, suggesting the role of NTN1 as the downstream effector of IGF1 protective activity towards neomycin-damaged cochlear HCs.

5.1. NTN1 is a mediator of IGF1 protective activity in the cochlea

In SCs, IGF1 activates PI3K/AKT and MEK/ERK, the two major signaling pathways downstream of IGF1R, which results in HC protection (Hayashi et al., 2013), suggesting that IGF1 did not act directly on HCs, but stimulated the expression of SC-derived molecules with protective activity towards HCs. In this study, we found that NTN1 induced by IGF1 both at the mRNA (Fig. 3E–H) and protein (Fig. 2A and B) levels provided significant support of neomycin-treated HCs, which was similar to that of IGF1 (Fig. 1). IGF1 effects were attenuated by an NTN1 blocking antibody (Fig. 2C–F), indicating that NTN1 mediated the pro-survival activity of IGF1 towards HCs. Moreover, given that IGF1 upregulates *Ntn1* expression through activation of the PI3K/AKT and MEK/ERK pathways (Hayashi et al., 2014) and that blockade of PI3K/AKT and MEK/ERK signaling did not attenuate NTN1 effects on HC survival (Fig. 6), NTN1 is likely to function downstream of the axis of IGF1-IGF1R-PI3K/AKT and MEK/ERK pathway. These results characterize NTN1 as an effector of IGF1 signaling in the protection of cochlear HCs.

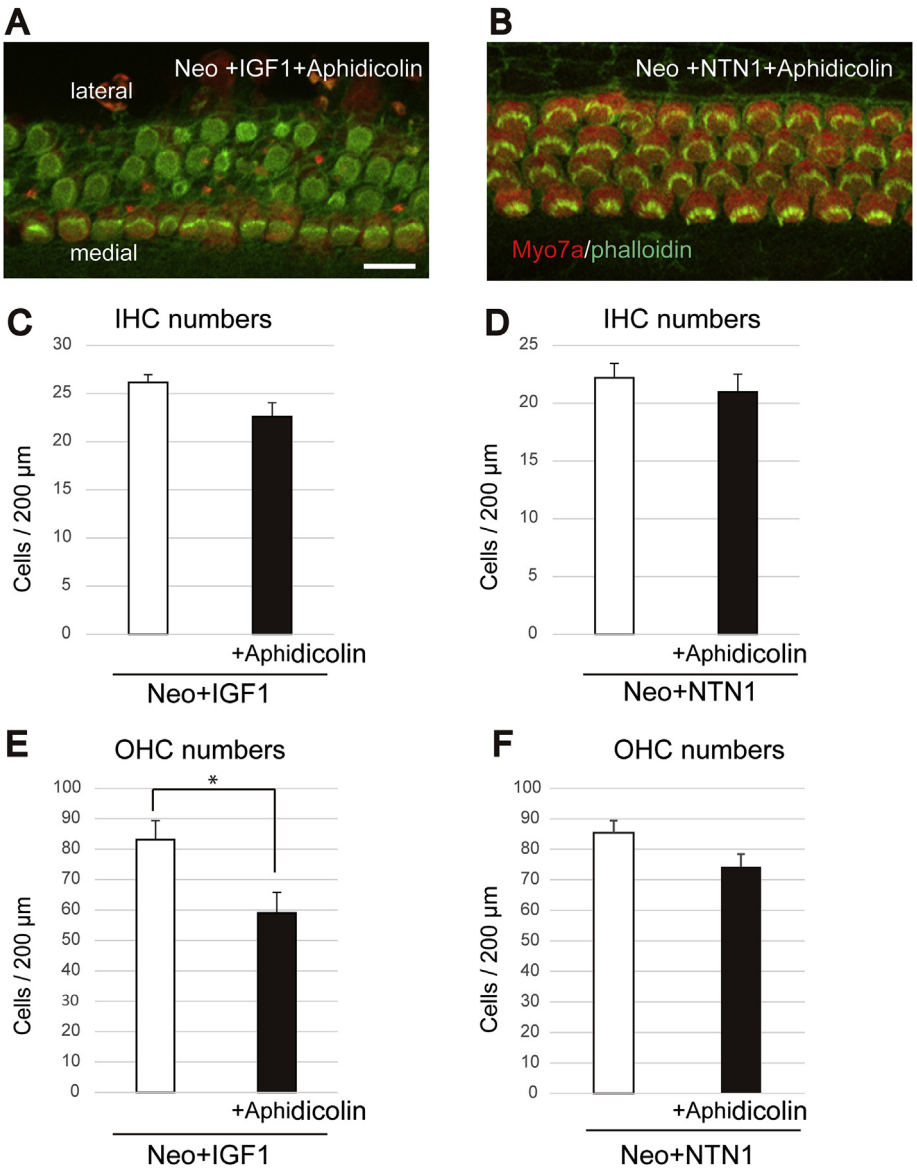

Fig. 9. NTN1 protection of neomycin-treated cochlear hair cells is not attenuated by cell cycle inhibition. **A**, **B**. Cochlear explants were treated with neomycin (Neo) + IGF1 + 2.8 mM aphidicolin (**A**) or Neo + NTN1 + 2.8 mM aphidicolin (**B**) and stained for myosin7a (Myo7a, red) and actin filament (phalloidin, green). **C**–**F**. Numbers of surviving inner hair cells (IHCs) and outer hair cells (OHCs) were counted. The addition of aphidicolin did not attenuate the protective effect of NTN1 on the IHCs (**D**) and OHCs (**F**), but reduced that of IGF1 on the OHCs (**E**). Scale bar, 10 µm. The data are expressed as the mean ± SE (**C**–**F**). *$p < 0.05$ by Student's *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5.2. SCs protect HCs from neomycin injury by secreting NTN1

We also determined the localization of IGF1-induced *Ntn1* expression in the cochlea subjected to the toxic effects of the aminoglycoside antibiotic. In the cochlea, IGF1R is expressed on the surface of the OHCs, IHCs, and all SCs, including pillar cells, Hensen's and Claudius' cells, inner sulcus cells, and Deiters' cells (Hayashi et al., 2013; Okano et al., 2011), as well as spiral ganglion cells (Sanchez-Calderon et al., 2010). Among these cell populations, *Ntn1* expression was significantly induced by IGF1 in all SCs and spiral ganglion cells, but weakly in HCs (Fig. 3). Given that the two main signaling pathways downstream of IGF1R, MEK/ERK and PI3K/AKT, are activated only in SCs (Hayashi et al., 2013), these results suggest that SCs are the major source of NTN1 that protects HCS in the cochlea.

Several studies have demonstrated that SCs play crucial roles in the protection of cochlear HCs (May et al., 2013; Sobkowicz et al., 1997). One of the SC-derived protective factors is heat shock protein 70 (HSP70). Thus, it has been shown that SCs of the utricle secrete HSP70 in response to heat stress to promote HC survival (May et al., 2013). Interestingly, avian species are able to regenerate acoustic HCs, which are differentiated from SCs, suggesting that SCs represent the stem or precursor cell population in the avian inner ear (Stone and Cotanche, 2007). Moreover, a recent study indicates that a part of mammalian SC population can act as HC precursors (Chai et al., 2012). Our present results show that in addition to their role as HC precursors, SCs can maintain HC population by secreting pro-survival factors, including NTN1.

IGF1 induced *Ntn1* expression in the sensory epithelium only when it was damaged by the aminoglycoside but not in the normal physiological conditions, and the mechanisms underlying these differential responses are not clear. NTN1 expression at both mRNA

and protein levels has been shown to be induced by stress factors in other organs. Thus, hypoxia upregulated *NTN1* mRNA expression in human epithelial cell lines CaCo-2 and T84 (Rosenberger et al., 2009). The expression of NTN1 and UNC5B proteins was induced in injured sciatic nerves, but could not be detected in normal nerves (Lee et al., 2007a), while renal tubular epithelial cells secreted NTN1 after ischemia reperfusion (Jayakumar et al., 2011). In contrast to these findings, *Ntn1* expression in the cochlear sensory epithelium was not significantly induced by neomycin treatment (Fig. 3A), but was upregulated by the combination of neomycin and IGF1 both in the cochlear sensory epithelium and in spiral ganglion cells (Fig. 3E and F). This expression pattern was different from that observed after IGF1 treatment, which induced *Ntn1* only in spiral ganglion cells (Fig. 3C), suggesting specific protective activity of IGF1 against aminoglycosides in the cochlear sensory epithelium. The mechanism underlying this differential regulation of *Ntn1* in the damaged cochlear sensory epithelium is unclear and remains to be elucidated.

Contrary to the cochlear sensory epithelium, in the spiral ganglion cells *Ntn1* expression was induced by IGF1 alone without neomycin, suggesting that NTN1 produced by spiral ganglion cells is not involved in the protection of cochlear HCs. NTN1 has originally been discovered as a molecule involved in axon guidance (Kennedy et al., 1994; Serafini et al., 1994). Indeed, NTN1 promoted neurite outgrowth in spiral ganglion cells (Lee and Warchol, 2008), confirming that the main role of IGF1-induced NTN1 in spiral ganglion cells is neurite development.

5.3. Interaction between NTN1 and its UNC5B receptor is the main mechanism underlying IGF1-induced HC protection

The following results of this study suggest the involvement of UNC5B in the protection of HCs by IGF1 or NTN1. First, only UNC5B among the six canonical NTN receptors was expressed in the basal-turn sensory epithelia of the cochlea where we observed HC protection (Fig. 4H–J). Second, the effects of UNC5B blockade demonstrated that IGF1-induced HC protection was largely mediated by the interaction between NTN1 and UNC5B (Fig. 5), although an off-target effect of UNC5b Ab should be considered. Finally, among the six canonical NTN1 receptors, UNC5B and DCC are called dependence receptors, as they promote apoptosis when not bound to NTN1 (Llambi et al., 2001; Mehlen et al., 1998), and our results indicate that NTN1 attenuated apoptosis triggered by neomycin in cochlear HCs (Fig. 7) at a neonatal stage. *Unc5b* expression was not detected at a later stage (P28) (Supplementary Fig. 2C). However, it is difficult to conclude that IGF1-NTN1 axis is not effective in the protection of the adult mice cochlea, because we did not exclude the possibility that *Unc5b* expression is induced after damage to the cochlea *in vivo*. *In vivo* study to examine the effect of IGF1-NTN1 axis in the damaged cochlea of adult animals will be the next step to apply current results to the clinical scene.

The inhibition of apoptosis in the cochlea by NTN1 observed in this study is consistent with previous reports. Thus, it has been shown that cell death regulation by NTN1 interaction with the receptors plays an important role in the development of the nervous system (Llambi et al., 2001). Furthermore it is known that aminoglycoside induces caspase 3- dependent apoptotic cell death (Cunningham et al., 2002), while NTN1 receptor-mediated signaling inhibits caspase and p53 activation and suppresses apoptosis (Llambi et al., 2001; Rajasundari et al., 2011; Wu et al., 2008).

However, we did not observe the effects of NTN1 on cell proliferation (Fig. 8), although IGF1 has been shown to induce the division of Hensen's and Claudius' cells (Hayashi et al., 2013). Previous findings suggest that proliferative effects of NTN1 are context-dependent. Thus, NTN1 overexpression in intestinal crypt epithelial cells of transgenic mice inhibited apoptosis, but did not induce cell proliferation (Mazelin et al., 2004); however, other studies described proliferative effects of NTN1 in Schwann cells (Lee et al., 2007a) and renal tubular epithelial cells (Wang et al., 2009). The analysis of cochlear HC protection against neomycin leads to the conclusion that NTN1 is involved in the major but not all mechanisms underlying IGF1 effects on cochlear HCs. Thus, NTN1 did not activate the main signaling pathways downstream of IGF1R and, while inhibiting HC apoptosis, did not affect Hensen's and Claudius' cell proliferation, which may be mediated by other IGF1 effectors. The lack of proliferative activity may be beneficial in clinical settings because it decreases the risk of tumorigenesis as an adverse effect. Differential signaling mechanisms activated by NTN1 and IGF1 should be taken into account while considering the use of these proteins in the treatment of SNHL.

6. Conclusion

The current study is the first report that describes the functional IGF1-NTN1 axis. IGF1 activates SCs to release NTN1, which binds to UNC5B, one of its canonical receptors expressed on HCs, and protects HCs against toxic effects of aminoglycoside by inhibiting HC apoptosis. Thus, NTN1 acts as an effector of IGF1 activity to support survival of cochlear HCs. Considering that NTN1 did not stimulate cell proliferation, it could be used as a more efficient and safe approach to treat SNHL caused by the damage of cochlear HCs.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2017.03.032.

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