Title: Insulin-like growth factor 1 inhibits hair cell apoptosis and promotes the cell cycle of supporting cells by activating different downstream cascades after pharmacological hair cell injury in neonatal mice

Abbreviated title: The mechanisms of hair cell protection by IGF-1

Author names: Yushi Hayashi, Norio Yamamoto, Takayuki Nakagawa, Juichi Ito

Affiliation: Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto, 606-8507, Japan

E-mail addresses:

Yushi Hayashi	y_hayashi@ent.kuhp.kyoto-u.ac.jp
NorioYamamoto	yamamoto@ent.kuhp.kyoto-u.ac.jp
Takayuki Nakagawa	tnakagawa@ent.kuhp.kyoto-u.ac.jp
Juichi Ito	ito@ent.kuhp.kyoto-u.ac.jp

Corresponding author:

Name: Norio Yamamoto

Address: Department of Otolaryngology-Head and Neck Surgery

Graduate School of Medicine

Kyoto University

54 Shogoin Kawahara-cho, Sakyo-ku

Kyoto city, Kyoto 606-8507, Japan

E-Mail: yamamoto@ent.kuhp.kyoto-u.ac.jp

Telephone: +81-75-751-3346

FAX: +81-75-751-7225

Abstract

Sensorineural hearing loss, which is mainly caused by cochlear hair cell damage, is an intractable disease, as cochlear hair cells and supporting cells are unable to proliferate in postnatal mammals. As a novel and potent treatment for sensorineural hearing loss, we have studied IGF-1 and found that it protects cochlear hair cells from the damage caused by noise and ischemic trauma. Through a clinical trial, we have also confirmed that IGF-1 is an effective treatment for idiopathic sudden sensorineural hearing loss. In the current study, we attempted to identify the downstream pathways of the IGF-1 signal and the mechanisms by which IGF-1 protects the neonatal mouse cochlear hair cells that have been damaged by neomycin. IGF-1 activated both the PI3K/Akt and MEK/ERK pathways to maintain the hair cell numbers in the injured cochlea. The PI3K/Akt pathway specifically protected the cochlear inner hair cells through the inhibition of apoptosis. In contrast, the MEK/ERK pathway induced the cell cycle promotion of Hensen's and Claudius' cells, the supporting cells that are located lateral to the outer hair cells of the cochlea. This cell cycle promotion of the supporting cells resulted in the maintenance of the outer hair

cell numbers. These results indicate that IGF-1 is a growth factor that efficiently regulates different mechanisms through different downstream cascades, thereby protecting cochlear hair cells.

Key words

Insulin-like growth factor 1, cochlea, hair cell, apoptosis, cell cycle promotion

Abbreviations

IGF-1: insulin like growth factor 1, HC: hair cell, IHC: inner hair cell, OHC: outer hair cell, SC: supporting cell, PI3K: phosphatidylinositol 3-kinase, MAPK: mitogen-activated protein kinases, ERK: extracellular signal-regulated kinases, MEK: MAPK/ERK kinase

Introduction

Insulin-like growth factor 1 (IGF-1) is a polypeptide endocrine hormone that functions after birth to facilitate cell growth; IGF-1 also controls cell proliferation, differentiation, and apoptosis (Jenkins and Bustin, 2004; Varela-Nieto et al., 2007). IGF-1 plays pivotal roles in various organs. For example, in the central nervous system, IGF-1 promotes neuronal growth, protects neurons and oligodendrocytes from cell death, and stimulates the proliferation of neuronal precursors (Bondy and Cheng, 2004; Popken et al., 2004; Varela-Nieto et al., 2003).

IGF-1 also has important roles in the hearing in mammals because defects in murine and human IGF-1 signaling cause severe impairments in sensorineural

hearing (Bonapace et al., 2003; Cediel et al., 2006; Walenkamp et al., 2005; Woods et al., 1996).

IGF-1 binds to its receptor, IGF-1 tyrosine kinase receptor (IGF-1R), and activates two main pathways by phosphorylation (Varela-Nieto et al., 2003). The first pathway is the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in which phosphorylated PI3K activates Akt to achieve cell survival (Datta et al., 1999) and cell cycle promotion (Medema et al., 2000; Zhou et al., 2001). The second pathway, the MEK/ERK pathway, is composed of Ras, Raf, and MEK, which activate the mitogen-activated protein kinases (MAPK) or extracellular signal-regulated kinases (ERK) 1/2, leading to an anti-apoptotic response (Finlay et al., 2000; Holmström et al., 1999) and cell proliferation (Pagès et al., 1993). PI3K also activates the MEK/ERK pathway through protein kinase C (PKC) (Chung et al., 2006).

The organ of Corti, which transduces sound signals to electrical signals in the cochlea, is composed of inner and outer hair cells (HCs) and supporting cells (SCs). After birth, the mammalian cochlear HCs cannot regenerate following injury because both HCs and SCs lose their ability to proliferate during

embryogenesis (Laine et al., 2010; Ruben, 1967). As cochlear HC damage is the cause of most sensorineural hearing loss (SNHL), a complete recovery from SNHL is difficult. Although glucocorticoid is an effective treatment for acute SNHL, this treatment improves hearing levels in only half of the patients treated (Hu and Parnes, 2009).

We have considered IGF-1 as an alternate treatment to glucocorticoid due to its relevance to hearing function, its cell proliferation potential on vertebrate inner ear HCs (León et al., 1995; Oesterle et al., 1997; Zheng et al., 1997), and its anti-apoptotic actions. Our group previously reported that, *in vivo*, IGF-1 could protect cochlear HCs against noise exposure (Iwai et al., 2006; Lee et al., 2007) and ischemic injury (Fujiwara et al., 2008). Furthermore, our clinical trial showed the effectiveness of IGF-1 treatment in patients with idiopathic sudden sensorineural hearing loss that is refractory to glucocorticoid treatment (Nakagawa et al., 2010).

However, the cellular mechanisms and downstream signals of IGF-1 action on damaged cochlear HCs still remain to be elucidated. To clarify these unknown

steps and factors, we took advantage of cochlear explant culture systems. In such systems, the activated downstream pathways can be easily detected using inhibitors that block various points of the IGF-1 signaling pathways; furthermore, such systems facilitate the observation of the death or promotion of cell cycles within the cochlea.

Results

IGF-1 protects cochlear hair cells against neomycin

In our previous studies, we found that IGF-1 protected hair cells from noise exposure (Iwai et al., 2006; Lee et al., 2007) and ischemic injury (Fujiwara et al., 2008). However, these insults cannot be applied to the explant culture system. Therefore, we used aminoglycoside (neomycin), which is commonly used in this type of study (Kikkawa et al., 2009; Yoshida et al., 2011) to injure hair cells of cochlear explants. The administration of 100 ng/ml of IGF-1 markedly protected both the inner and outer hair cells (IHCs and OHCs) from neomycin injury (Figure 1A and B). This effect was statistically significant for both the OHCs

and IHCs (p<0.0001 for OHCs and p=0.0018 for IHCs, one-way ANOVA) (Figure 1C and D) and was dose-dependent (Figure 1C and D).

Inhibition of the PI3K/Akt, MEK/ERK, or PKC pathways attenuates IGF-1 cochlear hair cell protection

To determine which downstream pathways were involved in the IGF-1 protection of the hair cells, we administered an inhibitor of each pathway in addition to IGF-1, based on the requirement that an inhibitor whose target pathway is involved in the IGF-1 effect should attenuate the hair cell-protection effect. As a preliminary experiment, we added each inhibitor to the cochlear explants and confirmed that none was toxic to the hair cells at the concentrations used in this study (data not shown).

The protection of the OHCs was significantly attenuated by PD98059 (ERK1 inhibitor, p=0.0024, Figure 2A and F) and U0126 (ERK1/2 inhibitor, p<0.0001 Figure 2B and F), where p<0.0033 is considered statistically significant by Bonferroni corrction. In contrast, only U0126 significantly attenuated the protection effect on IHCs (p=0.0005 Figure 2 B and G). The U0126 treatment

had a tendency to have greater attenuation effects than PD98059 even in OHCs (Figure 2A, B, and F). These findings indicated that the MEK/ERK pathway was involved in the IGF-1 hair cell protection effect and that both ERK1 and ERK2 or only ERK1 might contribute to this effect in OHCs. ERK2 might be important in the protection of IHCs and ERK1 is only involved in the protection of OHCs.

The treatment with LY294002, a PI3K inhibitor, significantly attenuated the IGF-1 protection of the OHCs and IHCs (OHC: p=0.0003 and IHC: p<0.0001, Figure 2C, F, and G), which indicated that PI3K is involved in IGF-1 protection in both OHCs and IHCs.

Two molecules, Akt and PKC, were reported to be activated in the downstream pathways of PI3K signaling (Chung et al., 2006). To identify which downstream pathways were involved in the IGF-1 protective effects on the cochlear explant cultures, we administered an inhibitor of Akt (Akt inhibitor VIII) or PKC (calphostin C) to the damaged explants in addition to IGF-1. The treatment with Akt inhibitor VIII caused the attenuation of the hair cell protection in the IHCs only (OHC: p=0.0372 and IHC: p=0.0002, Figure 2D, F, and G). In

contrast, the treatment with calphostin C caused the attenuation of hair cell protection in both the OHCs and IHCs, as observed with the ERK1/2 and PI3K inhibitors (OHC: $p<0.0001$ and IHC: $p=0.0002$, Figure 2E, F, and G). These findings indicated that the activation of PI3K by IGF-1 in the cochlear explants involved both of these signaling molecules (Akt and PKC). However, these molecules have different roles. Akt contributed to the protection of IHCs only; in contrast, PKC contributed to the IGF-1 protection of both the OHCs and IHCs against neomycin in the cochlea.

Activation of IGF-1 downstream signals occurred in the inner sulcus cells and the Hensen's and Claudius' cells

To determine which cell populations in the cochlear sensory epithelium are acted upon by IGF-1, we investigated the expression of the IGF-1 receptor, IGF-1R, in the cochlea using immunohistochemistry. The IGF family which is related to insulin includes IGF-1 and IGF-2, and although both of these molecules can bind to IGF-1R (Baserga et al., 2003), IGF-1 has the highest binding affinity to IGF-1R (Brodt et al., 2000).

Immunohistochemistry using cryosection and whole mounts showed that IGF-1R was expressed in the membranes of the OHCs (Figure 3A and B), IHCs (Figure 3A and B), pillar cells (Figure 3A and B), inner sulcus cells (asterisks in Figure 3A and B), and Hensen's and Claudius' cells (double asterisks in Figure 3A and B). The inner sulcus cells and the Hensen's and Claudius' cells are cell populations that are located medial to the IHCs and lateral to OHCs, respectively, in the cochlea. The pillar cells are located between the IHCs and OHCs. The pillar, Hensen's and Claudius' cells are considered SCs. Because our results suggest that IGF-1 may act on the OHCs, IHCs, and pillar, inner sulcus, and Hensen's and Claudius' cells, to specify which downstream signals of IGF-1 are activated in the various cell populations, we investigated the expression patterns of the activated forms of Akt (phosphorylated Akt, p-Akt) and ERK (phosphorylated ERK, p-ERK) using immunohistochemistry. We chose the phosphorylated forms of these two molecules because IGF-1/IGF-1R signaling converges on Akt and ERK. In addition to Akt and ERK, PKC is also involved in the protection of hair cells. As shown previously, PKC eventually activates ERK (Chung et al., 2006), suggesting that p-ERK also reflects the activation of PKC.

Signal detection was performed 15 min following the administration of neomycin with or without IGF-1.

When treated with neomycin only, we did not find any p-Akt or p-ERK signals in the cochlear epithelium (Figure 3C and E). As expected from our study using inhibitors, the addition of IGF-1 caused the activation of both Akt and ERK 15 min after the IGF-1 administration (Figure 3D and F). We found variability in the localization of each signal. p-Akt was positive in the inner sulcus cells only (asterisk in figure 3D), whereas p-ERK was positive in the Hensen's cells and Claudius' cells (double asterisk in Figure 3F). The results of the p-Akt immunohistochemistry suggested that the inner sulcus cells with activated Akt can protect the adjacent IHCs from neomycin. This supports the results obtained from the inhibitor experiment (Figure 2D) and indicates that Akt is an important signal for the protection of IHCs.

IGF-1 inhibits apoptosis in OHCs and IHCs

The maintenance of the number of HCs, i.e. HC protection, can be achieved via two possible cellular mechanisms: the inhibition of cell death and the promotion

of cell proliferation. Previous reports examining other organs including nervous systems, bones, and mammary glands clarified that IGF-1 regulates both mechanisms (Varela-Nieto et al., 2007). Thus, to determine whether IGF-1 inhibits the apoptosis of OHCs and IHCs that was induced by neomycin treatment, we evaluated the apoptosis status of the hair cells after the treatment with neomycin or with neomycin and IGF-1 by cleaved caspase-3 immunohistochemistry.

At 3 h following the administration of neomycin, the addition of IGF-1 did not cause a statistically significant decrease in the number of cleaved caspase-3-positive HCs (Figure 4A, B and E). However, the neomycin-treated group had a very low but some number of caspase-3-positive HCs (Figure 4A and E), which were not observed in the neomycin+IGF-1 group (Figure 4B and F). When we tested the status of apoptosis at 24 h following neomycin administration, we found significantly decreased number of caspase-3-positive-HCs ($p<0.0001$, Figure 4F) in the neomycin+IGF-1 group (Figure 4D) compared with the neomycin-only group (Figure 4C), and we found many cleaved caspase-3-positive HCs in both the OHCs and the IHCs in the

neomycin-only group. These results indicated that IGF-1 inhibited the neomycin-induced apoptosis of OHCs and IHCs.

IGF-1 promotes the cell cycles in Hensen's and Claudius' cells

Next, we considered another mechanism to explain the maintenance of the number of HCs, i.e., the promotion of HC or SC cell cycle. To test this hypothesis, we administered BrdU together with neomycin or with neomycin and IGF-1. BrdU is a synthetic nucleoside and an analog of thymidine that becomes incorporated into the nucleus during the S phase of mitosis.

When treated with neomycin, BrdU-positive cells were observed in none of cochlear HCs and in very few SCs (Figure 5A), indicating that almost no S phase entry occurred in these cell populations. The addition of IGF-1 caused a significant uptake of BrdU in the Hensen's and Claudius' cells (Figure 5B and C, p=0.0042). These data indicated that IGF-1 induced the S-phase entry of the Hensen's and Claudius' cells after neomycin administration. To confirm the cell cycle promotion by IGF-1, we examined expression of phophohistone H3 (pHH3), an M phase marker of the cell cycle. Immunohistochemistry of pHH3 6 h after

the treatment with neomycin or with neomycin and IGF-1 showed that the addition of IGF-1 caused significant increase of the pHH3 positive cell number in the Hensen's and Claudius' cells (Figure 5D–F). Combined with the result of BrdU uptake, this result indicates that IGF-1 induced promotion of the cell cycle in the Hensen's and Claudius' cells.

As the IGF-1 treatment caused the maintenance of the HC numbers, we hypothesized that the proliferation of the Hensen's and Claudius' cells, in addition to the inhibition of HC apoptosis, contributed to the maintenance of the HC numbers after the neomycin treatment. To test our hypothesis, we observed the IGF-1 protection of the HCs after inhibiting proliferation using aphidicolin or L-mimosine. Aphidicolin is a specific inhibitor of DNA polymerase α and δ and it blocks the cell cycle at early S phase (Hammond et al., 1987). L-Mimosine is a tyrosine analog that can arrest the cell cycle in the late G1 phase (Perry et al., 2005; Thompson et al., 1969). As a preliminary experiment, we determined that 2.8 μM of aphidicolin or 0.1 mM of L-mimosine was a sufficient concentration to inhibit BrdU uptake in the Hensen's and Claudius' cells even when the explants were treated with IGF-1 (supplementary figure 3A and B).

We also confirmed that neither the aphidicolin or L-mimosine treatment nor the combination of IGF-1 and aphidicolin or L-mimosine treatment was toxic to the cochlear explant cultures (data not shown, supplementary figure 3E–G).

The addition of aphidicolin or L-mimosine to the explants that were treated with neomycin and IGF-1 significantly attenuated the IGF-1 protection effect on the OHCs against neomycin (p=0.0274, Figure 6A and B for aphidicolin and p=0.0099, Figure 6C and D for L-mimosine). To exclude the possibility that the combination of neomycin, IGF-1, and aphidicolin induces the apoptosis of HCs, we examined the numbers of caspase-3-positive HCs in neomycin + IGF-1 group and neomycin + IGF-1 + aphidicolin group. We did not find difference in the numbers of caspase-3-positive HCs between these groups (data not shown). These results indicated that the cell cycle induction of the Hensen's and Claudius' cells with IGF-1 at least partly contributes to the maintenance of the number of OHCs.

Discussion

In this study we found that the application of IGF-1 attenuates the loss of HCs that is induced by neomycin. Although the positive effects of IGF-1 against HC injury were evident from the previous studies showing that IGF-1 could protect the HCs from noise exposure and ischemic injury (Fujiwara et al., 2008; Iwai et al., 2006; Lee et al., 2007) and the present study, the activated downstream cascades of IGF-1 in the cochlea and the cellular mechanisms of HC protection by IGF-1 have remained unknown. We clarified the downstream cellular mechanisms using explant cultures of the neonate mouse cochlea. IGF-1 controls cell proliferation, differentiation and apoptosis by binding to IGF-1R (Brodt et al., 2000). IGF-1R is a membrane receptor tyrosine kinase that transmits signals through several pathways, including the PI3K/Akt, MEK/ERK, and Janus kinases/signal transducers and activators of transcription (JAK/STAT) pathways (Brodt et al., 2000; Varela-Nieto et al., 2007).

Previous studies indicated that among these pathways, the PI3K/Akt and MEK/ERK pathways are involved in the protection of cochlear HCs within various contexts. The activity of the PI3K/Akt pathway diminishes after various

types of damage to HCs (Jiang et al., 2006; Selivanova et al., 2007) and the inhibition of the pathway worsens HC survival (Chung et al., 2006). The administration of dexamethasone, which is one of the most popular medications prescribed for the treatment of sensorineural hearing loss (Spear and Schwartz, 2011), exerts its effects through the activation of the PI3K/Akt pathway (Haake et al., 2009). The MEK/ERK pathway is also involved in the protection of HCs from aminoglycoside toxicity (Battaglia et al., 2003). The downstream signaling cascades of IGF-1 include both of these pathways that are beneficial for HC protection. Whether all or only a portion of the downstream signaling cascades are activated by IGF-1 signaling is context-dependent. In embryonic inner ears, IGF-1 exerts its effects only through the PI3K/Akt pathway, resulting in the IGF-1-induced protection of neural progenitor cells in the otic vesicle (Aburto et al., 2012) and the regulation of the timing of sensory cell differentiation (Okano et al., 2011). Here we determined that IGF-1 activated both the PI3K/Akt and MEK/ERK pathways (Figure 3C-F) and protected the HCs from neomycin-induced damage when it was administered to the postnatal cochlear explants that had been damaged by neomycin (Figure 2). These results suggest

that the treatment with IGF-1 is a more efficient method to protect HCs than the activation of a single signal cascade.

As demonstrated through anti-IGF-1R immunohistochemistry, IGF-1 can act on the OHCs, IHCs, inner sulcus cells, pillar cells and Hensen's and Claudius' cells (Figure 3A and B). However, using immunohistochemistry to identify phosphorylated IGF-1 downstream targets, we demonstrated that two downstream cascades of IGF-1 signaling were activated in the different cell populations (Figure 3C–F). p-Akt and p-ERK were detected in the inner sulcus cells (Figure 3D) and the Hensen's and Claudius' cells (Figure 3 H), respectively. The inhibition of Akt caused the attenuation of IHC protection but not OHC protection by IGF-1 (Figure 2D), supporting the result that the activation of Akt only occurred in the inner sulcus cells, which are located next to the IHCs.

The possible cellular mechanisms for IGF-1 protection of the HCs are the mechanisms that are needed to maintain the HC numbers. To maintain the cell numbers, the inhibition of cell death and/or an increase in the number of cells are necessary, i.e., the inhibition of apoptosis and the proliferation of cells or cell cycle promotion are possible mechanisms by which IGF-1 exerts its effects in

the cochlea. In this study we determined that both of these mechanisms were involved in maintaining the numbers HCs (Figure 4 – 6).

We were surprised by the detection of cell cycle promotion in the populations of Hensen's and Caludius' cells following the administration of IGF-1 to the neomycin-treated cochlear explants (Figure 5). We confirmed that this promotion of the cell cycle of the supporting cells contributed to the maintenance of the number of OHCs using an inhibitor of proliferation, aphidicolin (Figure 6A and B) and L-mimosine (Figure 6C and D). The administration of aphidicolin or L-mimosine reduced the number of OHCs but did not affect that of IHCs that were more distant from the Hensen's and Caludius' cells than the OHCs. This result suggested that the cell cycle promotion of the Hensen's and Claudius' cells contributed to the maintenance of the OHC numbers and that the proximity of these SCs to the OHCs caused this phenomenon to occur only in the OHCs. In birds, which can regenerate HCs, both the transdifferentiation of the SCs into HCs (Roberson et al., 2004; Shang et al., 2010) and the proliferation of the sensory epithelia (Corwin and Cotanche, 1988; Ryals and Rubel, 1988) occur in the basilar papillae, the hearing organ of

the bird, following aminoglycoside-induced HC damage. The maintenance of the OHC numbers in the mouse cochlea caused by the cell cycle promotion of the SCs suggested that both of the cell cycle promotion of SCs and their transdifferentiation into HCs are involved in the protection of the OHCs by IGF-1 in the postnatal mammal. This finding is astonishing because the natural regeneration of HCs has never been reported in the postnatal mammal due to the cessation of HC proliferation during the embryonic stage (Ruben, 1967). Here, we showed that OHC regeneration could be induced by IGF-1 treatment in neonatal mice, as reported in the avian inner ear (Oesterle et al., 1997). Although previous studies have reported the proliferation of postnatal mammalian vestibular sensory epithelial cells with various growth factors (Oesterle and Hume, 1999), our study is the first to report the induction of cell cycle promotion in the postnatal mammalian cochlear sensory epithelia using exogenous factors following HC damage. Myosin7a, a hair cell marker, did not co-localized with BrdU-positive cells (Figure 5B); therefore, it is possible that the SCs underwent cell cycle promotion and that they themselves transdifferentiated into OHCs, or that the SCs with cell cycle promotion

induced the transdifferentiation of other non-proliferating SCs into OHCs. Considering that we did not find any BrdU-positive OHCs (Figure 5B), the latter possibility is more likely. The cell autonomous transdifferentiation of Hensen's and Claudius' cells was induced by the inhibition of Notch signaling in the postnatal mouse cochlea (Yamamoto et al., 2006). Thus, a combination of IGF-1 treatment and inhibition of Notch signaling may achieve a more robust maintenance of the number of OHCs.

We also found that the maintenance of the IHC and OHC numbers was regulated by different cellular mechanisms. As already discussed, the cell cycle promotion only contributed to the maintenance of the OHC numbers because the inhibition of mitosis affected only the OHC numbers (Figure 6B and D). In contrast, the cleaved caspase-3 staining clearly indicated that the inhibition of apoptosis contributed to the maintenance of the numbers of both OHCs and IHCs.

As discussed above, the PI3K/Akt pathway was only involved in the maintenance of IHC numbers. The PI3K/Akt pathway appeared to exert its effects through the inhibition of apoptosis, even though its action was mediated

by the inner sulcus cells that were located medial to the IHCs, and no cell cycle promotion was observed around the IHCs (Figure 4C and 5B and E). Inner sulcus cells might provide some surviving factors to adjacent IHCs. In contrast, the MEK/ERK pathway was likely to be involved in the cell cycle promotion of the Hensen's and Claudius' cells for two reasons: p-ERK was detected in the Hensen's and Claudius' cells (Figure 3F) in which BrdU uptake and pHH3 were also detected, and the activation of MEK/ERK pathway causes the proliferation of SCs in the chicken basilar papillae, resulting in the regeneration of HCs (Bell and Oberholtzer, 2010).

In summary, we found that IGF-1 could activate both the PI3K/Akt and MEK/ERK pathways in the neomycin-injured postnatal mouse cochlea. The PI3K/Akt pathway maintained the number of IHCs through the inhibition of apoptosis. The MEK/ERK pathway was activated in the Hensen's and Claudius' cells and induced the cell cycle promotion in these cells that partly contributed to the maintenance of the OHCs. Our findings showed that IGF-1 can be used as an efficient medication against the sensorineural hearing loss caused by cochlear HC damage.

Materials and Methods

Animals

ICR mice were purchased from Japan SLC, Hamamatsu, Japan. All of the experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The Animal Research Committee of the Graduate School of Medicine, Kyoto University, approved all of the experimental protocols. Animal care was given under the supervision of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

Immunohistochemistry

The P2 mice cochleae were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C for 4 h, embedded in OCT compound (Tissue Tek, Miles Inc., IN, USA), and 10 μm mid-modiolar sections were then prepared. For whole-mount immunohistochemistry, the samples were fixed at room temperature (RT) for 15 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

The specimens were rinsed with phosphate-buffered saline (PBS) and then incubated in blocking solution at room temperature (30 min in 10% goat serum with 0.2% TritonX-100 for Myosin7a, IGF-1R, phospho-ERK1/2, and BrdU; and 15 min in 0.2% Triton X-100 and 15 min in 1% BSA in 0.2% Triton X-100 for phospho-Akt, cleaved caspase-3, and pHH3).

The primary antibodies used in this study were rabbit polyclonal anti-Myosin7a (1:1000; Proteus Bioscience Inc., Ramona, CA, USA), rabbit polyclonal anti-IGF-1R (1:100; Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal anti-p-Akt (Ser473) (1:50; Cell Signaling Technology), mouse monoclonal anti-p-ERK1/2 (1:1000; Sigma-Aldrich), rabbit polyclonal anti-cleaved caspase-3 (1:100; Cell Signaling Technology), mouse monoclonal anti-BrdU (1:50; BD Biosciences), and p-HH3 mouse monoclonal (1:100; Cell Signaling, #9706). The actin filaments were visualized with Alexa-546- or -633-labeled phalloidin (1:100; Invitrogen, Grand Island, NY, USA). The specificity of the anti-p-Akt and anti-p-ERK1/2 antibodies was confirmed using specific blocking peptides (p-Akt blocking peptide #1140 and p-ERK blocking peptide #1150 from Cell Signaling, Supplementary figure 1).

The primary antibodies were visualized with Alexa-488- or -546-conjugated anti-rabbit or anti-mouse goat IgG (1:1000; Invitrogen). The specimens for which nuclear staining was necessary were then incubated in PBS containing 2 mg/ml DAPI (4′ 6-diamidino-2-phenylindole·2HCl) (Invitrogen).

The omission of the primary antibodies was served as a negative control of immunohistochemistry.

The fluorescent images were captured using a Leica TCS-SPE confocal microscope (Leica Microsystems Inc., Wetzlar, Germany).

Preparation and treatment of cochlear sensory epithelium explant cultures

The explant cultures were established as previously described (Yamamoto et al., 2006). Briefly, postnatal day (P) 2 ICR mice were deeply anesthetized with carbon dioxide and then decapitated. The cochleae were dissected out of the heads with watchmaker forceps using a stereomicroscope. The organs of Corti were separated from the surrounding tissues and were placed onto cell culture inserts (Becton Dickinson, Franklin Lakes, NJ, USA). The cultures were incubated within 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 any growth factors or hormones contained in the serum, we used serum-free medium in all of the experiments. All of the experiments began following 24 h of incubation in the medium to stabilize the explants.

To determine the effects of IGF-1 against aminoglycoside treatment on the cochlear explant cultures, the explants were maintained for 24 more hours (n = 3–6) in medium containing neomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 2 mM with or without recombinant human IGF-1 at the specified concentration (Astellas Pharma Inc., Tokyo, Japan).

To determine the downstream effects of IGF-1 on the damaged cochlear HCs, various inhibitors of IGF-1 downstream signaling were added to the explant cultures. The explants were treated for 24 h with medium containing 2 mM neomycin, 100 ng/ml IGF-1, and various inhibitors, LY294002 (10 μM, PI3K inhibitor, Merck Chemicals, Darmstadt, Germany), Akt inhibitor VIII (10 μM, Akt inhibitor, Merck Chemicals), PD98059 (10 μM, ERK1 inhibitor, Merck Chemicals), U0126 (10 μM, ERK1/2 inhibitor, Merck Chemicals), and calphostin

C (100 nM, PKC inhibitor, Sigma-Aldrich) (n = 4–6). Specificity of each inhibitor was confirmed by immunohistochemistry of the specific target (Supplementary figure 2).

To specify the location of Akt or ERK activation within the cochlear epithelium, the explants were stained with anti-phospho-Akt or anti-phospho-ERK antibodies after incubation with 2 mM neomycin or both 100 ng/ml IGF-1 and 2 mM neomycin for 15 min (n = 3–5). We chose this time point because previous study showed that activation of phospho-Akt and phospho-ERK by IGF-1 occurred between 10 and 30 min after IGF-1 administration (Mairet-Coello et al., 2009).

The apoptotic status of the cochlear epithelium was estimated by immunostaining the explants with anti-cleaved caspase 3 antibodies after 3 or 24 h incubations with 2 mM neomycin or both 100 ng/ml IGF-1 and 2 mM neomycin (n = 5–7).

The promotion of the cell cycle by IGF-1 in the cochlear explants was visualized using BrdU and immunohistochemistry of phophohistone H3 (pHH3). BrdU (3 μg/ml, BD Biosciences, Franklin Lakes, NJ) was added to the explant cultures

during a 24 h treatment with 2 mM neomycin or both 100 ng/ml IGF-1 and 2 mM neomycin and the explants were double-immunostained with anti-BrdU and anti-Myosin7a antibodies (n = 3 or 4). Immunohistochemistry of pHH3 was performed 6 h after treatment with 2 mM neomycin or both 100 ng/ml IGF-1 and 2 mM neomycin. To confirm that the cell cycle promotion of the supporting cells contribute to the maintenance of the number of hair cells, explants were treated with 2 mM neomycin and 2.8 mM aphidicolin (Sigma-Aldrich) or 0.1mM L-mimosine (Calbiochem) ± 100 ng/ml IGF-1 for 24 h (n = 4–6). The specificity and the toxicity of aphidicolin and L-mimosine were examined (Supplementary figure 3).

Cell counts

The numbers of inner and outer HCs or SCs in the organ of Corti were counted along a 100 μm-longitudinal distance in the basal turn of each explant except pHH3-positive cells. For pHH3-positive cells, the number of pHH3-positive SCs in one high power field (275 μm × 275 μm) was counted. We analyzed the

basal turn of the cochlea in this study because hair cells in the apex are resistant to aminoglycosides. The hair cells with remaining stereocilia were counted as live hair cells, and the stereocilia were identified using phalloidin to detect the actin filaments. The cleaved caspase-3-positive cells were defined as those that had hair bundles stained with phalloidin, and only the BrdU or pHH3-positive cells that were co-stained with DAPI and that existed in the epithelial layer were counted.

Statistical analysis

The data were expressed as the mean ± standard errors. One-way ANOVA was used for the analysis of the protection effects of IGF-1 against neomycin. Post hoc tests were performed by the Bonferroni method. In the other experiments, the statistical analyses were performed using unpaired *t*-tests, and Bonferroni correction was applied on the multiple comparisons of the effects of various inhibitors of IGF-1 signaling. *p* values below 0.05 were considered statistically significant except in the cases analyzed with Bonferroni correction. *p* values below 0.0024 or 0.0033 was considered statistically significant in the analysis of

dose dependency of IGF-1 effects or various inhibitors of IGF-1 signaling, respectively.

Acknowledgments: This project was supported by KAKENHI (a Grant-in-Aid for Young Scientists (B) (22791595) and a Grant-in-Aid for Scientific Research (C) (24592545) to NY, and a Grant-in-Aid for Scientific Research (S) (23229009) to JI) from the Ministry of Education, Culture, Sports, Science and Technology in Japan and by the Japan Society for the Promotion of Science.

References

Aburto, M.R., Magariños, M., Leon, Y., Varela-Nieto, I., Sanchez-Calderon, H., 2012. AKT Signaling Mediates IGF-I Survival Actions on Otic Neural Progenitors. PLoS One 7, e30790.

Baserga, R., Peruzzi, F., Reiss, K., 2003. The IGF-1 receptor in cancer biology. Int J Cancer 107, 873-877.

Battaglia, A., Pak, K., Brors, D., Bodmer, D., Frangos, J.A., Ryan, A.F., 2003. Involvement of ras activation in toxic hair cell damage of the mammalian cochlea. Neuroscience 122, 1025-1035.

Bell, T.J., Oberholtzer, J.C., 2010. cAMP-induced auditory supporting cell proliferation is mediated by ERK MAPK signaling pathway. J Assoc Res Otolaryngol 11, 173-185.

Bonapace, G., Concolino, D., Formicola, S., Strisciuglio, P., 2003. A novel mutation in a patient with insulin-like growth factor 1 (IGF1) deficiency. J Med Genet 40, 913-917.

Bondy, C., Cheng, C., 2004. Signaling by insulin-like growth factor 1 in brain. Eur J Pharmacol 490, 25-31.

Brodt, P., Samani, A., Navab, R., 2000. Inhibition of the type I insulin-like growth factor receptor expression and signaling: novel strategies for antimetastatic therapy. Biochem Pharmacol 60, 1101-1107.

Cediel, R., Riquelme, R., Contreras, J., Díaz, A., Varela-Nieto, I., 2006. Sensorineural hearing loss in insulin-like growth factor I-null mice: a new model of human deafness. Eur J Neurosci 23, 587-590.

Chung, W.H., Pak, K., Lin, B., Webster, N., Ryan, A.F., 2006. A PI3K pathway mediates hair cell survival and opposes gentamicin toxicity in neonatal rat organ of Corti. J Assoc Res Otolaryngol 7, 373-382.

Corwin, J.T., Cotanche, D.A., 1988. Regeneration of sensory hair cells after acoustic trauma. Science 240, 1772-1774.

Datta, S.R., Brunet, A., Greenberg, M.E., 1999. Cellular survival: a play in three Akts. Genes Dev 13, 2905-2927.

Finlay, D., Healy, V., Furlong, F., O'Connell, F.C., Keon, N.K., Martin, F., 2000. MAP kinase pathway signalling is essential for extracellular matrix determined mammary epithelial cell survival. Cell Death Differ 7, 302-313.

Fujiwara, T., Hato, N., Nakagawa, T., Tabata, Y., Yoshida, T., Komobuchi, H., Takeda, S., Hyodo, J., Hakuba, N., Gyo, K., 2008. Insulin-like growth factor 1 treatment via hydrogels rescues cochlear hair cells from ischemic injury. Neuroreport 19, 1585-1588.

Haake, S.M., Dinh, C.T., Chen, S., Eshraghi, A.A., Van De Water, T.R., 2009. Dexamethasone protects auditory hair cells against TNFalpha-initiated apoptosis via activation of PI3K/Akt and NFkappaB signaling. Hear Res 255, 22-32.

Hammond, R.A., Byrnes, J.J., Miller, M.R., 1987. Identification of DNA polymerase delta in CV-1 cells: studies implicating both DNA polymerase delta and DNA polymerase alpha in DNA replication. Biochemistry 26, 6817-6824.

Holmström, T.H., Tran, S.E., Johnson, V.L., Ahn, N.G., Chow, S.C., Eriksson, J.E., 1999. Inhibition of mitogen-activated kinase signaling sensitizes HeLa cells to Fas receptor-mediated apoptosis. Mol Cell Biol 19, 5991-6002.

Hu, A., Parnes, L.S., 2009. Intratympanic steroids for inner ear disorders: a review. Audiol Neurootol 14, 373-382.

Iwai, K., Nakagawa, T., Endo, T., Matsuoka, Y., Kita, T., Kim, T., Tabata, Y., Ito, J., 2006. Cochlear protection by local insulin-like growth factor-1 application using biodegradable hydrogel. Laryngoscope 116, 529-533.

Jenkins, P., Bustin, S., 2004. Evidence for a link between IGF-I and cancer. Eur J Endocrinol 151 Suppl 1, S17-22.

Jiang, H., Sha, S.H., Schacht, J., 2006. Kanamycin alters cytoplasmic and nuclear phosphoinositide signaling in the organ of Corti in vivo. J Neurochem 99, 269-276.

Kikkawa, Y., Nakagawa, T., Tsubouchi, H., Ido, A., Inaoka, T., Ono, K., Ito, J., 2009. Hepatocyte growth factor protects auditory hair cells from aminoglycosides. Laryngoscope 119, 2027-2031.

Laine, H., Sulg, M., Kirjavainen, A., Pirvola, U., 2010. Cell cycle regulation in the inner ear sensory epithelia: role of cyclin D1 and cyclin-dependent kinase inhibitors. Dev Biol 337, 134-146.

Lee, K., Nakagawa, T., Okano, T., Hori, R., Ono, K., Tabata, Y., Lee, S., Ito, J., 2007. Novel therapy for hearing loss: delivery of insulin-like growth factor 1 to the cochlea using gelatin hydrogel. Otol Neurotol 28, 976-981.

León, Y., Vazquez, E., Sanz, C., Vega, J.A., Mato, J.M., Giraldez, F., Represa, J., Varela-Nieto, I., 1995. Insulin-like growth factor-I regulates cell proliferation in the developing inner ear, activating glycosyl-phosphatidylinositol hydrolysis and Fos expression. Endocrinology 136, 3494-3503.

Mairet-Coello, G., Tury, A., DiCicco-Bloom, E., 2009. Insulin-like growth factor-1 promotes G(1)/S cell cycle progression through bidirectional regulation of cyclins and cyclin-dependent kinase inhibitors via the phosphatidylinositol 3-kinase/Akt pathway in developing rat cerebral cortex. J Neurosci 29, 775-788.

Medema, R.H., Kops, G.J., Bos, J.L., Burgering, B.M., 2000. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature 404, 782-787.

Nakagawa, T., Sakamoto, T., Hiraumi, H., Kikkawa, Y.S., Yamamoto, N., Hamaguchi, K., Ono, K., Yamamoto, M., Tabata, Y., Teramukai, S., Tanaka, S., Tada, H., Onodera, R., Yonezawa, A., Inui, K.I., Ito, J., 2010. Topical insulin-like growth factor 1 treatment using gelatin hydrogels for glucocorticoid-resistant sudden sensorineural hearing loss: a prospective clinical trial. BMC Med 8, 76.

Oesterle, E.C., Hume, C.R., 1999. Growth factor regulation of the cell cycle in developing and mature inner ear sensory epithelia. J Neurocytol 28, 877-887.

Oesterle, E.C., Tsue, T.T., Rubel, E.W., 1997. Induction of cell proliferation in avian inner ear sensory epithelia by insulin-like growth factor-I and insulin. J Comp Neurol 380, 262-274.

Okano, T., Xuan, S., Kelley, M.W., 2011. Insulin-like growth factor signaling regulates the timing of sensory cell differentiation in the mouse cochlea. J Neurosci 31, 18104-18118.

Pagès, G., Lenormand, P., L'Allemain, G., Chambard, J.C., Meloche, S., Pouysségur, J., 1993. Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. Proc Natl Acad Sci U S A 90, 8319-8323.

Perry, C., Sastry, R., Nasrallah, I.M., Stover, P.J., 2005. Mimosine attenuates serine hydroxymethyltransferase transcription by chelating zinc. Implications for inhibition of DNA replication. J Biol Chem 280, 396-400.

Popken, G., Hodge, R., Ye, P., Zhang, J., Ng, W., O'Kusky, J., D'Ercole, A., 2004. In vivo effects of insulin-like growth factor-I (IGF-I) on prenatal and early postnatal development of the central nervous system. Eur J Neurosci 19, 2056-2068.

Roberson, D.W., Alosi, J.A., Cotanche, D.A., 2004. Direct transdifferentiation gives rise to the earliest new hair cells in regenerating avian auditory epithelium. J Neurosci Res 78, 461-471.

Ruben, R.J., 1967. Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. Acta Otolaryngol, Suppl 220:221-244.

Ryals, B.M., Rubel, E.W., 1988. Hair cell regeneration after acoustic trauma in adult Coturnix quail. Science 240, 1774-1776.

Selivanova, O., Brieger, J., Heinrich, U.R., Mann, W., 2007. Akt and c-Jun N-terminal kinase are regulated in response to moderate noise exposure in the cochlea of guinea pigs. ORL J Otorhinolaryngol Relat Spec 69, 277-282.

Shang, J., Cafaro, J., Nehmer, R., Stone, J., 2010. Supporting cell division is not required for regeneration of auditory hair cells after ototoxic injury in vitro. J Assoc Res Otolaryngol 11, 203-222.

Spear, S.A., Schwartz, S.R., 2011. Intratympanic steroids for sudden sensorineural hearing loss: a systematic review. Otolaryngol Head Neck Surg 145, 534-543.

Thompson, J.F., Morris, C.J., Smith, I.K., 1969. New naturally occurring amino acids. Annu Rev Biochem 38, 137-158.

Varela-Nieto, I., de la Rosa, E., Valenciano, A., León, Y., 2003. Cell death in the nervous system: lessons from insulin and insulin-like growth factors. Mol Neurobiol 28, 23-50.

Varela-Nieto, I., Hartl, M., Gorospe, I., León, Y., 2007. Anti-apoptotic actions of insulin-like growth factors: lessons from development and implications in neoplastic cell transformation. Curr Pharm Des 13, 687-703.

Walenkamp, M.J., Karperien, M., Pereira, A.M., Hilhorst-Hofstee, Y., van Doorn, J., Chen, J.W., Mohan, S., Denley, A., Forbes, B., van Duyvenvoorde, H.A., van Thiel, S.W., Sluimers, C.A., Bax, J.J., de Laat, J.A., Breuning, M.B., Romijn, J.A., Wit, J.M., 2005. Homozygous and heterozygous

expression of a novel insulin-like growth factor-I mutation. J Clin Endocrinol Metab 90, 2855-2864.
Woods, K.A., Camacho-Hübner, C., Savage, M.O., Clark, A.J., 1996. Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. N Engl J Med 335, 1363-1367.
Yamamoto, N., Tanigaki, K., Tsuji, M., Yabe, D., Ito, J., Honjo, T., 2006. Inhibition of Notch/RBP-J signaling induces hair cell formation in neonate mouse cochleas. J Mol Med 84, 37-45.
Yoshida, A., Kitajiri, S., Nakagawa, T., Hashido, K., Inaoka, T., Ito, J., 2011. Adipose tissue-derived stromal cells protect hair cells from aminoglycoside. Laryngoscope 121, 1281-1286.
Zheng, J.L., Helbig, C., Gao, W.Q., 1997. Induction of cell proliferation by fibroblast and insulin-like growth factors in pure rat inner ear epithelial cell cultures. J Neurosci 17, 216-226.
Zhou, B.P., Liao, Y., Xia, W., Spohn, B., Lee, M.H., Hung, M.C., 2001. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat Cell Biol 3, 245-252.

Figure Legends

Figure 1. IGF-1 protected cochlear hair cells against neomycin damage.

A and B. Representative images of cochlear explants immunostained with anti-myosin 7a (myo7a, green) and phalloidin (red) after the treatment with neomycin only (A) or neomycin and 100 ng/ml of IGF-1 (B). C and D. The number of surviving hair cells after neomycin and various concentrations of IGF-1 treatment were counted. Both the inner and outer hair cells (IHCs and OHCs) were destroyed by the presence of neomycin (A). The addition of 100

ng/ml IGF-1 dramatically improved the survival of the IHCs and OHCs (B). IGF-1 significantly attenuated the loss of the OHCs (C, $p<0.0001$) and IHCs (D, $p=0.0018$) in neomycin-damaged cochlear sensory epithelia. Lower concentrations of IGF-1 were less able to protect the cochlear hair cells.

The scale bar represents 25 μm. The bars represent the standard errors. *$p<0.001$; **$p=0.0011$; ***$p<0.003$ by Bonferroni method.

Figure 2. Blockage of PI3K/Akt, MEK/ERK or PKC attenuated the protective effect of IGF-1 on neomycin-induced cochlear hair cell damage.

A–E. PD98059 (A, 10 μM), U0126 (B, 10 μM), LY294002 (C, 10 μM), Akt inhibitor VIII (D, 10 μM) or calphostin C (E, 100 nM) were administered to the cochlear explants in addition to 2 mM neomycin and 100 ng/ml IGF-1 for 24 h. The specimens were immunostained with anti-myosin 7a (myo7a, green) and phalloidin (red), and the number of cochlear hair cells with remaining stereocilia was counted. F and G. The number of surviving OHCs (F) and IHCs (G) after neomycin treatment with various inhibitors of the IGF-1 downstream pathways was counted. When treated with U0126 (B), LY294002 (C), or

calphostin C (E), the protection of both the OHCs and IHCs was significantly attenuated (F and G). In contrast, when treated with PD98059 (A) or Akt inhibitor VIII (C), only the OHC or IHC protection was significantly attenuated, respectively (F and G). These findings indicated that the MEK/ERK or PI3K/PKC pathway and the PI3K/Akt pathway were involved in the protection of the HCs in different ways. Although off-target effects should be considered, these results suggest that the PI3K/Akt pathway protects only IHCs and the other pathways protect both the IHCs and OHCs. The scale bar in E represents 25 μm. The bars in F and G represent the standard errors. *p=0.0024; **p<0.0005; ***p<0.001 by Bonferroni correction.

Figure 3. Identification of cochlear cell populations that are protected by IGF-1.

A and B. Immunohistochemistry of IGF-1R (green) on a cross section (A) or whole mount (B) of postnatal day 2 (P2) mouse cochlea. C–F. Immunohistochemistry of phospho-Akt (green, C and D) and phospho-ERK (green, E and F) in the mouse cochlear explants grown in the neomycin

containing media with (D and F) or without (C and E) IGF-1. G and H. Primary antibody omission experiment for phospho-Akt (G) and phospho-ERK (H) grown in the neomycin containing media with IGF-1. The specimens were counterstained with phalloidin (red in A–D and purple in E and F) and DAPI (blue). IGF-1R was expressed in the OHCs, IHCs, inner sulcus cells (asterisks in A and B), pillar cells, and Hensen's and Claudius' cells (double asterisks in A and B). When the explants were treated with neomycin (Neo) only, neither phospho-Akt nor phospho-ERK positive cells were detected in the cochlear sensory epithelia. When the explants were treated with neomycin and IGF-1, the phospho-Akt and phospho-ERK were detected in the inner sulcus cells (asterisk in D) and in the Hensen's and Claudius' cells (double asterisk in F), respectively. * and ** indicate the area of the inner sulcus cells and the Hensen's and Claudius' cells, respectively. The scale bar in A represents 50 μm. The scale bars in B, C, E, G, and H represent 25 μm.

Figure 4. IGF-1 protected cochlear hair cells from apoptosis.

A–D. The explants were cultured for 3 (A and B) or 24 (C and B) hours in

neomycin (Neo) containing media with (B and D) or without (A and C) the addition of IGF-1. The specimens were immunostained with anti-cleaved caspase-3 antibody (green) and phalloidin (red), and the numbers of apoptotic cochlear hair cells were counted in the 3 (E) or 24 (F) h conditions. We detected very few cleaved caspase-3-positive hair cells in the Neo+IGF-1 samples (B and D), although we did detect cleaved caspase-3-positive cells in the neomycin-only samples (A and C). The difference in the number of cleaved caspase-3-positive hair cells was significant only in the 24 hour treatment experiments (E and F). The scale bar represents 25 μm. The bars represent standard errors. *$p<0.0001$ by Student's t-test.

Figure 5. IGF-1 induced cell cycle promotion in Hensen's and Claudius' cells.

A and B. BrdU incorporation was tested by immunohistochemistry in the cochlear explants treated with neomycin (Neo) (A) or with both Neo and IGF-1 (B) for 24 h. The specimens were immunostained with anti-BrdU antibody (green), anti-myosin7a antibody (myo7a, red) and DAPI (blue). C. The number of BrdU-positive cells was counted. D and E. Phospho-histone H3 (pHH3, green)

and myosin7a (myo7a, red) were immunostained in the cochlear explants treated with neomycin (Neo) (D) or with both Neo and IGF-1 (E) for 6 h. DAPI was used as counterstain of the specimens. F. The number of pHH3-positive cells was counted. The samples that were treated with neomycin contained only a small number of BrdU- or pHH3-positive cells (A, C, D, and F). The addition of IGF-1 resulted in a significantly increased number of BrdU- (B and C, p=0.0042) or pHH3- (E and F, p=0.0159) positive cells in the Hensen's and Claudius' cell region. The scale bars in B and E represents 25 μm. The bars represent standard errors. hpf = high power field (275 μm × 275 μm). *p=0.0042; **p=0.0159; by Student's t-test.

Figure 6. Inhibition of the cell cycle promotion attenuated the protective effect of IGF-1 on neomycin-induced cochlear hair cell damage.

A and C. Representative images of a cochlear explant culture treated with neomycin (Neo), IGF-1, and an inhibitor of proliferation, aphidicolin (A) or L-mimosine (C). The specimens were immunostained with anti-myosin7a (myo7a, green) and phalloidin (red). B and D. The numbers of surviving OHCs

and IHCs after the neomycin and IGF-1 treatment with or without aphidicolin (B) or L-mimosine (D) were counted. The addition of aphidicolin or L-mimosine caused a significant attenuation of the IGF-1 OHC protection effect (B, $p=0.0274$, or D, $p=0.0099$). The scale bars in A and C represents 25 μm. The bars represent standard errors. *$p=0.0274$; **$p=0.0099$; by Student's t-test.

Supplementary figure 1. Specificity of anti-phospho-Akt and anti-phospho-ERK antibodies was confirmed using specific blocking peptides.

After having been treated with 2 mM neomycin and 100 ng/ml IGF-1 for 15 min the cochlear explants were immunostained with anti-phospho-Akt (green in A) and anti-phospho-ERK (green in B) antibodies that were incubated with specific blocking peptides in advance. The specimens were counterstained with phalloidin (red in A and purple in B). The signals of phospho-Akt and phospho-ERK observed in inner sulcus cells and Hensen's and Claudius' cells, respectively (Figure 3 D and F), were not detected (asterisk in A and double asterisk in B). These findings indicated the specificity of these primary antibodies. * and ** indicate the area of the inner sulcus cells and the Hensen's and Claudius' cells, respectively. The scale bars represent 25 μm.

Supplementary figure 2. Specificity of PI3K/Akt and MEK/ERK inhibitors

The cochlear explants were treated with LY294002 (A and E, 10 μM), Akt inhibitor VIII (B and F, 10 μM), PD98059 (C and G, 10 μM) or U0126 (D and

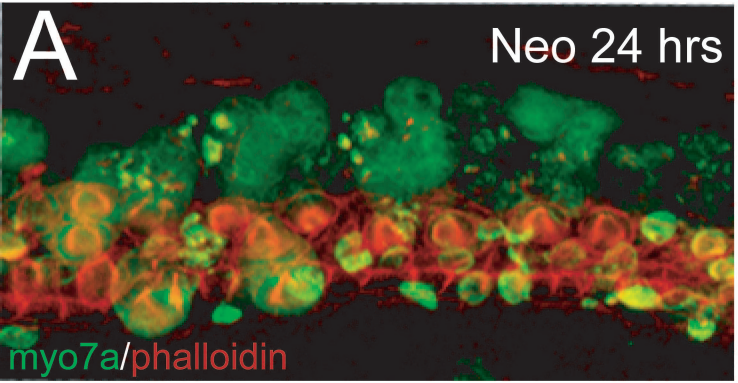
H, 10 μM) in addition to 2 mM neomycin and 100 ng/ml IGF-1 for 15 min. The specimens were immunostained with anti-phospho-Akt (green in A–D) or ERK (green in E–H) antibody and were counterstained with phalloidin (red in A–D and purple in E–H). Phospho-AKT signal were inhibited by PI3K (A) and AKT (B) inhibitors but not by MEK inhibitors (C and D). In contrast, phospho-ERK signals were inhibited by PI3K (E) and MEK (G and H) inhibitors but not by AKT inhibitors (F). Considering that PI3K can affect MEK/ERK signaling through PKC, these results clearly showed that each inhibitor specifically block its target. AKT inhibitor VIII can decrease the production of phospho-AKT by inhibiting AKT kinase. * and ** indicate the area of the inner sulcus cells and the Hensen's and Claudius' cells, respectively. The scale bars in A and E represent 25 μm.

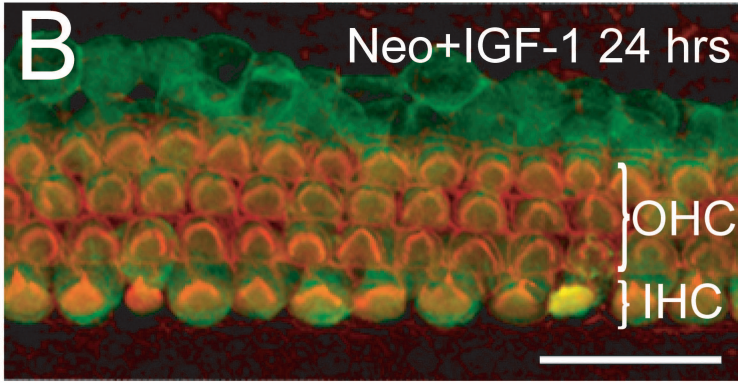
Supplementary figure 3. Specificity and toxicity of aphidicolin and L-mimosine.

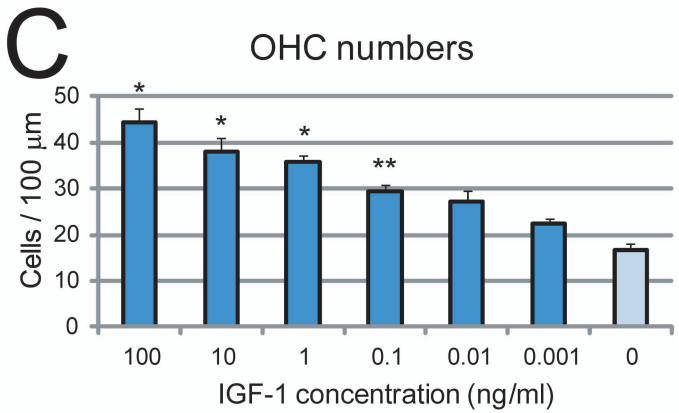
A and B. BrdU incorporation was tested by immunohistochemistry in the cochlear explants treated with 2 mM neomycin, 100 ng/ml IGF-1 and 2.8 mM aphidicolin or 0.1 mM L-mimosine for 24 h. The specimens were

immunostained with anti-BrdU antibody (green), anti-myosin7a antibody (myo7a, red) and DAPI (blue). C and D. The number of BrdU-positive cells was counted. Aphidicolin or L-mimosine treatment significantly decreased the number of BrdU positive Hensen's and Claudius' cells (*p=0.0009 in C and **p=0.0083 in D by Student's t-test). E–G. The cochlear explants were treated with 2.8 mM aphidicolin and 100 ng/ml IGF-1 (F) or 0.1 mM L-mimosine and 100 ng/ml IGF-1 (G) for 24 h. The non-treated specimen was presented as a control sample (E). The specimens were immunostained with anti-myosin 7a antibody (myo7a, green) and were counterstained with phalloidin (red). H and I. The number of cochlear outer and inner hair cells (OHC and IHC) with remaining stereocilia was counted. The numbers of OHC and IHC in the samples treated with aphidicolin or L-mimosine were not reduced in comparison with those of the control samples (H: p=0.0933 in OHC and p=0.6504 in IHC; I: p=0.2354 in OHC and p=0.7791 in IHC by Student's t-test). These findings indicated that aphidicolin or L-mimosine in this concentration with IGF-1 is not toxic to the hair cells in the cochlear explants. The scale bars in B and G represent 25 μm. The bars represent standard errors.

Figure 1


A
Neo 24 hrs
myo7a/phalloidin


B
Neo+IGF-1 24 hrs
OHC
IHC


C
OHC numbers
Cells / 100 µm
50
40
30
20
10
0
*
*
*
**
100
10
1
0.1
0.01
0.001
0
IGF-1 concentration (ng/ml)

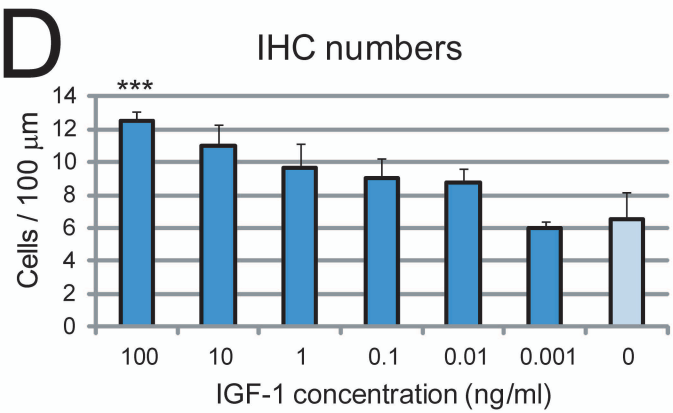
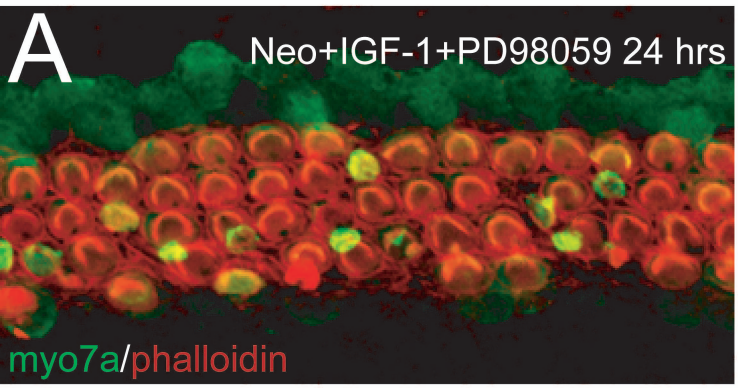
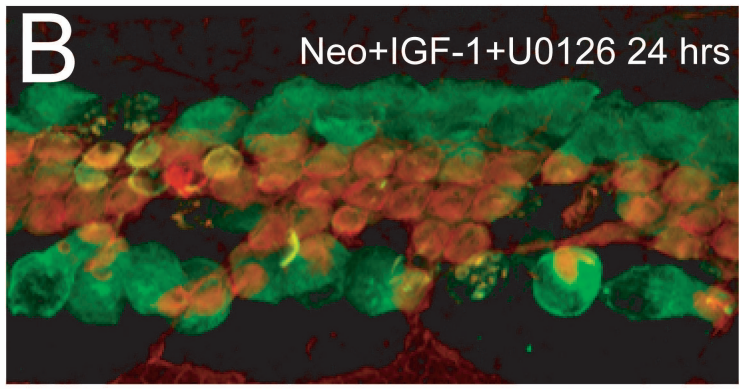
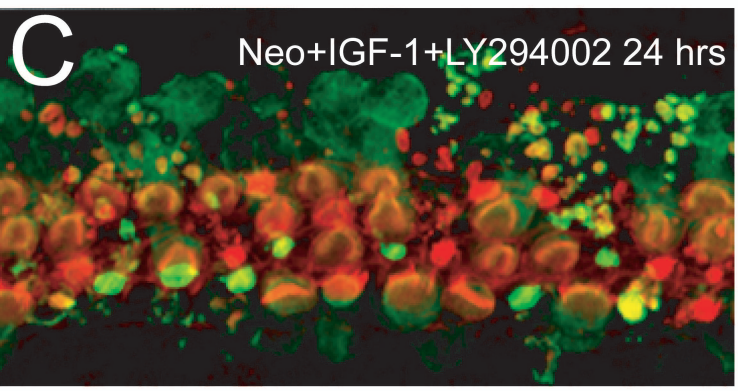
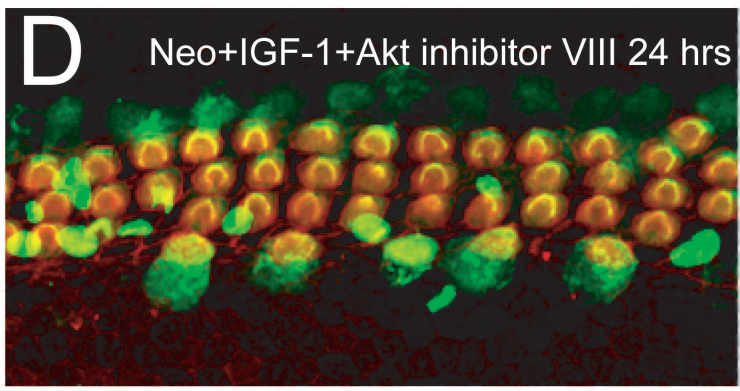

D
IHC numbers
Cells / 100 µm
14
12
10
8
6
4
2
0
***
100
10
1
0.1
0.01
0.001
0
IGF-1 concentration (ng/ml)

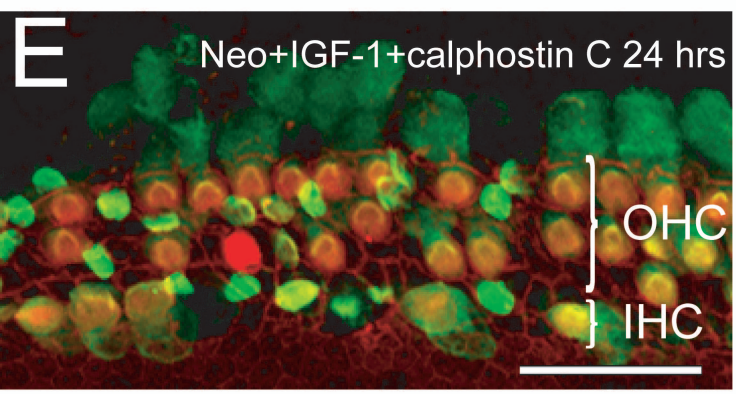
Figure 2

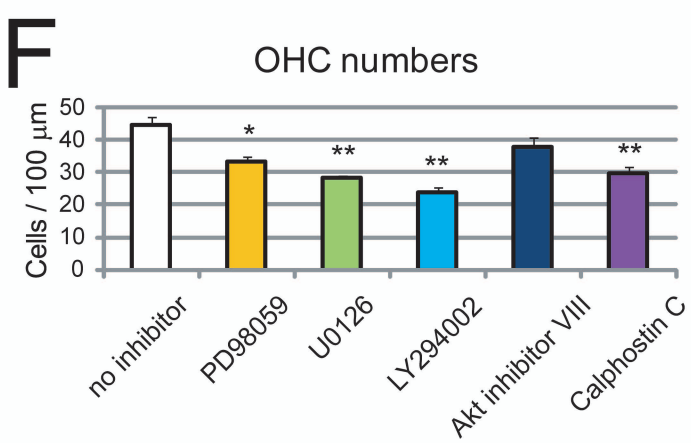

A
Neo+IGF-1+PD98059 24 hrs
myo7a/phalloidin


B
Neo+IGF-1+U0126 24 hrs


C
Neo+IGF-1+LY294002 24 hrs


D
Neo+IGF-1+Akt inhibitor VIII 24 hrs


E
Neo+IGF-1+calphostin C 24 hrs
OHC
IHC


F
OHC numbers
Cells / 100 µm
50
40
30
20
10
0
*
**
**
**
no inhibitor
PD98059
U0126
LY294002
Akt inhibitor VIII
Calphostin C

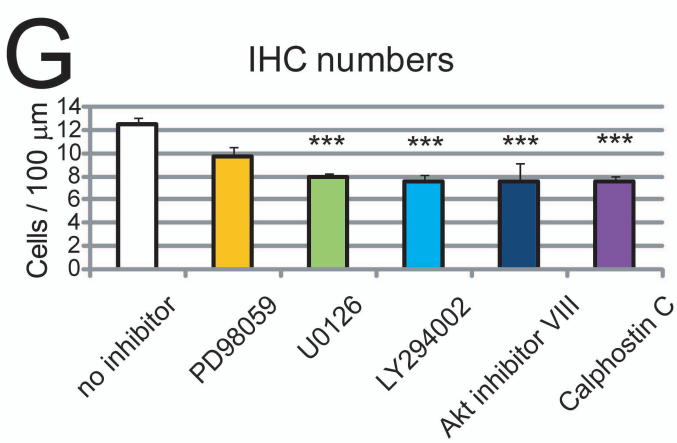

G
IHC numbers
Cells / 100 µm
14
12
10
8
6
4
2
0
***
***
***
***
no inhibitor
PD98059
U0126
LY294002
Akt inhibitor VIII
Calphostin C

Figure 3

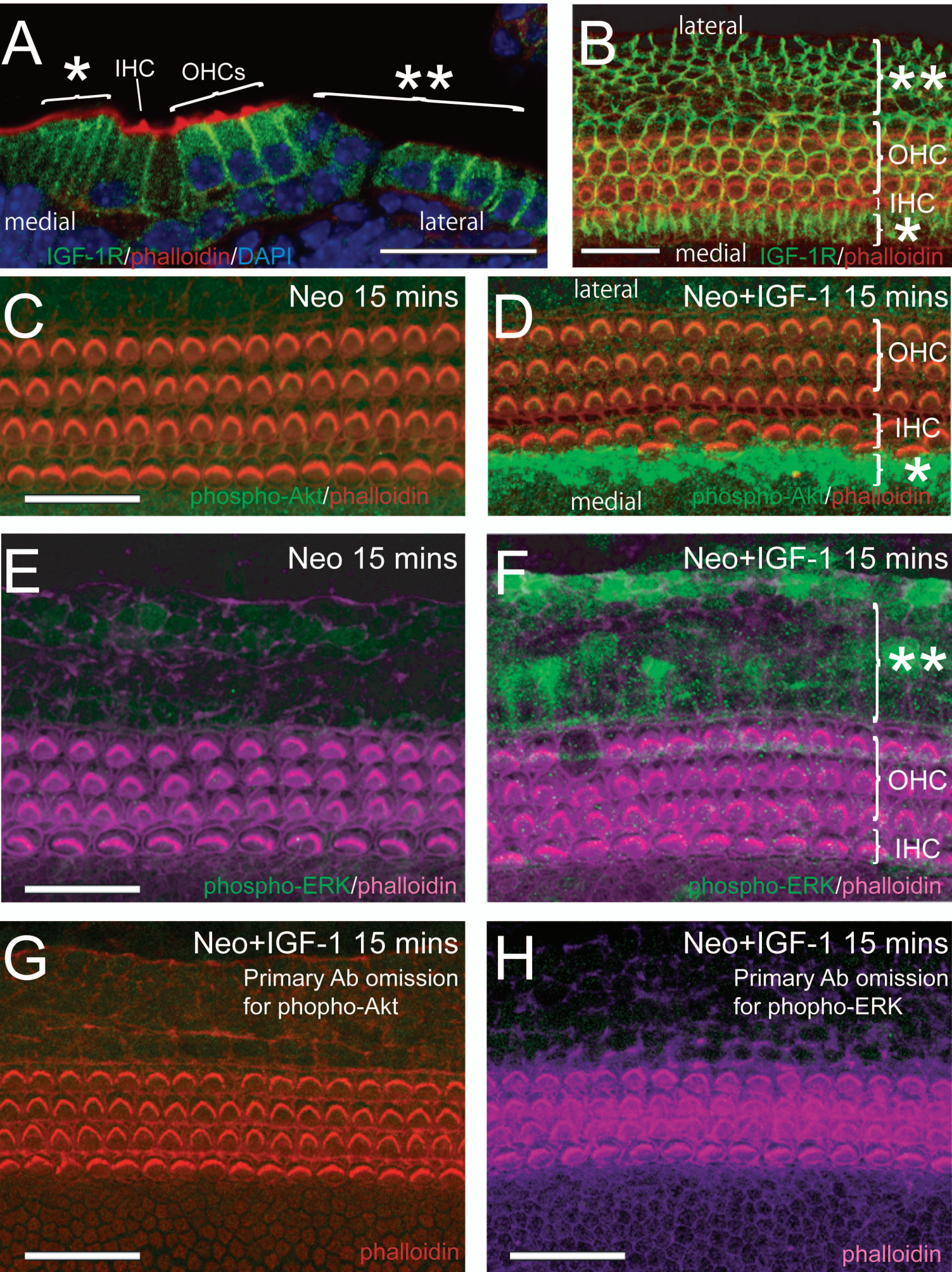
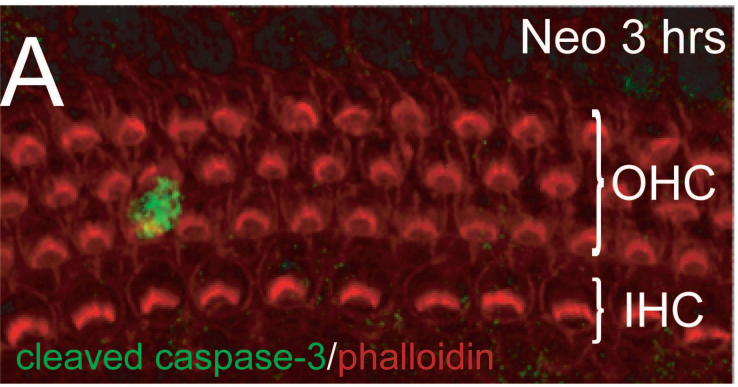
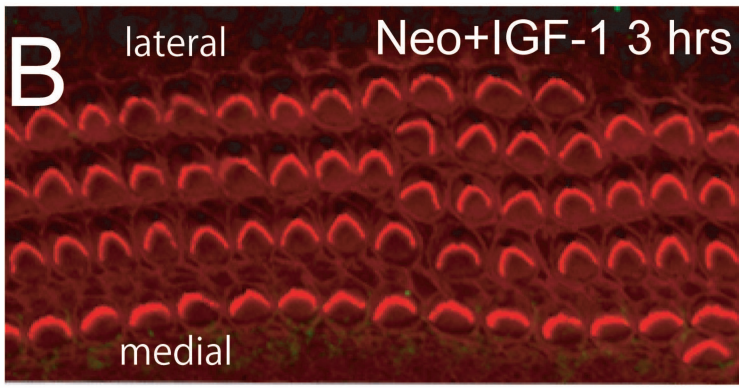
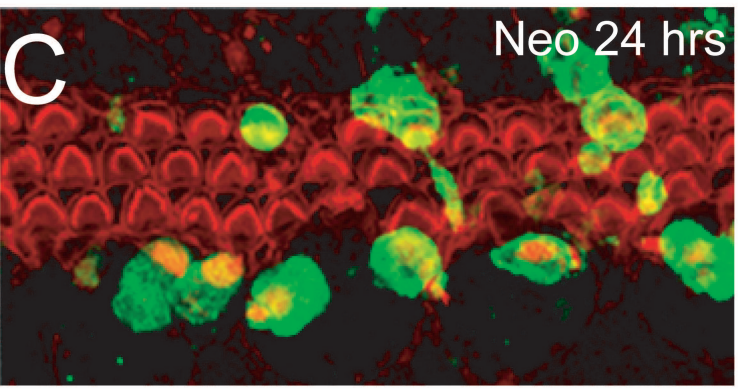
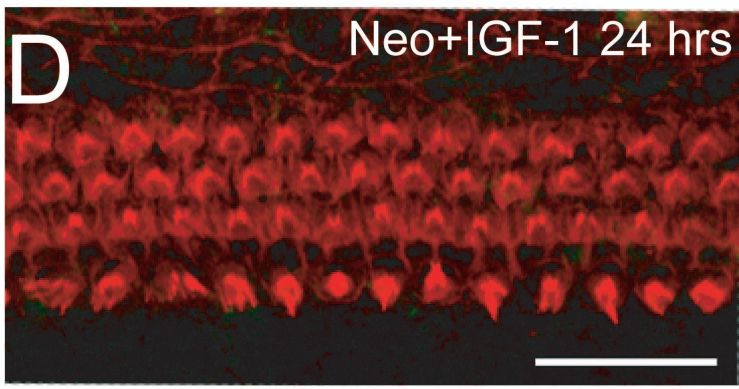

A
*
IHC
OHCs
**
medial
lateral
IGF-1R/phalloidin/DAPI
B
lateral
**
OHC
IHC
*
medial
IGF-1R/phalloidin
C
Neo 15 mins
phospho-Akt/phalloidin
D
lateral
Neo+IGF-1 15 mins
OHC
IHC
*
medial
phospho-Akt/phalloidin
E
Neo 15 mins
phospho-ERK/phalloidin
F
Neo+IGF-1 15 mins
**
OHC
IHC
phospho-ERK/phalloidin
G
Neo+IGF-1 15 mins
Primary Ab omission
for phopho-Akt
phalloidin
H
Neo+IGF-1 15 mins
Primary Ab omission
for phopho-ERK
phalloidin

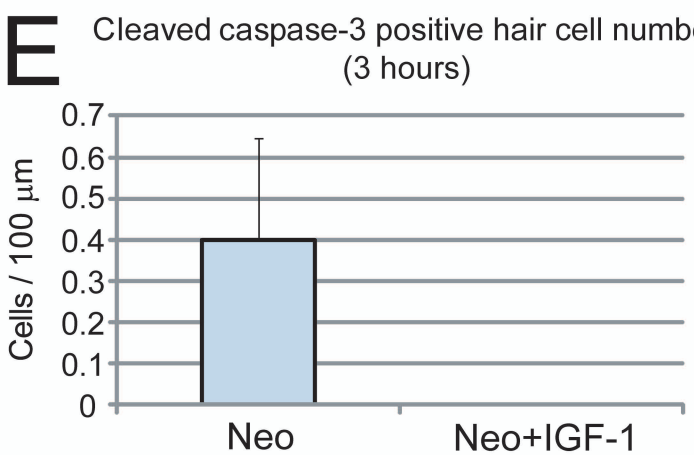
Figure 4


A
Neo 3 hrs
OHC
IHC
cleaved caspase-3/phalloidin


B
lateral
Neo+IGF-1 3 hrs
medial


C
Neo 24 hrs


D
Neo+IGF-1 24 hrs


E
Cleaved caspase-3 positive hair cell number
(3 hours)
Cells / 100 µm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Neo
Neo+IGF-1

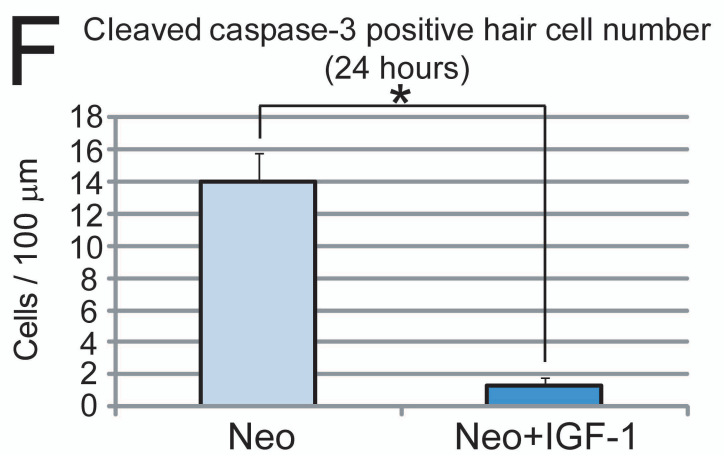
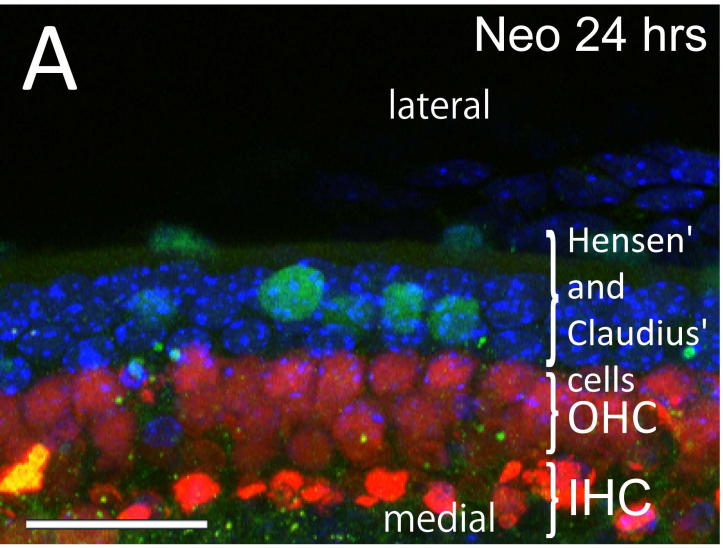
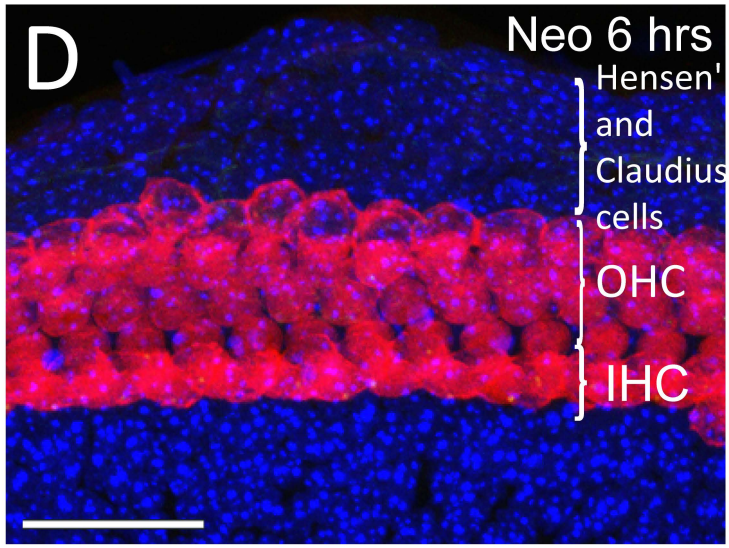
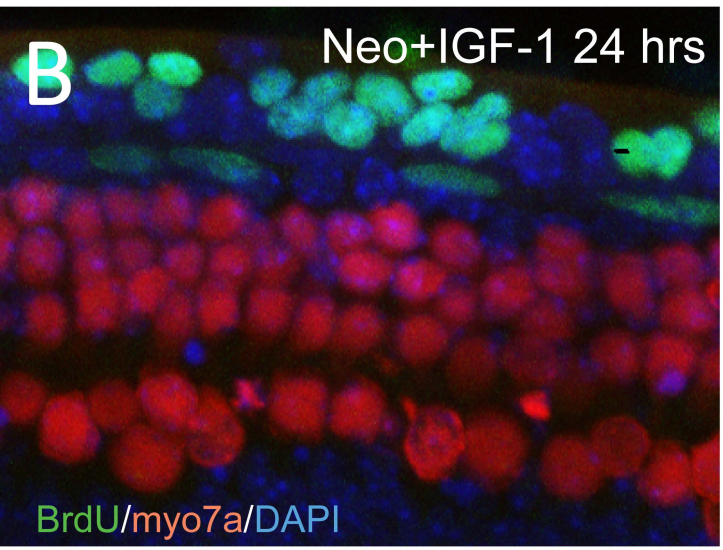
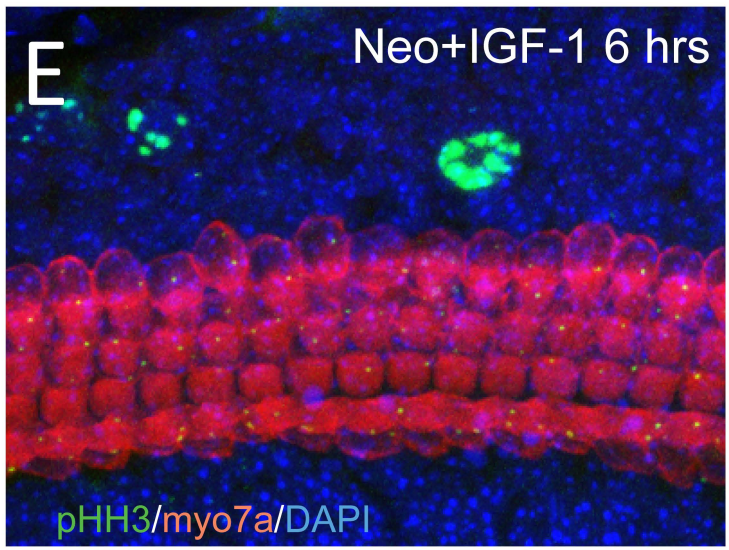

F
Cleaved caspase-3 positive hair cell number
(24 hours)
*
Cells / 100 µm
18
16
14
12
10
8
6
4
2
0
Neo
Neo+IGF-1

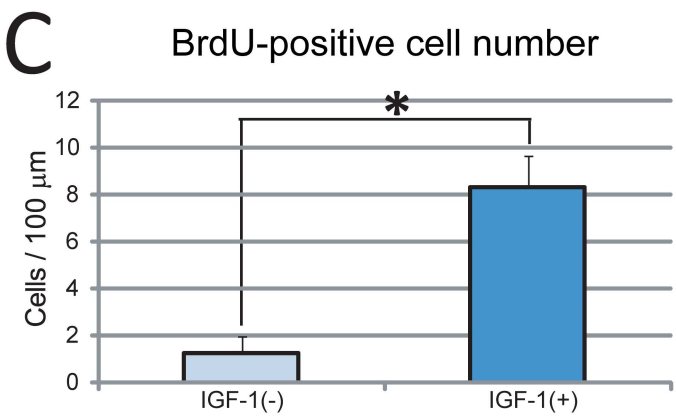
Figure 5


A
Neo 24 hrs
lateral
Hensen'
and
Claudius'
cells
OHC
IHC
medial


D
Neo 6 hrs
Hensen'
and
Claudius
cells
OHC
IHC


B
Neo+IGF-1 24 hrs
BrdU/myo7a/DAPI


E
Neo+IGF-1 6 hrs
pHH3/myo7a/DAPI


C
BrdU-positive cell number
Cells / 100 µm
12
10
8
6
4
2
0
*
IGF-1(-)
IGF-1(+)

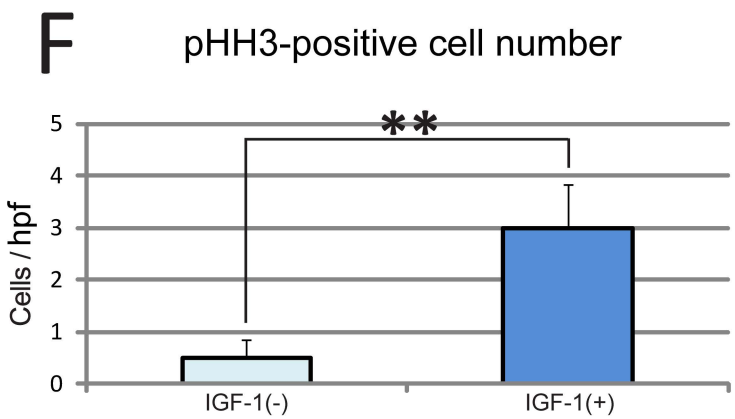
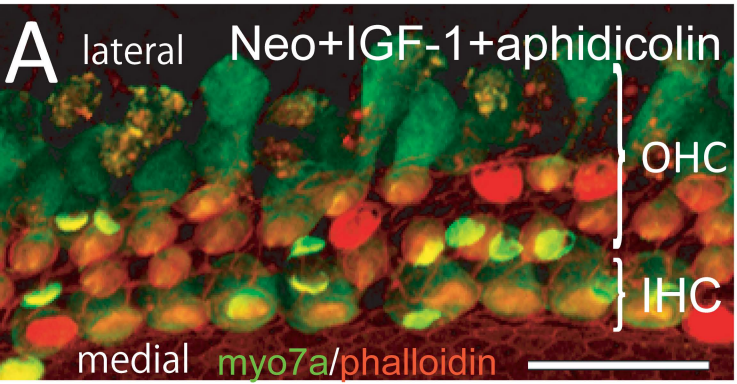
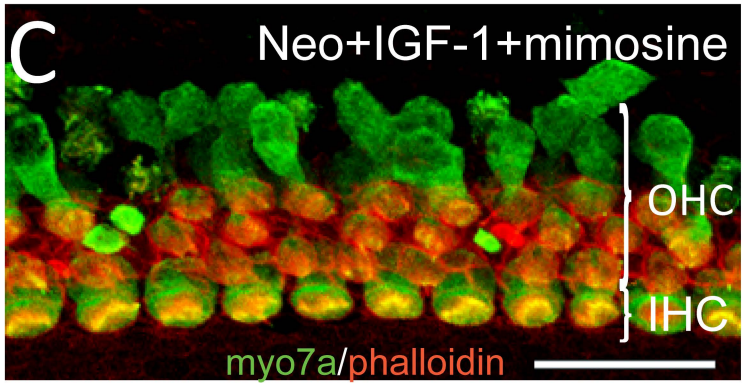
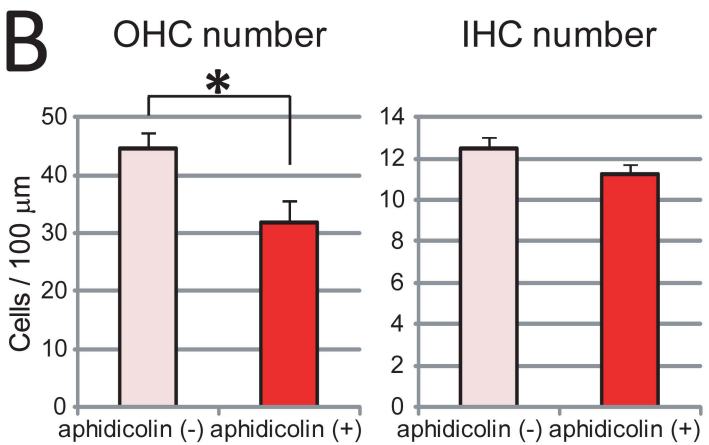
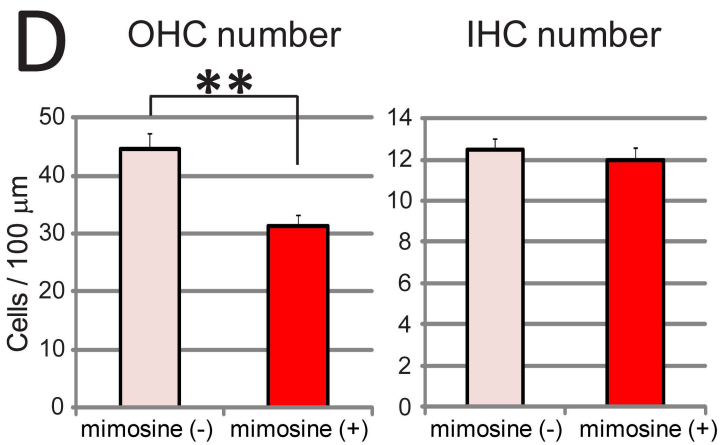

F
pHH3-positive cell number
Cells / hpf
5
4
3
2
1
0
**
IGF-1(-)
IGF-1(+)

Figure 6

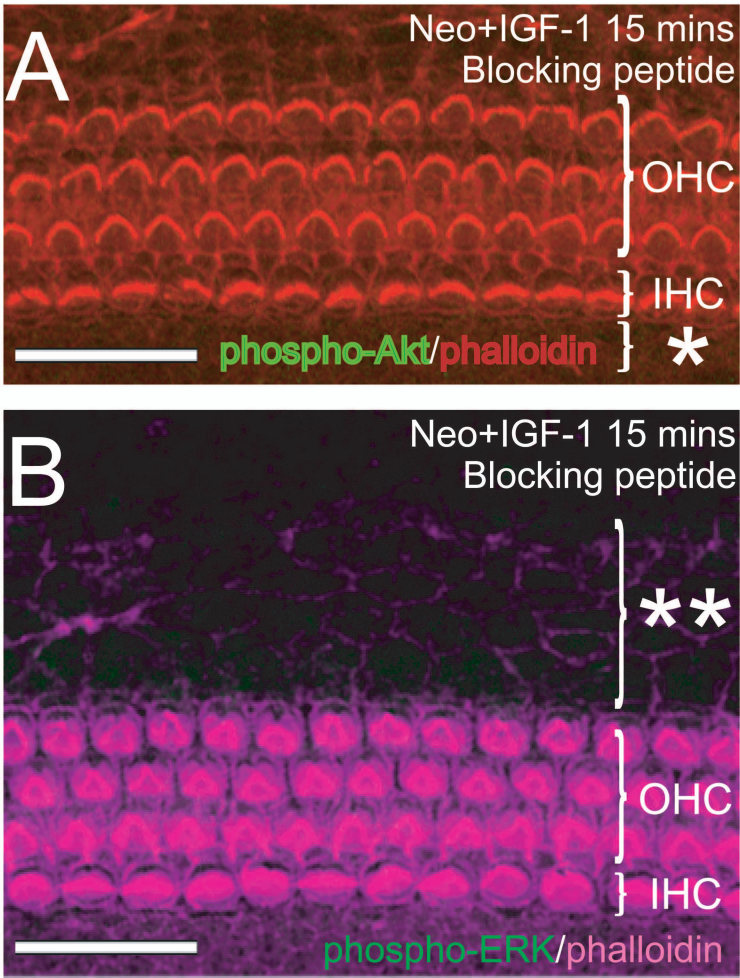

A
lateral
Neo+IGF-1+aphidicolin
OHC
IHC
medial
myo7a/phalloidin


C
Neo+IGF-1+mimosine
OHC
IHC
myo7a/phalloidin

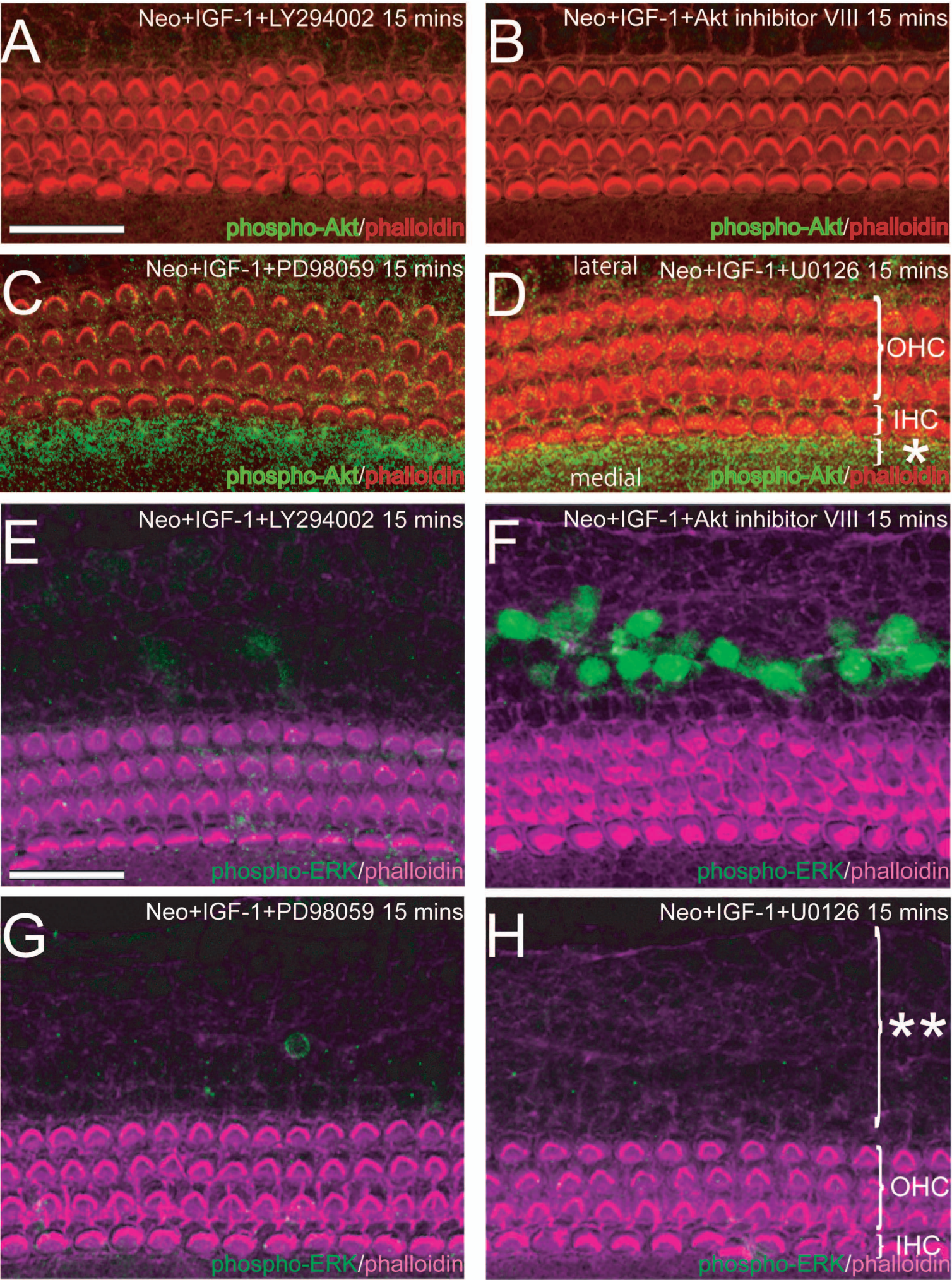
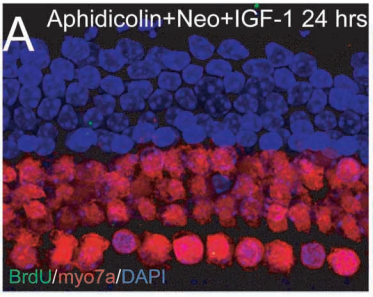
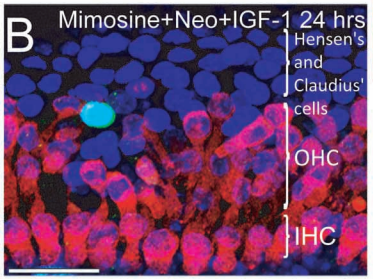
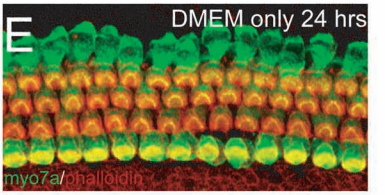
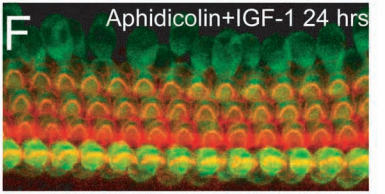
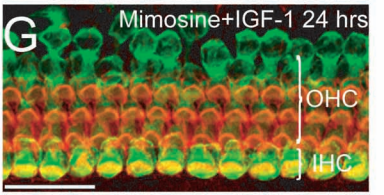
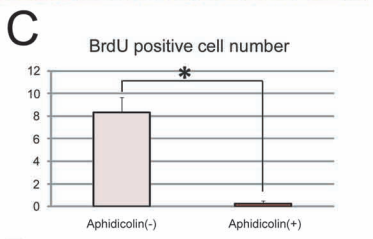
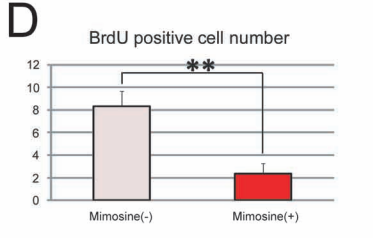
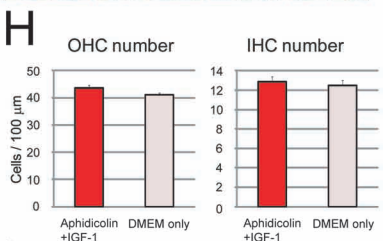
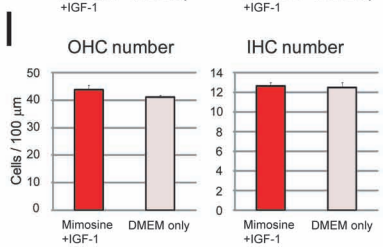

B
OHC number
IHC number
*
Cells / 100 µm
50
40
30
20
10
0
14
12
10
8
6
4
2
0
aphidicolin (-)
aphidicolin (+)
aphidicolin (-)
aphidicolin (+)


D
OHC number
IHC number
**
Cells / 100 µm
50
40
30
20
10
0
14
12
10
8
6
4
2
0
mimosine (-)
mimosine (+)
mimosine (-)
mimosine (+)

Supplementary figure 1



Supplementary figure 2



Supplementary figure 3