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TITLE PAGE

Inhibition of the JNK-mediated mitochondrial cell death pathway restores auditory function in sound exposed animals.

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Running Title. D-JNKI-1 rescues the cochlea after sound trauma

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ABBREVIATIONS: JNK/SAPK, c-Jun N-terminal Kinase/stress-activated protein kinase; MAPK, mitogen-activated protein kinase; CAP, compound action potential ; SEM, scanning electron microscopy ; TEM, transmission electron microscopy; IHCs, inner hair cells ; OHCs, outer hair cells; TTS, temporary threshold shift; PTS, permanent threshold shift; HA, hyaluronic acid; RWM: round window membrane.

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ABSTRACT

The therapeutic value of round window membrane delivered (RWM) D-JNKI-1 peptide (Bonny et al., 2001) against sound trauma-induced hearing loss is tested and characterized. Morphological characteristics of sound-damaged hair cell nuclei labeled by Hoechst staining show that apoptosis is the predominant mode of cell death following sound trauma. Analysis of the events occurring after sound trauma demonstrates that JNK/SAPK activates a mitochondrial cell death pathway (i.e. activation of Bax, release of cytochrome c, activation of procaspases and cleavage of fodrin). FITC-conjugated D-JNKI-1 peptide applied onto an intact cochlear RWM diffuses through this membrane and penetrates cochlear tissues with the exception of the stria vascularis. A time-sequence of fluorescence measurements demonstrate that FITC labeled-D-JNKI-1 remains in cochlear tissues for as long as 3 weeks. In addition to blocking JNK-mediated activation of a mitochondrial cell death pathway, RWM delivered D-JNKI-1 prevents hair cell death and development of a permanent shift in hearing threshold that is caused by sound trauma in a dose-dependent manner (EC_{50} = 2.05 μM). The therapeutic window for protection of the cochlea from sound trauma with RWM delivery of D-JNKI-1 extended out to 12 hours post- sound exposure. These results show that the MAPK/JNK signaling pathway plays a crucial role in sound trauma-initiated hair cell death. Blocking this signaling pathway with RWM delivery of D-JNKI-1 may have significant therapeutic value as a therapeutic intervention to protect the human cochlea from the effects of sound trauma.

The c-Jun N-terminal Kinase/stress-activated protein kinase (JNK/SAPK), a member of the mitogen-activated protein kinase (MAPK) family, can be activated by a variety of environmental stresses, such as UV irradiation or exposure to toxins (Kyriakis et al., 1994; Derijard et al., 1994). JNKs can phosphorylate a variety of cytoplasmic and nuclear proteins, e.g. c-Jun (Hibi et al., 1993, Mielke and Herdegen, 2000), activating transcription factor 2 (Gupta et al., 1995) and ETS-containing factors (Whitmarsh et al., 1995). In the cochlea, JNK can be activated by various forms of insults such as loss of trophic factor support (Scarpidis et al., 2003), drug ototoxicity (Ylikoski et al., 2002, Wang et al., 2003) and sound exposure (Pirvola et al., 2000; Wang et al., 2003). Blocking the MAPK/JNK cell death signal pathway with antisense oligonucleotides that target downstream target of phosphorylated JNK (i.e. c-Jun) can prevent the death of auditory neurons *in vitro* (Scarpidis et al., 2003). The peptide inhibitor CEP-1347 (derived from indolecarbazole, K252a), which blocks the MAPK/JNK cascade at the level of mixed lineage kinases (Maroney, et al., 2001) can prevent apoptosis of hair cells in organ of Corti explants exposed to a ototoxic level of neomycin, and protect auditory neurons from stress-initiated death in dissociated ganglion cell cultures following loss of trophic factor support (Pirvola et al., 2000). Systemic administration of CEP-1347 provides partial protection against hearing loss induced by sound trauma (Pirvola et al., 2000). Consistent with these observations, treatment of neomycin-exposed organ of Corti explants with D-JNKI-1, a chemically synthesized cell permeable JNK-ligand that blocks JNK-activation of c-Jun (Borsello and Bonny, 2004), prevents phosphorylation of c-Jun and protects the sensory hair cells (Wang et al., 2003). Perilymphatic perfusion of D-JNKI-1 *in vivo* resulted in almost complete protection of the cochlea from neomycin-induced hair cell and hearing loss (Wang et al., 2003). In another study, the MAPK/JNK signaling pathway was activated in response to cisplatin toxicity, but in this instance perilymphatic infusion of D-JNKI-1 did not protect against cisplatin ototoxicity, rather, it potentiated cisplatin's

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ototoxicity (Wang et al., 2004). In contrast, protection of both auditory hair cells and auditory function was observed with the D-JNKI-1 peptide in sound trauma-exposed animals (Wang et al., 2003). The specific downstream targets of MAPK/JNK signal-mediated cochlear hair cell death following sound trauma are at present unknown. Another problem is that to date there have been no studies which examine the efficacy of D-JNKI-1 treatment using a protocol that would be compatible with clinical applications: e.g. delivery onto the RWM of the cochlea and not infusion into the scala tympani via a cochleostomy. Finally, no data have been acquired concerning D-JNKI-1 treatment after the initial sound trauma exposure, which is can be important information for clinicians in their search for a treatment to rescue hearing after sound exposure has occurred.

The present study was designed to identify the downstream targets of MAPK/JNK signal-mediated hair cell death, and the therapeutic value of protection of hearing function when D-JNKI-1 is applied onto the RWM not only before, but also after exposure to a sound trauma.

MATERIALS AND METHODS

Pigmented guinea pigs (250 to 300g, Charles River, L'Arbresle, France) were used in this study. The care and use of animals followed the animal welfare guidelines of the « Institut National de la Santé et de la Recherche Medicale » (INSERM), under the approval of the French « Ministère de l'Agriculture et de la Forêt ». All efforts were made to minimize the number of animals used and any suffering caused by the experimental protocols. Table I summarizes the experimental protocol and details the number of animals used for each protocol.

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Drug preparation

Artificial perilymph solution (AP) consisted of the following: 137 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$; 1 mM $MgCl_2$; 1 mM $NaHCO_3$; 11 mM glucose; pH 7.4; osmolarity (304 + 4.3 mOsm/kg). 20 mM stock solution of D-JNKI-1 (Auris Medical AG, Lohn-Ammannsegg, under license from Xigen S.A., Lausanne, both Switzerland), 1 mM stock solution of the D form of TAT peptide (i.e. "TAT-empty") and the inactive mutant forms of JNKI-1 (i.e. JNKI-1-mut) and the FITC-conjugated D-JNKI-1 (Xigen S.A., Lausanne, Switzerland) were prepared in 0.1 M PBS at pH 7.2.

Before each experiment, the D-JNKI-1 was diluted in AP to a final concentration of either 0.01, 0.1, 1, 3.3, 10, 33 or 100 µM. JNKI-1-mut, TAT-empty and the FITC-conjugated D-JNKI-1 were diluted in AP to a final concentration of 100 µM.

Round window membrane drug delivery

30 minutes RWM delivery

To ensure that delivery onto the RWM allowed D-JNKI-1 to reach the sensory epithelium, we gently (3µl/min) infused the RWM niche with 100 µM of FITC-conjugated D-JNKI-1 over the course of 30 minutes using a glass pipette. The contralateral cochlea served as a control.

7 day RWM delivery via an osmotic minipump

Prior to surgery, the Alzet 2001 minipump (DURECT Corp., Cupertino, CA) was filled with 200 µl of AP containing either 0.01 to 100 µM of D-JNKI-1, or 100 µM of TAT-empty or 100 µM of JNKI-1-mut under sterile conditions. A glass micropipette was connected to the PE50 polyethylene tubing that connected to the osmotic pump. The pipette

and the tubing were then filled and connected to the flow moderator on the pump. The minipump flow rate was 1µL/h.

Any protective effect of D-JNKI-1 against sound trauma was tested by western blots, immunocytochemistry, scanning electron microscopy and functional evaluation. In all cases, D-JNKI-1 was applied onto the RWM via an osmotic minipump for 7 days, and each animal was implanted with a minipump 2 days prior to sound exposure. Furthermore, JNKI-1-mut and TAT-empty were used as negative controls to demonstrate the specificity of the peptide inhibitor.

We also evaluated the ability of D-JNKI-1 to rescue cochlear function from sound-induced hearing, when applied after the initial exposure to the sound trauma. Here, the 7 day minipump containing D-JNKI-1 was implanted 30 min before, or 30 min, 1h, 4 h, 6 h, 12 h or 24 h after the initial exposure to the sound trauma.

RWM delivery of D-JNKI-1 via a Hyaluronic Acid gel

Additional groups of animals were used to test the efficiency of D-JNKI-1 when applied after sound trauma using a hyaluronic acid (HA, molecular weight: 2.4 million, Genzyme Advanced Biomaterials, MA USA) gel. A final concentration of 100 µM of D-JNKI-1 was freshly prepared in a 2.6 % of HA gel before each experiment. The HA gel containing D-JNKI-1 was placed directly onto the RWM.

Surgery

Experiments were designed to record the compound action potentials (CAPs) in awake animals, from a connector plug fixed on the head of the animal during minipump or HA gel

implantation. This method has been extensively described elsewhere (Wang et al., 2002). Briefly, animals were anaesthetized with an intraperitoneal injection of 6% sodium pentobarbital (Sanofi, Montpellier, France; dose-0.3 ml/kg). Each bulla was opened under sterile conditions. The recording electrode was placed on the bony edge of the RWM leaving enough space to appose the infusion micro-catheter (tip diameter: 0.35 mm) or 2 µl of HA gel onto the RWM under microscopic control.

Functional assessment

Compound action potentials (CAPs) of the auditory nerve were elicited by tone bursts of alternating polarity (1 ms rise/fall, 8 ms duration) applied to the ear at a rate of 10/sec from 0 to 100 dB SPL in 5 dB steps in a free field via a JBL 075 earphone. Cochlear responses were amplified (gain 2000), averaged (128 samples) and stored on a Pentium PC computer operating at 100 megahertz (Dell Dimension, USA). CAP recordings were measured peak-to-peak, between the negative depression N1 and the subsequent positive wave P1. The threshold of the CAP was defined as the intensity in dB SPL needed to elicit a measurable response ($\geq$ 5 µV).

Sound trauma

The animals were then exposed to sound trauma (130 dB SPL for 15 min) under light anesthesia (Pentobarbital, dose-0.2 ml/kg). Sound trauma was induced by a continuous 6 kHz pure tone generated by a waveform synthesizer (Hewlett-Packard 8904A). Which was routed through a programmable attenuator and presented to the ears in free field via a JBL 075 earphone positioned 10 cm in front of the animal's head. The sound level was measured using a calibrated ½" Bruel and Kjaer microphone (model 4314) and a Bruel and Kjaer calibrating amplifier (model 2606).

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Morphological assessment

Confocal microscopy

Animals were killed with an overdose of pentobarbital. The cochleae were then rapidly removed and perfused with 4% paraformaldehyde in 0.1 M PBS, pH 7.3, post fixed for 2 hours in the same fixative at room temperature (RT) and rinsed in PBS. The surface of the organ of Corti was exposed by removing the otic capsule, stria vascularis, tectorial and Reissner's membranes. For all experiments, 10 µm thicknesses of cochlea cryostat sections were prepared after micro dissection, except for FITC fluorescence intensity evaluation of the cochleae infused with 100 µM of FITC-conjugated D-JNKI-1, in which the cryostat sections were performed after decalcification of the cochleae with 10 % of EDTA in PBS for 2 days at 4°C. All the observations were performed on a Leica DMRB microscope (blocks of filters N2.1 and A4).

Sensory hair cells and nerve endings: The assessment of hair cell and nerve ending integrity was performed on surface preparations from non-treated cochleae prior to and 1, 6, 12, 24, 48h, 5 days and 15 days after sound trauma. The immunostaining was performed with two primary antibodies, i.e. a rabbit polyclonal anti-calbindin-D-28K antibody (1:200 dilution; Sigma, France) to label the sensory hair cells and a mouse monoclonal antibody to NF 200 KDa (Clone: JG1; 1:500; Affinity BioReagents, CO, USA) to label the auditory nerve endings. Secondary antibodies were an Alexa 568 labeled goat anti-rabbit antibody together with an Alexa 488 labeled goat anti-mouse antibody (1:500; Molecular probes, OR, USA).

Nuclear morphology: Nuclear morphology of the cochlear cells were studied on surface preparations by staining the chromatin of the nuclei with Hoechst 33342 (0.002% wt:vol, Sigma Chemicals, St. Louis, MO, USA) at RT for 10 minutes.

Cytochrome c and Bax: Cytochrome c was detected in cryostat sections with a mouse anti-cytochrome c monoclonal antibody (1:200 dilution; Pharmingen International, San Jose, CA, USA). The secondary antibody was an Alexa 488 labeled goat anti-mouse antibody (1:500; Molecular probes, OR, USA). Bax was detected with rabbit polyclonal antibody to the Bax (1:750 Pharmingen, International, San Jose, CA, USA) and an Alexa 568 labeled secondary antibody (1:1000 dilution; goat anti-rabbit IgG antibody, Molecular Probes, OR). The sections were then counterstained with Hoechst 33342 (0.002% wt:vol, Sigma Chemicals, St. Louis, MO, USA) at RT for 10 min for staining DNA. All rinses were performed with PBS.

Cleaved fodrin by caspases: Cleavage of fodrin was detected in cryostat sections-with two primary antibodies: a monoclonal antibody against calbindin (1:600 dilution; Sigma, France) to identify hair cells, and a rabbit polyclonal antibody against cleaved α-Fodrin (Asp1185). This latter antibody recognizes only fodrin fragments that have been cleaved by caspases (1:400, Cell Signaling, San Diego, CA, USA). Secondary antibodies were an Alexa 488 labeled goat anti-rabbit antibody together with an Alexa 568 labeled goat anti-mouse antibody (1:500; Molecular probes, OR, USA). The sections were then counterstained with Hoechst 33342.

Diffusion and penetration of FITC-conjugated D-JNKI-1 peptides: The assessment of FITC-conjugated D-JNKI-1 peptides uptake by the sensory epithelium of the cochlea was

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performed on cryostat sections. Images of FITC fluorescence were captured using a Leica DMRB microscope, and the average pixel intensity in individual organ of Corti of all turns was measured using Image J1.34 software (National Institute of Health, USA). FITC fluorescence levels were measured using the histogram function in a 20 x 10 pixels box focused on organs of Corti of all turns. Measurements were repeated 9 times for each organ of Corti analyzed.

Scanning electron microscopy

Cochleae were fixed with a solution of 0.1 M phosphate buffer (pH = 7.4) containing 1% osmic acid, and immersed in the same fixative for one hour, washed in phosphate buffer (0.2 M) and subsequently dehydrated in a graded series of ethanol (30 to 100%). During the 70% ethanol stage, the bony capsules of each cochlea were dissected and the stria vascularis and Reissner's membranes were removed to expose the organ of Corti. Cochleae were then critical-point dried in CO_2, coated with gold-palladium, and observed with a Hitachi S4000 scanning electron microscope (SEM).

Quantification of SEM analysis was performed by counting missing hair cells from the apex to the base of each cochlea. The results were expressed as a percentage of remaining hair cells in the single row of inner hair cells (IHCs) and of the three rows of outer hair cells (OHCs) over the entire length of the cochlear duct. In control specimens, the guinea pig cochlea contains approximately 2000 IHCs and 7500 OHCs. For hair cell counts, the whole cochlea was divided into 20 segments (1 mm length of basilar membrane for each segment); each segment would be expected to contain about 100 IHCs and 375 OHCs. A hair cell was counted as absent when its cuticular plate was missing and it was replaced with a phalangeal scar.

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Transmission Electron Microscopy

Animals were decapitated during deep anesthesia, and their cochleae were prepared using our standard protocol for fixation and plastic embedding (Puel et al., 1994). Semithin and ultrathin radial sections of the plastic embedded organ of Corti were cut from the basal and middle turns and observed using a Hitachi 7100 electron microscope (Hitachi, Tokyo, Japan) at Centre de Ressources d'Imagerie Cellulaire de Montpellier.

Western Blotting

Tissue proteins were homogenized in Laemmli sample buffer separated on 10% SDS-PAGE and transferred to nylon membranes. Blots were incubated overnight at 4°C with rabbit polyclonal antibody to c-Jun (1:1000; Upstate biotechnology, NY) or rabbit polyclonal antibody to phosphoSer73-Jun (1:1000; Upstate biotechnology, NY) or rabbit polyclonal antibody to the N-terminal of Bax (1:750; Upstate Biotechnology, NY) that recognizes only the activated form of Bax, or a monoclonal antibody to 240/280 kDa α-fodrin (all spectin) (1:1000, Affiniti Research products, Devon, UK), or mouse monoclonal antibody to β-actin (1:500, Sigma Aldrich, St Louis, MO, USA) followed by incubation with alkaline phosphatase-conjugated secondary antibody (Sigma Aldrich, St Louis, MO, USA). Protein-antibody complexes were revealed with the BCIP/NBT-Purple Liquid Substrate (Sigma Aldrich, St Louis, MO, USA). Image scans of western blots was used to quantify phosphorylation levels of c-Jun and expression levels of c-Jun, Bax or α-fodrin.

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Statistics

Statistical analyses were performed using Sigma Plot 2000 for Windows (version 6.1). All comparisons between means were performed using Student's paired two-tail t tests or a nonparametric Wilcoxon rank test. Results are expressed as means ± SEM.

RESULTS

Sound induced hearing loss and hair cell death

Functional assessment

In contralateral (left) sound-exposed, untreated cochleae, the average hearing loss measured 20 min after sound trauma reached 65 dB in the higher frequencies of the CAP audiogram, with the maximum temporary hearing loss achieved between the frequencies of 12-16 kHz. During the first 2 days following sound exposure, there was a partial recovery in CAP thresholds, leading to a permanent hearing loss of 30 to 40 dB. No further improvement in hearing threshold levels was seen over the remaining 13 days of the experiment (Fig. 1A). These results are consistent with a previous study which used a similar paradigm of sound trauma (Dancer et al., 2001).

Morphology assessment

Confocal microscopic observations of the surface preparations of the organ of Corti were performed in sound-exposed and non-exposed cochleae from additional animals.

Sensory hair cell loss: Sensory hair cells were considered as missing when both calbindin and DNA staining were absent. One hour after sound exposure, a few of the first

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row OHCs were missing in the area of maximum sound-damaged located 14 to 16 mm from the cochlear apex (Fig. 1B and D). This area corresponds to the region of hair cells that encode for 8 to 14 kHz (see Cody et al., 1980 for the frequency map in the guinea pig cochlea). Six to twelve hours after sound exposure, some OHCs of all three rows were missing. In contrast, most of the IHCs, the auditory nerve fibers below the IHCs, and the nerve fibers crossing the tunnel to reach the OHCs were still visible. Two days after sound exposure, in the most affected area of the cochlea, most of the OHCs were lost and the remaining IHCs extensively damaged (Fig. 2D). By this stage of hair cell degeneration, most of the auditory nerve fibers had disappeared (Fig. 2D). At 5 days post sound trauma, almost all of the hair cells (both IHCs and OHCs) were missing in the sound damaged area, and no further hair cell loss was seen over the next 10 days of the experiment (i.e. day 15, Fig. 1D).

Apoptotic and necrotic nuclei: In order to study the nature of the cell death process, we used Hoechst 33342 to stain the cell nuclei. Cell nuclei from non-sound-exposed control cochleae appeared as bright spots with an intense blue color under fluorescent microscopy, indicating uniform staining of their DNA (Fig.1C_1). One hour after sound exposure, karyopyknosis changes (characteristic of apoptosis) were observed in the nuclei of some OHCs and IHCs present in the sound-damaged area (Fig. 1B and $C_{2,\ 3}$). Other nuclei showed characteristic changes of apoptosis such as a shrunken appearance and formation of micronuclei with a greatly increased intensity of Hoechst 33342 fluorescent staining (Fig. 1C_2 and C_3). Such changes were observed for up to 48 hours post-trauma. At this stage of hair cell degeneration, there were few but increasing signs of necrosis in the observed patterns of nuclear staining, e.g. enlarged nuclei, in the area near the maximally damaged region of the organ of Corti (13 mm from apex, Fig. 1C_4 and 2G).

The time course of hair cell death was determined by counting hair cells in 2 mm length portions around the sound-damaged area (14-16 mm from the apex) in the cochlea at 1, 6, 12, 24, 48 hours, 5 days and 15 days after sound trauma. In the control non-sound-exposed cochleae, a 1 mm length portion located 15 mm from the apex contains approximately 100 IHCs and 375 OHCs (this hair cell count is referred to as 100% hair cells present) (Fig. 2A). According to Kerr et al. (1972), cells with fragmented and condensed nuclei were counted as apoptotic cells and those with large and swollen nuclei as necrotic cells. Statistical analysis revealed a significant increase ($p < 0.001$) in apoptotic cell nuclei by 1 hour post sound exposure in the area of maximum damage (Fig. 1D). This increase in apoptotic hair cells reached a peak (i.e. 60% of hair cells in the damaged area) at 6 hours post-trauma (Fig. 1D). Two days after sound exposure, the majority of hair cells were missing in the area of maximum damage (Fig. 1D). In the regions directly adjacent to this traumatized zone, there was a slight increase in swollen and enlarged nuclei (i.e. less than 8% of the damaged cells, Fig. 2G), attesting to the occurrence of a necrotic process in these hair cells.

Ultrastructural analysis: In order to confirm the occurrence of apoptotic and necrotic processes within the hair cells, ultrastructural analysis was performed two days after sound exposure. When compared with hair cells from non-exposed control organ of Corti (Fig. 2B and C), the hair cells from sound-damaged organ of Corti showed features consistent with apoptosis such as distorted or shrunken cell bodies with electron-dense cytoplasm, fragmentation and condensation of the nuclear chromatin (Fig. 2E and F). There were also some hair cells with obvious signs of necrosis such as cell bodies with vacuolated cytoplasm and dispersed cellular debris, and swollen and enlarged nuclei (Fig. 2H and I). Interestingly, some individual hair cells demonstrated signs typical of both apoptosis (fragmented nucleus and chromatin compaction) and necrosis (cellular debris, disintegrated cytoplasmic

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membrane, Fig. 2F). Note the undamaged medial efferent endings after sound-induced injury in the OHC, the only changes being an increased synaptic vesicle density (Fig. 2I).

Mechanisms of sensory hair cell death

Phosphorylation of c-Jun by JNK and activation of Bax

Phosphorylation of c-Jun (serine 73) by JNK and activation of Bax were studied by Western Blots from control non-sound-exposed cochleae, and sound-exposed cochleae at 30 minutes, 3h, 6h, 12h, 48h and 5 days post sound exposure. When compared with non-sound-exposed control cochleae, sound trauma induced a phosphorylation of c-Jun (p-Jun) and a strong activation of Bax. Both events were detected between 30 minutes and 2 days post-trauma, with an apparent peak at 12 h (Fig. 3A, B and C), whereas the total levels of c-Jun protein or actin expression did not show any change (Fig. 3A, B and C). Application of a 10 or 100 µM concentration of D-JNKI-1, but not 100 µM of JNK-I-mut onto the RWM via an osmotic minipump prevented the phosphorylation of c-Jun and the activation of Bax observed 3 h and 12 h post sound-exposure (Fig. 3A, B and C)

Translocation of Bax and release of cytochrome c

Location of cytochrome c was determined by immunostaining cryostat sections. In non-sound-exposed cochleae, immunoreactivity to the anti-cytochrome c antibody localized to the hair cell cytoplasm, in a punctate pattern consistent with its mitochondrial localization (Fig. 4A). One hour after sound exposure, immunostaining for cytochrome c hair cells was diffuse and distributed uniformly throughout the cell (Fig. 4A). When compared with the pattern of cytochrome staining of non-sound-exposed control cochleae, the intensity of the staining was very weak, with sensory hair cells and supporting cells showing staining barely

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above background level. In contrast, the medial efferents connected to the OHCs still remained strongly labeled, attesting to the specificity of the diffuse cytochrome c labeling. This alteration in the pattern of cytochrome c staining of the sensory hair cells and supporting cells suggests that cytochrome c has been released from the mitochondria into the cytoplasm of damaged cells.

We also examined the activation and subcellular localization of Bax by using an antibody directed to Bax. Organ of Corti from non-sound-exposed control cochleae exhibited a diffuse pattern of weak immunostaining, indicating a cytoplasmic localization of Bax (Fig. 4B). Three hours post-trauma, the pattern of Bax immunostaining in the acoustically damaged cochlear cells changed to a dense, punctate pattern consistent with the activation of Bax in the affected sensory cells (Fig. 4B). At the same time, immunostaining for cytochrome c was no longer detectable in the sound-damaged cochlear cells. Together, these results suggest that activation of Bax contributed to the release of mitochondrial cytochrome c into the cytoplasm. The disappearance of cytochrome c staining in the sound-damaged cochlear cells by 3 hours post-trauma further indicates that, when released into the cytosol, cytochrome c is rapidly degraded (Fig. 4B). In addition, 100 µM D-JNKI-1 applied onto the RWM using an osmotic minipump implanted 2 days before sound exposure prevented both sound-induced activation of Bax and release of cytochrome C from the mitochondria to the cytoplasm (Fig. 4A and B).

Cleavage of fodrin

Expression of fodrin: The expression of fodrin (all spectin) was measured by Western Blots from control non-sound-exposed cochleae, and sound-exposed cochleae at 30 minutes, 3h, 6h, 12h 48h and 5 days post sound exposure. A strong decrease in fodrin expression levels

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was detected between 30 minutes and 5 days, with a maximum level of decrease reached at 3 h post-exposure (Fig. 3A and D). This could be prevented by a RWM of 10 or 100µM D-JNKI-1, but not 100 µM JNKI-1-mut in AP (Fig. 3A and D).

Location of fodrin cleaved by activated caspases: Fodrin is a major component of the cuticular plates of hair cells and a known substrate for effector caspases, e.g. caspase-3. Therefore we looked for the presence of cleaved fodrin in sound-exposed cochleae by using an antibody that is specific for the 150 kDa N-terminal large fragment of caspase-cleaved fodrin. In our experiments, no cleaved fodrin was detected in the cochlear cells from non-sound-exposed animals (Fig. 4C). Between 6 h and 2 days post sound exposure, there was a marked immunostaining of cleaved fodrin in the region of the cuticular plates, the cytoplasm of hair cells and in the pillar cells located in the main area of sound-damage (Fig. 4C). Other supporting cells from the organ of Corti, cells that compose the stria vascularis, spiral ligament and spiral ganglion cells did not stain for the presence of cleaved fodrin. Here again, 100 µM D-JNKI-1 applied on the RWM via an osmotic minipump prevented the occurrence of caspase-cleaved fodrin in sound trauma-exposed cochleae (Fig. 4C).

Diffusion and penetration of FITC-conjugated D-JNKI-1 peptides

FITC-conjugated D-JNKI-1 peptides were used to visualize the distribution of this inhibitory peptide, to ascertain whether this compound diffused through the RWM and reached cochlear tissues. At six hours and at three days after a 30 min delivery of 100 µM FITC-labeled peptide onto the RWM, FITC-labeled D-JNKI-1 was found to be distributed throughout the scala tympani, reaching the apical region of the cochlea. Distribution of this fluorescent peptide also extended to the scala vestibuli (Fig. 5A). The fluorescent signal

showed a base to apex gradient pattern of distribution (Fig. 5A and G) which is consistent with the FITC-labeled peptides being delivered at the base of the cochlea, where the RWM is located (Fig. 5A). All the cochlear cells, including the auditory hair cells and neurons, exhibited fluorescence, indicating that D-JNKI-1 applied onto RWM effectively reached these targeted sensory cells (Fig. 5A). The spiral ligament clearly labeled for the presence of FITC-labeled D-JNKI-1, whereas the stria vascularis did not (Fig. 5A to E).

Time-dependent fluorescence measurements revealed that the intensity of the FITC signal remained stable in all of the cochlear structures throughout the cochlear duct 6 hours, 3 days and 7 days after RWM delivery (Fig.5B to C, and H). A slight but not significant decrease in fluorescence occurred (i.e. 17%, Fig. 5D and H) in all the cochlear turns at 14 days and there was a limited amount of fluorescence in the tissue of the cochlear basal turn at 21 days after the initial delivery (Fig. 5E and H). Fluorescence from FITC-labeled D-JNKI-1 peptide was no longer observed in any of the cochlear turns at 28 days post-administration (Fig.5H).

Efficiency of RWM delivery of D-JNKI-1 peptide

Control experiments

Control experiments were designed to demonstrate the lack of non-specific effects due to surgery, minipump implantation, or the introduction into the cochlea of TAT vector or a peptide. In our studies, the implantation of a minipump containing D-JNKI-1, TAT-empty or JNKI-1-mut induced a slight high frequency hearing loss (5-10 dB) on the first day that had completely recovered within two days after implantation (not shown). To evaluate the functional consequences of drug delivery, treated right and untreated control left ears were exposed to sound trauma (6 kHz, 130 dB SPL, 15 min) via an earphone positioned 10 cm in

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front of the animal's head. Hearing losses were not significantly different between sound-exposed cochleae treated with TAT-empty or JNKI-1-mut, and the untreated control left ears (Fig. 6A). Consequently, the protective effects of D-JNKI-1 were therefore compared with the untreated-control left ear in the same animals.

Protective effects against sound trauma

Functional evaluation: We investigated the ability of D-JNKI-1 to protect hair cells against sound exposure when concentrations of increasing dosage levels of D-JNKI-1 (0.01 to 100 μM, 6 animals per doses) were applied onto the round window membrane. Sound-exposed, untreated left cochleae served as controls for the effectiveness of sound-induced hair cell and hearing loss. In these cochleae, the impairment reached a maximum of 35 to 40 dB in the higher frequencies (8-16 kHz) of the CAP audiogram (Fig. 6A) when measured 15 days after sound exposure. This represents a permanent hearing loss i.e. permanent threshold shift (PTS). When the round window membranes of the sound-trauma-exposed cochleae were infused with 100 μM of D-JNKI-1 in AP, there was still an immediate and significant elevation in hearing threshold (about 65 dB in the 8 to 16 kHz range) due to the initial effect of the sound trauma. However, this was followed by a clear improvement and almost complete recovery of the CAP thresholds when they were measured at 15 days after the initial sound exposure ($p<0.05$ between 4-12 kHz, Fig. 6A) – i.e. this was only a temporary threshold shift (TTS).

Statistical analysis was performed on the data obtained at 8 kHz at 15 days after sound trauma, since this was the frequency at which the maximal PTS was seen in the sound-exposed untreated cochleae. Significant improvement was seen in CAP thresholds for all concentrations of D-JNKI-1 above 1 μM (Fig. 6E), and there was a complete recovery to pre-

exposure hearing thresholds in response to treatment with 33 µM D-JNKI-1 (Fig. 6E). The effective concentrations of D-JNKI-1 required to prevent 50 % of the permanent threshold shift (EC_{50}) caused by exposure to the sound trauma was 2.05 µM for round window delivery (Fig. 6E).

Morphological evaluation: SEM observation of the surface morphology of each organ of Corti was performed at the end of the physiological assessment of hearing thresholds, i.e. 15 days after sound trauma. Counting all of the hair cells present over the entire length of the cochlear duct allowed us to construct cochleograms for the sound-exposed, contralateral untreated cochleae (Fig. 6B), and sound-exposed cochleae treated with 100 µM D-JNKI-1 in artificial perilymph (Fig. 6C). In the contralateral cochleae more than 80% of the hair cells were missing from the area representing the site of maximal damage, 14 to 16 mm from the apex (Fig. 6D). Application of 100 µM D-JNKI-1 resulted in protection of the hair cells from the effects of the sound trauma – there was no PTS in this group. Less than 15% of the hair cells were missing from the area of maximum sound-induced damage (Fig. 6D), which is consistent with the physiological data showing the development of almost no permanent loss of hearing.

Therapeutic window for effective D-JNKI-1treatment

Having demonstrated the efficacy of D-JNKI-1 as an otoprotective treatment applied during the sound trauma, we evaluated its ability to rescue cochlear function from a PTS, when applied after the initial exposure to the sound trauma. D-JNKI-1 was applied at a concentration of 100 µM in artificial perilymph onto an intact RWM using an osmotic minipump over 7 days. When compared with the 100% protective effect of D-JNKI-1 applied 30 min prior to sound trauma, delaying the onset of the treatment until 30 min after the trauma

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led to an 84% protection of hearing threshold (Fig. 6F). This protective effect against sound trauma-induced hearing loss continuously diminished as the time interval between the initial exposure to the damaging level of sound and the onset of treatment increased (Fig. 6F). Calculation of the percentage of protection shows 80%, 58%, 41%, 17% and 6% protection of hearing respectively with D-JNKI-1 RWM osmotic minipump therapy started 1, 4, 6, 12 and 24 hours post-exposure.

We also tested the ability of D-JNKI-1 to rescue the cochlea after sound exposure when it was administered onto the RWM in a HA gel. D-JNKI-1 delivered via this route enhanced functional recovery in a time-dependent manner (Fig. 6F). Furthermore, there was no significant difference in the protective effect on hearing between the D-JNKI-1 delivered via osmotic minipump or via a HA gel to the RWM (Fig. 6F).

DISCUSSION

Identification of sensory hair cell death

Several studies have implicated apoptosis in the death of hair cells following sound trauma (Hu et al., 2000; Pirvola, 2000; Wang et al., 2002; Cheng et al., 2005). However, the time course and proportion of apoptosis versus necrosis is only starting to be defined (Yang et al., 2004; Hu et al., 2006). Morphological changes in cell nuclei are the primary criteria for the differentiation of apoptosis and necrosis (Kerr et al., 1972). Condensed and fragmented nuclear DNA characterizes apoptosis, whereas swollen nuclei are characteristic of necrosis. Based on these criteria, we report a significant increase in apoptotic cell nuclei one hour after sound trauma, which concurs with the observations in several recent publications studying the biological effects of sound and impulse sound trauma (Hu et al., 2006; Yang et al., 2004). After two days, the number of apoptotic cells dramatically decreased, probably due to rapid

degeneration of cells in the damaged area, whilst necrotic nuclei reached a maximum of 8% of the total damaged cells. Ultrastructural investigations revealed typical features of apoptosis and of autolysis. Signs of necrosis were also occasionally seen in the traumatized area of the cochlea. Interestingly, some individual hair cells shared both necrotic and apoptotic features, which may be explained by the occurrence of a secondary necrotic process due to a metabolism deficiency resulting in an inability to maintain the apoptotic process in the damaged cells (Leist and Jaattela, 2001). Although probably several mechanisms of cell death occurred (see Leist and Jaattela, 2001; Lefebvre et al., 2002; Hetz et al., 2005; Krantic et al., 2005), our results suggest that apoptosis is the predominant mode of hair cell death following sound trauma. This mode of cell death may be linked to a defense mechanism which preserves the integrity of the cellular barriers, which avoid potassium-rich endolymph entering the sensory epithelia (Li et al., 1995). Finally, these degenerative mechanisms might have different time courses, which would explain the extended therapeutic window seen where D-JNKI-1 can be administered up to 12 hours post trauma and still protect against sound trauma-induced hearing loss.

JNK-mediated mitochondrial cell death pathway

The c-Jun NH2-terminal kinases represent one subgroup of mitogen-activated protein kinases that are primarily activated by cytokines and by exposure to environmental stress (Kyriakis et al., 1994; Dérijard et al., 1994). There is increasing evidence that a major role for this JNK subgroup of kinases is as mediators of apoptosis (Ip and Davis, 1998; Mielke and Herdegen, 2000). A major target of the JNK signaling pathway is the activator protein-1 transcription factor, activation of which is mediated in part by phosphorylation of c-Jun and related molecules.

In this study, sound trauma induced a significant increase in phosphorylation of the nuclear transcription factor c-Jun, the activation and translocation of Bax into the mitochondria, the release of cytochrome c from the mitochondria into the cytoplasm of a damaged cell, and the cleavage of fodrin by activated effector caspases. Interestingly, there was still a normal pattern of immunostaining for cytochrome-c for the medial efferent that connect to the OHCs, which concurs with previous ultrastructural studies reporting normal medial efferent appearance, even after severe sound-induced damage in the OHC (Spoendlin, 1970 and see Fig. 2I).

Blocking the JNK/SAPK-mediated mitochondrial cell death pathway with D-JNKI-1 prevented both hair cell death and the permanent loss of hearing induced by sound trauma. This supports previous results (Ghatan et al., 2000; Maundrell et al., 1997) which demonstrated that MAPK kinase regulates the activation and translocation of Bax to mitochondria. However, we cannot exclude that other pro-apoptotic proteins of the Bcl-2 family such as Bim may be implicated in the JNK-mediated cochlear cell death as shown in other systems (Putcha et al., 2003). We did not study the activation of Bim by JNKs in the stressed cochlea, and thus further experiments will be necessary to clarify this point.

The cleavage of fodrin by effector caspases is consistent with a previous study reporting that sound-induced apoptotic sensory hair cell death is associated with the activation of procaspase-3 (Hu et al., 2002a) and cleavage of F-actin in affected outer hair cells (Hu et al., 2002b). The results of this study suggest that JNK activates the intrinsic mitochondrial cell death pathway, and that this pathway is one of the major intracellular cascades by which hair cells respond to noise-induced damage.

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Efficacy of RWM delivery of D-JNKI-1

D-JNKI-1 inhibitory peptide contains a 10-amino-acid HIV-TAT transporter sequence to facilitate its entry into cells (Vives et al., 1997). FITC-conjugated D-JNKI-1 infused onto the RWM niche diffused rapidly through the RWM into the scala tympani. The fluorescent signal showed a gradient from base to apex in the scalas tympani, media and vestibuli. These results are consistent with those of Salt and Ma (2001), who used a trimethylphenylammonium-sensitive microelectrode to measure the concentration of this ion when applied onto the RWM. Most of the cellular subtypes within the organ of Corti, including the hair cells, exhibited fluorescence, indicating incorporation of FITC-labeled D-JNKI-1. The timed histological specimen study showed an initial cellular uptake of D-JNKI-1 as early as 30 min after its application onto the RWM and that this drug remained within cochlear cells for up to 3 weeks.

The fact that FITC-labeled D-JNKI-1 was still detectable in the cochlea at 3 weeks after only a single application offers interesting perspectives for a clinical application, since delivery of pharmacological agents onto the RWM in humans is already occurring in clinical practice (Seidman and Van De Water, 2003). Delivery of D-JNKI-1 onto the RWM preserved more than 80 % of the hair cells and prevented the occurrence of permanent deafness. Using RWM delivery, 2.05 μM of D-JNKI-1 was required to prevent 50 % of the permanent hearing loss caused by our sound trauma. Compared to intracochlear infusion, which has an EC_{50} of 2.31 μM (Wang et al., 2003), the RWM route for the delivery of D-JNKI-1 therapy is as effective as direct intracochlear infusion. This observation is of great clinical importance and highly relevant to the use of this drug in a clinical setting.

Most of the studies investigating JNK inhibitors have examined their effectiveness when treatment is commenced prior to sound trauma (Pirvola et al., 2000; Ylikoski et al.,

2002; Wang et al., 2003). Here, we report that D-JNKI-1 can not only prevent, but can also rescue the cochlea from the deleterious effects of sound trauma within a therapeutic window of 12 hours post-trauma. In addition to the clinical relevance of a 12 hour window of opportunity for the rescue of hearing, there are significant implications regarding the mechanism of cell death induced by sound trauma. This therapeutic window is consistent with the fact that JNK-mediated activation of the mitochondrial cell death pathway can occur between 30 minutes and 12 hours post sound exposure. Analysis of our data revealed that during this time period only 22% of the hair cells were missing and 40% of the hair cells were apoptotic in the area of sound damage. One possible explanation is that, when the apoptotic process is engaged, blockage of the JNK-activated mitochondrial signal pathway is no longer effective even if hair cells are still present, because JNK acts upstream in the apoptotic cascade (Jin et al., 2006). Further studies need to be undertaken to verify whether targeting a "down stream" event in the apoptotic cascade, such as activation of caspases, may prolong the window for initiation of therapy whilst retaining similar efficacy. If true, this might explain the 3 day therapeutic window reported by Yamashita et al. (2005) when using a combination of antioxidants (salicylate and trolox, intraperitoneally) against sound trauma.

This study shows that the activation of MAPK/JNK signal cascade by sound trauma regulates the intrinsic cell death pathway involving the mitochondria. A single dose of the peptide inhibitor D-JNKI-1 onto the RWM diffused into and remained within the auditory hair cells for as long as 3 weeks, the latter method of application being as potent as direct intracochlear perfusion of this peptide inhibitor of JNK in preventing sound-induced hair cell loss and deafness. The ability of D-JNKI-1 to diffuse through the RWM into auditory hair cells and neurons, the extended period of retention of this inhibitory compound within these neurosensory cells, and the finding of a 12 hour post-trauma window for effective treatment

provides an interesting clinical perspective and an opportunity to develop a realistic and effective therapy with which to treatment for cochlear injury. As has been demonstrated in this study, RWM delivery would be the preferred method of drug delivery because it avoids any unwanted side effects that could be associated with systemic administration, as well as avoiding any local risk to the cochlea if the cochlea had to be opened to be infused. The HA gel method of drug delivery at the RWM is particularly attractive because HA gels have been demonstrated to be non-ototoxic and it can be achieved using current surgical approaches without implantation of a pump system.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. ***sound induces loss of sensory hair cells and hearing threshold.*** **A**: The CAP threshold shift corresponds to the difference between the recordings measured before sound trauma and at 20 min (grey circles), 24 hrs (black circles), 48 hrs (squares), and 15 days (triangles) post-exposure. **B**: Fluorescent microscopic imaging of the maximally damaged region of the organ of Corti in cochleae at 1 hr post sound exposure. Surface preparations stained with calbindin (red), NF 200 kDa (green) and nuclear chromatin counterstained blue with Hoechst 33342 dye show that some first row OHCs (O, white asterisks) are missing. Pathological changes within the nuclei of some remaining first and second row OHCs are characterized by dense staining of chromatin fragments, and the presence of shrunken, pyknotic nuclei (arrowheads). Nerve fibers (arrows) below the IHCs (I) and OHCs were labeled with anti-NF 200 antibody. **C_1** to **C_4**: demonstrate different patterns of nuclear chromatin staining with Hoechst 33342. **C_1** shows a normal pattern of chromatin staining of a hair cell nucleus from a control non-sound-exposed organ of Corti. **C_2** to **C_4** show sound-induced changes in the staining of hair cell nuclei: chromatin condensation (**C_2**); pyknotic nucleus (**C_3**); and a swollen, enlarged nucleus (**C_4**). **D**: Time course of appearance of sound-induced apoptotic (circles) or necrotic (triangles) hair cell nuclei and hair cell loss (squares) from sound-exposed cochleae. The number of apoptotic, necrotic and missing hair cells is counted in a 2 mm length of maximally damaged cochlear duct from sound-exposed cochleae at 8 different time points (before and 1, 6, 12, 24, 48 hrs, 5 and 15 days post sound trauma). The results are expressed as the mean percentage of missing hair cells, or hair cell nuclei containing apoptotic or necrotic changes ± SEM (bars) for n = 4 independent experiments per time point. p: pillar cell. ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$. Scale bar: **B** = 20 µm, **C_1** to **C_4** = 4 µm.

Figure 2. ***Nature of sound trauma-induced death of auditory hair cells.*** **A**, **D** and **G**: Surface preparations of the organ of Corti, stained with calbindin (red) and NF 200 kDa (green), chromatin is counter-stained blue with Hoechst 33342 dye. **A**: The control non-sound-exposed cochlea. Note the auditory nerve fibers (white arrows) below the IHCs (I), and those crossing the tunnel to reach the OHCs (O). **D**: In this area of maximal damage (15 mm from apex), there is extensive loss of hair cells from all 3 OHC rows and from the single row of IHCs with extensive disruption of the auditory nerve fibers (white arrow). **G**: In an area near the site of maximum damage (13 mm from apex), the hair cell nuclei was swollen and enlarged (white arrow heads), whereas the nerve fibers (arrows) below the IHCs and OHCs are still clearly visible. **B**, **C**, **E**, **F**, **H** and **I** are transmission electron micrographs. **B** and **C**: An IHC (I, in **B**) and three OHCs (O_1, O_2, O_3 in **C**) from control non-sound-exposed animal. Note the IHC, OHCs, and their stereociliary bundles (arrows) and innervation (asterisks in **B**). **E** and **F**: Hair cells in the sound-damaged region of the organ of Corti. **E**: An IHC (I) showing characteristics of apoptosis, i.e. shrinkage of the cell body, and condensation of the cytoplasm with preservation of the cytoplasmic lateral membrane and swollen afferent dendrites (asterisks) at the basal pole of this damaged IHC. **F**: A degenerating OHC (O_1) with vacuolated cytoplasm, distorted and altered mitochondria (arrowheads), an electron-dense nucleus (n) due to chromatin compaction (signs of an advanced stage of apoptosis), and a disintegrated cytoplasmic membrane (arrow). **H** and **I**: Images of an area adjacent to the sound-damaged region of the organ of Corti. **H:** A degenerating IHC (I) showing characteristics of necrosis. **I:** A first row OHC (O_1) with vacuolated, swollen cytoplasm and a distended nucleus (n). Note the swollen afferent dendrites (asterisks) at the basal pole of the IHC in **H,** and the normal appearance of stereociliary bundles of the remaining OHCs (O_2, O_3, arrows) in **I**. ip: inner pillar cell, D: Deiter's cells, e: efferent fibers. Scale bar in **A, D, G** = 20 μm; in **B, C, E, F, H, I** = 10 μm.

Figure 3. ***JNK activation of c-Jun mediates the mitochondrial cell death pathway.*** **A**: Representative Western Blots using antibodies to phosphorylated c-Jun (p-c-Jun, Ser73) and the unactivated form of c-Jun, N-terminal of Bax that recognizes only the activated form of Bax or 240/280 kD α-fodrin (all spectin) or β-actin in control non-sound-exposed, sound-exposed, sound-exposed treated with D-JNKI-1 peptide, and sound-exposed treated with JNKI-1mut (inactive form). **B**: Histograms represent p-c-Jun/c-Jun ratios in sound-exposed cochleae expressed relative to the same ratio in non-sound-exposed cochleae, i.e. 0 hr specimens. **C**: Histograms represent the increase in Bax-NT post sound-exposure. Data expressed as activation with respect to non-sound-exposed ears, i.e. 0 hour specimens. **D**: Histograms represent the levels of α-fodrin post sound-exposure relative to control, non-sound-exposed ears (100%). In all cases, 10 or 100μM of D-DJNKI-1 prevented phosphorylation of c-jun, activation of Bax and the cleavage of fodrin 3 and 12h after sound exposure, whereas the inactive form of the peptide inhibitor (JNKI-1mut) had no effect ($p>0.05$). All values are presented as means ± S.D. for all experiments, ($n = 4$ per time point). ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$.

Figure 4. ***Release of cytochrome c, translocation of Bax, and cleavage of α-fodrin in damaged sensory hair cells.*** **A**: Confocal images of transverse cryostat sections of the organ of Corti labeled with antibody against cytochrome c. Left image shows a normal pattern of intense punctate staining for cytochrome-*c* in both the IHC and the OHCs in non-exposed cochleae. In contrast, the middle image shows a diffuse, pale pattern of immunostaining for cytochrome-*c* in both the IHCs and the OHCs at 1 hr post–exposure indicating a redistribution of cytochrome-*c* from the mitochondria to the cytoplasm. Note that there is still a normal pattern of immunostaining for cytochrome-c for the medial efferents that connect to the OHCs (white arrows and e). The right image is a sound-exposed cochlea that was treated with 100

µM of D-JNKI-1 peptide via the RWM. Note the normal pattern of intense punctate staining for cytochrome-*c* in both the IHC and the OHCs as in the non-exposed control cochleae. **B**: Triple labeling of non-exposed, sound-exposed untreated and sound-exposed D-JNKI-1 treated cochleae at 3 hrs post sound exposure. Cochlear cells were double labeled for Bax (red) and cytochrome c (green), counter-stained with Hoechst 33342 dye to label chromatin (blue). The left higher magnification image shows a diffuse pattern of Bax immunostaining and intense punctate staining for cytochrome-*c* in both the OHCs (O_1, O_2 and O_3) and Deiter's cells. There is a lack of co-localization of cytochrome c and Bax immunolabeling with a uniform pattern of DNA staining in all of the hair cell and support cell nuclei. In contrast, the middle higher magnification image shows intense and punctate pattern of immunostaining for Bax and the weak, pale pattern of immunostaining for cytochrome-*c* in the OHCs. The DNA staining revealed condensed, shrunken, and pyknotic nuclei in two OHCs (O1 & O2, asterisks) and one Deiters cell (white arrow head). Note that the damaged Deiter's cell (white arrow head) shows almost no cytochrome-*c* staining, whereas the intact Deiter's cells on either side display a punctate staining for this respiratory chain enzyme. The D-JNKI-1 treatment prevented the translocation of Bax in both the hair cells (IHCs, OHCs) and the support cells. **C**: Triple labeling of non-exposed, sound-exposed untreated and sound-exposed D-JNKI-1 treated cochleae at 12 hrs post sound exposure. Cochlear cells were double labeled for α-fodrin (green) and anti-calbindin antibody (red) and, counter-stained with Hoechst 33342 dye to label chromatin (blue). When compare with non-exposed cochlea (left image), sound exposed untreated cochlea displays a robust immunostaining for the cleaved form of α-fodrin in the cuticular plates (arrowheads) and the cytoplasm of both the OHCs and the IHC, and in the pillar cells (asterisk). The D-JNKI-1 treatment prevented caspase-induced cleavage of fodrin (right image). Dcs: Deiter's cells, e: efferent fibers. Scale bar in **A, B** and **C** = 10 µm, except left and middle images in **B** where the scale bar = 2 µm.

Figure 5. ***FITC-conjugated D-JNKI-1 delivered onto the round window diffuses into the cochlea***. **A** to **F**: FITC-conjugated D-JNKI-1 peptide (green) at a concentration of 100 μM in AP irrigated into the round window membrane (RWM) niche for 30 minutes. **A**: Green fluorescent staining is seen in the scala tympani (st), scala vestibuli (sv) and scala media (sm) of all cochlear turns 3 days post delivery. The FITC label shows a base to apex gradient of distribution. **B** to **E**: The intensity of the FITC signal remained intense and stable in the basilar membrane (bm), Reissner's membrane (Rm), the spiral limbs (sL), spiral ligament (sl), OHCs (O), IHCs (I), peripheral branches of the cochlear nerve fibers (nf) and spiral ganglion (sg), but not the cells of the stria vascularis (sv) at 3 days (in **B**) and 7 days (in **C**). In comparison there was a slight decrease of FITC D-JNKI-1 labeling in the cochlear turns at 14 days post delivery (in **D**) and a further decrease was observed at 21 days post application (in **E**). **F**: Higher magnification of organ of Corti at 3 days post delivery showing FITC-labeled D-JNKI-1 clearly visible in the OHCs and to a lesser extent within the IHC. **G** and **H**: Quantitative analysis of FITC fluorescence levels in the organ of Corti of all 4 cochlear turns from guinea pig cochleae removed at 3 days (in **G**), and in the basal turn of organ of Corti specimens at 6 hrs and 3, 7, 14, 21 and 28 days after RWM application of FITC-conjugated D-JNKI-1 peptide (in **H**). FITC fluorescence levels measurements were performed using a histogram function in a 20 x 10 pixel box focused on organs of Corti of all turns which were identified by light microscopy images with projections of fluorescence microscopy. Intensity of fluorescence is expressed as the mean of pixel intensity (mean ± SD). **G**: There is a base to apex gradient pattern of distribution of FITC-labeled D-JNKI-1 3 days post administration. **H**: FITC fluorescence persists up to 14 days after application of the FITC-conjugated D-JNKI-1 peptide. These fluorescence levels drastically decrease at 21 days and no difference was observed between the control untreated cochleae and the FITC-conjugated D-JNKI-1-RWM

cochleae at 28 days. ***: p<0.001, **: p<0.01, *: p<0.05. oC: organ of Corti, m: modiolus, pc: pillar cells. Scale bar: **A** = 170 µm; **B-E** = 60 µm**; F** = 30 µm.

Figure 6. D-JNKI-1 protects the cochlea against sound-induced hair cell loss and elevation of auditory threshold. **A**: Hearing thresholds measured at 15 days post exposure from contralateral untreated, sound-exposed, left cochleae (grey circles) and the sound-exposed right cochleae that were infused with D-JNKI-1 (black circles), JNKI-1-mut (black triangles) or D-TAT (black squares) onto the round window membrane (RWM). Contralateral untreated cochleae had a permanent hearing loss of 40 dB SPL (grey circles). Hearing loss was not significantly different in sound-exposed cochleae treated with D-TAT or JNKI-1-mut. In contrast, a significant improvement in the recovery of hearing threshold from an initial temporary threshold shift was seen in the D-JNKI-1 treated cochleae (black circles). **B** and **C**: Scanning electron micrographs of the area of greatest damage in untreated and D-JNKI-1 treated cochleae from the same animal. Untreated cochlea shows severe damage to the IHCs (I) and to the first row of OHCs (O) with a gradation of damage extending to the second and the third rows of OHCs (in **B**). Application of D-JNKI-1 onto the RWM effectively prevented nearly all sound trauma-induced hair cell loss (in **C**). **D**: Cochleograms representing the mean survival of hair cells as a function of the distance from the apex (in mm) in contralateral untreated cochleae (grey line) and in D-JNKI-1 treated cochleae (black line). In the sound-exposed untreated cochleae, more than 80% of the HCs were missing in the maximumally damaged area of the cochlea (14-16 mm from the apex). In contrast, less than 15% of the HCs were lost in D-JNKI-1 treated cochleae. **E**: Dose-response curve of D-JNKI-1 efficacy at day 15 post-exposure. Hearing thresholds in response to an 8 kHz pure tone stimulus were expressed as the percentage of recovery. Dose-response data were fitted to a curve using a non-linear least-square logistic equation and the Boltzman equation was

used for fitting the sigmoid curves. The EC_{50} was calculated as 2.05 μM for RWM delivery of D-JNKI-1. **F**: Time-dependent effect of RWM administration of D-JNKI-1 via an osmotic minipump (open circles), or via hyaluronic acid gel (HA gel; solid circles) on functional recovery of hearing thresholds. D-JNKI-1 treatments were commenced 0.5 before or 0.5, 1, 4, 6, 12 or 24 hrs after sound exposure. Note the efficacy of D-JNKI-1 when treatment was started within 6 hrs after the initial trauma. There is no significant difference in effectiveness with method of delivery, i.e. osmotic minipump/RWM vs. HA gel/RWM. Scale bars: **B** and **C** = 10 μm

Table I: Experimental protocol

SOUND INDUCED JNK-MEDIATED MITOCHONDRIAL CELL DEATH PATHWAY			
Experiments	**Methods**	**Markers**	**Number of animals**
Nature and time course of cell death	Immunocytochemistry TEM	calbindin, neurofilament, Hoechst	**n = 32** **n = 6**
Cell death mechanisms	western blots Immunocytochemistry	p-c-jun Bax fodrine cytochrome c cytochrome c-Bax-Hoechst cleaved fodrin- calbindin-Hoechst	**n = 28** **n = 18**
PROTECTIVE EFFECTS OF D-JNKI-1 AGAINST SOUND TRAUMA			
Experiments	**Methods**	**Drugs and time course**	**Number of animals**
Diffusion and penetration	Confocal imaging	100µM of FITC- D-JNKI-1	**n = 23**
Control	Functional assessment	100µM of TAT-empty, 100µM of JNK-mut	**n = 6** (3 per drugs)
Protective effects	Dose-response	0.01 to 100 µM of D-JNKI-1	**n = 42** (6 per doses)
	SEM	100µM of D-JNKI-1	**n=-6** (3 sound exposed untreated + 3 sound-exposed treated)
Therapeutic window	Time-response (minipump)	30 min before and 30 min, 1, 4, 6, 12, 24 hours after sound exposure.	**n = 42** (6 per time point)
	Time-response (HA gel)	30 min before and 30 min, 4, 12 hours after sound exposure.	**n = 24** (6 per time point)
	western blots	10µM of D-JNKI-1, 100µM of D-JNKI-1, 100µM of JNK-mut	**n=16** (4 per drugs and per time points)
	Immunocytochemistry	100µM of D-JNKI-1	**n=6**

p-c-jun: phosphorylated c-jun, TEM: transmission electron microscopy, SEM: scanning electron microscopy

Figure 1 (600 dots per inch, 21x 19 cm)

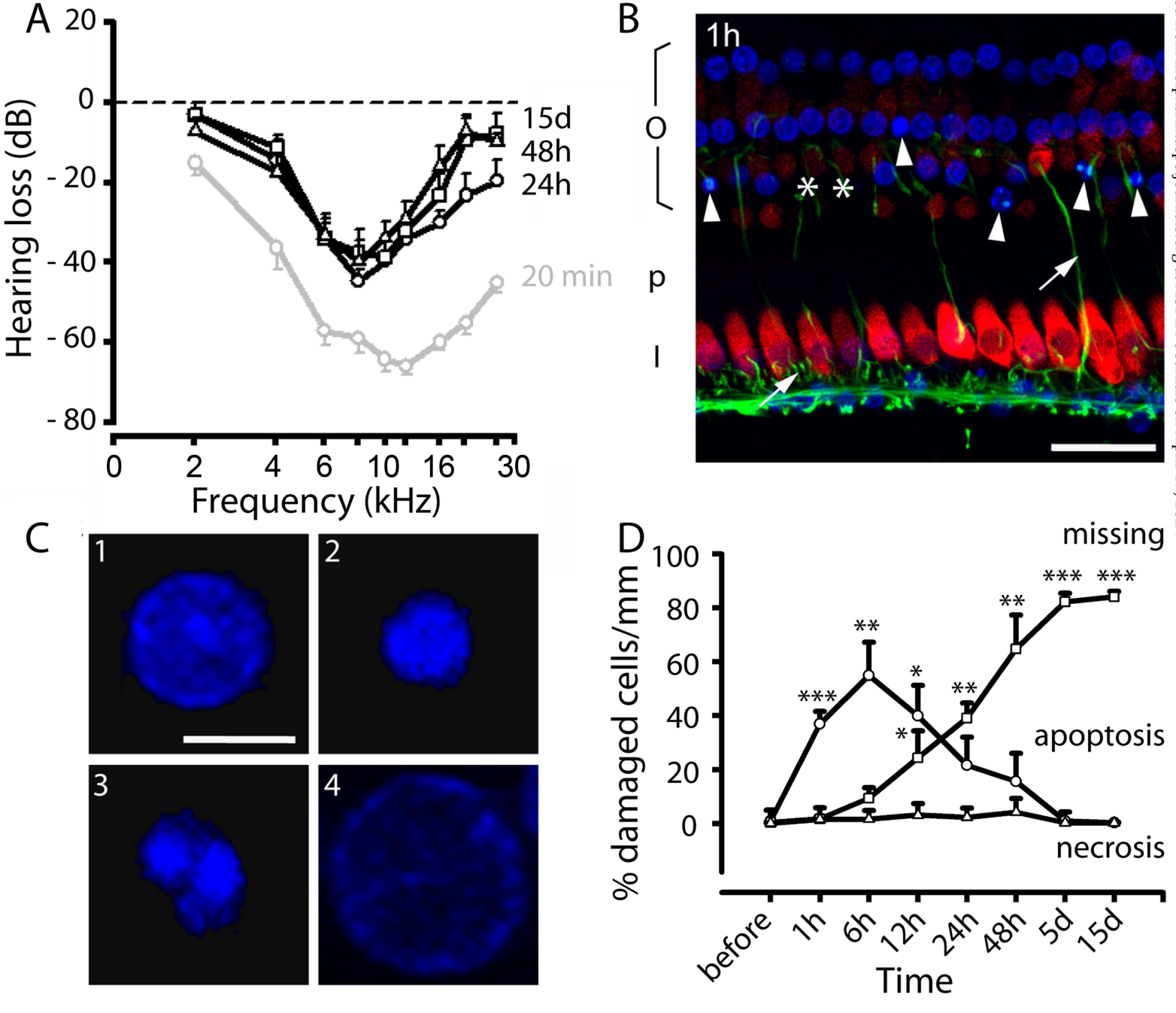

Figure 2 (600 dots per inch, 21 x 21.5 cm)

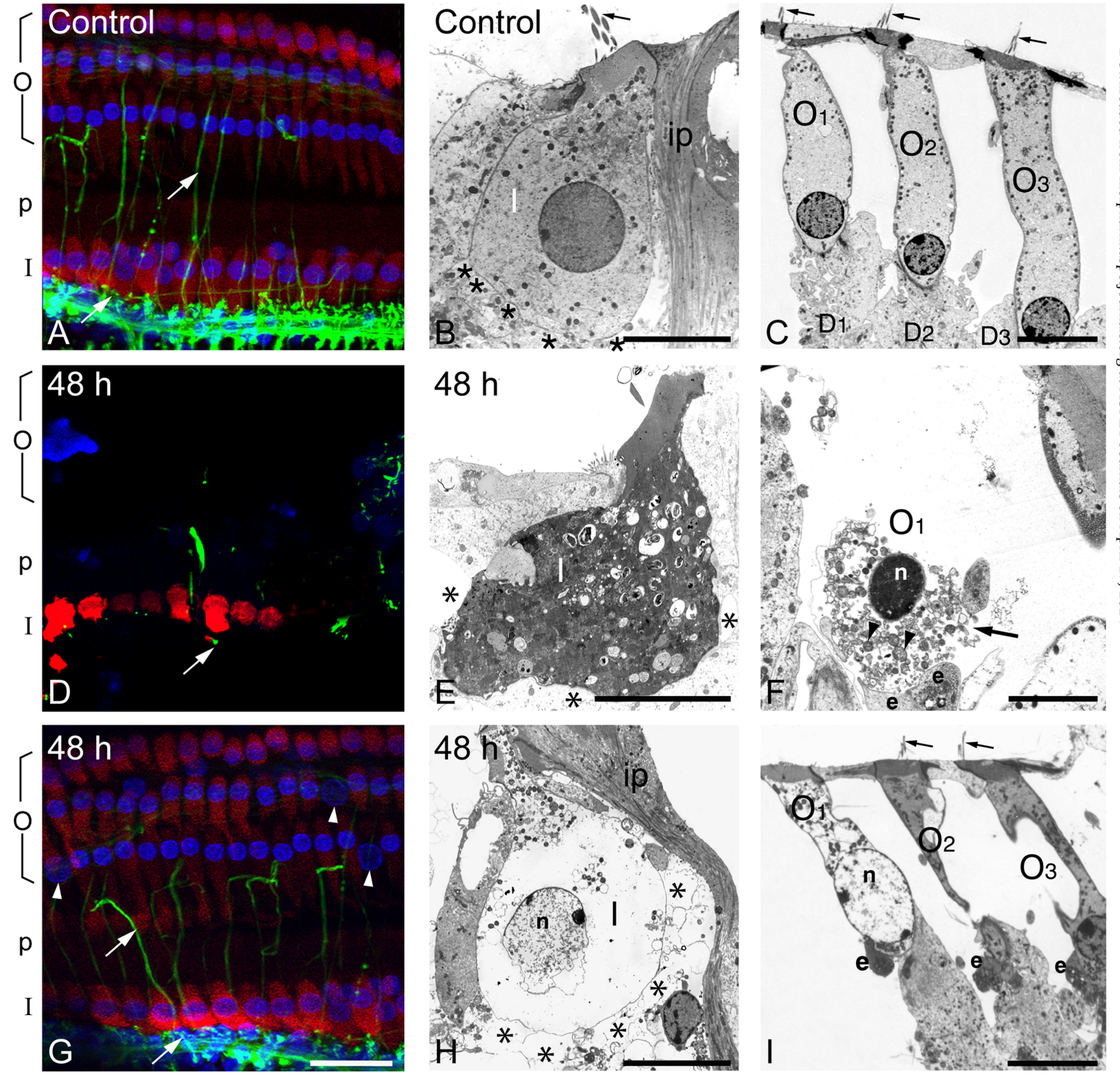

Figure 3 (600 dots per inch, 21 x 16 cm)

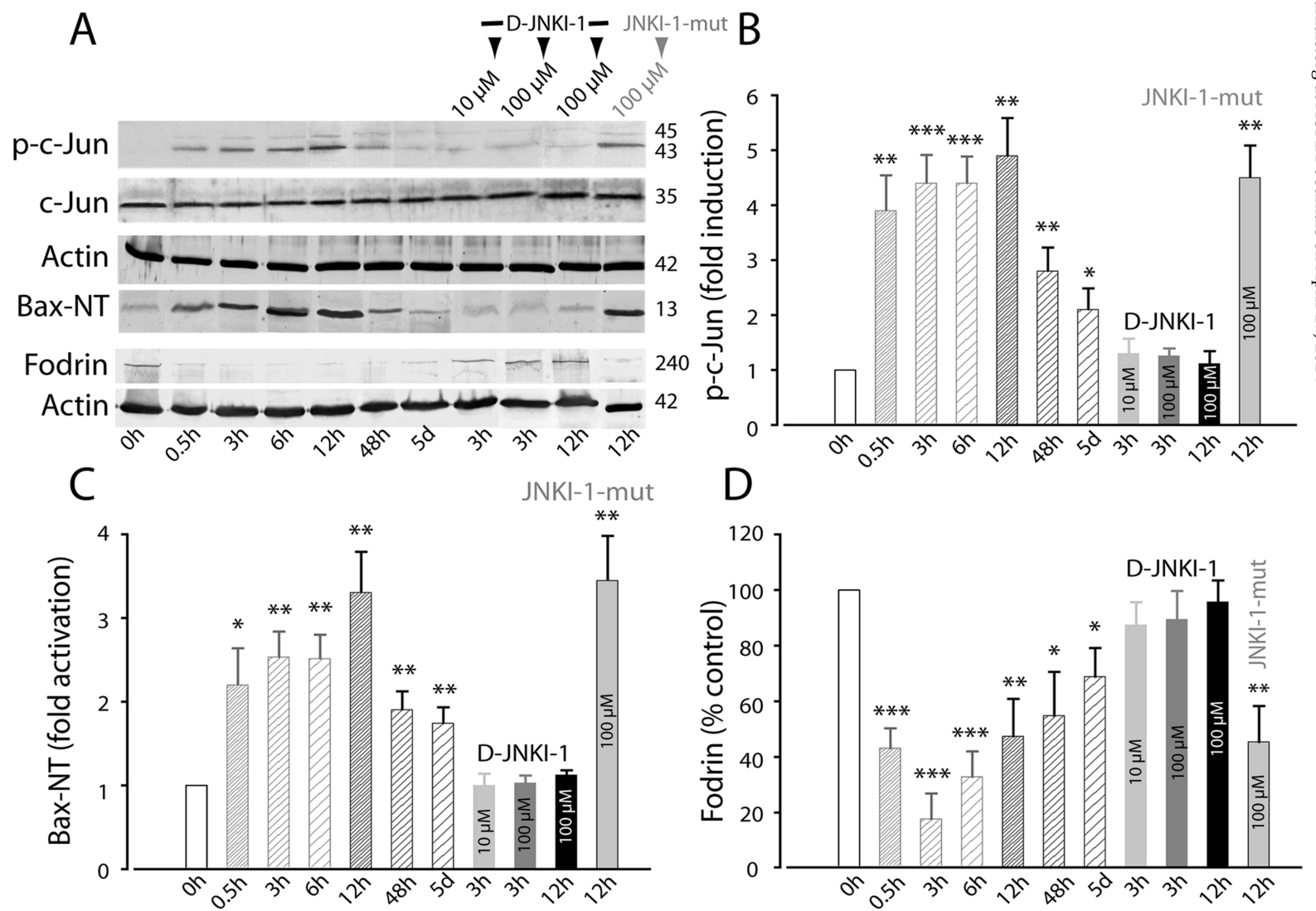

Figure 4 (600 dots per inch, 21 x 15 cm)

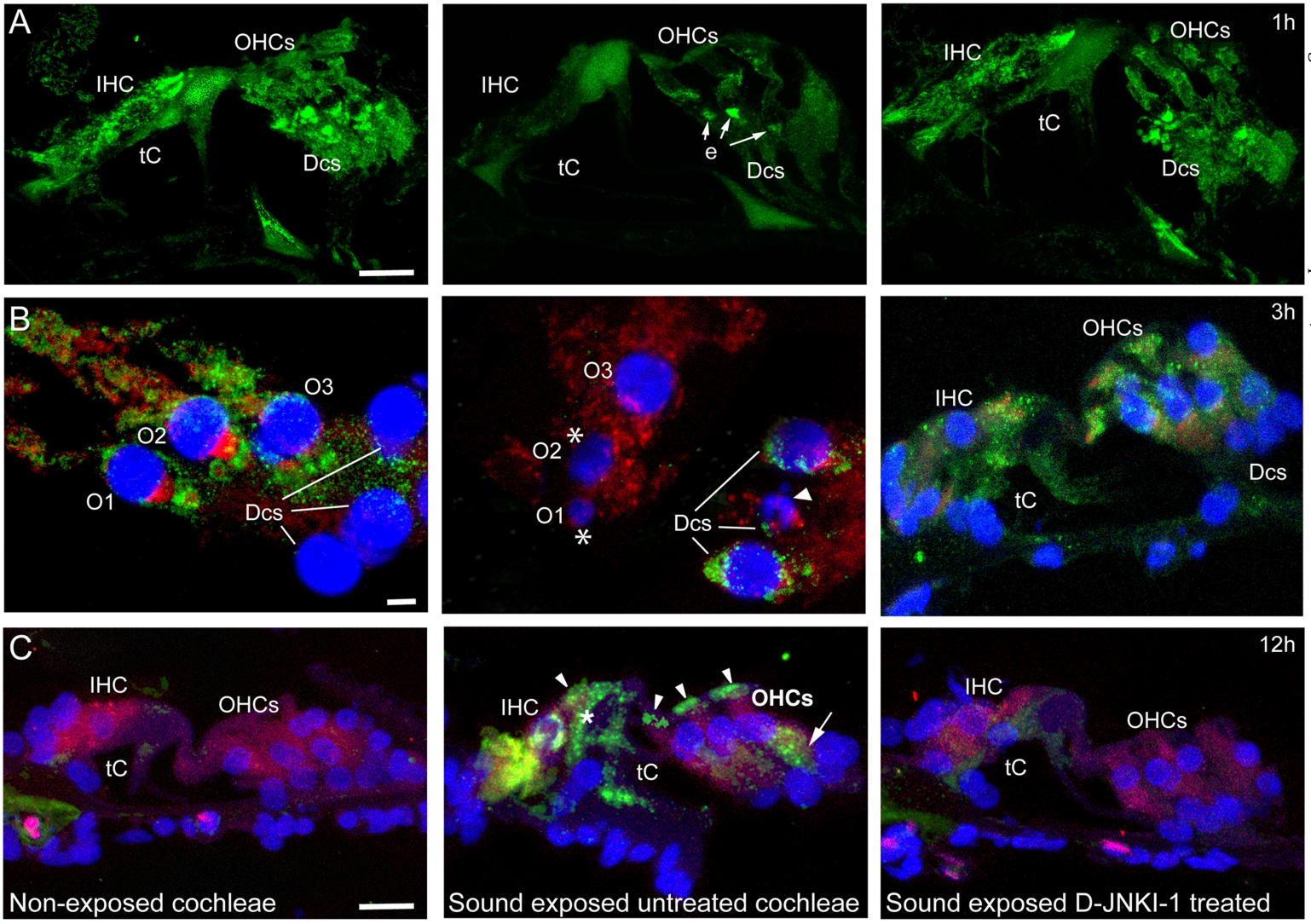

Figure 5 (600 dots per inch, 21 x 14 cm)

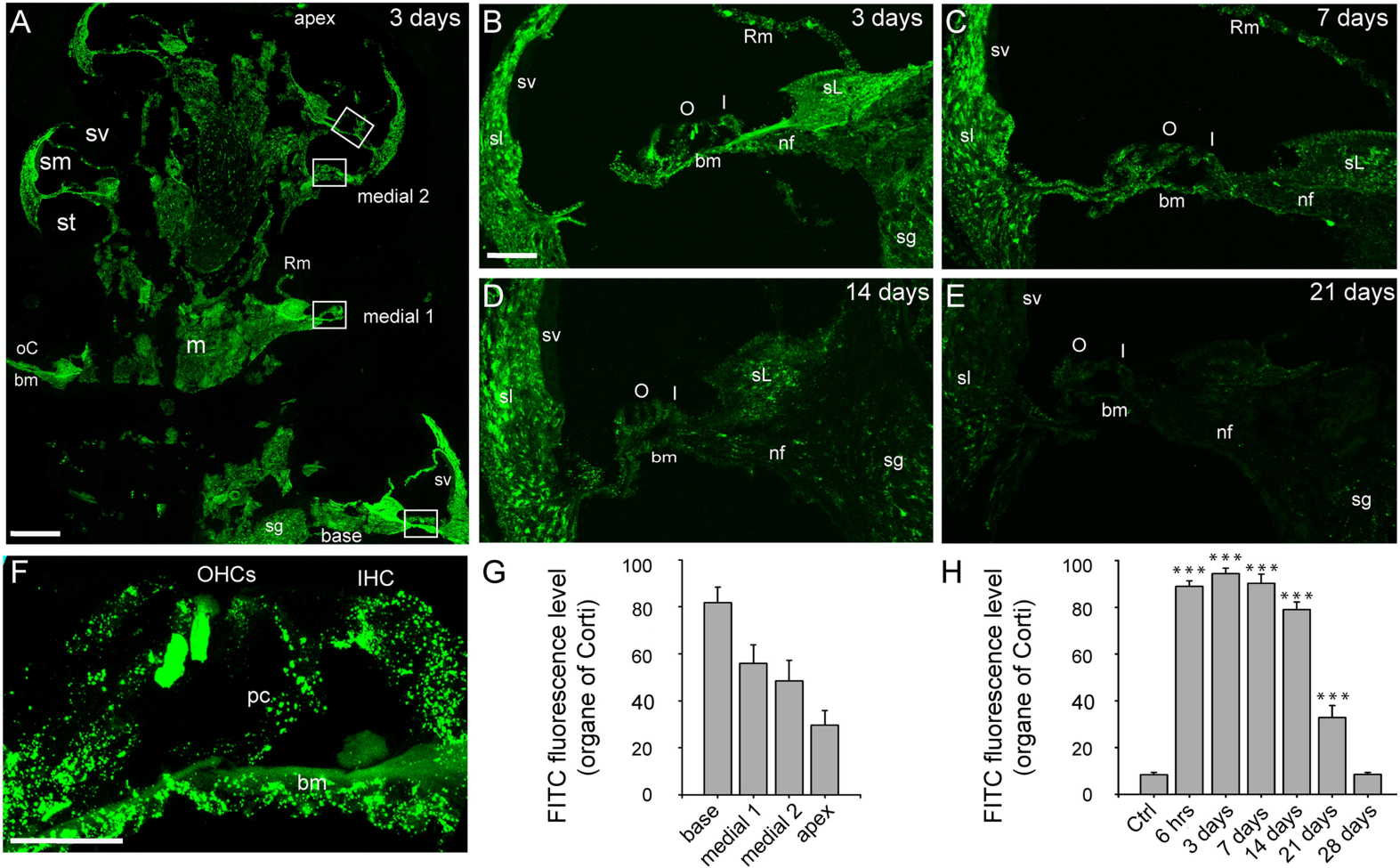

Figure 6 (600 dots per inch, 21 x 14 cm)