Hepatocyte Nuclear Factor-4 Alpha in Noise-Induced Cochlear Neuropathy

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ABSTRACT: Noise-induced hearing loss (NIHL) is a problem of profound clinical significance and growing magnitude. Alarmingly, even moderate noise levels, previously assumed to cause only temporary shifts in auditory thresholds (“temporary” NIHL), are now known to cause cochlear synaptopathy and subsequent neuropathy. To uncover molecular mechanisms of this neuropathy, a network analysis of genes reported to have significantly altered expression after temporary threshold shift-inducing noise exposure was performed. The transcription factor Hepatocyte Nuclear Factor-4 alpha (HNF4α), which had not previously been studied in the context of cochlear response to noise, was identified as a hub of a top-ranking network. Hnf4α expression and localization using quantitative RT-PCR and in situ hybridization, respectively, were described in adolescent and adult mice exposed to neuropathic noise levels in adolescence. Isoforms α3 and α12 in the cochlea were also identified. At every age examined, Hnf4α mRNA expression in the cochlear apex was similar to expression in the base. Hnf4α expression was evident in select cochlear cells, including spiral ganglion neurons (SGNs) and hair cells, and was significantly upregulated from 6 to 70 weeks of age, especially in SGNs. This age-related Hnf4α upregulation was inhibited by neuropathic noise exposure in adolescence. Hnf4α silencing with shRNA transfection into auditory neuroblast cells (VOT-33) reduced cell viability, as measured with the MTT assay, suggesting that Hnf4α may be involved in SGN survival. Our results motivate future studies of HNF4α in cochlear pathophysiology, especially because HNF4α mutations and polymorphisms are associated with human diseases that may include hearing loss.

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INTRODUCTION

The last decade has witnessed a dramatic increase in portable listening device (PLD) sales, particularly for music. The majority of people investing in PLDs are young, and, alarmingly, studies suggest that PLDs can cause temporary auditory threshold shifts (TTS) in young adults who have listened to music once for 4 hours at approximately 100 dB SPL (Le Prell et al., 2012). TTS was traditionally assumed to be associated with reversible changes in both auditory function, as evaluated through threshold audiometry, and anatomy, as reflected in temporary swelling of peripheral nerve terminals and bending of cochlear supporting cells (Nordmann et al., 2000). However, it is now apparent that TTS can cause permanent and substantial loss of spiral ganglion neurons (SGNs) (Kujawa and Liberman, 2009; Lin et al., 2011; Jensen et al., 2015), and functional difficulties when listening in noisy environments (Hickox and Liberman, 2014), despite return of auditory thresholds to normal and presence of morphologically intact hair cells. This noise-induced cochlear neuropathy is not revealed by conventional threshold testing and has therefore recently been termed “hidden hearing loss” (Liberman and Kujawa, 2014).

Surprisingly, while morphological and functional characteristics of TTS are well described, the underlying molecular mechanisms are only beginning to be elucidated (Yamashita et al., 2008; Meltser et al., 2010). Recently it has been recognized that TTS-inducing noise can be either neuropathic or non-neuropathic (Hickox and Liberman, 2014; Jensen et al., 2015). Neuropathic noise exposure causes permanent cochlear synapse and nerve loss, as assessed through synaptic counts and wave 1 of the auditory brainstem response (ABR), which represents the auditory nerve’s summed electrical activity. Non-neuropathic noise exposure causes TTS without permanently damaging cochlear synapses and neurons. Researchers have yet to characterize the molecular differences between neuropathic and non-neuropathic TTS-inducing noise levels.

Noise-induced TTS can activate similar molecular pathways as noise-induced permanent threshold shifts (PTS), which cause permanent elevation of ABR thresholds and multiple structural changes within the cochlea (Meltser et al., 2010). TTS-inducing noise causes cellular oxidative stress within 1 hour of exposure, and increases supporting cell levels of the proto-oncogene c-Fos that, along with c-Jun, is a part of the activator protein-1 (AP-1) complex (Shizuki et al., 2002). This TTS-associated cellular stress also induces endogenous corticosterone and nuclear factor kappa B (NF-κB) activation in SGNs (Tahera et al., 2006). NF-κB is vitally important for neuronal survival and synaptic plasticity (Lang et al., 2006). TTS noise causes acute synaptopathy, most likely due to glutamate excitotoxicity. This noise overexposure modulates post-synaptic glutamate receptors, possibly by regulating Ca^{2+} and phosphorylated c-Jun (JNK pathway) (Ruan et al., 2007). C-Jun is a member of the mitogen-activated protein kinases (MAPKs) (Bodmer et al., 2002). Noise-induced TTS activates different MAPK pathways than those activated by PTS (Meltser et al., 2010). At 24 hours following noise-induced TTS, the JNK and ERK pathways are upregulated in a delayed fashion; by contrast, levels increase immediately and settle faster in the PTS group. JNK pathway activation in the TTS group could be mediated by neurotrophins that promote SGN survival (Bodmer et al., 2002).

Given that TTS-inducing noise has been reported to alter expression of many molecules in the cochlea, we applied network theory to comprehensively analyze these molecules and identify central players. Network-based analysis is increasingly used across scientific fields because it provides deeper insight into complex biological systems and the underlying molecular interactions than the traditional linear, reductionist approach. We used Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com) for network analysis because it contains the largest curated database of biological and chemical interactions extracted from the literature. Our study focused on rodents because they are the most common animal models of noise-induced hearing loss (NIHL). The cochlear molecules reported in the literature to be differentially regulated by TTS were used as inputs into IPA software for network analysis. By analyzing hubs of the top ranking networks, we identified the transcription factor Hepatocyte Nuclear Factor-4 alpha (HNF4α) as a novel, potentially key molecular orchestrator of TTS. This computational result was validated using a combination of techniques, including real-time quantitative RT-PCR, in situ hybridization, exposure to neuropathic noise in vivo, and Hnf4α gene silencing in vitro.
to describe changes in Hnf4α expression with aging and noise exposure. Our data suggest that Hnf4α may be important for SGN survival.

**METHODS**

**PubMed Search**

A comprehensive literature search was performed in PubMed to identify genes implicated in cochlear response to noise using MeSH terms: “noise exposure,” “cochlea,” “gene change,” “gene expression,” “hearing,” “temporary threshold shift.” Studies in which rodents (varying species) were exposed to continuous broadband noise once for 1–5 hours with varying intensities were included. We excluded blast noise and consecutive noise exposures that would result in PTS because these insults are known to cause acute and widespread cellular destruction within the inner ear, and thus activate signaling pathways that differ from those activated in response to a single TTS-inducing noise exposure (Meltser et al., 2010). We studied rodents (mice, guinea pigs, rats, and chinchillas) collectively because physiological responses to noise are similar across rodents (Lin et al., 2011). In addition, combining rodents allowed us to maximize the number of genes eligible for network analysis (TTS is less studied than PTS). We included studies that describe gene and protein expression using microarray analysis, PCR, western blot, and immunohistochemistry. Only genes whose altered expression was validated or found to be statistically significant (p < 0.05) were included.

**Network Analysis**

IPA is based on the Ingenuity® Knowledge Base (IKB) that includes millions of individual interactions among genes, proteins, cells, tissues, drugs, and diseases. We uploaded into IPA software a data set containing a list of cochlear molecules identified through PubMed searches as having a significantly different level of expression due to TTS-inducing noise exposure. For each molecule, the fold change in expression due to TTS-inducing noise was specified. For molecules with unquantified directional change, a fold change of −1.5 or +1.5 was assigned for down- or upregulation, respectively. The IPA algorithm connected the input molecules into networks by providing additional molecules from IKB, while aiming to maximize the number of input molecules in any given network. Each network was characterized by the molecule with the most connections, called the hub. A core analysis was performed with IPA version 9.0, IKB version 3602 on June 1, 2011. When generating networks, both direct and indirect interactions were considered, referring to two molecules making or not making direct physical contact, respectively. Networks of up to 40 molecules were studied to allow for the possibility that nearly all input molecules belonged to the same network, and to facilitate visual inspection of the networks. IPA determined network significance using a right tailed Fisher’s exact test. The network score assigned by IPA is a negative logarithm of the p-value, and it reflects the likelihood that the associations forming the network are due to chance alone. We focused on highly significant networks with scores greater than 8.

**Animals and Noise Exposure**

We used CBA/CaJ mice (purchased from Jackson Laboratory, Bar Harbor, Maine) to study noise-induced changes in Hnf4α expression. About 6-week-old mice were exposed to noise levels that had previously been shown to cause neuropathic or non-neuropathic TTS (Jensen et al., 2015). Specifically, we played continuous octave-band noise (8–16 kHz) for 2 hours at either 97 dB sound pressure level (SPL) to cause neuropathic TTS, or 94 dB SPL to cause non-neuropathic TTS. Age-matched, unexposed animals served as controls. Animals were awake during exposures and were held unrestrained in small cages. Sound was created using a white-noise source and was filtered and amplified before being delivered through a horn attached to the top of the exposure booth. Exposure levels were measured in each cage with a 0.25-inch condenser microphone (Bruel and Kjær, Denmark). All procedures were approved by the Animal Care and Use Committee at Massachusetts Eye and Ear Infirmary.

**Real-Time Quantitative RT-PCR**

Animals were euthanized with CO2 and cochlear soft tissue was dissected from the bony otic capsule in RNAlater (Ambion). The tissue was then bisected into an apical piece and a basal piece. For in situ hybridization (below), livers were extracted from two animals at 6 weeks of age. Microdissected cochleae and liver samples were stored at −80°C until further use. After adding TRIzol (Invitrogen) to the tissue, total RNA was purified using the RNeasyMinElute Kit (Qiagen). RNA integrity was determined using the RNA 6000 Pico Assay Chip (Agilent Technologies) and the 2100 Agilent Bioanalyzer. Only samples with RNA integrity numbers (RIN) greater than 7 (with 10 being perfect) were used for cDNA synthesis. A 100 μL reaction included 5× first strand buffer, 50 mM MgCl₂, 2.5 mM dNTP Mix, 3 μg/μL random hexamers, SuperScript III Reverse Transcriptase, and 45 μL sample RNA. Reverse transcription was performed at 25°C for 10 min, 37°C for 60 min, and 95°C for 2 min. The resulting cDNA was rapidly cooled at −80°C. All cochlear samples were run in triplicates on the Applied Biosystems StepOnePlus™ Instrument. Each well of a 96-well plate contained 1 μL cDNA, 1 μL TaqMan® Hnf4α primer (see below), 4 μL 2× Platinum® qPCR Supermix (Invitrogen), and 4 μL water. Cycling conditions were 50°C for 2 min followed by 40 cycles of 95°C for 2 min, 95°C for 15 seconds (sec), and 60°C for 1 min.

We tested two Hnf4α primers (Applied Biosystems): TaqMan® Hnf4α primer targeting exons 3 and 4 common to all Hnf4α isoforms [Fig. 3(A)] (Hnf4α: Mm01247712_m1), and TaqMan® Hnf4α primer targeting exons 8 and 9 [Fig. 3(A)] (Mm00433964_m1). Level of gene expression was normalized relative to endogenous 18S rRNA. Groups were analyzed using the comparative threshold cycle (ΔCt) and
delta C_{\text{T}} (\Delta C_{\text{T}}) methods. Gene expression was calculated as $\Delta \Delta C_{\text{T}}$ relative to the unexposed apex at 6 weeks.

**Nested RT-PCR**

Cochlear cDNA was generated as described above. Nested RT-PCR involved two sets of primers that were used in two successive runs of PCR, with the second set intended to amplify a secondary target within the amplicon of the first set. The forward and reverse primer sequences [Fig. 3(B)] were designed to assay various murine *Hnf4a* isoforms [Fig. 3(C)], from alternative splicing of the 12 known exons [Fig. 3(A)]. Each of the 30 PCR cycles consisted of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. The PCR products were resolved in a 2% agarose gel [Fig. 3(D)].

**Fluorescent In Situ Hybridization Combined with Immunohistochemistry**

Animals were intracardially perfused with 4% paraformaldehyde (PFA), decapitated, and intracochlearly fixed with 4% PFA after opening the round and oval windows. Cochleae were decalcified in 0.12 M EDTA for 3 days at room temperature, serially dehydrated, embedded in paraffin, and cut into 10 μm sections. Mouse *Hnf4a* cDNA from nucleotide 116–640 (GenBank: NM008261) was cloned from liver cDNA, generated from 6-week-old C57Bl/6J mice, into the pBluescript II SK vector. The digoxigenin (DIG)-labeled single stranded antisense and sense RNA probes were prepared using T7 RNA polymerase and T3 RNA polymerase, respectively, with the presence of DIG-dUTP (digoxigenin DNA labeling mixture; Roche) according to the manufacturer’s protocol. After rehydration, cochlear sections were treated with 3% H2O2 for 20 min to reduce endogenous peroxidase activity, fixed with 4% PFA after opening the round and oval windows. Cochleae were immersed in triethanolamine and acetic acid (2:1) solution for 10 min before hybridization. The hybridization mixture, which contained the DIG-labeled antisense or sense probe, was applied to each section and incubated with 33% 0.2 M SSC and 67% TBS for 10 min, followed by Hoechst nuclear staining and section mounting with Vectashield (Vector Laboratories, #H-1000).

**HNF4a Gene Silencing in Cochlear Neuroblasts (VOT-33) and MTT Assay**

Cultured VOT-33 cells were transfected with *Hnf4a* shRNA retroviral plasmids (TL50970, OriGene) or a non-effective 29-mer scrambled shRNA control retroviral plasmid. To knockdown all isoforms of *Hnf4a*, shRNA retroviral plasmids included four different shRNAs against different exons of *Hnf4a*, which targeted exons 2, 5, 8, and 10 of *Hnf4a* and covered all isoforms of *Hnf4a*. After 48 hour, 10 μL of 12 mM MTT (Invitrogen) was added to each well to detect cell viability. The optical density (OD) at 540 nm was measured for each well using the SmartSpect™ Plus spectrophotometer (Bio-Rad). The average OD value of the VOT-33 cells transfected with the scrambled shRNA control was set as 100% and used to normalize OD values of *Hnf4a* transfection.

**Western Blot**

The procedure of western blot was described before (Kao et al., 2016). Briefly, VOT-33 cells were lysed in RIPA-DOC buffer (50 mM Tris buffer (pH 7.2), 150 mM NaCl, 1% Triton-X100, 1% deoxycholate, and 0.1% SDS) with protease inhibitors (Complete, #04693132001, Roche). Equal amounts of protein extract were loaded per lane, resolved by 4%–20% SDS–PAGE, and electro-transferred onto a PVDF membrane (Immobilon-P, IPVH00010, Millipore). Protein detection was performed using the primary antibodies against HNF4a (ab55223, Abcam) or β-actin (#4970, Cell Signaling Technology) at 4°C overnight. After incubation with secondary antibodies for 1 hour at room temperature, protein bands were detected using a chemiluminescence detection kit (#32106, Pierce). Images were quantified using ImageJ (NIH).

**Statistical Analysis**

Mean fold changes for noise-exposed and unexposed groups were compared using two-way ANOVA (GraphPad Prism 6 software) and post hoc Tukey’s test for multiple comparisons. Trends across time within the same group were calculated with one-way ANOVA and post hoc Tukey’s test for multiple comparisons. Results are expressed as mean ± standard error of the mean (SEM). Differences between means were considered statistical significant when $p < 0.05$.

**RESULTS**

**Network Analysis Implicates HNF4a as a Central Molecule in TTS**

Eleven studies focusing on TTS met our inclusion criteria, as detailed in the “Methods” section (Taggart et al., 2001; Shizuki et al., 2002; Caravelli et al., 2004; Cho et al., 2004; Vlajkovic et al., 2004; Tahera et al., 2006; Ruan et al., 2007; Miyao et al., 2008;
Yamashita et al., 2008; Matsunobu et al., 2009; Gratton et al., 2011). The studies identified 40 differentially expressed molecules in the cochlea due to TTS-inducing noise exposure (Table 1). These molecules were analyzed using IPA software. Network analysis revealed 6 significant networks ($p < 0.05$). The molecular hub (i.e., molecule with the most connections) of the top ranking network (score 32) was NF-κB [Fig. 1(A)], a transcription factor known to play a protective role in cochlear neurons’ stress response to low-level noise (Lang et al., 2006; Tahera et al., 2006), and in lateral wall fibrocytes’ response to PTS-inducing high-level noise (Adams et al., 2009).

Table 1: Genes Extracted from the Literature Implicated in Noise-Induced Temporary Threshold Shift (TTS)

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Species (Ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTN1</td>
<td>Actinin, alpha 1</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>ACTR1A</td>
<td>ARP1 actin-related protein 1 homolog</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>ANXA4</td>
<td>Annexin A4</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>ATP2A2</td>
<td>ATPase</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>BCL2L1</td>
<td>B CL2-like 1</td>
<td>Guinea pigs (Yamashita et al., 2008)</td>
</tr>
<tr>
<td>BTG2</td>
<td>BTG family, member 2</td>
<td>Rats (Cho et al., 2004)</td>
</tr>
<tr>
<td>CALM1</td>
<td>Calmodulin 1</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor 1A</td>
<td>Mice (Gratton et al., 2011)</td>
</tr>
<tr>
<td>CXCL10</td>
<td>Chemokine (C-X-C motif) ligand 10</td>
<td>Mice (Gratton et al., 2011), rats (Cho et al., 2004)</td>
</tr>
<tr>
<td>EGR1</td>
<td>Early growth response 1</td>
<td>Rats (Cho et al., 2004)</td>
</tr>
<tr>
<td>ENTPD1</td>
<td>Ectonucleoside triphosphate diphosphohydrolase 1</td>
<td>Rats (Vlajkovic et al., 2004)</td>
</tr>
<tr>
<td>ENTPD3</td>
<td>Ectonucleoside triphosphate diphosphohydrolase 3</td>
<td>Rats (Vlajkovic et al., 2004)</td>
</tr>
<tr>
<td>FOS</td>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
<td>Mice (Gratton et al., 2011), rats (Cho et al., 2004), guinea pigs (Shizuki et al., 2002)</td>
</tr>
<tr>
<td>GADD45G</td>
<td>Growth arrest and DNA-damage-inducible, gamma</td>
<td>Mice (Gratton et al., 2011)</td>
</tr>
<tr>
<td>GEFT1</td>
<td>Glutamine-fructose-6-phosphate transaminase 1</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>GPI</td>
<td>Glucose-6-phosphate isomerase</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>HIST1H2BD</td>
<td>Histone cluster 1, H2bd</td>
<td>Mice (Gratton et al., 2011)</td>
</tr>
<tr>
<td>HMOX1</td>
<td>Heme oxygenase (decycling) 1</td>
<td>Guinea pigs (Matsunobu et al., 2009)</td>
</tr>
<tr>
<td>HNRNPA2B1</td>
<td>Heterogeneous nuclear ribonucleoprotein A2/B1</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>HSPA9</td>
<td>Heat shock 70 kDa protein 9 (mortalin)</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intercellular adhesion molecule 1</td>
<td>Mice (Gratton et al., 2011), guinea pigs (Miyao et al., 2008)</td>
</tr>
<tr>
<td>JUN</td>
<td>Jun proto-oncogene</td>
<td>Rats (Ruan et al., 2007)</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
<td>Rats (Cho et al., 2004)</td>
</tr>
<tr>
<td>MDH2</td>
<td>Malate dehydrogenase 2, NAD (mitochondrial)</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>MYH9</td>
<td>Myosin, heavy chain 9, non-muscle</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>NEFL</td>
<td>Neurofilament, light polypeptide</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>NFKB1</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer</td>
<td>Mice (Tahera et al., 2006), guinea pigs (Miyao et al., 2008), chinchillas (Ruan et al., 2007)</td>
</tr>
<tr>
<td>NR3C1</td>
<td>Nuclear receptor subfamily 3, group C, member 1</td>
<td>Mice (Tahera et al., 2006)</td>
</tr>
<tr>
<td>NR4A1</td>
<td>Nuclear receptor subfamily 4, group A, member 1</td>
<td>Rats (Cho et al., 2004)</td>
</tr>
<tr>
<td>OTOS</td>
<td>Otosirpiralin</td>
<td>Guinea pigs (Caravelli et al., 2004)</td>
</tr>
<tr>
<td>PPIA</td>
<td>Peptidylprolyl isomerase A (cyclophilin A)</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>PPP1R15A</td>
<td>Protein phosphatase 1, regulatory subunit 15A</td>
<td>Mice (Gratton et al., 2011)</td>
</tr>
<tr>
<td>RGS16</td>
<td>Regulator of G-protein signaling 16</td>
<td>Mice (Gratton et al., 2011)</td>
</tr>
<tr>
<td>RPL3</td>
<td>Ribosomal protein L3</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>RPL35A</td>
<td>Ribosomal protein L35a</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>S100A1</td>
<td>S100 calcium binding protein A1</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>S100A10</td>
<td>S100 calcium binding protein A10</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>TCL1B</td>
<td>T-cell leukemia/lymphoma 1B</td>
<td>Mice (Gratton et al., 2011)</td>
</tr>
<tr>
<td>TUBB</td>
<td>Tubulin, beta class I</td>
<td>Chinchillas (Taggart et al., 2001)</td>
</tr>
<tr>
<td>WDR92</td>
<td>WD repeat domain 92</td>
<td>Mice (Tahera et al., 2006)</td>
</tr>
</tbody>
</table>

A total of 40 input genes were eligible for Ingenuity® Pathway Analysis (IPA) (gene name and symbol). The findings were based on 11 publications in the period from 2001 to 2011 in four different rodents (species). Names in parentheses refer to the associated study (references).
et al., 2011; Shen et al., 2015). The central hubs of the remaining networks were Jun in network 3 (score 29), ERK1/2 mitogen-activated protein kinase in network 4 (score 25), Phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K/Akt) in network 5 (score 17), and G protein-coupled receptor family (Gpcr) in network 6 (score 9). These molecules have been described in the cochlear response to noise. Specifically, Jun (JNK pathway) has been shown to become activated 24 hours after exposure to TTS-inducing noise, and a few hours after exposure to PTS-inducing noise (Meltser et al., 2010). ERK1/2 mitogen-activated protein kinase of the MAPK pathway demonstrates similar changes in activation after exposure to TTS- versus PTS-inducing noise (Meltser et al., 2010). PI3K/Akt enzymes have been shown to protect hair cells from apoptosis following ototoxicity (Kurioka et al., 2014), while Gpcr inhibitors can protect auditory hair cells from ototoxicity-induced apoptosis (Battaglia et al., 2003).

**Figure 1** The two most significant networks implicated in noise-induced temporary threshold shift (TTS) revealed by network analysis. Nuclear Factor kappa B (NF-κB) (A) and Hepatocyte Nuclear Factor-4 alpha (HNF4α) (B) were identified as the hubs of the first and second most significant networks, respectively. Pink arrow (A) points to the glucocorticoid receptor that interacts with NF-κB. The colored symbols represent molecules known to be either upregulated (red) or downregulated (green). Color intensity reflects level of expression. Different shapes signify different functional classes of molecules. The white symbols identify molecules provided by the IPA to generate networks whose molecules interact directly (solid line) or indirectly (dashed line). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**Hnf4α is Expressed in the Mature Cochlea**

Hnf4α is expressed in specific cochlear cells, as assessed using fluorescent in situ hybridization applied to cochlear cross sections of 6-week-old wild-type mice (N = 6 different animals) (Fig. 2). A representative slide illustrates Hnf4α expression in spiral ganglion neurons (SGNs), the organ of Corti, spiral ligament, and interdental cells of the spiral limbus [Fig. 2(A)]; a control slide exposed to the Hnf4α sense probe showed no signal [Fig. 2(B)]. A magnified view of SGNs [Fig. 2(C)] and the organ of Corti [Fig. 2(D)] provided additional cellular detail. The Hnf4α signal was cytoplasmic, as expected because the probes targeted mRNA. Concurrent immunostaining for Myosin 7a (green), a hair cell-specific marker, revealed Hnf4α expression in hair cells and the surrounding supporting cells [Fig. 2(D)] that are part of the cochlear epithelial gap-junction network (Kikuchi et al., 1995).
HNF4α is a complex gene with 12 exons and 9 known isoforms in mice and humans (Huang et al., 2009). It is transcribed from two different promoters, P1 and P2 [Fig. 3(A)]. Isoforms $a_1, a_2, a_7, a_8, a_{10}$, and $a_{11}$ are long forms, whereas isoforms $a_3, a_9,$ and $a_{12}$ are short forms. To identify cochlear Hnf4α isoforms, nested PCR was applied to cochlear cDNA using the primer pairs described in Figure 3(B). For the first PCR, we used primer pairs [Fig. 3(C)] spanning the following exons: 1A to 3, 1D1 to 3, 5 to 10, or 5 to 8+ (where 8+ is a slightly extended version of exon 8) (Huang et al., 2009) (Genbank accession number EF193391). Primers spanning the following exons were used for the second PCR: 1A to 3 to identify isoforms $a_1, a_2,$ and $a_3$; 1D2 to 3 to identify isoforms $a_7, a_8,$ or $a_9$ (shorter amplicon) or isoforms $a_{10}, a_{11},$ or $a_{12}$ (longer amplicon); 7 to 10 to identify isoforms $a_1, a_2, a_7, a_8, a_{10},$ or $a_{11}$; 7 to 8+ to identify isoforms $a_3, a_9,$ or $a_{12}$ (corresponding products from the first PCR were used as templates). This is illustrated in Figure 3(D) for both the liver (all isoforms) and the cochlea. Lane 1 suggested that isoforms $a_1, a_2,$ or $a_3$ might be present in the cochlea. Lane 2 showed that isoforms $a_7, a_8, a_9$ (not containing exon 1E) were absent from the cochlea and that isoforms $a_{10}, a_{11},$ and $a_{12}$ (containing exons 1D and 1E) might be present. Lane 3 suggested that exon 10 was absent, thereby excluding isoforms $a_1, a_2, a_7, a_8, a_{10},$ and $a_{11}$ from the cochlea. Lane 4 suggested that isoforms $a_3, a_9,$ or $a_{12}$ might be present in the cochlea. Taken together, the presence of isoforms $a_1, a_2, a_7, a_8, a_{10}, a_9,$ and $a_{11}$ was excluded (combined results in lanes 2 and 4) and only isoforms $a_3$ and $a_{12}$ were identified in the cochlea. By contrast, all isoforms were expressed in the liver [Fig. 3(D), left; Harries et al., 2009].
Hnf4α Expression Is Altered with Aging and Neuropathic Noise Exposure

Real-time quantitative RT-PCR was applied to micro-dissected cochlea to quantify changes in Hnf4α mRNA expression due to aging and TTS-inducing noise exposure in adolescence (Fig. 4). We have previously demonstrated that exposing 6-week-old mice to 8–16 kHz band noise for 2 hours is neuropathic for noise at 97 dB SPL and non-neuropathic for noise at 94 dB SPL (Jensen et al., 2015). The cochlear apex and base were analyzed separately because these noise parameters target the cochlear base, and produce delayed changes in the cochlear apex (Jensen et al., 2015). Consistent with the nested PCR results (Fig. 3), we found that real-time quantitative RT-PCR detected cochlear Hnf4α expression only when primers targeting exons 3 and 4, common to all isoforms, were used; primers targeting exons 8 and 9 did not detect cochlear Hnf4α as they were unable to detect isoforms 3, 9, and 12.

At each of the three ages we studied (i.e., 8, 10, and 70 weeks, representing unexposed controls for 2 weeks, 1 month, and 16 months after noise exposure), Hnf4α mRNA expression levels were similar in the cochlear apex and base (Fig. 4). As unexposed ears aged from 10 weeks (N = 5 mice) to 70 weeks (N = 3 mice), Hnf4α levels increased in the base by 33.4% ± 4.8% (p < 0.001) (Fig. 4). A similar trend was observed in the apex, but it did not meet our criterion for statistical significance.

Exposure to TTS-inducing neuropathic noise at 6 weeks of age resulted in a significant decrease in Hnf4α at 16 months after exposure in both the apex and base (Fig. 4). When comparing the neuropathic group (97 dB SPL; N = 11 mice) with the age-matched non-neuropathic group (94 dB SPL; N = 9 mice) and unexposed group (N = 9 mice), the interaction between exposure group and post-exposure time was significant in the base (F(4, 20) = 6.129,
p = 0.002) but not in the apex ($F(4, 21) = 2.360, p = 0.086$). At 16 months after exposure, Hnf4α levels in the 97 dB group were reduced by 34.30% ± 6.32% ($p < 0.01$) in the apex and by 35.83% ± 1.43% in the base ($p < 0.001$) compared with age-matched, unexposed controls. Non-neuropathic noise (94 dB SPL) did not cause statistically significant changes in Hnf4α expression in exposed mice relative to unexposed controls ($p > 0.05$).

The results of applying fluorescent in situ hybridization to characterize changes in cochlear Hnf4α expression due to aging and neuropathic noise exposure (Fig. 5) were consistent with the real-time quantitative RT-PCR experiment findings (Fig. 4). Cochlear Hnf4α expression increased from 6 weeks ($N = 5$ mice) [Fig. 5(A)] to 70 weeks of age ($N = 5$ mice) [Fig. 5(B)] in unexposed mice; this upregulation was most prominent in SGNs. The age-related increase in Hnf4α expression was inhibited by neuropathic noise exposure in adolescence ($N = 5$ mice) [Fig. 5(C)].

**Hnf4α Silencing Induces Cell Death in Cochlear Neuroblasts**

Because SGN degeneration due to noise-induced cochlear neuropathy is slow in vivo (Kujawa and Liberman, 2009; Jensen et al., 2015), we studied it in an accelerated model in vitro using the mouse auditory neuroblast cell line VOT-33 (Lawoko-Kerali et al., 2004). This cell line expressed the same isoforms (α3 and α12) as found in the cochlea [Fig. 6(A)]. VOT-33 cells were transfected with Hnf4α shRNA to knock down HNF4α expression and observe the effects of HNF4α deficiency on cell viability. VOT-33 cell transfection efficiency was approximately 30%. Quantitative western blot revealed a 30% decreased
in HNF4α protein expression due to gene silencing [Fig. 6(B)]. This, in turn, resulted in a 29.46% ± 12.78% reduction in cell viability relative to the control cells treated with scrambled shRNA (p = 0.031), as quantified using the MTT assay [Fig. 6(C)]. These \textit{in vitro} data demonstrate a quantitative agreement between the percentage of the VOT-33 transfected cells and the percent decrease in HNF4α protein expression and cell viability due to \textit{Hnf4a} knockdown, suggesting that HNF4α may play a role in cochlear neuroblast survival.

DISCUSSION

We report the first comprehensive network analysis of genes reported to be significantly altered by TTS-inducing noise trauma. Our \textit{in silico} analysis highlights the importance of a known protein, NF-κB, and points to a new candidate, HNF4α, as a potential novel orchestrator of TTS. NF-κB’s emergence as a key player in TTS validates our bioinformatics approach, given its established central role in the cochlear response to noise. NF-κB inhibition is known to activate the pro-apoptotic JNK pathway (via activation of phospho-Jun) within hours of exposure (Caelers et al., 2010), and NF-κB deficiency promotes auditory nerve degeneration and enhanced susceptibility to NIHL (Lang et al., 2006). Importantly, restraint stress activates glucocorticoid receptors and p38 (MAPKs), and protects against acoustic trauma (Meltzer et al., 2009). NF-κB activity can be modified by glucocorticoids \cite{pink arrow, Fig. 1(A)}, which are essentially the only compounds that can ameliorate human NIHL and sudden sensorineural hearing loss, if administered acutely (Zhou et al., 2013).

Cochlear Hnf4α Expression

HNF4α is one of the most ancient members of the steroid hormone receptor superfamily of ligand-dependent transcription factors that bind to regulatory regions of DNA in a species-specific fashion (Sladek et al., 1990). HNF4α is expressed in the liver, pancreatic epithelia, kidneys, stomach, and intestine, and has about 250 direct functional target genes in mouse and human (Babeu and Boudreau, 2014). The human HNF4α gene has 12+ exons and 2 promoters that produce 9 splice variants (isoforms) (Huang et al., 2009). Species-specific differences have been characterized (Harries et al., 2009). The functional significance of such a variety of isoforms remains unclear but important insights come from studying mice. Specifically, mice expressing only isoforms α1, α2, and α3 (P1) have impaired glucose metabolism while mice expressing only isoforms α7, α8, α9, α10, α11, and α12 (P2) have dyslipidemia and impaired liver function (Briancon and Weiss, 2006). Our results

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{\textit{Hnf4a} expression in murine spiral ganglion neurons changed 16 months after exposure to neuropathic noise. (A) \textit{Hnf4a} expression in a 6-week-old unexposed cochlea. \(N = 5\) mice. (B) \textit{Hnf4a} expression in a 70-week-old unexposed cochlea. \(N = 5\) mice. (C) \textit{Hnf4a} expression in a 70-week-old cochlea 16 months after exposure to 97 dB SPL noise. \(N = 5\) mice. \textit{Red}: \textit{Hnf4a} mRNA expression in fluorescence \textit{in situ} hybridization. Scale bars: 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
\end{figure}
indicate that only isoforms a3 and a12 are present in the murine cochlea, representing the P1 and P2 promoters, respectively. Functional consequences of cochlear Hnf4a’s limited repertoire of isoforms remain to be determined.

Hnf4a’s localization to SGNs is interesting because HNF4a is known to regulate neural stem cell differentiation into neurons (Wang et al., 2013), and has been implicated in neurodegenerative diseases (Potashkin et al., 2012). Moreover, Hnf4a’s existence in highly polarized hair cells and surrounding supporting cells is important given the critical role of HNF4a in epithelial polarization (Babeu and Boudreau, 2014) and regulation of tight junctions during hepatogenesis (Babeu and Boudreau, 2014).

In the cochlea, tight junctions between the epithelial cells lining the scala media are essential for normal hearing as they compartmentalize two chemically dissimilar inner ear fluids: endolymph and perilymph (Kikuchi et al., 1995). These tight junctions thereby facilitate establishment of the endocochlear potential, which drives transduction current through hair cells.

**Hnf4a in Cochlear Aging and Neuropathy**

Our findings that (1) Hnf4a expression is substantially upregulated in SGNs at 70 weeks of age relative to 6 weeks of age, (2) neuropathic noise inhibits this age-related increase, and (3) Hnf4a gene silencing in auditory neuroblasts decreases cell viability together suggest that Hnf4a may support SGN survival during aging and may be required for SGN survival after neuropathic noise exposure. Interestingly, the age-related percent increase in cochlear Hnf4a mRNA expression levels from 6 to 70 weeks was similar in magnitude and opposite in direction to the age-related percent decrease in ABR wave I amplitude and number of cochlear neurons that we had previously reported (Jensen et al., 2015). Specifically, ABR wave I amplitude, which is a sensitive measure of cochlear neuropathy, dropped 30% from 6 to 70 weeks of age (Jensen et al., 2015) while here we found that Hnf4a mRNA expression levels increased by 30% during the same period (in the same mouse strain). Taken together, these data suggest that age-related upregulation of Hnf4a expression may be a compensatory response. Further investigation is required to identify the exact mechanism underlying Hnf4a’s ability to regulate SGN survival. It is possible that HNF4a in the cochlea defends against reactive oxygen species (ROS), similar to its role in other cells (Marcil et al., 2010). Both aging and noise trauma are known to increase ROS in the cochlea (Henderson et al., 2006).

Importantly, there is a direct interaction between HNF4a and MAP3K3 (ERK pathway) in the IPA-generated network for HNF4a [Fig. 1(B)]. MAP3K3 (ERK pathway) is upregulated 24 hours after noise-induced TTS (Meltser et al., 2010) in cochlear SGNs and is thought to be part of a protective response to noise trauma.
noise trauma. We found that pathologic ERK activation in spiral ganglion cells contributes to the neurodegenerative phenotype due to osteoprotegerin deficiency, and may underlie the associated sensorineural hearing loss (Kao et al., 2013). To our knowledge, MAPKs activity has not been studied many months after noise exposure, such as in our study.

**HNF4α in Human Disease**

Mutations in human *HNF4α* cause Maturity Onset Diabetes of the Young (MODY) (Yamagata et al., 1996), whereas polymorphisms in the *HNF4α* promoter region are associated with an increased risk of type 2 diabetes (Gupta and Kaestner, 2004). While diabetes mellitus is known to be associated with increased risk of hearing loss (Bainbridge et al., 2011), hearing has not been studied in patients with MODY. Interestingly, the human *HNF4α* gene is located within the DFNB65 locus on chromosome 20 for nonsyndromic hearing loss—a causative gene for this hearing loss remains to be identified. This makes *HNF4α* an attractive candidate gene for human deafness, especially because this gene also emerged as a central player in the network analysis of human deafness genes (Stamatiou and Stankovic, 2013).

Our network analysis of TTS-associated genes admittedly represents a snapshot in time because the knowledge base for network analyses is constantly evolving in accordance with the latest research. We recognize that the *Hnf4α* mRNA transcriptional changes that we have focused on in real-time quantitative RT-PCR and *in situ* hybridization studies do not necessarily reflect similar changes at the protein level. Nonetheless, our finding that HNF4α protein reduction results in reduced viability of auditory neuroblasts *in vitro* supports our observation of transcriptional changes *in vivo*. Taken together, our study motivates future work in investigating specific roles of HNF4α in cochlear physiology and pathology.

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**AUTHOR CONTRIBUTIONS**

J.B.G., S-Y.K and K.M.S. designed research. J.B.G., S-Y.K. and M.C.B. performed experiments. J.B.G., S-Y.K and K.M.S. analyzed data. J.B.G. and K.M.S. wrote the manuscript. All authors critically edited and approved the final version of the manuscript.

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