Published in final edited form as:

Hear Res. 2010 December 1; 270(1-2): 21–27. doi:10.1016/j.heares.2010.10.008.

Ablation of mixed lineage kinase 3 (*MLK3*) does not inhibit ototoxicity induced by acoustic trauma or aminoglycoside exposure

Oksana Polesskaya^a, Lisa L. Cunningham^j, Shimon P. Francis^j, Anne E. Luebke^{d,e}, Xiaoxia Zhu^b, David Collins^a, Olga N. Vasilyeva^b, Julie Sahler^a, Emily A. Desmet^a, Harris A. Gelbard^{a,c,f}, Sanjay B. Maggirwar^a, Joseph P. Walton^b, Robert D. Frisina Jr.^{b,d,e,h,i}, and Stephen Dewhurst^{a,g}

^a Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY. USA

^b Department of Otolaryngology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY. USA

^c Department of Neurology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY. USA

^d Department of Biomedical Engineering, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY. USA

^e Department of Neurobiology and Anatomy, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY. USA

^f Center for Neural Development and Disease, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY. USA

^g James P. Wilmot Cancer Center, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY. USA

^h International Center for Hearing and Speech Research, Rochester, NY 14623

ⁱ National Technical Institute for the Deaf, Rochester Institute of Technology, Rochester, NY 14623

^j Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC

Abstract

Jun N-terminal kinase (JNK) is activated in cochlear hair cells following acoustic trauma or exposure to aminoglycoside antibiotics. Blockade of JNK activation using mixed lineage kinase (MLK) inhibitors prevents hearing loss and hair cell death following these stresses. Since current pharmacologic inhibitors of MLKs block multiple members of this kinase family, we examined the contribution of the major neuronal family member (MLK3) to stress-induced ototoxicity, using

Corresponding author: Stephen Dewhurst, Ph.D., Department of Microbiology and Immunology, University of Rochester Medical Center. 601 Elmwood Ave, Box 672, Rochester, NY 14642. USA, Phone: 585 275 3216; FAX: 585 473 2361, Stephen_Dewhurst@urmc.rochester.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Mlk3^{-/-} mice. Immunohistochemical staining revealed that MLK3 is expressed in cochlear hair cells of C57/BL6 mice (but not in *Mlk3*^{-/-} animals). After exposure to acoustic trauma there was no significant difference in DPOAE and ABR values between *Mlk3*^{-/-} and wild-type mice at 48 hours following exposure or 2 weeks later. Susceptibility of hair cells to aminoglycoside toxicity was tested by exposing explanted utricles to gentamicin. Gentamicin-induced hair cell death was equivalent in utricles from wild-type and *Mlk3*^{-/-} mice. Blockade of JNK activation with the pharmacologic inhibitor SP600125 attenuated cell death in utricles from both wild-type and *Mlk3*^{-/-} mice. These data show that MLK3 ablation does not protect against hair cell death following acoustic trauma or exposure to aminoglycoside antibiotics, suggesting that MLK3 is not the major upstream regulator of JNK-mediated hair cell death following these stresses. Rather, other MLK family members such as MLK1, which is also expressed in cochlea, may have a previously unappreciated role in noise- and aminoglycoside-induced ototoxicity.

Keywords

ototoxicity; aminoglycoside; noise; mixed lineage kinase; cochlea; utricle

1. Introduction

Exposure to aminoglycosides or acoustic trauma leads to the production of reactive oxygen species which trigger apoptotic signaling (Priuska and Schacht 1995; Murai, Kirkegaard et al. 2008) including activation of c-Jun-N-terminal kinase (JNK) (Pirvola, Xing-Qun et al. 2000; Ylikoski, Xing-Qun et al. 2002). Blockade of JNK prevents both aminoglycoside- and noise-induced ototoxicity (Pirvola, Xing-Qun et al. 2000; Ylikoski, Xing-Qun et al. 2002; Wang, Van De Water et al. 2003; Matsui, Gale et al. 2004; Sugahara, Rubel et al. 2006; Eshraghi, Wang et al. 2007; Wang, Ruel et al. 2007).

Mixed lineage kinases (MLK) represent a family of serine/threonine kinases that function as upstream regulators of both JNK and p38 MAPK (Sui, Fan et al. 2006; Hong and Kim 2007). In animal models, aminoglycoside- and noise-induced hearing loss can be inhibited by the pharmacologic MLK inhibitor, CEP-1347 (Pirvola, Xing-Qun et al. 2000; Ylikoski, Xing-Qun et al. 2002). MLK family members include dual leucine zipper kinase (DLK) as well as MLK1, MLK2 and MLK3 (Gallo and Johnson 2002; Wang, Besirli et al. 2004), all of which can be expressed in neurons (Xu, Maroney et al. 2001), and all of which are efficiently inhibited by CEP-1347 *in vitro*, with IC₅₀ values in the range of 20–50 nM (Maroney, Finn et al. 2001). Thus, while previous studies have implicated MLK-mediated JNK activation as an important signaling pathway in ototoxicity, the specific MLK family member involved in JNK activation and hair cell death remains unclear. We therefore conducted experiments to determine whether genetic ablation of the major neuronal MLK family member, MLK3, would inhibit aminoglycoside- and noise-induced ototoxicity.

We used *Mlk3*^{-/-} mice (Brancho, Ventura et al. 2005) and wild-type controls to examine the role of *Mlk3* in aminoglycoside- and noise-induced hair cell death and hearing loss. The susceptibility of wild-type C57/BL6 mice and *Mlk3*^{-/-} mice to noise-induced hearing loss was evaluated by distortion product otoacoustic emission amplitudes (DPOAEs) and auditory brainstem responses (ABRs). To study gentamicin-induced ototoxicity we used a well- characterized *ex vivo* model of the adult mouse utricle (Cunningham 2006). Toxicity was evaluated by measuring gentamicin-induced hair cell death. We found that ablation of *Mlk3* did not inhibit noise-induced hearing loss or gentamicin-induced hair cell death.

2. Methods

2.1. Animals and treatment

All animal procedures were approved by the University of Rochester Committee on Animal Resources. *Mlk3*^{-/-} mice were back-crossed to C57/BL6 animals (Jackson Laboratories) for 10 generations, and then maintained as a homozygous strain (Branchio, Ventura et al. 2005). Age- and gender- matched C57/BL6 mice (Jackson Laboratories) were used as controls. For immunostaining and for noise exposure experiments 4 weeks old male mice were used. Utricles were explanted from 8 week-old mice of both genders.

2.2. Cochlea dissection and immunostaining

Animals were anesthetized with ketamine/xylazine and then perfused with saline followed by 4% paraformaldehyde. Cochleae were then dissected and postfixed for 2 hours in 0.5% paraformaldehyde in 0.01M phosphate saline buffer. Decalcification was performed in 1 liter of 0.12M EDTA for 48 hours, after which cochleae were rinsed with 0.01M phosphate saline buffer and stored in 70% ethanol. Half-turns were dissected from decalcified cochleae with fine forceps and immunostained as wholemounts. Cochlear half-turns were incubated overnight in anti- *Mlk3* antibody (Abgent, cat# 7921a) diluted 1:1000, then for 1 hour in Alexafluor-488 donkey anti-rabbit (Invitrogen) antibodies diluted 1:1000 and counterstained with DAPI. Controls included elimination of primary antibody without otherwise altering the remaining steps of the procedure. The stained tissue sections were mounted onto glass slides with Vectashield (Vector Labs), and imaged using a Zeiss Axioplan upright microscope outfitted with Apotome (Zeiss) to allow for structured illumination.

2.3. Noise exposure

Unanesthetized mice were exposed to octave-band noise (8–16 kHz), for 1 hour at 106 dB SPL. Mice were placed in a small rodent cage with a custom top that allowed for a Panasonic leaf tweeter to be placed 6cm above the center of the cage. Noise intensities measured at the level of the pinna varied by less than 1.0 dB from 106 dB SPL.

2.4. Measurement of Auditory Brainstem Responses (ABRs) and Distortion Product Otoacoustic Emissions (DPOAEs)

Mice were anesthetized with ketamine and xylazine, and ABRs and DPOAE amplitudes and thresholds were then measured. All recording sessions were completed in a soundproof acoustic chamber with body temperature maintained with a hot water heating pad. Only mice with clear ear canals and tympanic membranes were included.

DPOAE—Tucker Davis hardware (TDT; Alachua, FL) was controlled via ActiveX from a custom Matlab R13 (The Mathworks; Natick, MA) graphical user interface. All sound stimuli were generated and signals acquired using Tucker Davis RP2.1 processors running at a sample rate of 195312.5 Hz. All signals were played through two electrostatic speakers (TDT EC1) connected by 4 cm tubes to a probe containing an ER10B+ microphone (Etymotic; Elk Grove Village, IL); the entire speaker and probe assembly was mounted in an adjustable vibration-isolating frame on a micromanipulator arm. All recorded signals were loaded into Matlab for analysis. Waveforms from each individual presentation were windowed using a Hamming window and high-resolution 390625-point FFTs (2x sample rate) were calculated. The resulting FFTs had a bin size of 0.5 Hz allowing for accurate measurement of signal level as a function of frequency. Frequency-domain averaging was used to minimize artifacts; FFTs for multiple repetitions of the same stimulus were averaged together before subsequent analysis.

The probe microphone was calibrated relative to a 1/4" B&K microphone (Type 4938, Bruel & Kjaer; Naerum, Denmark) by placing the probe tip into a coupler (0.1 ml, simulating the mouse ear canal) mounted to the B&K reference microphone. Tones were presented at approximately 65 dB SPL at all possible F1, F2, DP, and noise bin frequencies and the output of the ER10B+ preamp and B&K Measuring Amplifier (B&K Type 2610) was simultaneously captured on two RP2.1 A/D processors. The tones used for this and all subsequent calibrations and measurements were 490 ms in duration with rise/fall times of 5 ms and were repeated 5–10 times. FFTs were calculated to determine the signal level at the presentation frequency for each microphone. In this way, a list of the frequency-dependent differences between the two microphones was created that could be used to convert the ER10B+ output at each frequency to dB SPL. Speakers were calibrated by placing the probe tip into the coupler mounted to a B&K reference microphone. Tones were presented from both speakers at all F1 and F2 frequencies used in the experiment. The output of the ER10B+ was captured using a TDT RP2.1. The resulting dBV was converted to SPL based on the ER10B+ calibration.

DPOAE amplitudes were measured in the following manner: two primaries (F1 and F2) were generated at 65 and 50 dB SPL, respectively. The ratio of the two frequencies was 1.25 and the frequencies were based on geometric mean frequencies ranging from 5.6 to 44.8 kHz. Waveforms of the output of the ER10B+ probe microphone were captured on a TDT RP2.1. FFTs for each presentation were averaged together and the signal level at five frequencies was sampled: F1, F2, DP (2F1-F2), and two noise bins above and below the DP frequency. Following FFT sampling, dBV was converted to SPL based on the ER10B+ microphone calibration.

DPOAE threshold was defined as the F1 level required to produce a DP of 0 dB SPL (+/- 1 dB). We developed an automatic threshold search algorithm implemented in Matlab DP which was interleaved with the DP amplitude measures described above and began with two primaries of identical ratio and geometric mean as above at 65 and 50 dB SPL. Based on the distance of the DP from its target level of 0 dB SPL (distance henceforth: DP error), F1 and F2 level on the subsequent trial was incremented (or decremented) by 0.6 of this distance; e.g., if the DP was at 10 dB SPL, F1-F2 were decremented by 6 dB. F2 level was always F1-15 dB. The 0.6 "approach factor" was determined empirically to be an optimal rate of approach combining rapid acquisition of threshold and minimal oscillation around the target. Due to extremely steep DP I/O functions around 0 dB and the resulting overshoot of DP amplitude, occasionally on successive trials the DP amplitude did oscillate around 0 dB. In each case of oscillation, defined as three trials in which the sign of the DP error changed each trial, the approach factor was automatically made smaller by a factor of 1.5. This iterative procedure allowed rapid convergence on DP threshold while preventing overshoot. Once the DP was measured to be within 1 dB of 0 dB SPL, the identical F1 level was presented again for confirmation. Identification of thresholds required two successive trials of F1 and F2 levels that evoked a 0 dB SPL DP amplitude.

ABR—Needle electrodes were inserted at the vertex (non-inverted) and in the muscle posterior to the left pinna (inverted), with a ground inserted under the contralateral pinna. ABR waveforms were evoked with 5 ms tone pips (0.5-ms rise-fall times) with a $\cos^2$ onset envelope, delivered at 29/sec through a high-frequency leaf tweeter (Panasonic 100THD) placed 20 cm from the left pinna. The response was amplified (10,000 X), filtered (100 Hz – 3 kHz), and averaged using the BioSig (TDT, Gainesville, FL) data-acquisition system. A total of 200 responses were averaged (with stimulus polarity alternated), using an 'artifact reject', whereby response waveforms were discarded when peak-to-peak amplitude exceeded 7 μ V, to prevent contamination by muscle and cardiac activities. Intensity was varied in 5 dB steps starting at 80 dB and decreasing to at least 20 dB below threshold for

the specific test frequency. If no response was detected at 80dB, a value of 100dB was entered for the purpose of graph generation. Each intensity was replicated and threshold was defined as the lowest intensity at which a response was replicated.

2.5. Utricle explants, exposure to gentamicin and quantification of hair cell death

This was performed essentially as described (Cunningham 2006). Briefly, mice were euthanized by overdose of nembutal (Ovation Pharmaceuticals), and the auditory bulla was removed and carefully dissected under sterile conditions. Utricles were cultured free-floating in 24-well tissue culture plates. Culture medium consisted of dissecting medium (BME and EBSS, 2:1 v/v) supplemented with 5% fetal bovine serum. Gentamicin sulfate solution (Sigma) was added to culture medium to a final concentration of 5 mM. No gentamicin was added to control cultures. The JNK inhibitor SP600125 (Sigma) was added to some cultures (indicated in the pertinent Figures) at a final concentration of 10 μ M (Dinh and Van De Water 2009). Utricles were incubated at 37 °C in a 5% CO₂/95% air environment for 24 hours. At the end of the culture period, utricles were fixed overnight at 4°C in 4% paraformaldehyde, then washed in phosphate-buffered saline. For labeling of hair cells, otoconia were removed and utricles were incubated with fluorescently labeled antibodies against Myosin 7a (Proteus Biosciences) and then mounted on glass slides in Fluoromount-G (Southern Biotechnology, Birmingham, AL) and covered with a coverslip. Hair cell densities were measured by counting hair cells in boxes (30 μ m²) placed randomly over the epithelium using a Zeiss Axioplan fluorescent microscope and Zeiss Axiovision software.

2.6. Western blotting

Cochleae were dissected from 6 weeks old mice (*Mlk3*^{-/-} and wild-type) and homogenized in RIPA buffer (Millipore) with Complete protease inhibitor cocktail (Roche) and PhoStop phosphatase inhibitors (Roche) on ice, using a rotor-stator homogenizer. The lysate was centrifuged to remove the bony pellet, and the resulting supernatant was then aliquoted and stored at -20°C. For positive control, the following lysates were used, as recommended by manufacturer of antibodies: for MLK1 – lysate of A-375 cell line (derived from malignant melanoma, Imgenex), 10 ug/lane; for MLK2 – lysate of MOLT4 cells line (lymphoblastic leukemia, Abcam), 10 ug/lane; for DLK – lysate of whole mouse brain tissue, C57/BL6, 25 ug/lane. Protein lysates (25 ug/lane) were separated on 10% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes, and blocked in 1X TBS with 0.1% Tween-20 and 5% w/v nonfat dry milk. Membranes were probed overnight at 4°C with the following antibodies: anti-MLK1 (Abgent #AP7919a), anti-MLK2 (R&D systems, #AF5066), anti-DLK (Santa Cruz Biotechnology, #sc8125), anti-actin (Santa Cruz Biotechnology, #sc7210). After incubating with HRP-conjugated secondary antibodies immunoreactive bands were visualized by chemiluminescence (ECL Plus, GE Healthcare Life Sciences).

2.7. Statistical analysis

Statistical comparison was performed using 2-way ANOVA using Prism (Graph Pad software). The ABRs and DPOAEs curves (Fig. 2) were compared using multiple measures ANOVA. The protective effect of SP600125 against gentamicin-mediated hair cell death was tested by 3-way ANOVA. This statistical test was used because of the 3-way factorial design of the experiment shown in Fig. 4.

3. Results

3.1. MLK3 is expressed in hair cells in mouse cochlea

Whole mount preparations of cochleae from C57/BL6 and *Mlk3*^{-/-} mice were stained with antibodies against MLK3. Figure 1 shows MLK3 expression in cochlear hair cells. Both

outer and inner (not shown) hair cells showed MLK3 immunoreactivity in C57/BL6 cochleae while no staining was evident in *Mlk3*^{-/-} cochleae.

3.2. *Mlk3*^{-/-} mice are not protected against noise-induced hearing loss

Mlk3^{-/-} mice and wild-type mice, were exposed to acoustic trauma. Auditory brainstem responses (ABR) and distortion product otoacoustic emission amplitudes (DPOAE) were measured at baseline (prior to treatment) in all animals, and then 48 hours and 14 days after noise exposure. ABRs reflect the response of the auditory system to acoustic stimuli. DPOAEs measures acoustic energy, generated in the form of otoacoustic emissions (OAEs) that are produced by outer hair cells (OHCs) in the cochlea. We therefore measured ABRs and DPOAEs to perform a robust assessment of hearing impairment in mice.

Both *Mlk3*^{-/-} and wild type mice demonstrated significant temporary threshold shifts (TTSs), measured by ABR 48 hrs after noise exposure ($p = 0.0015$ and 0.0007 respectively, 2-way multiple measures ANOVA). In panel A of Figure 2 ABR thresholds are compared for C57/BL6 (left) and *Mlk3*^{-/-} (right) mice demonstrating that TTS of approximately 60 dB for both control and *Mlk3*^{-/-} mice 48 hours (triangles) following the noise exposure. Recovery from TTSs was assessed by measuring the ABR threshold 2 weeks post exposure. Wild type mice demonstrated a moderate recovery, on the order of 10 dB, of ABR thresholds after 2 weeks, however, no significant recovery was found for *Mlk3*^{-/-} mice ($p = 0.022$ and $p = 0.42$ respectively for the difference between ABRs measured in wild-type and *Mlk3*^{-/-} mice at 48 hrs and 2 weeks post exposure; 2-way multiple measures ANOVA). Both *Mlk3*^{-/-} and wild-type mice demonstrated significant permanent threshold shifts that were not statistically different from one another, indicating that ablation of *MLK3* provided no protection against noise trauma.

DPOAE testing showed similar results. Both *Mlk3*^{-/-} and wild type mice developed statistically significant DPOAE threshold shifts of approximately 40–50 dB 48 hrs after noise exposure ($p = 0.0039$ and 0.0010 respectively for *Mlk3*^{-/-} and wild-type mice) (Figure 2B). Two weeks later DPOAE measurements were not significantly changed in mice of either genotype. Although DPOAEs were not different for *Mlk3*^{-/-} and C57/BL6 mice at baseline, DPOAEs threshold shifts were slightly, but significantly greater in *Mlk3*^{-/-} mice than in wild type mice both 48 hours and 2 weeks post exposure ($p = 0.002$ and 0.0372 correspondingly), suggesting that outer hair cells of *Mlk3*^{-/-} mice were more susceptible to damage by acoustic trauma than those of wild-type mice.

Overall, these data indicate that *Mlk3*^{-/-} mice are not protected from either reversible (temporary) or permanent hearing loss induced by acoustic trauma. In addition, these data suggest that ablation of *MLK3* may increase susceptibility of outer hair cells to noise trauma.

3.3. Hair cells in utricles of *Mlk3*^{-/-} mice are not protected from gentamicin toxicity

The utricle is a part of the vestibular sensory organs of the inner ear. Utricular hair cells are similar to cochlear hair cells in their aminoglycoside sensitivity (Cunningham 2006). We tested whether hair cells in utricles isolated from *Mlk3*^{-/-} mice were resistant to gentamicin toxicity. Gentamicin-treated cultures were compared to control cultures; our results showed that gentamicin caused a robust decrease in hair cell number in utricle explants from both wild-type (9.05 ± 3.05 for gentamicin-exposed cultures vs. 18.29 ± 1.65 for control cultures, mean $\pm$ SD) and *Mlk3*^{-/-} mice (8.79 ± 2.27 for gentamicin-exposed cultures vs. 16.13 ± 1.37 for control cultures; mean $\pm$ SD) (Figure 3). In both cases, the gentamicin-induced hair cell loss was statistically significant when compared to untreated control cultures ($p < 0.0001$, 2-way ANOVA). In contrast, the magnitude of gentamicin-induced hair cell loss in

utricles from wild-type versus *Mlk3*^{-/-} animals was statistically indistinguishable ($p = 0.1088$, 2-way ANOVA). Thus, hair cells from utricles of *Mlk3*^{-/-} mice were not protected against gentamicin-mediated toxicity.

3.4. Hair cell death is mediated by JNK in both wild type and *Mlk3*^{-/-} utricles

It has been shown that gentamicin causes hair cell death by activating JNK (Pirvola, Xing-Qun et al. 2000; Ylikoski, Xing-Qun et al. 2002). We therefore wished to determine whether gentamicin acts through the same pathway in *Mlk3*^{-/-} hair cells. To do this, we evaluated the effect of the JNK inhibitor SP600125 on gentamicin-mediated hair cell death in utricles from wild-type and *Mlk3*^{-/-} mice genotypes. As expected, gentamicin caused robust hair cell loss in wild type and *Mlk3*^{-/-} utricles (Figure 4). SP600125 showed a statistically significant protective effect against gentamicin-induced hair cell loss in utricles from both wild type and *Mlk3*^{-/-} mice ($F_{1,45} = 5.40$, $p = 0.025$), although it did not prevent cell death completely (Figure 4). This protective effect was not statistically different in wild-type and *Mlk3*^{-/-} utricles ($F_{1,45} = 0.31$, $p = 0.578$). These data show that, even in absence of MLK3, gentamicin toxicity was still mediated by JNK.

3.5. Expression of MLK family members in cochlea

In light of these results, we examined whether other MLK family members (in addition to MLK3) are expressed in the inner ear and could therefore mediate toxic effects of acoustic trauma and gentamicin exposure. We performed western blots for MLK1, MLK2 and DLK. The tissue used for this analysis contained both cochlea and utricle. Our results (Figure 5) showed that, among these family members, only MLK1 was expressed in mouse cochlea and utricle. Unexpectedly, expression levels of MLK1 were lower in *Mlk3*^{-/-} than in wild-type mice. MLK2 and DLK were not detected in these tissues.

4. Discussion

Pharmacologic blockade of mixed lineage kinases (MLKs) protects against noise- and aminoglycoside-induced hearing loss (Pirvola, Xing-Qun et al. 2000; Ylikoski, Xing-Qun et al. 2002; Sugahara, Rubel et al. 2006), but it remains unclear which MLK family members contribute to antibiotic- and acoustic trauma-induced ototoxicity. MLK3 is the predominant neuronal MLK family member (Maroney, Finn et al. 2001), and thus a likely candidate to play a major role in this process. We therefore took advantage of an available *Mlk3* knockout mouse strain and performed a series of experiments aimed at examining the role of this specific MLK family member in contributing to the pathogenesis of antibiotic- and acoustic trauma-induced ototoxicity.

We demonstrated that *MLK3* is present in cochlear hair cells of wild-type mice. Activation of MLK3 leads to activation of JNK in neuronal cells (Gallo and Johnson 2002; Sui, Fan et al. 2006), and JNK is a well-known mediator of hair cell death caused by various insults, including noise and aminoglycoside treatment (Pirvola, Xing-Qun et al. 2000; Ylikoski, Xing-Qun et al. 2002; Wang, Van De Water et al. 2003; Eshraghi, Wang et al. 2007; Wang, Ruel et al. 2007). We therefore hypothesized that without functional MLK3 hair cells would be protected against death induced by noise or by gentamicin exposure. However, our results showed that *Mlk3* knockout mice were not resistant to noise-induced hearing loss, as measured by ABRs and DPOAEs. In fact, *Mlk3*^{-/-} mice showed a slight increase in susceptibility to noise trauma, with reduced recovery from temporary threshold shifts. Similarly, *ex vivo* experiments performed using utricles from *Mlk3*^{-/-} mice showed that *Mlk3*-deficient hair cells were not protected from gentamicin-related cell death. A specific pharmacologic inhibitor of JNK (SP600125) attenuated hair cell death in utricles from both

wild type and *Mlk3*^{-/-} mice, showing that gentamicin-mediated hair cell death proceeds through the JNK pathway - even in *Mlk3*^{-/-} animals.

Our data indicate that MLK3 is not the major upstream regulator of hair cell survival following exposure to either noise or aminoglycosides, and that other members of the MLK signaling pathway likely play an important role in this process. To address this possibility, we performed western blot analyses of inner ear tissue lysates using antibodies against MLK1, MLK2 and DLK. Only MLK1 was expressed in cochlea tissue, and it was present in both wild-type and *Mlk3*^{-/-} mice. This finding suggests that (i) there is no gross dysregulation of the expression of other MLK family members in the inner ear tissue of *Mlk3*^{-/-} mice (as might occur as a result of compensatory gene expression changes in response to deletion of MLK3), and (ii) that MLK1 may function as an upstream regulator of hair cell death in response to noise or aminoglycoside insult.

The possible role of MLK1 in noise- and antibiotic-induced ototoxicity is consistent with previous findings that have been interpreted as providing evidence for a requirement for MLK3 in these forms of stress-induced ototoxicity. These previous studies used the pharmacologic inhibitor CEP-1347 (Pirvola, Xing-Qun et al. 2000; Ylikoski, Xing-Qun et al. 2002), which is often referred to as an MLK3 blocker, but which in fact has similar activity against many MLK family members, including MLK1 (Maroney, Finn et al. 2001). Future experiments will be required to more directly address the role of MLK1 in noise- and aminoglycoside-induced ototoxicity.

In conclusion, our data show that MLK3 is not the major upstream mediator of hair cell death and hearing loss in response to noise trauma or aminoglycoside toxicity.

Acknowledgments

We thank Dr. Roger Davis (U. Massachusetts Medical Center) for generously providing the *Mlk3*^{-/-} mice, and Dr. Fu-Shing Lee (MUSC) for performing statistical analyses for this study. We also acknowledge the following grants for providing financial support for these experiments: a post-doctoral Fellowship from the Schmitt Program on Integrative Brain Research (OP), NIH T32 NS051152 (OP), NIH/NIDCD R01 DC007613 (LC), NIH/NIDCD R01 DC007613-S1 (LC), NIH R01 DC003086 (AL), NIH P01 MH064570 (OP, JS, ED, DC, HAG, SBM, SD), NIH P01 AG09524 (RF, JW, OV, XZ).

Abbreviations

JNK	c-Jun-N-terminal kinase
MLK	mixed lineage kinase
ABR	auditory brainstem response
DPOAE	distortion product otoacoustic emission amplitude

References

- Brancho D, Ventura JJ, et al. Role of MLK3 in the regulation of mitogen-activated protein kinase signaling cascades. *Mol Cell Biol* 2005;25(9):3670–3681. [PubMed: 15831472]
- Cunningham LL. The adult mouse utricle as an in vitro preparation for studies of ototoxic-drug-induced sensory hair cell death. *Brain Res* 2006;1091(1):277–281. [PubMed: 16569399]
- Dinh CT, Van De Water TR. Blocking pro-cell-death signal pathways to conserve hearing. *Audiol Neurotol* 2009;14(6):383–392. [PubMed: 19923808]
- Eshraghi AA, Wang J, et al. Blocking c-Jun-N-terminal kinase signaling can prevent hearing loss induced by both electrode insertion trauma and neomycin ototoxicity. *Hear Res* 2007;226(1–2): 168–177. [PubMed: 17098385]

- Gallo KA, Johnson GL. Mixed-lineage kinase control of JNK and p38 MAPK pathways. *Nat Rev Mol Cell Biol* 2002;3(9):663–672. [PubMed: 12209126]
- Hong HY, Kim BC. Mixed lineage kinase 3 connects reactive oxygen species to c-Jun NH(2)-terminal kinase-induced mitochondrial apoptosis in genipin-treated PC3 human prostate cancer cells. *Biochem Biophys Res Commun* 2007;362(2):307–312. [PubMed: 17707342]
- Maroney AC, Finn JP, et al. Cep-1347 (KT7515), a semisynthetic inhibitor of the mixed lineage kinase family. *J Biol Chem* 2001;276(27):25302–25308. [PubMed: 11325962]
- Matsui JI, Gale JE, et al. Critical signaling events during the aminoglycoside-induced death of sensory hair cells in vitro. *J Neurobiol* 2004;61(2):250–266. [PubMed: 15389694]
- Murai N, Kirkegaard M, et al. Activation of JNK in the inner ear following impulse noise exposure. *J Neurotrauma* 2008;25(1):72–77. [PubMed: 18355160]
- Pirvola U, Xing-Qun L, et al. Rescue of hearing, auditory hair cells, and neurons by CEP-1347/ KT7515, an inhibitor of c-Jun N-terminal kinase activation. *J Neurosci* 2000;20(1):43–50. [PubMed: 10627579]
- Priuska EM, Schacht J. Formation of free radicals by gentamicin and iron and evidence for an iron/ gentamicin complex. *Biochem Pharmacol* 1995;50(11):1749–1752. [PubMed: 8615852]
- Sugahara K, Rubel EW, et al. JNK signaling in neomycin-induced vestibular hair cell death. *Hear Res* 2006;221(1–2):128–135. [PubMed: 17005344]
- Sui Z, Fan S, et al. Inhibition of mixed lineage kinase 3 prevents HIV-1 Tat-mediated neurotoxicity and monocyte activation. *J Immunol* 2006;177(1):702–711. [PubMed: 16785569]
- Wang J, Ruel J, et al. Inhibition of the c-Jun N-terminal kinase-mediated mitochondrial cell death pathway restores auditory function in sound-exposed animals. *Mol Pharmacol* 2007;71(3):654–666. [PubMed: 17132689]
- Wang J, Van De Water TR, et al. A peptide inhibitor of c-Jun N-terminal kinase protects against both aminoglycoside and acoustic trauma-induced auditory hair cell death and hearing loss. *J Neurosci* 2003;23(24):8596–8607. [PubMed: 13679429]
- Wang LH, Besirli CG, et al. Mixed-lineage kinases: a target for the prevention of neurodegeneration. *Annu Rev Pharmacol Toxicol* 2004;44:451–474. [PubMed: 14744254]
- Xu Z, Maroney AC, et al. The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. *Mol Cell Biol* 2001;21(14):4713–4724. [PubMed: 11416147]
- Ylikoski J, Xing-Qun L, et al. Blockade of c-Jun N-terminal kinase pathway attenuates gentamicin-induced cochlear and vestibular hair cell death. *Hear Res* 2002;166(1–2):33–43. [PubMed: 12062756]

Figure 1. *Mlk3* is expressed in cochlear hair cells. Cochleae dissected from (A) C57/BL6 and (B) *Mlk3*^{-/-} mice were immunostained using anti- *MLK3* antibody (green) and counterstained with nuclear stain DAPI (red). Three rows of outer hair cells (OHC) are shown. Scale bar is 10 micrometers.

Figure 2. *Mik3*^{-/-} mice are not protected from noise-induced hearing loss. C57/BL6 and *Mik3*^{-/-} mice (N=3 each) were exposed to noise (1 hr, 106 dB SPL). ABR thresholds (**A**) and DPOAE thresholds (**B**) were measured before noise exposure (baseline), and then 48 hour and 2 weeks post exposure. Data represent mean values; error bars represent standard deviation.

Figure 3. Utricle hair cells from *Mik3*^{-/-} mice are not protected from gentamicin-induced toxicity. Utricles were isolated from C57/BL6 and *Mik3*^{-/-} mice and incubated with or without gentamicin. Hair cells were stained with anti-Myosin 7a antibody and counted. **(A)** Representative microphotographs of utricles incubated without (top) or with (bottom) gentamicin. Scale bar is 20 micrometers and applies to all panels. **(B)** Quantification of gentamicin damage to utricle hair cells from C57/BL6 and *Mik3*^{-/-} mice, N = 7–11 utricles per group. Data represent mean values; error bars represent standard error of the mean

Figure 4. JNK inhibitor SP600125 attenuates utricle hair cell death in both wild type and *Mlk3*^{-/-} mice. Utricles were isolated from C57/BL6 and *Mlk3*^{-/-} mice and incubated with or without gentamicin and SP600125. Hair cells were stained with anti-Myosin 7a antibody and counted. Quantification of gentamicin damage to utricle hair cells from (A) C57/BL6 and (B) *Mlk3*^{-/-} mice. N = 6–7 utricles per group. CNTR – not treated, GENT – gentamicin, SP – JNK inhibitor SP600125. Data represent mean values; error bars represent standard error of the mean.

Figure 5.

Western blot analysis of the expression of MLK family members in cochlea. On lane 1 positive controls are shown: for MLK1 - A375 cell line lysate; for MLK2 – MOLT4 cell line lysate; for DLK – mouse brain lysate. Lane 2 – cochlea lysate from C57/BL6 mice; lane 3 - cochlea lysate from *Mlk3*^{-/-} mice. Actin is shown for cochlear samples only to demonstrate equal loading.