Anatomical and functional recovery of the goldfish (*Carassius auratus*) ear following noise exposure

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Summary

Fishes can regenerate lateral line and inner ear sensory hair cells that have been lost following exposure to ototoxic antibiotics. However, regenerative capabilities following noise exposure have not been explored in fish. Moreover, nothing is known about the functional relationship between hair cell damage and hearing loss, or the time course of morphological *versus* functional recovery in fishes. This study examines the relationship between hair cell damage and physiological changes in auditory responses following noise exposure in the goldfish (*Carassius auratus*). Goldfish were exposed to white noise (170 dB re. 1 μPa RMS) for 48 h and monitored for 8 days after exposure. Auditory thresholds were determined using the auditory evoked potential technique, and morphological hair cell damage was analyzed using phalloidin and DAPI labeling to visualize hair cell bundles and nuclei. A TUNEL assay was used to identify apoptotic cells. Following noise exposure, goldfish exhibited a significant temporary threshold shift (TTS; ranging from 13 to 20 dB) at all frequencies tested (from 0.2–2 kHz). By 7 days post-exposure, goldfish hearing recovered significantly (mean TTS<4 dB). Increased apoptotic activity was observed in the saccules and lagenae between 0 and 2 days post-exposure. Immediately after noise exposure, the central and caudal regions of saccules exhibited significant loss of hair bundles. Hair bundle density in the central saccule recovered by the end of the experiment (8 days post-exposure) while bundle density in the caudal saccule did not return to control levels in this time frame. These data demonstrate that goldfish inner ear epithelia show damage following noise exposure and that they are capable of significant regenerative responses similar to those seen following ototoxic drug treatment. Interestingly, functional recovery preceded morphological recovery in the goldfish saccule, suggesting that only a subset of hair cells are necessary for normal auditory responses, at least to the extent that hearing was measured in this study.

Key words: hair cell, fish, saccule, ear, hearing, regeneration, threshold shift, noise exposure.

Introduction

Many vertebrates, including fishes and marine mammals, rely on acoustic signals for communication. In aquatic environments, where sound propagates five times faster than in air, acoustic signals are particularly important for communication over long distances or in low-light environments. Research on a variety of fishes, however, demonstrates sensitive hearing capabilities even in non-vocalizing fishes, leading to the hypothesis that hearing in fishes evolved for analysis of the complete auditory scene, including both biotic (e.g. conspecifics, predators, prey) and abiotic (e.g. rain, waves) components (Popper and Fay, 1993; Popper and Fay, 1997; Ladich, 1999; Ladich, 2000; Fay and Popper, 2000).

Anthropogenic activities such as the use of seismic air guns and sonar, and increased shipping traffic are introducing a significant amount of additional noise into the aquatic environment and are potentially affecting hearing and acoustic communication in fishes and marine mammals (Myrberg, Jr, 1990; Popper, 2003; Popper et al., 2004; Wartzog et al., 2004). Public and scientific interest has primarily focused on mammalian hearing and the effects of noise pollution on the mammalian auditory system (e.g. Erbe and Farmer, 2000; Au et al., 1997; Kastack et al., 1999; Costa et al., 2003; Nachtigall et al., 2003; Wartzog et al., 2004) but relatively few studies have been directed at understanding the effects of noise exposure on fishes (e.g. Hastings et al., 1996; McCauley et al.,

2003; Popper et al., 2005; Wysocki and Ladich, 2005; Wysocki et al., 2006).

Exposure to broadband noise can cause temporary threshold shifts (TTS) in goldfish (*Carassius auratus*) and catfish (*Pimelodus pictus*), both otophysan fishes with relatively sensitive hearing thresholds (Amoser and Ladich, 2003; Smith et al., 2004a; Smith et al., 2004b). Fishes with less sensitive hearing do not appear to be as susceptible to TTS under identical conditions (Scholik and Yan, 2002; Smith et al., 2004a), although exposure to much more intense sounds produced by a seismic air gun does produce TTS in at least some non-otophysan fishes (Popper et al., 2005).

The studies discussed above clearly show that fish hearing may be affected by exposure to sounds that are above the normal ambient levels to which the animals are exposed. However, only a few studies have examined inner ear morphology following noise exposure in fishes (Enger, 1981; Hastings et al., 1996; McCauley et al., 2003), and no studies have looked at the correlation between structural and functional damage. By contrast, the relationship between auditory function and structure following auditory trauma has been closely examined in birds and mammals (e.g. Boettcher et al., 1992; Saunders et al., 1992; Adler et al., 1993; Subramaniam et al., 1994; Saunders et al., 1995; Pourbakht and Yamasoba, 2003). These studies show that noise exposure causes significant morphological damage such as auditory hair bundle loss and hair cell death and significant physiological damage by various measures including eighth nerve compound action potentials (CAP), distortion product otoacoustic emissions (DPOAEs), and auditory evoked potentials (AEPs) (Boettcher et al., 1992; Saunders et al., 1992; Subramaniam et al., 1994; Pourbakht and Yamasoba, 2003).

Studies in the previous few decades have shown that birds, but not mammals, can repair or replace auditory hair cells damaged during sound exposure, and that morphological regeneration is correlated with functional recovery of auditory capabilities (Corwin and Cotanche, 1988; Stone and Cotanche, 1992; Stone and Cotanche, 1994; Cotanche, 1999; Smolders, 1999). Avian ears also exhibit regenerative capacity following exposure to aminoglycoside antibiotics and other ototoxic chemicals that appears similar to regeneration following acoustic overstimulation (Lippe et al., 1991; Janas et al., 1995; Stone et al., 1996; Roberson et al., 2004). Fishes, like birds, are able to regenerate sensory hair cells that are damaged by the application of ototoxic antibiotics (Yan et al., 1991; Lombarte et al., 1993) (P. Razdan, A.B.C. and A.N.P., unpublished data). However, it is unknown if regenerative capability is present in fish ears following intense noise stimulation.

Additional open questions concern the functional relationship between hair cell damage and hearing loss, and the time course of hair cell and functional recovery in fishes. To investigate these questions, we examined the physiological and morphological effects of exposure to continuous white noise on hearing in goldfish. Our goal was to determine the relationship between functional hearing ability and morphological damage and recovery in the auditory system of noise-exposed goldfish.

Materials and methods

Experimental animals

We chose goldfish *Carassius auratus* L. as a model species because they belong to the Superorder Otophysi, a taxonomic group of fishes having anatomical specializations (Weberian ossicles that connect the swim bladder to the inner ear) that enhance hearing sensitivity and bandwidth (von Frisch, 1938; Ladich and Wysocki, 2003). Goldfish are a popular model for fish hearing studies and show sophisticated auditory capabilities including tone discrimination and auditory scene segregation (Fay, 1992; Fay, 1998; Fay, 2000; Fay, 2005). As a result of their auditory sensitivity, goldfish and other otophysans (including the closely-related zebrafish, *Danio rerio*) are probably more susceptible to noise-induced auditory damage than fishes without specializations that enhance hearing sensitivity. Previous studies have shown that goldfish do indeed exhibit noise-induced hearing loss followed by a recovery in hearing capabilities (Smith et al., 2004a).

Goldfish were obtained from a local hatchery and maintained in multiple 76-l glass aquaria. Standard length means (± s.e.m.) for goldfish were 9.4±0.6 cm. All work was done under the supervision of the Institutional Animal Care and Use Committee of the University of Maryland.

White noise exposure

Fish were exposed to white noise with a bandwidth ranging from 0.1 kHz to 10 kHz at 170 dB re. 1 μPa RMS sound pressure level (SPL). The sound was generated using a Sony Portable MiniDisc Recorder (Model MZ R900) connected through an amplifier (5.2 Amp monoblock, AudioSource, San Francisco, CA, USA) to an underwater speaker (UW-30; Underwater Sound Inc., Oklahoma City, OK, USA) placed centrally on the bottom of a 19-l cylindrical chamber. White noise, defined as having a flat power spectrum across the entire bandwidth (i.e. all frequencies are presented at the same SPL), was computer-generated using Igor Pro software. This sound file was recorded previously to a MiniDisc and played back in a loop continuously for 48 h. Characteristics of the noise exposure (bandwidth and SPL) were similar in all experiments, with transduction in the chambers having little effect on the digitally generated flat 'white noise' spectra. These spectra were similar to those previously reported for experiments using a similar stimulus (Fig. 1) (Smith et al., 2004a; Smith et al., 2004b). The sound stimulus was recorded through a Type 10CT (GRAS Sound and Vibration, Denmark) hydrophone connected to a 5010B dual-mode amplifier (Kistler Instrument Corp., Amherst, NY, USA), and measured using a TDS 2012 oscilloscope (Tektronix, Inc., Beaverton, OR, USA). Total SPLs were calculated using the measured RMS voltages of the noise stimulus and a Type 42AC pistonphone calibrator. The exposure SPL of 170 dB re. 1 μPa RMS is equivalent to a power spectral density of approximately 124 dB re. 1 μPa^2 Hz^{-1}. The SPL of the noise exposure varied within the chamber from 170 dB re. 1 μPa RMS 1 cm directly above the speaker to 166–169 dB re. 1 μPa RMS at 8–14 cm above the
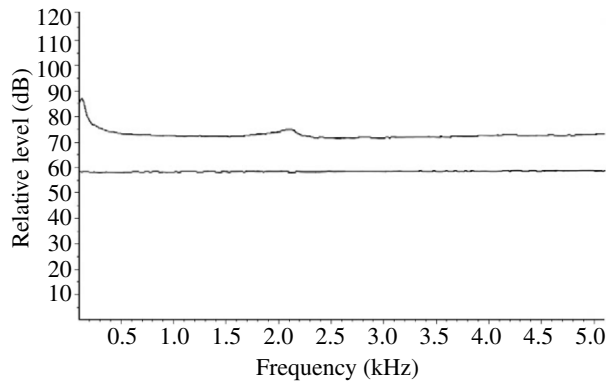
Fig. 1. The power spectra level of the 170 dB re. 1 μPa white noise stimulus (top curve) used for noise exposure experiments and control levels (bottom curve). The sounds were recorded by a hydrophone placed centrally within the noise exposure container at a depth of 23 cm under the water surface, 1 cm above the underwater speaker.

speaker. The SPL of the control chamber (see below) ranged from 110–125 dB re. 1 μPa RMS.

Fish were placed in each of two 19-l sound exposure chambers and exposed to the noise stimulus for 48 h. Following noise exposure, some fish were removed for immediate use and the remaining fish were transferred to 114-l aquaria for the appropriate recovery period (see below). Control fish were placed in the chamber for 48 h with the speaker in place but not active. Baseline fish, used to control for potential effects of handling or confinement stress, were kept in standard 38-l aquaria in a common fish holding facility prior to euthanasia and experimental use. An airstone was placed in each experimental and control chamber to provide proper aeration.

Auditory evoked potential technique

Hearing thresholds, determined using the auditory evoked potential (AEP) technique, were measured immediately after noise exposure (designated as day 0) and following 1, 2, 4 or 7 days of recovery (N=6 for controls and noise-exposed fish for each recovery day for a total of 36 fish).

AEP is a non-invasive method of measuring neural responses to auditory stimuli and is commonly used for measuring hearing in fishes and other vertebrates (Corwin et al., 1982; Kenyon et al., 1998; Higgs et al., 2001; Smith et al., 2004a; Smith et al., 2004b; Wysocki and Ladich, 2005). Each fish was lightly anesthetized with buffered MS-222 (tricaine methanesulfonate; Sigma-Aldrich, St Louis, MO, USA), restrained in a mesh sling, and suspended under water in a 19-L plastic vessel. The fish was suspended so that the top of the head was approximately 6 cm below the surface of the water and 22 cm above the underwater speaker.

Stainless steel subdermal electrodes (27 ga: Rochester Electro-Medical, Inc., Tampa, FL, USA) were used to record auditory evoked potentials. A reference electrode was inserted approximately 2 mm subdermally into the medial dorsal surface of the head between the anterior portion of the eyes, and a recording electrode was placed 2 mm into the dorsal midline surface of the fish approximately halfway between the anterior insertion of the dorsal fin and the posterior edge of the operculae, directly over the brainstem. A ground electrode was placed in the water near the body of the fish.

Sound stimuli were presented and AEP waveforms collected using SigGen and BioSig software running on a TDT physiology apparatus (Tucker-Davis Technologies Inc., Alachua, FL, USA). Sounds were computer-generated *via* TDT software and passed through a power amplifier connected to the underwater speaker. Tone bursts had a 2 ms rise and fall time, were 10 ms in total duration, and were gated through a Hanning window [similar to the conditions of other AEP studies (e.g. Mann et al., 2001; Higgs et al., 2001)]. Responses to each tone burst at each SPL were collected using the BioSig software package, with 1000 responses averaged for each presentation. The SPLs of each presented frequency were confirmed using a calibrated underwater hydrophone (calibration sensitivity of –195 dB re. 1 V/μPa; ±3 dB, 0.02–10 kHz, omnidirectional, GRAS Type 10CT, Denmark). Auditory thresholds were determined by visual inspection of auditory brainstem responses as has been done in previous studies (Higgs et al., 2001; Smith et al., 2004a; Smith et al., 2004b).

Morphological assays

Hair cell bundle loss and apoptotic cell death were quantified in epithelia of goldfish exposed to 48 h of white noise. Thirty-six additional fish were exposed to the noise stimulus as described for AEP above. Six additional control fish and two baseline fish were also used. Fish were killed with an overdose of buffered MS-222 immediately after noise exposure (day 0) and following 1, 2, 3, 5 or 8 days of recovery (N=6 fish per time point). The bony capsule surrounding the ear was opened and the heads were fixed for 1–4 h in 4% paraformaldehyde dissolved in 0.1 mol l^{-1} phosphate-buffered saline (both from Sigma). Ears were then removed from the head and the otolithic epithelia were carefully isolated.

Right ears (saccules, utricles, and lagenae) from each fish were processed using a TUNEL assay to label apoptotic cells (ApopTag peroxidase kit, Serologicals Corporation, Norcross, GA, USA). Processing followed the manufacturer's protocols with modification from Wilkins et al. (Wilkins et al., 2001). Left saccules from each fish were labeled with Oregon Green phalloidin (Molecular Probes/Invitrogen, Carlsbad, CA, USA) to visualize actin, the primary protein component of hair bundles. In all cases epithelia were mounted whole and a coverslip placed on top.

An additional double-labeling experiment was performed to determine the fate of hair cell bodies following noise exposure. Six additional goldfish were exposed to noise and killed either immediately following exposure (N=3 fish) or after 1 day of recovery (N=3 fish), and fixed as described above. Both saccules were removed from each fish and labeled with Oregon Green phalloidin and DAPI (both from Molecular Probes) for double-labeling of hair bundles and hair cell nuclei, respectively. Five

baseline (control) goldfish were sacrificed immediately after purchase and processed in an identical manner.

Epithelia were viewed with a Zeiss Axioplan (Germany) compound microscope equipped for epifluorescence (to view phalloidin and DAPI labeling) and differential interference contrast microscopy (for visualization of TUNEL label). Images were taken with a Zeiss MRc5 digital CCD camera and analyzed with Zeiss Axiovision software. For quantitative analysis of hair bundle loss, four regions of each saccule were chosen for study based on preliminary results that indicated differences in the degree of bundle loss across the rostral–caudal axis of the saccule. The four 2500 μm^2 areas were selected at positions of approximately 5%, 25%, 50% and 75% of the distance from the rostral to the caudal tip of each saccule. All hair bundles within each area were counted. Hair bundle counts were not performed for utricles and lagenae because pilot studies did not show a decrease in hair bundles in these epithelia. Cells were counted in double-labeled tissue (DAPI and phalloidin) in the same manner as for single-labeled phalloidin tissue except that the 5% position was not included. All DAPI-labeled nuclei were counted in the hair cell layer of each region. Double-labeled epithelia were viewed with a Zeiss Axioskop 2 microscope and analyzed with Northern Eclipse software (v. 7, Empix Imaging). For TUNEL-labeled tissue, all labeled cells were counted for each whole-mounted epithelium.

Statistical analysis

The effects of time following noise exposure on fish auditory threshold levels were tested using analysis of variance (ANOVA) with day post-noise exposure and frequency as factors. Tukey's *post-hoc* test was used to make pairwise comparisons between specific frequencies and days when significant main effects were found (Zar, 1984). Regression analysis was used to test for relationships between time following noise exposure and the resulting temporary threshold shift (TTS). These threshold shifts are defined as temporary since they decrease to near control levels after recovery from noise exposure. For this analysis, mean TTS for each day was averaged across the six frequencies at which hearing thresholds were determined (200, 400, 600, 800, 1000 and 2000 Hz), so that each point was calculated using 36 thresholds (N=6 fish ×6 frequencies).

Morphological changes in hair bundle number and apoptotic cell counts were tested using separate one-way ANOVAs. Tukey's *post-hoc* test was used to make pairwise comparisons when significant main effects were found. For hair bundles, a separate ANOVA was performed for each region of the saccule with day following noise exposure as the independent factor. For TUNEL-labeled cells, a separate ANOVA was performed for each sensory organ (saccule, lagena, and utricle) with day following noise exposure as the independent factor. To examine potential differences in numbers of hair cell bundles and nuclei in double-labeled epithelia, two-way ANOVAs were performed, with day post-noise exposure and hair cell component (bundle or nuclei) as factors.

Results

Effects of noise on auditory thresholds

Goldfish auditory sensitivity was the same for control and baseline fish, with best hearing between 400 and 1000 Hz, as found in previous studies (Fig. 2) (Smith et al., 2004a; Smith et al., 2004b). Control and baseline similarity demonstrated that no hearing loss was due to confinement in the exposure containers. Thus only control values were used for statistical analysis of auditory thresholds.

All post-exposure auditory thresholds were significantly higher than controls ($P \le 0.001$; Fig. 2A). Although auditory thresholds decreased with time of recovery ($P < 0.001$), the general shapes of the audiograms remained constant over time (i.e. there was no significant interaction between frequency and day post-exposure). TTS differed significantly across frequencies ($P = 0.02$), being greatest at 1000 Hz where control goldfish hearing sensitivity is greatest, and least at 100 and 2000 Hz (Fig. 2B).

Noise-exposed goldfish exhibited significant threshold shifts ($P < 0.001$) immediately after noise exposure, with a mean TTS of 16 dB averaged across all frequencies (Fig. 3). TTS decreased linearly with time of recovery so that TTS after 7 days of recovery was approximately 4 dB, a small but still significant ($P = 0.001$) threshold shift.
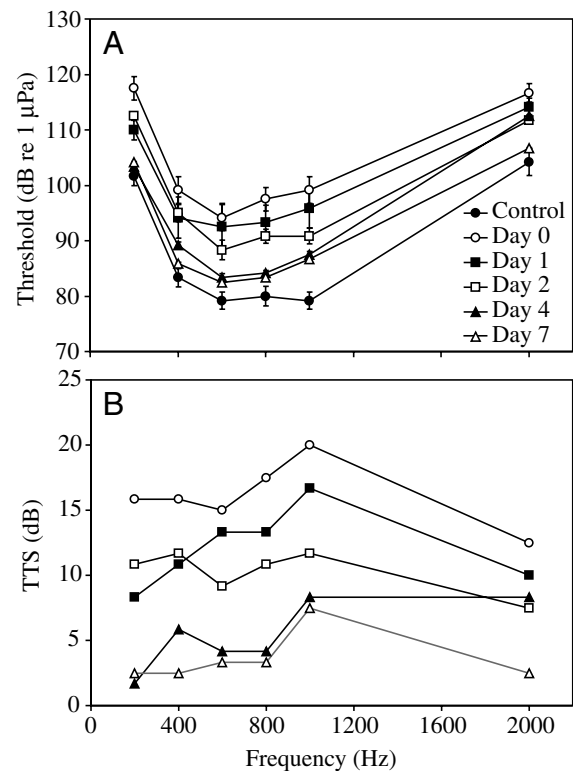

Fig. 2. (A) Auditory thresholds of control and experimental goldfish at various times following 48 h of white noise exposure. Day 0, goldfish tested immediately following exposure. (B) Mean TTS (temporary threshold shift) at each frequency calculated as threshold after noise exposure (from A) minus mean control levels. Values are means ± s.e.m.; N=6 per data point.
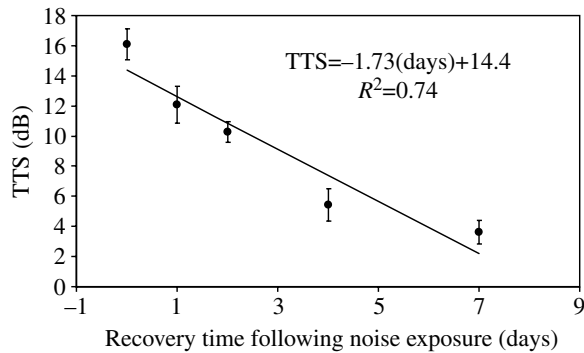
Fig. 3. Temporary threshold shift (TTS) of goldfish at various times following 48 h of white noise exposure. Day 0, goldfish were tested immediately following exposure. Values are means ± s.e.m.; N=6 per data point (one mean value of six fish for each of six frequencies). The line represents the linear regression equation for the data shown.

Fig. 4. (A) Drawing showing the four $2500~\mu m^2$ regions of the saccular macula where hair bundles were quantified (see Materials and methods for details). (B) Numbers of hair bundles in each saccular region by day; day 0 is immediately following 48 h of noise exposure; B, baseline animals that were killed prior to the experiment; C, control animals that were held in the experimental set-up for 48 h without the sound stimulus. Values are means ± s.e.m.; N=6 per data point for controls and days 0–8; N=2 for baseline. Asterisks indicate significant differences from baselines and controls (P<0.05).

Effects of noise on sensory epithelia

Exposure to white noise for 48 h caused significant hair bundle loss in the saccules of all goldfish relative to control and baseline fish. Hair bundle loss was greatest in the caudal region of the saccule (P<0.001; Fig. 4) and reached a maximum 24 h after the noise exposure was terminated, with 83.6% of hair bundles lost in this region (Figs 4, 5). Significant hair bundle loss was also seen in the center (50% region along the rostral-caudal axis) of the saccule (P<0.001, Fig. 4) reaching a maximum of 68% hair bundle loss at 5 days post-exposure. The double-labeling experiment showed that hair bundle numbers in all three epithelia regions examined (caudal, central, mid-rostral) were not significantly different from hair cell nuclear counts in either control or noise-exposed saccules (P>0.48 for each region, Fig. 6), indicating disappearance of the entire hair cell. There was no damage to the two rostral regions of the epithelium in the single-labeling experiment (P>0.5 for each region) although there was damage in the mid-rostral region (25% rostrocaudal axis) in the double-labeling experiment (P<0.01) immediately following noise exposure. There were no differences in hair bundle density between baseline and control animals (P>0.99 for each region, Fig. 4), demonstrating that holding the animals in the noise exposure chambers for 48 h did not damage inner ear epithelia.

Examination of saccular epithelia using fluorescence microscopy revealed characteristic signs of damage such as scar formations caused by abutting of adjacent supporting cells (arrow in Fig. 5C) (Li et al., 1995; Forge and Li, 2000). Intact cuticular plates with missing hair bundles were occasionally observed as well (arrowhead in Fig. 5C), although this may result from either acoustic trauma or dissection damage since this was seen occasionally in the control and baseline animals.

Hair bundle density in the caudal saccule increased by 37% following 8 days of recovery from noise exposure. However, bundle density was still significantly reduced as compared to control levels (P<0.001). In the central region of the saccule
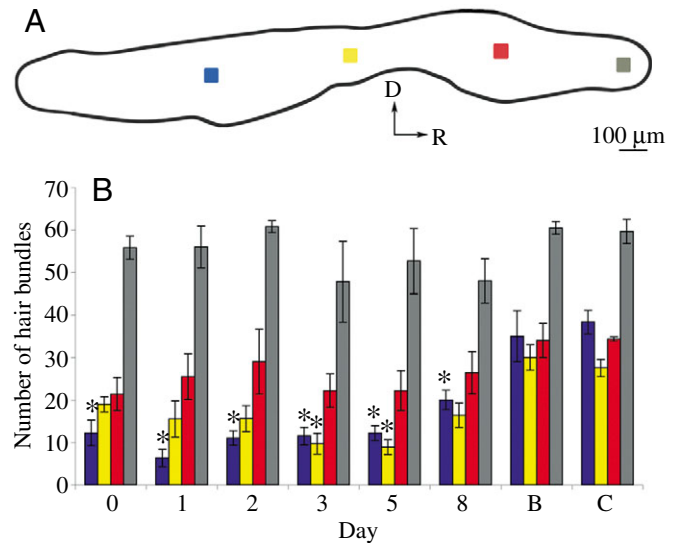
bundle density after 8 days of recovery was not significantly different from control levels (P=0.144).

Significant apoptosis was observed in the saccules of noise-exposed goldfish as compared to control and baseline fishes (P<0.001). Maximum apoptotic cell death occurred immediately following noise exposure (P<0.001) and decreased to control levels after 3 days of recovery (P=0.744; Fig. 7). There were no differences in the numbers of TUNEL-labeled cells between baseline and control fish, with an average of seven apoptotic cells seen in control saccules (Fig. 7).

Interestingly, there was also a significant increase in TUNEL-labeled cells in the lagena (P=0.037; Fig. 7). This increase was manifested as a spike in apoptotic cells after 24 h of recovery (day 1) as compared to later time points (P=0.038 when compared to day 5). There was no significant increase in apoptosis in the utricle at any time point as compared to control levels (data not shown).

Discussion

This study is the first to examine physiological and morphological changes simultaneously following noise-induced damage and recovery in a fish. Functional studies using noise exposure paradigms similar to those employed here have found significant TTS but have not examined the morphological correlates underlying these threshold shifts (Scholik and Yan, 2001; Amoser and Ladich, 2003; Smith et

al., 2004a; Smith et al., 2004b). We demonstrate here that exposure to white noise causes both significant TTS and morphological damage in goldfish. Signs of morphological damage include a significant increase in apoptotic cells and a significant decrease in hair bundle density in the noise-exposed goldfish saccule. Changes in hair bundle density paralleled changes in hair cell nucleus density, indicating that entire hair cells disappeared following noise exposure.

Although our white noise stimulus contained both sound pressure and particle motion components, our hydrophone
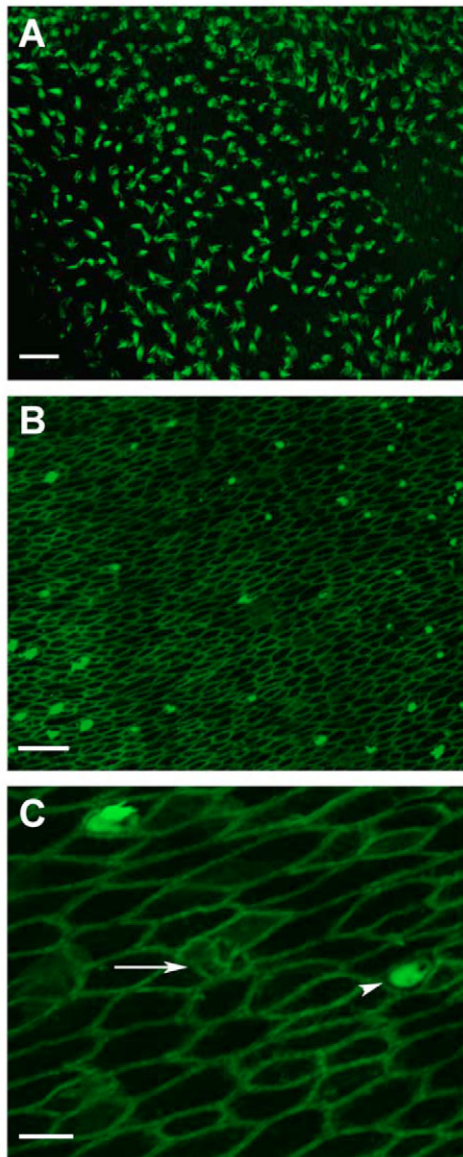

Fig. 5. Phalloidin-labeled saccular epithelia showing evidence of hair bundle loss following exposure to noise. (A) Control tissue from the caudal end of the saccule showing normal complement of hair bundles. (B) The same region of the saccule in an animal following 48 h of noise exposure and 1 day of recovery. (C) High magnification image of the saccule in B showing a scar formation characteristic of hair cell loss (arrow) and a bundleless cuticular plate (arrowhead). Scale bars, (A,B) 20 μm, (C) 5 μm.
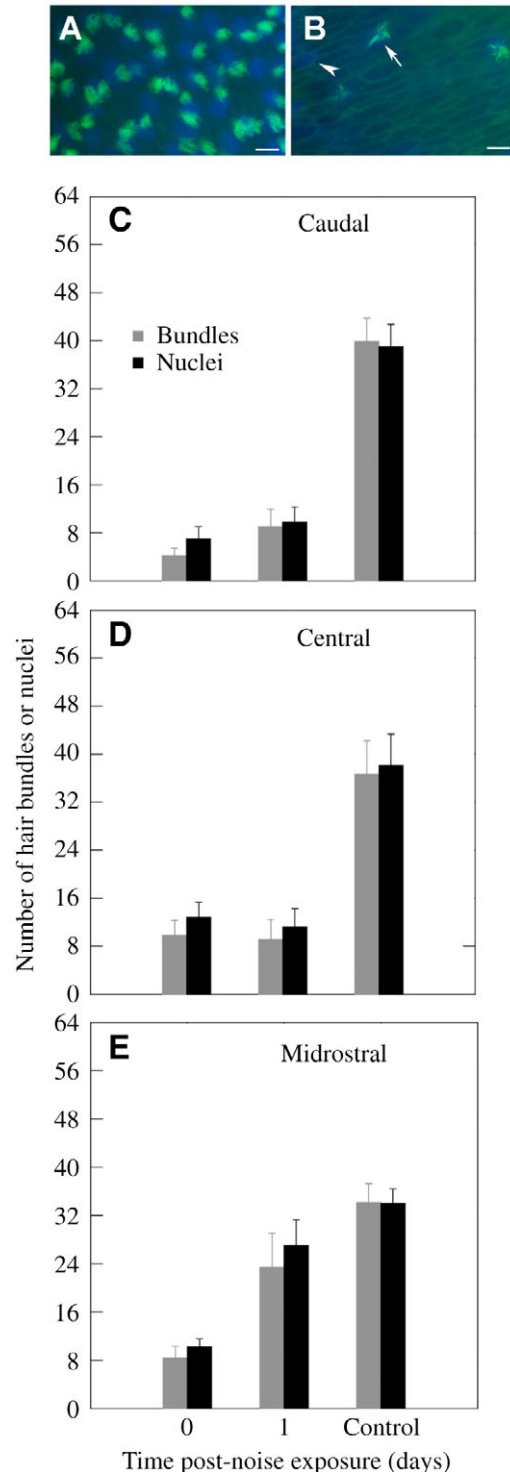

Fig. 6. (A) Caudal saccule from a control goldfish double-labeled with phalloidin and DAPI, showing hair cell nuclei (blue) and associated hair bundles (green). (B) Caudal saccule from a goldfish exposed to 48 h of white noise and then allowed to recover for 1 day. The arrow indicates a remaining hair bundle, and the arrowhead indicates a hair cell nucleus without a hair bundle. Scale bars, 10 μm. (C–E) Numbers of hair cell bundles and nuclei counted from 2500 μm^2 regions located at (C) 75% (caudal), (D) 50% (central) and (E) 25% (mid-rostral) along the rostrocaudal axis of the saccular macula following noise exposure. Values are means ± s.e.m.; N=3 (days 0–1); N=5 (controls).
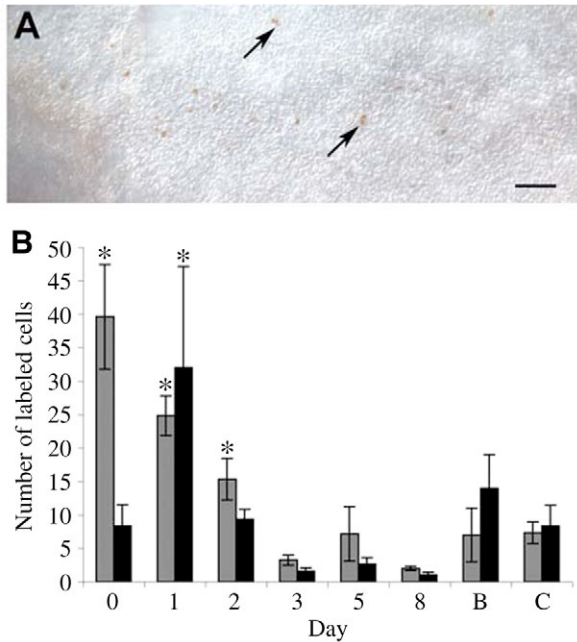
Fig. 7. (A) Caudal region of the saccular epithelium immediately after noise exposure (day 0). Arrows point to examples of TUNEL-labeled (apoptotic) cells visualized by brown DAB labeling. Scale bar, 50 μm. (B) TUNEL-labeled cells in the goldfish saccule (gray) and lagena (black) at specific days following 48 h white noise exposure. Labeled cells were counted for each whole-mount epithelium. Values are means ± s.e.m.; *N*=6 (controls + days 0–8); *N*=2 (baseline). Asterisks indicate significantly elevated cell counts relative to baseline (B) and control (C) values (*P*<0.05).

recorded only the pressure component, and we did not measure the particle motion sound field. Goldfish are more sensitive to the pressure component of sound than fishes without adaptations such as Weberian ossicles or other mechanical means of connecting the swim bladder to the ear. Therefore, it is likely that both the pressure and particle motion components of the stimulus contributed to the hair cell damage we observed.

After 48 h of white noise exposure, the average TTS (across all frequencies) was approximately 16 dB, with maximal TTS being 20 dB at 1 kHz. This threshold shift is comparable to the 21 dB TTS found by exposing goldfish to 21 days of white noise at similar intensities as used in this study (Smith et al., 2004a). This suggests that a maximal threshold shift occurs in goldfish within the first 48 h of noise exposure. Greater threshold shifts (>20 dB) have been observed in lake chub (*Couesius plumbeus*, another otophysan fish) exposed to much higher amplitude short-term seismic airgun signals (Popper et al., 2005) and in two other goldfish studies. A previous study exposed groups of goldfish to white noise ranging in intensity from 130 to 170 dB re. 1 μPa for 24 h (Smith et al., 2004b). They found that TTS was a linear function of noise SPL, with TTS being approximately 7 and 32 dB at intensities of 130 and 170 dB re. 1 μPa, respectively. Goldfish and catfish (*Pimelodus pictus*) exposed to 158 dB re. 1 μPa white noise for 24 h exhibited a maximal TTS of 26 and 32 dB, respectively (Amoser and Ladich, 2003). It is unclear why threshold shifts

were smaller in the current study compared to these other studies with noise exposures of equal or lesser sound pressure levels, but it is likely due to differences in sound sources and calibrations between studies.

In this study, TTS varied across frequencies, being greatest where control goldfish hearing thresholds were the lowest and least where the thresholds were the highest. This result follows the prediction of the Linear Threshold Shift (LINTS) hypothesis (Smith et al., 2004b), which states that TTS is a function of the difference between the baseline hearing threshold SPL at a particular frequency and the SPL of the noise exposure. Thus for a white noise stimulus with constant SPL across frequencies, the greatest TTS would be expected at the frequency where the fish is most sensitive.

After goldfish were allowed to recover from noise exposure, their hearing improved dramatically over the period of a week but did not quite return to control levels (mean TTS of 4 dB). In a previous study, goldfish exposed to white noise for 24 h also exhibited significant, but not complete, recovery of hearing 18 days post-noise exposure (Smith et al., 2004a). Surprisingly, after a longer-term (21 days) exposure at slightly lower noise intensities, goldfish had a TTS of 4 dB after a 7 days recovery (as in this study), but returned to control levels 14 days post-exposure (Smith et al., 2004a). In a similar study, goldfish were exposed to 158 dB re. 1 μPa white noise for up to 24 h and recovered from TTS after only 3 days (Amoser and Ladich, 2003), suggesting an inverse relationship between sound intensity, exposure time, and recovery time. Conversely, hearing thresholds in lake chubs exposed to 20 seismic air gun shots returned to control levels after 18 h of recovery, demonstrating that fast recovery from very intense noise is possible if that noise is of sufficiently short duration (Popper et al., 2005). Experiments in goldfish exposed to varying sound intensities with longer recovery times are necessary to fully explore the relationship between stimulus intensity and recovery from TTS.

Significant hair cell loss occurred in the caudal and central regions of the goldfish saccule, although the degree of damage was different between these two regions. In the caudal saccule, the greatest loss of hair cells occurred during the 48 h of noise exposure and continued following 1 day of recovery. This pattern of hair cell loss coincides with the period of maximum apoptosis, suggesting that hair cells in the caudal saccule are dying as a result of programmed cell death. In the central saccule, maximum bundle loss was observed after 3–5 days of recovery from noise, indicating ongoing degeneration following cessation of noise. Progressive post-exposure development of noise-induced morphological damage has also been noted in other teleost fishes and in the mammalian cochlea (Hastings et al., 1996; Bohne et al., 1999; McCauley et al., 2003; Yamashita et al., 2004). Significant apoptosis was only detected for 2 days after noise exposure, suggesting that some dying hair cells in the central saccule may have retained their bundles for one or more days before the bundle degenerated. Similar observations have been seen in the inner ears of birds and mammals following ototoxic drug administration, where dead

hair cells were ejected from the epithelium while the bundles were still intact (Forge and Li, 2000; Mangiardi et al., 2004).

Hair bundle (and presumably hair cell body) density in the caudal saccule, where the greatest degree of damage was observed, did not return to control levels by the end of this study (8 days after noise exposure). By contrast, significant morphological recovery occurred in the central saccule, a region showing lesser but still significant damage, after 8 days of recovery. Hair cell regeneration in amphibians and chicks occurs *via* both mitotic and non-mitotic mechanisms (Adler and Raphael, 1996; Adler et al., 1997; Baird et al., 2000; Roberson et al., 2004; Taylor and Forge, 2005). Studies with mitotic blockers show that hair cells that develop early in the recovery process (3–4 days after aminoglycoside exposure) arise by direct transdifferentiation of supporting cells into hair cells (Roberson et al., 2004). Hair cells that develop later in the recovery process (after 5 days) may be labeled with cell proliferation markers, indicating that these cells arise from supporting cells that undergo mitotic division (Roberson et al., 2004).

Direct transdifferentiation has not been specifically demonstrated in the ears of teleost fishes, but it is reasonable to hypothesize that supporting cell to hair cell conversion contributes to the early phase of recovery presently observed in the goldfish saccule. Mitotic generation of hair cells has been previously documented in fishes and this mechanism is most likely responsible for the later regenerative events (Lanford et al., 1996; Wilkins et al., 1999). However, complete repopulation of a severely damaged region such as the caudal saccule may take longer than the time course examined in the present study. In one ototoxic drug-damage study in the newt (*Notophthalmus viridescens*), new hair cells were observed after 12 days of recovery (Taylor and Forge, 2005), further indication that complete morphological recovery in the goldfish would likely take longer than the 8 days examined in this study. Additional studies using mitotic blockers and cell proliferation markers are needed to examine the contributions of each regenerative mechanism to hair cell recovery in the goldfish saccule.

It is noteworthy that maximum damage occurred in the caudal saccule, with less severe damage in the rostral end. Single-unit recording studies in the goldfish saccule show a crude level of tonotopic organization, although nowhere approaching the exquisite tonotopy found in birds and mammals. The caudal region responds to lower frequencies and lesser sound intensities than rostral hair cells (Furukawa and Ishii, 1967; Sento and Furukawa, 1987; Sugihara and Furukawa, 1989). Damage to the rostral region was observed only in the double-labeling experiment, where the presence of fewer fish in the exposure chamber may have lessened attenuation and therefore contributed to greater stimulus intensity. Therefore, it may be the case that the more sensitive hair cells were primarily damaged in the present study and that greater sound intensities may be necessary to severely damage rostral hair cells. Similarly, the saccule of the Atlantic cod *Gadus morhua* may also be tonotopically organized. When Enger (Enger, 1981) exposed cod to intense tones ranging from 50 to 350 Hz, low frequency tones damaged hair cells in the

caudal region, and higher frequency tones damaged hair cells in the rostral region of the saccule.

It is also possible that rostral hair cells sustained damage to extracellular bundle structures such as tip links, the putative site of mechanotransduction (Pickles et al., 1984; Assad et al., 1991). Moderate noise exposure causes tip link damage in birds and mammals (Clark and Pickles, 1996; Husbands et al., 1999). As tip links cannot be visualized using the fluorescent labeling method employed here, future studies may use scanning electron microscopy (SEM) to examine tip link structure under this noise exposure paradigm.

Although this study focused primarily on the saccule, apoptotic cells were counted in all three inner ear epithelia of each fish. Bundle density was not quantified in the utricle or lagena because significant bundle loss was not apparent during qualitative observation. The lack of hair bundle loss or increased apoptosis in the utricle supports the notion that this end organ may have decreased hearing sensitivity as compared to the saccule and/or that the utricle is primarily a vestibular end organ in otophysan fishes. The lagena, however, may be involved in directional hearing. It has recently been shown that lagenar nerve fibers have the potential to encode the direction of sound in sleeper goby *Dormitator latifrons* (Lu et al., 2003) and goldfish (Meyer et al., 2004), and significant apoptosis was observed in this end organ (albeit with a high degree of variability).

Previous morphological studies on the effects of noise damage in fishes have examined epithelial surface morphology using scanning electron microscopy (e.g. Hastings et al., 1996; McCauley et al., 2003). Minimal damage to the hair cells of the striolar region of the utricle and lagena was found in the oscar *Astronotus ocellatus* following exposure to a 300 Hz pure tone, with no damage to the saccule (Hastings et al., 1996). Noise exposure in that study was conducted in a flexible waveguide system, making it difficult to directly compare the results of this study with the present white-noise paradigm used in goldfish. Additionally, goldfish have much more sensitive hearing than the cichlid oscar (Kenyon et al., 1998). As hearing thresholds are considerably higher in tilapia, another cichlid, compared to goldfish, and since noise exposure has little effect on hearing in tilapia (Smith et al., 2004b), or other fish with poor hearing, such as bluegill sunfish *Lepomis macrochirus* (Scholik and Yan, 2002) it is not surprising that minimal hair cell damage was found in noise-exposed oscars.

Regeneration was not examined in the oscar study, although regeneration of the utricular and lagenar striola have been observed following ototoxic damage in the oscar (Yan et al., 1991; Lombarte et al., 1993). In another morphological study of noise damage in fishes (McCauley et al., 2003) pink snapper *Pagrus auratus* were exposed to repeated presentations of an air-gun stimulus with a source intensity of 223 dB re. 1 μPa (peak to peak). Exposure to this stimulus resulted in large holes in the saccular epithelium that were still present after 58 days of recovery (McCauley et al., 2003). At the same time, exposure of several species of fish, including an otophysan, to several blasts of a seismic air gun [much less cumulative exposure than in the pink snapper study (McCauley et al.,

2003)] showed no damage to hair cells viewed using SEM (A.N.P., M.E.S., J. Song, P. A. Cott, B. W. Hanna, A. O. MacGillivray, M. E. Austin and D. E. Mann, unpublished data), even when the same species showed TTS (Popper et al., 2005). Interestingly, morphological damage in the pink snapper saccule was most evident in the caudal region, similar to the present findings in the goldfish (McCauley et al., 2003).

A significant finding of the present study is that significant hearing recovery (as measured by AEP) occurred prior to full morphological recovery of the saccular epithelium. This observation suggests that a full complement of hair cells is not necessary for relatively normal hearing in the goldfish. Similar results have been seen in birds, and several authors have suggested that the early phase of recovery may depend more on regeneration of the tectorial membrane or restoration of micromechanical properties than on hair cell regeneration (McFadden and Saunders, 1989; Saunders et al., 1992; Adler et al., 1993; Niemiec et al., 1994; Müller et al., 1996). Although fishes do not have a tectorial membrane, they do have an analogous otolithic membrane that couples the hair bundles to the overlying otolith (Popper, 1977). The otolithic membrane was not examined in our noise-exposed fish and it is possible that recoupling of the surviving hair bundles to the otolith through a mechanism such as otolithic membrane repair contributed to the functional recovery we observed. However, it is also possible that complete functional recovery did not take place by the termination of our study. Neither the present study nor many of the avian studies measured sound source localization, tone discrimination, or other more complex auditory capabilities attributed to goldfish (e.g. Fay, 1984; Fay, 1998; Fay, 2005). Future experiments using multiple functional measures are necessary to confirm the extent of the recovery observed here.

In conclusion, exposure to 48 h of white noise causes significant physiological and morphological damage to goldfish ears. Significant functional recovery occurs after 7 days in quiet but morphological damage is still evident at this time. Although it is difficult to apply these results to other fishes owing to the great diversity in fish ear morphology and physiology, we suggest that similar noise-induced damage may be possible in other otophysan fishes such as the zebrafish, a popular developmental model species. Anthropogenic noise sources (e.g. shipping traffic, seismic surveys, sonar operations) of at least the intensity used in this experiment are found in fish habitats, suggesting that human-made aquatic sounds could cause significant morphological and functional damage to fish hearing, as well as the behavioral modification that has been reported. Hearing capabilities recover if given sufficient time in quiet, however, in a perpetually noisy environment, hearing damage may persist to the detriment of the animal.

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