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Acoustic Injury and TRPV1 Expression in the Cochlear Spiral Ganglion

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Abstract: Acoustic trauma not only produces temporary and permanent hearing loss but is a common cause of chronic tinnitus. Recent work indicated a possible role for the transient receptor potential channel vanilloid subfamily type 1 (TRPV1) in modulating the effects of cochlear injury. In our research, we investigated the effects of acoustic damage on TRPV1 expression in spiral ganglion neurons of adult rats. After exposing them unilaterally to noise, we extracted cochleas and processed the spiral ganglion for TRPV1 expression at four posttrauma intervals (2 hours, 24 hours, 12 days, and 16.9 months). We measured TRPV1 immunodensity in the apical, middle, and basal turns of the cochlea. We found a significant interaction (p = .039) between posttrauma interval and regional cochlear receptor expression: For survival intervals between 24 hours and 2 weeks, TRPV1 density increased in all cochlear regions; at the longest survival interval (16.9 months), TRPV1 density was dramatically reduced in the basal region. We also psychophysically tested the long-survival subjects, which showed evidence of 20-kHz tonal tinnitus. These results suggest that TRPV1 may participate after cochlear injury in a signal cascade that is responsible for the neuroplastic events leading to tinnitus and hyperacusis.

Key Words: acoustic trauma; animal model; spiral ganglion tinnitus; TRPV1; vanilloid receptor

The clinical and pathological similarities between neuropathic pain and chronic tinnitus are well-known [1,2]. Both phenomena are typically without objective stimulus correlates, follow neural injury, and are refractory to treatment. Neuropathic pain includes the pathological sensations of allodynia (pain produced by innocuous stimuli), hyperalgesia (increased responsiveness to noxious stimuli), and dysesthesia (perversion of normal sensations). Analogous auditory phenomena include tinnitus, recruitment, hyperacusis, and wind-up or kindling. Also well recognized is that neuropathic pain and chronic tinnitus have comorbid nonsensory features in common, such as depression, sleep disorder, and anxiety.

A cascade of events between peripheral injury and subsequent central pathophysiology has been implicated in the development of both neuropathic pain and chronic tinnitus. Peripheral somatic nerve damage initiates signals at the axonal site of injury. Subsequent contributions to the pain signal are generated in the dorsal root ganglia (DRG), the postsynaptic dorsal horn, and at more rostral levels of the somatic system. Loss of central inhibition may be a major source of pathological excitability within sensory pathways, both somatic [3] and auditory [4]. Phantom limb pain and postherpetic neuralgia are examples of persistent pain signals having a central component that does not respond to typical analgesic treatment [5–7]. Chronic tinnitus appears to be similar in many ways to these clinical syndromes. Quantitative laboratory studies have shown that tinnitus and the associated elevated central neural activity often develop slowly over time after auditory insult [8,9] and may persist for the remainder of an individual's life [10].

The transient receptor potential (TRP) family is a six-member group of nonspecific cation channel receptors that function as diverse cellular sensors in association with 28 different ion channels. TRP channels are widely expressed in a variety of organ systems, including

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the cochlea. TRP channel vanilloid subfamily type 1 (TRPV1) receptors are ubiquitous throughout the peripheral and central nervous system and have been implicated in the sensation of acute and chronic pain [11]. Peripheral sites include the DRG, trigeminal ganglia, vestibular ganglia, spiral ganglia (SG), and both sensory and supporting cells of the organ of Corti [12,13]. TRPV1 integrates the response of nociceptive fibers to a range of ligands. The receptor is activated by vanilloids (capsaicin, resiniferotoxin) and nonvanilloid endogenous ligands, such as protons (pH $<$ 5.9) [14], heat ($>$43°C) [15], and lipoxygenase metabolites [16]. A proposed role for TRPV1 is dynamic modulation of the neural response to injury that leads to nociception and hyperalgesia [17]. TRPV1 may also play a role in the development of neuropathic pain after nerve damage, including the abnormal sensory states of hyperalgesia and allodynia [18].

Sites of TRPV1 expression in the cochlea include the perivascular neural innervation of the spiral modiolar artery and radiating arterioles, inner hair cells (IHCs) and outer hair cells (OHCs), pillar cells [19], and SG cells [20]. Stimulation of cochlear TRPV1 receptors using the synthetic ultrapotent agonist resiniferotoxin reduced cochlear sensitivity, as indicated by decreased compound action potentials, cochlear microphonic, and electrically evoked otoacoustic emissions [19]. Cochlear blood flow and vascular permeability can be modulated by stimulation of the trigeminal ganglion via paravascular afferents that express TRPV1 [13,21].

That TRPV1 may modulate the effects of cochlear injury was suggested by its upregulation in response to kanamycin challenge [22]. These findings support the hypothesis that upregulation of TRPV1 mediates a cochlear response to injury. We hypothesized that TRPV1 may also mediate cochlear effects leading to hyperacusis and tinnitus after acoustic trauma. An initial step in testing this hypothesis required investigating the effect of acoustic insult on cochlear TRPV1 expression. Further confirmation would be to establish an association between TRPV1 and the presence of tinnitus. The primary objective of our study was to determine the relationship between acoustic trauma and TRPV1 density in cochleas of rats. A secondary objective, using a more limited data set, was to demonstrate a relationship between TRPV1 cochlear density and tinnitus.

SUBJECTS AND METHODS

Subjects

Subjects were 16 male Long-Evans rats (Harlan, Indianapolis, IN); 12 were 4–6 months old, and 4 were 20 months old at the time of sacrifice. The care, use, and experimental manipulation of animals were approved by the Southern Illinois University School of Medicine Laboratory Animal Care and Use Committee. Subjects were sacrificed at four time points after acoustic trauma: 2 hours, 24 hours, 12 days, and 16.9 months.

Acoustic Exposure

We unilaterally exposed all subjects to a traumatizing acoustic stimulus under anesthesia, an intramuscular mixture of ketamine HCl (24.6 mg/kg) and xylazine (3 mg/kg). Subjects were positioned in a modified stereotaxic head frame inside a sound-attenuation chamber and exposed once, unilaterally, for 60 minutes to an octave-band noise centered at 16 kHz, with a peak level of 115 dB (re 20 μPa). Acoustic brainstem-evoked response (ABR) thresholds were measured before trauma exposure, immediately after exposure, and before sacrifice. We obtained ABR measurements with an Intelligent Hearing Systems (HIS) Smart EP System, running IHS high-frequency software (v. 2.33) and using IHS high-frequency transducers (HFT9911-20-0035, Intelligent Hearing Systems, Miami, FL). Acoustic stimuli were presented directly to the entrance of the ear canal. Stainless steel needle electrodes were placed subcutaneously at the vertex and over the bullae with a reference electrode at the occiput. We obtained ABR thresholds for 5-msec-duration clicks and tone bursts presented at a rate of 50/sec. Tone bursts were gated using an exact Blackman envelope (2.5 msec rise/decay, 0 msec plateau) and presented in decreasing intensity series, beginning with intensity levels that elicited clear waveforms and progressing in 20-, 10-, and 5-dB (re 20 μPa) decrements, from above threshold to near threshold, respectively. Evoked potentials were amplified 200,000 times, bandpass filtered (100–3,000 Hz), and averaged over 1,024 sweeps. Recording epochs comprised the 12 msec after stimulus onset. We determined threshold as the lowest stimulus intensity that reliably produced a visually distinct evoked waveform.

Immunohistochemistry

After administration of a lethal anesthetic dose of sodium pentobarbital (26%), isopropyl alcohol (7.8%), and propylene glycol (20.7%; Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA), the animals were perfused transcardially with 0.9% normal saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). We extracted cochleas from the temporal bone, removed the stapes, and slowly perfused fixative through a small apical hole. DRG was harvested from the upper thoracic vertebral column. Cochleas and DRG were immersion-fixed for an additional 2–3 hours

and then stored in PBS until sectioned. Cochleas were decalcified on a rotator in 0.12 M EDTA at 4°C for 3–4 days. Once the cochleas were thoroughly decalcified, we placed them in 20% sucrose in PBS overnight. Specimens were blocked in a cryomold (No. 62534-15, Electron Microscopy Science, Hatfield, PA) with OCT embedding compound (Tissue-Tek, 4583, Electron) and sectioned at 10 μm at –17°C. Cochleas were transversely sectioned orthogonal to the modiolus, with all sections collected from the apex through the hook. We collected DRG sections and cochlear sections, dried them onto SuperfrostPlus slides (No. 12-550-15, Fisher Scientific, Pittsburgh, PA), and stored them at –0°C overnight. Sections were washed in PBS, blocked with PBS containing 0.025% Triton X-100 (PBS-T), 5% normal goat serum (S-1000, Vector Laboratories, Burlingame, CA), and 5% bovine serum albumin (Jackson Immuno Research Laboratories, West Grove, PA) for 1 hour, and then incubated with rabbit anticapsaicin receptor (Cat. No. PC420, Lot No. D29727, CalbioChem, La Jolla, CA) (1:1,000) in blocking solution for 24 hours (DRG) or 48 hours (cochleas). Every sixth section was separately processed for control staining. We incubated control sections in blocking solution without antibody. After triple washing in PBS-T, specimens were incubated with biotinylated anti–rabbit IgG (No. BA-1000, Vector) (1:200) in PBS-T with 2% BSA for 1 hour. After a second triple washing in PBS-T, we incubated specimens with Vectastain Elite ABC (PK-6100, Vector) for 1 hour. After incubation, the specimens were triple-washed a third time in PBS-T and developed them with Vector DAB (SK-4100, Vector) peroxidase substrate. After two washes in distilled water, we dehydrated the sections through ascending alcohols and cleared them in xylene. Slides were cover-slipped using Permount (Fisher Scientific). We collected and stained approximately 50 sequential sections per cochlea for quantitative densitometry.

Densitometry

We used serial transverse cochlear sections for densitometry to maintain a consistent cross-section of the sample regions from the apical, middle, and hook regions of the cochlea. The entire set of sections from each cochlea was equally divided into apical, middle, and lower thirds. We digitally photographed three randomly selected sections from each region (200×, Canon 5D 12.5 megapixel camera, Zeiss Photomicroscope III) and analyzed the images using Photoshop (CS2, Adobe, v. 9.1). We determined the density of the peroxidase reaction product in the SG by systematically scanning a 5 × 5–pixel intensity-measurement tool, cell by cell, in flattened gray-scale images, for 50 cells in each section, and by averaging the measures over the three sampled sections, to yield mean density per cochlear region. Density readings were entered into spreadsheets (Excel, Microsoft Corp, Redmond, WA) for archival storage, statistical analysis, and graphic depiction. To control for image background and anisotropy, cell density measures were normalized with respect to the density of proximal extracellular areas and were reported as differential density (i.e., cell density minus proximal extracellular density). We made comparisons between cochleas (i.e., exposed vs. unexposed) within subjects.

Tinnitus Testing

Animals exposed to acoustic trauma 16.9 months before histology were, in the interim, behaviorally trained and psychophysically tested for tinnitus using an operant suppression procedure shown to detect tinnitus in animals [10,23]. Briefly, we trained them in daily 1-hour sessions to lever-press for food in the presence of constant background (free-field) sound and to suppress lever pressing during randomly introduced silent periods. Substitution of variable pitch and loudness tones for some of the silent periods enabled us to derive psychophysical discrimination functions. The dependent measure, suppression ratio, is a measure of test-stimulus lever pressing relative to baseline pressing: 0 indicates no presses and 0.5 indicates pressing equivalent to baseline. In this paradigm, within the current parameters, tinnitus was evident using 20-kHz test stimuli (i.e., divergent psychophysical functions at specific frequencies indicate tinnitus because the endogenous signal [tinnitus] and external signal [test stimulus] maximally interact when similar). We compared psychophysical data of the four 16-month subjects to those of unexposed control subjects (N = 13) before trauma and 3 months after trauma [8].

RESULTS

Hearing Thresholds

We obtained ABR thresholds before trauma, immediately after trauma, and at the time of sacrifice. Baseline ABR thresholds obtained before trauma were similar for left and right ears within each experimental group (Fig. 1A).

Immediately after trauma, ABR thresholds were elevated 35–55 dB sound pressure level (SPL) in the exposed ears of all subjects (see Fig. 1B). The largest threshold shifts were at 16 and 20 kHz. The maximum immediate postexposure threshold elevation was comparable across all frequencies for subjects sacrificed 24 hours and 12 days after exposure (range, 71.3–76.3 dB). The maximum immediate threshold elevation in the long-term survival group was 90 dB at 16 kHz.

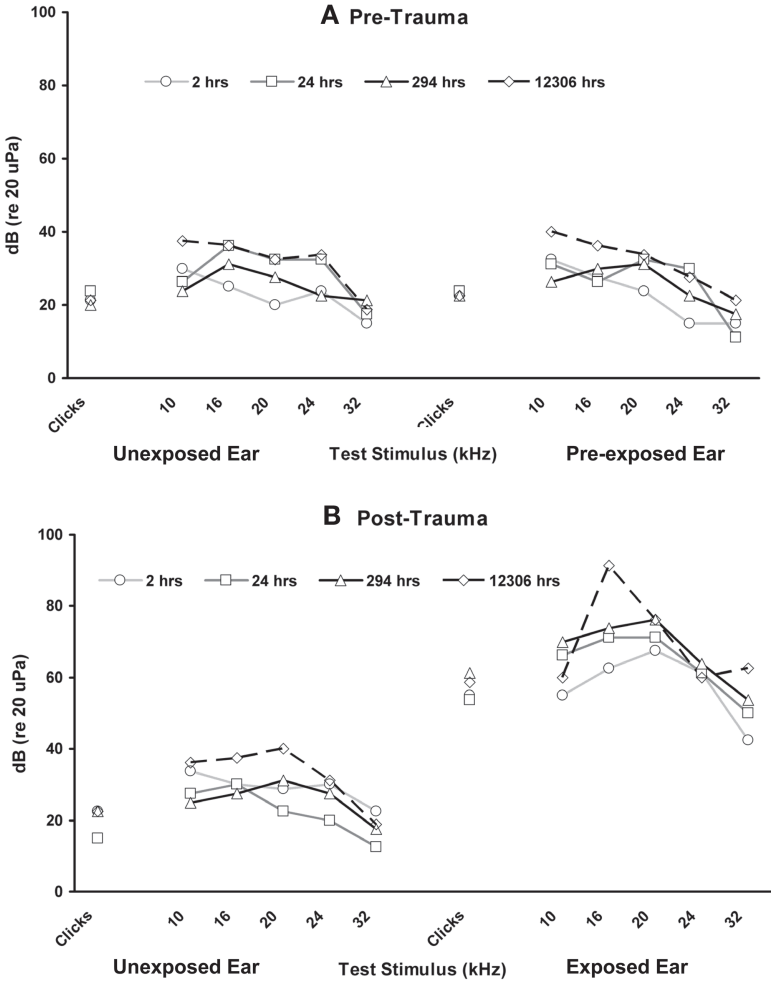

Figure 1. Immediate pretrauma (A) and posttrauma (B) group average acoustic brainstem-evoked potential thresholds for the four treatment groups (n = 4 subjects/group).

Thresholds at time of sacrifice for animals in the 2-hour group were only slightly elevated from thresholds immediately after exposure (49.3 dB and 57.8 dB, average thresholds, respectively). ABR thresholds of exposed ears for subjects sacrificed 24 hours and 12 days after trauma showed a progressive 20-dB loss over the period between exposure and sacrifice. In the 24-hour group, the maximum shift at sacrifice was at 20 kHz (mean threshold, 95 dB). The maximum shift in animals in the 12-day group was at 16 kHz (mean threshold, 93 dB). In contrast, ABR thresholds at sacrifice in the four subjects tested 16.9 months after acoustic trauma showed near-complete recovery of function to baseline pretrauma thresholds. Threshold averages across all frequencies (10–32 kHz) at time of sacrifice were 24 dB in the control ear and 24 dB in the exposed ear.

Evidence of Tinnitus

We measured frequency-specific psychophysical evidence of tinnitus in the long-survival subjects (Fig. 2). After unilateral acoustic trauma, the exposed subjects showed an upward shift of their 20-kHz discrimination functions as compared to those in the unexposed control group tested in parallel (see Fig. 2A). Differences in discrimination between exposed and control subjects were not evident when they were tested with either 10-kHz tones or broadband noise (Fig. 2B,C, respectively). The posttrauma upshift of the 20-kHz function in the exposed subjects demonstrates that their sensation of 20 kHz was affected by an endogenous factor not present in the control subjects, as the objective stimulus test conditions were equivalent for all subjects. The endogenous factor was likely to be tinnitus with features similar to that of 20-kHz tones.

Expression of TRPV1

TRPV1 expression was evident in multiple cell types within the cochlea, including OHCs, IHCs, pillar cells, and the SG. The typical cochlear pattern of immunostaining is illustrated in the midmodiolar section depicted

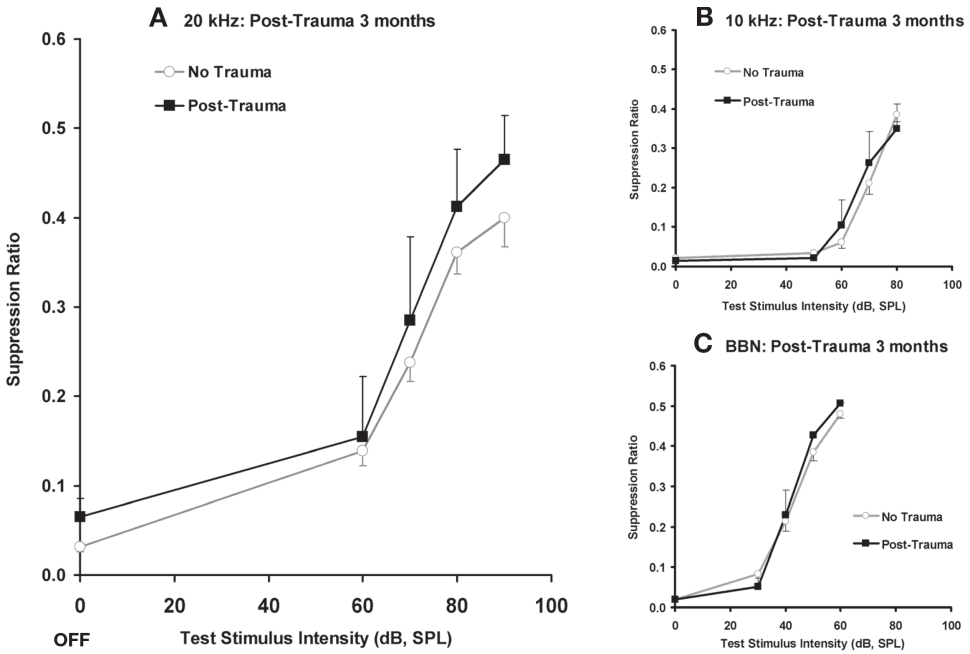

Figure 2. Psychophysical functions showing the discrimination performance of the four 16.9-month-survival subjects (group average; error bars indicate the standard error of the mean) as compared to a trained-and-tested in-parallel control group (n = 13). (A) Upshift of the posttrauma 20-kHz function provided evidence of tinnitus. Discrimination performance of the same subjects for 10-kHz tones (B) or broadband noise (C) were identical for the trauma and control groups. All functions were determined 3 months after trauma.

in Figure 3. We used serial transverse sections orthogonal to the modiolus for quantitative analysis (Fig. 4). TRPV1 density was altered by acoustic trauma in a pattern that was tonotopically and temporally differentiated. The change in TRPV1 density, as an interactive function of posttrauma survival time and cochlear region, was significant ($F = 1.88$; $df = 15; 72$, $p = .039$).

TRPV1 expression was not changed within 2 hours of acoustic trauma in any region of the cochlea. However, 24 hours after trauma, greater TRPV1 was evident in the apical region of exposed cochleas than in contralateral unexposed cochleas. A similar, but less pronounced, density difference was apparent in the middle-turn and hook regions (Fig. 5A–C). Subjects sacrificed 12 days (i.e., 294-hr mean) after trauma had only a slight elevation of TRPV1 density in the hook (see Fig. 5C) and apical (see Fig. 5A) regions of exposed cochleas as compared to unexposed cochleas. The elevated TRPV1 densities across the 24-hour to 12-day survival period were only weakly—and negatively—correlated with immediate ABR threshold elevations produced by the trauma exposure (Table 1). It is therefore unlikely that an adventitious correspondence between acute damage (as indicated by threshold shift) and survival interval accounted for the spatiotemporal pattern of TRPV1 densities across the 24-hour to 12-day survival interval.

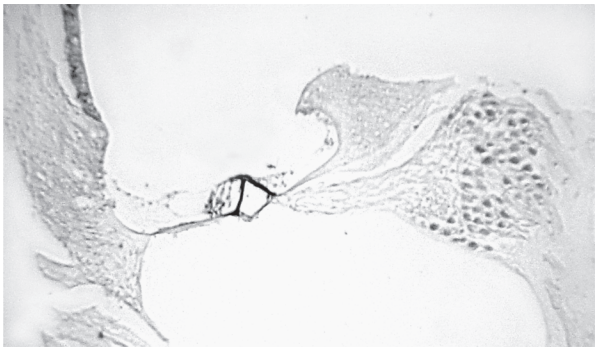

Figure 3. Midmodiolar cochlear section illustrating the typical pattern of TRPV1 antibody staining.

An unexpected large decrease in TRPV1 density was evident in the hook region of exposed cochleas of subjects sacrificed 16.9 months after the single exposure to acoustic trauma (see Fig. 5C). In addition to this large absolute decrement, there was progressive decrease, relative to the control ear, in TRPV1 density along the tonotopic gradient, from apex to hook: A small decrease in density was evident in the apical turn (5%), progressing to a 13% decrease in the middle turn and a 57% decrease in TRPV1 density in the hook region of the exposed cochleas (see Fig. 5).

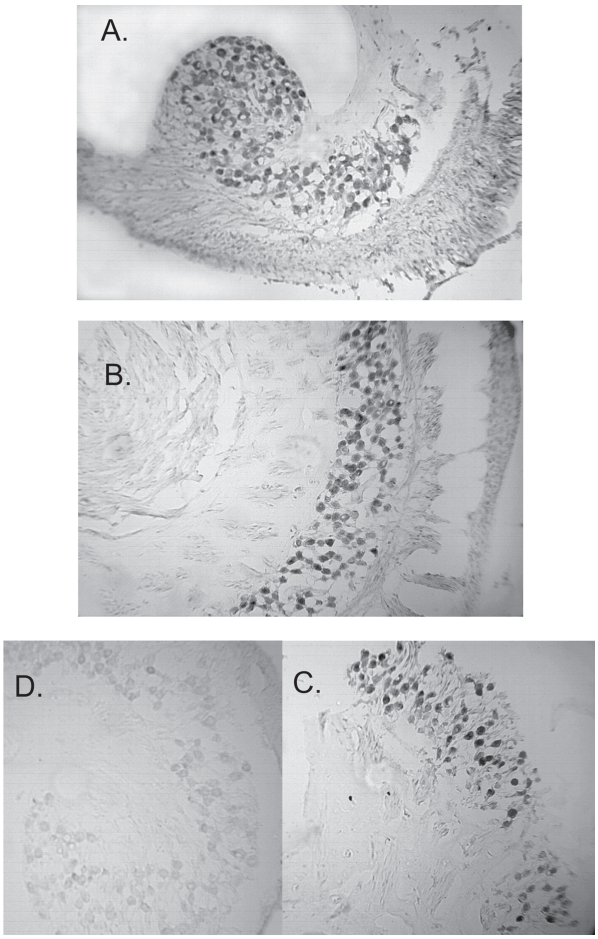

Figure 4. Representative transverse cochlear sections: (A) apical, (B) middle, and (C) hook regions, showing TRPV1-positive spiral ganglion neurons. (D) A typical antibody-negative control section.

DISCUSSION

Effect of Acoustic Trauma and Aging on TRPV1 Density

Unilateral acoustic trauma sufficient to induce a large temporary threshold shift and mild unilateral permanent threshold shift resulted in a transient upregulation of TRPV1 expression in the SG of rats. The increase in TRPV1 density was most evident 24 hours after exposure and was most pronounced in the apex, relative to unexposed cochleas. No increase in TRPV1 density was evident within 2 hours of exposure, and we could observe only a slight increase 12 days after exposure. After 16.9 months, trauma exposure resulted in a striking decrease in TRPV1 expression in the hook region of the exposed cochleas and a moderate relative decrease in the midregion. TRPV1 immunoreactivity of exposed cochleas did not correspond to either immediate posttrauma or at-sacrifice threshold elevations. The decreased

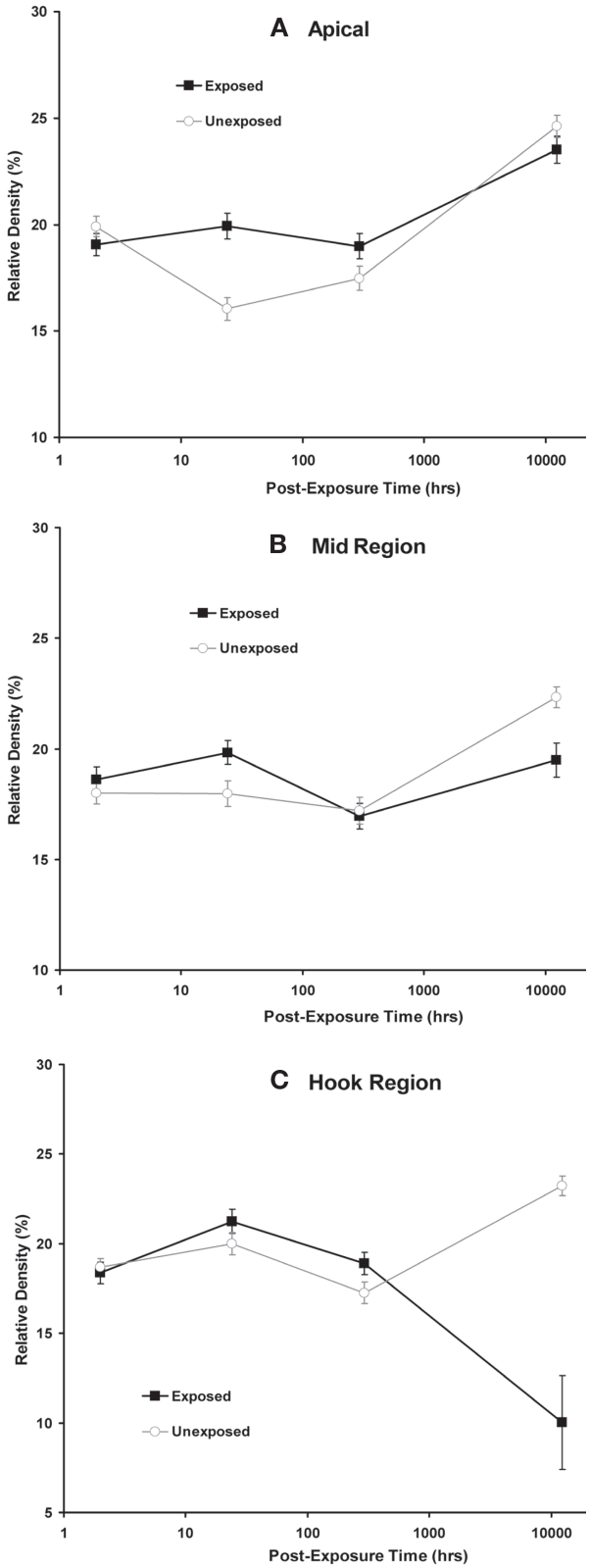

Figure 5. TRPV1 density in the spiral ganglion of trauma-exposed and unexposed (contralateral) cochleas, as a function of posttrauma survival time. The spatiotemporal density pattern is evident, comparing (A) apical, (B) middle, and (C) hook regions. We measured density relative to a proximal unstained region of the neuropil and reported it as percent difference.

Table 1. Correlation Matrix (*r*): TRPV1 Density Versus Immediate Posttrauma Acoustic Brainstem-Evoked Response Threshold Elevation During a 24- to 294-hr Survival Interval

Cochlear Region	Clicks	10 kHz	16 kHz	20 kHz	24 kHz	32 kHz
Apex	−0.172	−0.648	−0.170	−0.266	−0.530	−0.129
Midregion	−0.275	−0.666	−0.061	−0.316	−0.493	−0.182
Hook	−0.336	−0.533	−0.065	−0.199	−0.289	−0.392

TRPV1 immunoreactivity obtained at 16.9 months did not reflect a nonspecific aging effect, as the unexposed control cochleas from the same subjects showed TRPV1 immunoreactivity comparable to that of younger subjects (see Fig. 5).

Differential Effects of Acoustic Trauma and Kanamycin Toxicity

The effect of acoustic trauma on TRPV1 in the rats observed in our study was different from that reported for kanamycin toxicity in the mice. Sixteen months after acoustic trauma, our study found a large TRPV1 immunoreactivity decrease in the mid-to-hook region of rats. CBAJ mice treated with a 14-day course of systemic kanamycin, 700 mg/kg twice a day, had a sevenfold increase in SG TRPV1 mRNA and a fivefold increase in TRPV1 protein [22]. There are obvious species, manipulation, and time frame differences between the two studies: The kanamycin treatments used by Kitahara et al. [22] likely resulted in significant OHC damage. Although cochlear histology was not reported in their study, near-complete OHC loss in the basal half of the cochlea with intact IHCs throughout the cochlea was observed 2 weeks after a similar systemic kanamycin exposure [24]. Although hair cell damage was not measured in our study, an extensive database of quantitative cochlear histology in rats sacrificed 12–15 months after acoustic trauma with identical parameters used in our current study revealed only minimal OHC damage and no IHC loss. Significantly, a selective long-term loss of large-diameter, type-1 SG cell peripheral dendrites was observed in exposed cochleas, despite the absence of hair cell degeneration [25]. Furthermore, this large-diameter, type-1 SG cell degeneration occurred after a single acoustic exposure sufficient to induce tinnitus. This long-term neural degeneration may explain the long-term decrease in TRPV1 immunoreactivity obtained in our study. If neural degeneration is a key factor in any subsequent changes in TRPV expression, the time interval between exposure and sacrifice may have a significant impact on observed results.

It is also possible that the TRPV1 system responds differently to different modes of cell damage. In neuropathic pain, the mechanisms associated with toxic or metabolic injury appear to differ from those produced by mechanical nerve injury [26]. Postinjury hyperalgesia to thermal and mechanical stimuli is reduced in TRPV1 knockout mice, but chemonociception and hypersensitivity to inflammatory stimulation are maintained. Finally, there may be species-specific differences in the susceptibility of the TRPV1 system to toxins and trauma. For example, a genetic predisposition for the development of neuropathic pain has been established in different rat strains, and similar species differences may hold for TRPV1 [27].

Acoustic trauma clearly produces a complex change in the spatiotemporal expression of TRPV1 in rat cochleas. Whether the observations of an initial increase followed by a subsequent downregulation of the receptor are relevant to the onset or the persistence of acoustic trauma–induced tinnitus (or both) is not known. It is suggestive that, in our study, evidence of tinnitus was present in the four animals sacrificed 16.9 months after trauma exposure. Given the constraints of the psychophysical methods available, it is not known whether tinnitus was present in short-term survival subjects. As in the case of algesia caused by acute TRPV1 stimulation, acute cochlear injury may produce tinnitus through the activation of the TRPV1 receptor. This might be analogous to the tinnitus experienced for several hours after attending a loud concert or using firearms without ear protection. In contrast, when a critical subpopulation of TRPV1-positive neurons is lost over time, either through aging or cumulative acoustic trauma, the protective antinociceptive mechanism of TRPV1 may be lost, resulting in chronic tinnitus. An example would be the permanent tinnitus that emerges slowly, decades after exposure to loud music or gunfire.

Relevant to both TRPV1 expression and the effects of injury and inflammation on cochlear function is the observation that the enzymatic pathways for processing inflammatory mediators are present within the cochlea. SG neurons express both TRPV1 and 5-lipoxygenase, a key enzyme that converts arachidonic acid into inflammatory mediators [20]. A synthetic prostaglandin E_1 analog (misoprostol) has been reported effectively to reduce the rated loudness and annoyance of tinnitus in a double-blind placebo-controlled trial [28]. In that study, the tinnitus etiologies associated with the largest therapeutic response were sudden-onset tinnitus and tinnitus associated with acoustic trauma. In a similar study, Briner et al. [29] found the largest improvement from misoprostol in subjects with noise-induced tinnitus. In light of such clinical evidence combined with that of our laboratory study, the role of the cochlear vanilloid system of

neuromodulators in tinnitus should be explored. Potentially new avenues of treatment and prevention for chronic tinnitus may become available through these efforts.

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