Title

Discovery of a Biological Mechanism of Active Transport through the Tympanic Membrane to the Middle Ear

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The Sustained-Exposure Dexamethasone Formulation OTO-104 Offers Effective Protection against Noise-Induced Hearing Loss

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Key Words
Hearing loss · Acoustic trauma · Sustained exposure · Dexamethasone

Abstract
The otoprotective effects of OTO-104 were investigated both prior to and following acute acoustic trauma. Guinea pigs received a single intratympanic injection of OTO-104 and were assessed in a model of acute acoustic trauma. Doses of at least 2.0% OTO-104 offered significant protection against hearing loss induced by noise exposure when administered 1 day prior to trauma and up to 3 days thereafter. Otoprotection remained effective even with higher degrees of trauma. In contrast, the administration of a dexamethasone sodium phosphate solution did not protect against noise-induced hearing loss. Activation of the classical nuclear glucocorticoid and mineralocorticoid receptor pathways was required for otoprotection by OTO-104. The sustained exposure properties of OTO-104 were also superior to a steroid solution.

Introduction

Hearing can be compromised in a number of circumstances, including excessive noise exposure. This condition affects a significant segment of the population, in the order of 30 million in the USA alone. However, very little progress has been made to date in identifying effective therapeutic treatments and/or means of administration [Oshima et al., 2010; McCall et al., 2014].

Dexamethasone, a common anti-inflammatory corticosteroid, has been used by medical practitioners to treat various otic conditions involving a hearing loss component. This off-label approach has historically focused on the prescription of high doses of steroids administered systemically, but recently a shift towards a local delivery paradigm has emerged [Hu and Parnes, 2009]. Patients with inner ear disorders, such as Ménière's disease and sudden sensorineural hearing loss, have benefited from the administration of intratympanic steroids [Garduno-Anaya et al., 2005; Xenellis et al., 2006; Battaglia et al., 2008; Kitahara et al., 2008]. However, inconsistent clinical responses are commonly observed. These have often been attributed to variable and limited exposure to ste-

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roids in the inner ear, primarily due to the short residence time of aqueous solutions in the middle ear [Bird et al., 2007]. This has led to the hypothesis that better clinical efficacy can be achieved by maintaining therapeutic drug levels in the inner ear fluids for prolonged periods of time.

Sustained-exposure drug delivery to local compartments can be accomplished by increasing the residence time of the therapeutic agent in the targeted compartment. Previous studies have shown that dexamethasone formulated using a poloxamer hydrogel provides lasting exposure in the inner ear of guinea pigs and sheep for weeks to months with a single intratympanic injection [Wang et al., 2009, 2011a]. A proprietary otic formulation, OTO-104, was well tolerated locally and systemically in extensive toxicology studies conducted in guinea pigs [Piu et al., 2011]. Recently, results from a clinical study in patients with Ménière's disease have concluded that the intratympanic injection of OTO-104 was safe and well tolerated [Lambert et al., 2012].

Here, an evaluation of OTO-104 was conducted to investigate whether a single intratympanic injection of a poloxamer hydrogel containing dexamethasone could alleviate hearing loss in guinea pigs exposed to acute acoustic trauma.

Materials and Methods

Chemicals

Dexamethasone, dexamethasone sodium phosphate (DSP) and poloxamer 407 NF were purchased from Spectrum Chemicals. Mifepristone was obtained from Sigma Aldrich. Acepromazine, ketamine and xylazine were from MWI Veterinary Supply. OTO-104 consisted of a sterile suspension containing dexamethasone in 16% poloxamer 407 prepared as described previously [Piu et al., 2011]; 2.0% DSP sterile aqueous solution was also prepared as described previously [Wang et al., 2011b]. In brief, the DSP solution was prepared by dissolving DSP into a buffered solution (10 mM PBS, pH 7.4) and subsequent sterile filtration. Mifepristone was formulated as a 6.0% sterile suspension in 16% poloxamer 407. When OTO-104 and mifepristone were co-administered, the drugs were co-formulated in 16% poloxamer 407 at final concentrations of 6.0% of each agent. Briefly, all suspensions were prepared as follows: a 16% w/w solution of poloxamer 407 was prepared by slowly adding it to a cold buffered solution (10 mM PBS, pH 7.4). Heat-sterilized micronized dexamethasone and mifepristone, alone or in combination, were suspended with an appropriate amount of sterile poloxamer 407 solution using aseptic techniques. Samples were kept under refrigeration and resuspended before administration.

Animal Studies

All animal studies were conducted in accordance with the policies and recommendations of the US Department of Agriculture and the National Institute of Health guidelines for the handling and use of laboratory animals, and received approval from the Institutional Animal Care and Use Committee of Otonomy Inc. Female guinea pigs (Hartley, Charles River, n = 6 per group) weighing 200–300 g, of approximately 6–8 weeks of age, served as subjects for the experiments. The sample size of this experiment was not based on power calculation. This study was conducted as an exploratory study. The number of subjects per group was chosen based on prior experience (n = 6 appears sufficient to detect differences between treatment regimens), operational limitations (such as noise exposure), and concerns from the Institutional Animal Care and Use Committee of Otonomy Inc. to limit the number of animals in the study. Prior to any procedure, the animals were anesthetized using a combination of xylazine (10 mg/kg), ketamine (40 mg/kg), and acepromazine (0.75 mg/kg) for up to 1 h via the intramuscular route.

Intratympanic Injection. All animals were injected bilaterally. Each animal was positioned so that the head was tilted at an angle to favor injection towards the round window niche. Briefly, under visualization with an operating microscope, 50 µl of the formulation was injected using a 27- or 30-gauge blunt needle through the tympanic membrane into the superior posterior quadrant behind which the round window niche is located. To ensure adequate exposure to the round window, the animals were maintained in that position for 30 min. Subsequently, the second ear was dosed under the same conditions. Within the same treatment group, all animals received the same lot of formulations. During the procedure and until recovery, the animals were placed on a temperature-controlled heating pad (37–39°C). After consciousness was regained, the animals were returned to the vivarium.

Noise-Induced Hearing Loss. Awake animals (2 at a time) were placed in an enclosure and exposed to noise. The characteristics of the acoustic trauma were as follows: a single period of 2 h, sound pressure level (SPL) of either 105 or 110 dB SPL with a narrow band pass of 4–8 kHz. Noise was delivered using a Grason-Stadler white noise generator, filtered through a Krohn-Hite 3750 unit, amplified using a Crown D75 unit, and conveyed via a JBL speaker (model 2446H). Following treatment, the animals were returned to the vivarium. Noise exposure was calibrated over a range of 80–115 dB SPL across a wide range of frequencies using the Acoustical Interface Precision Microphone System and the Tucker-Davis Technologies System.

Auditory Brainstem Response Assessment. During the procedure, an additional dose of anesthetic (xylazine and ketamine) was administered if needed to maintain the depth of anesthesia sufficient to insure immobilization and relaxation. Auditory brainstem responses (ABRs) were recorded in an electrically and acoustically shielded chamber, one ear at a time. Needle electrodes were placed at the vertex (active) and immediately below the pinna of the test ear (reference) and contralateral ear (ground). Tucker-Davis Technologies System III hardware and SigGen/BioSig software (Tucker-Davis Technologies) were used to present the stimulus and record the ABR responses. Tones were delivered through a Tucker-Davis open-field ES1 driver placed 5 cm above the animal's ear. Acoustic calibration was performed with SigCal software (Tucker-Davis Technologies), and thresholds were expressed as decibel SPL in conditions identical to those of threshold recordings in animals. Stimulus presentations (tone bursts of 15 ms, with rise/fall times of 1 ms) were presented 10 per second. Up to 512 responses were averaged for each stimulus level. Responses were collected for stimulus levels in 5-dB decrement steps at 3 frequencies:

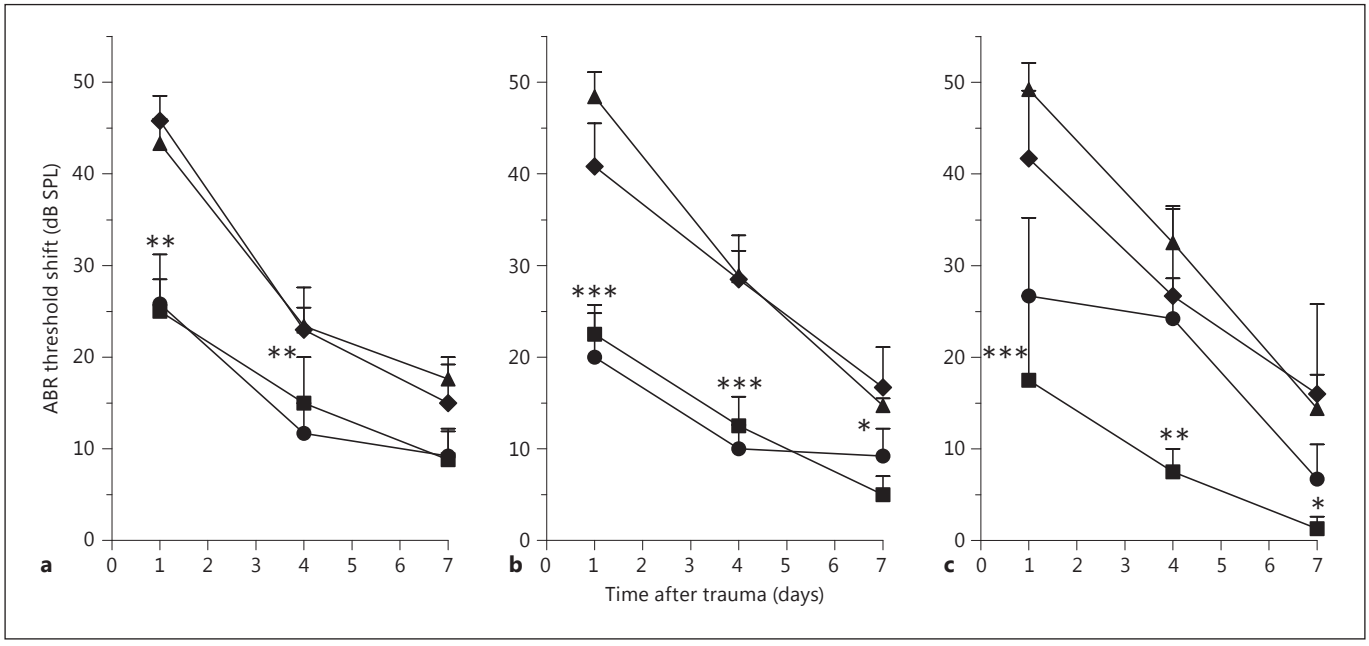

Fig. 1. OTO-104 protects against acute acoustic trauma when administered prior to noise exposure. Guinea pigs (n = 6) received a single bilateral intratympanic injection of various doses of OTO-104: poloxamer vehicle (triangles), 0.6% OTO-104 (diamonds), 2.0% OTO-104 (circles), and 6.0% OTO-104 (squares). One day later, the animals were exposed to narrow-band noise (4–8 kHz) for a period of 2 h at 105 dB SPL. ABR thresholds were monitored at the indicated times across 3 frequencies: 4 kHz (**a**), 8 kHz (**b**), and 16 kHz (**c**). Data are presented as means ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4, 8, and 16 kHz. Thresholds were interpolated between the lowest stimulus level where a response was observed and 5 dB lower, where no response was observed. The threshold was then reported as the mean value between these two stimulus conditions.

Data Analysis

Results are presented as means ± standard errors of the mean (SEM). Statistical analyses included a one-way ANOVA followed by Student's t test.

Results

OTO-104, a sustained-exposure hydrogel formulation of dexamethasone, was investigated in an acute acoustic trauma paradigm (fig. 1). One day prior to noise exposure (at 105 dB SPL), the guinea pigs received a single intratympanic injection of various OTO-104 doses, ranging from 0.6 to 6.0%. These doses were previously shown to provide therapeutic drug levels within the inner ear from days to weeks [Piu et al., 2011]. The therapeutic efficacy of dexamethasone, the active pharmaceutical ingredient in OTO-104, has been demonstrated to be optimal at concentrations of at least 10–50 ng/ml based on many in vitro and in vivo studies [Loew et al., 1986; O'Sullivan et al., 1997; Kim et al., 2009; Kim and Marcus, 2011]. Vehicle-treated (poloxamer) animals exhibited an initial hearing deficit at day 1 of 40–50 dB SPL across all 3 frequencies (4, 8, and 16 kHz), as measured by ABR. A spontaneous improvement in the control group was observed over time, resulting in a loss at day 7 of 15–20 dB SPL. The hearing deficit displayed by the 0.6% OTO-104 treatment group was comparable to that of the vehicle group. In contrast, doses of 2.0 and 6.0% OTO-104 provided significant otoprotection against noise-induced hearing loss. These benefits were evident throughout the course of the study and across frequencies. Interestingly, a very steep dose response was observed in the effects of OTO-104, with 0.6% showing no effect but 2.0 and 6.0% OTO-104 resulting in a similar degree of protection.

Next, the administration of dexamethasone as a solution was investigated. Intratympanically administered drugs delivered as solutions have a limited middle ear residence time, due to rapid drainage via the Eustachian tube. The guinea pigs were treated with a 2.0% aqueous DSP solution 1 day prior to noise exposure (at 105 dB SPL), and their hearing was monitored thereafter for a

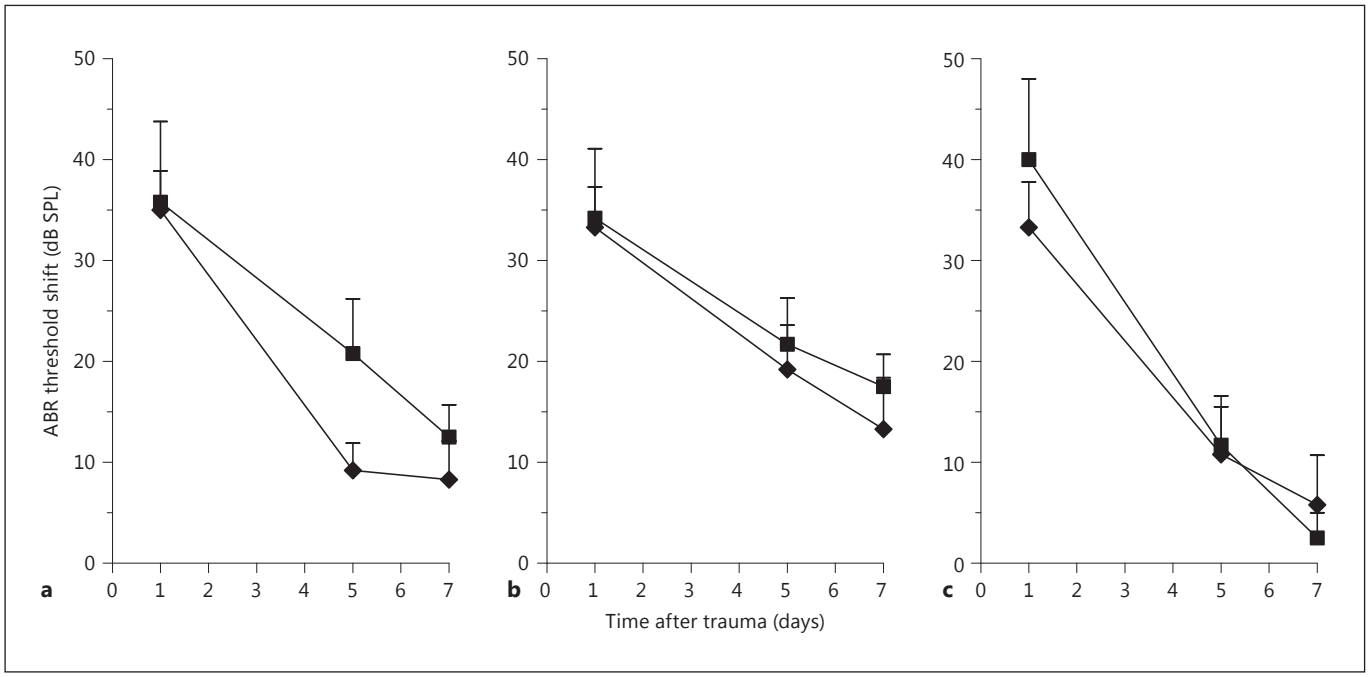

Fig. 2. An aqueous DSP solution does not protect against acute acoustic trauma when administered prior to noise exposure. Guinea pigs (n = 6) received a single bilateral intratympanic injection of either saline (squares) or 2.0% DSP solution (diamonds). One day later, the animals were exposed to narrow-band noise (4–8 kHz) for a period of 2 h at 105 dB SPL. Auditory function was monitored at the indicated times across 3 frequencies: 4 kHz (**a**), 8 kHz (**b**), and 16 kHz (**c**). Data are presented as means ± SEM.

7-day period. No differences between the vehicle control and the treated animals were noted at any of the time points across all 3 frequencies tested (fig. 2). The vehicle-treated animals displayed an initial deficit of 35–40 dB SPL across frequencies, which spontaneously improved by day 7 to 5–15 dB SPL. The animals treated with the DSP solution had a similar profile, with an initial deficit of 30–35 dB SPL, improving to 5–15 dB SPL at day 7. Under these experimental conditions, a steroid solution did not provide otoprotection against noise-induced hearing loss when administered 1 day prior to noise trauma.

The otoprotective potential of OTO-104 was next investigated as a function of the extent of the trauma (fig. 3). Intensities of either 105 or 110 dB SPL were compared. Differences in the recovery profile of the vehicle-treated guinea pigs exposed to different noise exposures were noted. At the pressure level of 105 dB SPL, the initial deficit of 40–50 dB SPL observed at day 1 improved spontaneously to 15–20 dB SPL by day 7 across frequencies. At the higher pressure level of 110 dB SPL, a similar initial ABR threshold shift was evident in the range of 40–55 dB SPL at day 1. However, spontaneous recovery was significantly less pronounced, with a deficit of 30–35 dB SPL observed by day 7. Following a single intratympanic injection of 6.0% OTO-104 one day prior to noise exposure, significant improvements in hearing were noted under both trauma conditions at all time points evaluated and across frequencies. The initial deficit was milder compared to the vehicle-treated animals (15–25 and 20–40 dB SPL in the acoustic trauma paradigms of 105 and 110 dB SPL, respectively). At day 7, complete recovery (lack of ABR threshold shift relative to naïve conditions prior to noise) was observed at the 8- and 16-kHz frequencies in the acoustic trauma paradigm of 105 dB SPL, and at the 16-kHz frequency in the acoustic trauma paradigm of 110 dB SPL.

To assess the ability of sustained release dexamethasone to reduce hearing loss from a prior noise exposure, the administration of OTO-104 was carried out starting 2, 3, or 4 days after acoustic trauma at 105 dB SPL. The guinea pigs received either a single intratympanic injection of poloxamer (vehicle) or 6.0% OTO-104. OTO-104 offered significant otoprotection when given 2 or 3 days following noise exposure. However, no differences were evident between the control and treated groups when the intratympanic administration was conducted 4 days after

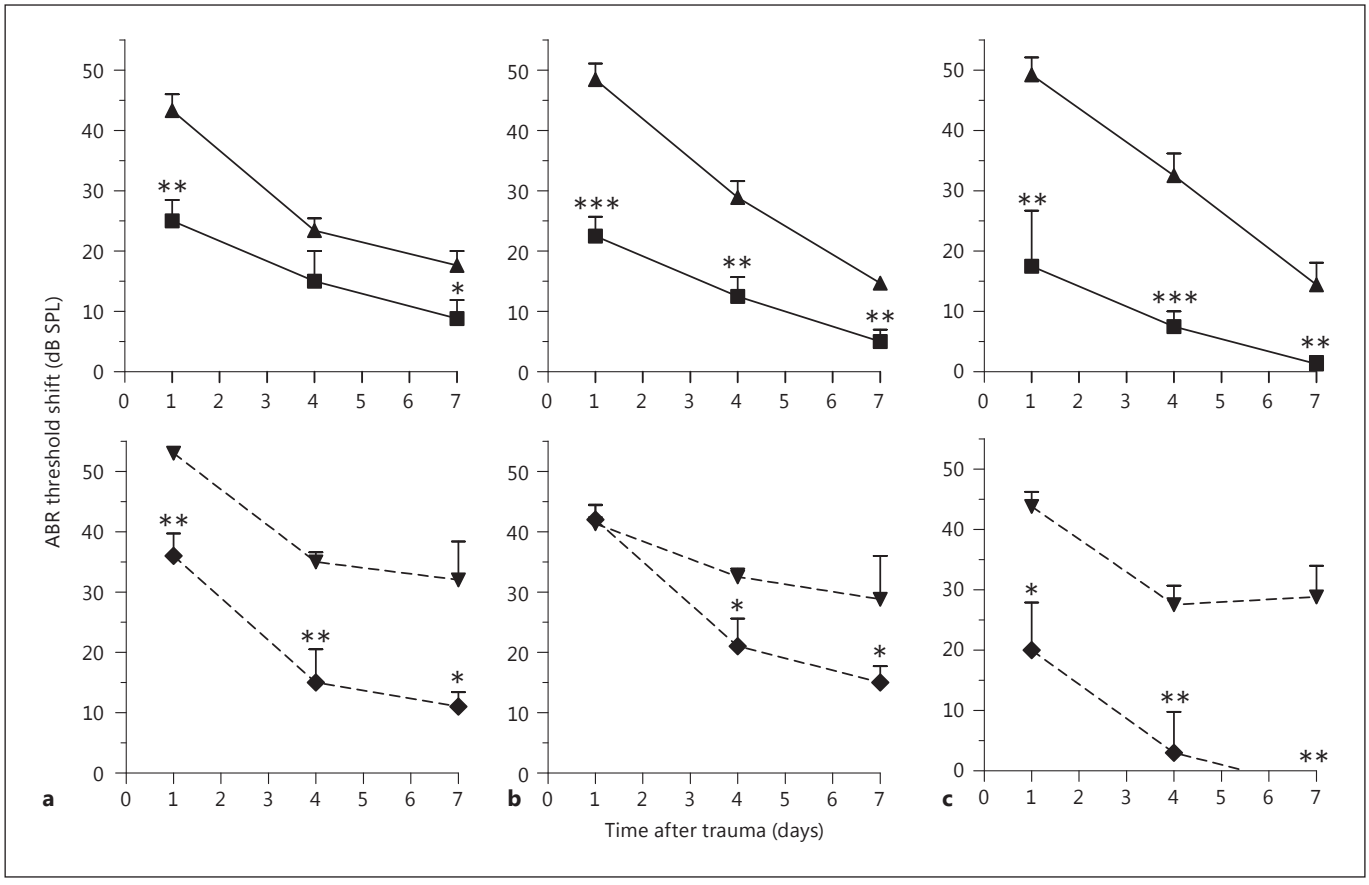

Fig. 3. OTO-104 protects against various degrees of acute acoustic trauma. Guinea pigs (n = 6) received a single bilateral intratympanic injection of either poloxamer vehicle (triangles) or 6.0% OTO-104 (diamonds). A day later, the animals were exposed to narrow-band noise (4–8 kHz) for a period of 2 h at a pressure level of either 105 dB SPL (solid lines) or 110 dB SPL (dashed lines). Auditory function was monitored at the indicated times across 3 frequencies: 4 kHz (**a**), 8 kHz (**b**), and 16 kHz (**c**). Data are presented as means ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

trauma (fig. 4). When given 2 days following noise exposure, the administration of OTO-104 resulted in a smaller ABR threshold shift of 10–20 dB SPL compared to the vehicle-treated animals across all 3 frequencies tested. When given 3 days after trauma, the difference between the treated and vehicle control groups was only of 5–10 dB SPL, but was statistically significant.

In contrast, the administration of an aqueous DSP solution 2 days after noise exposure did not offer any significant protection, even though a small trend in improvement was consistently noted across frequencies (fig. 5). The difference between the treated and vehicle control groups was 5–10 dB SPL, but was not statistically significant.

To assess the specificity of OTO-104 protection, treatment with the glucocorticoid and mineralocorticoid antagonist mifepristone was evaluated (fig. 6). The guinea pigs that received mifepristone alone had a hearing deficit following noise exposure that was comparable to the poloxamer control group, with values of 35–45 dB SPL across all 3 frequencies tested. Thus, steroid antagonism does not impact noise-induced hearing loss under these experimental conditions. As expected and demonstrated above, treatment with OTO-104 significantly reduced the hearing deficit in comparison to vehicle controls, with values of 15–25 dB SPL across all 3 frequencies. When mifepristone was co-administered with OTO-104, the otoprotective effect was completely abolished (deficit of 40–45 dB SPL across frequencies), suggesting that the mechanism of action of OTO-104 is primarily dependent upon the activation of classical nuclear receptor pathways.

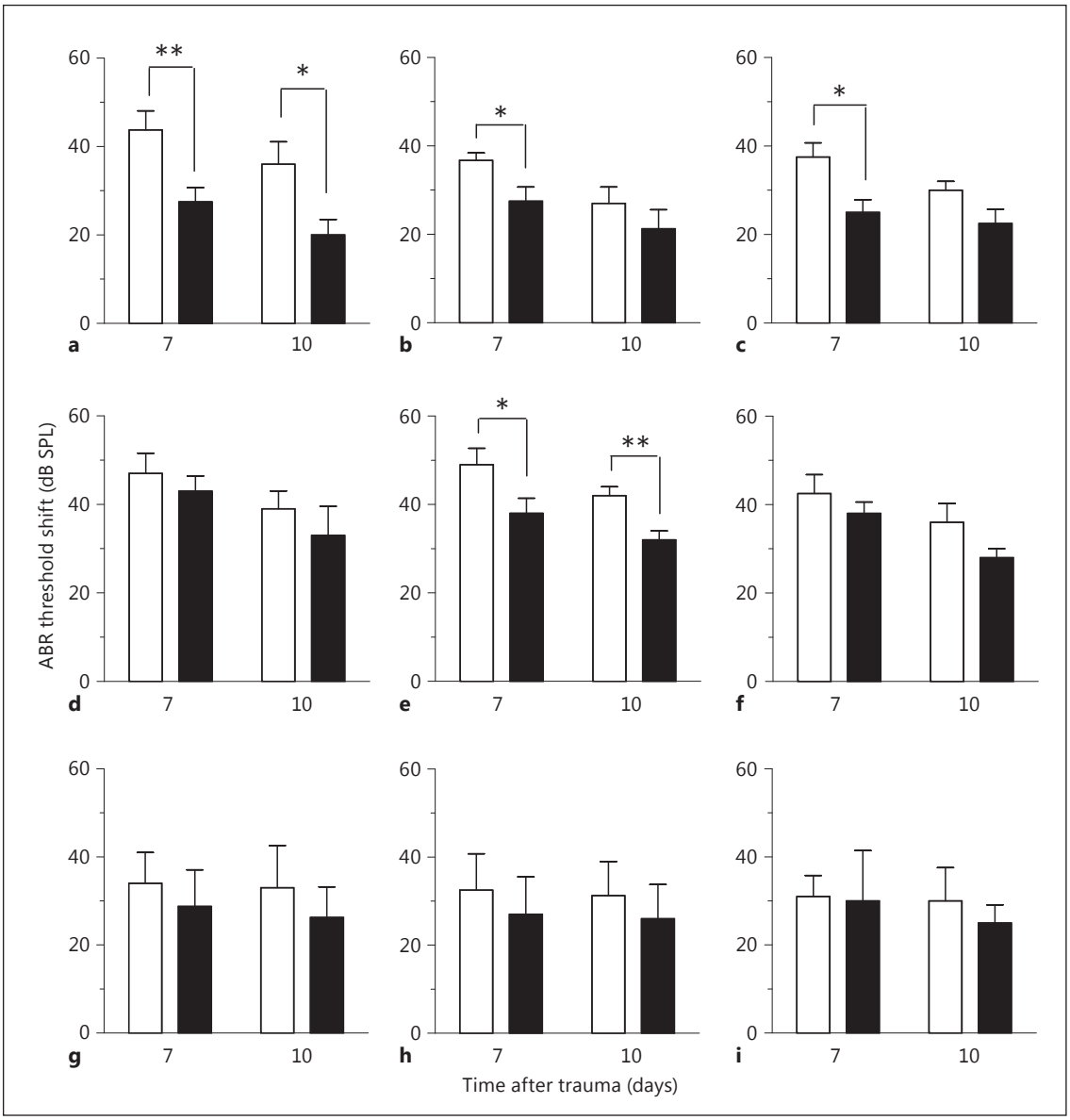

Fig. 4. OTO-104 protects against acute acoustic trauma when administered 2 or 3 days after noise exposure. Guinea pigs (n = 6) were exposed to narrow-band noise (4–8 kHz) for a period of 2 h at 105 dB SPL. At 2 days (**a–c**), 3 days (**d–f**), or 4 days (**g–i**) after trauma, the animals received a single bilateral intratympanic injection of either poloxamer vehicle (white bars) or 6.0% OTO-104 (black bars). Auditory function was monitored at the indicated times across 3 frequencies: 4 kHz (**a**, **d**, **g**), 8 kHz (**b**, **e**, **h**), and 16 kHz (**c**, **f**, **i**). Data are presented as means ± SEM. * $p < 0.05$; ** $p < 0.01$.

Discussion

The findings presented herein demonstrate that the administration of the corticosteroid dexamethasone directly to the ear in the form of a sustained-exposure hydrogel lends significant protection in a model of noise-induced hearing loss. Otoprotection was observed when OTO-104 was administered prior to as well as following trauma. This is in contrast to an aqueous dexamethasone solution that displayed no otoprotective benefit under the experimental conditions.

When a DSP solution was given intratympanically 1 day prior to acute acoustic trauma, no otoprotection was observed. This lack of protection following a single intra-

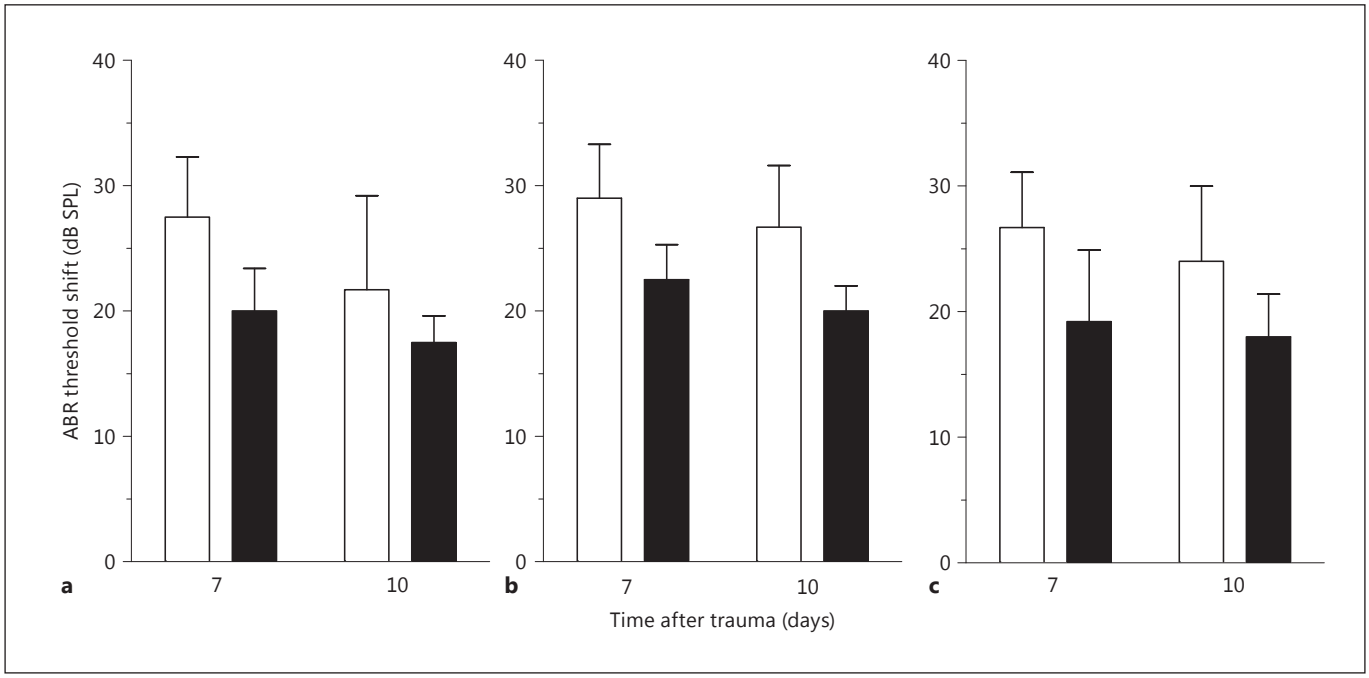

Fig. 5. An aqueous DSP solution does not protect against acute acoustic trauma when administered 2 days after noise exposure. Guinea pigs (n = 6) were exposed to narrow-band noise (4–8 kHz) for a period of 2 h at 105 dB SPL. Two days after trauma, the animals received a single bilateral intratympanic injection of either saline (white bars) or 2.0% DSP solution (black bars). Auditory function was monitored at the indicated times across 3 frequencies: 4 kHz (**a**), 8 kHz (**b**), and 16 kHz (**c**). Data are presented as means ± SEM.

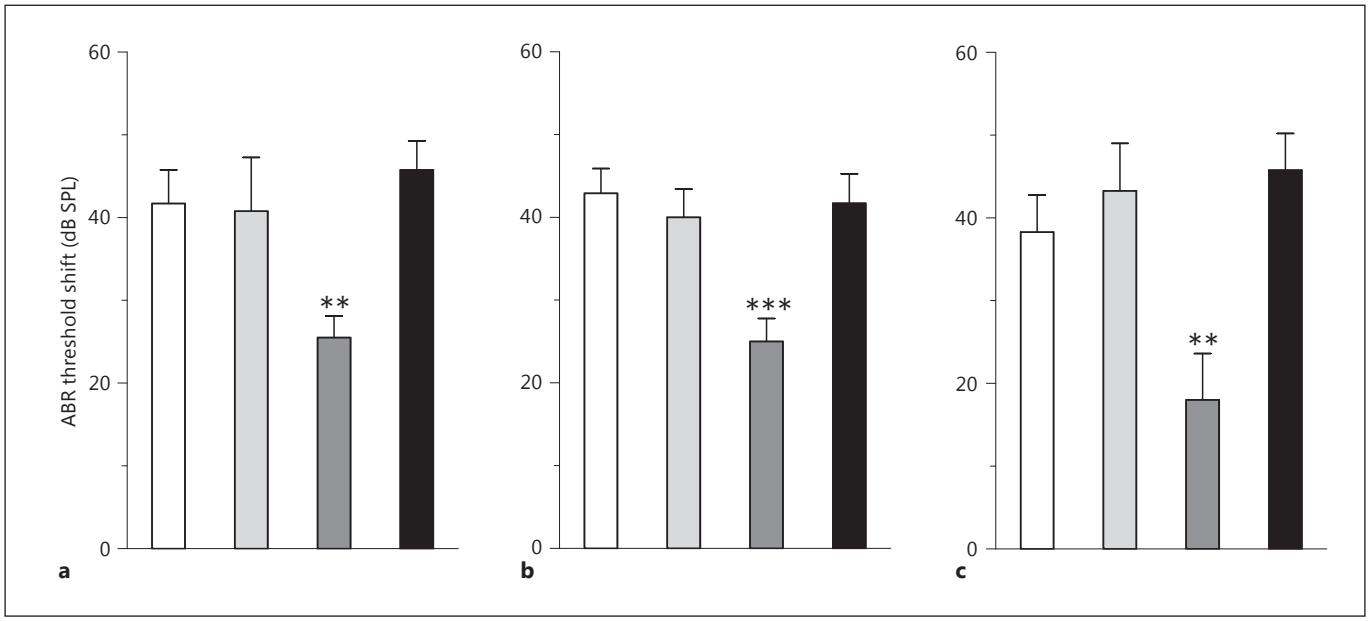

Fig. 6. Mifepristone antagonizes OTO-104 protection against acute trauma. Guinea pigs (n = 6) received a single bilateral intratympanic injection of either poloxamer vehicle (white bars), 6.0% mifepristone (light grey bars), 6.0% OTO-104 (dark grey bars), or 6.0% OTO-104 + 6.0% mifepristone (black bars). After 24 h, the animals were exposed to narrow-band noise (4–8 kHz) for a period of 2 h at 105 dB SPL. Auditory function was monitored 7 days post trauma across 3 frequencies: 4 kHz (**a**), 8 kHz (**b**), and 16 kHz (**c**). Data are presented as means ± SEM. ** $p < 0.01$; *** $p < 0.001$.

tympanic injection of dexamethasone solution in noise-induced hearing loss in rats has been reported by others [Yildirim et al., 2005]. These results are consistent with the short residence time of dexamethasone in the inner ear perilymphatic compartment when administered as a solution intratympanically. In guinea pigs, a DSP solution is cleared rapidly from the middle ear down the Eustachian tube, with detectable drug levels in the perilymph lasting for about 12 h at most [Wang et al., 2011a, b]. Further, Salt et al. [2011] have reported that the clearance rate of dexamethasone in the perilymph is quite rapid, in the order of 90 min. In contrast, the administration of sustained-release dexamethasone was very effective in preventing hearing loss following acute acoustic trauma. A single intratympanic injection of OTO-104 ensures the presence of dexamethasone in the inner ear compartment for days to weeks [Wang et al., 2009; Piu et al., 2011], maximizing the potential of dexamethasone to provide otoprotection against trauma over time. In support of this, investigators have reported that a dexamethasone solution can attenuate hearing loss, but only when the drug is delivered continuously to the inner ear via a perfusion pump [Takemura et al., 2004] or given intratympanically on multiple occasions following trauma [Han et al., 2015]. Taken altogether, these findings indicate that the therapeutic potential of dexamethasone is best achieved when the drug remains present in the inner ear compartment for extended periods of time – therapeutic properties that are clearly afforded by OTO-104.

Two different noise exposure paradigms were evaluated in this study. At the pressure level of 105 dB SPL, a significant improvement in hearing was noted in the untreated animals within 1 week, suggesting that this exposure is primarily associated with temporary threshold shift (TTS). In contrast, a much more modest recovery of hearing loss thresholds was noted in the untreated guinea pigs exposed to 110 dB SPL, consistent with the establishment of a permanent threshold shift (PTS). Interestingly, OTO-104 was equally effective in preventing against noise-induced hearing loss in these two treatment regimens, indicating that both TTS and PTS are improved by OTO-104. TTS is typically thought to result from reversible mechanical or biochemical damages to the cochlear hair cells and neurons, including inflammation immediately after noise trauma, whereas PTS develops later from irreversible damage to or loss of hair cells and spiral ganglion cells [Quaranta et al., 1998; Groschel et al., 2010]. Hair cell loss results from the activation of apoptotic and necrotic pathways. Apoptosis is an active process that converges towards the activation of caspases which lead the cell disassembly. In contrast, necrosis is a more passive process that can involve metabolic and inflammatory stress leading to disruption of the integrity of cell membranes. Researchers have suggested that, in acoustic trauma, apoptosis is the primary initial cell death pathway, followed subsequently by both apoptotic and necrotic processes [Hu et al., 2009]. Dexamethasone has been shown to protect against apoptosis of auditory hair cells by activating survival pathways [Haake et al., 2009]. Therefore, dexamethasone, by combining multiple modes of action from mitigating inflammatory responses to activating cell survival pathways, offers an effective means of protection against noise-induced hearing loss.

The administration of OTO-104 yielded protection to guinea pigs not only when given prior to noise exposure but also up to 3 days after trauma. These findings indicate that there exists a window of time during which OTO-104 can confer benefits. The temporal limit observed after noise exposure most likely involves irreversible changes occurring within the cochlea (and/or perhaps the central auditory system) during and after acoustic trauma. Indeed, it has been shown that irreversible damage to peripheral auditory structures can occur within a few days [Groschel et al., 2010], while during that same period there may also be primary degeneration within the central auditory pathway [Kujawa and Liberman, 2009]. The results presented herein are thus consistent with the notion that there exists a limited window of opportunity for dexamethasone to exert a protective effect prior to irreversible changes in the auditory system.

While a low dose of OTO-104 (0.6%) was not effective in protecting against noise-induced hearing loss, doses of 2.0% and above offered equivalent otoprotection. This steep dose response is consistent with the nature of the primary effectors of dexamethasone action – the glucocorticoid and mineralocorticoid nuclear receptors. These proteins belong to a family of ligand-activated transcription factors that modulate the expression of target genes on ligand binding. Transcription factors, via the formation of multi-protein complexes interacting with DNA motifs, can control the transition from transcriptionally repressive to active states, thereby providing a binary switch in transcriptional activation. This on/off mode of action can translate pharmacologically into a steep dose-response curve.

The ability of mifepristone, a glucocorticoid and mineralocorticoid antagonist, to antagonize the otoprotective effects of OTO-104 supports the notion that dexamethasone acts primarily by activating the nuclear receptors glucocorticoid and mineralocorticoid to alleviate noise-

induced hearing loss. These findings are consistent with published reports detecting the presence of glucocorticoid in the cochlea, in particular the spiral ligament, stria vascularis, and organ of Corti [ten Cate et al., 1993; Rarey and Curtis, 1996].

There is evidence that a local delivery approach to the treatment of noise-induced hearing loss is preferred over a systemic approach. A recent study from Han et al. [2015] compared the therapeutic effect of dexamethasone in a mouse paradigm of noise-induced hearing loss administered systemically versus intratympanically. The authors noted that both routes afforded protection against noise trauma but required multiple administration of dexamethasone (daily for 5 days for the systemic route). This is consistent with a recently published prospective randomized clinical trial in patients suffering from sudden sensorineural hearing loss [Rauch et al., 2011], which demonstrated comparable therapeutic benefits between a 19-day course of orally administered prednisolone and a regimen of 4 intratympanic injections of methylprednisolone. However, it has been recognized that the systemic route to treat otic disorders is inadequate owing to poor inner ear absorption and significant risks of adverse effects [Bird et al., 2007, 2011; Rauch et al., 2011].

The intratympanic administration of OTO-104 has been extensively studied both nonclinically [Wang et al., 2009, 2011a, b; Piu et al., 2011] and clinically [Lambert et al., 2012] in various species. It was not associated with adverse effects at doses that achieved sustained inner ear exposure both locally in the ear as well as systemically in animal studies. Systemic exposure resulting from intratympanic administration of OTO-104 is minimal in animals and negligible in humans, and was not associated with adverse events in a phase 1b clinical study in patients with Ménière's disease [Lambert et al., 2012]. In the otic compartment, an intratympanic injection of OTO-104 has no adverse effects on middle and inner ear integrity.

In conclusion, a single intratympanic administration of OTO-104, a sustained-exposure formulation of dexamethasone, provides significant protection against acoustic trauma. The therapeutic benefit was observed when the drug was given prior to and up to 3 days following trauma.

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