Adenosine amine congener mitigates noise-induced cochlear injury

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Abstract Hearing loss from noise exposure is a leading occupational disease, with up to 5% of the population at risk world-wide. Here, we present a novel purine-based pharmacological intervention that can ameliorate noise-induced cochlear injury. Wistar rats were exposed to narrow-band noise (8–12 kHz, 110 dB SPL, 2–24 h) to induce cochlear damage and permanent hearing loss. The selective adenosine A₁ receptor agonist, adenosine amine congener (ADAC), was administered intraperitoneally (100 µg/kg/day) at time intervals after noise exposure. Hearing thresholds were assessed using auditory brainstem responses and the hair cell loss was evaluated by quantitative histology. Free radical damage in the organ of Corti was assessed using nitrotyrosine immunohistochemistry. The treatment with ADAC after noise exposure led to a significantly greater recovery of hearing thresholds compared with controls. These results were upheld by increased survival of sensory hair cells and reduced nitrotyrosine immunoreactivity in ADAC-treated cochlea. We

propose that ADAC could be a valuable treatment for noise-induced cochlear injury in instances of both acute and extended noise exposures.

Keywords Adenosine · Adenosine receptors · Adenosine amine congener · Cochlea · Hearing · Treating hearing loss

Introduction

One of the most common causes of hearing loss is excessive exposure to noise. The problem is particularly common in the military and in industrial settings (construction workers, mining, forestry and airline industry). Some leisure activities (shooting, listening to loud music) may also lead to accidental noise-induced hearing loss. According to the World Health Organisation, hearing loss is the sixth-ranked cause of disease burden accounting for 3% of the disability-adjusted life years and significantly affecting over 250 million people. Hearing loss from noise exposure (noise-induced hearing loss, NIHL) is a leading occupational disease, with up to 5% of the population at risk world-wide. However, the traumatic noise experienced in some occupations such as in the military is very difficult to prevent [1]. The proportion of non-work related NIHL is also on the rise, with possible causes including increased levels of ambient noise in developed areas from motor vehicles and construction sites, as well as the increased use of portable music players (PMPs). The constant input of sounds at very high intensities could cause short- and long-term hearing loss. The Royal National Institute for Deaf People (RNID, UK) issued a serious warning that approximately two-thirds of 18- to 30-year-olds in the current population are unnecessarily exposed to dangerously high-

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intensity sounds (>85 dB) through PMPs which can cause hearing damage. Standards set by Occupational Safety and Health (OSH) indicate that exposure to noise over a daily 85 dBA/8 h dose will eventually harm hearing. “Safe” noise exposure time halves with every 3 dB increase in intensity. To address this, European Union legislation has now been implemented (2009) to mandate an 85-dB maximum initial level to PMPs (while providing a manual override option).

Hearing conservation programmes may be ineffective and there are many instances of unprotected exposure to excessive noise leading to hearing loss (particularly in the military and heavy industry). Hence, it is essential to develop therapies for NIHL that can ameliorate injury to the delicate structures of the inner ear and reduce hearing loss. Prosthetic rehabilitation via hearing aids and cochlear implants are the only current treatment strategies for hearing loss, whilst pharmacological therapies for NIHL have only recently been proposed [2]. The majority of hearing loss arises from injury to the sensory system of the inner ear. Whilst treatments exist for middle ear conditions, there are virtually no treatments that can ameliorate the damage to the inner ear and reduce the impact of sensorineural hearing loss. There is increasing evidence that oxidative stress and the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are key elements in the pathogenesis of cochlear injury due to noise exposure [3–7]. Compounds that target the mechanisms underlying oxidative stress thus offer considerable potential as therapies for hearing loss.

Adenosine receptor agonists were successfully used in the treatment of ischemic brain and cardiac injury [8, 9], demonstrating an extraordinary cytoprotective function. Adenosine receptors were identified and localised in the mammalian cochlea [10, 11], whilst prophylactic treatment with adenosine receptor agonists have been shown to reduce the cochlear damage from noise exposure [12–14]. These prophylactic medications were applied topically to the round window membrane (RWM) of the cochlea before noise exposure. Even though the RWM is the most surgically accessible route for drug delivery to the inner ear, it is still problematic because the substances placed onto the RWM do not distribute evenly through the cochlea, and it is difficult to use this route of administration for chronic clinical treatments [15].

This study investigates the therapeutic potential of the selective A_1 adenosine receptor agonist adenosine amine congener (ADAC) in the treatment of NIHL. ADAC was used in the past to provide neuroprotection in experimental models of cerebral ischemia and Huntington’s disease [16–18]. ADAC was found to be particularly advantageous due to reduced peripheral side effects [17] compared to other drugs acting upon adenosine A_1 receptors. Other A_1 receptor agonists show cardiovascular effects such as

bradycardia, hypotension and hypothermia [9]. The lack of side effects by ADAC and its high affinity for A_1 receptors in the brain is due to its modified chemical structure and increased ability to cross the blood–brain barrier [19, 20]. ADAC thus can be administered systemically avoiding the surgical procedures required to deliver the drug intratympanically or directly to the inner ear. The findings of the current study present novel evidence that post-noise treatment with ADAC can dramatically improve hearing thresholds in rats. We demonstrate that ADAC can attenuate noise-induced threshold shifts and ameliorate cochlear injury in instances of acute and extended noise exposure. This confers the adenosine signalling pathway in the cochlea as a principal route for the treatment of NIHL.

Materials and methods

Animals

Male Wistar rats (8–10 weeks old) were used in this study. The use of animals and experimental procedures were approved by the University of Auckland Animal Ethics Committee.

Treatments

ADAC was obtained from Dr Ken Jacobson (NIH, Bethesda, USA). ADAC (2.5 μ g) was dissolved in 100 μ L of 1 N HCl and then in 50 ml of 0.1 M PBS (pH 7.4), to prepare a 50 μ g/mL stock solution [16]. This solution was aliquoted and stored at -20°C for later use. When required, the ADAC aliquots were heated in a 37°C water bath for 30 min before administration. The ADAC injection dose (100 μ g/kg/day) was given intraperitoneally. The same volume of vehicle solution (200 μ L/100 g/day i.p.) was given to control animals. ADAC was administered as a single injection 6 or 24 h after noise exposure, or as five injections administered every 24 h commencing 6 h post-noise (chronic treatment). In the control group, injections of the drug vehicle were administered at the same intervals as ADAC. All experimental groups are shown in Table 1.

Body weight and temperature

To control for possible side effects of ADAC after systemic administration (e.g. weight loss, hypothermia), we have measured the body weight and rectal temperature in ADAC-treated and control animals. Body weight was measured immediately before noise exposure and 14 days after noise exposure. Rectal temperature was measured before ADAC administration and again 30 and 60 min after administration.

Table 1 Experimental groups

	Group number	Noise exposure (h)	Treatment	Treatment regime
	Group 1	24	ADAC	Single injection 6 h PE
	Group 2	24	Vehicle (control)	Single injection 6 h PE
	Group 3	24	ADAC	Single injection 24 h PE
	Group 4	24	Vehicle (control)	Single injection 24 h PE
	Group 5	24	ADAC	Multiple injections
	Group 6	24	Vehicle (control)	Multiple injections
<i>n</i> =8 animals per group	Group 7	2	ADAC	Multiple injections
<i>PE</i> post-exposure	Group 8	2	Vehicle (control)	Multiple injections

Noise exposure

Rats were exposed to 8–12 kHz band noise for 2 h, or 24 h at 110 dB SPL. Noise exposures were carried out in a custom built acoustic chamber (Shelburg Acoustics, Sydney, Australia) with internal speakers and external controls (sound generator and frequency selector). The sound intensity inside the chamber was measured using a calibrated Rion NL-40 sound level metre and there were minimal deviations of sound intensity (110 ± 1 dB SPL) in the vicinity of the animal cage. Up to four rats were placed in the chamber in a standard rat cage.

Auditory brainstem responses

ABR represents the activity of the auditory nerve and the central auditory pathways (brainstem/mid-brain regions) responding to the sound (clicks or pure tones). ABRs were obtained by placing fine platinum electrodes subdermally at the mastoid region of the ear of interest (active electrode), scalp vertex (reference) and mastoid region of the opposite ear (ground electrode). A series of auditory clicks or pure tones (4–28 kHz) presented at varying intensity and thresholds generate electrical activity reflecting differing levels of auditory processing. The threshold of the ABR complex (waves I–IV) were determined by progressively attenuating the sound intensity until the waveform could no longer be observed. The acoustic stimuli for ABR were produced and the responses recorded using a Tucker–Davis Technologies auditory physiology workstation (Alachua, FL, USA).

All ABR measurements were performed in a sound attenuating chamber (Shelburg Acoustics, Sydney, Australia). Rats were anaesthetised with the mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg) intraperitoneally, and then placed onto a heating pad, to maintain body temperature at 37°C. ABR potentials were evoked with digitally produced 5 ms tone pips (0.5 ms rise-fall time) at frequencies between 4 and 28 kHz in half-octave steps. Sound pressure level (SPL) was raised in 5 dB steps starting from 10 dB below threshold level to 90 dB SPL. Responses were averaged at each sound level (1024 repeats with stimulus polarity alternated), and response

waveforms were discarded when peak-to-peak amplitude exceeded 15 μ V. The ABR threshold was defined as the lowest intensity (to the nearest 5 dB) at which a response could be visually detected above the noise floor.

ABR thresholds were measured before and after noise exposure, and after ADAC/vehicle treatment. In the group exposed to noise for 24 h, post-noise ABR recordings were obtained 1 h before the rats received their first ADAC or vehicle injection. This was 5 h after noise exposure for groups 1, 2, 5 and 6 or 23 h for groups 3 and 4 (Table 1). The final ABR measurements were obtained 18 hours after the last ADAC/vehicle injection. In the group exposed to noise for 2 h, ADAC treatment commenced 6 h after the cessation of noise exposure, whilst ABRs were recorded 30 min and 14 days after noise exposure.

Hair cell counts

After the last ABR measurement, rats were euthanised with an overdose of anaesthetic (pentobarbitone, 100 mg/kg i.p.) and cochleae removed for histological analysis. After the overnight fixation in 4% paraformaldehyde (PFA), the cochlea was decapsulated and the organ of Corti removed. The organ of Corti was separated into the apical, middle and basal turns, and the tissues were permeabilised with 1% Triton X-100 for 1 h. Alexa Fluor 488 phalloidin (Invitrogen) dissolved in 0.1 M phosphate-buffered saline (PBS, pH 7.4) was used to stain the hair cells and their stereocilia. Tissues were incubated in 1% phalloidin (2U/ml) for 40 min, washed with 0.1 M PBS for 30 min and mounted onto glass slides using CitiFluor. The slides were visualised using a Zeiss epifluorescence microscope equipped with an Axiocam camera and Axiovision v3.1 software. Images were taken for the entire length of the cochlea, and the number of missing hair cells was counted for each turn and presented as a percentage of total number of hair cells.

Nitrotyrosine immunohistochemistry

Noise-induced production of RNS in the rat cochlea was examined by nitrotyrosine (NT) immunofluorescence six

days after 24 h exposure to the 110 dB SPL noise. After overnight fixation in 4% PFA, cochleae were decalcified in a 5% EDTA solution for 7 days and cryoprotected in a 30% sucrose (in 0.1 M PB) solution overnight. The cochleae were snap-frozen in *N*-pentane, and stored at -80°C until further processing. Frozen cochlear tissues were cryosectioned at 30 μm and transferred into 24-well plates (Nalge Nunc Int., Naperville, USA) containing the sterile 0.1 M PBS, and permeabilised with 1% Triton X-100 for 1 h. Non-specific binding sites were blocked with 10% normal goat serum (Vector Laboratories, Burlingame, CA). The nitrotyrosine antibody (BIOMOL Research Laboratories Inc., Plymouth, PA, USA) was diluted 1:750 in 1.5% normal goat serum and 0.1% Triton X-100 in 0.1 M PBS. Tissue sections were incubated with the primary antibody overnight at 4°C . The primary antibody was omitted in control wells. The secondary antibody Alexa 488 goat anti-mouse IgG conjugate (Invitrogen) was diluted 1:400 in a 0.1 M PBS solution containing 1.5% normal goat serum and 0.1% Triton X-100. Tissue sections were incubated with the secondary antibody for 2 h in the dark, then rinsed several times in PBS, mounted in fluorescence medium (DAKO Corporation, Carpinteria, CA, USA) and screened for NT specific immunofluorescence using a confocal microscope (TCS SP2, Leica Leisertechnik GmbH, Heidelberg, Germany). Image acquisition was controlled by Scanware software (Leica). A series of six to ten optical sections were collected for each specimen, and image analysis was performed on an optical section from the centre of the stack. The detection settings were pre-set to allow comparison of relative staining densities between control and ADAC-treated cochleae.

Statistical analysis

Results are presented as the mean $\pm$ S.E.M. The comparison of ABR thresholds and hair cell loss was performed using a Student's unpaired *t* test assuming unequal variances. Normality of the data was confirmed and the α level set at $P=0.05$.

Results

Body weight and temperature

ADAC treatment did not cause discernible behavioural changes in rats or alterations in body weight (Fig. 1a). Body weight increased equally in noise-treated and control groups during the measurement period. In addition, body temperature remained stable after administration of ADAC (Fig. 1b).

Fig. 1 Body weight and temperature in animals treated with ADAC (100 $\mu\text{g}/\text{kg}$). **a** Body weight was measured immediately before noise exposure and 14 days after noise exposure. **b** Rectal temperature ($^{\circ}\text{C}$) was measured before ADAC administration and 30 and 60 min after the injection. Data are expressed as means $\pm$ SEM. Number of animals: $n=8$ per group

Auditory thresholds after extended (24 h) noise exposure

Sound-evoked ABR responses were used to assess hearing of ADAC-treated rats. It is a clinical technique which is also used in rodents and other experimental animals to assess auditory function [21].

ABR thresholds were measured prior to noise exposure (baseline), post-exposure and after ADAC treatment. Baseline ABR thresholds were comparable in all groups (Fig. 2). Threshold shifts within 24 h after noise exposure ranged from 45 to 60 dB for auditory clicks and pure tones (Fig. 2). Animals treated with a single injection of ADAC showed significant recovery of ABR thresholds: 17–26 dB when the animals received early treatment (6 h after noise) and 6–11 dB in animals treated 24 h after noise exposure. Chronic treatment with ADAC (5 days) provided uniform recovery of ABR thresholds at all pure tone frequencies (22–28 dB). A similar effect was observed in response to auditory clicks which have been plotted as separate bar graphs in Fig. 2. The highest recovery of ABR thresholds was observed in the group that received multiple injections of ADAC (29 ± 3 dB) and the lowest in the group which received a single ADAC injection 24 h after noise exposure (8 ± 2 dB). In control groups treated with the vehicle solution, ABR responses were not statistically different from post-exposure thresholds.

Fig. 2 Auditory brainstem responses (ABRs) in rats exposed to 8–12 kHz band noise for 24 h at 110 dB SPL. ABR thresholds (expressed in dB SPL) were measured in response to pure tones (4–24 kHz) and auditory clicks. ADAC (100 µg/kg i.p.) was administered as a single injection 6 h after noise exposure (**a**, **b**) or 24 h after noise

exposure (**c**, **d**), or as five injections administered every 24 h commencing 6 h post-noise (**e**, **f**). In the control group (**g**, **h**), injections of the drug vehicle were administered at the same intervals as ADAC. Data are expressed as means ± SEM. Animal numbers: $n=8$ per group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; unpaired t test

Hair cell loss after extended noise exposure

In this study, phalloidin staining of F-actin was used for qualitative analysis of the organ of Corti morphology. Non-noise-exposed Wistar rats at that age (8–10 weeks) showed excellent preservation of the organ of Corti. Histological analysis of the organ of Corti exposed to noise (8–12 kHz, 110 dB SPL for 24 h) demonstrated damage mostly to the basal and middle turns, whilst the apical turn was less affected. Representative examples of the basal turn organ of Corti are shown in Fig. 3a,b. The organ of Corti in the control (noise-exposed cochlea treated with the vehicle solution) showed widespread outer hair cell loss particularly in the first row, and some inner hair cell loss (Fig. 3a). In contrast, the phalloidin-stained surface preparation of the organ of Corti from the ADAC-treated rat cochlea (Fig. 3b) showed well-preserved hair cell morphology, even though occasional loss of hair cells was also observed.

Nitrotyrosine immunohistochemistry after extended noise exposure

Nitrotyrosine immunohistochemistry staining was used for qualitative analysis of the RNS activity in the cochlea following 24 h noise exposure and 5 days ADAC treatment. All sections were taken from the middle turn. A comparison between ADAC-treated and vehicle-treated cochleae is shown for the groups where ADAC or vehicle injections were given for five consecutive days (Fig. 3c,d). The nitrotyrosine staining in the vehicle-treated cochlea (Fig. 3c) was primarily in the non-sensory epithelial cells lining scala media: inner sulcus cells, inner phalangeal cells, pillar, Deiters' and Hensen's cells of the organ of Corti. Interestingly, nitrotyrosine immunostaining was observed in the outer hair cells in only two out of eight animals, whilst no nitrotyrosine immunoreactivity was observed at the level of inner hair cells. Nitrotyrosine immunostaining in the ADAC-treated cochlea (Fig. 3d) was reduced to background levels observed in non-noise controls (data not shown). Immunostaining was also absent from controls where the primary antibody was omitted (data not shown). In summary, the nitrotyrosine immunostaining indicated that ROS/RNS activity in the cochlear partition was elevated for six days following the noise exposure, if ADAC treatment was omitted.

Auditory thresholds after acute noise exposure

In this study of the effects of acute noise exposure, rats were exposed to 8–12 kHz band noise presented for 2 h at 110 dB SPL. This noise treatment also produced significant hearing loss. We used the same ADAC treatment regime that was highly effective in the model of chronic noise

exposure: five ADAC injections given at 24-h intervals. ABR recordings were made before and after noise exposure (30 min and 14 days).

All noise-exposed animals showed comparable threshold shifts (32–60 dB) for auditory clicks and pure tones (4–28 kHz) 30 min post-noise. The highest threshold shifts (55–60 dB) were observed at 8–16 kHz frequencies representing the most damaged area. At the end point of the study (14 days post-noise), threshold shifts were significantly reduced in ADAC-treated animals compared to vehicle-treated controls (Fig. 4), which exhibited up to 30 dB greater permanent threshold shift at some frequencies. Threshold recovery was the greatest at pure tone frequencies ranging from 4 to 16 kHz. Thus, ADAC effectively ameliorated the hearing loss in rats exposed to acute traumatising levels of noise.

Hair cell loss after acute noise exposure

The outer and inner hair cells were counted in Alexa 488 phalloidin-labelled surface preparation of the organ of Corti in the basal, middle and apical turns and the percentage of missing hair cells was calculated for each turn. Quantitative analysis of the hair cell loss is shown in Fig. 5. The number of missing hair cells in control vehicle-treated animals varied between 23% and 34%, whilst the ADAC-treated animals showed on average 7–9% hair cell loss in the middle and basal cochlear turns, respectively. Chronic ADAC treatment thus reliably reduced cellular lesion in the organ of Corti after traumatic noise exposure.

Discussion

This study demonstrates the potency of the selective A₁ adenosine receptor agonist ADAC in alleviating the cochlear damage and resultant hearing loss from noise exposure. The most salient finding is that ADAC treatment can rescue noise-induced hearing loss and aid cochlear recovery from injury. To our knowledge, this study presents the most effective pharmacological strategy to date for treating noise-induced hearing loss post-trauma.

There has been much evidence that prophylactic antioxidant treatment can provide some protection from NIHL, but antioxidants have been shown to be only moderately effective when applied after noise exposure [1, 22]. The first successful attempt to attenuate hearing loss after noise trauma was presented by Lamm and Arnold [23]. Guinea-pigs exposed to hyperbaric oxygenation and anti-inflammatory agents (dexamethasone) in their study showed significant improvement of compound action potentials, auditory brainstem responses, and cochlear microphonics following noise exposure. Subsequently,

Fig. 3 Hair cell loss and reactive nitrogen species in the cochlea exposed to noise for 24 h. **a** The surface preparation of the organ of Corti of a noise-exposed rat treated with the vehicle solution for 5 days (control). **b** The organ of Corti of a noise-exposed rat treated with ADAC (100 μ g/kg i.p.) for 5 days. **c** Nitrotyrosine immunostaining in the control vehicle-treated cochlea. *Inset* high resolution image of the organ of Corti. **d** Nitrotyrosine immunostaining in the ADAC-treated cochlea. Abbreviations: *oc* organ of Corti, *sgn* spiral ganglion neurons, *sl* spiral ligament, *lim* spiral limbus, *isc* inner sulcus cells, *ipc* inner phalangeal cells, *pc* pillar cells, *dc* Deiters' cells, *hc* Hensen's cells. Scale bars 50 μ m

Yamashita et al. [2] demonstrated increased hair cell survival and improved auditory thresholds in noise-exposed guinea-pigs (4 kHz octave band noise at 120 dB SPL for 5 h) using a combination of salicylate and trolox (alpha-tocopherol analogue).

Adenosine receptor agonists have been trialled as a prophylactic treatment to prevent NIHL [12, 13]. The prevention of noise injury in the rodent cochlea was achieved using *R*-phenylisopropyladenosine (*R*-PIA), a broadly selective A_1 receptor agonist. Pretreatment with *R*-PIA attenuated noise-induced hearing loss in animals exposed to a 4-kHz octave band noise (105 dB SPL for 4 h) [13]. The reduction of permanent threshold shift was associated with reduced outer hair cell loss in *R*-PIA treated ears, suggesting that the activation of A_1 adenosine receptors facilitates the recovery of the outer hair cells after noise exposure. The combination of *R*-PIA with the antioxidant glutathione monoethylester was particularly effective against both impulse and continuous noise in the chinchilla cochlea [12]. The round window application of *R*-PIA in the chinchilla cochlea enhanced the activity of the two principal antioxidant enzymes in the cochlea, superoxide dismutase and glutathione peroxidase, and reduced the

levels of malondialdehyde, a marker of lipid peroxidation [10]. These findings support the otoprotective role of adenosine A_1 receptor activation.

Our results show that cochlear injury and hearing loss in rats exposed to narrow-band noise at 110 dB SPL for 2–24 h can be mitigated by ADAC administration after noise exposure. Early treatment starting 6 h after noise exposure provided greater recovery than late treatment commencing 24 h after noise exposure. The most sustainable treatment strategy, however, involved multiple injections of ADAC for 5 days after noise exposure. This therapy significantly attenuated noise-induced threshold shifts and improved hair cell survival. Qualitative analysis of hair cell morphology after prolonged (24 h) noise exposure demonstrated remarkable preservation of the organ of Corti in ADAC-treated animals compared to vehicle-treated controls. In studies with acute (2 h) noise exposure, hair cells were counted, and the data demonstrate significantly greater hair cell survival in the basal and middle turns of ADAC-treated cochleae.

This is consistent with the observation that free radical content in cochlear tissues remains elevated 7–10 days after noise exposure in non-treated animals [24], suggesting that

Fig. 4 ABR threshold shifts in rats after exposure to 8–12 kHz band noise for 2 h at 110 dB SPL. ABRs were measured in response to auditory clicks and pure tones (4–28 kHz) before and at time intervals (30 min and 14 days) after noise exposure. Five ADAC injections (100 µg/kg i.p.) were administered at 24 h intervals commencing 6 h post-noise. In the control group, injections of the vehicle solution (white bars) were administered at the same intervals as ADAC (black bars). Data are expressed as means ± SEM. Animal numbers: $n=8$ per group. * $p<0.05$; ** $p<0.01$; unpaired t test

an extended treatment is required. Our nitrotyrosine immunohistochemistry 6 days after noise damage further supports the sustained elevation of oxidative stress in the cochlea. Noise-induced ROS generation in cochlear tissues, particularly increased production of superoxide ions, is followed by formation of more toxic hydroxyl radicals that interact with polyunsaturated fatty acids in the cell membrane to form toxic aldehyde 4-hydroxynonenal (4-HNE). In addition, superoxide can react with nitric oxide to

generate peroxynitrite radical which reacts with cell membrane proteins to form nitrotyrosine (NT). NT is thus frequently used as a marker of ROS/RNS free radical damage in the cochlea [2, 25], and the overall intensity of NT immunostaining was reduced to a background level in the ADAC-treated cochlea.

ADAC also increased the survival of sensory hair cells. Hair cell loss is the hallmark pathology of NIHL, however, noise exposure can also cause injury to the fibrocytes of the spiral ligament, marginal cells of the stria vascularis, supporting cells in the organ of Corti and spiral ganglion neurones [4]. Even though ABR thresholds represent a functional correlate of neural activity from ascending auditory pathways, they depend on functioning of all structures in the auditory periphery [21]. Improved survival of sensory cells in ADAC-treated rats clearly contributes to improved ABR thresholds. The outer hair cells are thought to be particularly vulnerable due to their high metabolic demand required for active motility during sound transduction. The loss of hair cells is permanent, as these cell types are terminally differentiated and do not regenerate. Hair cell loss was reduced in animals which received chronic ADAC treatment, but the single injection of ADAC was less effective in preserving sensory hair cells. Drug diffusion to the cochlea may be a limiting factor in therapy, and multiple applications of ADAC may overcome this problem by providing even drug distribution across cochlear turns. Spiral ganglion survival following the noise treatments was not assessed in the present study, however, it would be an anticipated corollary of improved hair cell survival following ADAC treatment.

No signs of systemic side effects, such as the loss of body weight or hypothermia, have been observed with ADAC treatment. A feasibility of systemic administration is thus an important advantage of ADAC over other A_1 receptor agonists [17].

Fig. 5 The percentage hair cell loss in the cochlea exposed to noise for 2 h. Data presented as means ± SEM. Animal numbers: $n=8$ per group. * $p<0.05$, *** $p<0.001$; unpaired t test

The effect of ADAC on NIHL likely stems from the role of oxidative stress in determining permanent damage in the cochlea caused by loud sound [2]. The A₁ adenosine receptor stimulation can induce the activation of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase [26, 27]. ADAC may have a dual role, to reduce free radical production and increase the activity of key enzymes involved in free radical scavenging. Other mechanisms underlying cochlear protection by ADAC may include inhibition of glutamate release from the inner hair cell-spiral ganglion neuron synapse via presynaptic A₁R, and direct hyperpolarisation of postsynaptic neurones via G protein-activated inwardly rectifying K⁺ channels [28].

This study thus underpins an important role of adenosine signalling in mitigation of cochlear injury caused by oxidative stress. ADAC in particular emerges as an attractive pharmacological agent for therapeutic interventions in noise-induced cochlear injury.

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