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Aminoglycoside ototoxicity and hair cell ablation in the adult gerbil: A simple model to study hair cell loss and regeneration

Leila Abbas, Marcelo N. Rivolta*

Centre for Stem Cell Biology and Department of Biomedical Sciences, University of Sheffield, Sheffield S10 2TN, United Kingdom

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ABSTRACT

The Mongolian gerbil, *Meriones unguiculatus*, has been widely employed as a model for studies of the inner ear. In spite of its established use for auditory research, no robust protocols to induce ototoxic hair cell damage have been developed for this species. In this paper, we demonstrate the development of an aminoglycoside-induced model of hair cell loss, using kanamycin potentiated by the loop diuretic furosemide. Interestingly, we show that the gerbil is relatively insensitive to gentamicin compared to kanamycin, and that bumetanide is ineffective in potentiating the ototoxicity of the drug.

We also examine the pathology of the spiral ganglion after chronic, long-term hair cell damage. Remarkably, there is little or no neuronal loss following the ototoxic insult, even at 8 months post-damage. This is similar to the situation often seen in the human, where functioning neurons can persist even decades after hair cell loss, contrasting with the rapid, secondary degeneration found in rats, mice and other small mammals. We propose that the combination of these factors makes the gerbil a good model for ototoxic damage by induced hair cell loss.

1. Introduction

Since their introduction in the 1940s, aminoglycoside antibiotics have been recognised clinically for their off-target effects of ototoxicity. When used in combination therapy with a loop diuretic such as ethacrynic acid, the often-reversible deafness seen with the antibiotic alone was rapidly induced and permanent (Brown et al., 1974; Mathog et al., 1969). Such damage was found to be caused by the death of the sensory cells of the specialised hearing epithelium, the organ of Corti, located within the bony shell of the cochlea. The destruction of the three rows of outer hair cells and single row of inner hair cells would eventually lead to loss of the surrounding supporting cells and the replacement of the organ with a flattened epithelium of scar tissue, and accompanying profound deafness in the patient. However, as the field of regenerative medicine moves forward, this damaged epithelium becomes a potential target for therapeutic intervention, whether it be the idea of recreating the organ of Corti, or in its role as a model for cochlear implantation studies.

A sequela to the death of the organ of Corti is often the secondary loss of the spiral ganglion neurons (SGNs) which innervate the hair cells. This loss occurs with varying rapidity in different species. For instance in the guinea pig, a substantial abrogation of SGNs is observed 7 days after aminoglycoside treatment (Kong et al., 2010), whereas in human patients, remaining SGNs have been found several decades after hair cell loss is thought to have occurred (Ghorayer et al., 1980).

The gerbil is a well-established model for auditory research given its particular hearing physiology (Otto and Jürgen, 2012). On account of its ethology in the wild, the animal has a broad frequency range of hearing – low frequencies are used when ‘drumming’ with the hind limbs as a warning communication; at the other end of the auditory spectrum, animals ‘chirp’ at each other up to a level of around 25 kHz. This overlap with the human hearing range arguably makes the gerbil a more relevant model for hearing loss than high-frequency specialists such as the mouse or rat. Moreover, the species is surgically robust, with the relatively large cochlea easily accessed through the thin bone of the auditory bulla

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Abbreviations: SGN, Spiral Ganglion Neuron; ABR, Auditory Brainstem Response; PFA, Paraformaldehyde; EDTA, Ethylenediaminetetraacetic acid; PBS, Phosphate Buffered Saline; BSA, Bovine Serum Albumin; DAPI, 4,6-diamidino-2-phenylindole; MSBB, Methyl salicylate and Benzyl benzoate; ANOVA, Analysis of variance; RWM, Round window membrane; OHC, Outer hair cells; IHC, Inner hair cells; MBP, Myelin basic protein

* Corresponding author. Centre for Stem Cell Biology and Department of Biomedical Sciences, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK. Tel.: +44 (0) 114 2222385; fax: +44 (0) 114 222 2787.

E-mail address: m.n.rivolta@sheffield.ac.uk (M.N. Rivolta).

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making it particular well suited for experiments exploring therapeutic strategies requiring cell or drug delivery.

Remarkably though, while reliable protocols have been developed for the neuropathic damage of the spiral ganglion (Lang et al., 2005; Schmiedt et al., 2002), a simple and robust method to induce ototoxic lesions of the hair cells is not available for this species. Current protocols involve the topical application of aminoglycosides using slow-releasing gels or repeated application of aminoglycosides by transtympanic injections (Polgar et al., 2001; Wanamaker et al., 1999). Both methods are invasive and, at least in our hands, have proven unreliable.

Here we present data showing that the gerbil can be used as a model for rapid and permanent aminoglycoside-induced hearing loss using a 'one-shot' protocol, in which a single dose of kanamycin is accompanied by a dose of the loop diuretic furosemide. This is a refinement of experiments carried out in other species, where repeated, often toxic, dosage regimes have been employed.

2. Materials and methods

2.1. Animals

Mongolian gerbils from an in-house breeding colony (originally sourced from Charles River, Germany) were raised and aged between 3 and 6 months at the beginning of the protocol – no effect of age was noted on the initial hearing threshold measured. A mixture of males and females were used – no difference between sexes in hearing ability has been observed in our colony. All experiments were carried out in accordance with the Sheffield University Ethical Review Committee and under a Home Office Project Licence authority, conforming to UK and EU legislation.

2.2. Ototoxicity models

For systemic assays, animals were injected sub-cutaneously with a solution of gentamicin sulphate or kanamycin sulphate in normal saline (400–500 mg/kg; Sigma, Gillingham, U.K.) followed 20–30 min later by an intra-peritoneal injection of bumetanide (50 mg/kg; Sigma) or furosemide solution (100 mg/kg; Sigma). For topical application to the cochlea, anaesthesia was induced with isoflurane, the auditory bulla was accessed through a retro-auricular incision and windowed using a scalpel blade. Aminoglycoside and diuretic solutions (as above) were applied directly to the round window; in some cases, animals were implanted with a gelatin sponge (Cutanplast, Sheffield, U.K.) pre-soaked in an aminoglycoside/diuretic mixture. The bulla was sealed using a plug of fascia held in place with Vetbond adhesive (3 M, Bracknell, U.K.). The muscle layer and wound were closed with absorbable Vicryl suture material (Ethicon, Norderstedt, Germany) and the animal allowed to recover.

2.3. Auditory measurements

Animals were sedated with a ketamine/xylazine mix and placed on a heat mat to maintain body temperature. The System 3 digital signal processing package of hardware and software (Tucker Davies Technologies (TDT), Florida) was used to present click stimuli ranging from 110 dB to 20 dB at a rate of 20 s^{-1} in decreasing 10 dB steps. Tone stimuli were presented as pure tone pips lasting 5ms at 80 dB, at frequencies ranging from 6 kHz to 38 kHz in 4 kHz steps or at 2 kHz, 4 kHz, 8 kHz, 16 kHZ and 32 kHz at an intensity ranging from 20 dB to 90 dB. The sounds were presented to the animal via a 'closed field' method – a 3 mm diameter, 10 cm tube leading from the speaker was inserted into the auditory meatus until its tip was close to the tympanic membrane. Auditory brainstem response (ABR) measurements were recorded using 27G subdermal needle electrodes placed at the vertex of the skull (recording electrode) and the ipsilateral mastoid process (reference electrode), with a ground electrode placed dorsally adjacent to the tail. The differential voltage induced by the presentation of the sound stimulus was thus recorded. Each stimulus was presented 500 times, thus the wave generated represented an average response over this time. For each intensity, the amplitude of the response between the wave ii positive peak (P2) and the wave iii negative peak (N3), representing the neural activity within the cochlear nucleus and the superior olivary complex respectively, was measured, giving a 'read-out' of activity within the central auditory pathway (Burkard et al., 1993; Boettcher et al., 2006). The click ABR threshold was calculated as the sound level required to produce a voltage response two standard deviations above the mean noise level for each recording (May et al., 2002). All experiments were carried out in a sound-proof, custom-made audiology booth (AGS Noise Control Ltd, Melton Mowbray, U.K.).

2.4. Immunohistochemistry

Temporal bones were fixed by immersion in 4% paraformaldehyde (PFA; pH 7.4) for 24–48 h at 4 °C and decalcified in 0.5 M ethylenediaminetetraacetic acid (EDTA) pH8 for a week at 4 °C. Samples were taken through an ascending sucrose/PBS series (7.5%, 15%, 22.5%, 30; several hours/overnight in each stage). Following embedding in Cryo-M-Bed medium (VWR, Lutterworth, U.K.), 12 μm sections were taken on a Bright cryostat onto gelatine/chrome alum-coated glass slides. The tissue sections were defrosted, rehydrated and briefly re-fixed with 4% PFA, rinsed with 0.1% Triton/PBS and blocked in 5% donkey serum/1% B.S.A. before incubation overnight at 4 °C with the following antibodies: anti-αTubulin (1:150, mouse monoclonal; Sigma), anti-βIII–Tubulin (1:150, mouse monoclonal; Sigma), anti-espin (1:100, rabbit polyclonal; Sigma), anti-myelin basic protein (MBP; 1:75, mouse monoclonal; Millipore), anti-MyoVIIA (1:100, rabbit polyclonal; kind gift, C. Petit), anti-OCP2 (1:100, rabbit polyclonal, kind gift R. Thalmann), anti-Neurofilament 200 (NF-200; 1:200, rabbit polyclonal; Sigma), anti-Na, K-ATPase α3 subunit (NKAα3; Santa Cruz, Insight Biotechnology, Wembley, U.K.), anti-CtBP2 (BD Biosciences, Oxford, U.K.). Detection was carried out by incubating for 2 h at room temperature with anti-mouse Alexa 488 and anti-rabbit Alexa 568 (Life Technologies, Paisley, U.K.) and the tissue counter-stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma) before mounting in Vectashield (Vector Laboratories, Peterborough, U.K.). Images were taken on a Zeiss Axiophot microscope using Axiovision software and figures assembled using Adobe Photoshop 7.0. Whole-mount staining of intact cochleae was carried out essentially as described in MacDonald and Rubel (2008). Briefly, fixed cochleae were decalcified as above, rinsed in PBS and incubated with primary antibodies (anti-βIII–Tubulin and anti-MYOVIIA as above) for several days with agitation at 4 °C. After washing, samples were incubated at 4 °C with anti-mouse Alexa 488 and anti-rabbit Alexa 568 for 3–5 days. After washing, samples were dehydrated through an increasing ethanol gradient and cleared through MSBB, in a 5:3 ratio of methyl salicylate and benzyl benzoate (Sigma), for 2 days. Imaging was then carried out using a Zeiss LSM510 inverted confocal microscope. Images were analysed using FIJI (http://fiji.sc/Fiji) and figures assembled in Photoshop as above.

2.5. Phalloidin staining

Temporal bones were fixed and decalcified as described above. Cochlear epithelia were dissected out and washed in 0.1% Triton/PBS prior to incubation overnight in Alexa Fluor 568 phalloidin