

SAVING THE HAIR CELL

Investigating Apoptosis signal-regulating kinase 1 as a molecular target for preventing aminoglycoside induced hearing loss.

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Abstract

Aminoglycoside antibiotics are lifesaving medicines, crucial for the treatment of chronic or drug resistant infections. However, aminoglycosides can destroy the sensory hair cells of the ear. As a result, aminoglycoside treated individuals frequently experience a reduced quality of life, stemming from permanent high-frequency hearing loss and vestibular impairment. Currently, no alternate antibiotic substance has the same bactericidal profile or efficacy as aminoglycosides, and an adjuvant therapy capable of mitigating ototoxic outcomes does not exist.

In order to develop an otoprotective therapy, the mechanisms of aminoglycoside-induced hair cell death must be elucidated. With respect to this goal, the production of reactive oxygen species and subsequent c-Jun N-terminal kinase (JNK) and P38 mitogen-activated protein kinase (P38) phosphorylation has been extensively documented in aminoglycoside treated hair cells. However, strategies directly targeting ROS, JNK or P38 are limited by the importance of these molecules for normal cellular function. Notably, the upstream regulators of JNK or P38 have not been well studied in the ear. Therefore, this project aimed to elucidate the role of upstream regulator Apoptosis signal-regulating kinase 1 (ASK1) within the auditory system.

ASK1 is a key mediator of ROS induced JNK and P38 mediated disease. Importantly, ASK1 inhibition has previously been shown to reduce the pathological consequences of ROS, P38 and JNK signalling in varied disease models; without impeding the normal homeostatic cell function of these molecules. Therefore, ASK1 inhibition may represent a novel strategy for preventing aminoglycoside ototoxicity. However, the role of ASK1 as a mediator of drug-induced hair cell death has not been investigated. Moreover, the role of ASK1 in the development and function of auditory structures has not been explored.

This project first aimed to elucidate the importance of ASK1 in the inner ear by characterising the auditory phenotype of *Ask1* knockout mice. Histology indicated normal development of cochlear structures and the auditory brainstem response (ABR) demonstrated that *Ask1* knockout mice had hearing thresholds comparable to C57BL/6 controls. However, ABR peak analysis indicated that auditory signal

transduction is faster in *Ask1* knockout mice. In addition, the acoustic startle response showed *Ask1* knockout mice to be hypersensitive to auditory stimuli. Combined, this data indicates that ASK1 is important for neuronal function and that *Ask1* knockout mice have an auditory processing disorder.

This project then evaluated the importance of ASK1 as a mediator of drug-induced hair cell death. To test the hypothesis that ASK1 deficiency is protective against aminoglycoside-induced hair cell death, cochlear explants from *Ask1* knockout and C57BL/6 mice were treated with neomycin *in vitro*.

After six hours of 1 mM neomycin treatment, immunohistochemistry demonstrated that p-JNK staining was reduced in *Ask1* knockout explants when compared to C57BL/6 explants. Moreover, hair cell death was significantly attenuated in *Ask1* knockout explants. This data provides robust evidence that ASK1 has an important role in the process of hair cell death, and that ASK1 inhibition could limit aminoglycoside-induced hair cell death.

Therefore, the final aim of this project was to test ASK1 inhibition as an otoprotective strategy. ASK1 inhibition significantly attenuated neomycin-induced outer hair cell death in C57BL/6 cochlear explants. Immunohistochemistry and western blot analysis suggested that ASK1 inhibition attenuated both P38 and JNK phosphorylation in neomycin-treated explants. Importantly, ASK1 inhibition did not impact the efficacy of the aminoglycoside antibiotics against *P. aeruginosa* in the broth dilution test. This indicates that ASK1 inhibition may mitigate ototoxic outcomes without impairing the primary aminoglycoside treatment.

Overall, this thesis represents the first aural characterisation of the *Ask1* knockout mouse strain, providing valuable insight regarding the role of ASK1 in the auditory pathway. Moreover, this work has provided compelling evidence that ASK1 inhibition may be a useful strategy for the prevention of aminoglycoside induced hearing loss.

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Declaration

I certify that,

1. This thesis comprises of my original work towards the requirements of The Melbourne University Doctor of Philosophy degree, except where indicated in the preface.
2. Due acknowledgement has been made in the text to all other material.
3. This thesis is fewer than 100,000 words in length, exclusive of tables, figures, bibliographies and appendices.

Jacqueline Ogier

03 / 01 / 2020

Original signature removed for online thesis publication 28/04/2020

Preface

Some aspects of this thesis have been or are in the process of being published.

My contribution to each thesis chapter is declared in this section. Immediately following this outline, I have described my role for each individual publication.

Chapter 1: Introduction

I wrote all aspects of this chapter. However, some figures included are the work of others; for these figures the original source is clearly referenced in the figure legend. This chapter also includes some text and figures from my own publications. Specifically,

Figure 2 contains an image previously published in “An intronic mutation in *Chd7* creates a cryptic splice site, causing aberrant splicing in a mouse model of CHARGE syndrome” (*Sci Reports* 8, 5482, 2018). The full publication is attached to this thesis as Appendix 2. For this figure, X-ray μ CT imaging was performed by Dr. Benedicta Arhatari and I prepared the figure for publication.

Figure 5 contains images that have been published in “Organotypic Culture of Neonatal Murine Inner Ear Explants.” (*Frontiers in Cellular Neuroscience* 13, 895, 2019). The full publication is attached to this thesis as Appendix 1. These images are my original work.

Embedded within the latter sections of the introduction, is an invited literature review, entitled “ASK1 inhibition: a therapeutic strategy with multi-system benefits.” I wrote this manuscript, which was under second round review in the *Journal of Molecular Medicine* at the time of thesis submission. The manuscript has since been published.

Chapter 2: Methods

I wrote of the first draft and all subsequent revisions of this chapter.

Figure 14 and Figure 15 contain images of auditory brainstem response testing equipment and the acoustic startle response testing arena, that I have used previously in a short thesis presented to The University of Melbourne for the fulfillment of the Degree of Bachelor of Science, with Honours (Ogier 2013).

In addition, some methods described in this section have also been used in publications produced during my PhD candidature. These publications are:

1. Ogier et al. Intravenously delivered aminoglycoside antibiotics, tobramycin and amikacin, are not ototoxic in mice. *Hearing Research* (2019). (Chapter 3).
2. Ogier et al. Organotypic Culture of Neonatal Murine Inner Ear Explants. *Frontiers in Cellular Neuroscience*. (2019). (Appendix 1).
3. Ogier et al. An intronic mutation in Chd7 creates a cryptic splice site, causing aberrant splicing in a mouse model of CHARGE syndrome. *Scientific Reports* (2018). (Appendix 2).
4. Manning, J. et al. Effect of a pneumococcal whole cell vaccine on influenza A-induced pneumococcal otitis media in infant mice. *Vaccine* (2019). (Appendix 3).

Chapter 3: Investigating the role of ASK1 in the murine auditory system and as a mediator of aminoglycoside induced hair cell death.

I wrote the first draft of this chapter and all subsequent revisions. I performed all experimental work, all data analysis and prepared all figures. Experiments were designed with the guidance of Dr Rachel Burt, A/Prof Bryony Nayagam and A/Prof Paul J Lockhart. This chapter contains an original research manuscript entitled “Intravenously delivered aminoglycoside antibiotics, tobramycin and amikacin, are not ototoxic in mice”, which has been published in *Hearing Research* (Ogier, Lockhart, et al. 2019).

Chapter 4: Investigating the ASK1 inhibitor GS444217 as a protective strategy for preventing aminoglycoside-induced hair cell death

I wrote the first draft of this chapter and prepared all figures having performed all experiments and data analysis. Experimental design was performed under the guidance of Dr Eileen Dunne, Dr Rachel Burt, A/Prof Bryony Nayagam and A/Prof Paul J Lockhart. Collaborators A/Prof Greg H. Tesch and Dr. David J. Nikolic-Paterson also contributed to experiment conceptualisation.

Chapter 5: Discussion

I wrote all aspects of this chapter, including the first draft and subsequent revisions.

Publication contributions

I contributed to the following publications during my PhD candidature:

1. Ogier et al. Organotypic Culture of Neonatal Murine Inner Ear Explants. *Frontiers in Cellular Neuroscience* 13, 895 (2019). (Appendix 1).

I am the first author of this manuscript having contributed more than 50% of this work. I conducted all cochlear explant experiments producing original data for Figures 3–5. I wrote the manuscript draft, prepared all figures, and the video for publication. A/Prof Bryony Nayagam provided input to the text and performed cochlear dissections for use in Figure 1 and Supplementary Video 1. All sections pertaining to electrophysiology (electrophysiology method and data outlined in Figure 2) were supplied by Dr Rebecca Lim and Hannah Drury.

2. Ogier et al. An intronic mutation in *Chd7* creates a cryptic splice site, causing aberrant splicing in a mouse model of CHARGE syndrome. *Scientific Reports* 8, 5482 (2018). (Appendix 2).

My contribution accounted for more than 50% of this work. I am the first author of this manuscript having written the first draft and subsequent revisions based on feedback provided by co-authors and reviewers. Dr Benedicta Arhatari performed X-ray Micro-Computed Tomography (μ CT), supplied μ CT images and wrote the μ CT methods. *Trooper* mice were the result of an ethylnitrosourea screen performed by Dr Rachel Burt and Dr. Marina Carpinelli prior to the commencement of this PhD.

3. Manning, J. et al. Effect of a pneumococcal whole cell vaccine on influenza A-induced pneumococcal otitis media in infant mice. *Vaccine* (2019). (Appendix 3).

For this paper, I assisted with some experimental planning, animal perfusion, cochlear dissection and tissue preparation for histology. I am listed as the fifth of eleven authors.

4. Ogier et al. Intravenously delivered aminoglycoside antibiotics, tobramycin and amikacin, are not ototoxic in mice. *Hearing Research* 2019. (Chapter 3).

For this study, I conducted all experiments, prepared all figures and wrote the manuscript draft. I also wrote subsequent revisions based on feedback provided by co-authors and journal reviewers. I am the first author of this manuscript having contributed more than 50% of this work.

5. Ogier et al. ASK1 inhibition: a therapeutic strategy with multi-system benefits. *The Journal of Molecular Medicine* 2020. (Chapter 1).

I performed the literature search required for this review and wrote the manuscript draft. I am listed as first author, having contributed to more than 50 % of the work. (Chapter 1)

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Supervisors

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Advisory Committee

Thank you also, to the members of my advisory committee, Prof. Amanda Fosang, A/Prof. Shireen Lamande, Prof. John Christodoulou, and Dr. Sarah Stephenson. I feel incredibly lucky to have had the support of such awe-inspiring scientists. Your guidance ensured the success of my PhD.

Collaborators

Thank you, A/Prof Greg H. Tesch and Dr. David J. Nikolic-Paterson for kindly providing the *Ask1* knockout mice and inhibitor used in this work. I am grateful for your support, which has been impressive at every stage of this project.

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- 2019 The Society for Neuroscience Trainee Professional Development Award
- 2019 The Bio Techne Travel Grant
- 2018 The Australasian Neuroscience Society Student Conference Travel Award.
- 2018 The Murdoch Children's Research Institute Student Travel Award
- 2016 National Institutes of Health, Jackson Laboratory Conference Scholarship
- 2016 The Henry and Rachel Ackman Travel Scholarship

Abbreviations

α	Alpha
β	Beta
μ	Micro
ASK1	Apoptosis signal regulating kinase 1
ABR	Auditory brainstem response
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
AREST CF	Australian Respiratory Early Surveillance Team for Cystic Fibrosis
ASR	Acoustic startle response
ATTC	American Type Culture Collection
BM	Basilar membrane
BSA	Bovine serum albumin
Ca^{2+}	Calcium cation(s)
CAP	Compound action potential
CIAP	Clinical Information Access Portal
COPD	Chronic obstructive pulmonary disease
DAPI	4',6-diamidino-2-phenylindole
SPL	Sound pressure level
DMSO	Dimethyl sulfoxide
DN	Dominant-negative
DPOE	Distortion product otoacoustic emission
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
eGFR	glomerular filtration rate
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular receptor kinase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GLU	Glutamine

GRX	Glutaredoxin
GSH	Glutathione
H and E	Haematoxylin and eosin
HEI-OC1	House Ear Institute-Organ of Corti 1
HRP	Horseradish peroxidase
IHC	Inner hair cell(s)
IL	Interleukin
JNK	Jun N-terminal kinase
K ⁺	Potassium cation(s)
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAP2	Microtubule associated protein 2
Map3k5	Mitogen-activated protein kinase kinase kinase 5
MET	Mechanoelectrical transduction
MCP-1	Monocyte chemoattractant protein-1
MCRI	The Murdoch Children's Research Institute
MEM	Minimum essential media
MIC	Minimum inhibitory concentration
MIP-1 α	Macrophage inflammatory protein- 1 alpha
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
Na ⁺	Sodium cation(s)
NAC	N-acetyl cysteine
NaCl	Sodium chloride
NAFLD	Non-alcoholic fatty liver disease
NAPQI	N-acetyl-p-benzoquinone imine
NASH	Non-alcoholic steatohepatitis
OHC	Outer hair cell(s)
OAE	Otoacoustic emissions

P38	P38 mitogen-activated protein kinase
P2X ₂	P2X purinoceptor 2
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PPEF2	Protein phosphatase with EF-hand domain 2
PRDX1	Peroxiredoxin 1
RCH	The Royal Children's Hospital
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S.D	Standard Deviation
SDS	Sodium dodecyl sulphate
S.E.M	Standard error of the mean
SOD1	Superoxide dismutase
TBS(T)	Tris-buffered saline (Tween 20)
TLR	TLR9
TNF	Tumour necrosis factor
TRPV1	Transient receptor potential cation channel subfamily V member 1
TRX	Thioredoxin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
WEHI	The Walter and Eliza Hall Institute of Medical Research
WHO	The World Health Organization
X-ray μ CT	X-ray micro-computed tomography

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Chapter 1. Introduction

1.1. The auditory and vestibular systems

Audition is the intrinsic comprehension of vibration perceived as sound. To sense and understand sound, an intricate network of processes occur, whereby a complex stimulus is received and deciphered based on frequency, intensity and direction. This information is then used for auditory cognition, where information regarding the source is deduced. Fundamentally, the ability to hear is used to detect danger and communicate. However, the finely tuned sense of hearing is an integral part of human life. We are able to recognise a loved one's voice in a crowded and noisy environment. We receive critical emotive cues from the quality and tone of a voice. Importantly, we use our sense of hearing to find joy, whether experiencing a funny story or revelling in complex music and sound. The vestibular system is a complex series of structures in the ear that sense movement and orientation of the head. This sensory system provides fundamental information regarding our physical place within the environment. Vestibular cues are required for the coordination of postural and eye reflexes. In particular, perception of self-motion is critically important for navigation, bearing, and general control of body and eye movements (Britton & Arshad 2019). The effortless perception of movement allows for an individual to perform everyday actions such as standing up or raising a glass. Ultimately, vestibular sensation impacts every movement the body makes. However, more than postural control, vestibular function is critical for spatial awareness or the conscious sense of self in the first-person perspective, as considered by a knowledge of self-location and self-identification (Britton & Arshad 2019).

The processes that allow for the detection of sound and movement are ancient and complex. Firstly, peripheral sensation must occur, to receive the stimulus and encode a compound electrochemical signal. This signal is then subject to central processing, where cues are delineated to allow for higher order comprehension. These processes are outlined in this chapter, to provide the reader with a fundamental understanding of the auditory and vestibular pathways and to highlight the importance of the hair cell within these systems.

1.1.1. Peripheral auditory and vestibular sensation

The ear is a complex organ responsible for the collection and transduction of sound. The ear is divided into three structurally distinct regions, the outer, middle and inner ear (Figure 1)

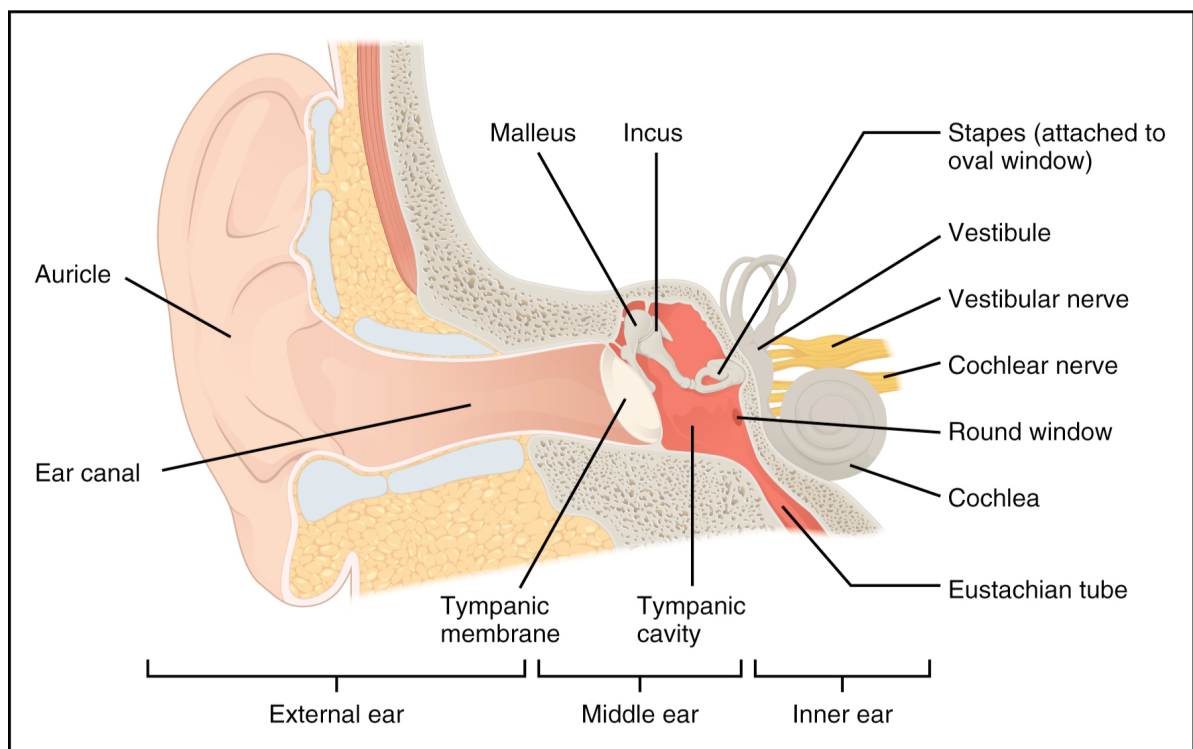


Figure 1. Anatomy of the Ear.

The auricle and ear canal comprise the outer ear. These structures collect and direct sound towards the tympanic membrane. The cartilage of the auricle is shaped, causing preferential transmission of high frequency stimuli, or sound emanating from an elevated position. The tympanic membrane of the middle ear causes the ossicular chain (malleus, incus and stapes) to vibrate. This process amplifies the signal. The middle ear is also connected to the pharynx by the Eustachian tube, which allows for pressure equilibrium to be maintained. Sound vibrations are then transferred to the inner ear, as the stapes footplate vibrates against the oval window of the cochlea, displacing the cochlea fluid. The inner ear consists of the cochlea and vestibular apparatus, where sound and movement are sensed, respectively. Image as published by Betts et al. 2012 (Betts et al. 2012).

1.1.1.1. The outer ear

The structures of the outer ear collect, amplify, and focus sound inwards towards the tympanic membrane. First, the cartilaginous grooves of the auricle, funnel sound towards the auditory canal. The shape of the auricle also causes preferential sound transmission, based on the location from which the stimulus originated (Yost 2007). This provides critical cues for subsequent auditory processing and cognition (Purves et al. 2001b). Once collected by the auricle, sound then travels through the auditory canal towards the tympanic membrane. The tympanic membrane then vibrates, causing synchronous vibrations of the ossicular chain. Overall, the shape of the auricle and auditory canal, combined with the mechanical profile of the tympanic membrane, selectively amplify higher frequency sounds that are particularly relevant to human speech (Zhang & Gan 2013; Yost 2007).

1.1.1.2. The middle ear

The middle ear is a small, air filled space containing the malleus, incus and stapes (ossicular chain), and the Eustachian tube. The malleus of the ossicular chain is attached to the tympanic membrane, so that vibrations of the membrane cause the ossicular chain to vibrate. The stapes footplate transfers the vibration through the oval window into the cochlea. Importantly, the size difference between the tympanic membrane and stapes footplate significantly amplifies sound pressure. The ossicles are also positioned to act as a lever, to enhance the signal further (Figure 2) (Yost 2007). This amplification process is critical for overcoming the high levels of impedance within the fluid filled cochlea. The Eustachian tube maintains pressure equilibrium in the middle ear by opening to the pharynx, thereby enabling efficient sound transduction through the ossicular chain (Betts et al. 2012; Yost 2007).

1.1.1.4. The cochlea

As its name suggests, the cochlea (Latin; *snail*) is a spiralling, osseous tube. The shape of the cochlea and the structures within it maximise sound amplitude and detection (Figure 3). Within the cochlea, there are three fluid filled segments; the scala vestibuli and scala tympani which contain a low potassium (~6.9 mM) / high sodium (~138 mM) solution, known as perilymph; and the scala media which is filled with endolymph, a high potassium (~145 mM) / low sodium (~2 mM) solution (Figure 4) (Bosher & Warren 1968; Berne et al. 2008). This creates an endocochlear potential of 80- 100 mV (Purves et al. 2001c). The endocochlear potential varies along the basilar membrane, decreasing in magnitude from the cochlear base to apex (W. Guo et al. 2012). Reisner's membrane separates the scala vestibuli and scala media, whilst the basilar membrane separates the scala media and scala tympani. The auditory neurosensory epithelium (organ of Corti) is located above the basilar membrane, within the scala media (Figure 4). The organ of Corti contains three rows of outer hair cells and one row of inner hair cells. Stereocilia on the apical aspect of outer hair cells are embedded within the tectorial membrane.

As sound reverberates through the cochlea fluid, propagating from the base towards the apex, the basilar membrane is displaced. The basilar membrane is narrow and rigid at its base, becoming wider and more flexible towards the apex, which causes the sound wave to increase in amplitude and decrease in velocity. Ultimately a point of maximum displacement is reached, specific to the frequency of the sound wave. At this point, the specialised hair cells perform mechanotransduction (Figure 6) (Purves et al. 2001c). For high frequency sounds, this occurs in the base of the cochlea, whilst low frequencies cause maximum displacement in the cochlear apex (Figure 3).

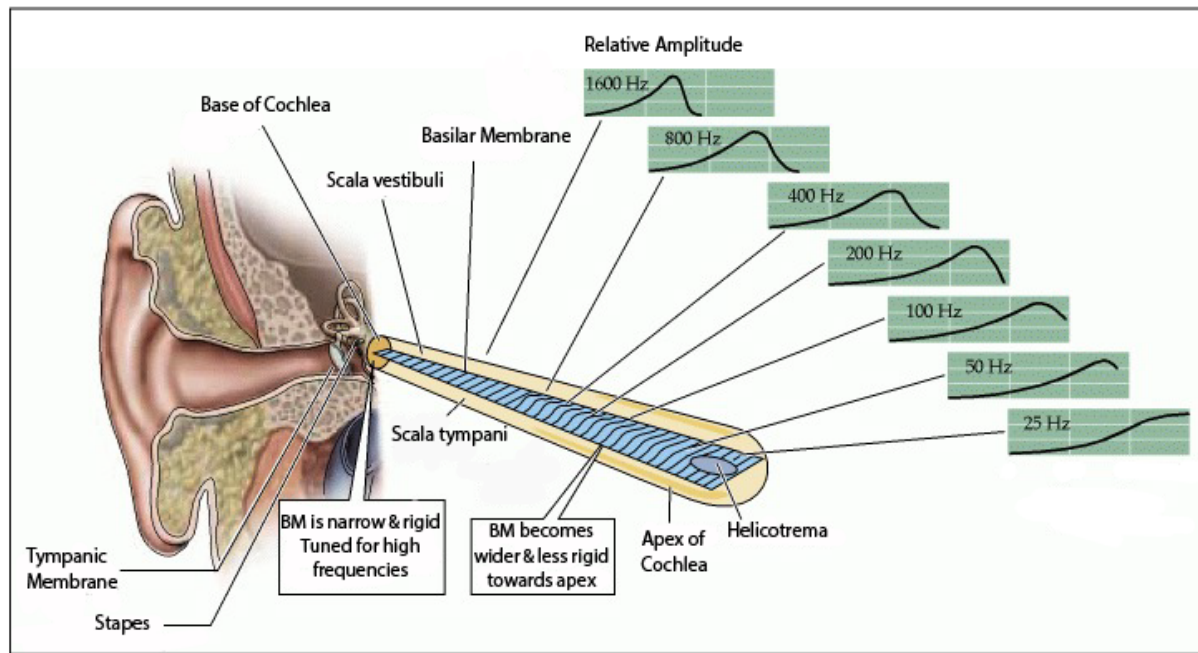


Figure 3. The shape and structures of the cochlea allow for highly tuned sound perception.

The cochlea is a spiral shaped tube, presented here in an ‘unwound’ fashion. The mammalian cochlea is highly tuned, with information transmitted from different cochlear regions. This is driven by changes in the structure of the basilar membrane (BM), which becomes heavier, wider, and less rigid towards the cochlear apex. As a sound wave propagates from the base of the basilar membrane towards the apex, the amplitude of the wave increases and the velocity decreases. Ultimately a point of maximum displacement is reached. Frequencies are detected at the point where the traveling sound wave reaches its maximum amplitude (Purves et al. 2001c). This baso-apical frequency gradient is enhanced further by the activity of the outer hair cells and the specific discharge rates of individual auditory nerves (Purves et al. 2001c). The helicotrema, situated at the apex of the cochlea, is a small opening connecting the scala tympani and vestibuli. Figure adapted from Betts et al. 2012 (Betts et al. 2012).

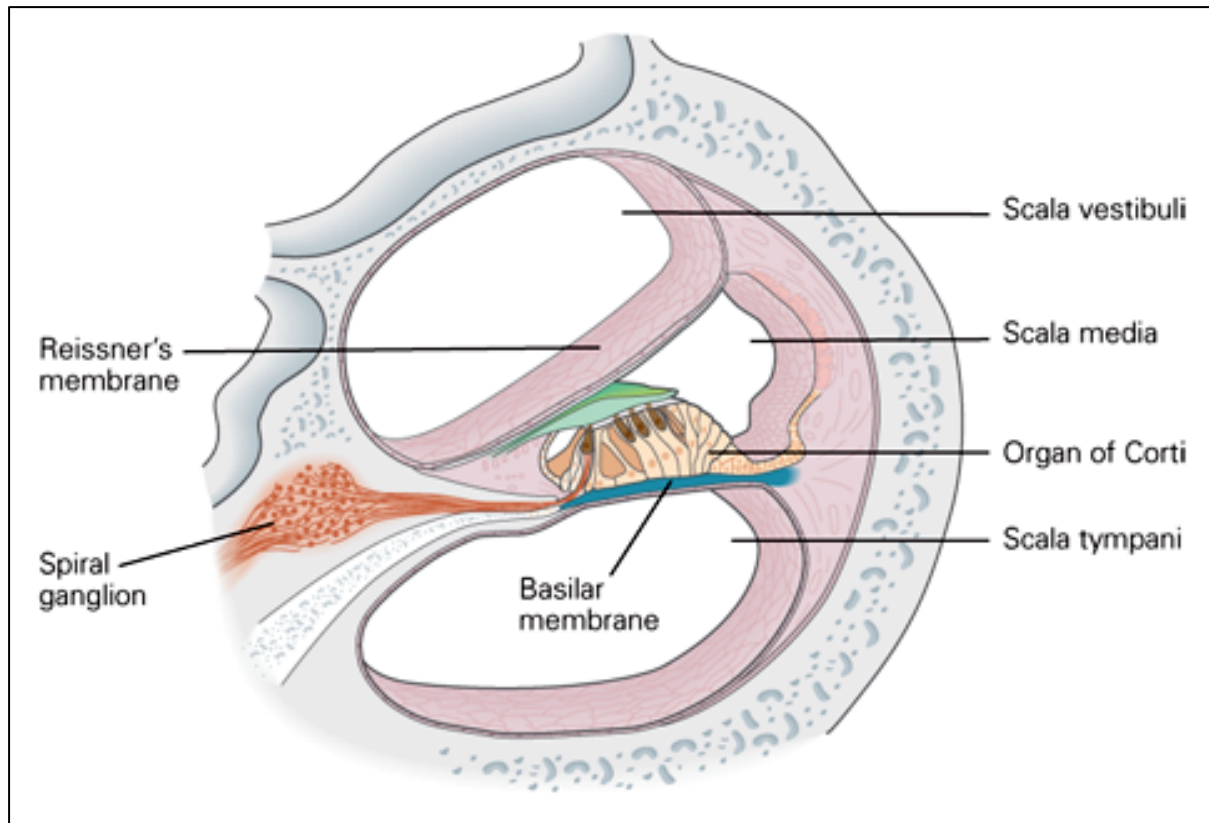


Figure 4. A cross section of one cochlear turn.

The cochlea is a tube, divided into three compartments. The scala vestibuli and scala tympani contain perilymph (138 mM Na⁺, 6.9 mM K⁺), whereas the scala media contains endolymph (2 mM Na⁺, 145 mM K⁺) (Bosher & Warren 1968; Berne et al. 2008). Reissner's membrane separates the scala vestibuli and scala media. The scala media and scala tympani are separated by the basilar membrane. The organ of Corti is positioned on top of the basilar membrane. Electrochemical signals created by the hair cells are received by neurons of the spiral ganglion. Figure adapted from Kandel et al. 2013 (Kandel et al. 2013).

1.1.1.5. The organ of Corti

The organ of Corti contains numerous supporting cell types and approximately 15,000 hair cells per cochlea. Hair cells are usually patterned with one row of inner hair cells and three rows of outer hair cells (Figure 5). However, a fourth row of outer hair cells is not uncommon. Fine actin bundles, known as stereocilia are arranged in a staircase pattern (rows of increasing height) at the apical point of each hair cell (Figure 5). The stereocilia of inner hair cells are positioned in a relatively linear fashion, whereas outer hair cell stereocilia are arranged in V or W shape and embedded within the tectorial membrane. Stereocilia are intricately connected by tip links and top connectors, which are linked to mechanoelectric transduction (MET) channels. Cochlear hair cells are specialised auditory sensory receptors that perform mechanotransduction (Figure 6).

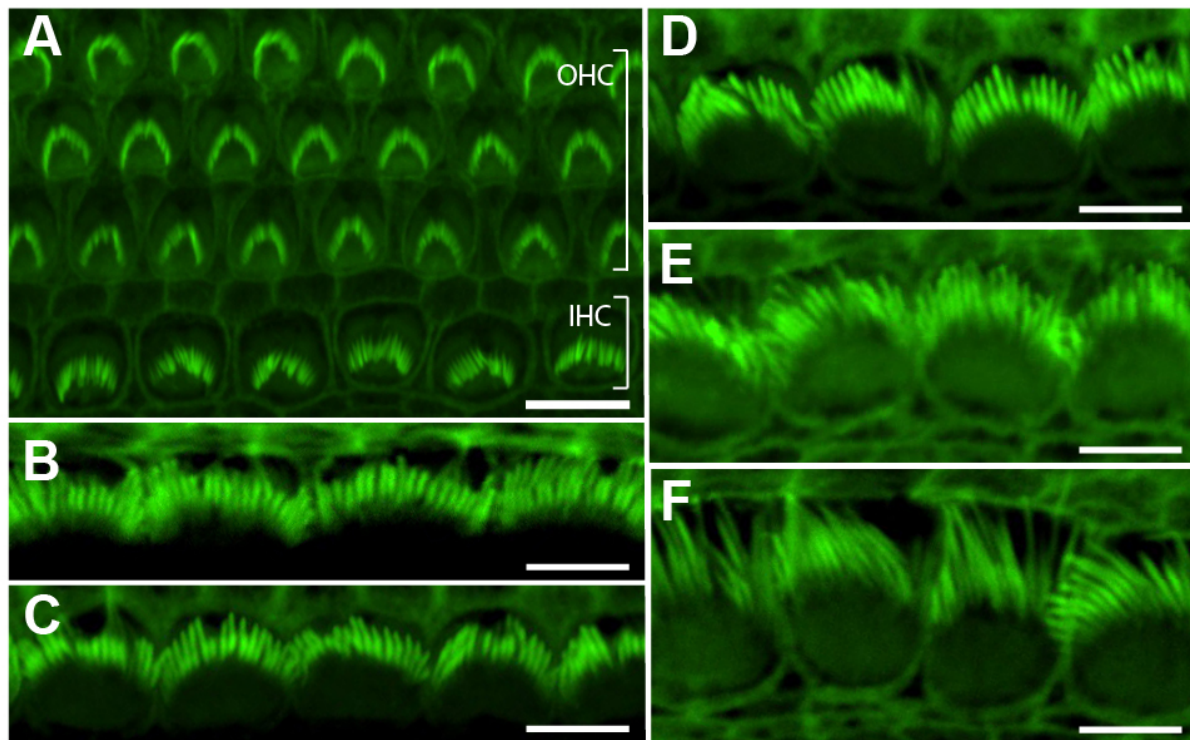


Figure 5. Phalloidin stained stereocilia.

A) One medial row of inner hair cells (IHC) and three more laterally located rows of outer hair cells (OHC) illustrating normal polarity and patterning of cochlear stereocilia bundles. Scale bar = 10 μm . **B-F)** The length of inner hair cell stereocilia (green) increases from the basal to apical regions of the cochlea. Conversely, stereocilia width decreases from the cochlear base to apex. Images were progressively collected from the basal region to apical regions of a neonatal mouse cochlea explant, to demonstrate the graded change in stereocilia length and width. **B)** Basal inner hair cells. **C)** Upper basal inner hair cells **D)** Mid section inner hair cells **E)** Upper mid section inner hair cells. **F)** Apical inner hair cells. Scale bar = 5 μm . Figure adapted from Ogier et al. 2019.

1.1.1.6. Cochlear hair cell mechanotransduction

Mechanotransduction is the process of converting a mechanical stimulus into an electrochemical signal. For auditory sensation, this means converting a sound pressure wave into an electrochemical signal that can be decoded by the brain. As sound propagates through the cochlear fluid, the basilar and tectorial membranes move, creating a shearing force. This force displaces the stereocilia, causing tension in the tip links. It is thought that this mechanically opens the hair cell MET cation channels. Due to the endocochlear potential, potassium and calcium rapidly enter through the MET channels causing depolarisation of the hair cell. Depolarisation of the hair cell is unusual, as an action potential is not created within the cell. Instead, hair cells are subject to a potassium driven, graded receptor potential. When the hair cell depolarises, voltage gated calcium channels activate in the hair cell membrane. Calcium influx then triggers neurotransmitter release at the base of the hair cell, which diffuses into the nerve terminal. At this point, an action potential is created in the nerve (spiral ganglion), and transmitted to the brain via the vestibulocochlear nerve bundle (LeMasurier & Gillespie 2005).

Both inner and outer hair cells perform mechanotransduction, however the role of each cell type differs. Inner hair cells sense and transmit auditory information to the brain (Figure 7). Whereas the primary function of outer hair cells is to amplify the signal received by inner hair cells (Fettiplace 2017). Outer hair cells contain large amounts of the motor protein prestin (Ashmore 2008; Zheng et al. 2000). Therefore, outer hair cells are able to elongate or contract within milliseconds of a membrane potential change (Brownell et al. 1985; Goutman et al. 2015; Liberman et al. 2002; Zenner 1986). Such movements enhance or dampen the signal within the cochlea. In particular, contractile movement of the depolarized outer hair cells enhances basilar membrane vibration and further displaces the cochlear endolymph. This increases the stimulation of inner hair cells, achieving a 40 dB SPL increase in perceived sound (Frolenkov 2006; Liberman et al. 2002). For this reason, outer hair cell function is often referred to as the cochlear amplifier. However, efferent signals to the outer hair cells can result in hyperpolarisation, reducing contractile movement and dampening the effects of the cochlear amplifier (Frolenkov 2006; Brownell et al. 1985). The cochlear amplifier is particularly important for sound localisation, speech recognition in noisy environments, and protection from noise-induced hearing loss (Winslow & Sachs 1987; Andéol et al. 2011; Maison et al. 2013; Dallos et al. 2008; Ashmore 2008; Goutman et al. 2015).

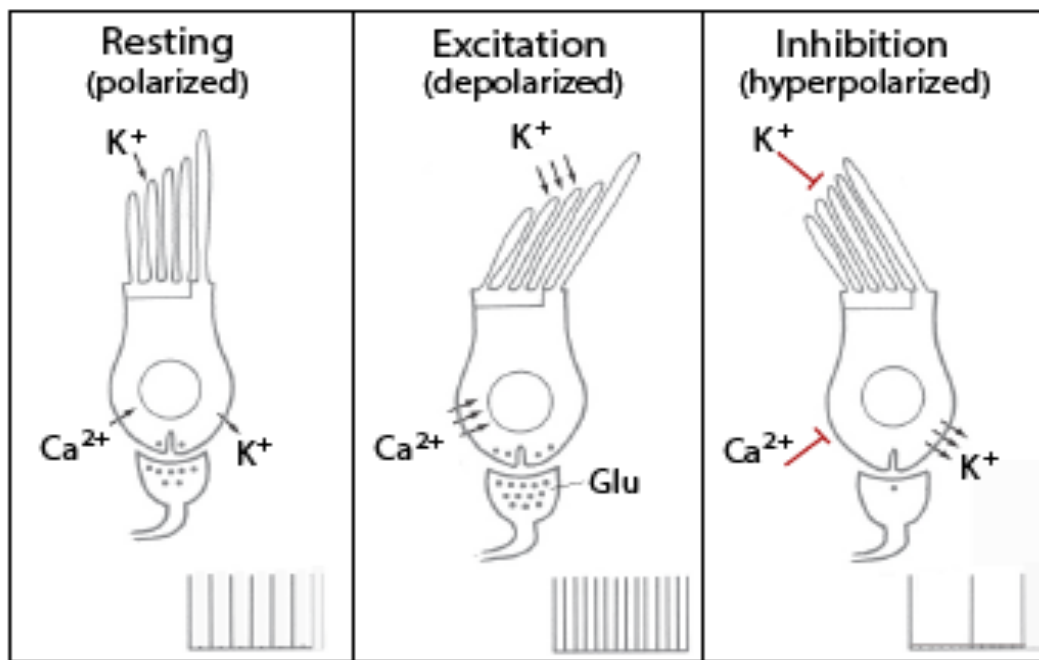


Figure 6. Hair cells convert a mechanical stimulus into an electrochemical signal.

The hair cell stereocilia are bathed in potassium (K^+)-rich endolymph of the scala media. The hair cell base is embedded within the basilar membrane and exposed to perilymph. As a result, the hair cell has a resting potential (approximately -45 mV for inner hair cells, -75 mV for outer hair cells), which facilitates the entry of K^+ into the hair cell through MET channels. Hair cells are therefore subject to biphasic, graded receptor potentials (Purves et al. 2001a). At rest, MET channels are slightly open. Small amounts of potassium and calcium enter the cell causing axon terminals to release a limited amount of neurotransmitter (Glutamate (Glu)), and cause action potentials at a basal (spontaneous) rate. However, sound induced deflections towards the tallest stereocilia cause MET channels to open fully, allowing for rapid K^+ and calcium (Ca^{2+}) entry into the hair cell. As a result, the hair cell depolarises and neurotransmitter is released. Conversely, deflection of the stereocilia in the opposite direction will close the MET channels, resulting in hyperpolarisation of the cell and inhibition of basal axon activity. Image adapted from Emanuel et al. 2009 (Emanuel et al. 2009). For simplicity, this image depicts only a few stereocilia. In reality, each hair cell can have between 20 and 300 stereocilia, depending on its location within the ear and host species (Tilney et al. 1992; Tilney & Saunders 1983; Narayanan et al. 2015; Tilney & M. S. Tilney 1988).

1.1.1.7. The MET channel

It is estimated that each stereocilia bundle contains 50-100 functional MET channels (Goutman et al. 2015; Ricci et al. 2003; Denk et al. 1995). The MET channel is a non-selective cation channel, with an estimated pore size of 1.2 nm (Fettiplace 2017; Farris et al. 2004) and a high single-channel conductance (greater than 100 pS) (Goutman et al. 2015; Ricci et al. 2003; LeMasurier & Gillespie 2005). However, MET channel conductance is variable, decreasing from base to apex within the cochlea, further enhancing cochlear tuning and preferential high frequency specificity (Fettiplace 2017; Ricci et al. 2003). The ultrastructure of MET channels is an area of active research. Recently, the association of MET channels with tip links has been confirmed, key structural proteins have been identified, and the location of MET channels at both the apex and base of stereocilia bundles has been clarified (reviewed in Goutman et al. 2015 and Qiu & Muller 2018). Nevertheless, MET channel structure and functioning remains poorly understood. In particular, the protein subunits required to form the MET channel pore have not been elucidated (Cunningham & Muller 2019).

1.1.2. Cochlear hair cell innervation

Hair cells receive both afferent and efferent synapses, however the patterns of innervation differ between the two hair cell types and is related to their function. Inner hair cells are directly innervated by large afferent projections from the type I spiral ganglion neurons and receive an indirect efferent connection from the lateral olivocochlear nucleus. On the other hand, outer hair cells are directly innervated by both afferent type II spiral ganglion neurons and efferent medial olivocochlear neurons (Fuchs & Lauer 2019; Spoendlin 1985).

1.1.3. The spiral ganglion and afferent hair cell innervation

The spiral ganglion neurons connect the hair cells of the cochlea to the cochlear nucleus, receiving auditory signals from hair cells in the form of neurotransmitter release (Coate et al. 2019). Acoustic information is then encoded as action potentials that travel along the cochlear portion of the VIII nerve for central processing and cognition (Nayagam et al. 2011). In humans, approximately 32,000 afferent auditory

neurons extend a peripheral dendrite project to the base of hair cells and a central axon into the cochlear nucleus (Berne et al. 2008; Spoendlin & Schrott 1989). As the inner hair cells are the primary source of auditory information, the vast majority of afferent fibres contact inner hair cells (Figure 7).

There are two types of afferent neurons within the cochlea, type 1 and type 2 which were first identified due to a marked difference in anatomical features (Liberman 1982; Berglund & Ryugo 1987; Kiang et al. 1982). Type 1 neurons are large in diameter and myelinated, whereas the processes of type 2 neurons are smaller and unmyelinated (Vyas et al. 2019; Liu et al. 2015). Numerous distinctions have now been identified relating to protein expression, organelle number and most importantly, function (Liberman 1982; Vyas et al. 2019; Liu et al. 2015). Additionally, subtypes of the type 1 afferents have now been identified, with specific electrical properties that further enhance cochlear frequency tuning and unique transcriptional profiles (Petitpre et al. 2018; Burns et al. 2015).

1.1.3.1. Type 1 spiral ganglion neurons

Approximately 90% of the spiral ganglion neurons are type 1. Type 1 projections are usually unbranched, contacting a single hair cell with an unmyelinated terminal at the ribbon synapse. The inner hair cell afferent dendrite synapses are glutamatergic. Each inner hair cell is innervated by 10-20, mostly unbranched afferent auditory nerve fibres (Liberman 1982). Type I afferents relay information regarding the frequency, intensity, and timing of the acoustic signal.

1.1.3.2. Type 2 spiral ganglion neurons

Type 2 neurons make up only ~10% of the total afferent projections within the cochlea. Each type 2 afferent contacts up to 20 outer hair cells. The function of type 2 afferents is still being delineated. It is believed that type 2 afferents provide vital information regarding efferent negative feedback control, which is important for the autonomous reflex suppression of the cochlear amplifier (Froud et al. 2015). Alternatively, it has been suggested that type 2 neurons combine sound signals in a different way to type 1 neurons, enhancing the sensitivity of auditory processing (Reid et al. 2004). However,

the fact that type 2 spiral ganglion neurons resemble pain-sensing fibres from the dorsal root ganglion, combined with observations that type 2 neurons only signal in response to extremely loud or damaging stimuli, suggests that type 2 neurons sense noise induced ‘pain’ in the ear (Vyas et al. 2019; Weisz et al. 2009; Liu et al. 2015; Flores et al. 2015; Fuchs & Glowatzki 2015; Zhang & Coate 2017).

1.1.4. Efferent hair cell innervation

Cochlear efferent signals can derive from either the auditory nerve-cochlear nucleus reflex arc or the medial and lateral nuclei of the superior olivary complex (LeMasurier & Gillespie 2005; Fuchs & Lauer 2019; Goutman et al. 2015; Guinan 2010). Efferent neurons primarily release acetylcholine, which activates a calcium-dependent potassium current that inhibits hair cells by hyperpolarisation (Fuchs & Lauer 2019). However, alternate neurotransmitters including dopamine and gamma-aminobutyric acid are also present in efferent terminals (Goutman et al. 2015; Eybalin 1993).

Efferent projections do not directly contact inner hair cells. However, the unmyelinated lateral olivocochlear efferent fibres contact the peripheral dendrites of type 1 neurons, dampening afferent inner hair cell signals (Figure 7). Conversely, the outer hair cells receive efferent projections which terminate at the hair cell base, alongside afferent terminals (Figure 7) (Berne et al. 2008). As a result, descending signals from the olivary nucleus can directly modulate the motile response of outer hair cells, which in turn regulates tuning and amplification effects. This enhances auditory transduction, assisting with sound localisation and signal to noise differentiation (Fuchs & Lauer 2019; Fettiplace 2017). As a result, efferent feedback may also play a role in protection from noise-induced hearing loss (Fuchs & Lauer 2019).

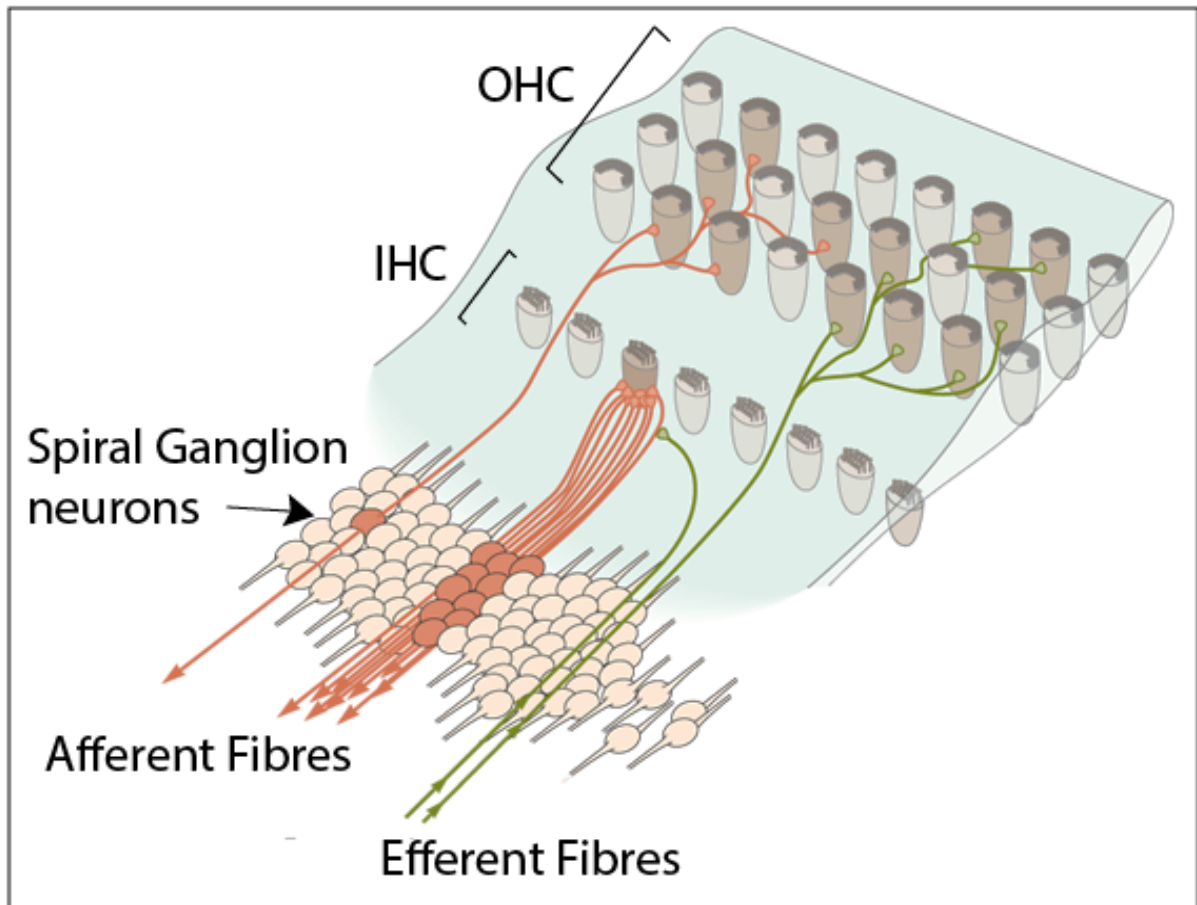


Figure 7. Afferent and efferent innervation patterns in the cochlea.

Inner hair cells sense and transmit auditory information to the brain. Each inner hair cell provides input to approximately 10 afferent auditory nerve fibres (orange). In contrast, few sensory axons transmit information from the outer hair cells. Instead, outer hair cells primarily receive efferent signals (green). Efferent feedback modulates outer hair cell electromotility, driving the cochlear amplifier. Some efferent fibres directly dampen afferent activity. Figure adapted Kandell et al. 2013 (Kandel et al. 2013).

1.1.5. The vestibular apparatus

The vestibular apparatus is the sensory organ for the detection of movement. It consists of three semicircular canals and the adjoining vestibule (Figure 2) (Yost 2007). The semicircular canals, vestibule and cochlea are interconnected, allowing for endolymph exchange. The saccule, utricle, and semicircular ducts are filled with endolymph, whilst perilymph fills the remaining space (Ekdale 2013). Like the cochlea, the vestibular apparatus contains hair cells that convert a mechanical stimulus into an electrochemical signal. However, vestibular hair cells are unique, in both structure and function.

1.1.5.1. Hair cells in the ampullae detect head rotation

Head rotation around the horizontal axis is detected by a neurosensory epithelium (crista) that lines each ampulla (Figure 8). The ampullae are located at the base of each semicircular canal. Like the cochlear organ of Corti, the crista contains specialised hair cells and supporting cell types. However, the stereocilia of the hair cells in the crista are embedded in the cupula, which is a gelatinous structure sensitive to fluid movements. When the head is rotated, inertia causes the fluid in the semi-circular canal to lag. As a result, the cupula deflects the stereocilia, thus inducing hair cell mechanotransduction (Figure 6). The fluid in each semicircular canal is most sensitive to linear movement along the axis that most closely resembles the canals trajectory. Thus, the combination of different signals generated by each ampullae provide precise information regarding head movement.

1.1.5.2. The vestibule

The vestibule is the largest part of the vestibular apparatus. The vestibule contains the otolith organs, known as the utricle and saccule (Figure 2). In the otolith organs, hair cell stereocilia are embedded within the otolithic membrane (Figure 9A). This fibrous membrane contains dense, crystalline calcium carbonate otoliths (Greek; *ear-stones*), which increase the overall membrane density. As a result, the membrane is responsive to linear or gravitational force (Figure 9B & C). When gravity or inertia act on the membrane, the hair cell stereocilia deflect, inducing hair cell mechanotransduction (Figure 6). The signals produced by hair cells in the otolith organs detect head tilt (from vertical orientation) and linear movements.

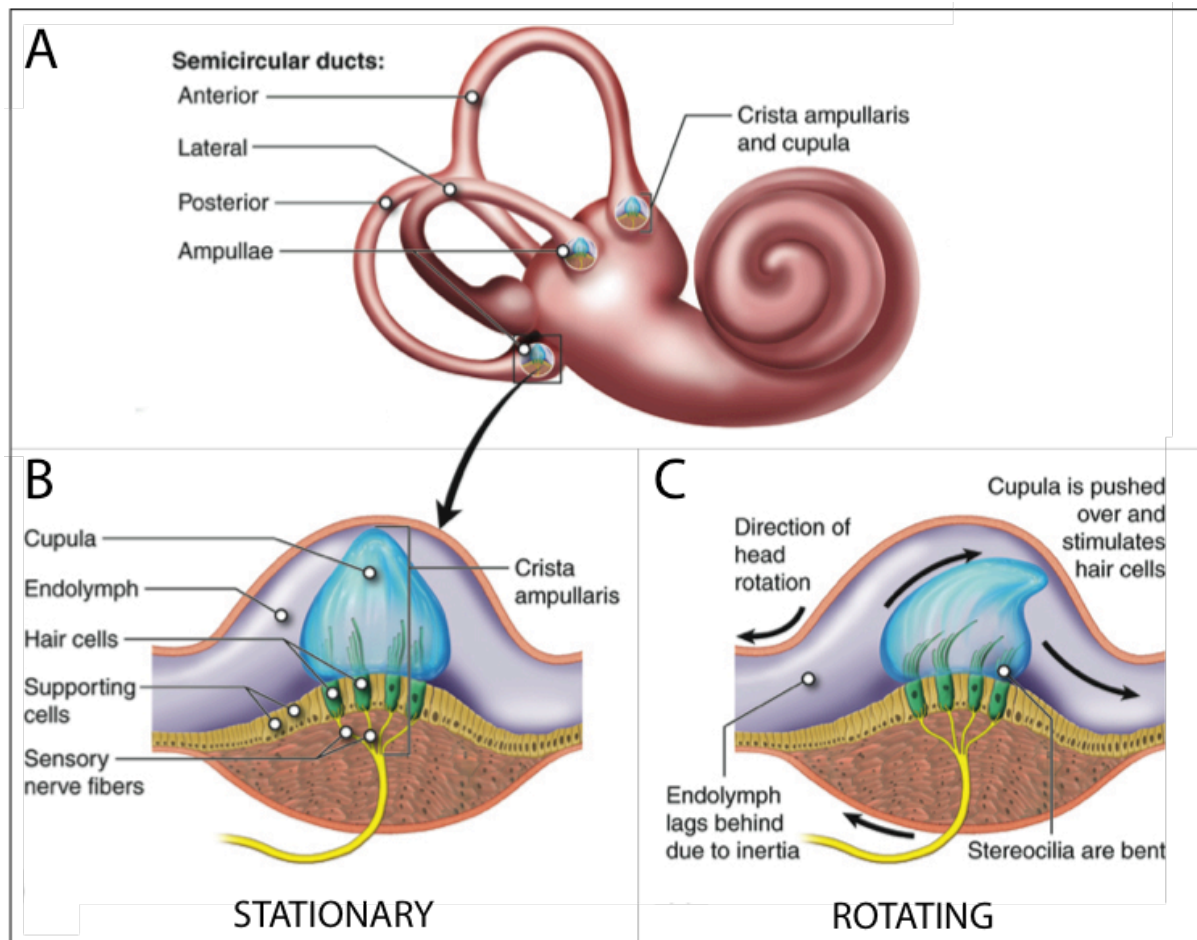


Figure 8. Horizontal head movements are sensed by vestibular hair cells in the crista of the semicircular canals.

A) The base of each semicircular canal is enlarged, housing the ampullae. **B)** Within each ampulla there is a gelatinous structure known as the cupula. The neurosensory epithelium that detects head rotation (crista) lines the ampulla, with hair cell stereocilia embedded in the cupula. **C)** As the head rotates, inertia causes fluid to lag in the semi-circular canals. The cupula deflects the stereocilia, inducing hair cell mechanotransduction. Figure adapted from Carnegie Mellon University (CMU 2019).

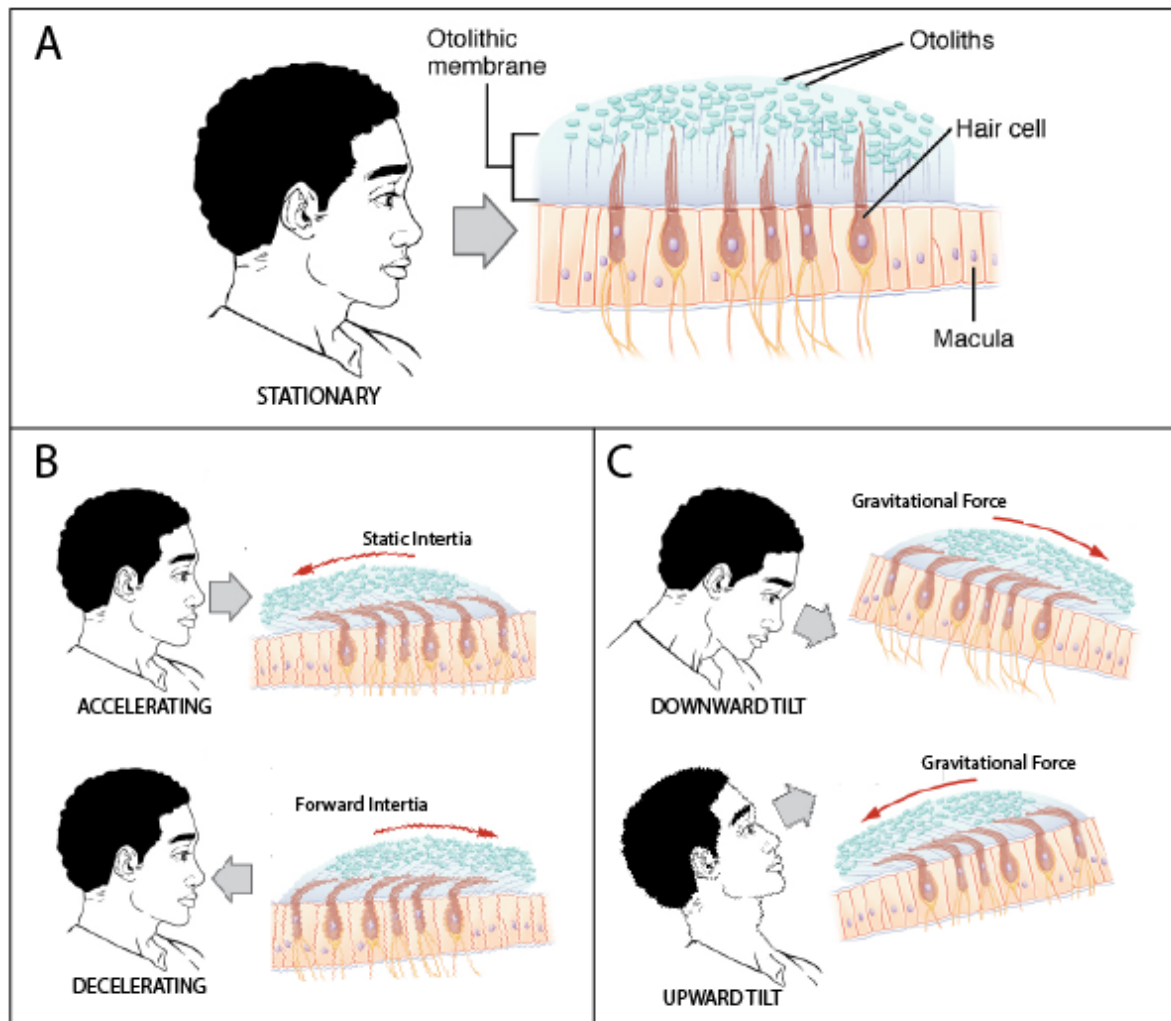


Figure 9. Hair cells within the Otolith organs sense linear acceleration, deceleration and static head position.

A) The sensory epithelia of the otolith organs is the macula. The stereocilia of macular hair cells are embedded within an otolithic membrane, which contains calcium carbonate otoliths. When gravity or inertia act on the membrane, the hair cell stereocilia deflect, inducing hair cell mechanotransduction. **B)** Due to inertia, the otolithic membrane of the utricle drags when the head moves in a linear direction. Inertia also causes the otolithic membrane to continue moving when the linear movement is halted. **C)** Gravitational force causes otolith movement in the saccule when the head is tilted. Figure adapted from Betts et al. 2012 (Betts et al. 2012).

1.1.5.3. Vestibular hair cells and mechanotransduction

There are a number of similarities between cochlear and vestibular hair cells. In particular, mechanotransduction of vestibular hair cells is analogous to that of cochlear hair cells (Figure 6). The two types of vestibular hair cell, type I and type II, are frequently compared with the inner and outer hair cells of the cochlea. However, the exact functions of vestibular hair cells are not well defined. Electrophysiological differences suggest that type I hair cells may detect acceleration during high frequency head movements better than type II hair cells (Burns & Stone 2017). Type I hair cells are flask-shaped with longer stereociliary bundles (Figure 10) and they are innervated by large cup-shaped afferents. Conversely, type II hair cells have shorter stereocilia and a cylindrical shape. Type II hair cells synapse with small afferent terminals. Type I and II hair cells are spread equally throughout the respective vestibular organs, along a polarity axis (Figure 10) (Burns & Stone 2017).

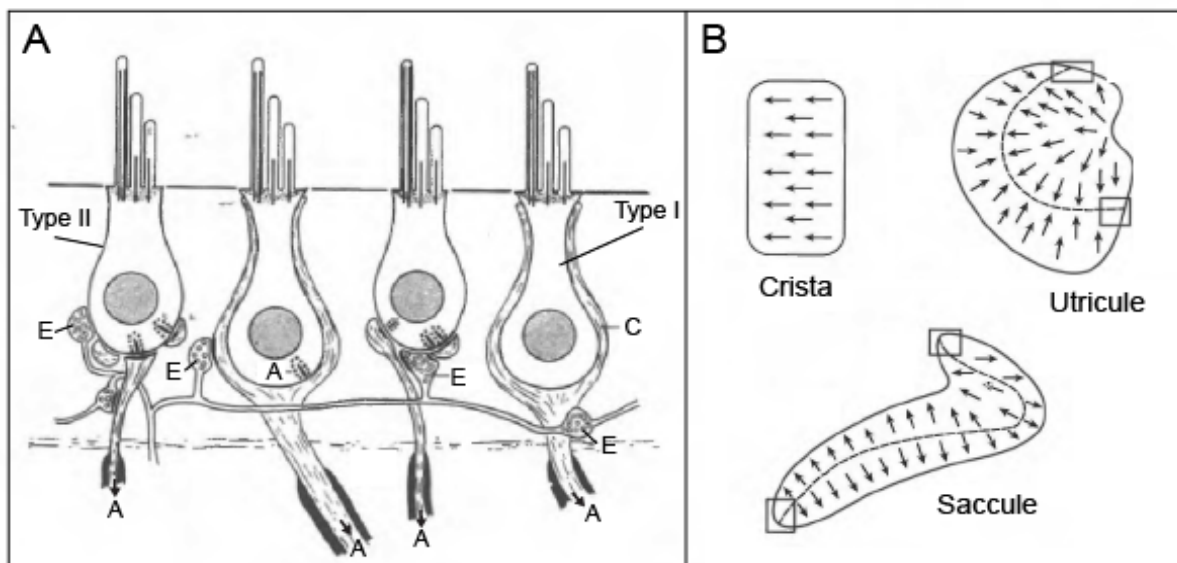


Figure 10. Vestibular hair cell innervation and polarity.

A) Flask-shaped type I hair cells are innervated by the afferent (A) calyx (C). Smaller afferent boutons contact the cylindrical type II hair cells. Efferent fibres (E) contact type II hair cells and the calyx of type I cells. **B)** Arrows indicate the morphological polarization of hair cells in the sensory epithelia. Dashed lines represent the point of polarity transition (striola). In each crista, hair cells follow the same orientation. In the macula of the utricle, hair cells face inwards towards the polarity axis. Hair cells in the macula of the saccule are oriented in opposing directions, pointing away from the polarity axis. Figure adapted from Goldberg et al. 2012 (Goldberg 2012).

1.1.6. Central Processing

1.1.6.1. The vestibulocochlear nerve

The VIII cranial or vestibulocochlear nerve conveys neural signals from both the vestibular apparatus and cochlea. However, the signals are transported by separate, parallel nerve fibre bundles (Betts et al. 2012). The sequence and distribution of responses within the auditory nerve relate to the physical properties of the sound (Ehret & Romand 1997), which are decoded during central auditory processing.

1.1.7. Auditory processing

The central auditory pathway contains multiple nuclei, arranged in a hierarchical manner (Figure 11). Each nucleus contains uniquely distributed neurons with specialised firing properties. The neuronal sub-types respond to various aspects of the auditory signal, allowing for the progressive decoding of complex auditory information (Ehret & Romand 1997).

In brief, the trajectory of an auditory signal is as follows:

Information from the cochlea is received by the cochlear nucleus via the vestibulocochlear nerve (Nayagam et al. 2011). Auditory nerve fibres terminate within the cochlear nucleus and synapse with secondary auditory neurons. Neuronal responses in the cochlear nucleus are variable, receiving both excitatory and inhibitory input, including integrative somatosensory signals (Lauer et al. 2013; Meltzer & Ryugo 2006; Ryugo et al. 2003). The combination of these inputs is processed and then tonotopically projected to higher order nuclei (Muniak & Ryugo 2014; Doucet & Ryugo 2003; Doucet et al. 2002). The signal is projected directly to the ipsilateral superior olivary complex and indirectly to the contralateral superior olivary complex (via large axons that cross the midline). The superior olivary complex is the first point at which auditory signals from each ear converge. Based on this binaural input, the source of a sound can be localised (Ehret & Romand 1997; Tirko & Ryugo 2012). The auditory signal is then relayed tonotopically through the lateral lemniscus, further delineating binaural spatial cues (Shneiderman et al. 1999; Aitkin et al. 1970; Siveke et al. 2019; Ehret & Romand 1997; Kelly et al. 1998). The signal then reaches the inferior colliculus, which acts as a relay point between the auditory brainstem and forebrain, receiving both excitatory and inhibitory inputs (Pannese et al. 2015). Axons from the cochlear nucleus, superior olivary complex and inferior lateral lemniscus terminate at the

inferior colliculus (Ono & Ito 2015; Ehret & Romand 1997; Milinkeviciute et al. 2017). Therefore, this nucleus reintegrates multiple signals, allowing for complex frequency analysis and calculations based on differences between bilateral signals (Tsukano et al. 2017). Thus, the inferior colliculus deciphers vocalisation cues (i.e. consonants and vowels) and is also connected to the visual system, the somatosensory cortex, the cerebellum and other regions relevant to vocalisation, attention, and learning (Pannese et al. 2015). From the inferior colliculus, ascending afferents relay a complex signal to the medial geniculate body of the auditory thalamus, which selectively filters auditory information (Tsukano et al. 2017). The medial geniculate body processes and directs components of the auditory signal for cognition in distinct cortical regions. For example, the medial geniculate body performs preliminary analysis of communicative signals before interacting with the amygdala for the rapid interpretation of emotion (Pannese et al. 2015). The final aspect of the auditory pathway is the auditory cortex, where afferent auditory signals terminate. At this point, the complex signal from each ear has been sequentially decoded and directed to the appropriate cortical layer, allowing for cognitive processing and comprehension at the highest level. Ultimately, the auditory cortex integrates with the complex sensory networks and other modalities of the brain to achieve complete perception, effecting cognition such as prediction, decision-making, and learning (King et al. 2018). Efferent projections also descend from the auditory cortex and all other nuclei along the auditory pathway. As a result, each nucleus provides feedback control over the corresponding lower order nuclei (Ehret & Romand 1997; Milinkeviciute et al. 2017; Meltzer & Ryugo 2006). The reciprocal connectivity of the ascending and descending pathways allows for parallel signal processing and modulation to occur. In addition, distinct synaptic tracts exist between the auditory cortex and cochlea, allowing for rapid modulation of the ascending auditory signal (Huffman & Henson 1990). Whilst the efferent auditory pathway has not received the same attention as the ascending tract, recent studies suggest that descending signals are critical for auditory perception, controlling cochlear gain, compression, noise masking and frequency tuning (Fletcher et al. 2016; Drga et al. 2016; Yasin et al. 2014; Mishra & Biswal 2019; Yakunina et al. 2019). In general, these effects are thought to protect the ear from noise-induced damage and enhance auditory perception in noisy environments (Lopez-Poveda 2018). However, the efferent pathway also interconnects cognitive centres related to attention and emotion (Kimura et al. 2007; Janak & Tye 2015; Rosen et al. 1991).

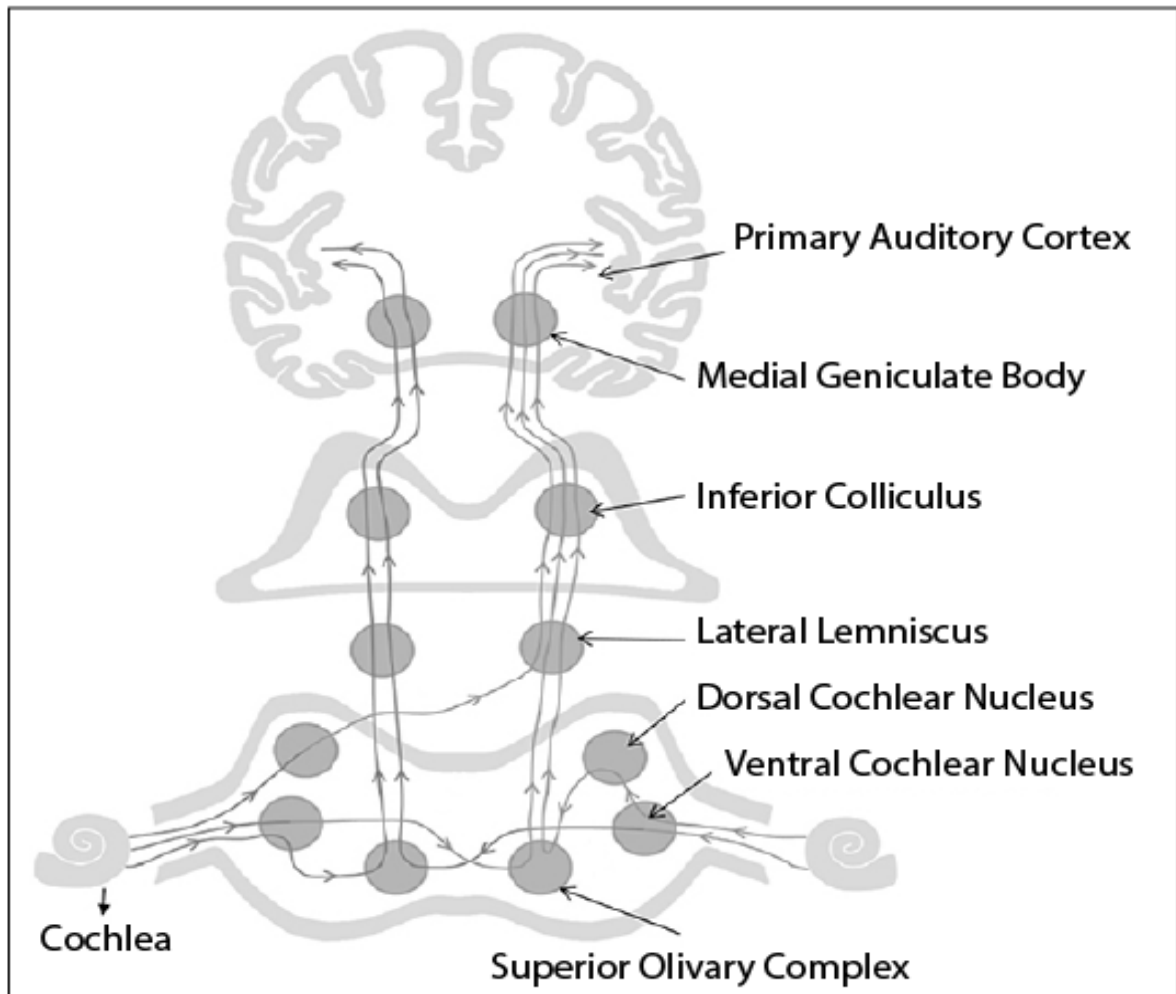


Figure 11. A schematic representing the major ascending aspects of the central auditory pathway.

Afferent fibres synapse onto the inner hair cells of the cochlea. Signals from the cochlea are then conveyed via the auditory nerve to the cochlear nucleus. The signal is projected directly to the ipsilateral superior olivary complex and indirectly to the contralateral superior olivary complex. The now binaural signal is then relayed through the lateral lemniscus, the inferior colliculus and the medial geniculate body of the auditory thalamus, before reaching the auditory cortex. The auditory cortex communicates with other sensory networks to achieve higher order cognition. Complex auditory information is progressively decoded throughout the multiple nuclei of the central auditory pathway, however not all projections follow the pathway in a linear fashion. Efferent projections that occur in reverse, from the auditory cortex to each lower order nucleus, provide feedback control of the auditory signal. However, these projections are not displayed. Figure adapted from Jayakody et al. 2018 (Jayakody et al. 2018).

1.1.8. Vestibular processing

Vestibular signals are received and processed in four vestibular nuclei, located in the brainstem (Figure 12). The signal undergoes secondary processing in the thalamus and higher order processing in the cerebral cortex. However, unlike auditory signals, vestibular information is not processed in a hierarchical manner. Instead, vestibular signals are simultaneously conveyed and integrated widely throughout the brain, with more than 10 cortical regions receiving vestibular information (Lopez 2013). In particular, vestibular cues are intricately connected with ocular and motor control centres.

Initially, hair cells within the vestibular apparatus synapse with bipolar afferent neurons of Scarpa's ganglion. The central processes of these neurons convey the signal via the vestibular portion of the vestibulocochlear nerve. The nerve enters the brainstem just below the cochlear nucleus and integrates with the vestibular complex. A small number of primary afferents also project directly to the cerebellar cortex. The vestibular complex contains four major neuronal clusters, the inferior, medial, lateral, and superior vestibular nuclei (Figure 12). A network of transverse fibres extensively link these nuclei. Secondary fibres project widely from the vestibular complex, particularly towards motor nuclei such as the inferior olive, extra-ocular, trochlear, and abducens nuclei. As a result, vestibular cues are rapidly integrated into complex sensory reflexes, including compensatory eye movements, postural control, and balance.

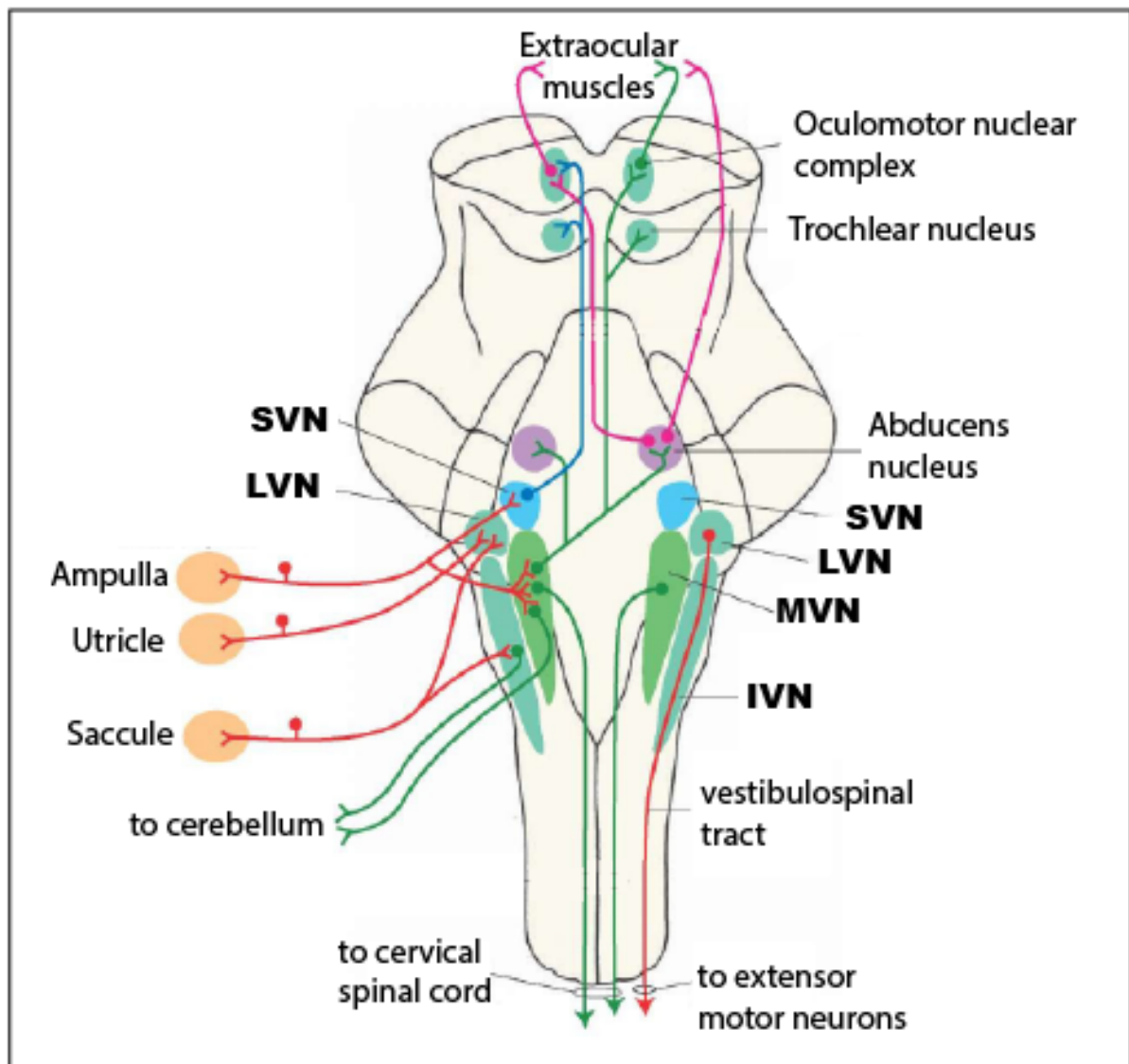


Figure 12. Central vestibular processing is intricately connected with ocular and motor circuitry.

Hair cells within the ampulla, utricle, and saccule synapse with afferent neurons in Scarpa ganglion. The signal is conveyed via the vestibulocochlear nerve to the vestibular complex. The vestibular complex consists of four major nuclei; the inferior (IVN), medial (MVN), lateral (LVN) and superior (SVN) vestibular nuclei. The vestibular complex is intricately connected with ocular and motor specific nuclei. Figure adapted from online resource (Crankshaft-Publishing 2019).

1.2. Hearing loss and vestibular dysfunction

Hearing loss is a major public health issue. Globally, the World Health Organization estimates that 466 million people have hearing loss greater than 30 dB SPL (Bitner-Glindzicz et al. 2009). Moreover, it is projected that by 2030, 630 million individuals will be affected. The current economic burden of hearing loss is calculated to be over 750 billion international dollars, and this cost is rising (Bitner-Glindzicz et al. 2009). In Australia, approximately one in every thousand babies is born with hearing impairment. But even those born with normal hearing will most likely acquire hearing loss over their lifetime (Table 1) (Access-Economics 2006). In 2005, a conservative estimate suggested that one in six Australians had hearing loss (2.6 million Australians in total) (Access-Economics 2006). However, this estimate has now been updated, to over 3.6 million Australians affected. Currently, the financial burden of hearing loss in Australia is estimated to be \$33.3 billion AUD, due to productivity loss, carer costs, medical costs, and overall disease burden (Deloitte 2017).

Hearing loss can have a profound effect on individuals. It is a barrier for communication that influences education, employment, interpersonal relationships, emotional development, and self esteem (Rieffe 2012). Individuals with impaired hearing also experience a high cognitive load, expending significant energy on auditory perception (Wayne & Johnsrude 2015). Mental fatigue can ensue, which then impairs higher-level cognitive processing, such as memory or decision making (reviewed by (Wayne & Johnsrude 2015)). Compensatory neurological changes also occur in response to long-term deprivation of sensory input (Wingfield & Grossman 2006; Pelle et al. 2011). Overall, cognitive decline occurs more rapidly in those with hearing loss (Wayne & Johnsrude 2015; Lin et al. 2011; Lin et al. 2013). Other co-morbidities of hearing loss include an increased risk of falling, impaired self-awareness, loss of personal identity, and higher mortality rates (Lin & Ferrucci 2012; Fisher et al. 2014). Overall, the burden of disease can be very high for individuals with hearing impairment, resulting in disproportionate rates of depression and loneliness (Cosh et al. 2018; Hay-McCutcheon et al. 2018; Li et al. 2014; Gopinath et al. 2009). Whilst the

use of hearing aids can reduce depression and cognitive decline somewhat, the social stigmas relating to hearing aid use can be problematic and overall quality of life remains lower (Wallhagen 2010; Lin et al. 2013; Boi et al. 2012; Mener et al. 2013).

Table 1. The prevalence of hearing loss in Australia.

One in every thousand babies is born with impaired hearing. Those born with unimpaired hearing may acquire hearing damage over their lifetime. Data adapted from Access Economics, based on source material from 1997 - 2005 (Access-Economics 2006; Wilson 1997). These figures are predicted to double by 2050 (Access-Economics 2006).

Age (years)	Number of Hearing Impaired Persons	% of age group
0-14	10,268	0.26%
15-50	534,121	5.10%
51-60	741,902	29.50%
61-70	955,575	58.40%
71+	1,303,364	74.00%
Total	3,545,231	17.40%

1.2.1. Aetiologies of hearing loss

Hearing loss is a complicated condition. Generally, impaired hearing is categorised as conductive or sensorineural. However, numerous aetiologies and diverse pathologies exist within these categories (Table 2). More than 50% of all juvenile hearing loss cases have an underlying genetic basis. However, most people born with unimpaired hearing will acquire hearing loss over their lifetime (Table 1). Acquired hearing loss is usually caused by aging, acoustic trauma, disease or exposure to ototoxic agents.

Table 2. Categories of hearing loss and common aetiologies.

Conductive hearing loss is defined by poor sound wave propagation through the outer and middle ear. Sensorineural hearing loss can involve impaired sensory receptor function (hair cells) or poor signal transduction from the cochlea to brain. Some individuals have mixed hearing loss, with elements of both conductive and sensorineural hearing loss. Auditory processing disorders in the brain interfere with hearing cognition, however these stem from a neurological condition rather than disrupted auditory sensation.

CONDUCTIVE	SENSORINEURAL
<u>Ossicle Chain Dysfunction</u> Congenital malformation Otitis Media / Infection Otosclerosis / Bone overgrowth Trauma/ Breakage Eustachian tube dysfunction <u>Tympanic Membrane Dysfunction</u> Tympanosclerosis / Calcification of the tympanic membrane Perforation from infection, rapid air pressure changes or trauma <u>Blockage of the Ear Canal</u> Stenosis / Abnormal canal narrowing Wax / Foreign Objects Infection/ Swelling Exostasis / Bony growths Congenital malformations Tumour	<u>Hair Cell Death</u> Acoustic Trauma / Noise Ototoxic medicines Aging Genetics Physical trauma Infection or Disease <u>Cochlear Degeneration</u> Cell death Ionic or Pressure imbalance Trauma Aging Infection <u>Auditory Nerve Damage</u> Acoustic neuroma / Tumour Neurodegeneration / Cell death

1.2.2. Acquired hearing loss

Acquired hearing loss is a global health issue. The World Health Organization estimates that 40-50% of acquired hearing loss cases are preventable (Bitner-Glindzicz et al. 2009). Generally, acquired hearing impairment is caused by the destruction of hair cells. However, neuronal damage at any point from the spiral ganglion to the brain, can also impair signal transduction. The predominant causes of acquired hearing loss are aging, noise exposure, and exposure to ototoxic substances. There are numerous substances and compounds which are ototoxic, meaning toxic to the ear (Table 3) (Hoshino et al. 2015; Clerici et al. 1996; Ton & Parng 2005).

1.2.3. Ototoxic drugs are a significant cause of hearing loss

Ototoxicity, the phenomenon whereby a substance damages any aspect of the cochlea, vestibular system or auditory nerve was first recorded almost 1000 years ago. The Persian philosopher Ibni Sina (Avicenna) noted that medicinal mercury vapour caused hearing loss (Hamidi et al. 2008). More recently, the industrial revolution of the 18th and 19th centuries resulted in an increased incidence of chemical ototoxicity, whilst medical progress in the 20th century led to an increase in the utilisation of ototoxic medicines. In this time, ototoxic side effects, including acquired deafness, tinnitus, ataxia, nausea, and vertigo have been extensively studied. However, despite considerable research of ototoxic outcomes, there is currently no preventative therapy. Recently, the World Health Organization highlighted ototoxicity as a problematic cause of hearing loss in the 2017 report entitled *Addressing the rising prevalence of hearing loss* (WHO 2018). Within this report, the incidence of ototoxicity is described as *alarming*, with a clear statement that ototoxic drug induced hearing loss must be addressed (WHO 2018). Indeed, despite lifesaving outcomes, these ototoxic medicines have damaged the hearing of millions of people worldwide, including approximately 1,280,000 children (WHO 2016). There are more than one hundred medicines with ototoxic properties (Table 3), many of which are listed on the World Health Organization's list of critical medicines. In some cases, these medicines cause mild ototoxic outcomes such as transient dizziness or ringing in the ears. However, some ototoxic drugs can cause permanent damage, resulting in debilitating hearing loss, tinnitus or vestibular dysfunction. Two ototoxic drug classes are particularly problematic. These include the platinum based chemotherapeutics, which are used to treat a range of metastatic and refractory tumours in both adults and children, and the aminoglycoside antibiotics, used to manage chronic or multi-drug resistant infection (Xie et al. 2011; Kranzer et al. 2015).

Table 3 Ototoxic medicines. Based on TGA Approved Australian Product Information, Australian Medicines Handbook and British National Formulary, accessed through the Clinical Information Access Portal (CIAP) 26/4/15. Adapted from (Driscoll 2018).

NSAIDs	Antibiotics	Diuretics	Cardiac Medicines	Antidepressants Smoking Cessation	CNS Medicines	Antihistamines
celecoxib	amikacin	acetazolamide	Antihypertensives	agomelatine	aripiprazole	cetirizine
diclofenac	amphotericin b	amiloride	amlodipine	amitriptyline	baclofen	cycloheptadine
etoricoxib	azithromycin	bumetanide	atenolol	clomipramine	bromocriptine	dexchlorpheniramine
ibuprofen	aztreonam	eplerenone	carvedilol	citalopram	buspirone	diphenhydramine
indomethacin	capreomycin	ethacrynic acid	clonidine	desvenlafaxine	caffeine	famotidine
ketoprofen	ciprofloxacin	frusemide	diltiazem	doxepin	carbamazepine	loratadine
ketorolac	clarithromycin	hydrochlorothiazide	enalapril	duloxetine	eletriptan	pheniramine
meloxicam	dapsone	indapamide	fosinopril	escitalopram	gabapentin	promethazine
naproxen	doxycycline		irbesartan	imipramine	galantamine	
piroxicam	erythromycin	Local Anaesthetics	losartan	mianserin	lacosamide	Eye Drops
sulindac	ganciclovir		metoprolol	moclobemide	lithium	
tioprofenic acid	gentamicin	articaine	nifedipine	nortriptyline	paliperidone	brinzolamide
	imipenem	bupivacaine	nimodipine	paroxetine	pramipexole	dorzolamide
Aspirin Like	itraconazole	lignocaine	olmesartan	reboxetine	risperidone	flurbiprofen
	lincomycin	prilocaine	perindopril	sertraline	selegiline	timolol
aspirin (100mg/day may be ok)	mebendazole	ropivacaine	prazosin	trimipramine	topiramate	& combination drops
choline salicylate	metronidazole		propranolol	tranylcypromine	valproic acid	travoprost
mesalazine	minocycline	Cytotoxics (i.e chemotherapies)	ramipril	venlafaxine	ziprasidone	
salicylic acid	moxifloxacin		telmisartan		zolpidem	Other
sulfasalazine	posaconazole	anagrelide	terazosin	bupropion	zolmitriptan	
	roxithromycin	carboplatin	trandolapril	varenicline	zonisamide	acitretin
	streptomycin	cisplatin	valsartan		zuclopenthixol	isotretinoin
Opiates + antagonists	sulfadiazine	cyclophosphamide	verapamil	Immuno- suppressives		Interferons ± ribavirin
	sulfamethoxazole/ trimethoprim	ifosfamide	Other Cardiac		Quinines	omeprazole
buprenorphine		methotrexate	atorvastatin	cladribine		pantoprazole
hydromorphone	teicoplanin	paclitaxel	cilostazol	cyclosporine	chloroquine	sildenafil
oxycodone	terbinafine	vincristine	flecainide	mycophenolate	hydroxychloroquine	tadalafil
tramadol	tobramycin		nicorandil	penicillamine	mefloquine	varafenafil
naltrexone	vancomycin		nitroprusside	tacrolimus	quinine	Protease Inhibitors & antiretrovirals
	valganciclovir		ticlopidine		atovaquone/proguanil	

1.2.3.1. Platinum based chemotherapy

Platinum based compounds such as cisplatin, oxaliplatin and carboplatin are used extensively in anti-cancer treatment regimens, due to their efficacy against neoplasms including lymphoma, sarcoma, carcinoma and germ cell tumors (Dasari & Tchounwou 2014; Rybak et al. 2007). Cisplatin alone is used in almost 50% of all anti-tumour therapies (Galanski et al. 2005). However, permanent high frequency hearing loss and tinnitus are commonly reported side effects (Rybak et al. 2009; Kopelman et al. 1988; Nitz et al. 2013; Dammeyer et al. 2014; Berg et al. 1999). A high frequency threshold shift between 40 and 80 dB SPL can occur within hours of treatment, or manifest up to 3 months after treatment (Knight et al. 2005; Rybak et al. 2009; Kopelman et al. 1988; Berg et al. 1999). Such high frequency hearing loss particularly interferes with speech recognition (Berg et al. 1999; Kopelman et al. 1988). However, tinnitus is considered a particularly uncomfortable and common outcome of cisplatin therapy (Kopelman et al. 1988). The ototoxicity of platinum based compounds is attributed to the production of reactive oxygen species (ROS) and simultaneous inhibition of free radical scavenging within hair cell (Kopke et al. 1997; Husain et al. 1996; Rybak et al. 2009).

1.2.3.2. Aminoglycoside antibiotics

Broad spectrum aminoglycosides are the most commonly used antibiotics worldwide (Xie et al. 2011; Hermann 2007). Aminoglycosides are particularly important for treating drug resistant infections, which are a significant and increasing problem (Seddon et al. 2012; Bitner-Glindzicz et al. 2009). In particular, drug resistant strains of *Pseudomonas aeruginosa* or tuberculosis require aminoglycoside interventions to preserve life (Seddon et al. 2012; J. Xie et al. 2011). However, an unfortunate consequence of prolonged or high dose treatment is ototoxicity, manifesting as irreversible damage to the hair cells of the cochlea and vestibular apparatus (Ratjen et al. 2009). Individuals with cystic fibrosis are particularly vulnerable to ototoxicity, as chronic lung infections (primarily caused by *Pseudomonas aeruginosa*) require long term aminoglycoside treatment (Ratjen et al. 2009).

Aminoglycosides penetrate nearly every cell within the cochlea, persisting for over 6 months (Imamura & Adams 2003). It is well established that the subsequent aminoglycoside-induced hair cell death is predominantly caused by excessive ROS production and the inhibition of ionic regulating mechanisms (Xie et al. 2011; Leitner et al. 2011; Selimoglu 2007; Clerici et al. 1996; Hirose et al. 1997) . Unfortunately, aminoglycoside antibiotics are often needed in conjunction with platinum based chemotherapeutics. The dual use of these two drug classes, with different mechanisms of creating ROS, amplifies the extent of ototoxicity (Knight et al. 2005). No adequate aminoglycoside substitution exists for many conditions where the treatment is currently required. Therefore, there is considerable interest in how aminoglycoside ototoxicity can be avoided or mitigated (Xie et al. 2011; Ding et al. 2002) .

1.2.4. The prevalence of ototoxic outcomes

Within the literature, the correlation of ototoxic outcomes and drug dosage is a controversial topic. For both platinum based chemotherapeutics and aminoglycoside antibiotics, there is considerable disparity between reported ototoxic outcomes. For example, Berg et al. 1999 reported hearing loss in 26% of paediatric patients receiving cisplatin and found that drug dosage, whether cumulative or singular, had no effect on the likelihood of developing drug induced sensorineural hearing loss (Berg et al. 1999). Kopelman et al. 1988 also reported no correlation between cisplatin dose and hearing loss (Kopelman et al. 1988). However, the Kopelman study utilised a more rigorous hearing assessment and found that 100% of patients had high frequency hearing loss. A systematic review performed by The American Speech-Language-Hearing Association highlighted a similar disparity within 48 studies of aminoglycoside ototoxicity. The reported prevalence of gentamycin-induced hearing damage ranged from 0-20% of treated individuals (across 7 studies), despite the same dosage being used (4-5 mg/kg/day). Amikacin ototoxicity reports were even more variable with 0-64% of patients experiencing hearing loss (across 10 studies), again utilising the same dosage (15 mg/kg/day). The observed variability of ototoxic outcomes associated with aminoglycoside use may be due to the individual rRNA binding affinity of each antibiotic (Rybak & Ramkumar 2007; Kitasato et al. 1990; Hoshino et al. 2015; Clerici et al. 1996; Ton & Parng 2005; Cabanas et al. 1978).

However, inconsistent study design is likely to have played a significant role in the variable results described. For example, researchers or medical practitioners use different definitions of ototoxicity, use different drug administration routes, and use a different battery of hearing assessments (Huth et al. 2011; Rybak et al. 2008). Notably, even rigorously controlled animal studies report variable ototoxic outcomes in littermate control mouse and guinea pig experiments (Santi et al. 1982).

These observations suggest that a significant proportion of variance in ototoxic outcomes is likely to be caused by individual susceptibilities to ototoxic drugs. For example, the severity of ototoxicity can depend on the age and ethnicity of the individual, concurrent drug administration, and other co-morbidities such as anaemia or kidney disease (Rybak et al. 2009; Knight et al. 2005; Blakley 1997; Xie et al. 2011; Warady et al. 1993). Additionally, a well-characterised genetic mutation in the mitochondrial 12S ribosomal RNA gene has been shown to enhance susceptibility to aminoglycoside ototoxicity (Prezant et al. 1993). Over 50% of individuals treated with ototoxic drugs experience delayed onset, high frequency hearing loss. Therefore, it is widely considered that the short follow-up times and simple hearing assessments utilised in many studies have caused drug-induced hearing loss to be significantly underestimated and underreported (Fausti et al. 1992; Choung et al. 2009; Xie et al. 2011; Huth et al. 2011).

1.2.5. The mechanisms of ototoxicity

The stria vascularis contains numerous capillaries, which allow for the passage of ototoxic drugs from the blood to the endolymph within the otic capsule (Hiel et al. 1992). Once suspended in the endolymph, ototoxic drugs are able to rapidly enter the vulnerable hair cells. This was clearly demonstrated in a seminal experiment performed by Shun-ichi Imamura and Joe C. Adams (2003). During the experimental time frame, labelled gentamycin migrated rapidly from the treated guinea pig's blood, across the stria vascularis and into the cochlear lymph. Initially, all cochlear cell types were labelled, but within 24 hours most gentamycin was concentrated within the hair cells (Imamura & Adams 2003). Hair cells were shown to retain gentamycin for over six months, with the cuticular plate (the site at which the stereocilia anchor to the apical end of the hair cell) showing particularly strong gentamycin accumulation. This study identified the stria vascularis as the cochlear point of entry for aminoglycosides

and also highlighted the susceptibility of hair cells to both drug uptake and retention. It is not clear why hair cells are so susceptible to the accumulation of ototoxic drugs. However, the MET channel is predicted to facilitate rapid drug entry. For example, *Mariner* zebrafish have a missense mutation in the myosin VIIa gene which causes defective hair cell mechanotransduction. Hair cell uptake of gentamycin is significantly reduced in *Mariner* zebrafish, suggesting that functioning MET channels are required for drug entry (Wang & Steyger 2009). Supporting cells within the mammalian cochlea that do not have MET channels are also less prone to aminoglycoside damage (Sha et al. 2001). The observation that basal hair cells take up more labelled gentamycin than apical hair cells also implicates MET channels in aminoglycoside uptake, as MET channel conductance is greatest at the base of the cochlea (Hiel et al. 1992). In addition, designer aminoglycosides that cannot pass through MET channels have significantly reduced ototoxic effects (Huth et al. 2015). Therefore, it is likely that the MET channel is the entry point for ototoxic drugs, allowing for large quantities to rapidly enter the hair cell. However, it remains unclear why aminoglycosides persist in hair cells, when other cell types can clear the compounds.

1.2.6. Aminoglycosides and platinum-based drugs impair mitochondrial function

Platinum based compounds and aminoglycosides prevent mitochondrial protein synthesis (Marullo et al. 2013). Cisplatin does this by forming adducts with mitochondrial DNA (Marullo et al. 2013), whereas aminoglycosides target the bacterial ribosome, inhibiting translation and causing mRNA mismatching (Cabanas et al. 1978). Shared ancestry (prior to endosymbiosis) means that the mitochondrial ribosome is similar to the bacterial ribosome, which renders it susceptible to aminoglycosides (Prezant et al. 1993). Additionally, a well-characterised human mitochondrial mutation (A to G, nt 1555 within the 12S ribosomal RNA gene) changes the structure of the mitochondrial ribosome sub-unit interface to resemble that of the bacterial ribosome more closely. This enhances aminoglycoside binding affinity and increases an individual's susceptibility to drug induced hearing loss (Prezant et al. 1993). The m.1555 A to G mutation is found in 10-34% of patients with aminoglycoside induced ototoxicity (Min-Xin 2006; Z. Gao et al. 2017), whereas the mutation

frequency in the general population is much lower (~0.2%) (Gopel et al. 2014; Bitner-Glindzicz et al. 2009; Vandebona et al. 2009)

Once aminoglycosides or platinum-based compounds have impaired mitochondrial protein synthesis, mitochondrial dysfunction ensues. As a result, ROS are created in amounts unable to be managed by cellular homeostatic processes, leading to hair cell destruction. Mitochondria produce ROS during normal cellular respiration or thermoregulation. However, when this process is compromised mitochondrial ROS production greatly increases (Murphy 2009).

1.2.7. Reactive oxygen species induce hair cell death

For many years, drug induced hair cell death has been attributed to oxidative stress and the ability of ROS to cause extensive cellular damage. Indeed, an increase in ROS production has been extensively documented in hair cells, secondary to ototoxic drug exposure. However, there are competing hypotheses as to what role/s ROS play in hair cell death (Figure 13).

The traditional theory is that ototoxic drugs directly inhibit mitochondrial respiration causing the elevated production of ROS (Figure 13A). These ROS then damage the cell beyond repair, resulting in apoptosis. However, antioxidants such as glutathione (GSH) are deliberately compartmentalised by the mitochondria or expelled from the hair cell before ROS production increases. This efflux of GSH plays a significant role in the execution phase of apoptosis (Franco & Cidlowski 2006; Franco & Cidlowski 2009). Furthermore, during the early stages of apoptosis, cytochrome c activity stimulates mitochondrial production of ROS (Murphy 2009). These observations suggest that apoptotic signalling has already begun before ROS production has increased. Indeed, the use of synthetic antioxidants to remove ROS from the stressed cochlea results in hair cells producing alternate cell death signals. Therefore, a more recent hypothesis of the mechanism of hair cell death has been proposed, suggesting that ROS are not the primary mechanism for ototoxicity, but rather an important aspect of the cell-death signalling cascade (Figure 13B). This alternate theory predicts that cellular mechanisms quickly recognise mitochondrial dysfunction, and that ROS are purposely enhanced as part of a cell death signalling cascade (Franco & Cidlowski

2009). Further work is required elucidate exactly how and why mitochondria produce ROS in the damaged hair cell, particularly with respect to the potential redox signalling that occurs. However, ROS production is considered to be an integral component of the apoptotic pathway (Kagan et al. 2005), in addition to necrosis and extrinsic (receptor-based) cell death (Qin et al. 2015; Kamogashira et al. 2015).

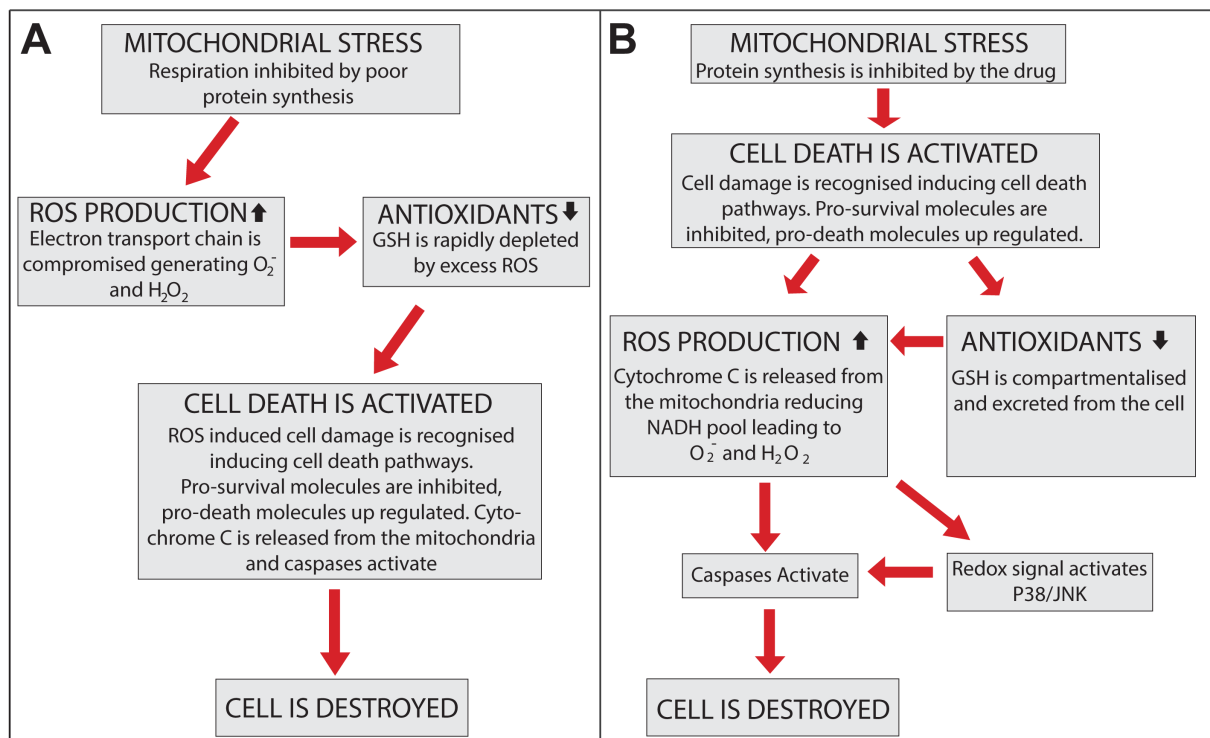


Figure 13. There are alternate proposed mechanisms of ROS-mediated-aminoglycoside-induced hair cell death.

A) The predominant theory regarding aminoglycoside toxicity is that stressed mitochondria output ROS. These ROS deplete antioxidants and directly damage the cell. In response to extensive cell damage, cell death pathways activate. **B)** A more modern theory suggests that impaired mitochondrial protein synthesis is quickly recognised and cell death is activated, with ROS purposely produced as a redox signal for activation of executioner molecules.

1.3. Preventing Ototoxicity

Minimising or avoiding the use of ototoxic medicines are the only known approaches for preventing drug-induced hearing damage. However, neither of these options are particularly helpful in the current clinical setting. Minimising the dose used may reduce ototoxic outcomes in the individual, however, even a single aminoglycoside treatment can cause permanent damage to the ear (Ahmed et al. 2012). Furthermore, the use of an alternate drug is not always feasible. Clinicians will consider the risk of hearing loss against the risk to life when using a less effective drug, justifying their decision in part as “better deaf than dead” (Seddon et al. 2012). Therefore, numerous avenues of investigation have been pursued for preventing drug-induced hearing loss. The association of oxidative stress with ototoxicity has caused significant focus to be placed on antioxidant or free radical scavenger molecules for the prevention of ototoxicity. However, other exciting potential interventions include preventing drug uptake into the hair cell, inducing hair cell regeneration, and co-therapy with compounds that inhibit cell death pathways.

1.3.1. Preventing hair cell-specific drug uptake

Aminoglycosides enter the hair cell via non-selective cation MET channels. Each hair cell has up to 100 MET channels, which facilitates rapid drug uptake (estimated at 9000 molecules per second) (Marcotti et al. 2005; Goutman et al. 2015; Ricci et al. 2003; Denk et al. 1995). To address this issue, Huth et al. 2015 created nine synthetic aminoglycosides with physical properties that prevented their passage through MET channels (Huth et al. 2015). Basic amine sites in sisomicin were converted into carboxamides or sulphonamides. This reduced the drugs’ positive charge, which was predicted to decrease ototoxic effects by preventing drug entry through the MET channels. Notably, sisomicin is not a commonly used aminoglycoside, however it was selected for this study due to it being commercially available at a higher purity than other common aminoglycosides such as gentamycin (Huth et al. 2015).

In total, nine sisomicin derivatives were tested *in vitro*, with variable reductions in outer hair cell death observed. The least ototoxic compound (N1MS) was then tested in live mice. A combined dose of 175 mg/kg sisomicin and 300 mg/kg furosemide, resulted in significant ototoxic outcomes, evidenced by large changes in auditory

brainstem responses (ABRs), distortion product otoacoustic emissions (DPOAEs), and hair cell number in cochlear whole mount preparations. Conversely, N1MS and furosemide treatment resulted in small ABR and DPOAE threshold shifts at higher frequencies and hair cell death was markedly reduced in cochlear whole-mount sections.

Notably, the synthetic aminoglycoside did not have the same antibiotic profile as unaltered sisomicin. N1MS was better at treating extended spectrum β lactamase producers but less effective against *P. aeruginosa* and *S. aureus*, which represent the more prevalent, persistent and pernicious bacterial infections found in hospitals (Nandalal & Somashekar 2007; Wisplinghoff et al. 2004). Overall, Huth et al. 2015 proved in principle that careful drug design can reduce the ototoxicity of an aminoglycoside. However, further work is required to determine if commonly used antibiotics, such as amikacin or tobramycin, could be similarly modified to reduce ototoxicity without impairing antibiotic efficacy.

1.3.2. Replacing lost hair cells.

Ototoxic drugs primarily induce hearing loss via the death of auditory hair cells. Whilst many cell populations within the body are maintained by replacing damaged cells, this is not the case for adult mammalian hair cells.

Some spontaneous hair cell regeneration has been observed in embryonic mammals, but regenerative capabilities are lost at the onset of hearing (Cox et al. 2014; Kelley et al. 1995; Bramhall et al. 2014). For mice this occurs approximately two weeks after birth, whereas in humans it occurs at approximately five months gestation. Interestingly, non-mammalian vertebrates such as fish (Mackenzie et al. 2012; Popper & Hoxter 1984; Corwin 1981; Thomas & Raible 2019), amphibians (Stone 1933; Stone 1937; Wright 1947) and most avians (Hashino et al. 1992; Cruz et al. 1987) can regrow adult hair cells. Therefore, understanding the mechanisms that enable non-mammalian hair cell regeneration may be key for regenerating mammalian cells. Indeed, significant efforts have been made to understand and mimic hair cell regenerative processes from non-mammalian vertebrates in mammalian systems. It is now known that, when avian hair cells are damaged, the adjacent supporting cells can re-enter the cell cycle and differentiate into new hair and supporting cells (Corwin &

Cotanche 1988; Balak 1990). In addition, supporting cells can undergo trans-differentiation and convert into a functional hair cell (Adler & Raphael 1996; Cotanche & Corwin 1991; Tucci & Rubel 1990; Duckert & Rubel 1993). Similar trans-differentiation of supporting cells has also been observed in the salamander, with the supporting cells requiring around six months to become functional hair cells (Jones & Corwin 1993; Taylor & Forge 2005; Steyger et al. 1997; Tucci & Rubel 1990; Duckert & Rubel 1993).

To date, one of the most important developments regarding hair cell regeneration, is the identification of ATOH1 (*Math1* in mice) as an essential gene for hair cell generation (Bermingham et al. 1999). Subsequently, complex ATOH1 interactions with SOX2, WNT and NOTCH have emerged (Samarajeewa et al. 2019; Kelly et al. 2012). NOTCH inhibition or *Atoh1* overexpression has been shown to induce new hair cell generation in both the cochlea and vestibular apparatus (Jen et al. 2019; Izumikawa et al. 2005; Gubbels et al. 2008; Chen et al. 2013; Kawamoto et al. 2003; Ren et al. 2016). The new hair-cell-like cells appear to be the result of trans-differentiation, deriving from supporting cells, border cells, pillar cells, deiters cells or other non-sensory epithelial cells (Jen et al. 2019; Zheng & Gao 2000; McGovern et al. 2019). However, this exciting development is not without limitations. The regenerative potential of ATOH1 or NOTCH is greatest in neonatal tissues, with mature cell types resistant to NOTCH and ATOH1 signals (Maass et al. 2016; Yamamoto et al. 2006; Maass et al. 2015; Bramhall et al. 2014). Furthermore, newly generated hair-cell-like cells tend to be immature and resemble outer hair cells or vestibular type 2 hair cells, neither of which are the primary sensory cells for hearing or balance (Gubbels et al. 2008). Perhaps the greatest challenge of all, is achieving strict hair cell patterning in the newly generated hair cells, as hair cell orientation and size, stereocilia patterning, number and length, and tonotopic neural connections are all critical factors for the specificity and sensitivity of human hearing. Because of this, it is hypothesised that there are mechanisms actively blocking hair cell regeneration in the mammalian ear (Groves 2010). These mechanisms are thought to have evolved in conjunction with the evolution of mammalian specific hearing sensitivities. The rationale being, that in order to maintain the highly structured hair cell patterns required for such sensitive hearing, hair cell regeneration is blocked. Regeneration is proposed to potentially interrupt this organised cell layout and hinder our ability to decipher complex sounds.

An excellent example of how hair cell regeneration could be problematic is observed in newly generated hair cells, such as those established using ATOH1 over expression. These newly generated hair cells often form stereocilia with incorrect or even reversed orientation (Zhao et al. 2011). Such changes in polarity would inevitably cause aberrant hair cell signalling that would compromise central auditory processing and cognitive capabilities. ATOH1 or NOTCH-induced hair cell growth is also ectopic, with hair-cell-like cells growing outside of the organ of Corti (Izumikawa et al. 2005). These ectopic cells are also problematic, as they can attract auditory neurons and again interfere with normal auditory signals (Kawamoto et al. 2003; Gubbels et al. 2008).

The research performed to date indicates that very specific WNT and NOTCH activity is required for the development and patterning of hair cells in the cochlea (Samarajeewa et al. 2019; Lush et al. 2019). WNT and NOTCH have also been reported to prevent trans-differentiation of adult supporting cells, potentially contributing to the impaired regenerative capabilities of mammalian hair cells (Samarajeewa et al. 2019; McGovern et al. 2018; Maass et al. 2016; Yamamoto et al. 2006; Maass et al. 2015; Bramhall et al. 2014). Despite the limited evidence of efficacy, ATOH1 therapy was tested in humans with severe-to-profound bilateral hearing loss (NCT02132130) (Plontke 2015). A recombinant adenovirus vector (CGF166) containing cDNA encoding human ATOH1 was tested in a Phase 1 clinical trial of 45 people, to determine its safety and tolerability. Changes in pure tone audiometry (compared to pretreatment values) were assessed as an additional outcome. In 2018, the lead investigator was quoted as saying *“The best results are with patients who still have some residual hearing”* (Cook 2018 and 2020). However, the trial was suspended in 2020, with an investigator quoted as saying *“We were not getting the results we’d hoped for”*. To date, there is no publicly available data to corroborate or interrogate these statements more thoroughly.

The trial did however raise the importance of residual hearing for putative regeneration therapies. Notably, the damaged organ of Corti begins to systematically collapse in people with drug induced hearing loss (Santi et al. 1982). Initially, hair cell death causes rapid disintegration of supporting cell structures (Mellado Lagarde et al. 2014). Then, degeneration of neuronal connections occurs (Perez & Bao 2011; Kujawa & Liberman 2009) followed by cognitive changes in the brain (Lin et al. 2013).

Furthermore, high doses of aminoglycosides can kill supporting cells outright and damage the auditory nerve (Xie et al. 2011). In such cases the pool of remaining supporting cells may be insufficient for the induction of transdifferentiation and hair cell regeneration. Moreover, hair cell regeneration alone may not achieve improved functional hearing or balance.

Despite these limitations, exciting progress is being made toward hair cell regenerative therapies. Numerous steps must still be ascertained and then recapitulated in mammalian cells to ensure correct hair cell localisation and patterning. However, progress is evident in new protocols for guided cell differentiation and human hair cell organoid growth (Koehler et al. 2017; Mattei et al. 2019). In the meantime, preventing hair cell death is an important option. Crucially, hair cells that survive insult can be repaired *in situ*, which may allow an individual to retain intrinsic functional hair cells (Baird et al. 2000; Tang & Watson 2015; Rzadzinska et al. 2004).

1.3.3. Antioxidants for preventing hair cell death

In 1997, Su-Hua Sha and Jochen Schacht discussed the recent findings that ROS were produced in excess during aminoglycoside use and suggested that antioxidants could be otoprotective (Sha & Schacht 1997; Priuska & Schacht 1995; Sha & Schacht 1999b). Since then, researchers worldwide have intensively studied free radical scavengers for the prevention of ototoxicity. Seminal work by Sha et al. 1999 showed that the antioxidant salicylate could attenuate hearing loss in guinea pigs (Sha & Schacht 1999a). Subsequently, a human trial was initiated at the Xijing Hospital and Air Force Chengdu Hospital between 1999 and 2003 (Sha et al. 2006). A cohort of 195 patients receiving gentamicin was divided into a treatment group, which was given 3 g of aspirin per day, for 14 days and a control group, given a placebo. The aspirin did not alter gentamicin efficacy. However, gastric complaints were a significantly common adverse event, and three patients were removed from the trial due to gastric bleeding. Approximately 3% of the treatment group experienced moderate hearing loss, as opposed to 13% of the placebo group at 5-7 weeks post treatment. The trial did not assess vestibular outcomes, or long-term hearing. However, the trial outcomes supported the concept of antioxidant/aminoglycoside co-delivery as an otoprotective

strategy. A complementary trial performed at the Besat Hospital in Hamadan, Iran, achieved comparable results (Naeem et al. 2010).

Subsequently, numerous antioxidant therapies have been investigated, including, N-acetyl-cysteine (NAC), L-ascorbic acid, minocycline, vitamins E and C, resveratrol, and curcumin (Kopke et al. 2015; Wu et al. 2015; Tokgoz et al. 2012; Celebi et al. 2013; Noack et al. 2017; Fetoni et al. 2014; Fetoni et al. 2015). Many of these initial studies reported promising results, however reproducibility has been problematic, with conflicting results in the literature. For example, NAC has garnered much attention in the last decade as an otoprotective antioxidant, but a consensus on the compound's efficacy has not been reached. Kocyigit et al. 2015 used oral NAC in conjunction with amikacin and vancomycin and reported that NAC not only attenuated ototoxicity in humans, but may have a "*curative effect on impaired hearing functions*" (Kocyigit et al. 2015; Tokgoz et al. 2011). In contrast, Yoo et al. 2014. identified only two patients in a cohort of 11 had better hearing in an ear treated with transtympanic NAC whilst undergoing cisplatin therapy. In a rat model, intraperitoneal NAC co-treatment moderately reduced gentamycin ototoxicity but in the guinea pig, intraperitoneal NAC has been reported to *increase* kanamycin ototoxicity (Yoo et al. 2014; Somdas et al. 2015; Bock et al. 1983). Subsequently, a systematic review of NAC trials revealed a high degree of potential bias and rated the evidence of otoprotection as low (Kranzer et al. 2015).

After three decades of research, numerous studies have suggested that antioxidant therapy could reduce ototoxicity. However, none of the studies have translated to a change in medical care. Antioxidants are able to reduce ROS within the ear, which has a moderately protective effect against hair cell death (Jiang et al. 2005; Sha & Schacht 2000; McFadden et al. 2003; Ton & Parng 2005). However, there are still many unanswered questions regarding antioxidant use in a clinical setting. Experimental inconsistencies have been problematic, and antioxidants proven efficacious *in vitro* were unable to prevent ototoxicity *in vivo* (Oishi et al. 2014). The safety of antioxidants in conjunction with lifesaving treatments also remains questionable. Aspirin mediated gastric bleeding is one example, but a more significant outcome is the possibility that antioxidants can reduce chemotherapeutic efficacy (Salganik 2001; Berndtsson et al. 2007). Aspirin use is considered safe and is widely implemented in healthy individuals,

but the individuals requiring aminoglycoside antibiotics or platinum-based chemotherapy are in a compromised state of health. Direct delivery of antioxidants to the ear could overcome important issues such as drug interactions and gastric discomfort. However, intra-tympanic NAC delivery reportedly causes extreme acute pain in treated individuals, and can result in severe inflammation or ear infection (Riga et al. 2013).

An additional limitation of therapeutic strategies targeting ROS, is that normal ROS production is an integral part of cellular and physiological function. Therefore, antioxidant therapies can induce damaging reductive cell stress (Henkel et al. 2019; Villanueva & Kross 2012). Overall, the failure of antioxidant trials to produce a change in clinical practice has highlighted the need for additional basic research, aimed at understanding hair cell death at a cellular level, and to clarify the role of ROS as an apoptotic signal in this process.

1.3.4. Blocking cell death pathways

At the cellular level, a complex interplay of cell death pathways appears to contribute to ototoxicity. Both apoptotic and necrotic hair cells have been observed in the aminoglycoside treated organ of Corti, although sometimes lacking traditional cell death markers (Jiang et al. 2006; Lenoir et al. 1999; Vago et al. 1998; M.-J. Choi et al. 2019). Subsequent to aminoglycoside treatment, hair cells can exhibit highly variable cell death time frames, ranging from 90 minutes to 24 hours. Moreover, the interactions of pro-death and pro-survival molecules allow for alternate pathways to activate when others are inhibited (Owens et al. 2009; Coffin et al. 2013; Choi et al. 2013). Understanding hair cell death is further complicated by the apparent involvement of different cell death pathways in response to various ototoxic drugs. For example, BAX inhibition protects hair cells against neomycin, but not gentamycin induced hair cell death (Coffin et al. 2013). In contrast, elevated levels of BCL2 protects against gentamycin but not neomycin treatment (Coffin et al. 2013). Numerous alternative mediators of apoptosis such as caspases 3, 8 and 9, caspase independent mitochondrial enzyme Endonuclease G, c-Jun N-terminal kinase, tumour suppressor P53 and μ -calpain have been reported in various models of ototoxicity, suggesting that the pathways to hair cell death are variable, interchangeable and compensatory in

different situations (reviewed in Sedo-Cabezón et al. 2013). Therefore, delineating the predominant pathogenic pathways of hair cell death may be challenging. However, identification of key hair cell death mediators may provide novel drug targets and opportunities for preventing ototoxicity.

1.3.5. MAP kinase-induced caspase activation drives hair cell death.

Many cell death pathways have been implicated in ototoxic hair cell loss. However, it has been predicted that each pathway ultimately induces caspase activity (Cheng et al. 2005). Indeed, general caspase inhibition reduces aminoglycoside and cisplatin induced hair cell death *in vitro* (Matsui et al. 2002; Liu et al. 1998; Shimizu et al. 2003). Further investigation *in vitro* and *in vivo* has shown that caspases 3, and 9 are particularly important for hair cell specific execution (Matsui et al. 2003; Cunningham et al. 2002; Devarajan et al. 2002; Lee et al. 2004; Matsui et al. 2004). Notably, the initiator caspase 9 is upstream of the executioner caspase 3, however inhibition of either is otoprotective (Cunningham et al. 2002; Shimizu et al. 2003; Wang et al. 2004). Given the important role of caspase activity in hair cell death, recent studies have aimed to elucidate the upstream mechanisms driving caspase activation. As a result, there is now significant evidence that mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK), P38 mitogen-activated protein kinase (P38) and c-Jun N-terminal kinase (JNK) are activated in dying hair cells, inducing caspase activity. However, the reported role of each MAPK in hair cells treated with ototoxic drugs is quite variable (So et al. 2007; Pirvola et al. 2000; Sugahara et al. 2006).

1.3.6. JNK is an important mediator of aminoglycoside induced hair cell death

Immunostaining of murine and avian cochlear and vestibular explants has demonstrated significant hair cell JNK phosphorylation in response to neomycin, anisomycin, gentamycin or cisplatin treatment (Yang et al. 2016; Pirvola et al. 2000; Wang et al. 2003; Francis et al. 2013; Nicholas et al. 2017; Matsui et al. 2004; Sugahara

et al. 2006; Brancho et al. 2005). For rat cochlear explants, JNK phosphorylation has been observed as 2.5 times higher in gentamycin treated explants than control explants, when quantified by ELISA (Bas, Van de Water, et al. 2012). Likewise, elevated phospho-JNK has been observed in the nuclei of hair cells collected from gentamycin treated guinea pigs (Ylikoski et al. 2002). Furthermore, D-JNKI-1, a cell permeable peptide that blocks the JNK signalling pathway prevents neomycin induced cell death in cochlear explants. *In vivo*, local application of D-JNKI-1 into the scala tympani is able to prevent neomycin induced hair cell death and hearing loss in mice, as well as noise induced or electrode induced hair cell trauma and hearing loss (Wang et al. 2003; Eshraghi et al. 2007; J. Wang et al. 2007). Conversely, D-JNKI-1 intracochlear perfusion was reported to increase cisplatin toxicity in guinea pigs, suggesting that cisplatin and aminoglycosides induce divergent cell death pathways (Wang et al. 2004). Similar observations were made by So et al. 2007 and Lee et al. 2010, who found that unlike ERK inhibition, JNK or P38 inhibition was not protective against cisplatin induced cell death in the House Ear Institute-Organ of Corti 1 (HEI-OC1) (So et al. 2007; Lee et al. 2010). However, these findings were contradicted by Nichols et al. 2007, who reported that JNK inhibition was indeed protective against cisplatin ototoxicity in mouse cochlear explants (Nicholas et al. 2017).

1.3.7. ERK is an important survival molecule for hair cells.

The consensus within the literature is that ERK phosphorylation does not occur as part of a pro-apoptotic pathway in aminoglycoside treated hair cells. In particular, Bas et al. 2012 utilised a cell-based ERK1/2 ELISA to show that gentamycin treatment did not induce ERK phosphorylation in rat organ of Corti explants when compared to saline treated control explants (Bas, Van de Water, et al. 2012). Furthermore, RNA sequencing of hair cells that had been isolated from the mouse organ of Corti demonstrated that ERK expression was reduced in gentamycin treated hair cells (Tao & Segil 2015). Further to this, chemical inhibition of ERK with UO126 has been shown to induce outer hair cell death in un-treated cochlear explants, and also enhances gentamicin ototoxicity (Battaglia et al. 2003). Thus, the role of ERK in hair cell apoptosis appears to be that of a pro-survival molecule (Lahne & Gale 2008; Kurioka et al. 2015).

Notably, contradictory results have been reported in studies that utilise the House Ear Institute-Organ of Corti 1 (HEI-OC1) auditory cell line. For example, Kalinec et al. 2005, found that ERK phosphorylation was significant in gentamycin treated HEI-OC1, whereas JNK activation was not (Kalinec et al. 2005). Importantly, HEI-OC1 cells do not represent a specific auditory cell type, nor do they perform specialised mechano-electric transduction, which likely alters aminoglycoside drug uptake. Genetic drift may also contribute to these contradictory results, with reports of HEI-OC1 cells developing drug resistance and losing key hair cell characteristics such as prestin expression (Kalinec et al. 2016; Walters et al. 2015; Cederroth 2012). Therefore, the ERK observations in gentamycin and cisplatin treated HEI-OC1 cells that contradict those made in studies using inner ear explants or live animals suggest that HEI-OC1 specific cell death pathways are at play (Kalinec et al. 2005).

1.3.8. P38 activity contributes to hair cell death

P38 has also been implicated in hair cell death. For example, immunostaining and ELISA have demonstrated significant P38 phosphorylation in the hair cells of gentamycin treated rat cochlear explants (Wei et al. 2005; Bas, Van de Water, et al. 2012). Inhibition of P38 protects against gentamycin induced hair cell death in cochlear explants, however co-inhibition of P38 and caspase 3 achieves better hair cell survival rates than either treatment in isolation (Wei et al. 2005). In contrast, Sugahara et al. 2006, found P38 inhibitor SB203580 did not protect utricular hair cell explants from neomycin induced death (Sugahara et al. 2006).

1.3.9. ASK1 is an important mediator of ROS induced P38 and JNK activation.

Phosphorylated ASK1 is thought to be a key regulator of prolonged and pro-apoptotic P38 and JNK activation. A review of current literature that discusses the role of ASK1 in numerous disease models and the potential of ASK1 inhibition as a strategy for preventing P38 or JNK mediated cell death and disease is provided below. During the examination of this thesis, the manuscript underwent second round review in The Journal of Molecular Medicine. It has subsequently been published, as described in the thesis preface.

REVIEW



ASK1 inhibition: a therapeutic strategy with multi-system benefits

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Abstract

p38 mitogen-activated protein kinases (P38 α and β) and c-Jun N-terminal kinases (JNK1, 2, and 3) are key mediators of the cellular stress response. However, prolonged P38 and JNK signalling is associated with damaging inflammatory responses, reactive oxygen species-induced cell death, and fibrosis in multiple tissues, such as the kidney, liver, central nervous system, and cardiopulmonary systems. These responses are associated with many human diseases, including arthritis, dementia, and multiple organ dysfunctions. Attempts to prevent P38- and JNK-mediated disease using small molecule inhibitors of P38 or JNK have generally been unsuccessful. However, apoptosis signal-regulating kinase 1 (ASK1), an upstream regulator of P38 and JNK, has emerged as an alternative drug target for limiting P38- and JNK-mediated disease. Within this review, we compile the evidence that ASK1 mediates damaging cellular responses via prolonged P38 or JNK activation. We discuss the potential benefits of ASK1 inhibition as a therapeutic and summarise the studies that have tested the effects of ASK1 inhibition in cell and animal disease models, in addition to human clinical trials for a variety of disorders.

Keywords Apoptosis signal-regulating kinase 1 · MAP3K5 · Clinical trial · ROS · MAPK · p38 · JNK

Mitogen-activated protein kinase kinase kinase 5 (MAP3K5), commonly known as apoptosis signal-regulating kinase 1 (ASK1), has emerged as a target for preventing p38 mitogen-activated protein kinase (MAPK14, 11, and 12/P38 α , β , and γ) and c-Jun N-terminal kinase (MAPK8, 9, and 10/JNK1, 2, and 3)-mediated cell death and disease. Both P38 and JNK are associated with reactive oxygen species (ROS)-induced disease¹, and numerous studies have demonstrated that P38 and JNK inhibition ameliorates cell

death [3–7]. However, complete inhibition of P38 or JNK in vivo is problematic, given that these ubiquitously expressed proteins are also critical for cell survival and homeostatic and/or metabolic functions [8–11]. This is highlighted by the embryonic lethality of homozygous *P38 α* and *Jnk1/2* knockout mice [12, 13]. In addition, homozygous *P38 β* knockout mice exhibit defective skeletal development [14] and homozygous *Jnk1* knockout mice spontaneously develop intestinal tumours [15]. Negative outcomes have also been reported due to partial *P38* or *Jnk* expression, with heterozygous *P38 α* knockout mice developing progressive renal dysfunction [16] and heterozygous *Jnk1* knockout mice exhibiting altered weight gain, hyperinsulinaemia, insulin resistance, inflammatory cytokine disruption, and reduced viability [17]. Serious side effects have also been observed when pharmacological inhibition of P38 or JNK is pursued in vivo [14, 18, 19]. For example, pamapimod, a P38 (α and β) inhibitor, did not significantly reduce joint swelling or improve mobility in individuals with rheumatoid arthritis in a phase II clinical trial. However, 35% of the participants receiving daily pamapimod (300 mg) experienced infection, 20% developed a skin rash, 15% became dizzy, and 13% had elevated hepatic enzymes indicative of liver damage [20].

The pro-survival or pro-death outcomes of P38/JNK activity are largely dependent on the duration of activation. Short-term activation is protective, inducing cellular repair

¹ In mammals, four isoforms of P38 have been identified (α , β , γ , and δ) and three isoforms of JNK (1, 2, and 3) (reviewed in [1, 2] respectively). However, the literature does not always specify which specific isoform(s) has been analysed. Throughout this review, specific isoforms are named if they are reported in the primary literature. If isoform-specific information is not available, we have used the general nomenclature P38 or JNK.

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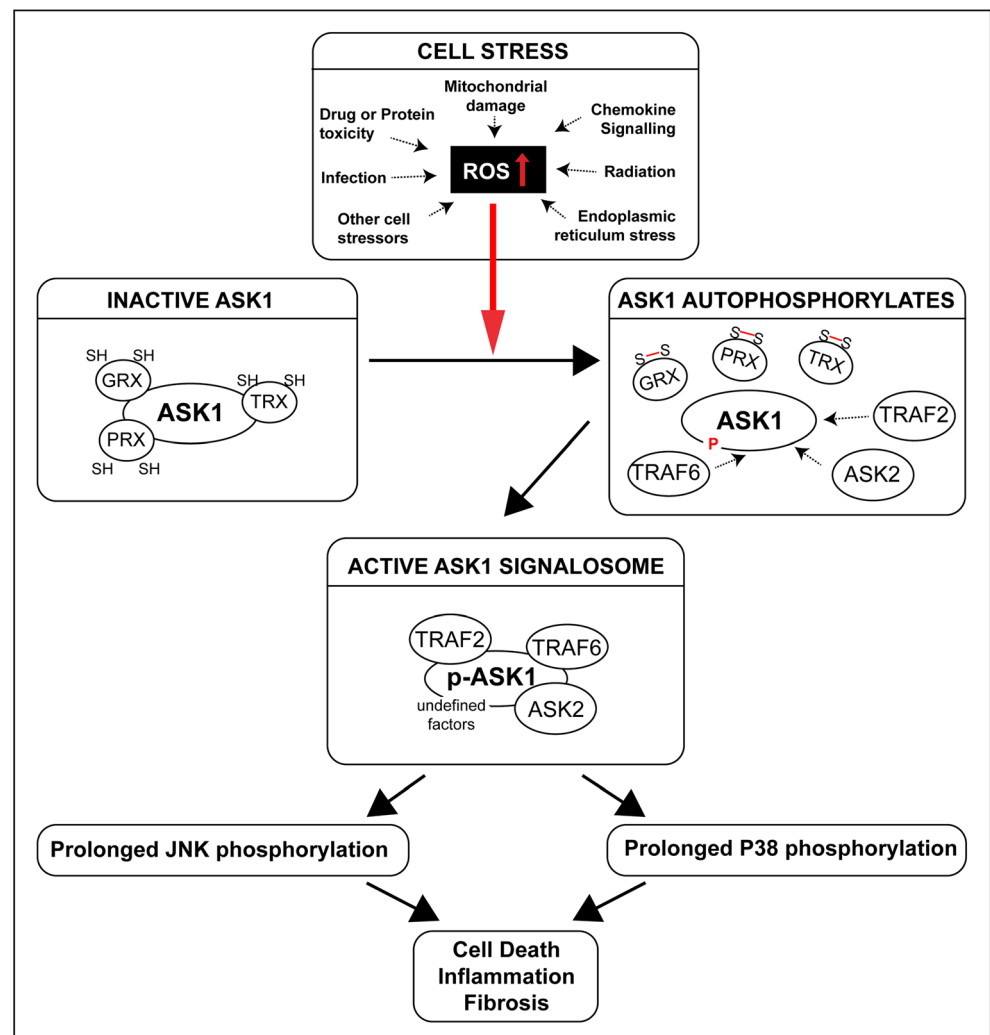
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mechanisms, whereas sustained P38/JNK phosphorylation initiates apoptotic and necrotic cell death cascades [21–26]. Therefore, an alternative therapeutic approach that targets a common, upstream molecule that is only activated by cell stress and capable of regulating both the P38 and JNK pathways is desirable. As an upstream converging point of cell stress signalling, ASK1 meets these criteria. Similar to P38 and JNK, ASK1 is ubiquitously expressed [27]. However, unlike P38 and JNK, ASK1 is primarily activated in response to cell stress (reviewed in Shiizaki et al. [28]). In particular, ASK1 activation is tightly controlled by redox signalling, due to the nature of its dithiol oxidoreductase binding partners, thioredoxin (TRX), glutaredoxin (GRX), and peroxiredoxin 1 (PRX1) [29–31] (Fig. 1). TRX, GRX, and PRX1 have redox active sites consisting of two cysteine residues that act as molecular switches [32]. When the cell is redox neutral, TRX, GRX, and PRX1 remain in a reduced state, bound to, and inactivating ASK1. TRX and PRX1 bind at the N-terminal domain of ASK1, and GRX at the C-terminus [30, 33–35]. Bound ASK1 is a substrate for ubiquitination and

degradation [36]. Alternatively, cellular oxidative imbalance induces modifications of the cysteine sulphur atom of TRX, GRX, and PRX1. As a result, disulphide bonds form between the cysteine residues, inactivating TRX, PRX1, and GRX [37, 38]. Inactivated TRX, PRX1, and GRX dissociate from ASK1. Unbound ASK1 is then activated by auto-phosphorylation and a large multicomponent complex forms, referred to as the ASK1 signalosome [39]. This complex and associated ASK1 activity initiates the P38 and JNK signalling cascades [27, 40]. Importantly, ASK1 promotes the sustained and pro-apoptotic activation of P38 and JNK, without impacting short-term P38/JNK activity [41–43]. Therefore, ASK1 inhibition is unlikely to affect the pro-survival, homeostatic activities of P38 and JNK. This hypothesis is supported by the viability of *Ask1* knockout mice, which are healthy and long-lived, and show no developmental abnormalities [42, 44–48].

The function of ASK1 as an upstream regulator of P38/JNK-mediated disease has received considerable attention in recent literature. ASK1 is widely expressed in many tissues, including the kidney, liver, brain, heart, and lung [49]. Whilst ASK1 is

Fig. 1 In the redox neutral cell, dithiol oxidoreductases TRX, GRX, and PRX bind to and inactivate ASK1. However, cell stressors can induce cellular oxidative imbalance, which causes disulphide bonds to form between the cysteine residues of TRX, GRX, or PRX. As a result, TRX, PRX, and GRX dissociate from ASK1. Unbound ASK1 is then activated by auto-phosphorylation and a large multicomponent complex forms, referred to as the ASK1 signalosome. The ASK1 signalosome promotes the sustained activation of the P38 and JNK signalling cascades which have been associated with damaging inflammatory responses, cell death, and fibrosis in multiple tissues



primarily activated in response to oxidative stress, other factors such as calcium overload, endoplasmic reticulum stress, infection, and receptor-mediated inflammatory signals, including lipopolysaccharide (LPS) and tumour necrosis factor (TNF) can also induce ASK1 signalling (reviewed in Shiizaki [28]). As a result, the role of ASK1 has been extensively investigated in disease models, with therapeutic potential observed in diseases of the kidney [50], liver [51], central nervous system [52, 53], joints [54], and cardiovascular [47, 55, 56]. Subsequently, ASK1 inhibitors have been tested as a therapeutic in various disease settings, ranging from *in vitro* cellular studies to *in vivo*

animal models and large clinical trials (Table 1). Herein, we discuss the potential of ASK1 inhibition for preventing disease, combining evidence from *Ask1* knockout models, and research utilising ASK1 inhibitory compounds.

Kidney disease: ASK1 modulation limits renal inflammation, apoptosis, and fibrosis

Several models of kidney injury indicate that ASK1 activation drives renal damage via the ROS-P38 and JNK signalling

Table 1 A number of ASK1 inhibitors have been developed. The information regarding each is varied. Some have not been extensively studied, whilst others have been used to treat cell lines, small animals, and humans. *NA* indicates information was not available

Inhibitor	Supplier	Notable observations	IC50	Formula	MW	Ref
BPyO-34	Otava Chemicals	NA	520 nM	C ₃₂ H ₂₉ FN ₂ O ₅ S	572.65 g/mol	[57]
GS-444217	Gilead Sciences	ATP competitive inhibitor. Reduced pathological remodelling of the pulmonary vasculature and the right ventricle. Halts progression of pulmonary hypertension in rats. Reduces renal injury in multiple rodent models	2.9 nM	C ₂₃ H ₂₁ N ₇ O	411.46 g/mol	[58–61]
GS-459679	Gilead Sciences	ATP competitive inhibitor. Protects against paracetamol-induced liver injury in mice. Reduces myocardial infarct size and cardiomyocyte apoptosis after acute myocardial ischemia/reperfusion in rats	6.1 nM	NA	NA	[43, 62, 63]
GS-4997 (selonsertib)	Gilead Sciences	ATP competitive inhibitor. Antagonises multidrug resistance in ABCB1- and ABCG2-overexpressing cancer cells. Reduces the progression of liver damage in rodents Phase II clinical trials against diabetes and kidney disease	3.2 nM	C ₂₄ H ₂₄ FN ₇ O	445.502 g/mol	[64–69]
GS-627	Gilead Sciences	Phase III clinical trials for NASH Decreased joint damage and inflammation in a rat model of collagen-induced arthritis	4.3 nM	NA	NA	[70]
K811	Kyowa Hakko Kirin Co. Ltd	Nitrogen-containing heterocyclic derivative with high IC50 values for other kinases. Slows disease progression and enhances survival in a mouse model of amyotrophic lateral sclerosis. Limits proliferation of gastric cancer cells and reduces xenograft tumour size in mice	6 nM	C ₂₉ H ₂₆ N ₄ O ₅	510.19 g/mol	[71, 72]
K812	Kyowa Hakko Kirin Co. Ltd	Slows disease progression and enhances survival in a mouse model of amyotrophic lateral sclerosis. Note: lower specificity than K811	6nM	C ₃₀ H ₁₃ F ₃ N ₆ O	509.56 g/mol	[71, 72]
MSC2032964A	Tocris Biosciences	Suppressed autoimmune inflammation in both the spinal cord and optic nerves of a mouse model of autoimmune encephalomyelitis	93 nM	C ₁₆ H ₁₃ F ₃ N ₆ O	362.31 g/mol	[52]
SRT-015	Seal Rock Therapeutics	Targeted-liver specific ASK1 inhibitor reduces hepatomegaly, fibrosis, and steatosis of the liver	NA	NA	NA	In development
TC ASK 10	Tocris Biosciences	Prevents aberrant smooth muscle growth in patients with chronic obstructive pulmonary disease	14 nM	C ₂₁ H ₂₁ N ₅ O ₂ HCl	432.35 g/mol	[73, 74]

cascades [48, 50, 58]. Moreover, studies in animal models of acute kidney injury have shown that *Ask1* knockout or inhibition protects against tubular apoptosis, inflammation, and fibrosis [48, 50]. For example, kidney function, as measured by blood urea nitrogen and serum creatinine levels, is superior in *Ask1*^{−/−} mice following renal ischaemia or unilateral ureteric obstruction when compared to wild-type mice [48, 50]. Lower leukocyte and macrophage infiltration rates also indicate that inflammation is reduced in the damaged kidneys of *Ask1* knockout mice [48, 50]. In rat models of kidney injury induced by auranofin, nephrotoxic serum injection, or unilateral ureteral obstruction, pre-treatment with the ASK1 inhibitor GS-444217 (30 mg/kg via oral gavage) suppresses the ROS-ASK1-P38/JNK response [59, 60]. As a result, kidney function is preserved, due to reduced tubular cell death, inflammation, and fibrosis. Similarly, GS-444217 slows the progression of renal disease in mice with streptozotocin-induced diabetic kidney disease [58]. Streptozotocin treatment damages pancreatic beta cells, causing insulin-dependent diabetes and development of diabetic kidney disease [75]. However, early intervention with GS-444217 reduces renal dysfunction and scarring. Late intervention (up to 15 weeks post diabetes onset) improves renal function and halts the progression of glomerular scarring, tubular injury, and renal inflammation [58]. These observations in the late intervention group suggest that a large treatment window exists for diabetic kidney disease [58]. This study also highlights the tolerability and bioavailability of GS-444217 when delivered in standard mouse chow (0.1% w/w), achieving plasma levels of approximately 20 μmol/L.

The benefits of GS-444217 treatment are even more pronounced in the db/db *eNOS*^{−/−} mouse model of progressive diabetic kidney disease. Endothelial nitric oxide synthase (*eNOS*) dysfunction is associated with diabetic nephropathy in humans and *eNOS*^{−/−} mice have an enhanced susceptibility to kidney damage [76]. When crossed with genetically obese leptin receptor-deficient (db/db) mice, the resultant db/db *eNOS*^{−/−} progeny robustly model type II diabetic nephropathy. However, daily GS-444217 treatment reduces glomerular scarring, podocyte death, and renal fibrosis, preventing glomerular filtration changes and albuminuria in db/db *eNOS*^{−/−} mice [59].

Overall, multiple murine models with differing mechanisms of injury have provided evidence that pharmacological inhibition of ASK1 is able to preserve renal function via decreased cell death, inflammation, and fibrosis. These compelling results have subsequently led to a clinical trial testing ASK1 inhibition in humans with diabetic kidney disease (NCT02177786). In 2016, 333 participants took selonsertib (GS-4997) or a placebo, orally, once per day for 48 weeks as part of a phase II clinical trial [64]. The primary outcome measure was a change in glomerular filtration rate (eGFR) from baseline at week 48. This endpoint was not achieved,

due to unexpected confounding factors, such as selonsertib-mediated inhibition of creatinine secretion. However, post hoc analysis indicated that the 18 mg selonsertib treatment group had a 71% eGFR reduction and a dose-dependent reduction of p-P38 was noted in all selonsertib treatment groups. This outcome highlights the difficult nature and apparent pitfalls of clinical trial design. However, the results do suggest that selonsertib has a protective effect that warrants further clinical investigation. Therefore, a phase III clinical trial is now underway, to evaluate whether selonsertib can slow the decline of kidney function, reduce the risk of kidney failure, or reduce the risk of death due to kidney disease in participants with diabetic kidney disease (NCT04026165). Notably, these outcome measures are broader than previous selonsertib trials, which will allow for the identification of improvements that may have otherwise been missed using narrower parameters (such as eGFR) to measure kidney function.

Liver disease: ASK1 modulation prevents hepatocyte death, inflammation, and fibrosis in the liver

Oxidative stress is a primary pathogenic cause of liver disease. For example, excessive ROS production has been associated with alcoholic liver disease, non-alcoholic fatty liver disease, hepatic fibrosis, hepatitis C-induced damage, and poor surgical recovery (reviewed in Lach and Mikalak [77]). Recently, reports of paracetamol-induced, ROS-mediated liver injury have increased significantly, with drug toxicity now a leading cause of acute liver failure [78, 79]. At low doses, paracetamol metabolism generates the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is detoxified via glutathione (GSH) conjugation. However, high paracetamol doses cause NAPQI production at a rate exceeding the liver's detoxification capabilities. NAPQI then forms mitochondrial DNA and protein adducts, inducing oxidative stress [80]. This redox signal causes ASK1-mediated, sustained JNK activation which leads to hepatocyte death [81, 82]. As such, ASK1 inhibition is proposed as a potential therapy for reducing paracetamol-induced liver injury [41].

In a proof of principle study, Nakagawa et al. [41] demonstrated that paracetamol-induced cellular damage in primary hepatocyte cultures was the result of ASK1-mediated JNK activation. Then, studies utilising *Ask1* knockout mice demonstrated that ASK1 deficiency was protective against paracetamol-induced liver toxicity in vivo. Notably, the protection conferred in *Ask1* knockout mice was greater than that observed in *Jnk1* and *Jnk2* knockout mice, suggesting that compensatory mechanisms may also be blocked by ASK1 inhibition [41]. Overall, three important observations were made regarding ASK1 activation in paracetamol-treated hepatocytes. First, transient P38 and JNK phosphorylation

occurred in *Ask1* knockout mice, yet prolonged phosphorylation (3–6 hours post treatment) was significantly reduced. This supports previous reports that ASK1 activation is required for the sustained, pro-apoptotic activation of JNK, whilst the pro-survival role of JNK is not impacted [41]. Secondly, long-term JNK activation was not completely inhibited in *Ask1* knockout mice, illustrating that ASK1-independent mechanisms of JNK activation exist [41]. However, the *partial* reduction of ASK1-mediated JNK1 or JNK2 activation achieved significant liver protection [41]. This suggests that incomplete ASK1 inhibition will be beneficial *in vivo*, even if functional limitations such as poor drug uptake in a target tissue prevent strong ASK1 inhibition.

The benefit of ASK1 inhibition was then demonstrated in wild-type C57BL/6 mice given hepatotoxic doses of paracetamol. ASK1 inhibition with GS-459679 reduced JNK phosphorylation and centrilobular necrosis [43]. As a result, mRNA levels of inflammatory factors (tumour necrosis factor (TNF- α), interleukin 6 (IL-6), and interleukin 6 1 beta (IL-1 β)) remained low and paracetamol-treated mice maintained alanine aminotransferase (ALT) serum levels—which is a strong indicator of liver function [43, 62]. Importantly, GS-459679 was protective when given 90 minutes *after* hepatotoxic paracetamol treatment [62]. This would suggest that a reasonable window of opportunity exists for liver protection in humans experiencing paracetamol overdose, particularly as paracetamol-induced liver damage occurs more slowly in humans than mice [62]. However, this hypothesis remains to be assessed clinically [62].

Beyond paracetamol-induced liver injury, ASK1 deficiency also reduces inflammation and hepatocyte death in a mouse model of obstructive cholestatic injury. In this model, surgical bile duct ligation causes inappropriate bile flow. The subsequent inflammatory response causes fibrosis and cirrhosis of the liver [83]. However, the inflammatory response and overall liver damage induced by bile duct ligation are much lower in *Ask1* knockout mice when compared to controls. For example, infiltrating neutrophil and macrophage activity is reduced in the damaged livers of *Ask1* knockout mice. Notably, *Ask1* knockouts exhibit superior survival when compared to controls receiving the same surgery (78% vs. 51% surviving 21 days post surgery). Overall, serum levels of ALT, aspartate aminotransferase (AST), bilirubin, and bile acid are significantly lower in *Ask1* knockout mice, which also suggests improved liver function when compared to controls [51]. These results indicate that ASK1 inhibition may prevent severe liver damage whilst diagnosis and treatment strategies are implemented in humans with cholestasis.

To date, ASK1 inhibition has predominantly been tested as a therapeutic strategy for limiting non-alcoholic steatohepatitis (NASH). NASH is the most severe form of non-alcoholic fatty liver disease (NAFLD) and the

predominant cause of chronic liver failure. NAFLD affects over 25% of adults worldwide and 30% of people with NAFLD will develop NASH [84]. Hepatic oxidative stress, lipotoxicity, and inflammation drive the progression of NAFLD to NASH, causing cirrhosis, fibrosis, or hepatic carcinoma [84]. Currently, there is no approved pharmacological intervention for NASH. Instead, treatment is focused on reducing body weight and increasing physical activity. However, there is a growing need for alternate interventions as the prevalence of obesity and liver disease is increasing [84].

Similar to humans, mice consuming a high-fat diet develop heavy and fibrotic livers, laden with accumulating collagen and triglyceride [85]. However, homozygous *Ask1* knockout mice fed a high-fat diet have healthy livers, comparable to wild-type normal-diet-fed mice [85]. In particular, liver weight and triglyceride content do not increase in *Ask1* knockout mice fed a high-fat diet [85]. ASK1 inhibition also prevents P38 phosphorylation and caspase-3 cleavage in an alternate mouse model of NASH, the nod-like receptor family pyrin domain containing 3 (*Nlrp3*) knock-in transgenic mouse [65]. *Nlrp3* KI mice develop hepatic inflammation and fibrosis due to aberrant NLRP3-mediated inflammation. However, ASK1 inhibition with selonsertib significantly reduces liver fibrosis in *Nlrp3* KI mice (as demonstrated by a 33.5% reduction in hydroxyproline levels when compared to untreated *Nlrp3* KI controls). Moreover, serum ALT levels of untreated *Nlrp3* KI mice are twice as high as the selonsertib-treated *Nlrp3* KI mice, indicating that liver function is retained when selonsertib is used [65]. Selonsertib has now been tested as a treatment for NASH and liver fibrosis in humans.

In 2016, a phase II clinical trial determined that selonsertib was safe and effective in NASH patients with stage 2 or 3 liver fibrosis. In this trial, participants received 24 weeks of open-label, oral selonsertib treatment (6 or 18 mg, daily). Outcomes were assessed by comparing pre- and post-treatment liver biopsies, collagen content, fat content, magnetic resonance elastography, and serum markers of liver injury. Most study participants had stage 3 fibrosis, hepatocellular ballooning, and diabetes mellitus at the beginning of the trial. However, at the trial's conclusion, the progression of liver disease was notably reduced in selonsertib-treated participants. After 24 weeks of treatment, 13 of 30 individuals in the 18 mg selonsertib group and 8 of 27 in the 6 mg selonsertib group had a one or more stage reduction in fibrosis. Reduced fibrosis was also associated with decreased liver stiffness, collagen content, and lobular inflammation [66, 67]. The observed reduction of fibrosis was particularly exciting, as fibrosis is a strong predictor of liver failure in NASH [67]. Overall, the trial provided evidence that selonsertib treatment is safe and efficacious for individuals with NASH [67]. Selonsertib subsequently progressed to a larger phase

III clinical trial for NASH-induced compensated cirrhosis (NCT03053063). This trial was designed to last 240 weeks. However, the trial was discontinued after 48 weeks. The reasoning for this discontinuation is unknown, as the trial results are not yet available. Two other clinical trials utilising selonsertib for NASH treatment are still in progress; a phase II trial for NASH-induced bridging fibrosis (NCT03053050), and a phase II trial combining selonsertib, cilofexor (GS-9674, a non-steroidal FXR agonist), and firsocostat (GS-0976, an ACC inhibitor) for NASH-induced fibrosis (NCT03449446).

Brain disorders: ASK1 deficiency limits neurodegeneration

Neurodegenerative disorders are a significant and growing health and economic burden. For example, 50 million people globally are living with dementia and this number is expected to increase to 131 million by 2050, due to the ageing population [86, 87]. Of all the neurodegenerative disorders, Alzheimer's disease is the most prevalent, accounting for approximately 70% of dementia cases. The primary pathological features of Alzheimer's are the presence of plaques and neurofibrillary tangles in the brain, composed predominantly of beta-amyloid and phosphorylated Tau, respectively. Beta-amyloid impairs mitochondrial function, resulting in excessive ROS production (reviewed in Reddy and Beal [88] and Spuch et al. [89]). An *in vitro* study of primary neuronal cells derived from embryonic mice suggested that this altered redox state mediates cell death via ASK1-JNK activation [53]. Furthermore, a comparison of neuronal cultures derived from wild-type and homozygote *Ask1* knockout mice indicated that ASK1 deficiency affords remarkable protection against beta-amyloid-induced cell death. After three days of exogenous beta-amyloid treatment, only 20% of the wild-type neurons remained viable compared with 70% of the *Ask1*^{-/-} neurons [53]. In this model, ASK1-JNK activation is the primary effector of neuronal cell death. However, 30% of *Ask1*^{-/-} cells still died, showing that there are ASK1-independent pathways for neuronal cell death [53]. *In vivo* studies utilising the 5XFAD mouse model of Alzheimer's disease suggest that P38 activation also mediates beta-amyloid-induced neurodegeneration. 5XFAD transgenic mice express a mutant form of the amyloid precursor protein (APP) containing five familial Alzheimer's mutations. These mutations have an additive effect, driving beta-amyloid overproduction, and the transgene's *Thy1* promoter ensures brain-specific expression [90]. As a result, 5XFAD mice develop beta-amyloid plaques, which cause progressive neuronal cell death. By four months of age, 5XFAD mice have significant memory deficits [90, 91]. However, this deficit is reduced in homozygous *Ask1* knockout 5XFAD mice, as measured by

the passive avoidance test. P38 phosphorylation is strikingly lower in the 5XFAD/*Ask1*^{-/-} mouse cerebrum, when compared to 5XFAD/*Ask1*^{+/+} controls. Interestingly, JNK phosphorylation did not change, which contrasts with the JNK activation observed in cultured, beta-amyloid-treated neurons. However, it is likely that the alternate activation of P38 and JNK is due to different *in vitro/in vivo* cell culture conditions. Alternatively, it may indicate that neurodegeneration is driven by more than one apoptotic pathway. Nevertheless, ASK1 deficiency reduced both P38- and JNK-mediated forms of neurodegeneration, which highlights the strength of ASK1 as a common upstream target.

Parkinson's disease is another common neurodegenerative disease characterised by oxidative stress and activation of the ASK1-P38 and JNK pathways. *In vitro*, Hu et al. [92] observed significant and persistent ASK1 phosphorylation in dopaminergic neurons (MN9D cell line) treated with the neurotoxic compound oxidopamine. However, targeted ASK1 knockdown, using lentiviral-delivered short hairpin RNA, significantly enhances oxidopamine-treated MN9D survival. *In vivo*, the same lentiviral shRNA against *Ask1* is protective against oxidopamine toxicity. ShRNA was injected into the mouse substantia nigra pars compacta, reducing ASK1 levels by ~80% in tyrosine hydroxylase-positive dopaminergic neurons (as measured by western blot analysis). Three weeks after *Ask1* knockdown, 3 µg of oxidopamine was injected into the left striatum. Seven days after oxidopamine treatment, immunoblotting showed that phosphorylation of both P38 and JNK was significantly reduced in the ASK1-deficient pars compacta. Moreover, histology indicated that twice as many dopaminergic neurons survived in the knockdown group. Behavioural testing, including the corner test and apomorphine-induced rotation test, showed that motor function in the oxidopamine-treated *Ask1* knockdown mice was significantly better when compared to oxidopamine-treated wild-type mice [92]. The benefits afforded by incomplete *Ask1* knockdown (80%) in oxidopamine-treated mice indicate that partial ASK1 inhibition may represent a powerful therapeutic tool, even if complete inhibition is difficult to achieve in a target tissue.

As ASK1-mediated activation of P38 and JNK had been consistently observed in neurons after oxidopamine treatment, in both the MN9D cell line and mice, Hu et al. then examined whether this pathway was active in post-mortem tissue derived from individuals with Parkinson's disease. Immunofluorescence analysis demonstrated significantly more p-ASK, p-JNK, and p-P38 staining in the Parkinson's affected tissue compared to matched control samples [92]. Collectively, the observations of p-ASK1, p-P38, and p-JNK in Parkinson's model systems and human tissue provide strong evidence that ASK1 is a relevant therapeutic target for Parkinsonian disorders.

Inflammatory disease: ASK1 modulation limits damaging immune responses

Recent studies have associated ASK1-P38 and JNK with the damaging inflammatory responses underlying immune-mediated disease, such as multiple sclerosis, amyotrophic lateral sclerosis, and arthritis [52, 54, 70, 71, 93].

Neuroinflammation

Multiple sclerosis (MS) is a disease characterised by immune-mediated destruction of the myelin sheath within the central nervous system. In contrast, amyotrophic lateral sclerosis (ALS) is caused by degeneration of the motor neurons within the brain and spinal cord. The aetiopathogenesis of ALS and MS are thought to be quite different; however, the progression of both diseases is characterised by a strong immune response, which may include microglia and astrocyte activation and monocyte or T cell immune responses (reviewed in Zhao et al. [94]).

In a mouse model of MS, where myelin oligodendrocyte glycoprotein (MOG) injections are used to induce T cell-mediated autoimmune encephalomyelitis, ASK1 deficiency affords significant protection against several disease-associated phenotypes. For example, MOG-treated wild-type mice develop significant visual impairment, whereas similarly treated *Ask1* knockout mice do not [52]. Optic nerve histology shows significantly reduced infiltrating inflammatory cells and axonal degeneration in *Ask1*^{−/−} samples [52]. Spinal cord histopathology also indicates that CNS inflammation and glial activation is drastically reduced in MOG-treated *Ask1*^{−/−} mice. In vitro experiments using LPS-treated astrocytes and microglia suggest that the underlying mechanism of MOG treatment is toll-like receptor 4 and 9 (TLR4 and TLR9)–induced P38 signalling. The TLR-P38 pathway also enhanced pro-inflammatory TNF α and nitric oxide synthase activity, which drives demyelination. Both TLR4 and TLR9 are associated with ROS production, providing a logical mechanism for ASK1-mediated P38 activation. The proposed TLR-ROS-ASK1-P38-chemokine pathway to demyelination requires further investigation in vivo. However, chemokine production, inflammation, and demyelination are reduced in MOG-treated *Ask1*^{−/−} mice, supporting the mechanism proposed. Overall, it seems likely that the reduced axonal degeneration and demyelination observed in *Ask1*^{−/−} mice with MOG-mediated autoimmune encephalomyelitis is the result of reduced P38-mediated inflammation [52]. Importantly, ASK1 inhibition (MSC2032964A) achieves similar attenuation of demyelination within the same mouse model and also blocks lipopolysaccharide-binding protein–induced ASK1-P38 activation in cultured mouse astrocytes, providing evidence that ASK1 inhibition may be able to slow the progression of MS [52].

ASK1 deficiency also modulates disease progression in the SOD1 transgenic mouse model of ALS. SOD1 mice carry the familial Cu/Zn-superoxide dismutase (SOD1) mutation, associated with ALS. In this model, aggregates of mutant SOD1 accumulate in the mitochondria causing ROS production, ASK1 phosphorylation, and neuronal cell death mediated by P38 but not JNK [95–97]. *Ask1* knockout does not alter the age of disease onset or prevent ALS lethality in the SOD1 mouse model. However, ASK1 deficiency does mitigate motor neuron death as the disease progresses. As a result, *Ask1*^{−/−} SOD1 mice survive, on average, one month longer than *Ask1*^{+/+} SOD1 mice [98]. ASK1 inhibitors K811 and K812 also reduce ALS pathology in vitro and in vivo. For example, primary spinal cord cultures derived from wild-type mice were infected with lentivirus expressing either a wild-type or mutant form of SOD1. The number of surviving motor neurons in the spinal cord cultures expressing mutant SOD1 was significantly lower than those expressing wild-type SOD1. However, treatment with K811 or K812 completely inhibited mutant SOD1-induced motor neuron death. Similarly, K811 or K812 treatment reduced glial activation and prevented motor neuron death in the SOD1 mouse model. This improved the life expectancy of SOD1 mice by three weeks [71]. The extended lifespan of SOD1 mice treated with ASK1 inhibitors is of particular significance, as riluzole and edaravone, the FDA-approved ALS treatments, do not alter the lifespan of SOD1 transgenic mice [99, 100]. If ASK1 inhibition is able to achieve a similar reduction of motor neuron death in ALS-affected individuals as observed in the ALS mouse model, then the therapeutic effect of ASK1 inhibition would be significantly greater than that of either riluzole or edaravone. However, ASK1 inhibition has not yet been investigated in human tissues derived from individuals with ALS.

Joint inflammation, arthritis, and bone repair

Rheumatoid arthritis is an autoimmune disease characterised by chronic inflammation, leading to bone and cartilage destruction within the joints. Upregulated P38 activity is a key contributor to this inflammatory response and a number of P38 inhibitors have been tested as potential treatments [6, 54, 101]. However, P38 inhibitors have failed to achieve significant disease attenuation in vivo, possibly because P38 has both pro- and anti-inflammatory effects [11, 24]. Alternatively, low doses may have been used to avoid the liver toxicity frequently described when P38 inhibitors are used in vivo, and such doses may be insufficient to achieve meaningful disease attenuation.

Interestingly, experiments utilising synoviocytes derived from individuals with rheumatoid arthritis indicate that JNK activation occurs concurrently with P38 activation and that both must be inhibited to effectively limit inflammatory responses [70]. Therefore, Mninch et al. [54] characterised a

mouse model of rheumatoid arthritis to assess the role of ASK1 as an upstream regulator of P38/JNK-mediated inflammation. In this model, serum from arthritic transgenic K/BxN mice is transferred to naive mice. The host's inflammatory cells, including neutrophils and mast cells, activate in response to the transferred antibodies. This results in elevated levels of inflammatory cytokines, causing significant injury and arthritis within days [102, 103]. However, serum levels of inflammatory cytokines are reduced in treated *Ask1* knockout mice compared to C57BL/6 controls. As a result, *Ask1* knockouts experience significantly less swelling of the joint and reduced cartilage destruction and bone damage when compared to K/BxN-injected wild-type mice [54]. ASK1 inhibition has also been tested in the collagen-induced arthritis rat model [70]. Like the K/BxN serum transfer model, immunisation with type II collagen induces a significant inflammatory response in the host animal, causing arthritis symptoms to manifest within three weeks [104]. However, treatment with the ASK1 inhibitor GS-627 significantly reduces joint damage and swelling in collagen-treated rats [70]. Combined, these two murine models of arthritis suggest that ASK1 inhibition can mitigate the damaging inflammatory response underpinning rheumatoid arthritis.

Whilst rheumatoid arthritis is a systemic inflammatory disease, osteoarthritis tends to manifest as age- or injury-related cartilage destruction. When the joint is initially injured, ROS, cytokines, and growth factors are released into the joint space. As a result, chondrocytes become hypertrophic and proliferative, and the extracellular matrix is degraded. The slow degradation of cartilage increases mechanical stress within the joint, eventually leading to bone remodelling and complete cartilage destruction. Oxidative stress is strongly associated with chondrocyte hypertrophy, mediated by ASK1-P38 and JNK [105, 106]. However, ASK1 inhibition using exogenous thioredoxin blocks the TNFR1–ASK1–P38/JNK signalling pathway in cultured synovial fibroblasts derived from human osteoarthritis knee tissue [93, 106, 107]. In vivo, chondrocyte hypertrophy is significantly reduced in untreated two-year-old *Ask1*^{−/−} mice, when compared to age-matched *Ask1*^{+/+} mice [93]. As a result, *Ask1*^{−/−} mice experience significantly less age-related cartilage degeneration, proteoglycan loss, and calcification of the joints, compared to wild-type controls [93]. Similarly, two surgically induced mouse models of osteoarthritis—one severe (partial meniscectomy) and one mild (joint destabilisation/injury)—suggest that ASK1 deficiency prevents cartilage damage and enhances bone repair [93]. In the severe osteoarthritis model, wild-type mouse joints exhibit extensive cartilage degradation, proteoglycan loss, fibrosis, and inflammatory infiltrates at four weeks post surgery. However, both heterozygous and homozygous *Ask1* knockout mice have significantly reduced bone thinning, cartilage degradation, and fibrosis [93]. Similar results were achieved in the milder joint destabilisation model, with

Ask1^{−/−} mice showing reduced cartilage hypertrophy and proteoglycan loss eight weeks after surgery, when compared to controls.

Human cartilage collected from individuals with both osteoarthritis and rheumatoid arthritis demonstrate significant ASK1 phosphorylation [70, 93]. This suggests that the benefits of ASK1 deficiency in murine models of arthritis may also be relevant to human disease. Collectively, the research performed to date suggests that ASK1 inhibition is a potential therapeutic strategy to reduce joint degradation in rheumatoid and osteoarthritis. Inflammation and chondrocyte activity are also important for bone formation and repair. Therefore, the observation that ASK1 induces P38/JNK-mediated chondrocyte hypertrophy and death also suggests that ASK1 inhibition may be useful for enhancing fracture repair [93, 107]. Indeed, bone mineralisation and formation is accelerated in *Ask1*^{−/−} mice [107]. However, further studies are required to ascertain the viability of ASK1 inhibition for enhancing human bone repair.

Cardiopulmonary disease: ASK1 mediates heart and lung phenotypes associated with invasive smooth muscle cells

Smooth muscle cells have an important role for maintaining airway structure and function. However, aberrant smooth muscle activity can cause chronic obstructive pulmonary disease (COPD). In particular, smooth muscle remodelling can impede airway contractility and relaxation, causing thick masses that obstruct airflow (reviewed in Yan et al. [108]). Smooth muscle cells in individuals with COPD show remarkably high p-ASK1 protein levels [73]. Therefore, ASK1 inhibition may represent a therapeutic intervention to limit COPD airway remodelling. In vitro studies have shown that ASK1 inhibition, using the compound TC ASK 10 or siRNA *Ask1* knockdown, is able to prevent smooth muscle growth and migration by inhibiting P38 and JNK1 and 2 signalling [73, 74]. The benefits of ASK1 inhibition have not yet been investigated in COPD animal models. Instead, animal studies have focused on the pathological role of ASK1-mediated smooth muscle cell activity during cardiovascular remodelling.

Smooth muscle cells maintain the structural integrity and dynamic properties of blood vessels [109]. However, smooth muscle cells can also cause vascular thickening, known as neointimal hyperplasia after a surgical vascular injury [55]. Similarly, smooth muscle remodelling after myocardial injury can damage the ventricle, causing catastrophic cardiac dysfunction [47]. In both cases, p-ASK1-JNK-mediated apoptosis plays an essential role in the remodelling process and subsequent dysfunction. However, ASK1 inhibition limits pathological vascular remodelling

in animal models. For example, when a balloon catheter is inserted into an artery in rats, the intimal endothelial lining is mechanically removed, causing a distending mural injury [110]. Within two minutes of injury, ASK1 phosphorylation occurs, with maximal levels observed five minutes after injury. Notably, ASK1 phosphorylation is elevated seven-fold compared to uninjured controls [55]. ASK1-mediated P38 and JNK activation subsequently promotes vascular smooth muscle cell migration and proliferation. As a result, rats with vascular injury develop neointimal hyperplasia within two weeks [55]. However, pre-treatment infusion of adenovirus vectors expressing a dominant-negative ASK1 mutant (DN-ASK1) in the target artery reduces smooth muscle infiltration and prevents vascular thickening after injury [55]. This outcome has been replicated in mice, with neointimal formation significantly reduced in *Ask1*^{-/-} mice subsequent to an experimental vascular injury induced by cuff placement around the femoral artery [55]. In other mouse models of myocardial infarction or pressure overload-induced cardiac injury, post-injury remodelling impairs cardiac contractility, and changes the ventricular dimensions of wild-type mice [47]. However, *Ask1*^{-/-} mice retain cardiac contractility and exhibit reduced fibrosis compared to wild-type controls [47]. ASK1 inhibition (GS-444217) also prevents pathological vasculature remodelling in two independent rodent models of pulmonary arterial hypertension. In the first model, a single injection of the plant toxin monocrotaline is used to induce pulmonary vascular remodelling. Four weeks after the injection, a peak increase in pulmonary pressure and right ventricle hypertrophy are observed in wild-type rats. However, pulmonary pressure and right ventricle hypertrophy are significantly reduced in mice treated with GS-444217 (0.2% in chow). Importantly, both early (day 7) or late (day 14) interventions are able to significantly improve heart function. Likewise, GS-444217 prevents right ventricle remodelling and pulmonary hypertension in rats injected with the cardiotoxic tyrosine-kinase inhibitor semaxanib and subjected to four weeks of hypoxia [61].

In humans, ASK1 and P38 α have also been associated with the pathophysiology of pulmonary arterial hypertension by enhancing the proliferation of fibroblasts and smooth muscle cells [7, 61]. Combined, these findings indicate that ASK1 inhibition may be able to limit pathogenic cardiac or vascular smooth muscle cell remodelling and slow the onset of heart failure. However, a phase II clinical trial, testing selonsertib as a therapeutic for pulmonary arterial hypertension did not achieve its primary endpoint, which was a reduction in baseline pulmonary vascular resistance, as measured by right heart catheterisation [68]. The 24-week randomised trial (NCT02234141) compared three daily doses (2 mg, 6 mg, or 18 mg) of selonsertib to placebo in 150 individuals with pulmonary arterial hypertension. There was a correlation of

reduced pulmonary vascular resistance in the higher dose groups (6 mg and 18 mg); however, this was confounded by an increase from baseline vascular resistance in the low-dose group. No treatment groups showed a statistically significant change; however, this may have been due to the large standard deviation observed within each group. For most secondary measures, there was no change from baseline in people who received selonsertib. However, the number of participants that had an improved WHO functional class (a system for defining the severity of an individual's symptoms and how they impact on day-to-day activities) was notably higher in all selonsertib groups (14–19%) when compared to the placebo group (3%).

Discussion

The stress-activated MAP kinases P38 and JNK have been associated with apoptosis, inflammation, and fibrosis in multiple disease states affecting the kidney, liver, brain cardiovascular systems, and joints. In particular, ROS-mediated, prolonged activation of P38 or JNK is thought to induce damaging cellular responses. In the past, numerous attempts have been made to prevent ROS-mediated disease through the use of antioxidants, small molecule inhibitors, or RNA interference. However, the therapies directed at ROS, P38, or JNK have been disappointing, with a number of limitations being observed. For example, dysregulated ROS production is cytotoxic. However, ROS signalling remains an integral part of normal cellular and physiological function. It is therefore important to target redox stress, without affecting off-target ROS activity. Likewise, P38 and JNK phosphorylation is important for both pro-survival and pro-death signalling, largely dependent on the duration of activity. Therefore, inappropriate inhibition of transient P38/JNK can have critical implications for cellular homeostasis in otherwise normal functioning cells.

In some disease states, P38 and JNK appear to be interchangeably activated, emphasising the potential limitations associated with direct inhibition of either molecule in isolation, whereas ASK1 inhibition has repeatedly been shown to selectively regulate prolonged, apoptotic P38 and JNK activation, but not pro-survival mechanisms [47, 48, 50]. Similarly, ASK1 inhibition is a promising alternative to antioxidant supplementation, as it can reduce the pathological consequences of oxidative stress, without impacting physiological ROS activity.

Many ASK1 inhibitors possessing good potency and selectivity profiles have now been developed (Table 1). These inhibitors are primarily available as small molecules with excellent oral bioavailability and are capable of achieving stable plasma concentrations, with a long half-life and widespread distribution across tissues, including the brain [52, 59, 62, 73]. A number of clinical trials have shown that ASK1 inhibition is

well tolerated in humans [64, 66, 68]. There is, however, one major point of concern that is relevant to any therapeutic target within an apoptotic pathway—the potential for aberrant cell survival. For example, reduced ASK1 expression has been associated with aggressive hepatic tumours [111, 112]. Alternatively, enhanced ASK1 expression has a role in the carcinogenesis of human gastric cancer [113]. ASK1 inhibition slows the growth of gastric cancer xenografts in mice and *Ask1*^{−/−} mice have fewer and smaller tumours than wild-type controls in the *N*-methyl-*N*-nitrosourea chemically induced gastric tumorigenesis model [72, 113]. Moreover, platelet-specific deletion of ASK1 prevents tumour metastasis in mice that have established lung cancer, induced by the injection of either melanoma or Lewis lung carcinoma cells [69, 114]. Thus, it is apparent that ASK1 can function as both a tumour suppressor or tumour promoter, depending on the cellular responses induced by ASK1 modulation. It is also worth noting that *Ask1*^{−/−} mice are healthy and long-lived, suggesting that pharmacologically mediated ASK1 inhibition is unlikely to have serious side effects [42, 44–48]. In humans, the ASK1 inhibitor selonsertib has been administered for 12 months in clinical trials, with no increase in malignancies or other significant detrimental effects reported [64]. It seems likely that compensatory cell death mechanisms can prevent neoplastic growth during ASK1 inhibition; however, the potential of carcinogenesis must be considered. It is conceivable however, that individuals with tumours susceptible to ASK1 inhibition could benefit from an adjuvant ASK1 inhibitory therapy that not only enhances the anti-neoplastic effects of chemotherapy, but also affords protection against the frequently observed toxic effects that platinum-based compounds have in the brain, kidney, and ear.

Overall, the evidence available suggests that pro-carcinogenic outcomes resulting from reduced ASK1 activation are unlikely to represent an immediate concern. However, some potential for enhanced bleeding, altered heat production, and oxygen consumption has been observed in *Ask1*^{−/−} mice [115, 116]. These observations suggest that ASK1 inhibition may impact post-operative care and may not be appropriate for individuals with metabolic disease. However, another noteworthy observation is that ASK1 deficiency attenuates lung injury in ventilated mice [117]. Ventilated *Ask1*^{−/−} mice also maintain a more stable heart rate and better oxygen saturation than wild-type controls [117]. Therefore, ASK1 inhibition may provide benefits at the acute and post-ventilation stages of treatment, preventing acute respiratory distress syndrome [117]. Further investigation should determine if similar outcomes are observed in humans. However, it is clear that the potential benefits of ASK1 inhibition will be achieved in a tightly controlled, disease-specific context.

As discussed in this review, ASK1 inhibition has not met defined endpoints in some clinical trials. It is not yet clear

what caused these outcomes; however, it may be that ASK1 inhibition had a positive effect that was not captured due to trial methodology. The species effect is another factor, and it is possible that a *Homo sapiens*-tailored approach is required to reap the full benefits of ASK1 inhibition. For this to occur, the publication of full clinical trial results is required. Alternatively, compensatory mechanisms may be at play. For example, numerous studies have shown that ASK1 inhibition significantly reduces unwanted cell death, but in most cases P38 or JNK activity was not completely blocked [50, 53]. At least 14 MKKKs exist that activate the JNK pathway [118], so it is unlikely that ASK1 inhibition is able to completely prevent cell death. Additional MAPKK-independent cell death signals may also be driving apoptosis. Notably, the fundamental interaction of p-ASK1 with specific P38 and JNK isoforms has been limited by a lack of tools for discerning between highly similar proteins. Further investigation will be required to address this issue. Nevertheless, the benefits of ASK1 inhibition or deficiency in pre-clinical disease models are clear. ASK1 does not have to be completely inhibited to have a strong effect and ASK1 inhibition has successfully attenuated disease in models with established tissue damage [58, 62, 92]. Such favourable outcomes suggest that an achievable ‘treatment window’ exists and that ASK1 modulation may benefit human health.

Summary

ASK1 is a widely expressed and evolutionarily conserved mediator of cell death, fibrosis, and inflammation [49]. The reported beneficial effects of ASK1 deficiency are widespread, indicating that ASK1 inhibition may be a particularly useful therapy in multi-system disorders, such as diabetes-induced tissue damage. Indeed, ASK1 inhibition is beneficial in models of diabetes or high-fat diet-related damage of the cardiovascular system [85, 119], liver [85], kidney [58], and brain [45]. Furthermore, ASK1 inhibition or deficiency has repeatedly limited pathologic activation of P38 or JNK in a disease-specific manner [46–48, 50, 55, 59].

Thus far, the argument for ASK1 inhibition against ROS-P38/JNK-mediated disease is convincing. However, additional disease-specific research is required to fully elucidate the potential benefits of ASK1 inhibition. Within this review, we have illustrated the benefits afforded by ASK1 deficiency in numerous and varied disease models. Many examples now suggest that ASK1 inhibition can limit damaging cell death, inflammation, and fibrosis of the kidney, liver, central nervous system, heart, and lungs. Furthermore, ASK1 inhibition may slow the progression of inflammatory diseases such as multiple sclerosis, arthritis, and amyotrophic lateral sclerosis. Overall, significant protective effects have been observed in numerous tissue types. ASK1 inhibition has great potential

and may ultimately be used to achieve lasting, improved human health.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

- Escós A, Risco A, Alsina-Beauchamp D, Cuenda A (2016) p38 γ and p38 δ mitogen activated protein kinases (MAPKs), new stars in the MAPK galaxy. *Front Cell Dev Biol* 4:4555
- Bogoyevitch MA (2006) The isoform-specific functions of the c-Jun N-terminal kinases (JNKs): differences revealed by gene targeting. *Bioessays*. 28(9):923–934
- Bodmer D, Brors D, Bodmer M, Ryan AF (2002) Rescue of auditory hair cells from ototoxicity by CEP-11004, an inhibitor of the JNK signaling pathway. *Laryngo-rhino-otologie*. 81(12):853–856
- Eshraghi AA, Wang J, Adil E, He J, Zine A, Bublik M, Bonny C, Puel JL, Balkany TJ, van de Water T (2007) Blocking c-Jun-N-terminal kinase signaling can prevent hearing loss induced by both electrode insertion trauma and neomycin ototoxicity. *Hear Res* 226(1–2):168–177
- Wang J, Van de Water TR, Bonny C, de Ribaupierre F, Puel JL, Zine A (2003) A peptide inhibitor of c-Jun N-terminal kinase protects against both aminoglycoside and acoustic trauma-induced auditory hair cell death and hearing loss. *J Neurosci* 23(24):8596–8607
- Kanashiro A, Franchin M, Bassi GS, Reis Santana DA, Cunha TM, Cunha FQ, Ulloa L, Rodrigues GJ (2018) Inhibition of spinal p38 MAPK prevents articular neutrophil infiltration in experimental arthritis via sympathetic activation. *Fundam Clin Pharmacol* 32(2):155–162
- Church AC, Wadsworth R, Bryson G, Welsh DJ, Peacock AJ (2012) p38 MAPK: an important pathway in the pathobiology of pulmonary hypertension and pulmonary vascular remodelling. *Thorax*. 67(Suppl 2):A19
- Lamb JA, Ventura J-J, Hess P, Flavell RA, Davis RJ (2003) JunD mediates survival signaling by the JNK signal transduction pathway. *Mol Cell* 11(6):1479–1489
- Sancho R, Nateri AS, de Vinuesa AG, Aguilera C, Nye E, Spencer-Dene B, Behrens A (2009) JNK signalling modulates intestinal homeostasis and tumorigenesis in mice. *EMBO J* 28(13):1843–1854
- Hull-Thompson J, Muffat J, Sanchez D, Walker DW, Benzer S, Ganfornina MD, Jasper H (2009) Control of metabolic homeostasis by stress signaling is mediated by the lipocalin NLaz. *PLoS Genet* 5(4):e1000460
- Lee J, Sun C, Zhou Y, Lee J, Gokalp D, Herrema H, Park SW, Davis RJ, Ozcan U (2011) p38 MAPK-mediated regulation of Xbp1s is crucial for glucose homeostasis. *Nat Med* 17(10):1251–1260
- Mudgett JS, Ding J, Guh-Siesel L, Chartrain NA, Yang L, Gopal S et al (2000) Essential role for p38 α mitogen-activated protein kinase in placental angiogenesis. *Proc Natl Acad Sci* 97(19):10454–10459
- Kuan CY, Yang DD, Samanta Roy DR, Davis RJ, Rakic P, Flavell RA (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron*. 22(4):667–676
- Greenblatt MB, Shim J-H, Zou W, Sitara D, Schweitzer M, Hu D et al (2010) The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice. *J Clin Invest* 120(7):2457–2473
- Tong C, Yin Z, Song Z, Dockendorff A, Huang C, Mariadason J et al (2007) c-Jun NH2-terminal kinase 1 plays a critical role in intestinal homeostasis and tumor suppression. *Am J Pathol* 171(1):297–303
- Maruyama M, Yagasaki Y, Sudo T, Osada H (2003) Renal abnormalities in mice caused by insufficiency of p38 α . *J Recept Signal Transduct Res* 23(2–3):173–183
- Tuncman G, Hirosumi J, Solinas G, Chang L, Karin M, Hotamisligil GS (2006) Functional in vivo interactions between JNK1 and JNK2 isoforms in obesity and insulin resistance. *Proc Natl Acad Sci* 103(28):10741–10746
- Lim AKH, Ma FY, Nikolic-Paterson DJ, Ozols E, Young MJ, Bennett BL, Friedman GC, Tesch GH (2011) Evaluation of JNK blockade as an early intervention treatment for type 1 diabetic nephropathy in hypertensive rats. *Am J Nephrol* 34(4):337–346
- Ijaz A, Tejada T, Catanuto P, Xia X, Elliot SJ, Lenz O et al (2009) Inhibition of c-Jun N-terminal kinase improves insulin sensitivity but worsens albuminuria in experimental diabetes. *Kidney Int* 75(4):381–388
- Cohen SB, Cheng T-T, Chindalore V, Damjanov N, Burgos-Vargas R, Delora P, Zimany K, Travers H, Caulfield JP (2009) Evaluation of the efficacy and safety of pamapimod, a p38 MAP kinase inhibitor, in a double-blind, methotrexate-controlled study of patients with active rheumatoid arthritis. *Arthritis Rheum* 60(2):335–344
- Bradham CA, Stachlewitz RF, Gao W, Qian T, Jayadev S, Jenkins G et al (1997) Reperfusion after liver transplantation in rats differentially activates the mitogen-activated protein kinases. *Hepatology*. 25(5):1128–1135
- Chang L, Kamata H, Solinas G, Luo J-L, Maeda S, Venuprasad K et al (2006) The E3 ubiquitin ligase itch couples JNK activation to TNF α -induced cell death by inducing c-FLIP(L) turnover. *Cell*. 124(3):601–613
- Ventura J-J, Hubner A, Zhang C, Flavell RA, Shokat KM, Davis RJ (2006) Chemical genetic analysis of the time course of signal transduction by JNK. *Mol Cell* 21(5):701–710

24. Tomida T, Takekawa M, Saito H (2015) Oscillation of p38 activity controls efficient pro-inflammatory gene expression. *Nat Commun* 6:8350
25. Liu H, Lo CR, Czaja MJ (2002) NF-kappaB inhibition sensitizes hepatocytes to TNF-induced apoptosis through a sustained activation of JNK and c-Jun. *Hepatology*. 35(4):772–778
26. Shinohara M, Ybanez MD, Win S, Than TA, Jain S, Gaarde WA et al (2010) Silencing glycogen synthase kinase-3beta inhibits acetaminophen hepatotoxicity and attenuates JNK activation and loss of glutamate cysteine ligase and myeloid cell leukemia sequence 1. *J Biol Chem* 285(11):8244–8255
27. Ichijo H, Nishida E, Irie K, Dijke ten P, Saitoh M, Moriguchi T et al (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science*. 275(5296):90–94
28. Shiizaki S, Naguro I, Ichijo H (2013) Activation mechanisms of ASK1 in response to various stresses and its significance in intracellular signaling. *Adv Biol Regul* 53(1):135–144
29. Jarvis RM, Hughes SM, Ledgerwood EC (2012) Peroxiredoxin 1 functions as a signal peroxidase to receive, transduce, and transmit peroxide signals in mammalian cells. *Free Radic Biol Med* 53(7):1522–1530
30. Nadeau PJ, Charette SJ, Landry J (2009) REDOX reaction at ASK1-Cys250 is essential for activation of JNK and induction of apoptosis. *Mol Biol Cell* 20(16):3628–3637
31. Kekulandara DN, Nagi S, Seo H, Chow CS, Ahn Y-H (2018) Redox-inactive peptide disrupting Trx1-Ask1 interaction for selective activation of stress signaling. *Biochemistry*. 57(5):772–780
32. Fra A, Yoboue ED, Sitia R (2017) Cysteines as redox molecular switches and targets of disease. *Front Mol Neurosci* 10:167–167
33. Kim SY, Kim TJ, Lee K-Y (2008) A novel function of peroxiredoxin 1 (Prx-1) in apoptosis signal-regulating kinase 1 (ASK1)-mediated signaling pathway. *FEBS Lett* 582(13):1913–1918
34. Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, Ichijo H (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* 17(9):2596–2606
35. Song JJ, Lee YJ (2003) Differential role of glutaredoxin and thioredoxin in metabolic oxidative stress-induced activation of apoptosis signal-regulating kinase 1. *Biochem J* 373(Pt 3):845–853
36. Liu Y, Min W (2002) Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. *Circ Res* 90(12):1259–1266
37. Mullen L, Hanschmann E-M, Lillig CH, Herzenberg LA, Ghezzi P (2015) Cysteine oxidation targets peroxiredoxins 1 and 2 for exosomal release through a novel mechanism of redox-dependent secretion. *Mol Med* 21(1):98–108
38. Kylarova S, Kosek D, Petrvalska O, Psenakova K, Man P, Vecer J, Herman P, Obsilova V, Obsil T (2016) Cysteine residues mediate high-affinity binding of thioredoxin to ASK1. *FEBS J* 283(20):3821–3838
39. Tobiume K, Saitoh M, Ichijo H (2002) Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of pre-formed oligomer. *J Cell Physiol* 191(1):95–104
40. Kim H, Oh Y, Kim K, Jeong S, Chon S, Kim D, Jung MH, Pak YK, Ha J, Kang I, Choe W (2015) Cyclophilin A regulates JNK/p38-MAPK signaling through its physical interaction with ASK1. *Biochem Biophys Res Commun* 464(1):112–117
41. Nakagawa H, Maeda S, Hikiba Y, Ohmae T, Shibata W, Yanai A, Sakamoto K, Ogura K, Noguchi T, Karin M, Ichijo H, Omata M (2008) Deletion of apoptosis signal-regulating kinase 1 attenuates acetaminophen-induced liver injury by inhibiting c-Jun N-terminal kinase activation. *Gastroenterology*. 135(4):1311–1321
42. Tobiume K, Matsuzawa A, Takahashi T, Nishitoh H, Morita K-I, Takeda K et al (2001) ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep* 2(3):222–228
43. He P, Zeng B, Zhang X-L, Fang D-L, Zhou X-Q, Wan K-Q, Tian WG (2016) Protective effect of apoptosis signal-regulating kinase 1 inhibitor against mice liver injury. *Asian Pac J Trop Med* 9(3):283–287
44. Izumiya Y, Kim S, Izumi Y, Yoshida K, Yoshiyama M, Matsuzawa A, Ichijo H, Iwao H (2003) Apoptosis signal-regulating kinase 1 plays a pivotal role in angiotensin II-induced cardiac hypertrophy and remodeling. *Circ Res* 93(9):874–883
45. Toyama K, Koibuchi N, Hasegawa Y, Uekawa K, Yasuda O, Sueta D et al (2015) ASK1 is involved in cognitive impairment caused by long-term high-fat diet feeding in mice. *Sci Rep* 5:10844
46. Hasegawa Y, Toyama K, Uekawa K, Ichijo H, Kim-Mitsuyama S (2018) Role of ASK1/p38 cascade in a mouse model of Alzheimer's disease and brain aging. *J Alzheimers Dis* 61(1):259–263
47. Yamaguchi O, Higuchi Y, Hirotsu S, Kashiwase K, Nakayama H, Hikoso S et al (2003) Targeted deletion of apoptosis signal-regulating kinase 1 attenuates left ventricular remodeling. *Proc Natl Acad Sci* 100(26):15883–15888
48. Terada Y, Inoshita S, Kuwana H, Kobayashi T, Okado T, Ichijo H, Sasaki S (2007) Important role of apoptosis signal-regulating kinase 1 in ischemic acute kidney injury. *Biochem Biophys Res Commun* 364(4):1043–1049
49. Tobiume K, Inage T, Takeda K, Enomoto S, Miyazono K, Ichijo H (1997) Molecular cloning and characterization of the mouse apoptosis signal-regulating kinase 1. *Biochem Biophys Res Commun* 239(3):905–910
50. Ma FY, Tesch GH, Nikolic-Paterson DJ (2014) ASK1/p38 signaling in renal tubular epithelial cells promotes renal fibrosis in the mouse obstructed kidney. *Am J Physiol Renal Physiol* 307(11):F1263–F1273
51. Noguchi H, Yamada S, Nabeshima A, Guo X, Tanimoto A, Wang K-Y, Kitada S, Tasaki T, Takama T, Shimajiri S, Horlad H, Komohara Y, Izumi H, Kohno K, Ichijo H, Sasaguri Y (2014) Depletion of apoptosis signal-regulating kinase 1 prevents bile duct ligation-induced necroinflammation and subsequent peribiliary fibrosis. *Am J Pathol* 184(3):644–661
52. Guo X, Harada C, Namekata K, Matsuzawa A, Camps M, Ji H, Swinnen D, Jorand-Lebrun C, Muzerelle M, Vitte PA, Ruckle T, Kimura A, Kohyama K, Matsumoto Y, Ichijo H, Harada T (2010) Regulation of the severity of neuroinflammation and demyelination by TLR-ASK1-p38 pathway. *EMBO Mol Med* 2(12):504–515
53. Kadowaki H, Nishitoh H, Urano F, Sadamitsu C, Matsuzawa A, Takeda K, Masutani H, Yodoi J, Urano Y, Nagano T, Ichijo H (2005) Amyloid beta induces neuronal cell death through ROS-mediated ASK1 activation. *Cell Death Differ* 12(1):19–24
54. Mnich SJ, Blanner PM, Hu LG, Shaffer AF, Happa FA, O'Neil S, Ukairo O, Weiss D, Welsh E, Storer C, Mbalaviele G, Ichijo H, Monahan JB, Hardy MM, Eda H (2010) Critical role for apoptosis signal-regulating kinase 1 in the development of inflammatory K/BxN serum-induced arthritis. *Int Immunopharmacol* 10(10):1170–1176
55. Izumi Y, Kim S, Yoshiyama M, Izumiya Y, Yoshida K, Matsuzawa A, Koyama H, Nishizawa Y, Ichijo H, Yoshikawa J, Iwao H (2003) Activation of apoptosis signal-regulating kinase 1 in injured artery and its critical role in neointimal hyperplasia. *Circulation*. 108(22):2812–2818
56. Toyama K, Koibuchi N, Uekawa K, Hasegawa Y, Kataoka K, Katayama T et al (2014) Apoptosis signal-regulating kinase 1 is a novel target molecule for cognitive impairment induced by chronic cerebral hypoperfusion. *Arterioscler Thromb Vasc Biol* 34(3):616–625

57. Starosyla SA, Volynets GP, Lukashov SS, Gorbatiuk OB, Golub AG, Bdzhola VG et al (2015) Identification of apoptosis signal-regulating kinase 1 (ASK1) inhibitors among the derivatives of benzothiazol-2-yl-3-hydroxy-5-phenyl-1,5-dihydro-pyrrol-2-one. *Bioorg Med Chem* 23(10):2489–2497
58. Tesch GH, Ma FY, Han Y, Liles JT, Breckenridge DG, Nikolic-Paterson DJ (2015) ASK1 inhibitor halts progression of diabetic nephropathy in Nos3-deficient mice. *Diabetes*. 64(11):3903–3913
59. Liles JT, Corkey BK, Notte GT, Budas GR, Lansdon EB, Hinojosa-Kirschenbaum F, Badal SS, Lee M, Schultz BE, Wise S, Pendum S, Graupe M, Castonguay L, Koch KA, Wong MH, Papalia GA, French DM, Sullivan T, Huntzicker EG, Ma FY, Nikolic-Paterson DJ, Altuhami T, Yang H, Fogo AB, Breckenridge DG (2018) ASK1 contributes to fibrosis and dysfunction in models of kidney disease. *J Clin Invest* 128(10):4485–4500
60. Amos LA, Ma FY, Tesch GH, Liles JT, Breckenridge DG, Nikolic-Paterson DJ, Han Y (2018) ASK1 inhibitor treatment suppresses p38/JNK signalling with reduced kidney inflammation and fibrosis in rat crescentic glomerulonephritis. *J Cell Mol Med* 22(9):4522–4533
61. Budas GR, Boehm M, Kojonazarov B, Viswanathan G, Tian X, Veeroju S et al (2018) ASK1 inhibition halts disease progression in preclinical models of pulmonary arterial hypertension. *Am J Respir Crit Care Med* 197(3):373–385
62. Xie Y, Ramachandran A, Breckenridge DG, Liles JT, Lebofsky M, Farhood A, Jaeschke H (2015) Inhibitor of apoptosis signal-regulating kinase 1 protects against acetaminophen-induced liver injury. *Toxicol Appl Pharmacol* 286(1):1–9
63. Gerczuk PZ, Breckenridge DG, Liles JT, Budas GR, Shryock JC, Belardinelli L et al (2012) An apoptosis signal-regulating kinase 1 inhibitor reduces cardiomyocyte apoptosis and infarct size in a rat ischemia-reperfusion model. *J Cardiovasc Pharmacol* 60(3):276–282
64. Chertow GM, Pergola PE, Chen F, Kirby BJ, Sundry JS, Patel UD (2019) Effects of selonsertib in patients with diabetic kidney disease. *J Am Soc Nephrol* 30(10):1980–1990
65. Schuster S, McGeough MD, Johnson CD, Zagorska A, Budas G, Hoffman HM et al (2017) Apoptosis signal-regulating kinase 1 (ASK1) inhibition reduces liver fibrosis and apoptosis in a NLRP3 mutant model of NASH. *J Hepatol* 66(1):S608–S609
66. Younossi ZM, Stepanova M, Lawitz E, Charlton M, Loomba R, Myers RP et al (2018) Improvement of hepatic fibrosis and patient-reported outcomes in non-alcoholic steatohepatitis treated with selonsertib. *Liver Int* 38(10):1849–1859
67. Loomba R, Lawitz E, Mantry PS, Jayakumar S, Caldwell SH, Arnold H, Diehl AM, Djedjos CS, Han L, Myers RP, Subramanian GM, McHutchison J, Goodman ZD, Afdhal NH, Charlton MR, GS-US-384-1497 Investigators (2018) The ASK1 inhibitor selonsertib in patients with nonalcoholic steatohepatitis: a randomized, phase 2 trial. *Hepatology*. 67(2):549–559
68. Rosenkranz S, Feldman J, McLaughlin V, Rischard F, White J, Ebrahimi R et al (2017) The ARROW study: a phase 2, prospective, randomized, double-blind, placebo-controlled study of selonsertib in subjects with pulmonary arterial hypertension. *Eur Respir J* 50(suppl 61):OA1983
69. Ji N, Yang Y, Cai C-Y, Lei Z-N, Wang J-Q, Gupta P et al (2019) Selonsertib (GS-4997), an ASK1 inhibitor, antagonizes multidrug resistance in ABCB1- and ABCG2-overexpressing cancer cells. *Cancer Lett* 440–441:82–93
70. Nygaard G, Di Paolo JA, Hammaker D, Boyle DL, Budas G, Notte GT et al (2018) Regulation and function of apoptosis signal-regulating kinase 1 in rheumatoid arthritis. *Biochem Pharmacol* 151:282–290
71. Fujisawa T, Takahashi M, Tsukamoto Y, Yamaguchi N, Nakoji M, Endo M, Kodaira H, Hayashi Y, Nishitoh H, Naguro I, Homma K, Ichijo H (2016) The ASK1-specific inhibitors K811 and K812 prolong survival in a mouse model of amyotrophic lateral sclerosis. *Hum Mol Genet* 25(2):245–253
72. Hayakawa Y, Hirata Y, Sakitani K, Nakagawa H, Nakata W, Kinoshita H, Takahashi R, Takeda K, Ichijo H, Maeda S, Koike K (2012) Apoptosis signal-regulating kinase-1 inhibitor as a potent therapeutic drug for the treatment of gastric cancer. *Cancer Sci* 103(12):2181–2185
73. Eapen MS, Kota A, Vindin H, McAlinden KD, Xenaki D, Oliver BG, Deshpande DA, Sohal SS, Sharma P (2018) Apoptosis signal-regulating kinase 1 inhibition attenuates human airway smooth muscle growth and migration in chronic obstructive pulmonary disease. *Clin Sci* 132(14):1615–1627
74. Terao Y, Suzuki H, Yoshikawa M, Yashiro H, Takekawa S, Fujitani Y et al (2012) Design and biological evaluation of imidazo[1,2-a]pyridines as novel and potent ASK1 inhibitors. *Bioorg Med Chem Lett* 22(24):7326–7329
75. Lenzen S (2008) The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia*. 51(2):216–226
76. Nakayama T, Sato W, Kosugi T, Zhang L, Campbell-Thompson M, Yoshimura A, Croker BP, Johnson RJ, Nakagawa T (2009) Endothelial injury due to eNOS deficiency accelerates the progression of chronic renal disease in the mouse. *Am J Physiol Renal Physiol*. 296(2):F317–F327
77. Cichoż-Lach H, Michalak A (2014) Oxidative stress as a crucial factor in liver diseases. *World J Gastroenterol* 20(25):8082–8091
78. Ostapowicz G, Fontana RJ, Schiodt FV, Larson A, Davern TJ, Han SHB et al (2002) Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Ann Intern Med* 137(12):947–954
79. Larson AM, Polson J, Fontana RJ, Davern TJ, Lalani E, Hynan LS et al (2005) Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology*. 42(6):1364–1372
80. Davern TJ, James LP, Hinson JA, Polson J, Larson AM, Fontana RJ et al (2006) Measurement of serum acetaminophen-protein adducts in patients with acute liver failure. *Gastroenterology* 130(3):687–694
81. McGill MR, Sharpe MR, Williams CD, Taha M, Curry SC, Jaeschke H (2012) The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. *J Clin Invest* 122(4):1574–1583
82. Hanawa N, Shinohara M, Saberi B, Gaarde WA, Han D, Kaplowitz N (2008) Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. *J Biol Chem* 283(20):13565–13577
83. Tag CG, Sauer-Lehnen S, Weiskirchen S, Borkham-Kamphorst E, Tolba RH, Tacke F et al (2015) Bile duct ligation in mice: induction of inflammatory liver injury and fibrosis by obstructive cholestasis. *J Vis Exp* 96
84. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M (2016) Global epidemiology of nonalcoholic fatty liver disease—meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*. 64(1):73–84
85. Yamamoto E, Dong Y-F, Kataoka K, Yamashita T, Tokutomi Y, Matsuba S, Ichijo H, Ogawa H, Kim-Mitsuyama S (2008) Olmesartan prevents cardiovascular injury and hepatic steatosis in obesity and diabetes, accompanied by apoptosis signal-regulating kinase-1 inhibition. *Hypertension*. 52(3):573–580
86. (2019) Global, regional, and national burden of Alzheimer's disease and other dementias, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol* 18(1): 88–106
87. World Alzheimer Report 2016: improving healthcare for people living with dementia. [Internet]. Alzheimer's Disease International; 2016 [cited 2019 May 17]. Available from: www.alz.co.uk/research/WorldAlzheimerReport2016.pdf

88. Reddy PH, Beal MF (2008) Amyloid beta, mitochondrial dysfunction and synaptic damage: implications for cognitive decline in aging and Alzheimer's disease. *Trends Mol Med* 14(2):45–53
89. Spuch C, Ortolano S, Navarro C (2012) New insights in the amyloid-beta interaction with mitochondria. *J Aging Res* 2012: 324968–324968
90. Eimer WA, Vassar R (2013) Neuron loss in the 5XFAD mouse model of Alzheimer's disease correlates with intraneuronal Abeta42 accumulation and Caspase-3 activation. *Mol Neurodegener* 8:2
91. Jawhar S, Trawicka A, Jenneckens C, Bayer TA, Wirths O (2012) Motor deficits, neuron loss, and reduced anxiety coinciding with axonal degeneration and intraneuronal Abeta aggregation in the 5XFAD mouse model of Alzheimer's disease. *Neurobiol Aging* 33(1):196.e29–196.e40
92. Hu X, Weng Z, Chu CT, Zhang L, Cao G, Gao Y et al (2011) Peroxiredoxin-2 protects against 6-hydroxydopamine-induced dopaminergic neurodegeneration via attenuation of the apoptosis signal-regulating kinase (ASK1) signaling cascade. *J Neurosci* 31(1):247–261
93. Zhang Q-S, Eaton GJ, Diallo C, Freeman TA (2016) Stress-induced activation of apoptosis signal-regulating kinase 1 promotes osteoarthritis. *J Cell Physiol* 231(4):944–953
94. Zhao W, Beers DR, Appel SH (2013) Immune-mediated mechanisms in the pathogenesis of amyotrophic lateral sclerosis. *J NeuroImmune Pharmacol* 8(4):888–899
95. Veglianesi P, Coco Lo D, Bao Cutrona M, Magnoni R, Pennacchini D, Pozzi B et al (2006) Activation of the p38MAPK cascade is associated with upregulation of TNF alpha receptors in the spinal motor neurons of mouse models of familial ALS. *Mol Cell Neurosci* 31(2):218–231
96. Holasek SS, Wengenack TM, Kandimalla KK, Montano C, Gregor DM, Curran GL, Poduslo JF (2005) Activation of the stress-activated MAP kinase, p38, but not JNK in cortical motor neurons during early presymptomatic stages of amyotrophic lateral sclerosis in transgenic mice. *Brain Res* 1045(1–2):185–198
97. Wengenack TM, Holasek SS, Montano CM, Gregor D, Curran GL, Poduslo JF (2004) Activation of programmed cell death markers in ventral horn motor neurons during early presymptomatic stages of amyotrophic lateral sclerosis in a transgenic mouse model. *Brain Res* 1027(1–2):73–86
98. Nishitoh H, Kadowaki H, Nagai A, Maruyama T, Yokota T, Fukutomi H, Noguchi T, Matsuzawa A, Takeda K, Ichijo H (2008) ALS-linked mutant SOD1 induces ER stress- and ASK1-dependent motor neuron death by targeting Derlin-1. *Genes Dev* 22(11):1451–1464
99. Hogg MC, Halang L, Woods I, Coughlan KS, Prehn JHM (2018) Riluzole does not improve lifespan or motor function in three ALS mouse models. *Amyotroph Lateral Scler Frontotemporal Degener* 19(5–6):438–445
100. Ito H, Wate R, Zhang J, Ohnishi S, Kaneko S, Ito H, Nakano S, Kusaka H (2008) Treatment with edaravone, initiated at symptom onset, slows motor decline and decreases SOD1 deposition in ALS mice. *Exp Neurol* 213(2):448–455
101. Brown KK, Heitmeyer SA, Hookfin EB, Hsieh L, Buchalova M, Taiwo YO et al (2008) P38 MAP kinase inhibitors as potential therapeutics for the treatment of joint degeneration and pain associated with osteoarthritis. *J Inflamm* 5:22–22
102. Christensen AD, Haase C, Cook AD, Hamilton JA (2016) K/BxN serum-transfer arthritis as a model for human inflammatory arthritis. *Front Immunol* 7:213–213
103. Aya K, Alhawagri M, Hagen-Stapleton A, Kitaura H, Kanagawa O, Novack DV (2005) NF-(kappa)B-inducing kinase controls lymphocyte and osteoclast activities in inflammatory arthritis. *J Clin Invest* 115(7):1848–1854
104. Brand DD, Latham KA, Rosloniec EF (2007) Collagen-induced arthritis. *Nat Protoc* 2(5):1269–1275
105. Morita K, Miyamoto T, Fujita N, Kubota Y, Ito K, Takubo K et al (2007, 204) Reactive oxygen species induce chondrocyte hypertrophy in endochondral ossification. *J Exp Med. The Rockefeller University Press* (7):1613–1623
106. Tsou H-K, Chen H-T, Chang C-H, Yang W-Y, Tang C-H (2012) Apoptosis signal-regulating kinase 1 is mediated in TNF-alpha-induced CCL2 expression in human synovial fibroblasts. *J Cell Biochem* 113(11):3509–3519
107. Eaton GJ, Zhang Q-S, Diallo C, Matsuzawa A, Ichijo H, Steinbeck MJ et al (2014) Inhibition of apoptosis signal-regulating kinase 1 enhances endochondral bone formation by increasing chondrocyte survival. *Cell Death Dis* 5:e1522
108. Yan F, Gao H, Zhao H, Bhatia M, Zeng Y (2018) Roles of airway smooth muscle dysfunction in chronic obstructive pulmonary disease. *J Transl Med* 16(1):262
109. Metz RP, Patterson JL, Wilson E (2012) Vascular smooth muscle cells: isolation, culture, and characterization. *Methods Mol Biol* 843:169–176
110. Tulis DA (2007) Rat carotid artery balloon injury model. *Methods Mol Biol* 139:1–30
111. Nakagawa H, Hirata Y, Takeda K, Hayakawa Y, Sato T, Kinoshita H, Sakamoto K, Nakata W, Hikiba Y, Omata M, Yoshida H, Koike K, Ichijo H, Maeda S (2011) Apoptosis signal-regulating kinase 1 inhibits hepatocarcinogenesis by controlling the tumor-suppressing function of stress-activated mitogen-activated protein kinase. *Hepatology*. 54(1):185–195
112. Jiang C-F, Wen L-Z, Yin C, Xu W-P, Shi B, Zhang X, Xie WF (2016) Apoptosis signal-regulating kinase 1 mediates the inhibitory effect of hepatocyte nuclear factor-4α on hepatocellular carcinoma. *Oncotarget*. 7(19):27408–27421
113. Hayakawa Y, Hirata Y, Nakagawa H, Sakamoto K, Hikiba Y, Kinoshita H et al (2011) Apoptosis signal-regulating kinase 1 and cyclin D1 compose a positive feedback loop contributing to tumor growth in gastric cancer. *Proc Natl Acad Sci* 108(2):780–785
114. Kamiyama M, Shirai T, Tamura S, Suzuki-Inoue K, Ehata S, Takahashi K et al (2017) ASK1 facilitates tumor metastasis through phosphorylation of an ADP receptor P2Y12 in platelets. *Cell Death Differ* 24(12):2066–2076
115. Naik MU, Patel P, Derstine R, Turaga R, Chen X, Golla K, Neeves KB, Ichijo H, Naik UP (2017) Ask1 regulates murine platelet granule secretion, thromboxane A2 generation, and thrombus formation. *Blood*. 129(9):1197–1209
116. Hattori K, Naguro I, Okabe K, Funatsu T, Furutani S, Takeda K et al (2016) ASK1 signalling regulates brown and beige adipocyte function. *Nat Commun* 7:11158–11158
117. Makena PS, Gorantla VK, Ghosh MC, Bezawada L, Kandasamy K, Balazs L, Luellen CL, Thompson KE, Parthasarathi K, Ichijo H, Waters CM, Sinclair SE (2012) Deletion of apoptosis signal-regulating kinase-1 prevents ventilator-induced lung injury in mice. *Am J Respir Cell Mol Biol* 46(4):461–469
118. Johnson GL, Nakamura K (2007) The c-Jun kinase/stress-activated pathway: regulation, function and role in human disease. *Biochim Biophys Acta* 1773(8):1341–1348
119. Liu Z, Shi S, Zhu H, Chen Y, Zhang Y, Zheng Z et al (2018) Novel ASK1 inhibitor AGI-1067 attenuates AGE-induced fibrotic response by suppressing the MKKs/p38 MAPK pathway in human coronary arterial smooth muscle cells. *Int Heart J* 59(6):1416–1424
120. Gujral JS, Knight TR, Farhood A, Bajt ML, Jaeschke H (2002) Mode of cell death after acetaminophen overdose in mice: apoptosis or oncotic necrosis? *Toxicol Sci* 67(2):322–328

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1.3.10. ASK1 inhibition for preventing P38 and JNK induced hair cell death

The consensus of a large number of *in vitro* and *in vivo* studies is that JNK is the predominant MAPK driving hair cell death (Bodmer et al. 2002; Wang et al. 2003; Coleman et al. 2007; Sugahara et al. 2006; Pirvola et al. 2000; Ylikoski et al. 2002; Tao & Segil 2015). P38 has been implicated to lesser extent and the role of ERK appears minimal (Bas, Van de Water, et al. 2012; Tao & Segil 2015; Wei et al. 2005). As discussed above, aminoglycoside-induced hair cell death can be reduced by inhibition of P38 or JNK. However, both JNK and p38 are important for normal cellular function. Consequently, therapies that broadly inhibit JNK or P38 can have toxic effects in off-target-tissues (Bubici & Papa 2014; Ma et al. 2009). Therefore, it is important to ascertain what upstream signals are driving the apoptosis-specific roles of JNK and P38 in hair cells. To address this issue, Tao et al. 2015 examined the transcriptomic changes in the hair cells of gentamycin treated neonatal murine cochlear explants (Tao & Segil 2015). This analysis indicated that the gene encoding mitogen-activated protein kinase kinase kinase 5 (MAP3K5) was the only mitogen-activated protein kinase kinase kinase to show significantly increased expression as a result of gentamycin treatment (Tao & Segil 2015). MAP3K5, known as apoptosis signal-regulating kinase 1 (ASK1) is a key mediator of ROS induced JNK and p38 mediated disease. As discussed above, ASK1 phosphorylation only occurs in response to cell stress and is particularly responsive to excess ROS. Therefore, ASK1 inhibition potentially represents a viable strategy for preventing ototoxicity, particularly that of aminoglycosides that induce ROS mediated P38 and JNK signalling, without impeding the homeostatic functions of these kinases. Commercially available ASK1 inhibitors have shown excellent bio-availability and tolerability parameters in phase 1 clinical trials, and have been safely used in humans (Tesch et al. 2016; Younossi et al. 2018; Loomba et al. 2018; Chertow et al. 2019). Interestingly, the role of ASK1 in hair cell death has not previously been investigated.

1.4. Summary

There are multiple theoretical treatment strategies that have the potential to prevent ototoxicity. Ideally, non-ototoxic compounds would be developed with the same antibiotic profile as aminoglycosides. Indeed, the mass production of an effective and non-ototoxic aminoglycoside derivative would be transformative for patients and lucrative for the developer. However, such medicines are yet to be developed and validated. Significant progress has been made towards hair cell regeneration. However, achieving the complex hair cell patterns required for functional hearing is a conspicuous limitation. Supporting cell types can be artificially driven to proliferate and transdifferentiate. However, directing this differentiation to produce the appropriate type of mature hair cell, in the right place, with correct stereocilia orientation, and innervation poses a substantial challenge. Therefore, an adjuvant intervention that could prevent ototoxicity, by blocking notable cell death pathways presents as an important option. Notably, destroyed hair cells cannot regenerate, but those that survive insult may be repaired *in situ* (Baird et al. 2000; Tang & Watson 2015; Rzadzinska et al. 2004). Therefore, an adjuvant therapy that prevented aminoglycoside-induced hair cell death would circumvent the issues of hair cell patterning and innervation in regenerative therapies, as treated individuals could retain intrinsic functional hair cells.

Global research efforts towards understanding hair cell death and developing otoprotective interventions are ongoing. Studies to date have demonstrated that redox status drives hair cell death. Research at the cellular level indicates that ROS production in the dying hair cell is part of an elaborate and complex signalling cascade. However, antioxidant compounds continue to dominate clinical research in the prevention of ototoxicity, despite equivocal results to date. In order to achieve legitimate therapeutic progress, research efforts must be expanded to include studies investigating the basic mechanisms of cell death within the ear. Understanding the underlying pathogenesis of hair cell loss will identify novel therapeutic targets, which may ultimately be used to prevent drug-induced hearing loss.

To date, MAPKs have been strongly associated with the pathogenic mechanism underlying aminoglycoside induced hair cell death. JNK has been identified as the primary MAPK driving hair cell death, however P38 has also been implicated to lesser extent. Conversely, ERK may be important for hair cell survival (Lee et al. 2010; So et al. 2007). Based on current knowledge, ASK1 has emerged as a potential upstream target for preventing ROS induced hair cell death due to its key role in regulating JNK and P38 activity. Furthermore, the reported activation of P38 and JNK subsequent to acoustic trauma, post operative trauma and inflammation suggest that ASK1 may be broadly relevant to the pathogenesis of multiple forms of acquired hearing loss. This project will specifically investigate ASK1 as a novel therapeutic target for the prevention of aminoglycoside induced hair cell death.

1.5. Project Aims

Aminoglycosides are the most commonly used antibiotics worldwide and represent a significant cause of acquired hearing loss. Currently, there is no antibiotic substitute with the bactericidal profile of aminoglycosides and an adjuvant therapy capable of mitigating ototoxic outcomes does not exist. As a result, individuals requiring aminoglycoside treatment can experience the debilitating loss of auditory and motor sensation. To develop a therapy that mitigates these side-effects, it is crucial that the pathogenesis of aminoglycoside induced hair cell death is well defined.

Within the literature, the JNK and P38 signalling cascades have been identified as important mediators of aminoglycoside induced hair cell death. Moreover, redox stress is a well-defined contributor to the pathway resulting in apoptosis in aminoglycoside treated hair cells. Based on these observations, the primary hypothesis of this project is that ASK1 is an important mediator of hair cell death and therefore, ASK1 inhibition will attenuate aminoglycoside induced hair cell death.

Specifically, this project aims to:

1. Investigate the role of ASK1 in the murine auditory system by characterising the hearing phenotype of *Ask1* knockout mice.
2. Investigate the importance of ASK1 in the apoptotic response of hair cells to aminoglycoside-induced damage.
3. Investigate ASK1 inhibition as a potential therapeutic for preventing aminoglycoside-induced hair cell death.

Chapter 2. Methods

2.1. Mice

2.1.1. Ethics approval

All experimental procedures were approved in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 2013 (NHMRC 2013). The Murdoch Children's Research Institute (MCRI) Animal Ethics Committee approved all procedures described under project numbers A764, A766, A839, A844, A875 and A904.

2.1.2. Animal husbandry

Mice were maintained at the MCRI animal facility, with a controlled ambient temperature of 23 °C, relative humidity between 30-60%, and an automated light cycle scheduled for 12 hours on, 12 hours off. Mice were group-housed in individually ventilated micro-isolator cages (Tecniplast, Buguggiate, VA, Italy), with free access to standard Barastoc mouse chow (Ridley AgriProducts, Melbourne, VIC, Australia) and sterilised water.

2.1.3. Provenance of experimental animals

A colony of *Ask1*^{-/-} mice was established at MCRI, founded by two *Ask1*^{-/-} breeding pairs that were kindly provided by A/Prof Greg H. Tesch and Dr. David J. Nikolic-Paterson (Monash Medical Centre, Australia). The initial Monash *Ask1*^{-/-} colony was generated by the Australian Phenomics Network (Monash University) using a cloned *Ask1* (*Map3k5*) knockout mouse embryonic cell line (C57BL/6N) from the European Conditional Mouse Mutagenesis Program (Ma et al. 2014) and *Ask1* knockout was confirmed using real time PCR analysis of mRNA expression. C57BL/6 mice were purchased from the Walter and Eliza Hall Institute of Medical Research (Parkville, Australia).

2.1.4. Genotyping

Genotyping was performed using standard polymerase chain reaction (PCR) genotyping protocols.

2.1.4.1. DNA extraction

DNA was extracted from mouse ear clips and precipitated using the isopropanol method (Laird et al. 1991). Tissue was disrupted on rotation in a hybridisation oven (Thermo Scientific) at 55 °C overnight, in 500 µl lysis buffer containing 100 mM TrisHCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 200 mM NaCl, 1% Sodium dodecyl sulfate and 0.4 µg Proteinase K (in Milli-Q purified H₂O). Lysed samples were then centrifuged (10 min, 10,000 g, 8 °C). The supernatant was mixed with 500 µl ice cold isopropanol and placed on wet ice until DNA precipitation was complete (approximately 10 min). The sample was then centrifuged (20 min, 16,000 g, 8 °C) and the supernatant discarded. 250 µl of 70% ethanol was added to the pellet and the sample centrifuged again (5 min, 16,000 g, 8 °C). Ethanol was then discarded and the sample dried at 50 °C on a heat block (Ratek Instruments). The DNA pellet was then dissolved in 50 µl Tris-EDTA TE buffer (10 mM Tris, 1 mM EDTA) at 55 °C in the hybridisation oven.

2.1.4.2. PCR conditions

DNA in TE was diluted 1/10 in Ultra pure H₂O (Life Technologies). Two PCR reaction mixes were prepared as per Table 4 and cycled in an Applied Biosystems Veriti Thermal Cycler (Life Technologies) as per Table 5. The amplified product was then separated in a 2% agarose gel (Bioline). Before loading, 10 µl PCR product was mixed with 2 µl loading dye, containing 1x Gel Red nucleic acid stain (Biotium). A 100 bp plus Gene Ruler DNA Ladder (Thermofisher) was also loaded to indicate product size. Electrophoresis was performed for 1 hour at 80 V (5 V per cm) in 0.5 x TBE. Gels were visualised using a G:Box-Chemi-XR5 (Syngene). For the wild type allele, a visual band 563 bp in size was observed, whereas a visual band at 312 bp indicated the mouse was positive for a knockout allele.

Table 4. The PCR reaction mix used to genotype *Ask1*^{-/-} mice.

Wild Type Reaction (Band Size 563 bp)		Knockout Reaction (Band Size 312 bp)	
Primer 1 (10 µM) cttgatctgctgggtccacgtcc	0.80 µl	Primer 1 (10 µM) cttgatctgctgggtccacgtcc	0.80 µl
Primer 2 (10 µM) gacactaaacccaccctgcctcc	0.80 µl	Primer 3 (10 µM) caacgggttcttctgttagtcc	0.80 µl
Titanium <i>Taq</i>	0.06 µl	My <i>Taq</i>	0.06 µl
Titanium <i>Taq</i> 10x Buffer	2.00 µl	My <i>Taq</i> 5x Buffer	1.50 µl
H ₂ O	10.94 µl	H ₂ O	15.84 µl
DNTPs (2.5 mM)	0.40 µl	Dilute DNA	1.00 µl
Betaine	4.00 µl		
Dilute DNA	1.00 µl		

Table 5. The thermocycle program used for *Ask1*^{-/-} mouse PCR genotyping

TEMPERATURE	TIME	CYCLES
95°C	3 minutes	1
94°C	30 seconds	35
61°C	30 seconds	
72°C	45 seconds	
72°C	10 minutes	1
4°C	Hold as Required	1

2.2. Behavioural Testing

2.2.1. Auditory brainstem responses

Auditory brainstem responses (ABRs) were performed as previously described, to assess the auditory function of experimental mice (Ogier et al. 2014; Carpinelli et al. 2013; Ogier et al. 2018). Briefly, mice were anesthetised with 80 mg/kg ketamine and 20 mg/kg xylazine via intraperitoneal injection. Refresh nighttime eye ointment was applied to protect the mouse's eyes from drying and a heat cabinet (Tecniplast) was used to maintain the mouse's body temperature at 37 °C. Sedation was sufficient when the mouse no longer exhibited the Pedal withdrawal reflex. Another half dose was given 10 minutes after the initial dose if the mouse retained a pedal reflex. The mouse was then placed within a custom Coulbourn habitest isolation cubicle containing a faraday cage (Figure 14A). Sub-dermal stainless-steel electrode needles (4mm, S06666-0, Rochester Electro-Medical) were inserted at the vertex of the mouse's skull (positive), left cheek (negative) and left hind leg (ground) (Figure 14B). Computer generated pulses of sound were produced by an evoked potentials workstation (Tucker Davis Technologies) in conjunction with the BioSigRP Stimulate/Record System v4.4.1. Clicks (white noise of mixed frequency spectrum 0-50 kHz) or pure tones (4 kHz, 8 kHz, 16 kHz and 32 kHz) were presented rapidly in short bursts (100 µs in duration, repeated 512 times). The ABR signal was calculated as the average of all 512 repeats. Sound was presented through a free-field magnetic speaker (model FF1, Tucker Davis Technologies) 10 cm from the left ear of the mouse. The maximum intensity of sound tested was 100 dB SPL, decremented in 5 dB SPL steps. Hearing thresholds were defined as the lowest sound pressure level capable of eliciting a visible ABR.

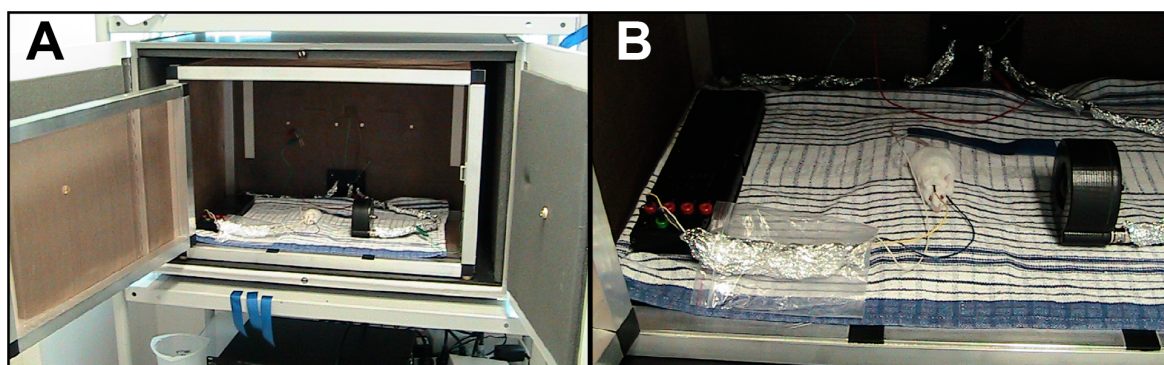


Figure 14. The ABR testing arena.

A) The sound attenuating ABR cabinet is lined with grey, acoustic dampening foam. Inside the cabinet is a custom faraday cage, to minimise electrical interference. **B)** Within the faraday cage, an anaesthetised mouse is placed on a heat pad. Sub-dermal electrodes are inserted at the vertex of the mouse's skull (positive), left cheek (negative) and left hind leg (ground). To further minimise interference, wires connected to the battery-powered preamplifier, speaker, and electrodes are encased in foil and grounded to the faraday cage.

2.2.2. Acoustic startle responses

Acoustic startle responses (ASRs) were measured using the 'plug and play' SR-LAB system (San Diego Instruments). Mice were restrained in clear restraint capsule, before being placed in a sound attenuating isolation box (Figure 15). The restraint capsule is a plastic cylinder (four cm in diameter) mounted on a flat plastic base, with a piezoelectric accelerometer attached to the underside. After a one-minute habituation period of background white noise (70 dB SPL), pulses of varying sound level were delivered, according to a pre-programmed, pseudo-randomised test. Six pulses of white noise were delivered at background levels (70 dB SPL), 85, 90, 95, and 100 dB SPL, and 16 pulses at 115 dB SPL. The time between each pulse varied from three to eight seconds. Sound was delivered through a speaker built into the cabinet. The piezoelectric accelerometer created a voltage output based on the mouse's response to each sound, which was recorded using SR - Lab software (San Diego Instruments). Data was manipulated with the highest and lowest response for each sound level deleted before the average was taken from all other startle responses.

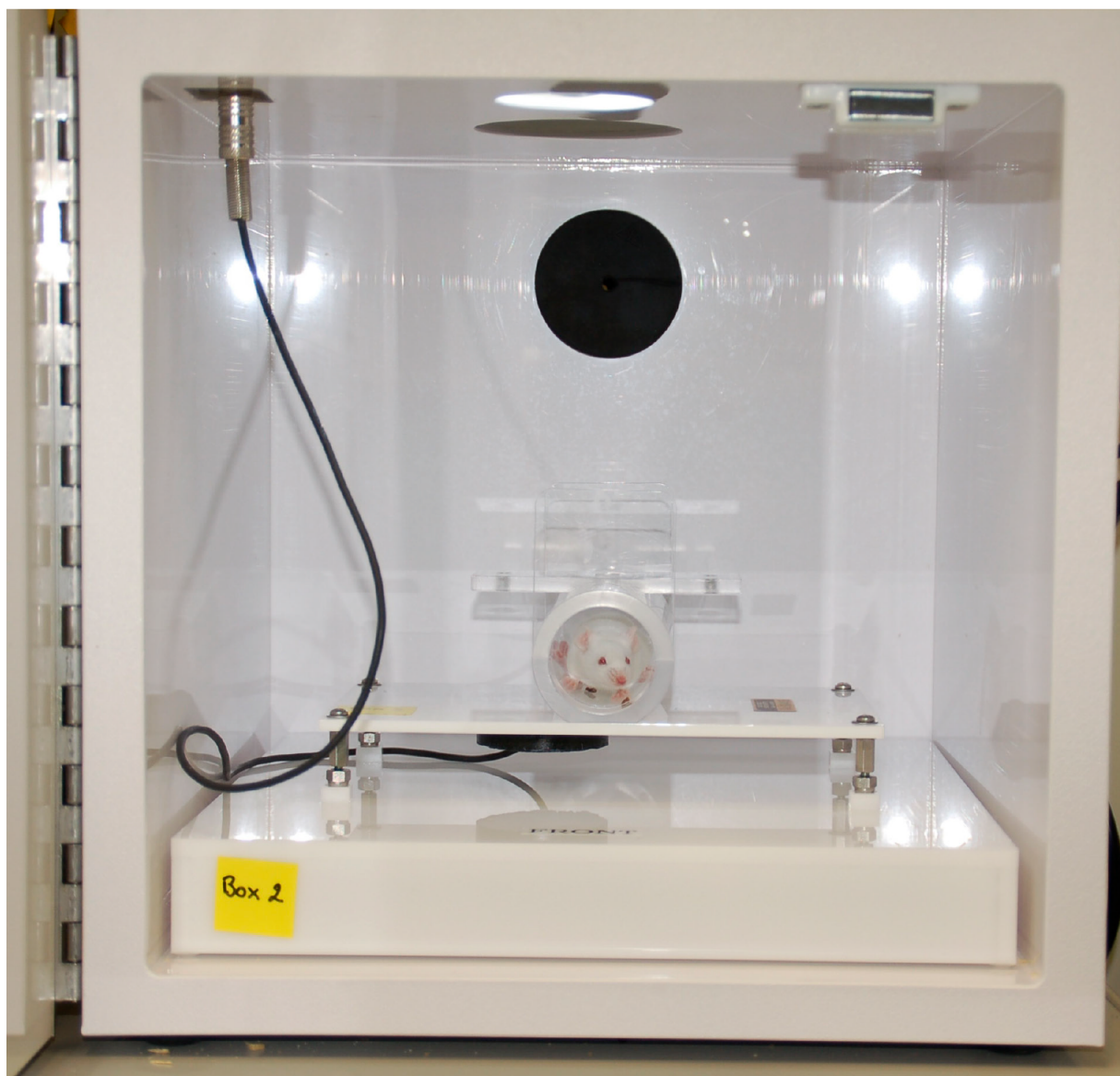


Figure 15. The sound attenuating ASR chamber.

The conscious mouse is restrained in a Perspex cylinder mounted atop a flat plastic base. A piezoelectric accelerometer attached to the underside of this restraint capsule converts the mouse's movements to voltage, which is recorded using SR Lab software.

2.2.3. Rotarod performance test

Motor performance was assessed using the Rota-Rod for Mice (47600, Ugo-Basile) as previously described (Stephenson et al. 2018; Ogier, Lockhart, et al. 2019). Mice were acclimatised to the testing room in their home cages for one hour before testing. Testing was performed on consecutive days, with the first day performed as habituation/training. Trials on the second day were recorded. Mice were placed on the

Rota rod, facing away from the handler. The rod accelerated from 4-40 rpm during each five-minute trial. The trial ended when the mouse fell from the rotating rod, rotated with the rod two consecutive times, or when 300 seconds was reached. Latency to fall was recorded by the Rota-Rod CUB software. Each mouse underwent three trials, with 30 minutes rest in a home cage between trials.

2.3. Tissue collection and analysis

2.3.1. Perfusion

Adult mice were euthanised by anaesthetic overdose (320 mg/kg ketamine and 80 mg/kg xylazine via intraperitoneal injection). Once the pedal response could no longer be elicited, the mouse was monitored until breathing ceased. At this point the thoracic cavity was quickly opened to expose the beating heart. Using micro scissors, a hole was cut in the right atrium and the tip of a 21 guage needle placed in the left ventricle. Phosphate Buffered Saline (PBS) was flushed through the circulatory system via the needle for 5 minutes, followed by 4% paraformaldehyde (PFA) for another 5 minutes. Cochleae, brains and kidneys were collected and stored in 4% PFA. Kidneys were sent to the Australian Phenomics Network (APN), Department of Anatomy and Neuroscience, University of Melbourne, Parkville, where sectioning, staining, and expert analysis was provided. Cochleae and brains underwent further processing in house, as described below.

2.3.2. Cochlea histology

Cochlea histology was performed to examine the general structure of the cochlea, the organ of Corti and Rosenthal's canal. Cochleae collected from perfused mice were post fixed for one hour then washed three times in PBS on rotation (five minutes per wash), before being decalcified in 10% EDTA (in PBS, pH 7.4) on rotation for 10 days at 4 °C. Decalcified cochleae were either dissected in order to isolate the organ of Corti, or embedded in paraffin for whole cochlea sectioning.

2.3.3. Organ of Corti isolation

Decalcified adult cochleae were dissected according to the Lieberman protocol, Eaton-Peabody Laboratories, Massachusetts, USA (Lieberman 2015). Each cochlea was

bisected from the apex to the round window and then rotated approximately 90°. Parallel incisions were then made superiorly and inferiorly to the organ of Corti at each cochlear turn. The modiolus and any remaining cochlear wall was removed. Cochlear sections were placed on polytetrafluoroethylene (PTFE) printed microscope slides (ProSciTech). Immunohistochemistry was performed as outlined in section 2.3.7.

2.3.4. Paraffin embedding, cryosectioning, and staining of whole adult cochleae

Decalcified cochleae were oriented in 1% low melt agarose in PBS gel within a 10 mm x 10 mm x 5 mm cryomold. Once oriented, samples were embedded in paraffin wax for sectioning and delivered to the WEHI Histology Service, Parkville. The sample was cut into 2 µm sections and stained with Haematoxylin and eosin before being mounted with 10 sections per slide. Sections were viewed using a Leica stereo microscope and pictures acquired using the accompanying digital camera and LAS software.

2.3.5. Neonatal mouse neurosensory epithelium dissection and culture

Neurosensory epithelium dissection, culture, and immunohistochemistry methods are described in a stepwise manner in Ogier et al. 2019 (Appendix 1) (Ogier, Burt, et al. 2019). Mouse pups (P3-5) were euthanised by decapitation, and stored briefly on ice. Under sterile conditions within a laminar flow hood, each skull was bisected, and temporal bones removed into ice-cold minimum essential media containing non-essential amino acids and penicillin-streptomycin (all Life Technologies). Initial skull and cochlea dissection was carried out in ice-cold minimal essential medium (MEM). Dissected cochleae were then placed in ice-cold neurobasal-A medium (containing N2 Supplement, L-glutamine and D-glucose, all Life Technologies). All further dissection steps and culture were performed in this neurobasal media. The vestibular apparatus, otic capsule, cochlear wall, and stria vascularis were removed before the neurosensory epithelium could be gently unwound from the cochlear modiolus. Neurosensory epithelium explants were cultured on organotypic membranes (Merck Millipore Milli Cell) at 37 °C, 5% CO₂. For *Ask1*^{-/-} and C57BL/6 comparisons, explants were cultured overnight before fresh neurobasal media was added containing neomycin or DMSO

control. This process was repeated daily for longer treatments, with a maximum culture time of 4 days post dissection. For explants used to investigate ASK1 inhibition, dissected explants were cultured for 3-4 hours, before being treated with GS444217. Neomycin (Life Technologies) and GS444217 (Gilead) were initially suspended in DMSO (Sigma Aldrich), before being diluted into neurobasal media for the treatment of hair cells in culture. GS444217 pre-treatment was 16 hours, before fresh media was added containing GS444217 and the associated treatment (neomycin or DMSO control). Explants were fixed in 4% PFA for 5 minutes and washed 3 times in PBS. A scalpel was used to cut the membrane surrounding the explant, and the section moved to a well upon PTFE printed microscope slides (ProSciTech). Immunohistochemistry was performed as outlined in section 2.3.7.

2.3.6. *In vitro* FM 46-4x treatment

FM 46-4x was used to investigate if GS444217 could block hair cell MET channels. FM dyes rapidly enter hair cells via the MET channel and result in extensive fluorescent staining within the cytoplasm, whereas fluorescent staining of other cell types within the organ of Corti is limited to outer cell membranes (Meyers et al. 2003; Gale et al. 2001). Cochlear explants were treated overnight with either vehicle (DMSO) or 100 μ M GS444217. Then, 400 μ l neurobasal media containing 100 μ M FM 46-4x was added to the apical surface of hair cells in culture. After 120 seconds, hair cells were washed twice with neurobasal media, for two minutes. Explants were then fixed in 4% PFA for five minutes and washed three times in PBS. A scalpel was used to cut the membrane surrounding the explant, and the section moved to a well upon PTFE printed microscope slides (ProSciTech). Immunohistochemistry was performed as outlined in section 2.3.7.

2.3.7. Immunocytochemistry

The reagents used for immunofluorescence, including suppliers and optimal dilutions are listed in Table 6. To distinguish sensory hair cells, a rabbit anti-mouse Myosin VIIa antibody was used in conjunction with a fluorescently conjugated goat anti-rabbit secondary antibody. Alternatively, a conjugated Phalloidin stain was used to highlight stereocilia. The *in situ* cell death detection kit was used to identify apoptotic cells. FM 4-64x was used to identify hair cells with functional MET channels. Prolong Gold Anti-

Fade with DAPI was used as a mounting medium and to stain the nuclei of all cells within the explant.

The protocol used for staining is described in a stepwise manner in Ogier et al. 2019 (Appendix 1) (Ogier, Burt, et al. 2019). In brief, explants were blocked in a solution of 0.1% triton-X in PBS containing 2% normal goat serum for 30 minutes at room temperature. The initial blocking solution was removed from each well and replaced with 20 μ L of blocking solution containing primary antibody. The antibody was diluted and incubated as per Table 6. Explants were washed in blocking solution (three x five min washes) and the secondary antibody added as per Table 6, protected from light. When the *in situ* cell detection kit was also used, explants were again washed in blocking solution before 7 μ L of the cell death detection solution was added to each explant incubated at 37 °C for 1 h. Explants were then washed in blocking solution. If phalloidin was used, it was always added after all other immunohistochemistry reactions had been performed. After a brief phalloidin incubation, explants were washed three times with 1x PBS and then mounted on slides using ProLong Gold antifade (Life Technologies). Slides were air-dried overnight at room temperature and the cover glass edges were sealed with clear nail varnish.

2.3.8. Confocal imaging

General confocal images were taken using a Laser Scanning Microscope 780 (Zeiss) in conjunction with Zen Black digital imaging software (Zeiss). Images were acquired using standard built in photomultiplier tubes (LSM-T-PMT, Zeiss).

2.3.9. Hair cell counts

An Olympus IX70 fluorescent microscope with Evolution VF cooled monochrome 12 bit camera was used, to capture 20-30 images from the base to apex of each neurosensory epithelium, at 20x magnification (imaging software Qcapture pro V 6.0). Adobe Photoshop CC.2015 was then used to photo-stitch images together, creating a large image of the entire explant (Figure 16). Images were then randomised and de-identified. Fiji software was used to draw two boxes (0.18mm x 0.09mm) that were rotated to match the explant curve (Schindelin et al. 2012) . The boxes were then overlaid onto the image, either side of the explant mid-point, which was determined by measuring from the furthest aspects (apex and base) of the cochlear explant in the photo stitched image (Figure 16). Hair cell counts were manually performed using the

Fiji cell count tool. Hair cells within each rectangle were counted, including partial cells with more than half the cell body inside the rectangle boundary. The average inner and outer hair cell number of the two boxes was then recorded for each explant. Inner and outer hair cells were identified by stereotypic anatomical position. However, a stronger Myosin VIIa signal indicated the inner hair cell type and the DAPI counterstain showed the shape and underlying cell types in the explant. This was particularly useful when significant hair cell death occurred. The key for re-identification was provided after hair cell counts had been completed.

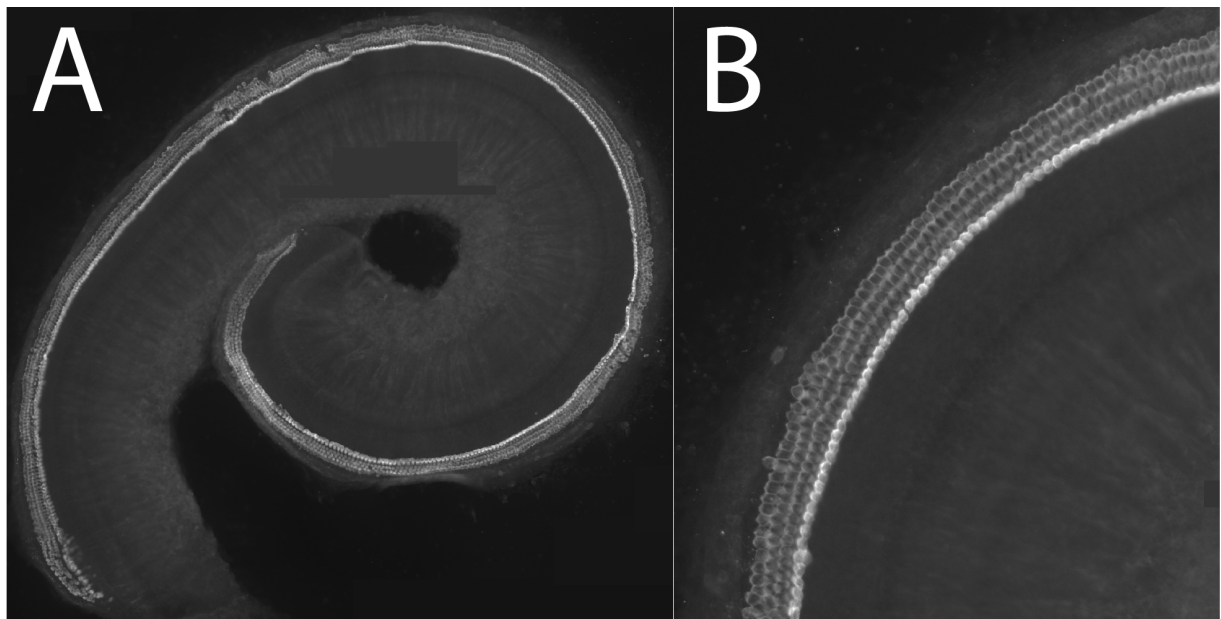


Figure 16. An example explant stained with a Myosin VIIa primary antibody and a fluorescently conjugated secondary antibody.

Multiple images were used and then stitched together using Adobe Photoshop CC.2015. The photo-stich function shows the entire explant, so that the quality of dissection and explant orientation can be observed. However, the image remains clear when it is enlarged, allowing for hair cell counting. **A)** An example photo-stitch explant image. **B)** A section of the explant enlarged for hair cell visualisation.

Table 6. Antibodies, stains and kits used for immunofluorescence

Antibody/Target	Host information	Catalogue Number	Supplier	Dilution	Incubation
ASK1 antibody	Rabbit monoclonal	AB45178	Abcam	1/50	Overnight at 4 °C
FM 4-64FX	Lipophilic Styryl Dye	F34653	Thermo Fisher	To 100 µM	Two min at 37 °C <i>on live cells</i>
Goat Secondary Anti-Rabbit (conjugated Alexa Fluor 488 or 594)	Goat polyclonal	AB150085, AB150080	Sapphire Bioscience	1/500	Two hours at room temp or overnight at 4 °C
<i>In situ</i> cell death detection kit	Terminal transferase & Fluorescein-12- dUTP	11684795910	Sigma Aldrich	1:9 Enzyme to label solution	One hour at 37 °C
Myosin-VII antibody	Rabbit polyclonal	PTS-25-6790-C050	Sapphire Bioscience	1/500	One hour at room temp or overnight at 4 °C
Phalloidin Alexa Fluor 488 or 594	Peptide isolated from <i>A. phalloides</i> , selectively labels F- actin	A12379, A12381	Invitrogen	1/180	8-10 minutes at room temp
Phospho-JNK 1,2 & 3 antibody (conjugated Alexa Fluor 488)	Rabbit monoclonal	AB201862	Abcam	1/100	Overnight at 4 °C
Phospho-P38	Rabbit polyclonal	#9211	Cell Signalling Technology	1/250	Overnight at 4 °C

2.3.10. Western blot analysis

2.3.10.1. Protein extraction

Three explants from each treatment were pooled and submerged in protein extraction buffer (10 mM Tris-HCL, pH 7.5, 2% SDS and 1x Protease inhibitor (Sigma-Aldrich, Cat# S8820) on wet ice. A 1 ml syringe with 23G needle was used to begin homogenisation. Lysates were then sonicated using a probe sonicator (Branson). Total protein concentration of each sample was then estimated using the bicinchoninic acid assay (BCA) (Pierce, Cat# 23225). The assay was performed as per the manufacturer's instructions, in a 96 well flat bottomed plate, in triplicate. Plates were incubated at 37 °C for 30 minutes before absorbance was measured at 562 nm using a Fluorostar Optima microplate plate reader (BMG Labtech). A Bovine Serum Albumin (BSA) standard curve was used to estimate relative protein concentration. Three explants combined produced ~ 1 mg/ml protein sample.

2.3.10.2. SDS-PAGE and immunoblot analysis

Samples were heated at 95 °C for five minutes in 1x Novex™ Tris-Glycine SDS Sample Buffer (Thermo Scientific, Cat#LC2676) and 1% β -mercaptoethanol (Sigma-Aldrich, Cat#M6250). Denatured proteins were separated in a 4-20% gradient Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad Cat#4561093), in 1x Novex™ Tris-Glycine SDS Running Buffer (Thermo Scientific, Cat#LC2675). The SeeBlue™ pre-stained protein standard was also loaded for protein size determination (Life Technologies, Cat#LC5625). Protein was transferred from the gel onto a 0.45 μ m pore PVDF membrane (Immobilon-P, Cat#IPVH00010) using an overnight 10 V wet transfer.

Transfer buffer consisted of 20% ethanol and 1x Novex™ Tris-Glycine SDS Running Buffer). Membranes were then blocked in 5% skim milk/ TBST (10 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.05% Tween-20) for two hours at room temperature. Membranes were then washed three times (10 minute wash) in wash buffer (2.5% BSA and 0.01% Sodium Azide in TBST). The primary experimental antibody was diluted in wash buffer and incubated on the membrane overnight at 4 °C (Table 7). The membrane was washed three times (10 minute wash) before the secondary antibody was added (diluted in wash buffer) and incubated at room temperature for one hour. The membrane was then washed three times (10 minute wash). Immediately prior to

visualisation, the membrane was treated with an Enhanced Chemiluminiscence Substrates kit (Bio-Rad, Cat#170-5061). Protein distribution on the membrane was imaged using the Image Quant LAS4000 imager (GE Healthcare). The membrane was then washed three times (10 minute wash) in wash buffer, before the staining and imaging process was repeated using the control antibodies (Table 7).

Table 7. Antibodies used for western blot analysis

Antibody	Catalogue Number	Supplier	Dilution	Role
Phospho-JNK 1,2 & 3	AB201862	Abcam	1/1000	Primary (experimental)
Phospho-P38	#9211	Cell Signalling	1/1000	Primary (experimental)
Vinculin	AB192002	Abcam	1/10,000	Primary (control)
B-tubulin	AB18207	Abcam	1/4000	Primary (control)
Goat anti-rabbit (HRP conjugate)	#111035003	Jackson Immuno	1/20,000	Secondary

2.4. Antibiotic minimum inhibitory concentrations

To ascertain whether GS444217 impacts antibiotic efficacy, the minimum inhibitory concentrations (MICs) of amikacin, tobramycin and neomycin against *Pseudomonas aeruginosa* were determined using the resazurin salt indicator of cell viability, in conjunction with the broth dilution test. Resazurin salt is weakly fluorescent, appearing blue in colour. However, viable cells metabolise resazurin, producing resorufin, which is bright pink and highly fluorescent. The MIC was defined as the amount of antibiotic required to prevent bacterial activity in the broth dilution test, as measured by resorufin fluorescence.

2.4.1. Provenance of *Pseudomonas* isolates

Antibiotic efficacy was tested against three *P. aeruginosa* isolates. A blood isolate reference strain (American type culture collection, 27853) was kindly provided by the Department of Microbiology at The Royal Children's Hospital. Two clinical isolate strains (designated 0307, and 0315) were provided by the Department of Respiratory and Sleep Medicine at The Royal Children's Hospital. Isolates 0307 and 0315 were collected by the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF). Sputum was collected during routine hospital visits and *P. aeruginosa* was subsequently isolated by the Royal Children's Hospital pathology laboratory. All aspects of the AREST CF study were approved by the RCH Human Research Ethics Committee (Approval 25054).

2.4.2. The broth dilution test

To reanimate frozen stocks of *P. aeruginosa*, a sterile loop was used to streak colonies onto Luria Bertani agar plates. Plates were then incubated overnight at 37 °C, 5% CO₂. A single colony from each plate was used to inoculate 10 mL fresh Luria-Bertani broth. The inoculated broth was incubated overnight and then diluted 1:100 in fresh broth. The diluted culture was incubated until an optical density of 0.1 at 600 nm (approximately 582 cells per ml) was achieved. At this point, the broth was used to inoculate a 96 well plate containing serial 1:2 dilutions of amikacin, tobramycin or neomycin and GS444217. Antibiotic concentrations ranged from 0-512 µg/ml and GS444217 concentrations were 0-100 µM, diluted in broth containing 700 µM of resazurin (Sapphire bioscience). A 30 µl *P. aeruginosa* inoculum was added to each

well, to a final volume of 200 µl LB. Plates were incubated at 37 °C for 16 hours. Fluorescence was measured at 540 nm excitation and 580 nm emission using an Infinite M200 Pro plate reader (Tecan life Sciences).

2.5. Statistical analysis

ABR and ASR data was compared between groups for each frequency tested. Multiple unpaired t-tests were performed in conjunction with the Holm Sidak method of correction for multiple comparisons. Analysis was performed in GraphPad software (version 7.0a for Mac).

Hair cell counts were first analysed with a three-way analysis of variance, before pairwise Fisher comparisons were made. Fisher individual tests for differences of means was performed to ascertain specific p-values for strain/ treatment. Sue Finch provided guidance regarding statistical analysis as part of the Melbourne University Statistical Consulting Platform. Analysis was performed in MINITAB Statistical Software (version 17 for Windows).

A standard two-way ANOVA was used to identify significant changes in antibiotic minimum inhibitory concentrations. Post Hoc t-tests were performed as required using the two-stage step-up false discovery method of Benjamini, Krieger and Yekutieli (Benjamini et al. 2006) in GraphPad software (version 7.0a for Mac)

Chapter 3. The role of ASK1 in the murine auditory system and as a mediator of aminoglycoside induced hair cell death.

Aminoglycosides are the most commonly used antibiotics worldwide, (Xie et al. 2011; Hermann 2007), used in the treatment of life threatening, drug resistant infections (Seddon et al. 2012; Bitner-Glindzicz et al. 2009; Xie et al. 2011). These antibiotics save lives, but a significant and common side effect is the death of sensory hair cells in the ear (Ratjen et al. 2009). As a result, aminoglycoside treated individuals frequently experience permanent high-frequency hearing loss and vestibular impairment. Currently, no treatment exists for the prevention of aminoglycoside-induced hair cell death. Instead, clinicians aim to minimise or avoid aminoglycoside use. However, in some cases, an alternate drug may not be suitable and one single aminoglycoside treatment can cause permanent damage to the ear (Ahmed et al. 2012; Seddon et al. 2012). Therefore, research efforts have focused on elucidating the mechanisms driving aminoglycoside induced hair cell death, in order to identify potential therapeutic targets to mitigate aminoglycoside ototoxicity. These studies have established that aminoglycoside-induced damage causes excessive mitochondrial ROS production in hair cells (Xie et al. 2011; Leitner et al. 2011; Selimoglu 2007; Clerici et al. 1996; Hirose et al. 1997) . Enhanced ROS production causes cellular oxidative stress, which subsequently induces JNK or P38 mediated cell death (Yang et al. 2016; Pirvola et al. 2000; Wang et al. 2003; Francis et al. 2013; Nicholas et al. 2017; Matsui et al. 2004; Sugahara et al. 2006; Branchio et al. 2005; Bas, Van de Water, et al. 2012; Ylikoski et al. 2002; Wei et al. 2005). However, the upstream regulators of JNK and P38 are still being defined in different cell and tissue types.

One example is ASK1, a key mediator of ROS induced JNK and P38 activation (reviewed in chapter 1, page 45). ASK1 may be a useful target for preventing aminoglycoside ototoxicity. However, its role as a mediator of drug-induced hair cell death has not been investigated. Therefore, this project aimed to elucidate whether ASK1 is an important molecule that facilitates aminoglycoside-induced sensory hair cell death. A three part proof-of-principle study using *Ask1* knockout mice was

designed to test whether the down regulation of ASK1 can attenuate aminoglycoside induced hair cell death and hearing loss. Each of the three parts is presented separately within this chapter.

The first aspect of this study involved an analysis of the auditory structures and functional hearing of *Ask1*^{-/-} mice. This characterisation was necessary to ascertain whether ASK1 deficiency causes a notable hearing phenotype and to establish the suitability of *Ask1*^{-/-} hair cells for subsequent experiments. Notably, the overall importance of ASK1 in the inner ear is largely unknown and, prior to this study, *Ask1* knockout mice had not been characterised from an auditory perspective. For the second part of this chapter, the hair cell response to aminoglycoside-induced damage was tested *in vitro* using control and *Ask1*^{-/-} cochlear explants. These experiments were designed to test whether reduced ASK1 is protective against aminoglycoside-induced hair cell death. Finally, the effect ASK1 deficiency with respect to aminoglycoside induced hearing loss was to be tested *in vivo*, using *Ask1* knockout mice. Notably, the final arm of this study required the development of a murine model of systemic aminoglycoside administration, which closely mimicked clinical practice in humans. This component is presented in the form of an original research paper (Ogier, Lockhart, et al. 2019).

3.1. Characterising the *Ask1* knockout hearing phenotype.

The first component of this study was to ascertain whether *Ask1*^{-/-} mice have normal hearing and to determine the suitability of the strain for hair cell specific research. It was hypothesised that *Ask1*^{-/-} mice would have a normal hearing phenotype based on the evidence that ASK1 is primarily activated in response to cellular stress. In order to test this hypothesis, functional hearing assessments were performed, including auditory brainstem response (ABR) and acoustic startle response (ASR) analysis. In addition, cochlear histology was used to investigate overall cochlear structure and organ of Corti explants were collected from neonatal mice to examine hair cell structures *in vitro*.

3.1.1. *Ask1*^{-/-} cochlear morphology appears normal

To ascertain whether ASK1 deficiency impacted normal cochlear development, cochleae were dissected from eight week old C57BL/6 and *Ask1*^{-/-} mice. The cochleae were de-calcified, embedded in agar and sectioned through the mid-modiolar plane. Sections were then stained with Haematoxylin and eosin. No gross deformities were noted during dissection, nor in sectioned *Ask1*^{-/-} cochleae, when compared to C57BL/6 controls (Figure 17). The outer cochlear wall appeared uniform and unremarkable. Likewise, the fluid filled cochlea compartments, Reissner's membrane, spiral ligament and tectorial membrane were observed to be normal. Closer inspection of the organ of Corti also indicated that inner and outer hair cells were present and typically patterned. Other supporting cell types such as Pillar cells, Border cells, Deiter's cells and cells of Claudius were present. Neurons of the spiral ganglion were numerous, myelinated and showed normal projections extending to the organ of Corti (Figure 17 C and D). Overall, cochlear histology indicated that *Ask1* knockout is not detrimental for the development of cochlear structures. However, important features including stereocilia could not be visualised. Therefore, additional histology was performed using *Ask1*^{-/-} neonatal cochlear explants, to interrogate fine auditory structures, such as hair cell patterning, polarity and stereocilia formation.

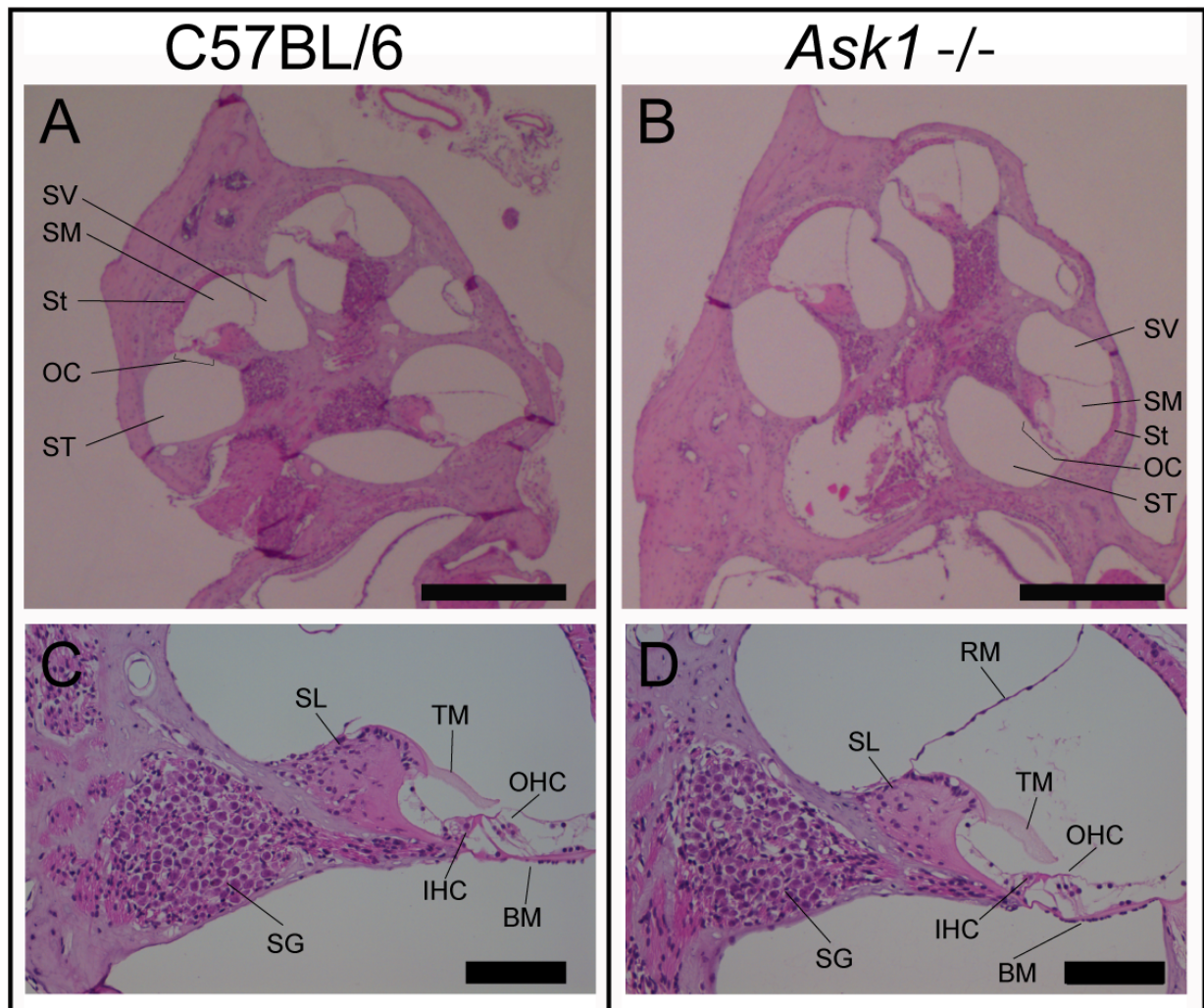


Figure 17. Histology showed normal cochlear morphology in *Ask1*^{-/-} mice.

A) A representative mid modiolar section of a C57BL/6 cochlea **B)** a representative mid modiolar section of an *Ask1*^{-/-} cochlea. **C)** Higher magnification of a single mid-turn in the C57BL/6 mid modiolar section, showing typical organ of Corti and Rosenthal's canal. **D)** Higher magnification of a single mid-turn in a mid modiolar section of an *Ask1*^{-/-} cochlea. Sections were stained with Haematoxylin and eosin. SV: scala vestibuli, SM: scala media, St: stria vascularis, OC: organ of Corti, ST: scala tympani. SL: spiral limbus, TM: tectorial membrane OHC: outer hair cells, SG: spiral ganglion (neurons,) IHC: inner hair cell, BM: basilar membrane, RM: Reissner's membrane. N= 3 mice per genotype. Histology performed in eight week old mice. Scale bar (A-B) = 500 μ m. Scale bar (C-D) = 100 μ m.

3.1.2. *Ask1*^{-/-} cochlear hair cells show typical patterning

Hair cell patterning and polarity is critically important for normal auditory perception. Therefore, cochlear explants were dissected from neonatal (P4) *Ask1*^{-/-} and C57BL/6 mice, to further investigate whether *Ask1* knockout impacts hair cell morphology. Myosin VIIa, a hair cell specific protein routinely used to identify and localise hair cells was used to highlight inner and outer hair cells in C57BL/6 (Figure 18 A) and *Ask1*^{-/-} cochlear explants (Figure 18 B). Hair cells were observed in typical formation, with one row of inner hair cells and three rows of outer hair cells in both control and *Ask1*^{-/-} explants. Very occasionally, four rows of outer hair cells were observed in both *Ask1*^{-/-} and C57BL/6 explants, which is not unusual at this age (Parker et al. 2010). Hair cell counts showed no difference in hair cell number between *Ask1*^{-/-} and C57BL/6 explants ($p = 0.8$) (Figure 25). However, hair cell stereocilia could not be clearly visualised using Myosin VIIa. As stereocilia are critical for hair cell function and highly patterned, further staining was performed to investigate stereocilia morphology. The highly specific F-actin stain phalloidin was used to visualise hair cell stereocilia, because the stereocilia are fine actin bundles. Stereocilia were subsequently observed with correct polarity and in typical staircase formation on the hair cells of both C57BL/6 controls (Figure 19 A-B) and *Ask1*^{-/-} explants (Figure 19 B-C). Phalloidin staining also re-confirmed that inner and outer hair cell patterning was normal in *Ask1*^{-/-} explants (Figure 19 E).

Collectively, the observations made in neonatal and adult cochlear tissues suggest that no gross structural or morphological change occurs as a result of *ASK1* deficiency in *Ask1* knockout mice. Functional testing was then utilised, to further validate these findings.

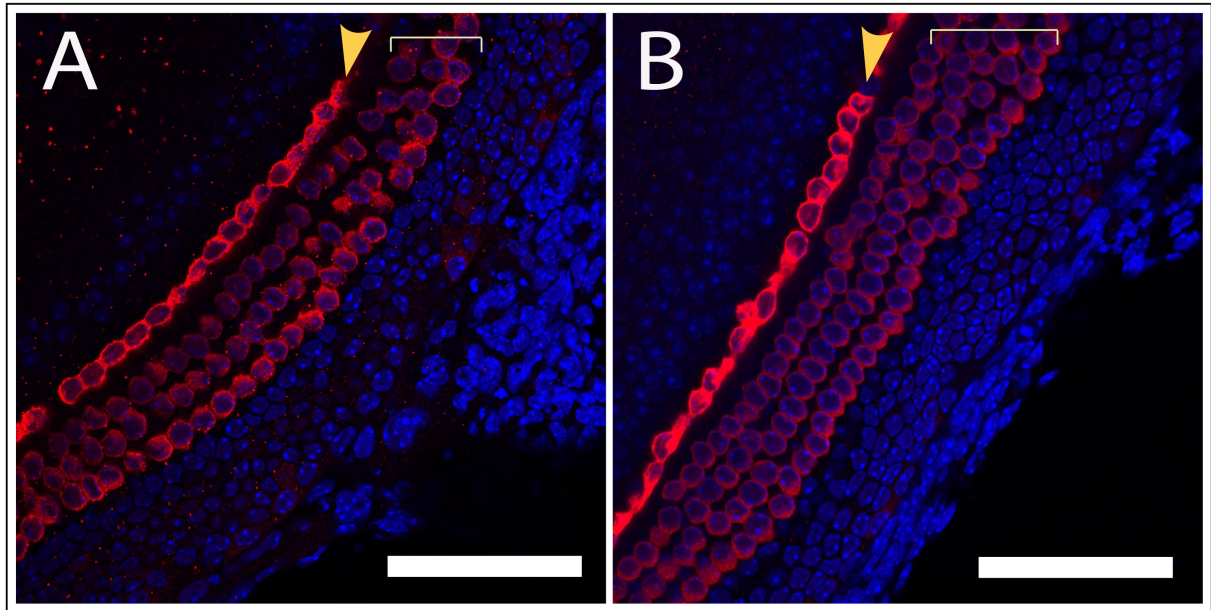


Figure 18. Typical hair cell patterning observed using Myosin VIIa staining in neonatal cochlear explants

Representative confocal images show typical hair cell patterning, with one clear row of inner hair cells (yellow arrow), and three rows of outer hair cells ([]) in **A)** C57BL/6 control explants and **B)** *Ask1*^{-/-} explants, dissected at P4 and cultured overnight. Myosin VIIa (red), DAPI nuclear stain (blue). Scale bars are 70 μm .

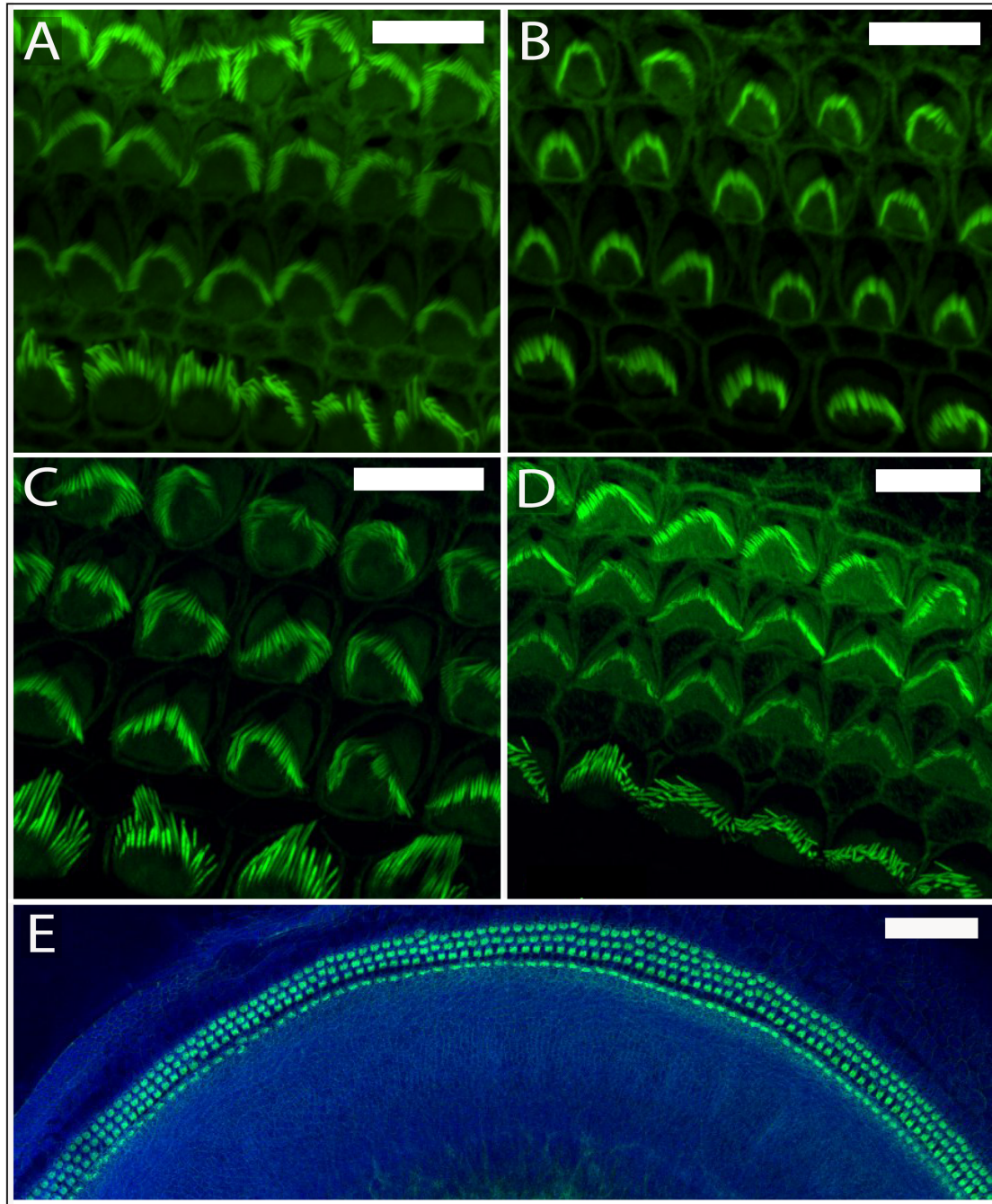


Figure 19. Phalloidin staining of cochlear explants highlights stereocilia.

Stereocilia and hair cells highlighted by phalloidin (green) show correctly oriented bundles, with typical staircase patterning and graded changes in length and width, from apex to base, in explants derived from C57BL/6 controls and *Ask1*^{-/-} mice (P3). **A)** Stereocilia on apical hair cells of the C57BL/6 cochlear explant and **B)** stereocilia in the C57BL/6 basal region. **C)** Stereocilia from apical hair cells in the *Ask1*^{-/-} cochlear explant and **D)** stereocilia in the basal region of an *Ask1*^{-/-} cochlear explant. Scale bars in A-D represent 10 μm. **E)** Phalloidin staining of the upper-mid region of an *Ask1*^{-/-} explant, counterstained with DAPI (blue). Scale bar = 70 μm.

3.1.3. *Ask1*^{-/-} mice have normal hearing thresholds

The auditory brainstem response (ABR) was used to investigate functional hearing in *Ask1*^{-/-} mice. ABR testing provides a quantitative measure of neuronal firing in the auditory pathway, in response to short auditory stimuli.

ABR testing demonstrated that the hearing thresholds of *Ask1*^{-/-} mice were comparable to C57BL/6 controls (Figure 20). Specifically, there was no significant difference between *Ask1*^{-/-} and C57BL/6 control hearing thresholds when measured between 4 and 32 kHz at 4, 10 and 24 weeks of age. Notably, *Ask1*^{-/-} and C57BL/6 controls both exhibited similar high frequency, age-related hearing loss that is characteristic of the C57BL/6 strain. Male and female data was pooled, as hearing thresholds were not affected by gender. The unremarkable hearing thresholds observed in *Ask1*^{-/-} mice indicate that the peripheral structures of auditory perception, from the outer to inner ear are not significantly altered by ASK1 deficiency. However, more subtle changes in auditory perception may not be detected when utilising auditory thresholds alone. Therefore, further analysis of the ABR data was performed, including peak amplitude and peak latency evaluation.

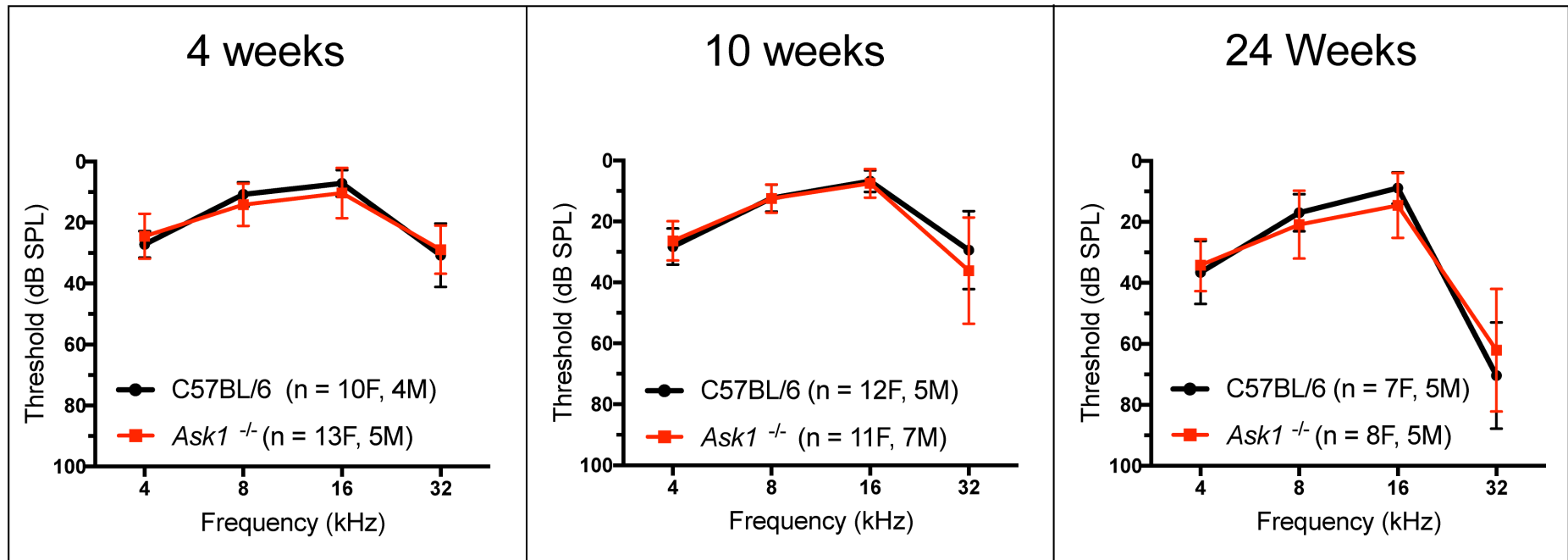


Figure 20. Auditory Brainstem Response thresholds of *Ask1*^{-/-} and C57BL/6 mice.

There was no significant difference between the average hearing thresholds of *Ask1*^{-/-} mice and C57BL/6 mice at 4, 10 and 24 weeks of age. P values for comparisons were 0.2, 0.1, 0.2 and 0.6 (at 4, 8, 16 and 32 kHz respectively) in 4 week old mice; 0.4, 0.9, 0.6 and 0.2 at 10 weeks of age; and 0.5, 0.3, 0.1 and 0.3 at 24 weeks of age (t-test using the Holm-Sidak method for multiple comparison correction). Age related, high frequency hearing loss was evident in both *Ask1*^{-/-} mice and C57BL/6 mice at 24 weeks of age. F = female, M = male, n = sample size. Error bars represent standard deviation.

3.1.4. Auditory signal transduction differs between *Ask1*^{-/-} and C57BL/6 mice

The rapid and synchronous conduction of auditory signals from the cochlea, along the VIIIth nerve and brainstem is a critical aspect of auditory processing and cognition. Therefore, to further interrogate the hearing abilities of *Ask1*^{-/-} mice, peak analysis of ABR waveforms was performed, including the recording of amplitude and latency for each ABR peak (peaks I-V). In the ABR, each recorded peak represents auditory nuclei (Figure 11). Hence, ABR peak analysis provides information regarding auditory signal transduction from the VIIIth cranial nerve to higher order nuclei. Specifically, peak latencies are indicative of auditory signal travel time between auditory nuclei (neuronal conduction velocity) and ABR peak amplitudes are indicative of the number of neurons simultaneously firing.

Peak analysis suggested that neuronal conduction throughout the auditory pathway was different in *Ask1*^{-/-} mice compared to C57BL/6 controls (Figure 21A). Specifically, peak latency (time from the initial auditory stimulus, that the peak occurs) was reduced in *Ask1*^{-/-} mice when compared to C57BL/6 controls, indicating that neural conduction of the ascending auditory signal is faster in *Ask1*^{-/-} mice (Figure 21). This trend was present at peak I, however a cumulative effect was noted, with the difference between C57BL/6 and *Ask1*^{-/-} ABR peak latencies increasing for each subsequent peak. The trend was present in ABR data collected from mice with newly matured hearing, at four weeks of age (Figure 21B) and mice at 10 weeks of age (Figure 21C). Peak latencies recorded in 10 week old C57BL/6 and *Ask1*^{-/-} mice indicated faster signal transduction when compared to the corresponding 4 week latency data, suggesting that signal transmission is faster in the mature mice. However, the trend of faster signal transduction in *Ask1*^{-/-} mice when compared to C57BL/6 controls was still present in the mature cohort (Figure 21C).

Peak amplitude analysis also showed notable ABR differences between *Ask1*^{-/-} and C57BL/6 mice, with the amplitudes of peak II, III and V lower in *Ask1*^{-/-} mice (Figure 22). These peak amplitudes indicate that neuronal activity is reduced in the cochlear nucleus, lateral lemniscus, and the inferior colliculus of *Ask1*^{-/-} mice. Notably, peak III, which is associated with signal transduction from the superior olivary complex to lateral lemniscus, was the most significantly and consistently affected, as determined using student's unpaired t-test (Figure 22 A and B). Interestingly, there was no amplitude difference between *Ask1*^{-/-} and C57BL/6 ABR peak I, suggesting that a comparable auditory signal was produced in the VIIIth nerve. However, ABR peak analysis suggests that auditory signal processing is altered by *Ask1* deficiency. To further investigate the potential of an auditory processing disorder in *Ask1* knockout mice, additional behavioural testing (ASR) was utilised.

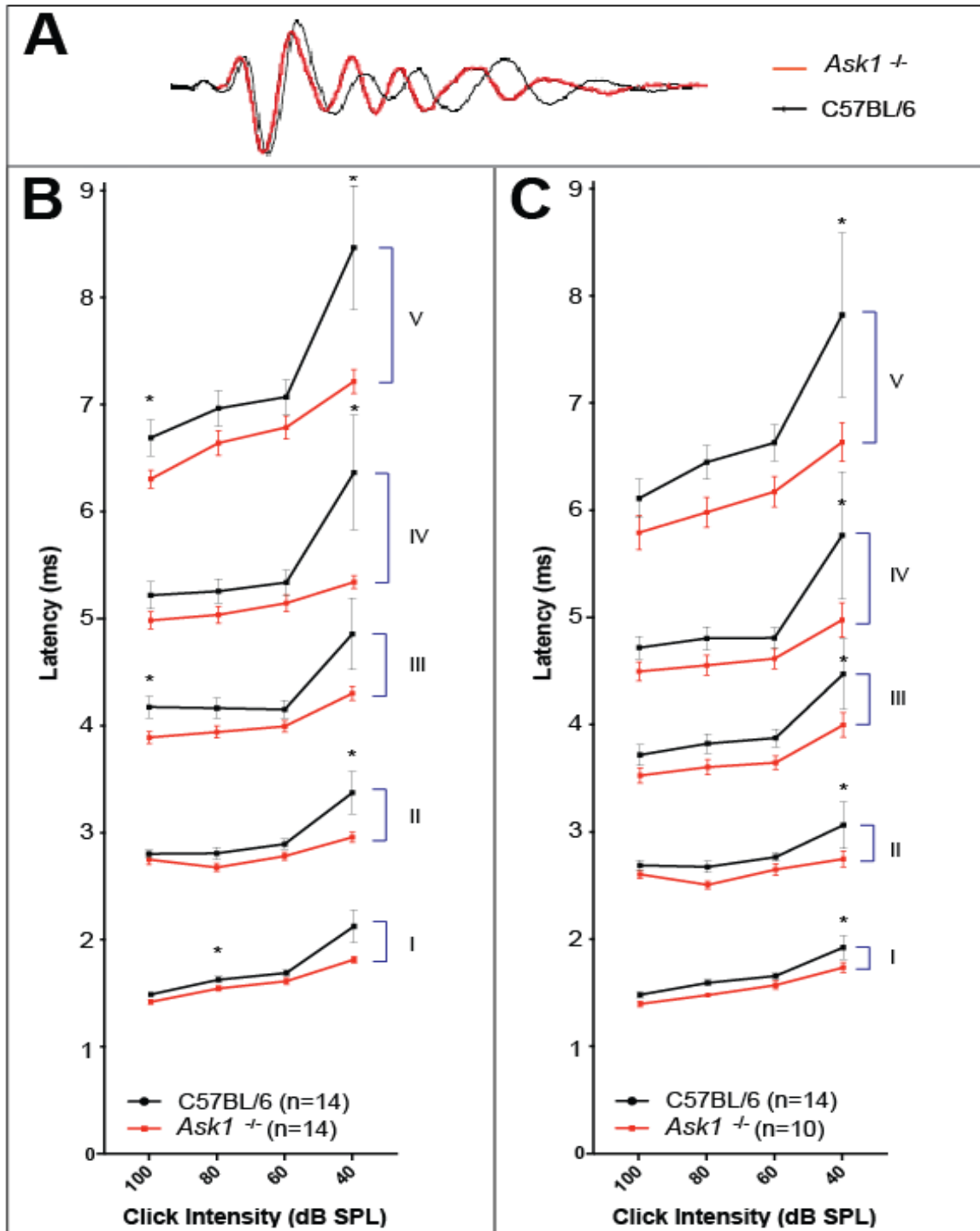


Figure 21. Auditory signal conduction is faster in *Ask1*^{-/-} mice than C57BL/6 controls.

A) An example overlay of ABRs elicited from one four-week old female *Ask1*^{-/-} (red) and C57BL/6 (black) mouse at 80 dB SPL, indicating unique peak heights and signal transduction times. Average ABR peak latencies were subsequently quantified for each mouse strain **B)** Average peak latencies in 4 week old *Ask1*^{-/-} and C57BL/6 mice. **C)** Average peak latencies in 10 week old *Ask1*^{-/-} and C57BL/6 mice. Significance was tested using Student's unpaired t-test, * indicates $p < 0.05$. Error bars represent S.E.M.

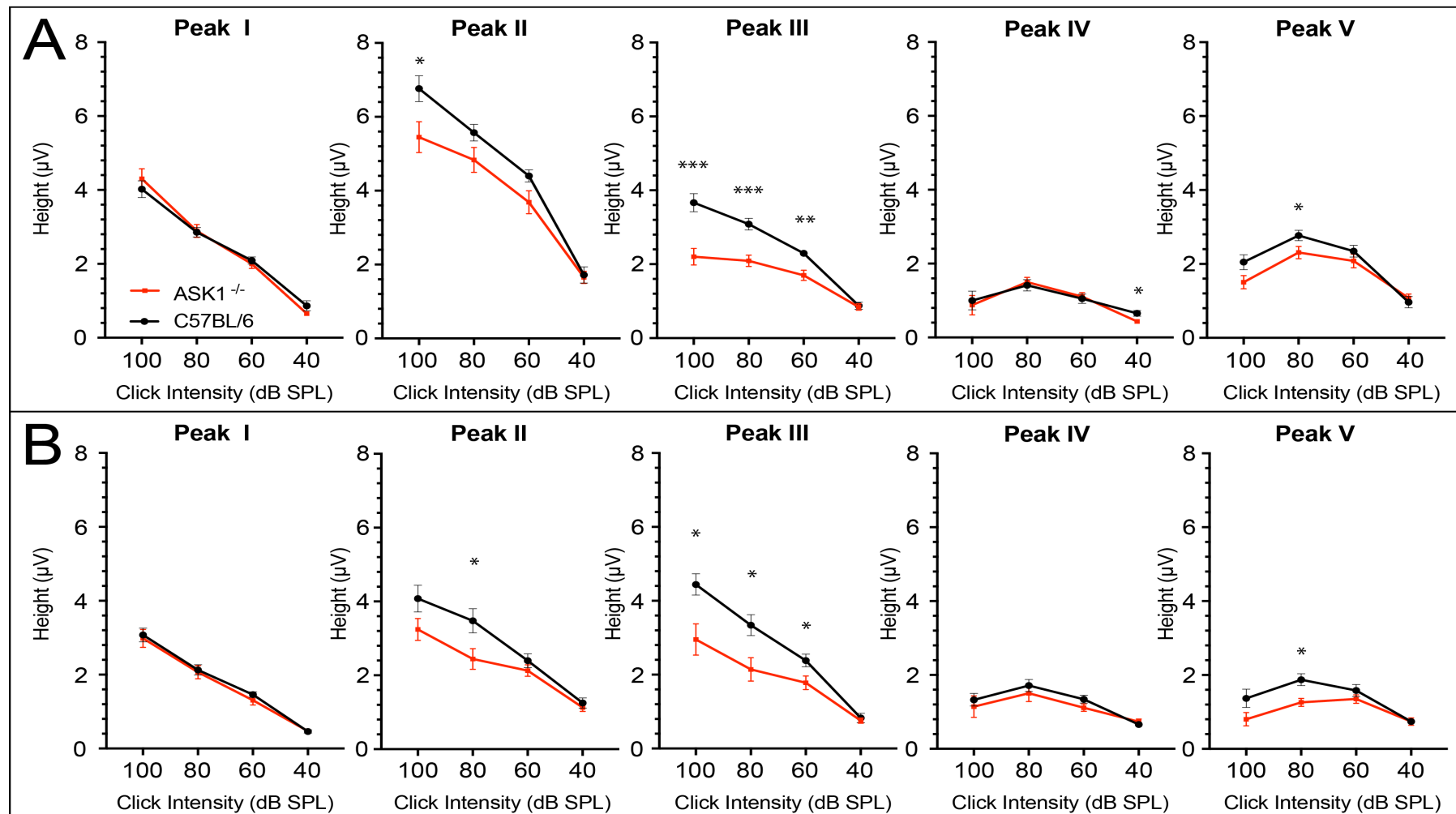


Figure 22. ABR peak amplitudes.

A) ABR peak heights of *Ask1*^{-/-} (n = 14) and C57BL/6 (n = 14) mice at 4 weeks of age. **B)** Peak heights of *Ask1*^{-/-} (n = 10) and C57BL/6 (n = 14) mice at 10 weeks of age. Significance was detected using Student's unpaired t-test, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001. Error bars represent S.E.M.

3.1.5. *Ask1*^{-/-} mice have a stronger startle response than C57BL/6 controls

Having observed unusual auditory signal transduction in *Ask1*^{-/-} mice, the acoustic startle response (ASR) was used to further evaluate auditory processing. The acoustic startle response is an involuntary, evolutionarily conserved muscular reflex induced by sudden sounds. ASR testing quantifies the strength of this response, and is often used to identify dysfunctional neural processing in auditory or psychiatric disorders (Poli & Angrilli 2015). Low ASR's have been associated with hearing loss, depression, bipolar disorder, stress, and reduced motor function, whereas increased ASRs indicate anxiety, hyperacusis, tinnitus or reduced inhibitory signals from the amygdala (Beck 2013; Knudson & Melcher 2016).

In *Ask1*^{-/-} mice, the ASR was notably higher than C57BL/6 controls, suggesting that *Ask1*^{-/-} mice are acoustically hypersensitive (Figure 23). For the male cohort, the difference was statistically significant (p values were 0.0003, 0.0009, 0.02, 0.01, and 0.007 for comparisons at 85, 90, 95, 100 and 115 dB SPL respectively). The same trend was present when comparing female *Ask1*^{-/-} and C57BL/6 mice, however it did not achieve statistical significance (p values were 0.7, 0.2, 0.07, 0.08, and 0.09 for comparisons at 85, 90, 95, 100 and 115 dB SPL respectively). In general, female *Ask1*^{-/-} and C57BL/6 mice had a higher startle response than males, which is not uncommon in mice with a C57BL/6 background (Willott et al. 2003). Overall, the startle response was higher in *Ask1*^{-/-} mice, compared to C57BL/6 controls. The acoustic hypersensitivity observed in *Ask1*^{-/-} mice, combined with the previously described unusual ABR signal transduction, strongly indicate abnormal auditory processing in *Ask1*^{-/-} mice.

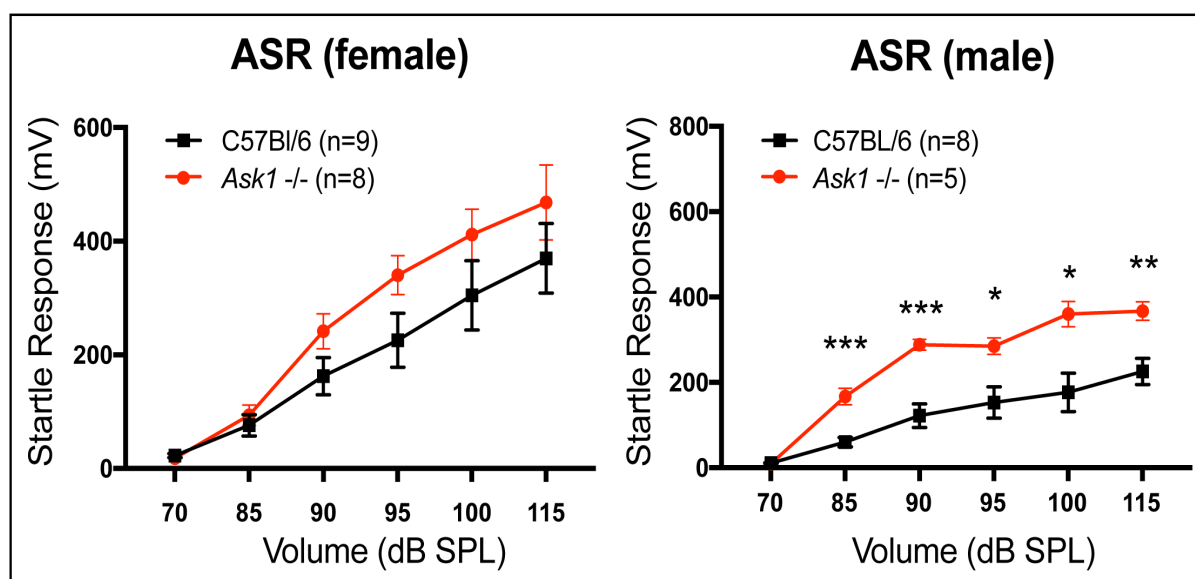


Figure 23. *Ask1*^{-/-} mice may be hypersensitive to loud auditory stimuli.

A trend for *Ask1*^{-/-} mice to have a greater startle response than C57BL/6 controls was present in both male and female mouse cohorts. However the difference was only statistically significant in the male cohort, as detected using multiple unpaired t-tests, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$. Error bars represent S.E.M.

3.1.6. Discussion

In this section, morphological and structural analysis of the cochlea was performed *ex vivo* and functional techniques were used *in vivo* to test if *Ask1* knockout compromises the murine auditory system. Qualitative histology from adult whole cochleae and neonatal cochlear explants did not identify any significant differences in the cochlea and neurosensory epithelium of *Ask1*^{-/-} mice when compared to C57BL/6 age matched controls. In addition, quantitative ABR testing showed that *Ask1*^{-/-} mice had normal hearing thresholds, indicative of a fully functioning ear. However, ABR peak analysis indicated that neural transduction differed between *Ask1*^{-/-} and C57BL/6 control mice. Peak analysis demonstrated that auditory signal transduction is more rapid in *Ask1*^{-/-} mice, and that the signal amplitude is reduced in the cochlear nucleus, superior olivary complex and the inferior colliculus of *Ask1*^{-/-} mice. These nuclei are integral for sensory-gating based on efferent feedback, which filters and improves the auditory signal (Terreros & Delano 2015; Suthakar & Ryugo 2017; Aedo et al. 2016).

In humans, reduced peak amplitudes have been associated with VIIIth nerve fibre number and auditory synaptopathy, in individuals with otherwise normal hearing

thresholds (Verhulst et al. 2016). Higher peak amplitudes are observed in individuals with speech delay of unknown origin (Abadi et al. 2016). Overall, aberrant signal transduction in human ABR's is indicative of an auditory processing disorder caused by nerve dysfunction, myelin disorder, synaptopathies or other unknown factors (Rattay & Danner 2014; Moller et al. 1988; Sininger et al. 1997; Mehraei et al. 2016). Similarly, the ABR data collected in this thesis indicates that the loss of *Ask1* potentially results in central auditory processing disorder in *Ask1* knockout mice. Therefore, ASR testing was used to explore the *Ask1*^{-/-} auditory transduction phenotype. In mice, ASR testing is generally used to assess neural processing dysfunction in auditory or psychiatric disorders (Poli & Angrilli 2015). As a complex reflex arc, the startle response is dependent on efferent feedback systems integrated with the central auditory system. However, other factors such as muscle strength, emotional status and memory function can contribute to an unusual ASR (Le Duc et al. 2016; Decker et al. 1995; Angrilli et al. 1996).

For *Ask1*^{-/-} mice, ASR testing showed that male mice had a much stronger startle response than that of C57BL/6 controls. A similar trend was present in the female cohort. An overall gender bias was present, with both C57BL/6 and *Ask1*^{-/-} females having a stronger startle response than males. This gender bias correlates with previous reports that female mice on a C57BL/6 background have a higher startle than males (Willott et al. 2003). Likewise, ASR is stronger in women than men (Kofler et al. 2001; Swerdlow et al. 1993; Swerdlow et al. 1999).

Collectively, the ABR and ASR data suggests that there is some form of auditory processing disorder in *Ask1*^{-/-} mice. Impaired sensory gating could account for the fast transduction in *Ask1*^{-/-} mice, with reduced signal control causing sensory overload and acoustic hypersensitivity. Interestingly, impaired sensory gating has been observed in people with schizophrenia, and variants in the gene encoding ASK1 or protein phosphatase with EF-hand domain 2 (PPEF2, a modulator of ASK1) have been reported in humans with schizophrenia (Morris & Pratt 2014; Jones et al. 2016). A recent genome wide meta-analysis also correlated an ASK1 polymorphism with poor cognitive empathy in males, which is a common trait in schizophrenia (Warrier et al. 2018). Additionally, Kumakura et al. 2010 identified that ASK1 is important for regulating locomotor activity, memory, and motor coordination mediated by dopaminergic transmission (Kumakura et al. 2010). In the Kumakura study, *Ask1*^{-/-}

mice were hyperactive, with enhanced motor coordination. Furthermore, dopamine and the dopamine metabolite DOPAC increased in *Ask1*^{-/-} mice (Kumakura et al. 2010). These observations suggest that ASK1 has an important role in neural circuitry, providing further evidence in support of an auditory processing disorder. However, similar to many human auditory processing disorders, delineating the neural mechanisms involved will be a challenge. In particular, auditory processing involving efferent feedback control and sensory gating is very difficult to study (Lopez-Poveda 2018). Ostensibly, the ASR neural circuit is simple and well defined. However, the integration of auditory cues with other sensory modalities in the brain is incredibly complex and the efferent interactions of these modalities with ascending auditory cues and other auditory based reflex arcs remains poorly understood (Hironaka 2008; Steube et al. 2016; Yeomans & Frankland 1995).

Collectively the data produced in this chapter, suggests that *Ask1* knockout does not result in gross morphological alterations to the peripheral auditory system. However, the functional data does suggest that ASK1 deficiency results in a subtle but significant alteration of auditory transmission. Based on this observation, it appears that the initial hypothesis: ASK1 deficiency does not affect the development and function of the auditory pathway in mice, must be rejected. However, it is important to note that this data was not generated using littermate controls. Therefore, the strain effect is a potential confounding variable.

The strain effect is a term used to describe phenotypic changes caused by unknown genetic differences between two inbred mouse strains, likely caused by genetic drift. Therefore, littermate control comparisons are considered to be the gold standard for mouse experimentation. Not only does this ensure a level of genetic homogeneity, but it also avoids epigenetic influences. In this study, the *Ask1*^{+/-} breeding pairs available did not produce sufficient offspring for the phenotyping experiments required and small litters often lacked either *Ask1*^{+/+} or *Ask1*^{-/-} genotypes. With insufficient littermate controls available, *Ask1*^{-/-} mice were compared with C57BL/6 controls. The *Ask1*^{-/-} mice used in this study were initially generated using a cloned *Ask1* knockout mouse embryonic cell line (C57BL/6N) and subsequently backcrossed with C57BL/6J mice from the Walter and Eliza Hall Institute of Medical Research (WEHI). To minimise strain effects, C57BL/6^{WEHI} mice were used as controls in this study. However, it is possible that C57BL/6^{WEHI} sub-colonies exist. Therefore, whilst every

effort was made to limit genetic differences, the strain effect may have influenced experimental outcomes. As a result, it is difficult to ascertain whether the differences observed between *Ask1*^{-/-} mice and C57BL/6 mice are caused by ASK1 deficiency or the strain effect. Importantly, the poor reproductive performance of the initial *Ask1*^{-/+} breeding mice does not appear to be a genotype specific phenotype. *Ask1*^{-/+} breeding pairs readily reproduced, and subsequent *Ask1*^{-/+} mice have been productive. Data collection is now ongoing in littermate *Ask1*^{-/-} and *Ask1*^{+/+} mice. Notably, the preliminary data collected from *Ask1*^{+/+} littermate controls is comparable to that collected from C57BL/6 mice in the experiments described above.

Importantly, the central auditory processing phenotype observed in the *Ask1*^{-/-} mice does not compromise the research questions addressed in this thesis. Specifically, the next component of this study (3.1.2) investigates the role of ASK1 in aminoglycoside-induced sensory hair cell death, utilising *Ask1*^{-/-} mice. The morphological and functional observations made in this chapter indicate that *Ask1*^{-/-} hair cells are functional, and therefore suitable for this purpose.

3.1.7. Future Directions

ABR and ASR characterisation of *Ask1*^{-/-} mice is now being performed and compared to littermate controls, to eliminate the potentially confounding strain effect. Additionally, mouse brains have been collected for serial segmentation and staining. Initial investigations will focus on neuron density and myelination in auditory specific nuclei, using an anti-myelin basic protein antibody (o8402, Biolegend) in conjunction with haematoxylin and eosin staining. There is some evidence that ASK1 has a role in neuronal development. For example, ASK1 mediated P38 expression is thought to contribute to the regulation of neural stem cell differentiation (Song et al. 2013). Furthermore, suppression of ASK1 (using ASK1-small interfering RNA) increases Microtubule Associated Protein 2 (MAP2) expression in neural stem cells derived from mice (Song et al. 2013). MAP2 has been heavily associated with dendrite elongation and anchoring (Harada et al. 2002; Caceres et al. 1984; Teng et al. 2001). Thus, it is possible that dendrite length is altered in *Ask1*^{-/-} mice, causing the faster signal transduction phenotype. However, this remains to be tested.

Further functional assessment of *Ask1*^{-/-} mice could include an analysis of acoustic reflexes and otoacoustic emissions (OAE). This would provide information regarding efferent feedback control over the stapedial reflex, central masking effects, and olivocochlear bundle function (Kumar & Barman 2002; Karabulut et al. 2019; Ciuman 2010; Collet et al. 1990; Smith et al. 2017). For example, efferent signals (from the olivocochlear bundle) have an inhibitory effect on outer hair cell motility, so stimulation of the olivocochlear bundle can reduce the VIII nerve response, basilar membrane motility, and OAE amplitude (Ciuman 2010; Collet et al. 1990). For acoustic threshold analysis, noise is used to provide contralateral suppression of the acoustic reflex thus reducing otoacoustic emissions. Electrocochleography (for measuring summing potential and the cochlear microphonic) is another way that the auditory signal produced in the inner ear could be analysed. For example, CAP measurements can identify cochlear synaptopathy, or an abnormal CAP combined with a strong cochlear microphonic or otoacoustic emissions (OAEs) could indicate auditory neuropathy (Pienkowski et al. 2018). Overall a combination of these tests may assist in delineating the effects of ASK1 deficiency in the auditory pathway.

Anterograde and retrograde tracers could also prove useful *in vivo*, highlighting the architecture of the auditory circuit in *Ask1*^{-/-} mice. Alternatively, individual efferent fibre responses can be measured with careful electrode placement. However, efferent fibre recordings tend to represent the medial olivocochlear fibres, because the unmyelinated and narrow lateral fibres are difficult to access (Lopez-Poveda 2018). Overall, a battery of functional and mechanical tests is available to further interrogate the role of ASK1 in the development and function of the murine auditory system.

3.1.8. Concluding Remarks

Ask1^{-/-} mice potentially have an interesting auditory processing phenotype that warrants further investigation. However, ABR hearing thresholds, cochlear histology and immunohistochemistry suggest that *Ask1*^{-/-} cochlear hair cells are normal. Therefore, *Ask1*^{-/-} mice are a suitable model for use in the context of hair cell specific research.

3.2. Investigating the effect of ASK1 deficiency on resistance to aminoglycoside-induced sensory cell death *in vitro*.

Aminoglycosides are the most commonly used antibiotics worldwide and significantly contribute to the problem of ototoxicity (Xie et al. 2011; Hermann 2007; Kranzer et al. 2015). Therefore, a strategy for mitigating aminoglycoside ototoxicity is needed (Xie et al. 2011; Ding et al. 2002). It is well established that aminoglycosides readily penetrate and persist in cochlear hair cells (Imamura & Adams 2003). As a result, mitochondrial protein synthesis is disrupted in these cells, causing excessive ROS production that ultimately results in hair cell death (Xie et al. 2011; Leitner et al. 2011; Selimoglu 2007; Clerici et al. 1996; Hirose et al. 1997). However, the exact mechanisms of hair cell death are not well defined. Delineating these mechanisms, and identifying key hair cell death mediators may provide novel opportunities for preventing aminoglycoside ototoxicity.

Previous studies have shown that the inhibition of P38 or JNK phosphorylation is protective against aminoglycoside-induced hair cell death. However, JNK or P38 inhibition can have toxic effects in off-target-tissues (Bubici & Papa 2014; Ma et al. 2009). Therefore, upstream signals and molecules that drive the apoptosis-specific roles of JNK and P38 in hair cells might function as better targets for ameliorating hair cell loss due to aminoglycoside use. One candidate target is ASK1, an upstream mitogen-activated protein kinase kinase kinase (MAP3K), specifically activated by ROS and subsequently activating downstream MAP kinases (JNK and P38 in particular) (Hayakawa et al. 2012; Obsil & Obsilova 2017). Notably, a recent study demonstrated that ASK1 was the only MAP3K with significantly increased RNA expression after gentamycin treatment in hair cells, suggesting a functional role for ASK1 in the aminoglycoside damaged hair cell (Tao & Segil 2015). However, beyond this observation, the role of ASK1 in hair cell death has not been investigated.

Collectively, the available data is consistent with the hypothesis that ASK1 is activated in response to redox signals in aminoglycoside-treated hair cells, which induces JNK

phosphorylation, resulting in hair cell death. To test the hypothesis that reduced ASK1 might be protective against aminoglycoside-induced hair cell death, organ of Corti explants were collected from neonatal *Ask1*^{-/-} and C57BL/6 mice and cultured on organotypic membranes, with or without neomycin treatment. Hair cell death was observed using an immunofluorescent TUNEL stain and hair cell survival was quantified based on counts of Myosin VIIa positive cells. Immunohistochemistry was used to investigate the effect of *Ask1* knockout on JNK activation in neomycin treated explants.

3.2.1. *Ask1*^{-/-} neurosensory epithelia are resistant to neomycin ototoxicity

Due to the limited timeframe of hair cell viability *in vitro* (approximately 1 week), most explant studies use extremely high doses (1 mM) of neomycin to quickly induce hair cell death. In this study, 1 mM of neomycin quickly induced outer hair cell death in wild type C57BL/6 explants, as indicated by visible TUNEL staining, 20 hours after neomycin treatment commenced (Figure 24A). Qualitatively there was a notable loss of Myosin VIIa-positive hair cells and after 45 hours of 1 mM neomycin treatment, TUNEL staining was prevalent. By 69 hours, this treatment caused near complete loss of hair cells, with very few Myosin VIIa positive hair cells remaining (Figure 24 E). Notably, the remaining Myosin VIIa positive hair cells in C57BL/6 explants, displayed cellular condensation or fragmentation, indicating that apoptosis was occurring.

Ask1^{-/-} explants showed markedly different responses to 1 mM neomycin treatment when compared with C57BL/6 controls. There was no visible TUNEL stain at the 20 hour time-point (Figure 24B), very little at the 45 hour time-point (Figure 24D) and none at the 69 hour time-point (Figure 24F). At all time-points observed, there appeared to be more Myosin VIIa-positive hair cells in the *Ask1*^{-/-} explants compared to C57BL/6 explants. Furthermore, the nuclear compartment of remaining hair cells appeared less fragmented at the 20 and 45 hour time-point in the *Ask1*^{-/-} group in comparison to neomycin treatment and time-matched controls. However, at the 69

hour time-point, the nuclei of Myosin VIIa-positive hair cells were significantly condensed or fragmented in the *Ask1*^{-/-} explants. This observation suggests that hair cell death is delayed, but not prevented in neomycin-treated *Ask1*^{-/-} explants. This observation also indicated that TUNEL staining was not the most suitable means to assess hair cell death, because hair cells that were lost were not always TUNEL-positive at the time points captured. In contrast, Myosin VIIa immunohistochemistry provided robust and specific hair cell labelling. As a result, the subsequent methodology for quantifying hair cell survival was based on Myosin-VIIa staining.

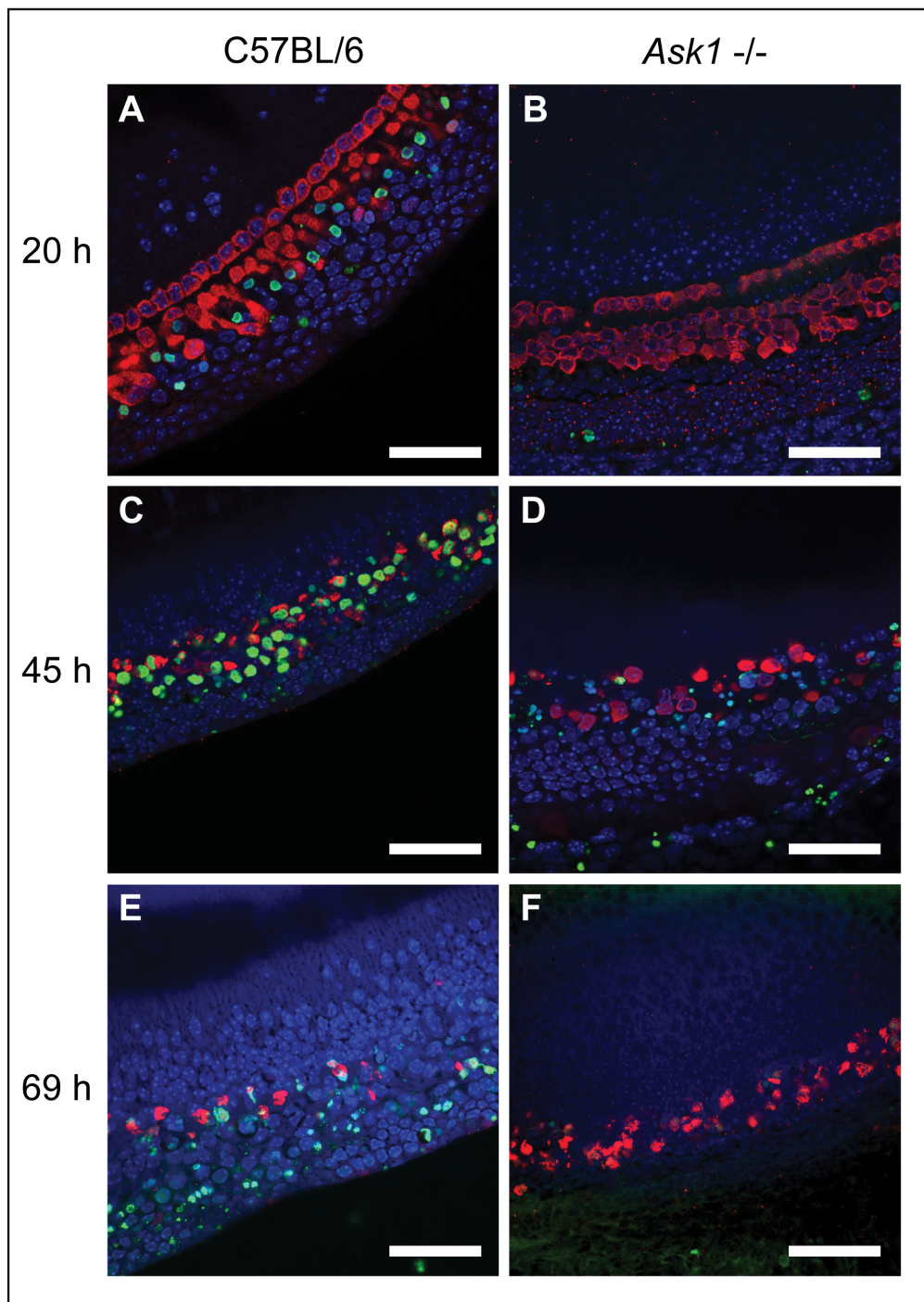


Figure 24. The effect of 1 mM neomycin on hair cell survival *in vitro*.

Representative images of C57BL/6 cochlear explants (left) compared with *Ask1*^{-/-} cochlear explants (right) treated for 20, 45 and 69 hours with 1 mM of neomycin. Green = TUNEL, Blue = DAPI, Red = Myosin VIIa (hair cells). N = 3-5 explants per treatment group, images were collected from the mid-turn of each explant.

Scale bar = 50 μM.

3.2.2. Quantification of hair cell survival

To quantify hair cell survival, organ of Corti explants were collected from neonatal *Ask1*^{-/-} and C57BL/6 mice and cultured on organotypic membranes as described previously (Ogier, Burt, et al. 2019). Explants were cultured overnight before neomycin or control treatment (DMSO) was added. The explants were then cultured for a further 24, 48 or 72 hours, with the allotted treatment. Repeated hair cell cultures were performed as C57BL/6 or *Ask1*^{-/-} neonatal pups became available. Therefore, to limit experimental variation, explants from each litter were divided and subject to different treatments. Ultimately, data was collected from 5-9 explants per treatment group, derived from 3-4 mouse litters. After culturing for the appropriate time period, explants were stained with Myosin VIIa and the number of Myosin VIIa positive cells in two 180 µm x 90 µm fields of view were counted in the explant mid-turn, with the average of these recorded. The explant mid-turn was utilised in this experiment, because hair cells of the basal turn can sustain damage as a result of mechanical dissection and apical hair cells are highly resistant to aminoglycoside death. The data is presented below, in two formats. First, as raw hair cell counts for each group (ie, DMSO or neomycin treatment groups for both strains). Then, hair cell counts from neomycin treated explants were calculated as a percentage survival ($\pm$ S.E.M), using hair cell counts from the DMSO treated explants as the expected survival number. In text, these numbers are rounded to the nearest whole number. The investigator was blinded to genotype during counting.

3.2.2.1. *Ask1*^{-/-} hair cells are resistant to high-dose-neomycin-induced cell death.

Treatment with 1 mM of neomycin resulted in a progressive loss of both inner and outer hair cells in *Ask1*^{-/-} and C57BL/6 cochlear explants over three days (Figure 25). For inner hair cells, there was no difference between the hair cell counts from C57BL/6 and *Ask1*^{-/-} explants, that had been treated with vehicle only ($p = 0.4$). However, after 24 hours of 1 mM neomycin treatment, there were significantly more Myosin VIIa-positive inner hair cells remaining in the *Ask1*^{-/-} explants ($91\% \pm 2\%$) in comparison to C57BL/6 controls ($77\% \pm 3\%$) (mean $\pm$ S.E.M, $p=0.006$, $n = 8$ *Ask1*^{-/-} and 7 C57BL/6) (Figure 25). In contrast, there was no significant difference between *Ask1*^{-/-}

and C57BL/6 inner hair cell counts at the 48 and 72 hour time-points ($p = 0.8$ and 0.7 respectively). For outer hair cells, the number of Myosin VIIa-positive cells declined during the neomycin treatment, in both C57BL/6 and *Ask1*^{-/-} explants. However, the rate of hair cell death was significantly different, with *Ask1*^{-/-} outer hair cells being more resistant to neomycin toxicity, evidenced by a slower rate of loss. After 24 hours treatment, $77\% \pm 6\%$ of *Ask1*^{-/-} hair cells remained, compared with $52\% \pm 7\%$ of C57BL/6 hair cells ($p = 0.02$). The difference was not significant at the 48 hour time point, although the trend remained, with $26\% \pm 3\%$ *Ask1*^{-/-} outer hair cells persisting compared with $19\% \pm 4\%$ of C57BL/6 outer hair cells ($p = 0.2$). After three days of neomycin treatment, only $3\% \pm 1\%$ of the outer hair cells remained in C57BL/6 explants compared to $16\% \pm 2\%$ in the *Ask1*^{-/-} explants ($p = 0.0002$).

Collectively, the data shows that inner hair cells are less susceptible to neomycin-induced cell death than outer hair cells, in this model. This observation was consistent for both C57BL/6 and *Ask1*^{-/-} hair cells. Interestingly, *Ask1* knockout provided protection against neomycin mediated inner hair cell death at the earliest time-point (24 hours), but not the latter time-points (48-72 hours). Conversely, *Ask1* knockout provided significant protection against neomycin mediated outer hair cell death at both the early (24 hours) and late (72 hours) stages of the time-course.

It is important to note that the 1 mM neomycin treatment utilised in this experiment is a very high dosage. Aminoglycoside use in humans is generally maintained at the lowest effective dose possible, over a number of weeks. Although not formally reported in the literature, it is extremely unlikely that aminoglycoside concentrations would reach such high levels in the human ear – as aminoglycosides must cross the stria vascularis, which presents an important blood-barrier *in vivo*. For example, kinetic studies of gentamicin in rats show that a gentamycin plasma level of 6.2-6.5 $\mu\text{g/ml}$ achieves only 1.5 $\mu\text{g/ml}$ in the perilymph and 1.2 $\mu\text{g/ml}$ in the endolymph (Tran et al. 1983). Therefore, a dose response experiment was performed to determine if *Ask1* knockout provided greater resistance to neomycin induced hair cell death, using lower neomycin concentrations.

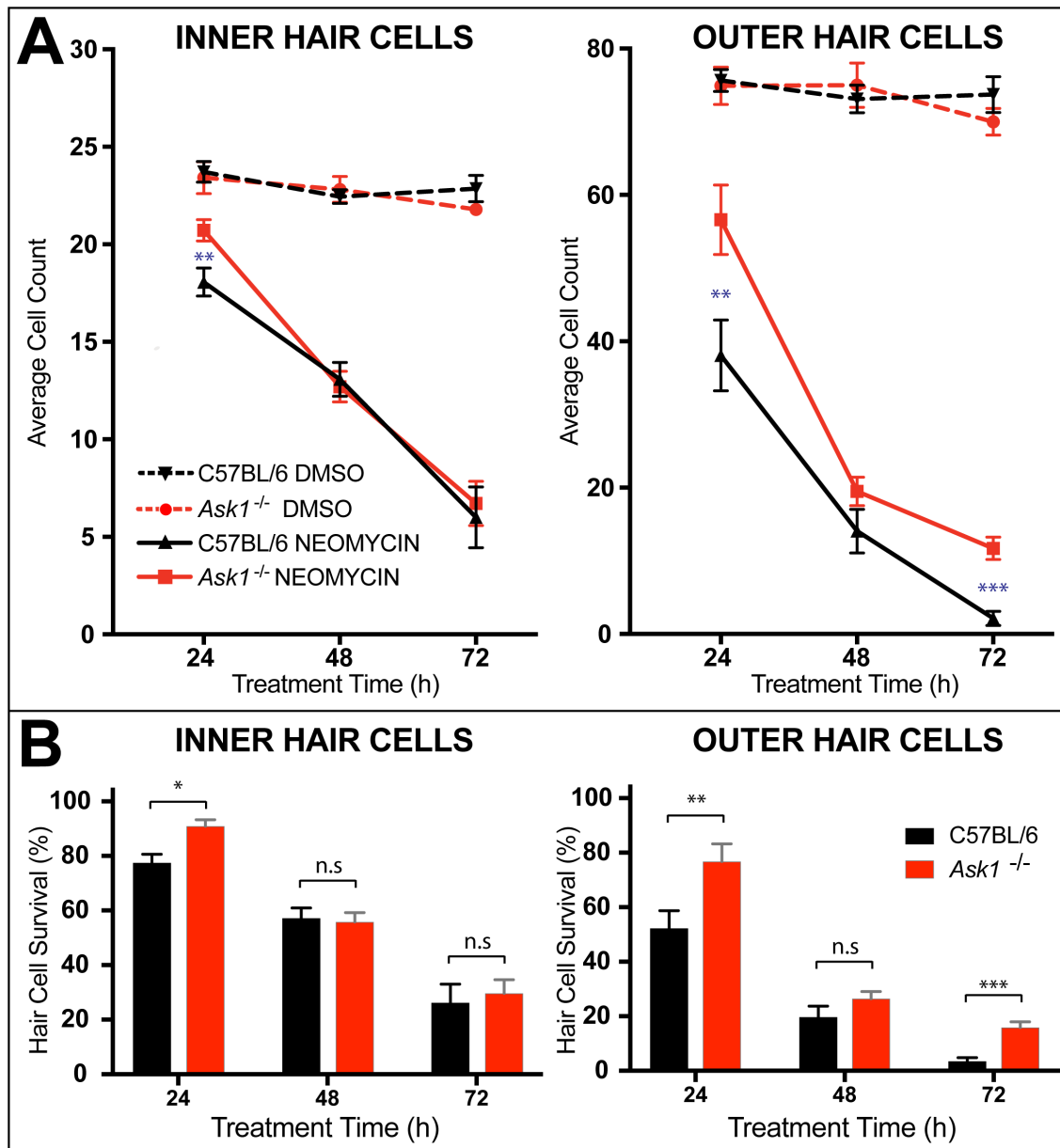


Figure 25. *Ask1*^{-/-} hair cells are resistant to neomycin-induced cell death compared to C57BL/6 controls.

A) Average numbers of Myosin VIIa-labelled inner (left) and outer (right) hair cells after 24, 48 or 72 hours treatment with either vehicle (DMSO) or 1 mM neomycin. **B)** Hair cell counts presented as percentage survival based on average hair cell counts in the DMSO control group. Mean with standard error of the mean shown (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s = not statistically significant as per a standard unpaired t-test). N (for 24, 48 and 72 h timepoints respectively) = 7, 9, 7 (C57BL/6 DMSO), 6, 8, 7 (*Ask1*^{-/-} DMSO), 8, 7, 6 (C57BL/6 neomycin) and 7, 5, 7 (*Ask1*^{-/-} neomycin). Cell counts represent the averages of two 180 μm x 90 μm fields of view taken from each explant in the mid-turn region.

3.2.2.2. *Ask1*^{-/-} hair cells are resistant to moderate-dose-neomycin-induced cell death.

In vivo, aminoglycosides are thought enter hair cells from the endolymph, via the large, non-ion-selective MET channels (previously discussed in chapter 1, page 32). Notably, aminoglycoside levels in the endolymph do not reach that of plasma concentrations and therefore, the commonly utilised experimental neomycin dose of 1 mM represents an extreme and nonphysiological condition (Tran et al. 1983).

Therefore, to ascertain whether the protective effect of ASK1 deficiency is more pronounced against lower aminoglycoside doses, a dose response experiment was performed to identify conditions that would achieve significant hair cell death within the experimental time frame of three days, but without complete loss of all inner ear hair cells on the neurosensory epithelium. C57BL/6 cochlear explants were treated with neomycin ranging from 100 μ M up 800 μ M (Figure 26). The lowest treatment tested (100 μ M neomycin) induced no notable hair cell death within a three-day treatment period. Conversely, 600-800 μ M neomycin-resulted in complete hair cell loss within three days (Figure 26). A neomycin concentration of 400-500 μ M induced near complete outer hair cell death, whilst a proportion of inner hair cells remained. Based on these observations, 400 μ M neomycin was selected as the lowest, most readily quantifiable dose for *in vitro* neomycin treatment.

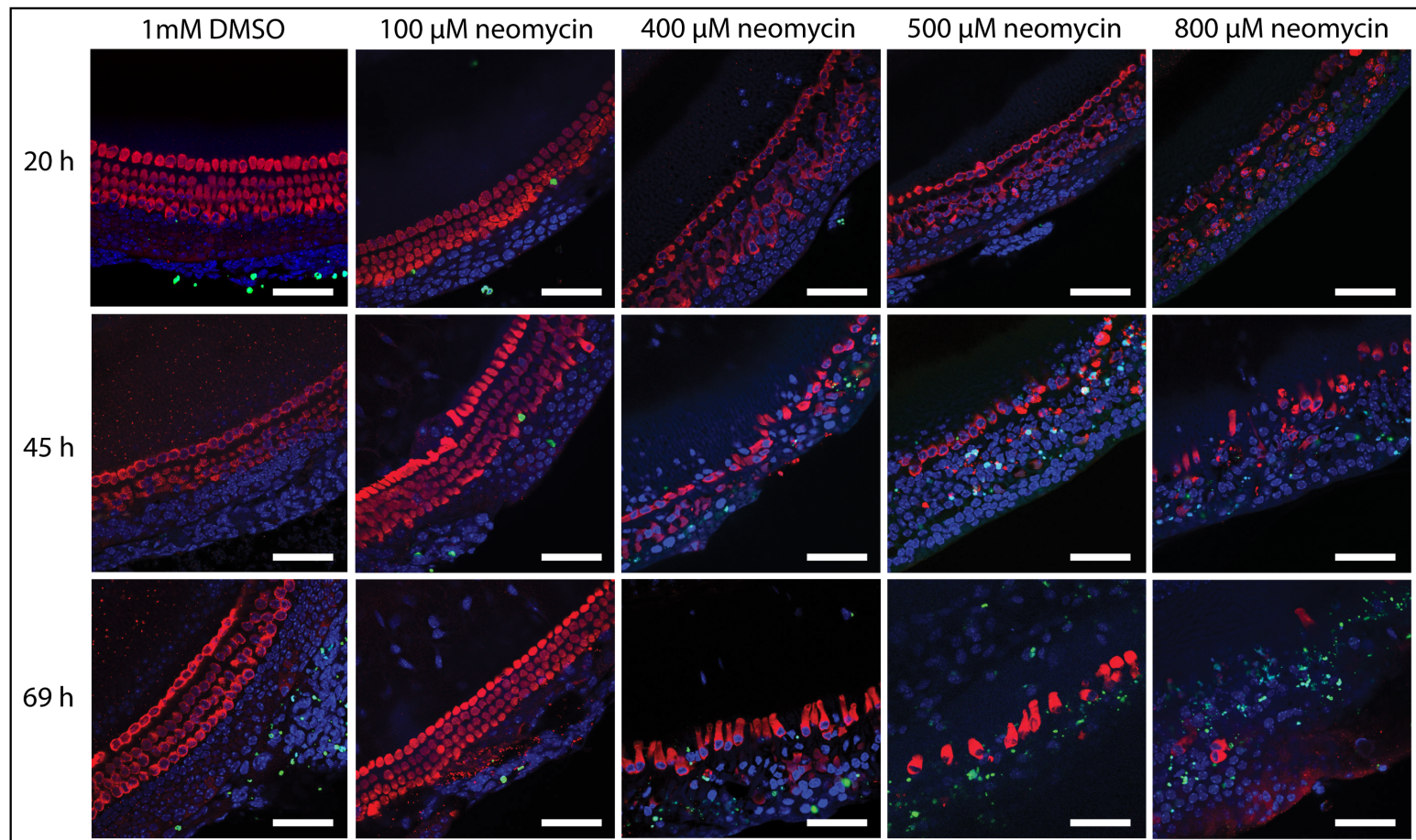


Figure 26. Dose dependant loss of hair cells in C57BL/6 explants treated with neomycin over 69 hours *in vitro*.

Representative images show increasing neomycin dosage (left to right), starting with a vehicle-only control on the left, to 800 μ M neomycin on the right. Neomycin exposure time is represented top to bottom, with imaging performed at 20 hours treatment (top) increasing to 69 hours treatment (bottom). DMSO controls showed good hair cell survival over 69 hours. Alternatively, there was a marked dose-dependant effect on hair cell survival. Green = TUNEL, Blue = DAPI, Red = Myosin VIIa. N = 2-4 per treatment, scale bar = 50 μ M.

Ask1^{-/-} neurosensory epithelium explants were next challenged with a 400 μ M neomycin dose (Figure 27). Hair cells were labelled using Myosin VIIa and quantified in the same manner as for the previously described experiment utilising 1 mM of neomycin (Page 103). Counts were performed blinded to genotype, in 5-9 explants (per treatment group), derived from 3-4 mouse litters. As previously seen in the 1 mM neomycin experiments (Figure 25), the inner hair cells of both C57BL/6 and *Ask1*^{-/-} explants were more resistant to the neomycin treatment than outer hair cells (Figure 28B). However, 400 μ M neomycin did cause progressive loss of both inner and outer hair cells in both *Ask1*^{-/-} and C57BL/6 cochlear explants.

In particular C57BL/6 explants showed a progressive decline in outer hair cell number until few outer hair cells remained (Figure 27A, C and E). In contrast, neomycin-induced outer hair cell death appeared to be reduced in *Ask1*^{-/-} explants (Figure 27B, D and F). Notably, at the 69 hour time point many outer hair cells were lost in the C57BL/6 explants treated with 400 μ M neomycin but numerous outer hair cells were positive for Myosin VIIa in the *Ask1*^{-/-} explants (Figure 27E and F). Hair cell quantification demonstrated that there were significantly more Myosin VIIa outer hair cells in *Ask1*^{-/-} explants when compared with C57BL/6 control explants at the 24 hour and 72 hour time-points ($p = 0.02$ for both time points). In particular, $60\% \pm 5\%$ of *Ask1*^{-/-} outer hair cells survived three days of culture with 400 μ M neomycin compared to $39\% \pm 5\%$ of C57BL/6 outer hair cells. There was no significant difference between *Ask1*^{-/-} and C57BL/6 outer hair cell counts at the 48 hour time-point ($p = 0.2$), which corresponds with hair cell data from the previous high dose experiments. Inner hair cells withstood 24 hours of 400 μ M of neomycin treatment in both *Ask1*^{-/-} and C57BL/6 explants. However, after 48 hours, C57BL/6 inner hair cell counts were lower than *Ask1*^{-/-} hair cell counts. This trend strengthened at the 72 hour time point, but was not statistically significant ($p=0.067$).

Notably, the lower neomycin dose tested did not appear to improve experimental outcomes. However, the combined data obtained from both 400 μ M and 1 mM neomycin treatments consistently indicates that ASK1 deficiency significantly reduces hair cell death in *Ask1*^{-/-} cochlear explants.

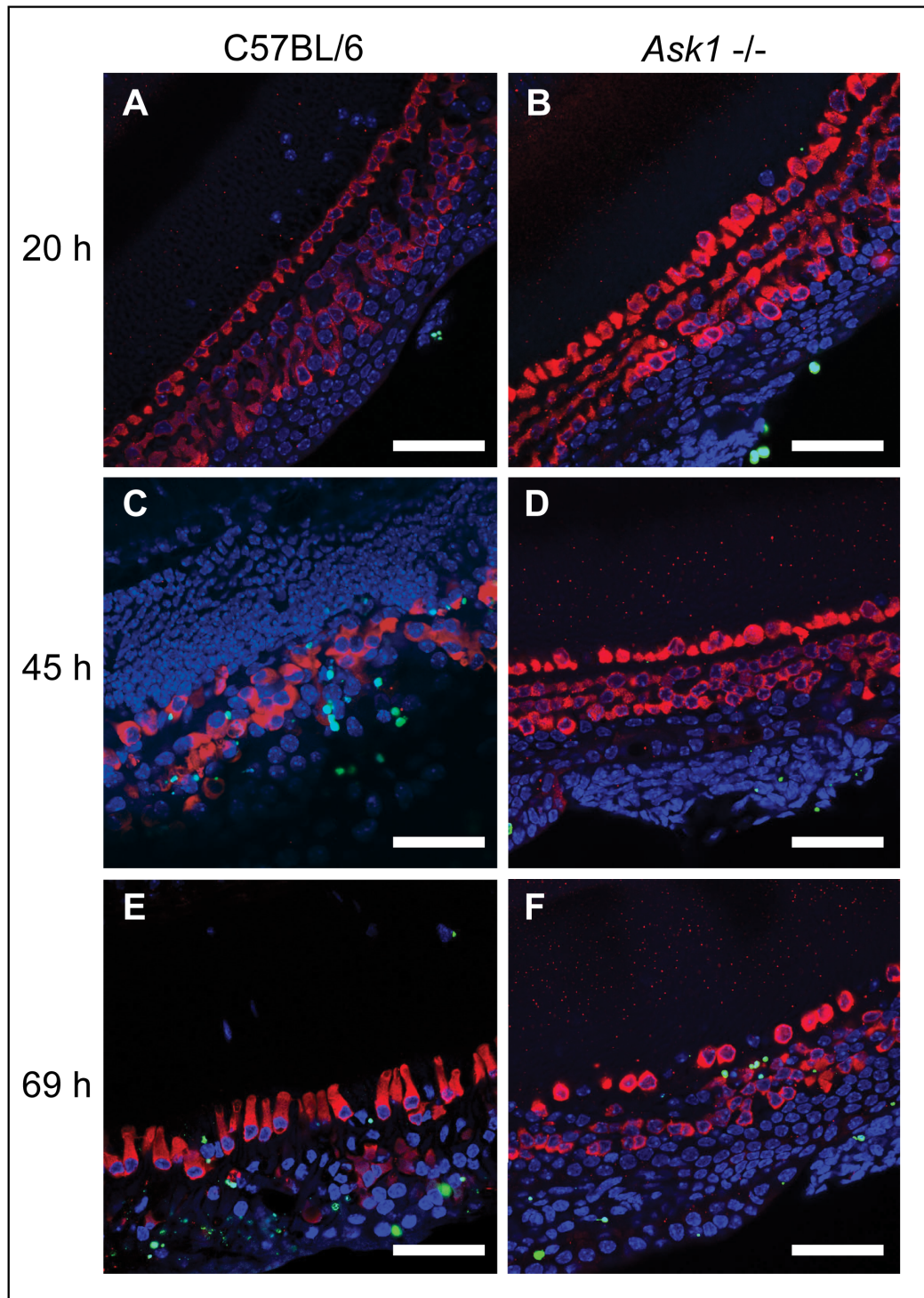


Figure 27. C57Bl/6 and *Ask1*^{-/-} explants treated with 400 μM neomycin over 69 hours *in vitro*.

C57Bl/6 control explants (left) compared with *Ask1*^{-/-} explants (right) treated for 20, 45 and 69 hours with 400 μM neomycin. N = 3-5 explants per treatment group, images collected at 40x magnification, scale bar = 50 μM. Green = TUNEL, Blue = DAPI, Red = myosin VIIa (hair cells).

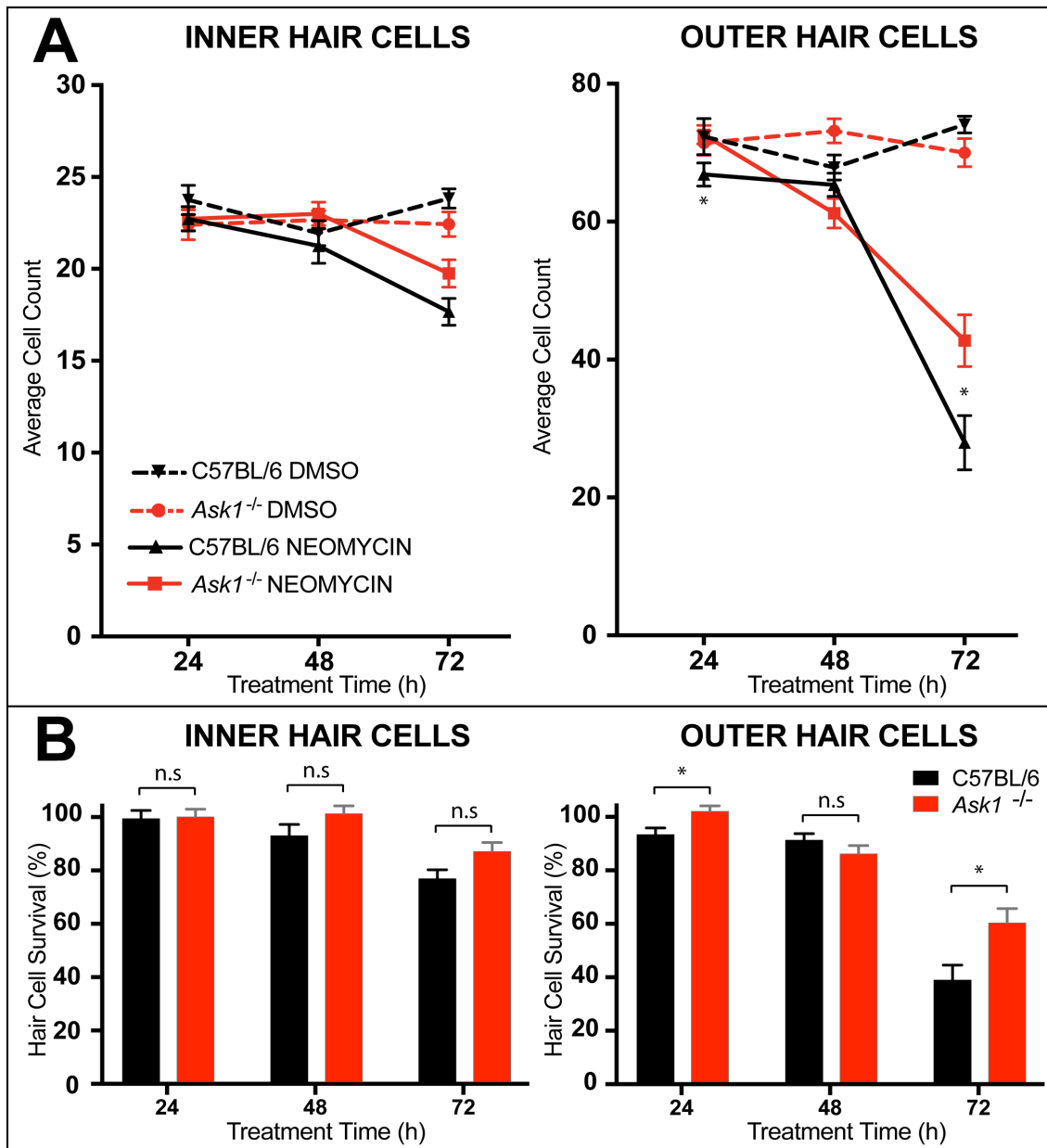


Figure 28. Hair cell counts from explants treated with DMSO or 400 μ M neomycin.

A) Average number of Myosin VIIa-labelled inner (left) and outer (right) hair cells after a 24, 48 or 72 h treatment with either vehicle or 400 μ M neomycin. Cell counts are the averages of two 180 μ m x 90 μ m fields of view taken from each explant in the mid-turn region **B)** Hair cell counts presented as percentage survival based on average hair cell counts in the DMSO control group. Mean with S.E.M shown (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s = not statistically significant). N (for 24, 48 and 72 h timepoints respectively) = 6, 8, 6 (C57BL/6 DMSO), 5, 6, 7 (*Ask1*^{-/-} DMSO), 9, 6, 9 (C57BL/6 neomycin), 9, 7, 6 (*Ask1*^{-/-} neomycin).

3.2.3. Activation of JNK-mediated apoptosis appears reduced in *Ask1*^{-/-} hair cells treated with neomycin

Previous studies have demonstrated that JNK is a key mediator of aminoglycoside induced hair cell death (Bodmer et al. 2002; Wang et al. 2003; Ylikoski et al. 2002; Tao & Segil 2015) and ASK1 is a known mediator of JNK phosphorylation (reviewed in chapter 1). Therefore, reduced JNK phosphorylation was hypothesised to be the likely mechanism by which *Ask1* knockout explants are protected against neomycin toxicity. To test this hypothesis, cochlear explants were treated with neomycin and analysed with an antibody targeting phosphorylated JNK.

Immunohistochemistry of cochlear explants treated with 1 mM neomycin for six hours showed notable staining for phosphorylated JNK (Figure 29). Qualitatively, the signal appeared much stronger in C57BL/6 explants than *Ask1*^{-/-} explants. Higher magnification images showed punctate or dappled staining, which may indicate a mitochondrial association. Confocal imaging also indicated that the p-JNK signal was concentrated in the nuclei of neomycin-treated C57BL/6 hair cells, but not *Ask1*^{-/-} hair cells. For this evaluation, the *Ask1*^{-/-} and C57BL/6 explants were cultured, stained, and imaged concurrently to minimise inter-experimental variation. However, further controlled experimentation is required to replicate and quantify these observations.

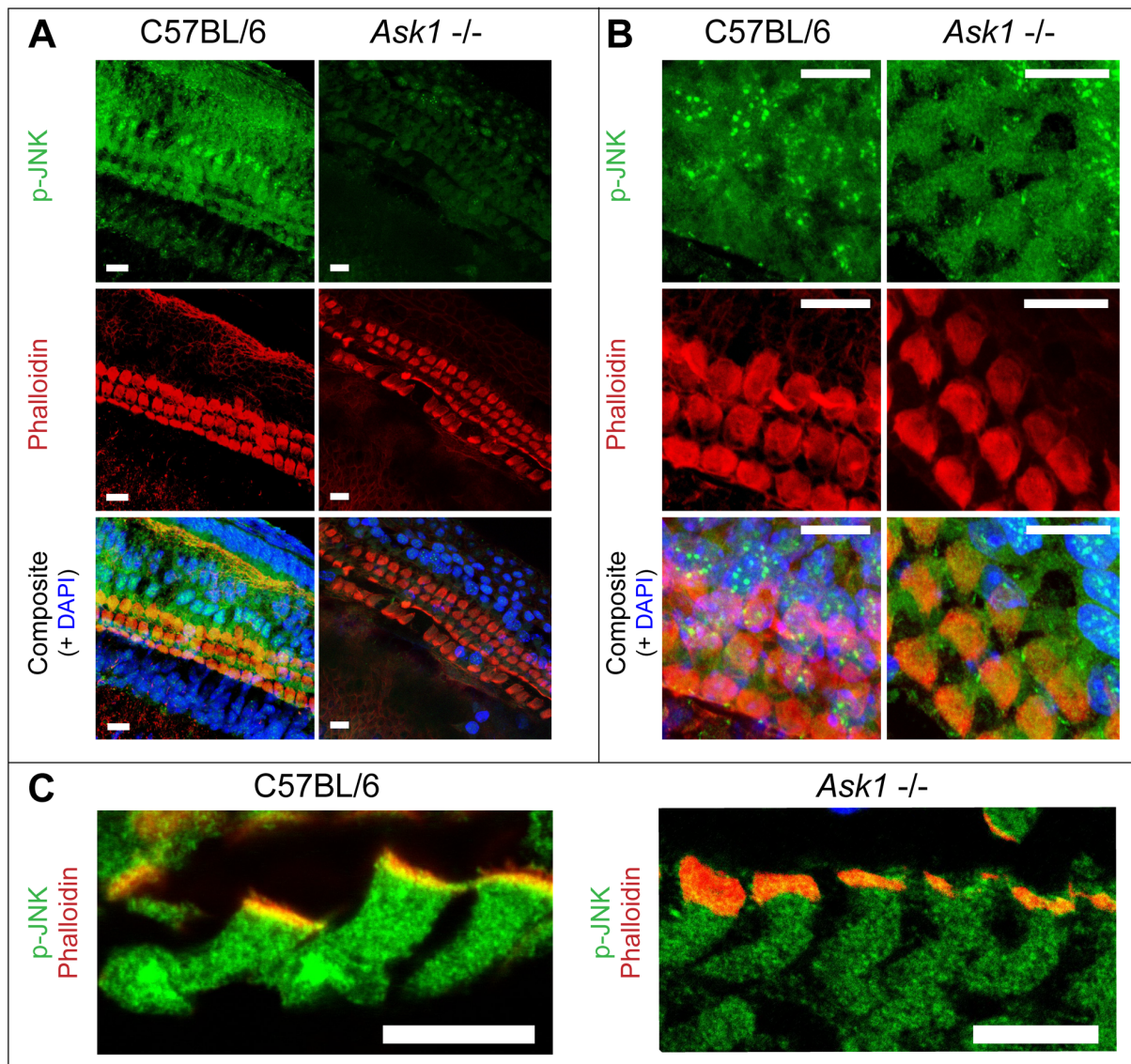


Figure 29. JNK phosphorylation in neomycin-treated hair cells.

Cochlear explants were treated with 1 mM neomycin for six hours, and then co-stained with anti-p-JNK (green), phalloidin (red) and DAPI (blue). **A)** Neomycin treated C57BL/6 and *Ask1*^{-/-} explants were positive for p-JNK staining, including notable hair cell staining. However, the signal appeared weaker in *Ask1*^{-/-} explants when compared with C57BL/6 explants. **B)** Higher magnification imaging showed strong puncta of p-JNK staining in C57BL/6 explants. **C)** p-JNK staining was particularly strong in the nuclei of C57BL/6 hair cells. Scale bars = 10 μm. Images representative of 4-5 explants per treatment taken from littermate controls.

3.2.4. Discussion

Collectively, the data presented in this chapter suggests that ASK1 is a potential therapeutic target to reduce ROS-mediated JNK activation in aminoglycoside-treated hair cells. Hair cell death in cochlear explants from neonatal *Ask1*^{-/-} and C57BL/6 mice was investigated by exposure-time and neomycin dosage to test the hypothesis that ASK1 deficiency is protective against neomycin-induced, JNK mediated, hair cell death *in vitro*. These experiments demonstrated that *Ask1*^{-/-} hair cells were resistant to cell death induced by both high (1 mM) and moderate (400 μ M) neomycin treatment compared to C57BL/6 controls. Notably, inner hair cells were more resistant to neomycin toxicity than outer hair cells—regardless of genotype. This is an observation that has been frequently reported in the literature and is thought to be caused by greater drug uptake in the outer hair cells (Ding et al. 2002; Santi et al. 1982; Alharazneh et al. 2011; Hiel et al. 1992; Lee et al. 2013; Rybak & Ramkumar 2007; Stepanyan et al. 2011). Conversely, neomycin treatment caused conspicuous outer hair cell death. ASK1 deficiency however, conferred significant protection, with markedly higher surviving hair cell counts at the 24, and 72 hour time points, for both 400 μ M and 1 mM neomycin treatments.

Interestingly, outer hair cell counts of *Ask1*^{-/-} and C57BL/6 explants at the 48 hour time-point were very similar, in both neomycin treatments. This is difficult to explain with the data available, given that there was a significant difference in survival at both the 24 and 72 hour time-points. One potential explanation is that ASK1 deficiency is affecting different mechanisms of cell death. For example, immunohistochemistry suggests that JNK phosphorylation and nuclear translocation is reduced in neomycin-treated *Ask1*^{-/-} hair cells. It may be that compensatory mechanisms then activate to ensure damaged hair cells are destroyed. For example, caspase activation can occur independently of MAPK phosphorylation in hair cells (Dinh et al. 2015). Hair cells can also emit ‘eat me’ signals, inducing phagocytosis by resident macrophages or supporting cells (Hirose et al. 2017; Kaur et al. 2015). An alternate possibility is that the initial protective effect of ASK1 deficiency is due to direct inhibition of apoptosis in hair cells, whereas the delayed protective effect is indirectly caused by greater survival of supporting cell types. For instance, hair cells are particularly susceptible to neomycin uptake, due to the non-selective nature of the MET channel. However, after three days of neomycin treatment, it is likely that toxic effects will occur in other cell

types. At this point, ASK1 deficiency may be protective in many cell types, improving the overall structural integrity of the explant and further supporting hair cell survival. Thus, the observed hair cell death at the 72 hour time-point may be related to both direct hair cell death *and* other supporting cell types succumbing to toxicity.

3.2.5. Future Directions

In order to clarify the mechanisms underlying hair cell death in these experiments, further immunohistochemistry would be useful. For example, p-JNK staining is punctate, suggesting mitochondrial co-localisation. However, co-localisation with a mitochondrial specific stain such as a fluorescently tagged streptavidin would be required to confirm this. Furthermore, a time course study, highlighting p-JNK, p-P38 and other pro-apoptotic molecules in cochlear explants would be valuable for delineating the time-frame of activation and the localisation of these pro-apoptotic factors. Further immunohistochemistry may also provide information regarding the protection afforded by ASK1 deficiency, such as the delayed onset of p-JNK phosphorylation or the activation of compensatory pathways. Additional immunohistochemistry experiments will also facilitate the quantification of cell specific fluorescent signals.

Similarly, western blot analysis provides a method to quantify protein levels in treated explants at different time-points. However, numerous explants must be pooled to extract sufficient protein. Importantly, western blotting of cochlear explants does not provide hair cell specific information, rather data that represents protein levels in the entire explant. Therefore, it can be difficult to relate pro-apoptotic protein changes to a specific auditory cell type. Normalising the signal with a Myosin VIIa loading control could help overcome this issue. However, treatment and hair cell death may alter Myosin VIIa levels in wild type explants, potentially confounding the analysis. In the context of interrogating hair cell death pathways, both cochlear immunofluorescence and western blots are limited in the number of proteins that can be assessed at any time. Therefore, an ideal analysis would use in depth single cell proteomics. This would provide vital information regarding hair cell death and may identify novel apoptosis mechanisms specific to the hair cell. Furthermore, hair cells from different cochlear regions could be compared, and responses specific to inner or outer hair cells could be identified. This form of analysis would not require as many cochlear explants, however

it would be a complex and expensive endeavour. Cultured explants would likely need to be sectioned, to ensure comparisons were being made between comparable cochlear regions. Then hair cells would need to be specifically labelled using proteins with expression profiles specific to inner and outer hair cell types (such as calretinin, calbindin or oncomodulin)(Simmons et al. 2010). The explant sections would then need to be dissociated for fluorescence activated cell sorting (FACS), which could be problematic, as the process of cell isolation has the potential to change cellular miRNA's and their target transcripts (Richardson et al. 2015). Alternatively, a laser capture technique could be utilised to isolate hair cells. However, separating the hair cell from the underlying epithelial structures would be difficult. Finally, cells would need to undergo single cell transcription profiling.

Recent technological advances have made DNA and RNA profiling possible using nanopore sensors, but these have not yet translated to proteomic studies. Excitingly, high throughput mass spectrometry has developed to the point that up to 100 proteins can be profiled in a single cell (Marx 2019; Specht et al. 2019). The current proteomic technologies available represent the genesis of what is likely to be a rapidly evolving field, limited mainly by technological advances. As more robust proteomic systems become available, single cell protein analysis will revolutionise our understanding of aminoglycoside-induced hair cell death, likely identifying novel therapeutic targets.

3.2.6. Concluding Remarks

The results produced in this section demonstrate that a genetic-mediated reduction in ASK1 affords significant protection against neomycin-induced hair cell death *in vitro*. Based on these results, there appears to be therapeutic potential for ASK1 as a molecular target for preventing aminoglycoside-induced hearing loss *in vivo*. Notably, in humans, aminoglycosides are delivered over a prolonged period at a lower dose than those used in this study. Therefore, the protective effect observed *in vitro* may be enhanced or reduced *in vivo*. Therefore, the next section aimed to develop an appropriate *in vivo* murine model of ototoxicity, to further test the potential of ASK1 as a mediator of aminoglycoside induced ototoxicity.

3.3. Testing whether ASK1 deficiency attenuates aminoglycoside ototoxicity *in vivo*

The comparison of hair cell survival after neomycin treatment in cochlear explants taken from C57BL/6 mice and *Ask1*^{-/-} knockout mice demonstrated that ASK1 deficiency afforded significant protection against neomycin toxicity *in vitro* (page 103). In order to ascertain whether the protection afforded by ASK1 deficiency *in vitro* translates to a relevant clinical outcome, such as the preservation of hearing *in vivo*, a protocol for modelling aminoglycoside-induced hearing loss in mice is required. However, a review of the literature indicated that the use of mice for modelling human ototoxic outcomes is controversial. The preponderant method for inducing ototoxicity in the mouse utilises very high doses of kanamycin in conjunction with the loop diuretic furosemide (Hirose & Sato 2011; Oesterle et al. 2008; Hartman et al. 2009; Ruan et al. 2014; Li et al. 2011). The reported effects of this method vary greatly and it is difficult to relate and compare these ototoxic outcomes to human ototoxicity. Furthermore, the high mortality of mice undergoing such treatment is difficult to justify ethically. Additionally, a notable research gap was identified, in that intravenous (i.v.) aminoglycoside delivery had not been reported in mice, despite this being the drug delivery route used in humans. In order to address this and potentially to develop a more appropriate *in vivo* model of aminoglycoside-induced ototoxicity, a study was performed to test the tolerability of repeated i.v. injections in mice, followed by a trial using various aminoglycoside regimens. The data obtained from these experiments has been collated as an original research article, which is provided overleaf. This manuscript was *in press*, having been accepted for publication in Hearing Research (Ogier, Lockhart, et al. 2019) prior to the examination of this thesis. It was published during the thesis examination period.



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Intravenously delivered aminoglycoside antibiotics, tobramycin and amikacin, are not ototoxic in mice

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Resistant

ABSTRACT

Many drugs on the World Health Organization's list of critical medicines are ototoxic, destroying sensory hair cells within the ear. These drugs preserve life, but patients can experience side effects including permanent hearing loss and vestibular dysfunction. Aminoglycoside ototoxicity was first recognised 80 years ago. However, no preventative treatments have been developed. In order to develop such treatments, we must identify the factors driving hair cell death. *In vivo*, studies of cell death are typically conducted using mouse models. However, a robust model of aminoglycoside ototoxicity does not exist. Previous studies testing aminoglycoside delivery via intraperitoneal or subcutaneous injection have produced variable ototoxic effects in the mouse. As a result, surgical drug delivery to the rodent ear is often used to achieve ototoxicity. However, this technique does not accurately model clinical practice. In the clinic, aminoglycosides are administered to humans intravenously (i.v.). However, repeated i.v. delivery has not been reported in the mouse. This study evaluated whether repeated i.v. administration of amikacin or tobramycin would induce hearing loss. Daily i.v. injections over a two-week period were well tolerated and transient low frequency hearing loss was observed in the aminoglycoside treatment groups. However, the hearing changes observed did not mimic the high frequency patterns of hearing loss observed in humans. Our results indicate that the i.v. delivery of tobramycin or amikacin is not an effective technique for inducing ototoxicity in mice. This result is consistent with previously published reports indicating that the mouse cochlea is resistant to systemically delivered aminoglycoside ototoxicity.

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1. Introduction

Ototoxic drugs destroy the sensory hair cells of the auditory and vestibular systems. As a result, treated individuals may acquire irreversible hearing loss, ataxia, nausea and vertigo. Presently, the only way to reduce ototoxic effects is to avoid using these medicines. However, in most cases, ototoxic drugs are part of a life saving therapy. One example, is the family of broad spectrum aminoglycoside antibiotics, which are the most commonly used antibiotic worldwide (Xie et al., 2011; Hermann, 2007). Prolonged or high dose aminoglycoside treatment damages the hair cells of the cochlea and vestibular apparatus (Ratjen et al., 2009). Individuals

with Cystic fibrosis are particularly vulnerable to ototoxic effects, as chronic lung infections (primarily caused by *Pseudomonas aeruginosa*) require long term aminoglycoside treatment (Ratjen et al., 2009). For individuals acquiring permanent hearing loss, ototoxic effects diminish quality of life. In adults with hearing loss, isolation and depression are common, whilst children can suffer from impaired language and social development (Roland et al., 2016; Rajendran and Roy, 2010; Cosh et al., 2018; Hay-McCutcheon et al., 2018). For clinicians, such quality of life implications may impact medical decision-making (Al-Malky, 2016).

A strategy to mitigate drug induced hearing loss would be revolutionary, but efforts towards this goal have been hampered by limitations of the mouse for studies of ototoxicity. Rodent models are utilised for *in vivo* mammalian research and the technologies for the manipulation and analysis of the mouse genome have advanced significantly. Hundreds of mouse strains exist that are valuable for investigating hair cell death. However, these resources

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cannot be effectively utilised without a method to reproducibly induce hearing loss that mimics the human experience. In humans, aminoglycosides are administered intravenously, and the capillaries of the *stria vascularis* then facilitate passage of the drug from the blood into the cochlea. However, in rodent models, drugs tend to be delivered via sub cutaneous (s.c.) injection, intra peritoneal (i.p.) injection, or directly into the cochlea via more invasive processes.

Of the currently utilised techniques to model ototoxicity, *trans*-tympanic injections are considered to be the most minimally invasive form of drug delivery to the cochlea. However, there is an increased risk of infection and perforations of the tympanic membrane may not heal. Repeated drug delivery can exacerbate these problems and the procedure alone can induce permanent hearing threshold shifts (Oishi et al., 2013; McCall et al., 2010). Alternatively, the round window niche (RWN) technique is often used. For this procedure, the middle ear is accessed surgically and the bony tympanic bulla is partially unroofed. A liquid or soaked gelfoam pad is then applied topically to the round window membrane (Stevens et al., 2015). This technique avoids cochlear perforation, however temporal bone drilling and perilymphatic fistulas can cause acoustic or mechanical damage (Stevens et al., 2015). Like *trans*-tympanic injections, the RWN technique is limited to a single treatment.

Both *trans*-tympanic injections and RWN techniques rely on passive drug diffusion, which causes a base to apex drug gradient within the cochlea (Stevens et al., 2015; Wanamaker et al., 1998, 1999; Lee and Kimura, 1991; Zhai et al., 2010; Jero et al., 2001). Other anatomic factors can further impair diffusion, such as Eustachian drainage, round window pseudomembranes, interstitial fluid and wax plugs (McCall et al., 2010; Alzamil and Linthicum, 2000). A third alternative, cochleostomy with perilymphatic perfusion, ensures consistent drug delivery to target tissues. However, this technique can directly damage the ear and cause permanent hearing loss (Stevens et al., 2015; Chen et al., 2006). The advantage of methods involving direct drug delivery to the cochlea is that systemic toxicities including nephrotoxicity, encephalopathy and peripheral neuropathy can be avoided. However, surgeries are more suited to large rodents such as the gerbil (Wanamaker et al., 1999; Schmiedt et al., 2002), rat (Guner et al., 2017; Iwai et al., 2006), chinchilla (Coleman et al., 2007) or guinea pig (Zhai et al., 2010). Extensive surgical training and anesthesia is required (Jero et al., 2001; Borkholder et al., 2014). In addition, the use of surgical implements such as micropump infusers, tubing or antibiotic soaked gel pads increase the risk of infection or inflammation (Stevens et al., 2015; Schmiedt et al., 2002; Borkholder et al., 2014).

Surgical techniques were developed because the adult mouse cochlea appears to be resistant to the effects of systemically delivered aminoglycosides (Murillo-Cuesta et al., 2017; Poirrier et al., 2010). However, within the literature, there are conflicting reports regarding the effects of systemically delivered ototoxic drugs in mice (Table 1). A number of studies indicate that mice are *not* susceptible to aminoglycoside induced hearing loss, whilst others suggest that high aminoglycoside doses must be used due to the mouse's high metabolic rate (Nair and Jacob, 2016; Wu et al., 2001). The most commonly reported successful ototoxic protocol in mice uses 800 mg/kg of kanamycin, given twice daily, s.c., for 15 days (Wu et al., 2001; Horvath et al., 2019; Murillo-Cuesta et al., 2010; Francis et al., 2011). However, the ototoxic outcomes achieved using this protocol vary between studies and some researchers have not been able to replicate ototoxic outcomes using this method (Poirrier et al., 2010; Severinsen et al., 2006). Importantly, kanamycin is not particularly relevant in the modern clinic and the high doses described have toxic effects in mice, causing skin lesions and increased mortality that is difficult to justify

ethically (Wu et al., 2001; Murillo-Cuesta et al., 2010; Francis et al., 2011). In contrast, immediate intravenous (i.v.) aminoglycoside toxicity is rarely observed in humans (Gonzalez and Spencer, 1998). Children and adults routinely receive daily aminoglycoside infusions in the clinic – a practice which has not been accurately modelled in the mouse (Gonzalez and Spencer, 1998; Antibiotic clinical practice guidelines, 2020; Aminoglycoside Dosing Gui, 2020; Beaucaire, 2000). In the mouse, researchers favour i.p. or s.c. routes of drug delivery. This is because i.v. injections in mice are more difficult than in humans. The mouse lateral tail vein is very small, with a diameter of approximately 300 μ m (Berry-Pusey et al., 2013). To facilitate injection, a mouse must first be warmed to encourage vasodilation and restrained to minimise movement. The process is time consuming and requires technical dexterity. Thus, repeated i.v. injections in mice are not routinely performed. However, the i.p. and s.c. modes of delivery are not ideal for aminoglycoside administration. Aminoglycosides are polar molecules, which hinders membrane penetration and drug uptake (Gonzalez and Spencer, 1998; van Gennip et al., 2009). In contrast, i.v. injection achieves immediate drug uptake and increases the drug's half life (Bunke et al., 1983). In humans, plasma levels of the aminoglycoside tobramycin achieved by i.v. infusion are double those achieved using i.p. injection (Bunke et al., 1983). Therefore, we considered that the reported aminoglycoside resistance in mice may be driven by the preponderant use of i.p. and s.c. drug administration (Table 1).

The aim of this study was to develop a murine model of aminoglycoside ototoxicity that mirrored clinical practice and outcomes. We hypothesised that repeated, daily i.v. injection of ototoxic aminoglycosides would induce high frequency hearing loss in mice that mimicked human ototoxic pathologies. However, information regarding mouse specific aminoglycoside dosing is limited. Very few studies have used repeated daily i.v. injections and those that have, reported poor outcomes (Thomas and Atkinson, 1994). Human to mouse conversion guidelines, based on body surface area suggest that very high doses of aminoglycosides would be required to achieve the animal equivalent of a human dose, but published LD₅₀ values indicated such doses i.v. would prove lethal (Nair and Jacob, 2016; Sharma and McNeill, 2009; Reagan-Shaw et al., 2008). Notably, significant aminoglycoside induced mortality rates are frequently reported in mice, even when non-systemic routes of delivery are used (Table 1).

At present, people with cystic fibrosis have a heightened risk of developing aminoglycoside induced hearing loss, due to the frequent high dose aminoglycoside treatments that they may require (Zettner and Gleser, 2018; Garinis et al., 2017). For this patient cohort, tobramycin and amikacin are the most frequently used antibiotics (Ranganathan, 2006; Smyth et al., 2017; Safi et al., 2016). This was reflected in a survey of cystic fibrosis centers in America, in which 98.4% reported tobramycin use and 34.4% reported amikacin use (Prescott, 2014). However, beyond this patient cohort, amikacin is one of the most commonly used antimicrobial agents (Ramirez and Tolmasky, 2017; Leibovici et al., 2001; Edson and Terrell, 1987). Therefore, amikacin and tobramycin were selected for use in this study. We first assessed the feasibility of performing repeated daily i.v. injections in mice and then tested whether daily i.v. treatment with amikacin or tobramycin could induce hearing loss in C57BL/6 mice.

2. Methods

2.1. Mice

Groups of mice were housed in individually ventilated micro-isolator cages (Tecniplast, Buguggiate, VA, Italy) at the Murdoch

Table 1

Examples of aminoglycoside use in mice. Modern aminoglycosides such as tobramycin and amikacin were poorly represented in studies of ototoxicity, however examples of their use exist in alternate fields of study. Ototoxicity and mortality caused by amikacin, gentamycin, neomycin and kanamycin vary. Abbreviations used in the table are: amikacin (amik), gentamycin (gent), kanamycin (kan), neomycin (neo), tobramycin (tob), intravenous (i.v.), intramuscular (i.m.), subcutaneous (s.c.), intraperitoneal (i.p.), round window niche (RWN) and intratympanic (i.t).

MAX DOSE	DRUG	ROUTE	SCHEDULE	DURATION	OTOTOXICITY	ADVERSE REACTIONS	REF & YEAR
2 mg/kg	amik	i.v.	2x per week	21 days	Not tested.	None.	(Ehlers et al., 1996)
15 mg/kg	amik	i.p.	3x per day	1 day	Not tested.	None.	(Mathe et al., 2007)
15 mg/kg	amik	i.p.	2x per day	3 days	Not tested.	None.	(Dinc et al., 2015)
25 mg/kg	amik	s.c.	6x per week	30 Days	Not tested.	None.	(Vrioni et al., 1998)
80 mg/kg	amik	i.v.	3x per week	28 Days	Not tested.	None.	(Dhillon et al., 2001)
150 mg/kg	amik	s.c.	5x per week	40 days	Not tested.	None.	(Zheng et al., 2013)
160 mg/kg	amik	i.v.	N/A	N/A	Not tested.	Immediately lethal 100%.	(Dhillon et al., 2001)
160 mg/kg	amik	i.m.	3x per week	28 Days	Not tested.	None.	
500 mg/kg	amik	s.c.	1x daily	15 days	No ototoxic effect.	25% mortality.	(Murillo-Cuesta et al., 2010)
1200 mg/kg	amik	i.p.	Single dose	1 day	Not tested.	Extensive skin lesions.	(Kaynar et al., 2007)
						Nephrotoxicity (granulovacuolar tubular degeneration). One unexpected death.	
3072 mg/kg	amik	s.c.	4x daily	1 day	Not tested.	None.	(Zuluaga et al., 2015)
3072 mg/kg	amik	s.c.	1x daily	1 day	Not tested.	None.	(Craig et al., 1991)
0.56 M	gent	i.t	1x daily	1 day	Reduced synaptic ribbons and spiral ganglion neurons. No significant hair cell death. High frequency hearing loss reported.	None.	(Oishi et al., 2015)
25–200 mg/kg	gent	RWN	1x daily	1 day	Dose dependent stereocilia loss, extending the length of the cochlea.	Hair cell death in sham treated animals. Animals euthanised due to severe vestibular damage.	(Heydt et al., 2004)
200 mg/kg	gent	i.p.	1x daily	7 days	No ototoxic effect.	50% mortality.	(Horvath et al., 2019)
300 mg/kg	gent	i.m.	1x daily	15 days	No ototoxic effect.	50% mortality.	(Murillo-Cuesta et al., 2010)
300 mg/kg	gent	s.c.	1x daily	28 days	Small and variable threshold shifts.	None.	(Wu et al., 2001)
300 mg/kg	gent	s.c.	Continuousmini pump	14 days	No ototoxic effect.	None.	
300 mg/kg	gent	s.c.	2 x daily	28 days	Variable hearing loss at 24 kHz.	None.	
350 mg/kg	gent	s.c.	2 x daily	5 days	Not tested.	100% lethal.	
400 mg/kg	gent	s.c.	1x daily	28 days	Not tested.	Catatonic mice/difficulty recovering.	
500 mg/kg	gent	s.c.	NA	NA	Not tested.	Immediate death.	
600 mg/kg	kan	s.c.	1x daily	15 days	No ototoxic effect.	25% mortality.	(Murillo-Cuesta et al., 2010)
700 mg/kg	kan	s.c.	Continuousmini pump	28 days	No ototoxic effect.	None.	(Wu et al., 2001)
700 mg/kg	kan	s.c.	2x daily	7 days	No ototoxic effect.	None.	(Jiang et al., 2005)
700 mg/kg	kan	s.c.	2x daily	14 days	No ototoxic effect.	Slight increase in mortality.	(Poirrier et al., 2010)
700 mg/kg	kan	i.p.	2x daily	14 days	No ototoxic effect.	Slight increase in mortality.	
800 mg/kg	kan	s.c.	2x daily	15 days	25 dB SPL ABR threshold shift (click).	33% mortality.	(Murillo-Cuesta et al., 2010)
800 mg/kg	kan	i.m.	2x daily	15 days	25 dB SPL ABR threshold shift (click).	30% mortality.	
800 mg/kg	kan	s.c.	2x daily	15 days	Hair cell death, elevated ABR thresholds.	Reduced weight gain.	
800 mg/kg	kan	s.c.	2x daily	15 days	Hair cell death, elevated DPOE thresholds, no significant ABR change.	None.	(Horvath et al., 2019)
800 mg/kg	kan	s.c.	2x daily	14 days	Hair cell death and elevated ABR thresholds at all frequencies.	25% mortality.	(Francis et al., 2011)
850 mg/kg	kan	s.c.	2x daily	15 days	Outer hair cell death and elevated ABR thresholds at 24 and 32 kHz.	None.	(Jiang et al., 2019)
900 mg/kg	kan	s.c.	2x daily	15 days	No hair cell loss.	None.	(Severinsen et al., 2006)
900 mg/kg	kan	s.c.	2x daily	15 days	Extensive cochlear and vestibular hair cell death.	Some mice died prematurely.	(Wu et al., 2001)
1000 mg/kg	kan	s.c.	1x daily	21 days	Hearing loss in 1 of 5 mice.	None.	
1400 mg/kg	kan	i.p.	2x daily	14 days	No ototoxic effect.	Slight increase in mortality.	(Poirrier et al., 2010)
300 mg/kg	neo	i.p.	1x daily	15 days	No ototoxic effect.	71% mortality.	(Murillo-Cuesta et al., 2010)
400 mg/kg	tob	i.p.	Every 4 h	1 day	Not tested.	None.	(Louie et al., 2013)

Childrens Research Institute (MCRI), on a 14 h light/10 h dark cycle. Animals had standard Barastoc mouse chow (Ridley AgriProducts, Melbourne, VIC, Australia) and sterilised water *ad libitum*. Enrichment provided included cardboard toys and sunflower seeds. The Murdoch Childrens Research Institute Animal Ethics Committee approved experimental procedures in project number A844, conforming to the Australian Code of Practice for the Care and Use of

Animals for Scientific Purposes, 8th edition, 2013.

2.2. Intravenous injections

Group housed mice were placed in a temperature controlled Ventilated Cabinet (Tecniplast, Buguggiate, VA, Italy) in their home cage at 30 °C, for 45 min. Individual mice were then restrained in a

Perspex restraint capsule and injected with saline, or antibiotic saline solutions (Hospira, Australia). To prevent scarring or hematomas, injections were performed distally, with each subsequent injection performed closer to the body and on alternate lateral tail veins. Daily i.v. injections were performed by an experienced researcher under veterinary supervision. Post injection, mice were rewarded with 100% organic fruit puree (Raffertys Garden, banana, pear and mango puree).

2.3. Mouse health monitoring

Animal technicians performed daily general health checks and a veterinarian inspected the mice weekly. Before each injection, the researcher checked each mouse for signs of tail irritation, weight loss or general discomfort. Strict criteria were observed for continuation of the experiment (S1 Fig). Post injection, mice were monitored for clinical signs of aminoglycoside toxicity such as convulsions, prostration, hypoactivity, polydipsia, dyspnoea and ataxia.

2.4. Experimental mouse cohorts

Pilot Repeated i.v. Injection Cohort.

A cohort of 10 six week old C57BL/6 mice were injected daily with 200 μ l of saline for 14 consecutive days.

2.5. Aminoglycoside Injection Cohorts

A baseline ABR recording was collected from four week old mice. At six weeks of age, mice received an i.v. aminoglycoside injection daily for six consecutive days, followed by one day of rest and then another six days of treatment. Further ABR testing was performed at 10 and 20 weeks of age. Rotor Rod balance assessments were performed at 19 weeks of age.

Treatment groups comprised of the following:

Saline Control (n = 10)
40 mg/kg amikacin (n = 10)
80 mg/kg amikacin (n = 5)
120 mg/kg amikacin (n = 10)
145 mg/kg amikacin (n = 5)
40 mg/kg tobramycin (n = 5)

Antibiotics were diluted in saline to 6 mg/ml for 40 mg/kg dosing, 12 mg/ml for 80 mg/kg dosing, 18 mg/ml for 120 mg/kg dosing and 21.75 mg/ml for 145 mg/kg dosing. For all doses each mouse received 6.66 μ l per gram of body weight. For example, a 30 g mouse received 200 μ l i.v.

2.6. Auditory brainstem response testing

ABR testing was performed as previously published (Ogier et al., 2014, 2018). Briefly, mice were anaesthetised using a solution containing 100 mg/kg ketamine and 20 mg/kg xylazine in saline, delivered i.p (10 μ l anaesthetic solution per gram bodyweight). Once unconscious, REFRESH night time was applied to the mouse's eyes to prevent drying (Allergan, NSW, Australia). A heatpad was used to prevent hypothermia in anaesthetised mice. A free-field magnetic speaker (model FF1, Tucker Davis Technologies) was placed 10 cm from the left pinna. Computer-generated clicks (100 μ s duration, with a spectrum of 0–50 kHz) and 3 ms pure tone stimuli of 4, 8, 16 and 32 kHz were presented with maximum intensities of 100 dB SPL. Subdermal needle electrodes (S06666-0, Rochester Electro-Medical, Inc., Lutz, FL, USA) were positioned at the apex of the skull (+ve), the left cheek (–ve) and the left hind leg

(ground). ABRs were viewed using an evoked potentials workstation (Tucker Davis Technologies, Alachua, FL, USA) as previously described (Al-Malky, 2016). The average of 512 stimuli repeats was used to calculate ABR traces in BioSig software (Tucker Davis Technologies). ABR Thresholds were detected by visual analysis, at the lowest intensity stimulus that reproducibly evoked an ABR.

2.7. Rotor rod testing

Motor performance was assessed using the Rota-Rod for Mice (47600, Ugo-Basile, Italy) as previously described (Stephenson et al., 2018). Home cages were placed in the testing room, one hour before trials commenced. Then, up to five mice were placed on the Rota rod, facing away from the handler. The rod accelerated from 4 to 40 rpm during each five-minute trial. The trial ended when the mouse fell from the rotating rod, rotated with the rod more than two consecutive times, or when 300 s was reached. Each mouse underwent three trials, with a period of 30 min between trials. Mice were returned to home-cages when resting. Testing was performed twice on consecutive days, with the first day performed as habituation/training. Trials on the second day were recorded. Latency to fall was recorded by the Rota-Rod CUB software.

2.8. Tissue collection and analysis

At 20 weeks of age (immediately post ABR test), anaesthetised mice were euthanised by i.p injection of 400 mg/kg ketamine and 80 mg/kg xylazine in saline. After cessation of breathing, the thoracic cavity was carefully opened. A cannula was inserted into the left ventricle and a small perforation was made in the right atrium. PBS was perfused through each animal for five minutes, followed by a five-minute flush of 4% paraformaldehyde.

Kidneys and cochleae were immediately dissected and stored in 4% paraformaldehyde.

2.8.1. Kidney histology

Multiple levels were cut through each kidney along the frontal plane, to capture the cortex, medulla and papilla. Sections were cut and stained with Haematoxylin and Eosin (H&E) by the Australian Phenomics Network Histopathology and Organ Pathology Service, University of Melbourne. Sections were analysed by two, senior veterinary pathologists.

2.8.2. Cochlear histology

Cochleae were washed in tris-buffered saline and decalcified in 10% EDTA for 10 days at 4 °C with gentle rolling. After a 1hr PBS wash, each cochlea was dissected into six segments and labelled as previously described (Lieberman, 2019; Ogier et al., 2019). Whole mount preparations were washed with a detergent solution of PBS +0.1% Triton X, for 5 min and then labelled using a fluorescently tagged phalloidin (Invitrogen), diluted 1:80 in detergent solution. After 3 PBS washes the preparations were mounted on PTFE printed slides (Pro Sci Tech), under coverslips (Pro Sci Tech) using Prolong Gold anti-fade mounting medium with DAPI (Life Technologies) and sealed with clear nail varnish.

2.9. Statistical analysis

All statistical analyses were completed in Prism 6 software (GraphPad Software Inc.) The Holm–Sidak method was used to correct for multiple T-test comparisons and Dunnett's method was used to correct for multiple ANOVA comparisons.

3. Results

3.1. Daily i.v. saline injections are well tolerated by mice

To assess the safety of daily i.v. injections in mice, we performed a pilot experiment using saline only. Ten C57BL/6 mice ($n = 5$ females and 5 males) received saline i.v. once per day for 14 consecutive days. During the pilot, mice receiving saline did not lose weight and tails were visibly unchanged over the two-week period, save for one, very small haematoma on one tail. No adverse reactions were noted in this group. From a user perspective however, visualisation of veins became more difficult towards the end of the two-week treatment. As a result, more time was required to find an adequate injection site.

3.2. Daily i.v. aminoglycoside injections cause dose specific adverse effects

3.2.1. Amikacin

After each injection, mice treated with 40 or 80 mg/kg were moderately inactive for a short period of time (less than 5 min). Mice given 120 mg/kg commonly exhibited rapid breathing and prostration (approximately 30 s), followed by a brief period of hypoactivity. These effects occurred for each daily injection, with mice recovering quickly each time. However, two mice were removed from the 120 mg/kg cohort (2 of 10) due to localised swelling of the tail. Treatment with 145 mg/kg of amikacin was immediately lethal to two mice (2 of 2). As a result, treatment with 145 mg/kg was not continued in the remaining cohort.

3.2.2. Tobramycin

Mild prostration and rapid breathing was observed in the 40 mg/kg tobramycin cohort (approximately 30 s) followed by hypoactivity. Higher tobramycin doses were not tested, as the prostration observed at 40 mg/kg was similar to that seen in the highest tolerable amikacin treatment group.

3.3. Aminoglycosides induced mild low frequency hearing loss

Hearing thresholds were assessed using the Auditory Brainstem Response (ABR) at two and twenty weeks post treatment. Two weeks post-treatment, average hearing thresholds were unchanged in the low dose amikacin group at all frequencies tested. In contrast, the 80 and 120 mg/kg amikacin groups, and the 40 mg/kg tobramycin group showed altered hearing thresholds when compared with controls (Fig. 1). The small, threshold shift was only present at low frequencies (4–8 kHz). At twenty weeks no significant differences in auditory function were observed between control and treatment groups. Cochlear histology was also unremarkable (Fig. 2).

3.4. Aminoglycoside treatment did not impair Rota-Rod performance

In humans, ototoxic aminoglycosides frequently cause vestibular impairment. In some cases, aminoglycoside toxicity may be greater in the vestibular apparatus than the cochlea (Seemungal and Bronstein, 2007). However, there are contrasting reports regarding the vestibular toxicity of amikacin and tobramycin (Nakashima et al., 2000; Jiang et al., 2017). Generally, amikacin and tobramycin are considered to be more cochleotoxic than vestibulotoxic, however there is certainly evidence within the literature showing that both are able to induce permanent vestibular damage (Zagolski, 2008; Edson et al., 2004; Matz, 1986; Prayle et al., 2010; Munckhof et al., 1996; Scheenstra et al., 2009). In humans,

Electronystagmography is the gold standard of vestibular analysis (Gupta and Mundra, 2015). However, measuring the vestibulo-ocular reflex in mice is more difficult and rarely attempted. Instead, the accelerating rotor rod is commonly used as a measure of gross motor coordination and balance in rodent models. Poor performance in this test has been validated as a measure of dysfunction of the hair cells or stereocilia (Isgrig et al., 2017; Mathur et al., 2015; Tung et al., 2014). In this mouse study, the accelerating rotor rod was used to assess whether vestibular function was impaired by aminoglycoside treatment. On average, the mice in all treatment and control groups performed well, balancing on the accelerating apparatus for between three and four minutes (Fig. 3). There was no significant difference between groups (one way ANOVA $p = 0.61$).

3.5. Nephrotoxicity was not observed in aminoglycoside treated mouse kidneys

In humans, aminoglycoside antibiotics can cause subclinical kidney damage, leading to chronic kidney disease (CKD) which manifests at the level of the glomerulus and tubules (Prayle et al., 2010). To identify acute injury, creatine levels are often measured. However, this measure has previously underrepresented chronic kidney damage (Prayle et al., 2010; Mehta et al., 2015). Alternatively, histological analysis provides a clear indication of drug induced kidney damage (Mehta et al., 2015). Similarly, in mice, histological signs of CKD can be present with no changes in creatinine and BUN (reviewed in Rabe et al. (Rabe and Schaefer, 2016)). Therefore, kidney histology was used to ascertain whether the daily i.v. aminoglycoside treatments performed in this study resulted in damage to the mouse kidney (Fig. 4A–C). Histological changes expected in a kidney damaged by aminoglycosides include extensive tubular damage, scarring or interstitial fibrosis. Dilated atrophic tubules may also be present due to incomplete focal regeneration (Hottendorf and Williams, 1986). Within this study, no such morphological changes were present in the nephrons of aminoglycoside treated mice (Table 2). Protein casts were observed in two mice, one from the 80 mg/kg amikacin group, and one from the 120 mg/kg amikacin group. These casts were few in number and considered by the expert veterinary pathologist to be incidental findings. Similarly, very mild pyelitis was observed in one saline control, which is a common incidental finding of no clinical significance.

4. Discussion

Mice are generally considered to be resistant to aminoglycoside induced ototoxicity, however a mouse model of repeated i.v. aminoglycoside injections that mimic the delivery of aminoglycosides in the clinic has not been reported. This study assessed the feasibility of daily i.v. injections and tested for aminoglycoside induced hearing loss in C57BL/6 mice, using the i.v. drug delivery method.

To mirror clinical practice, the modern aminoglycoside antibiotics amikacin and tobramycin were given i.v. once per day. Information regarding mouse specific aminoglycoside dosing within the literature was limited, so we first assessed a low dose of 40 mg/kg amikacin and steadily increased to 145 mg/kg, which was lethal in mice. We then assessed mice for functional evidence of ototoxicity using the auditory brainstem response and rotor rod testing.

The results of this study provide some evidence that i.v. aminoglycoside injections can accelerate hearing loss in the low-mid frequency auditory range of C57BL/6 mice (4–8 kHz). A small, transient threshold shift of approximately 5 dB SPL was observed two weeks post treatment with amikacin or tobramycin. Similarly, in humans, drug-induced hearing loss first occurs at the same

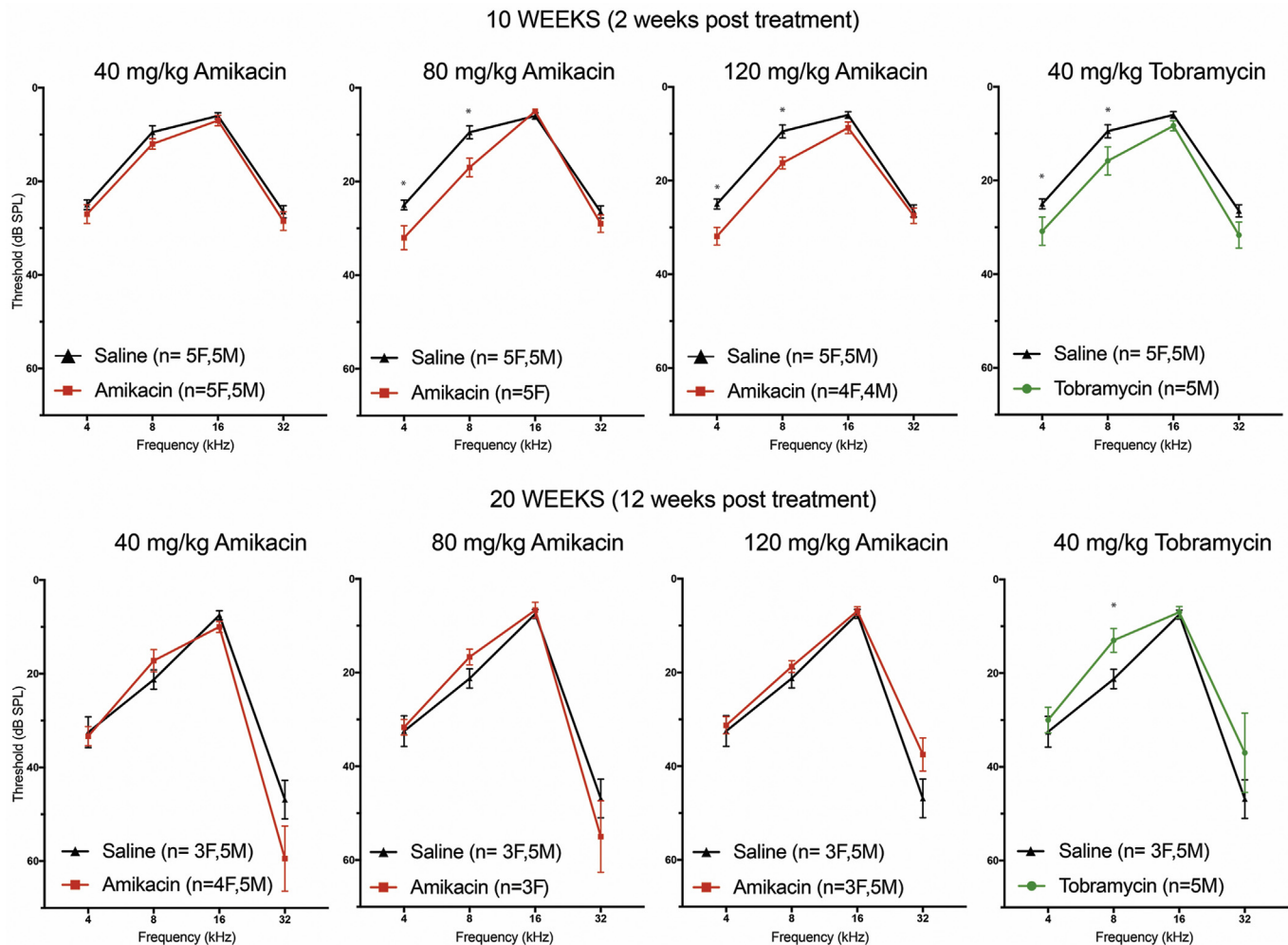


Fig. 1. Hearing thresholds of mice at 10 and 20 weeks of age following treatment with amikacin, tobramycin or saline. Each graph illustrates mean ABR thresholds to tone-pip stimuli at either 10 or 20 weeks of age following treatment with different doses of aminoglycoside or saline. Two weeks post treatment there was no difference between the hearing thresholds of mice treated with 40 mg/kg of amikacin and the saline treated control group. A small, but statistically significant difference was observed when comparing the thresholds of mice treated with 80 or 120 mg/kg of Amikacin or 40 mg/kg of tobramycin with saline controls. At 20 weeks these threshold differences were no longer present, with comparable ABR thresholds between all treatment groups. * $P < 0.05$ (values outlined in S1 Tab.); Error Bars = S.E.M; F = female, M = male. Y-axis is inverted.

frequency (4–8 kHz). From this, it is tempting to suggest that hair cells correlating with frequency specific perception are more susceptible to ototoxicity. However, ototoxic hearing loss in humans has a distinct pattern, with hair cells in the base of the cochlea dying at a greater rate than those situated towards the apex. Due to cochlear tonotopy, this cell death pattern causes high-frequency hearing loss, which is above 2000Hz in humans. *In vitro* studies utilising mouse cochlear explants have shown a similar basal to apical pattern of cell death in response to aminoglycoside treatment. Therefore, a similar pattern of cell death *in vivo* would be expected to induce hearing loss above 16 kHz in mice, because the auditory perception range of mice is higher than that of humans. Furthermore, the small differences observed between the hearing thresholds of treated and un-treated mice at 10 weeks were no longer present at 20 weeks of age. And, imaging indicated that hair cells were present in the apical turn of cochleae collected from aminoglycoside treated mice. Therefore, the ABR results obtained at 10 weeks, although statistically significant, must be viewed with caution.

Generally, low frequency hearing loss may be indicative of a conductive issue, or the result of inner ear hydrops. However, in both cases a much larger threshold shift than 5 dB SPL is expected

(Ogier et al., 2014; Zhang et al., 2016; Tian et al., 2016; MacArthur et al., 2006). Furthermore, a condition such as hydrops would be expected to cause severe progressive hearing loss and vestibular impairment, which we did not observe in aminoglycoside treated mice (Ataman and Enache, 2007; Ding et al., 2016a). It is possible that the small differences initially observed, were compounded by the limitations of ABR Testing. In this study, ABR thresholds were measured to the nearest 5 dB SPL. Therefore, a mouse with a hearing threshold between 31 and 35 dB SPL would record a threshold of 35 dB SPL. This can result in the magnification of insignificant threshold shifts. However, the trend towards low frequency hearing changes was consistent in the 10 week old aminoglycoside treated mice and there is some previous evidence that low frequency hearing loss has been caused by aminoglycoside treatments in mice (Hirose and Sato, 2011; Li et al., 2016). Nevertheless, the small and short-lived differences observed in this study would likely prove inadequate when testing oto-protective substances experimentally. Very large animal cohorts would be required to detect appreciable differences between treatment groups and given the labour intensive nature of i.v. injections, such cohorts would be difficult to generate and manage.

There are however, more sensitive methods than ABR testing

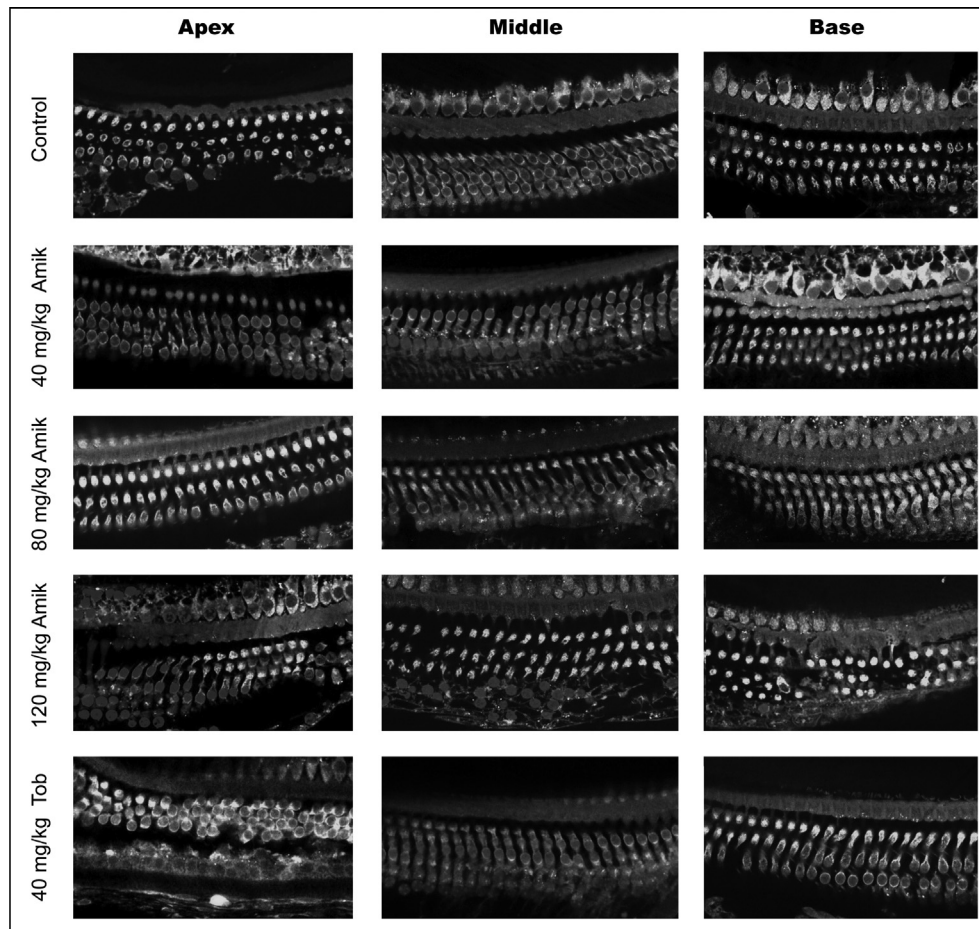


Fig. 2. Representative images of phalloidin labelled hair cells from mouse cochlea whole mount preparations (collected 2 weeks treatment). Hair cells of the apical, middle and basal turns were present, with minimal hair cell loss observed in any treatment group ($n = 1-2$ per treatment). Abbreviations used are, Amik for amikacin, and Tob for tobramycin.

available for assessing cochlear damage in the mouse. For example, otoacoustic emissions that have been produced by the outer hair cells as they act to amplify a sound wave within the cochlea can be recorded in the ear canal by Distortion Product Otoacoustic Emission (DPOAE) testing. These emissions provide a more sensitive measure of outer hair cell function (Kemp, 1978; Martin et al., 2006). Additionally, scanning electron microscopy or histology combined with immunohistochemistry has previously been used to investigate subtle cell death patterns (Severinsen et al., 2006; Heydt et al., 2004; Ruan et al., 2014). However, the goal of this study was to achieve robust ABR threshold shifts similar to those observed in humans to ensure a clinically relevant model of ototoxicity. In humans, the reported prevalence of aminoglycoside ototoxicity can be quite variable, affecting between 21% and 83% of individuals (Zettner and Gleser, 2018; Al-Malky et al., 2011, 2015; Handelsman et al., 2017; Pedersen et al., 1987; Moore et al., 1984; Gleser and Zettner, 2018). However, when hearing threshold shifts are observed, they are usually greater than 20 dB SPL and often in more than one frequency (Al-Malky et al., 2011, 2015; Pedersen et al., 1987; Gleser and Zettner, 2018). For amikacin specifically, Javadi et al. reported ototoxic outcomes in 70% of treated individuals that had received a single course of 500 mg (fixed dose) daily, for an average of 35 days (Javadi et al.). Alternatively, Black et al. observed ototoxic outcomes in only 24% of treated individuals that had received 22.5 mg/kg daily for 19 days. However, the hearing loss measured in these individuals was greater, with an

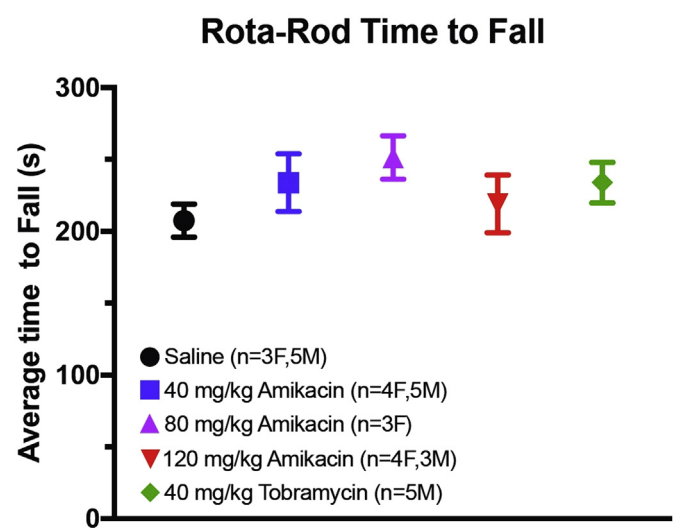


Fig. 3. Aminoglycoside treatment did not impair Rota-Rod performance. The accelerating rotor rod test was used to assess motor coordination of mice. No statistical differences were observed between control and treatment groups (one way ANOVA $p = 0.61$). P values for each post-hoc comparison of treatment against saline control are 0.59 for 40 mg/kg amikacin, 0.45 for 80 mg/kg amikacin, 0.71 for 120 mg/kg amikacin, and 0.70 for 40 mg/kg tobramycin (Dunnett's multiple comparisons test) Error Bars = S.E.M; F = female, M = male.

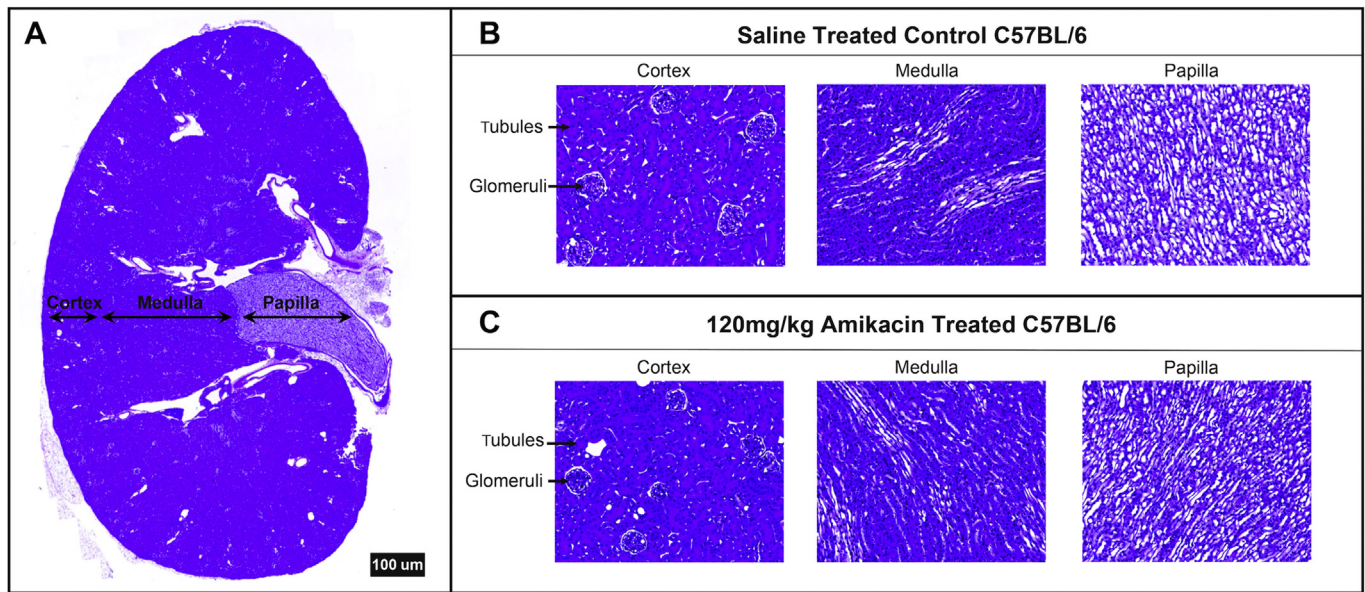


Fig. 4. Kidney samples collected from aminoglycoside treated mice did not show signs of nephrotoxicity. A) Light micrograph of a representative H&E stained kidney frontal section collected from a saline control mouse (1.2x magnification). The cortex, medulla and papilla could be visualised in each segment. B) Light micrographs of the cortex, medulla and papilla of a saline treated control mouse at 20x magnification. C) Light micrographs of the cortex, medulla and papilla of a mouse receiving the highest aminoglycoside dose within this study (120 mg/kg amikacin) at 20x magnification. All segments appeared normal, with no indicators of toxicity, such as tubular damage or scarring observed.

average threshold shift of 37–39 dB SPL at the higher frequencies (4–8 kHz) (Black et al., 1976). For tobramycin, Pederson et al. reported that 15 mg/kg daily for 14 days induced a threshold shift of 15–30 dB SPL, also in two high frequencies, for 32% of those treated. Such large ABR threshold shifts were not induced by the aminoglycoside dosing regimens tested in this study, which were 5–7 times higher than the maximum recommended human dose.

Notably, allometric scaling suggests that even higher doses of aminoglycosides would be required to match mouse and human dosing. For example, human to mouse conversion guidelines based on body surface area, suggest that a human dose must be multiplied by 12.3 to achieve an equivalent dose in the mouse (outlined in Nair and Jacob, 2016) (Nair and Jacob, 2016; Sharma and McNeill, 2009; Reagan-Shaw et al., 2008). However, such conversions remain controversial and in our hands, high doses were immediately lethal. Some previous studies have shown that mice tolerate higher aminoglycoside doses when given via s.c. and i.p. injection (Table 1). However, this may reflect the slower drug uptake

achieved using i.p./s.c. injections (Turner et al., 2011), and an overall reduced drug uptake due to the poor membrane penetration of aminoglycosides (Gonzalez and Spencer, 1998; van Gennip et al., 2009). Indeed, tobramycin plasma levels achieved by i.v. infusion are double those achieved using i.p. injection in humans (Bunke et al., 1983). Notably, high mortality is still a frequent outcome when mice are given aminoglycosides i.p. or s.c. (Table 1).

Such immediate life threatening responses to aminoglycosides rarely occur in humans. This may indicate that humans have an intrinsic ability to tolerate systemic aminoglycosides when compared to mice, and as a result only susceptible tissues such as the kidney and ear are damaged. Alternatively, infusion rate is an important factor to consider. In this study, mice received daily i.v. injections, which were relatively rapid (approx. 30–40 s). In contrast, daily aminoglycosides treatments can be given via i.v. infusion over 30 min in humans. This is an aspect of human treatment that is particularly difficult to recapitulate in mice. Implantable infusion pumps can provide constant and slow i.v. drug

Table 2
Reported pathology of kidney samples collected from mice treated with amikacin (amik), tobramycin (tob) or saline (n = 2–4 per treatment group). Incidental findings were reported, however no significant lesions were observed in any sample.

Sex	Treatment	Macro Observation	Pathology Report
F	Saline	NO LESIONS OF SIGNIFICANCE	No significant findings.
M	Saline	NO LESIONS OF SIGNIFICANCE	Mild, focal chronic pyelitis with a lymphoplasmacytic infiltrate.
M	40 mg/kg tob	NO LESIONS OF SIGNIFICANCE	No significant findings.
F	40 mg/kg amik	NO LESIONS OF SIGNIFICANCE	No significant findings.
M	40 mg/kg amik	NO LESIONS OF SIGNIFICANCE	No significant findings.
F	80 mg/kg amik	NO LESIONS OF SIGNIFICANCE	Several tubular protein casts characterised by markedly dilated lumina filled with proteinaceous material and lined by attenuated epithelium.
F	120 mg/kg amik	NO LESIONS OF SIGNIFICANCE	Small protein casts with minimal luminal distension.
M	120 mg/kg amik	NO LESIONS OF SIGNIFICANCE	No significant findings.

delivery in mice. However, these pumps have a small reservoir volume (100–200 μ l), which limits their capacity for aminoglycoside delivery.

Overall, the utility of mice to model aminoglycoside ototoxicity remains controversial. Some studies have reported success using kanamycin, whereas a number have found mice to be resistant to ototoxic drugs (Poirrier et al., 2010; Wu et al., 2001; Murillo-Cuesta et al., 2010; Jiang et al., 2005). Our finding, that daily i.v. injections of tobramycin or amikacin did not induce significant high frequency hearing loss in C57BL/6 mice offers further support to the hypothesis that mice are resistant to aminoglycoside ototoxicity.

There may be a number of reasons why systemic aminoglycosides are unable to cause hearing loss in mice. It has been proposed that the mouse's high metabolic rate and rapid drug clearance prevents serum levels reaching ototoxic levels (Poirrier et al., 2010; Wu et al., 2001). However, Wu et al. previously suggested that kanamycin induced hair cell damage is greatest in BALB/c mice, even though these mice clear kanamycin from their system faster than C57BL/6 and CBA mice (Wu et al., 2001). Furthermore, i.v. injections provide immediate systemic drug distribution and increase the half life of aminoglycosides (Bunke et al., 1983). This should ensure rapid drug delivery to the ear. Therefore, an alternate hypothesis regarding ototoxic resistance is that the blood-endolymph barrier in mice is less permeable to aminoglycosides than in other mammals.

The blood-endolymph barrier within the *stria vascularis* is one of the strongest tissue-blood barriers in mammals. However, intrinsic tissue components can alter the integrity of this barrier. For example, melanin expression in the ear is linked with external pigmentation and animals with darker pigmentation are reportedly more resistant to ototoxicity (Wu et al., 2001; Murillo-Cuesta et al., 2010; Conlee et al., 1989; Meyer zum Gottesberge, 1988). This is of potential relevance, as the capillaries of the *stria vascularis* are flanked by melanocytes, which excrete melanin and control vascular permeability. These capillaries are the critical point at which blood to endolymph drug transport occurs. Melanocyte expression of pigment epithelial-derived factor impacts the expression of tight- and adherens-junction proteins which ultimately control the permeability of the blood-endolymph barrier (Zhang et al., 2016). Furthermore, excreted melanin is in itself a free radical scavenger capable of binding to aminoglycosides (Wu et al., 2001; LaFerriere et al., 1974; Hilding and Ginzberg, 1977; Tachibana, 1999; Lyttkens et al., 2009; Rozanowska et al., 1999; Wrzesniok et al., 2012).

Therefore, it is possible that this protocol may have stronger effects in white mouse strains such as BALB/c. However, evidence regarding the susceptibility of BALB/c mice to ototoxic drugs within the literature is conflicting. Wu et al. report that twice daily subcutaneous injections of kanamycin cause significant high frequency threshold shifts in five-week old BALB/c mice (Wu et al., 2001). However, this result could not be replicated by Poirrier et al. in nine-week old BALB/C mice (Poirrier et al., 2010). The same dosing regimen (2x s.c. 900 mg/kg kanamycin) failed to alter hair cell number in the BALB/c utricle (Severinsen et al., 2006). Whilst it may be worth determining whether daily i.v. administration of aminoglycosides can induce measurable ABR threshold shifts in white strains of mice, the pigmented C57BL/6 strain is the most commonly used mouse in biomedical research and many genetically altered strains have been developed from mice with pigmented colouring (Bryant, 2011). However, with advances in gene editing technology such as CRISPR/cas9 it is becoming easier to modify strains with a desirable genetic background.

For this study, we used amikacin and tobramycin, which are the most frequently used ototoxic aminoglycosides in clinical practice. However, some studies have suggested that kanamycin is the most

ototoxic aminoglycoside in mice (Wu et al., 2001; Murillo-Cuesta et al., 2010). It is possible that intravenous kanamycin may have had a stronger affect than amikacin or tobramycin. However, as discussed above, the reported ototoxicity of kanamycin in mice is controversial. Alternatively, co-administration of loop diuretics such as furosemide, have been used to disrupt the blood-endolymph barrier and enhance ototoxic outcomes in mice (Hirose and Sato, 2011; Ruan et al., 2014; Oesterle et al., 2008; Hartman et al., 2009; Li et al., 2011). Loop diuretics cause vasoconstriction within the *stria vascularis* and *strial* ischemia follows. This causes dysregulation of the blood-endolymph barrier, allowing aminoglycosides to accumulate within the cochlear endolymph (Ding et al., 2016b; Rybak, 1985, 1993). It is likely that furosemide co-administration would enhance the ototoxicity of i.v. administered amikacin or tobramycin. Indeed, the ototoxic effects of furosemide-aminoglycoside co-treatments appear to be consistent and easily reproduced, and a recent study has reported hearing impairment in juvenile mice treated with an amikacin-furosemide combination (P14-21, n = 4) (Zadrozniak et al., 2019). However, the use of furosemide can be confounding for a number of reasons, particularly when assessing otoprotective substances. Furosemide is an ototoxic compound, causing rapid, transient hearing loss (Ding et al., 2016b; Rybak, 1985; Schwartz et al., 1970). However, permanent furosemide ototoxicity can occur (Rifkin et al., 1978; Gallagher and Jones, 1979; Quick and Hoppe, 1975). Importantly, combined furosemide-kanamycin treatments in mice do not achieve ototoxicity that mimics human aminoglycoside induced hair cell death. Instead, extensive cochlear damage and a marked inflammatory response results in hearing loss across all frequencies (Hirose and Sato, 2011). Furthermore, furosemide significantly increases aminoglycoside serum levels, which often leads to unacceptable mortality (up to 75% mortality depending on dose) (Hirose and Sato, 2011; Li et al., 2011; Rybak, 1985; Kaka et al., 1984; Ohtani et al., 1978; Ju et al., 2017). Such mortality is difficult to justify when the extensive cochlear destruction achieved implies an alternate mechanism of injury. As a result, the benefits afforded by otoprotective compounds in a furosemide ototoxicity model may be related to a number of factors beyond hair cell protection.

Mice are also considered to be resistant to aminoglycoside nephrotoxicity (Schmitz et al., 2002; Suzuki et al., 1994, 1995), with pigmented strains significantly more resistant to nephropathy than non-pigmented strains (Borza et al., 2013; Huang et al., 2013; Sato et al., 2004; Qi et al., 2005; Ma and Fogo, 2003). Previous studies using the highly nephrotoxic aminoglycoside gentamycin have induced kidney damage in white mice (Suleimani et al., 2018; Whiting et al., 1981; Aldahmash et al., 2016). And, the use of furosemide in conjunction with aminoglycosides has also induced nephropathy in mice (Ohtani et al., 1978; Adelman et al., 1979). However, we observed no evidence of kidney damage in amikacin or tobramycin treated C57BL/6 mice, despite frequently observing signs of immediate systemic toxicity such as significant prostration. The absence of kidney damage may be due to the intrinsic resistance of C57BL/6 mice to nephropathy, or may be due to the significantly lower nephrotoxic properties of tobramycin and amikacin (Sweileh, 2009; Hottendorf and Gordon, 1980). It is possible that a longer treatment period could enhance the oto- and nephrotoxicities of aminoglycosides in this i.v. model. However, tail veins can become difficult to visualise after a number of injections. Presently, the surgical approach of directly applying compounds to the mouse's ear remains a consistent way to deafen mice with ototoxic aminoglycosides. This technique bypasses the *stria vascularis*, allowing for drug entry via the round window, reducing systemic toxicities and mortality. However, confounding issues such as physical damage, the inflammatory response, variable drug diffusion and the level of skill required are problematic in an

experimental setting, limiting the utility of this model.

Studies utilising fluorescently labelled aminoglycosides have also highlighted the importance of the stria capillary network and marginal cells as an entry point for aminoglycosides (Wang and Steyger, 2009; Huth et al., 2011; Imamura and Adams, 2003; Tran Ba Huy et al., 1986). However, the process by which aminoglycosides are trafficked from capillary, to marginal cells and finally the endolymph is not yet understood. Future research regarding the blood-endolymph barrier in mice may elucidate the mechanism underlying ototoxicity resistance and provide further opportunity for developing otoprotective strategies.

5. Summary

The aim of this study was to develop a murine model of aminoglycoside ototoxicity that mimicked clinical practice. Previous studies have suggested that mice are not susceptible to aminoglycoside induced hearing loss. However, the standard care methodology utilised for human aminoglycoside infusions have not been accurately modelled in the mouse. In humans, aminoglycosides are predominantly given i.v., whereas i.p. or s.c. routes of drug delivery have been used in the mouse. We hypothesised that repeated, daily i.v. injection of ototoxic aminoglycosides would induce high frequency hearing loss similar to that observed in aminoglycoside treated humans. We demonstrated that daily i.v. injections are achievable but C57BL/6 mice do not appear to be a suitable model for systemic aminoglycoside ototoxic investigations (*in vivo*). Daily i.v. aminoglycoside injections of 80–120 mg/kg of amikacin and 40 mg/kg tobramycin induced significant signs of systemic toxicity, such as rapid breathing and prostration in treated mice. Higher doses given i.v. were immediately lethal. This regimen was unable to achieve strong ABR threshold shifts in mice mimicking the high frequency hearing loss that occurs in aminoglycoside treated humans. For example, tobramycin has been shown to induce a hearing loss of ~39 dB SPL at two high frequencies in humans after 14 days of treatment (Pedersen et al., 1987) and for some individuals, ototoxic outcomes can worsen over time (Fee, 1983). In this study, no significant high frequency hearing loss was observed in aminoglycoside treated C57BL/6 mice at either 2 or 12 weeks post treatment. However, age related hearing loss typical of the C57BL/6 strain was observed in both the control and aminoglycoside treated mice at 20 weeks of age. We conclude that daily i.v. injections of amikacin or tobramycin do not achieve a robust model of ototoxicity in C57BL/6 mice.

Author contributions statement

JMO, PJJ and RAB conceived the experiments. JMO conducted experiments, wrote the manuscript and prepared figures. All authors reviewed the manuscript.

Declaration of competing interest

The authors have declared that no competing interests exist.

CRediT authorship contribution statement

Jacqueline M. Ogier: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Paul J. Lockhart:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition. **Rachel A. Burt:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heares.2019.107870>.

References

- Adelman, R.D., Spangler, W.L., Beasom, F., Ishizaki, G., Conzelman, G.M., 1979. Furosemide enhancement of experimental gentamicin nephrotoxicity: comparison of functional and morphological changes with activities of urinary enzymes. *J. Infect. Dis.* 140, 342–352.
- Al-Malky, G., 2016. Audiological monitoring in ototoxicity - are we doing enough? *ENT and Audiology News* 25.
- Al-Malky, G., Suri, R., Dawson, S.J., Sirimanna, T., Kemp, D., 2011. Aminoglycoside antibiotics cochleotoxicity in paediatric cystic fibrosis (CF) patients: a study using extended high-frequency audiometry and distortion product otoacoustic emissions. *Int. J. Audiol.* 50, 112–122.
- Al-Malky, G., Dawson, S.J., Sirimanna, T., Bagkeris, E., Suri, R., 2015. High-frequency audiometry reveals high prevalence of aminoglycoside ototoxicity in children with cystic fibrosis. *J. Cyst. Fibros.* 14, 248–254.
- Aldahmash, B.A., El-Nagar, D.M., Ibrahim, K.E., 2016. Reno-protective effects of propolis on gentamicin-induced acute renal toxicity in swiss albino mice. *Nefrologia* 36, 643–652.
- Alzamil, K.S., Linthicum, F.H.J., 2000. Extraneous round window membranes and plugs: possible effect on intratympanic therapy. *Ann. Otol. Rhinol. Laryngol.* 109, 30–32.
- Aminoglycoside dosing guideline, Stanford health care, Santa Clara, USA. Available at: http://med.stanford.edu/bugsanddrugs/dosing-protocols/_jcr_content/main/panel_builder/panel_0/download_2/file.res/Aminoglycoside%20Dosing%20Guide%202017-08-23.pdf. Accessed: 11 July 2018.
- Antibiotic clinical practice guidelines, Royal childrens hospital, Parkville, Australia. Available at: https://www.rch.org.au/clinicalguide/guideline_index/Antibiotics/. Accessed: 11 July 2018.
- Ataman, T., Enache, A., 2007. Our experience with the medical treatment of endolymphatic hydrops. *Int. Tinnitus J.* 13, 138–142.
- Beaucaire, G., 2000. Does once-daily dosing prevent nephrotoxicity in all aminoglycosides equally? *Clin. Microbiol. Infect.* 6, 357–362.
- Berry-Pusey, B.N., et al., 2013. A semi-automated vascular access system (VAS) for preclinical models. *Phys. Med. Biol.* 58, 5351–5362.
- Black, R.E., Lau, W.K., Weinstein, R.J., Young, L.S., Hewitt, W.L., 1976. Ototoxicity of amikacin. *Antimicrob. Agents Chemother.* 9, 956–961.
- Borkholder, D.A., Zhu, X., Frisina, R.D., 2014. Round window membrane intracochlear drug delivery enhanced by induced advection. *J. Control. Release* 174, 171–176.
- Borza, D.-B., Zhang, J.-J., Beck Jr., L.H., Meyer-Schwesinger, C., Luo, W., 2013. Mouse models of membranous nephropathy: the road less travelled by. *Am. J. Clin. Exp. Immunol.* 2, 135–145.
- Bryant, C.D., 2011. The blessings and curses of C57BL/6 substrains in mouse genetic studies. *Ann. N. Y. Acad. Sci.* 1245, 31–33.
- Bunke, C.M., Aronoff, G.R., Brier, M.E., Sloan, R.S., Luft, F.C., 1983. Tobramycin kinetics during continuous ambulatory peritoneal dialysis. *Clin. Pharmacol. Ther.* 34, 110–116.
- Chen, Z., Mikulec, A.A., McKenna, M.J., Sewell, W.F., Kujawa, S.G., 2006. A method for intracochlear drug delivery in the mouse. *J. Neurosci. Methods* 150, 67–73.
- Coleman, J.K.M., Littlesunday, C., Jackson, R., Meyer, T., 2007. AM-111 protects against permanent hearing loss from impulse noise trauma. *Hear. Res.* 226, 70–78.
- Conlee, J.W., Gill, S.S., McCandless, P.T., Creel, D.J., 1989. Differential susceptibility to gentamicin ototoxicity between albino and pigmented Guinea pigs. *Hear. Res.* 41, 43–51.
- Cosh, S., et al., 2018. The relationship between hearing loss in older adults and depression over 12 years: findings from the Three-City prospective cohort study. *Int. J. Geriatr. Psychiatry* 1654–1661. <https://doi.org/10.1002/gps.4968>.
- Craig, W.A., Redington, J., Ebert, S.C., 1991. Pharmacodynamics of amikacin in vitro

- and in mouse thigh and lung infections. *JAC (J. Antimicrob. Chemother.)* 27, 29.
- Dhillon, J., Fielding, R., Adler-Moore, J., Goodall, R.L., Mitchison, D., 2001. The activity of low-clearance liposomal amikacin in experimental murine tuberculosis. *J. Antimicrob. Chemother.* 48, 869–876.
- Dinc, G., et al., 2015. Antimicrobial efficacy of doripenem and its combinations with sulbactam, amikacin, colistin, tigecycline in experimental sepsis of carbapenem-resistant *Acinetobacter baumannii*. *New Microbiol.* 38, 67–73.
- Ding, C.-R., et al., 2016. Effect of endolymphatic hydrops on sound transmission in live Guinea pigs measured with a laser Doppler vibrometer. *Neural Plast.* 8648297 (2016).
- Ding, D., et al., 2016. Ototoxic effects and mechanisms of loop diuretics. *J. Otolaryngol.* 11, 145–156.
- Edson, R.S., Terrell, C.L., 1987. The aminoglycosides: streptomycin, kanamycin, gentamicin, tobramycin, amikacin, netilmicin, and sisomicin. *Mayo Clin. Proc.* 62, 916–920.
- Edson, R.S., et al., 2004. Vestibular toxicity due to inhaled tobramycin in a patient with renal insufficiency. *Mayo Clin. Proc.* 79, 1185–1191.
- Ehlers, S., et al., 1996. Liposomal amikacin for treatment of *M. avium* infections in clinically relevant experimental settings. *Zentralblatt Bakteriologie* 284, 218–231.
- Fee Jr., W.E., 1983. Gentamicin and tobramycin: comparison of ototoxicity. *CID* 5, S304–S313.
- Francis, S.P., et al., 2011. Celastrol inhibits aminoglycoside-induced ototoxicity via heat shock protein 32. *Cell Death Dis.* 2, e195.
- Gallagher, K.L., Jones, J.K., 1979. Furosemide-induced ototoxicity. *Ann. Intern. Med.* 91, 744–745.
- Garinis, A.C., et al., 2017. The cumulative effects of intravenous antibiotic treatments on hearing in patients with cystic fibrosis. *J. Cyst. Fibros.* 16, 401–409.
- Gleser, M.A., Zettner, E.M., 2018. Negative hearing effects of a single course of IV aminoglycoside therapy in cystic fibrosis patients. *Int. J. Audiol.* 57, 917–924.
- Gonzalez, L.S., Spencer, J.P., 1998. Aminoglycosides: a practical review. *Am. Fam. Physician* 58, 1811–1820.
- Guner, E.A., et al., 2017. Cochlear and vestibular effects of combined intratympanic gentamicin and dexamethasone. *J. Int. Adv. Otol.* 13, 47–52.
- Gupta, S.K., Mundra, R.K., 2015. Electronystagmography a very useful diagnostic tool in cases of vertigo. *Indian J. Otolaryngol. Head Neck Surg.: Off. Publ. Assoc. Otolaryngol. India* 67, 370–374.
- Handelsman, J.A., Nasr, S.Z., Pitts, C., King, W.M., 2017. Prevalence of hearing and vestibular loss in cystic fibrosis patients exposed to aminoglycosides. *Pediatr. Pulmonol.* 52, 1157–1162.
- Hartman, B.H., et al., 2009. Hes5 expression in the postnatal and adult mouse inner ear and the drug-damaged cochlea. *J. Assoc. Res. Otolaryngol.* 10, 321–340.
- Hay-McCutcheon, M.J., Reed, P.E., Cheimariou, S., 2018. Positive social interaction and hearing loss in older adults living in rural and urban communities. *J. Speech Lang. Hear. Res.* 61, 2138–2145.
- Hermann, T., 2007. Aminoglycoside antibiotics: old drugs and new therapeutic approaches. *Cell. Mol. Life Sci.* 64, 1841–1852.
- Heydt, J.L., Cunningham, L.L., Rubel, E.W., Coltrera, M.D., 2004. Round window gentamicin application: an inner ear hair cell damage protocol for the mouse. *Hear. Res.* 192, 65–74.
- Hilding, D.A., Ginzberg, R.D., 1977. Pigmentation of the stria vascularis. The contribution of neural crest melanocytes. *Acta Otolaryngol.* 84, 24–37.
- Hirose, K., Sato, E., 2011. Comparative analysis of combination kanamycin-furosemide versus kanamycin alone in the mouse cochlea. *Hear. Res.* 272, 108–116.
- Horvath, L., Bachinger, D., Honegger, T., Bodmer, D., Naldi, A.M., 2019. Functional and morphological analysis of different aminoglycoside treatment regimens inducing hearing loss in mice. *Exp. Ther. Med.* 18, 1123–1130.
- Hottendorf, G.H., Gordon, L.L., 1980. Comparative low-dose nephrotoxicities of gentamicin, tobramycin, and amikacin. *Antimicrob. Agents Chemother.* 18, 176–181.
- Hottendorf, G.H., Williams, P.D., 1986. Aminoglycoside nephrotoxicity. *Toxicol. Pathol.* 14, 66–72.
- Huang, L., Scarpellini, A., Funk, M., Verderio, E.A.M., Johnson, T.S., 2013. Development of a chronic kidney disease model in C57BL/6 mice with relevance to human pathology. *Nephron extra* 3, 12–29.
- Huth, M.E., Ricci, A.J., Cheng, A.G., 2011. Mechanisms of aminoglycoside ototoxicity and targets of hair cell protection. *Int. J. Otolaryngol.* 2011, 19.
- Imamura, S.-I., Adams, J.C., 2003. Distribution of gentamicin in the Guinea pig inner ear after local or systemic application. *J. Assoc. Res. Otolaryngol.* 4, 176–195.
- Isgrig, K., et al., 2017. Gene therapy restores balance and auditory functions in a mouse model of usher syndrome. *Mol. Ther.* 25, 780–791.
- Iwai, K., et al., 2006. Cochlear protection by local insulin-like growth factor-1 application using biodegradable hydrogel. *The Laryngoscope* 116, 529–533.
- Javadi, M. R. et al., 2019. The incidence of amikacin ototoxicity in multidrug-resistant tuberculosis patients. *Iran. J. Pharm. Res. (IJPR)* 10, 905–911.
- Jero, J., et al., 2001. Cochlear gene delivery through an intact round window membrane in mouse. *Hum. Gene Ther.* 12, 539–548.
- Jiang, H., Sha, S.-H., Schacht, J., 2005. NF-kappaB pathway protects cochlear hair cells from aminoglycoside-induced ototoxicity. *J. Neurosci.* 25, 644–651.
- Jiang, M., Karasawa, T., Steyger, P.S., 2017. Aminoglycoside-induced cochleotoxicity: a review. *Front. Cell. Neurosci.* 11, 308.
- Jiang, M., et al., 2019. Inflammation up-regulates cochlear expression of TRPV1 to potentiate drug-induced hearing loss. *Sci. Adv.* 5, eaaw1836.
- Ju, H.M., Lee, S.H., Choi, J.S., Seo, Y.J., 2017. A simple model for inducing optimal increase of SDF-1 with aminoglycoside ototoxicity. *BioMed Res. Int.* 2017, 4630241.
- Kaka, J.S., Lyman, C., Kilarski, D.J., 1984. Tobramycin-furosemide interaction. *Drug Intell. Clin. Pharm.* 18, 235–238.
- Kaynar, K., et al., 2007. Amikacin-induced nephropathy: is there any protective way? *Ren. Fail.* 29, 23–27.
- Kemp, D.T., 1978. Stimulated acoustic emissions from within the human auditory system. *J. Acoust. Soc. Am.* 64, 1386–1391.
- LaFerriere, K.A., Arenberg, I.K., Hawkins, J.E.J., Johnsson, L.G., 1974. Melanocytes of the vestibular labyrinth and their relationship to the microvasculature. *Ann. Otol. Rhinol. Laryngol.* 83, 685–694.
- Lee, K.S., Kimura, R.S., 1991. Effect of ototoxic drug administration to the endolymphatic sac. *Ann. Otol. Rhinol. Laryngol.* 100, 355–360.
- Leibovici, L., et al., 2001. Departmental consumption of antibiotic drugs and subsequent resistance: a quantitative link. *J. Antimicrob. Chemother.* 48, 535–540.
- Li, Y., Ding, D., Jiang, H., Fu, Y., Salvi, R., 2011. Co-administration of cisplatin and furosemide causes rapid and massive loss of cochlear hair cells in mice. *Neurotox. Res.* 20, 307–319.
- Li, S., Hang, L., Ma, Y., 2016. FGF22 protects hearing function from gentamycin ototoxicity by maintaining ribbon synapse number. *Hear. Res.* 332, 39–45.
- Liberman, M.C. Video tutorial: cochlear dissection. Available at: www.masseyeandear.org/research/otolaryngology/investigators/laboratories/eaton-peabody-laboratories/epl-histology-resources/video-tutorial-for-cochlear-dissection. Accessed: 26 May 2019.
- Louie, A., Liu, W., Fikes, S., Brown, D., Drusano, G.L., 2013. Impact of meropenem in combination with tobramycin in a murine model of *Pseudomonas aeruginosa* pneumonia. *Antimicrob. Agents Chemother.* 57, 2788–2792.
- Lyttkens, L., Larsson, B., Göller, H., Engleson, S., Stahle, J., 2009. Melanin capacity to accumulate drugs in the internal ear: a study on lidocaine, bupivacaine and chlorpromazine. *Acta Otolaryngol.* 88, 61–73.
- Ma, L.-J., Fogo, A.B., 2003. Model of robust induction of glomerulosclerosis in mice: importance of genetic background. *Kidney Int.* 64, 350–355.
- MacArthur, C.J., Hefeneider, S.H., Kempton, J.B., Trune, D.R., 2006. C3H/HeJ mouse model for spontaneous chronic otitis media. *The Laryngoscope* 116, 1071–1079.
- Martin, G.K., Stagner, B.B., Lonsbury-Martin, B.L., 2006. Assessment of cochlear function in mice: distortion-product otoacoustic emissions. *Curr. Protoc. Neurosci.* Chapter 8, Unit8.21C.
- Mathe, A., Szabo, D., Anderlik, P., Rozgonyi, F., Nagy, K., 2007. The effect of amikacin and imipenem alone and in combination against an extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* strain. *Diagn. Microbiol. Infect. Dis.* 58, 105–110.
- Mathur, P.D., et al., 2015. A study of whirlin isoforms in the mouse vestibular system suggests potential vestibular dysfunction in DFNB31-deficient patients. *Hum. Mol. Genet.* 24, 403–4030.
- Matz, G.J., 1986. Aminoglycoside ototoxicity. *Am. J. Otolaryngol.* 7, 117–119.
- McCall, A.A., et al., 2010. Drug delivery for treatment of inner ear disease: current state of knowledge. *Ear Hear.* 31, 156–165.
- Mehta, R.L., et al., 2015. Phenotype standardization for drug-induced kidney disease. *Kidney Int.* 88, 226–234.
- Meyer zum Göttesberge, A.M., 1988. Physiology and pathophysiology of inner ear melanin. *Pigment Cell Res.* 1, 238–249.
- Moore, R.D., Smith, C.R., Lietman, P.S., 1984. Risk factors for the development of auditory toxicity in patients receiving aminoglycosides. *J. Infect. Dis.* 149, 23–30.
- Munckhof, W.J., Grayson, M.L., Turnidge, J.D., 1996. A meta-analysis of studies on the safety and efficacy of aminoglycosides given either once daily or as divided doses. *J. Antimicrob. Chemother.* 37, 645–663.
- Murillo-Cuesta, S., Contreras, J., Cediell, R., Varela-Nieto, I., 2010. Comparison of different aminoglycoside antibiotic treatments to refine ototoxicity studies in adult mice. *Lab. Anim.* 44, 124–131.
- Murillo-Cuesta, S., et al., 2017. A comparative study of drug delivery methods targeted to the mouse inner ear: bullostomy versus transtympanic injection. *J. Vis. Exp.* 54951.
- Nair, A.B., Jacob, S., 2016. A simple practice guide for dose conversion between animals and human. *J. Basic Clin. Pharm.* 7, 27–31.
- Nakashima, T., Teranishi, M., Hibi, T., Kobayashi, M., Umemura, M., 2000. Vestibular and cochlear toxicity of aminoglycosides—a review. *Acta Otolaryngol.* 120, 904–911.
- Oesterle, E.C., Campbell, S., Taylor, R.R., Forge, A., Hume, C.R., 2008. Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear. *J. Assoc. Res. Otolaryngol.* 9, 65–89.
- Ogier, J.M., et al., 2014. CHD7 deficiency in 'Loopier', a new mouse model of CHARGE syndrome, results in ossicle malformation, otosclerosis and hearing impairment. *PLoS One* 9, e97559.
- Ogier, J.M., et al., 2018. An intronic mutation in Chd7 creates a cryptic splice site, causing aberrant splicing in a mouse model of CHARGE syndrome. *Sci. Rep.* 8, 5482.
- Ogier, J.M., Burt, R.A., Drury, H.R., Lim, R., Nayagam, B.A., 2019. Organotypic culture of neonatal murine inner ear explants. *Front. Cell. Neurosci.* 13, 895.
- Ohtani, I., Ohtsuki, K., Omata, T., Ouchi, J., Saito, T., 1978. Potentiation and its mechanism of cochlear damage resulting from furosemide and aminoglycoside antibiotics. *ORL* 40, 53–63, 03011569.
- Oishi, N., Chen, F.-Q., Zheng, H.-W., Sha, S.-H., 2013. Intra-tympanic delivery of short interfering RNA into the adult mouse cochlea. *Hear. Res.* 296, 36–41.
- Oishi, N., et al., 2015. XBP1 mitigates aminoglycoside-induced endoplasmic reticulum stress and neuronal cell death. *Cell Death Dis.* 6, e1763.

- Pedersen, S.S., Jensen, T., Osterhammel, D., Osterhammel, P., 1987. Cumulative and acute toxicity of repeated high-dose tobramycin treatment in cystic fibrosis. *Antimicrob. Agents Chemother.* 31, 594–599.
- Poirrier, A.L., et al., 2010. Ototoxic drugs: difference in sensitivity between mice and Guinea pigs. *Toxicol. Lett.* 193, 41–49.
- Prayle, A., Watson, A., Fortnum, H., Smyth, A., 2010. Side effects of aminoglycosides on the kidney, ear and balance in cystic fibrosis. *Thorax* 65, 654–658.
- Prescott, W.A.J., 2014. A survey of extended-interval aminoglycoside dosing practices in United States adult cystic fibrosis programs. *Respir. Care* 59, 1353–1359.
- Qi, Z., et al., 2005. Characterization of susceptibility of inbred mouse strains to diabetic nephropathy. *Diabetes* 54, 2628–2637.
- Quick, C.A., Hoppe, W., 1975. Permanent deafness associated with furosemide administration. *Ann. Otol. Rhinol. Laryngol.* 84, 94–101.
- Rabe, M., Schaefer, F., 2016. Non-transgenic mouse models of kidney disease. *Nephron* 133, 53–61.
- Rajendran, V., Roy, F.G., 2010. Comparison of health related quality of life of primary school deaf children with and without motor impairment. *Ital. J. Pediatr.* 36, 75–75.
- Ramirez, M.S., Tolmasky, M.E., 2017. Amikacin: uses, resistance, and prospects for inhibition. *Molecules* 22, 2267.
- Ranganathan, S., 2006. *Melbourne Handbook for the Management of Children with Cystic Fibrosis*.
- Ratjen, F., Brockhaus, F., Angyalosi, G., 2009. Aminoglycoside therapy against *Pseudomonas aeruginosa* in cystic fibrosis: a review. *J. Cyst. Fibros.* 8, 361–369.
- Reagan-Shaw, S., Nihal, M., Ahmad, N., 2008. Dose translation from animal to human studies revisited. *FASEB J.* 22, 659–661.
- Rifkin, S.I., de Quesada, A.M., Pickering, M.J., Shires, D.L.J., 1978. Deafness associated with oral furosemide. *South. Med. J.* 71, 86–88.
- Roland, L., et al., 2016. Quality of life in children with hearing impairment: systematic review and meta-analysis. *Otolaryngol. Head Neck Surg.* 155, 208–219.
- Rozanowska, M., Sarna, T., Land, E.J., Truscott, T.G., 1999. Free radical scavenging properties of melanin interaction of eu- and pheo-melanin models with reducing and oxidising radicals. *Free Radic. Biol. Med.* 26, 518–525.
- Ruan, Q., et al., 2014. Topographic and quantitative evaluation of gentamicin-induced damage to peripheral innervation of mouse cochlea. *Neurotoxicology* 40, 86–96.
- Rybak, L.P., 1985. Furosemide ototoxicity: clinical and experimental aspects. *The Laryngoscope* 95, 1–14.
- Rybak, L.P., 1993. Ototoxicity of loop diuretics. *Otolaryngol. Clin. N. Am.* 26, 829–844.
- Safi, K.H., Damiani, J.M., Sturza, J., Nasr, S.Z., 2016. Extended-Interval aminoglycoside use in cystic fibrosis exacerbation in children and young adults: a prospective quality improvement project. *Glob. Pediatr. Health* 3, 2333794X16635464–2333794X16635464.
- Sato, N., et al., 2004. Acute nephrotoxicity of aristolochic acids in mice. *J. Pharm. Pharmacol.* 56, 221–229.
- Scheenstra, R.J., Rijntjes, E., Tavy, D.L.J., Kingma, H., Heijerman, H.G.M., 2009. Vestibulotoxicity as a consequence of systemically administered tobramycin in cystic fibrosis patients. *Acta Otolaryngol.* 129, 4–7.
- Schmiedt, R.A., Okamura, H.-O., Lang, H., Schulte, B.A., 2002. Ouabain application to the round window of the gerbil cochlea: a model of auditory neuropathy and apoptosis. *J. Assoc. Res. Otolaryngol.* 3, 223–233.
- Schmitz, J., et al., 2002. Megalin deficiency offers protection from renal aminoglycoside accumulation. *J. Biol. Chem.* 277, 618–622.
- Schwartz, G.H., David, D.S., Riggio, R.R., Stenzel, K.H., Rubin, A.L., 1970. Ototoxicity induced by furosemide. *N. Engl. J. Med.* 282, 1413–1414.
- Seemungal, B.M., Bronstein, A.M., 2007. Vestibular function is also vulnerable. *BMJ Br. Med. J. (Clin. Res. Ed.)* 335, 952–952.
- Severinsen, S.A., Kirkegaard, M., Nyengaard, J.R., 2006. 2,3-Dihydroxybenzoic acid attenuates kanamycin-induced volume reduction in mouse utricular type I hair cells. *Hear. Res.* 212, 99–108.
- Sharma, V., McNeill, J.H., 2009. To scale or not to scale: the principles of dose extrapolation. *Br. J. Pharmacol.* 157, 907–921.
- Smyth, A.R., Bhatt, J., Nevitt, S.J., 2017. Once-daily versus multiple-daily dosing with intravenous aminoglycosides for cystic fibrosis. *Cochrane Database Syst. Rev.* 3, CD002009–CD002009.
- Stephenson, S.E.M., et al., 2018. Generation and characterisation of a parkin-Pacrg knockout mouse line and a Pacrg knockout mouse line. *Sci. Rep.* 8, 7528.
- Stevens, S.M., Brown, L.N., Ezell, P.C., Lang, H., 2015. The mouse round-window approach for ototoxic agent delivery: a rapid and reliable technique for inducing cochlear cell degeneration. *J. Vis. Exp.* 53131.
- Al Suleimani, Y.M., et al., 2018. The effect of the dipeptidyl peptidase-4 inhibitor sitagliptin on gentamicin nephrotoxicity in mice. *Biomed. Pharmacother.* 97, 1102–1108.
- Suzuki, S., Hatashima, S., Shinzawa, Y., Niwa, O., Tamatani, R., 1994. Toxicity of neomycin on enzyme activities of kidney and duodenal mucosa in vivo: organ specificity and species difference between rats and mice. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* 109, 77–92.
- Suzuki, S., et al., 1995. Comparison of gentamicin nephrotoxicity between rats and mice. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* 112, 15–28.
- Sweileh, W.M., 2009. A prospective comparative study of gentamicin- and amikacin-induced nephrotoxicity in patients with normal baseline renal function. *Fundam. Clin. Pharmacol.* 23, 515–520.
- Tachibana, M., 1999. Sound needs sound melanocytes to be heard. *Pigment Cell Res.* 12, 344.
- Thomas, B., Atkinson, J., 1994. A 14 day toxicity study of trehalose in the albino mouse, unpublished report No. 434. Frederick Res. Centre 1–32.
- Tian, C., Harris, B.S., Johnson, K.R., 2016. Ectopic mineralization and conductive hearing loss in Enpp1asj mutant mice, a new model for otitis media and tympanosclerosis. *PLoS One* 11, e0168159–e0168159.
- Tran Ba Huy, P., Bernard, P., Schacht, J., 1986. Kinetics of gentamicin uptake and release in the rat. Comparison of inner ear tissues and fluids with other organs. *J. Clin. Invest.* 77, 1492–1500.
- Tung, V.W.K., Burton, T.J., Dababneh, E., Quail, S.L., Camp, A.J., 2014. Behavioral assessment of the aging mouse vestibular system. *J. Vis. Exp.* 51605.
- Turner, P.V., Brabb, T., Pekow, C., Vasbinder, M.A., 2011. Administration of substances to laboratory animals: routes of administration and factors to consider. *J. Am. Assoc. Lab. Anim. Sci.* 50, 600–613.
- van Gennip, M., et al., 2009. Augmented effect of early antibiotic treatment in mice with experimental lung infections due to sequentially adapted mucoid strains of *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 64, 1241–1250.
- Vrioni, G., Nauciel, C., Kerharo, G., Matsiota-Bernard, P., 1998. Treatment of disseminated *Mycobacterium genavense* infection in a murine model with ciprofloxacin, amikacin, ethambutol, clarithromycin and rifabutin. *J. Antimicrob. Chemother.* 42, 483–487.
- Wanamaker, H.H., Gruenewald, L., Damm, K.J., Ogata, Y., Slepecky, N., 1998. Dose-related vestibular and cochlear effects of transtympanic gentamicin. *Am. J. Otol.* 19, 170–179.
- Wanamaker, H.H., Slepecky, N.B., Cefaratti, L.K., Ogata, Y., 1999. Comparison of vestibular and cochlear ototoxicity from transtympanic streptomycin administration. *Am. J. Otol.* 20, 457–464.
- Wang, Q., Steyger, P.S., 2009. Trafficking of systemic fluorescent gentamicin into the cochlea and hair cells. *J. Assoc. Res. Otolaryngol.* 10, 205–219.
- Whiting, P.H., Petersen, J., Simpson, J.G., 1981. Gentamicin-induced nephrotoxicity in mice: protection by loop diuretics. *Br. J. Exp. Pathol.* 62, 200–206.
- Wrzesniok, D., Buszman, E., Miernik-Biel, E., 2012. Amikacin, kanamycin and tobramycin binding to melanin in the presence of Ca(2+) and Mg(2+) ions. *Acta Pol. Pharm.* 69, 1035–1041.
- Wu, W.J., et al., 2001. Aminoglycoside ototoxicity in adult CBA, C57BL and BALB mice and the Sprague-Dawley rat. *Hear. Res.* 158, 165–178.
- Xie, J., Talaska, A.E., Schacht, J., 2011. New developments in aminoglycoside therapy and ototoxicity. *Hear. Res.* 281, 28–37.
- Zadrozniak, M., Szymanski, M., Luszczki, J.J., 2019. Vitamin C alleviates ototoxic effect caused by coadministration of amikacin and furosemide. *Pharmacol. Rep.* 71, 351–356.
- Zagolski, O., 2008. Vestibular system in infants after systemic therapy with amikacin. *J. Otolaryngol. Head Neck Surg.* 37, 534–539.
- Zettner, E.M., Gleser, M.A., 2018. Progressive hearing loss among patients with cystic fibrosis and parenteral aminoglycoside treatment. *Otolaryngol. Head Neck Surg.* 159, 887–894.
- Zhai, F., Liu, J.-P., Dai, C.-F., Wang, Q., Steyger, P.S., 2010. Evidence-based modification of intratympanic gentamicin injections in patients with intractable vertigo. *Otol. Neurotol.* 31, 642–648.
- Zhang, Y., Liu, B., Wang, R., Jia, R., Gu, X., 2016. Characteristics of the cochlear symptoms and functions in meniere's disease. *Chin. Med. J.* 129, 2445–2450.
- Zheng, M., et al., 2013. A comparative study on the activities of six combination regimens against multidrug-resistant *Mycobacterium tuberculosis* infection in BALB/c mice. *J. Chemother.* 25, 81–86.
- Zuluaga, A.F., Rodriguez, C.A., Agudelo, M., Vesga, O., 2015. Pharmacodynamics of nine generic products of amikacin compared with the innovator in the neutropenic mouse thigh infection model. *BMC Res. Notes* 8, 546.

Supplementary Figure 1. The health monitoring sheet used to ensure animal wellbeing during daily i.v. aminoglycoside treatment.

Aminoglycoside Monitoring Sheet

Investigator Name	AEC Project Number: A844
Animal – ID Number:	Species/Strain: C57BL/6

Aminoglycoside Treatment:

	1		2		3		4		5		6		REST	
	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm
Activity														
Alertness														
Mobility														
Movement/Gait														
Fur condition														
Injection site														
Weight (g)														
Weight % change														

	7		8		9		10		11		12		RECOVER	
	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm
Activity														
Alertness														
Mobility														
Movement/Gait														
Fur condition														
Injection site														
Weight (g)														
Weight % change														

Scoring Matrix

Signs	0	1	2	3
Activity	Normal	Reduced activity	Huddled/Inactive	Unconscious
Alertness	Normal	Dull or Depressed	Little response to handling	Unconscious

Supplementary Figure 1 (continued). The health monitoring sheet used to ensure animal wellbeing during daily i.v. aminoglycoside treatment.

Mobility	Normal	Slower movement	Very little movement	Immobile
Movement/Gait	Normal	Slight ataxia	Ataxic	Limb dragging or None
Respiration	Normal	Slight panting	Panting or irregular breathing	Ragged breathing / gasping
Fur Condition	Normal	Slightly ruffled fur	Very ruffled fur	Hunched; sunken or dehydrated appearance; urine or faeces on fur
Injection Site	Normal	Slight irritation at injection site	Inflamed or open wound at injection site	Infection at injection site
Weight	Normal or gain	0 – 10% weight loss	11-20% weight loss	> 20% weight loss

ACTION PLAN FOR INDIVIDUAL SCORES

Score	Action
1	If animal scores 1 in any category, increase monitoring to twice daily If animal scores 1 in three or more categories, cease treatment and consider if euthanasia necessary
2	Report to chief investigator- Cease treatment and consider if euthanasia necessary
3	Report to chief investigator – Euthanise

Supplementary Table 1. P values reported as the result of unpaired t-tests comparing the average ABR hearing threshold of control and treated mouse groups. Doses given in mg/kg.

ABR's of control vs aminoglycoside treated mice					
10 week timepoint			20 week timepoint		
40 Amikacin	Frequency (kHz)	P value	Frequency (kHz)	P value	
	4	0.8278	4	0.8278	
	8	0.2248	8	0.2248	
	16	0.1246	16	0.1246	
	32	0.1526	32	0.1526	
80 Amikacin	Frequency (kHz)	P value	Frequency (kHz)	P value	
	4	0.0095	4	0.8851	
	8	0.0083	8	0.2346	
	16	0.3169	16	0.6618	
	32	0.2899	32	0.3435	
120 Amikacin	Frequency (kHz)	P value	Frequency (kHz)	P value	
	4	0.0039	4	0.7438	
	8	0.0028	8	0.3170	
	16	0.0567	16	0.6420	
	32	0.6344	32	0.1058	
40 Tobramycin	Frequency (kHz)	P value	Frequency (kHz)	P value	
	4	0.0455	4	0.6059	
	8	0.0469	8	0.0293	
	16	0.0684	16	0.7511	
	32	0.0774	32	0.2635	

3.3.1. Manuscript Epilogue and Future Directions

The utility of mice as a model of human ototoxicity remains a controversial topic. However, this attempt to induce ototoxic outcomes using repeated, intravenous injections of tobramycin or amikacin provides further evidence that mice are resistant to systemic aminoglycoside ototoxicity.

Having been unable to establish an *in vivo* mouse model that appropriately replicates the standard aminoglycoside treatment and ototoxic outcomes in humans, the hypothesis that *Ask1*^{-/-} mice are resistant to aminoglycoside-induced hearing loss remains to be fully tested.

In order to test the hypothesis that *Ask1*^{-/-} mice are resistant to aminoglycoside-induced hearing loss, current experiments are utilising cochlear surgery to achieve unilateral neomycin-induced hearing loss in mice. By surgically accessing one mouse cochlea and applying 5% neomycin (soaked on a Gelfoam sponge) to the round window membrane, it is possible to bypass the stria blood barrier and achieve high neomycin concentrations in the ear. This surgical approach produces ototoxic outcomes in mice and reduces aminoglycoside-induced mortality. However, there are still important limitations regarding the protocol. In particular bone drilling is required, which can cause acoustic or mechanical damage, and there is the potential of perilymphatic fistulas developing, which affects the mouse's hearing in ways unrelated to aminoglycoside toxicity (Stevens et al. 2015). Whilst these confounding factors are limitations to the technique, the surgical approach is currently one of the most consistent techniques available for inducing aminoglycoside ototoxicity in mice.

Other methods of drug delivery could be useful in mice, such as intratympanic injections. However, there are alternate animal models, such as the guinea pig, that may be more suited to modelling human ototoxic outcomes. The auditory range of guinea pigs overlaps with human hearing more so than that of mice (Figure 30) (Heffner & Heffner 2007) and human ototoxicity has been modelled in guinea pigs for over 50 years (Brummett et al. 1971; Wersall et al. 1969). Notably, systemic delivery of tobramycin or amikacin, which did not cause hearing loss in mice during this study, causes significant ototoxic effects in guinea pigs (Akiyoshi 1978; Kitasato et al. 1990;

Aran et al. 1982; Brummett et al. 1971; Dirain et al. 2018). However, the utility of guinea pigs for research is limited by their comparatively large size, long gestational period, and limited reproductive capacity. As a result, many organisations do not have facilities suitable for guinea pig research and the robust technologies available for genetic manipulation in rodents are limited for guinea pig specific studies. The discovery and development of CRISPR/cas9 gene editing technology may change this. Nevertheless, there are currently no *Ask1* knockout guinea pigs reported in the literature. However, it may be possible to use a pharmacological method such as small molecule ASK1 inhibitors in wild type guinea pigs. This would also be a good opportunity to interrogate what plasma levels of an inhibitor are required to achieve meaningful attenuation of aminoglycoside toxicity *in vivo*. Therefore, the second component of this thesis investigated a pharmacological method for down regulating ASK1 activity *in vitro*, to establish the pre-clinical evidence needed for the justification of inhibitor based *in vivo* modelling.

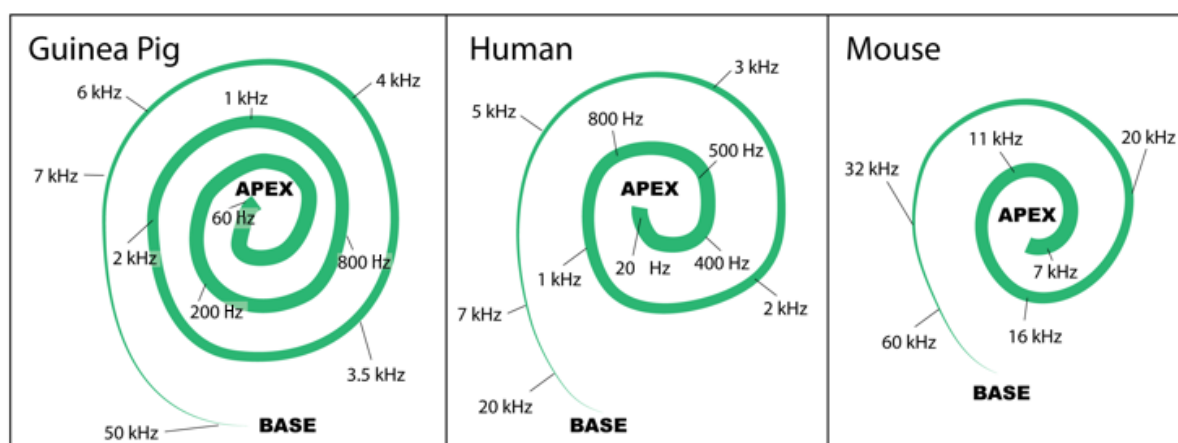


Figure 30. Tonotopic frequency determination in the cochlea of guinea pigs, humans, and mice.

Tonotopic organisation is similar across all vertebrates (high to low frequency determination from the cochlear base to apex). Mice can hear sounds between approximately 1 and 100 kHz whereas humans and guinea pigs hear much lower sounds. Human audition is between 20 Hz and 20 kHz and guinea pigs have the broadest hearing range, from 60 Hz to 50 kHz (Culler et al. 1943; Heffner & Heffner 2007; Purves 2001c). The guinea pig cochlea consists of approximately three and half turns, the human cochlea has two and three quarter turns, and the mouse cochlea has two turns.

Chapter 4. Pharmacological inhibition of ASK1 as a protective strategy for preventing aminoglycoside-induced hair cell death

The data generated in Chapter 3 provides compelling evidence that reduced ASK1 is protective against aminoglycoside ototoxicity. Specifically, neomycin-treated *Ask1*^{-/-} hair cells had significantly better survival rates when compared with neomycin-treated C57BL/6 controls. It was therefore predicted that ASK1 inhibition using a small molecule inhibitor would protect hair cells against neomycin toxicity *in vitro*. Notably, commercially available ASK1 inhibitors have shown excellent bio-availability and tolerability parameters in clinical trials, having been safely used in humans with diabetic kidney disease and non-alcoholic fatty liver disease (Tesch et al. 2016; Younossi et al. 2018; Loomba et al. 2018). In particular, GS444217 and its analogue GS4997 (Figure 31) have successfully moved to phase III clinical trials in humans (reviewed in chapter 1). In animal models of kidney damage, GS444217 has been shown to specifically impede the ASK1 mediated P38/JNK response to cellular oxidative stress (Liles et al. 2018; Tesch et al. 2015; Amos et al. 2018). Therefore, GS444217 was tested as a potential therapeutic for preventing aminoglycoside induced hair cell death.

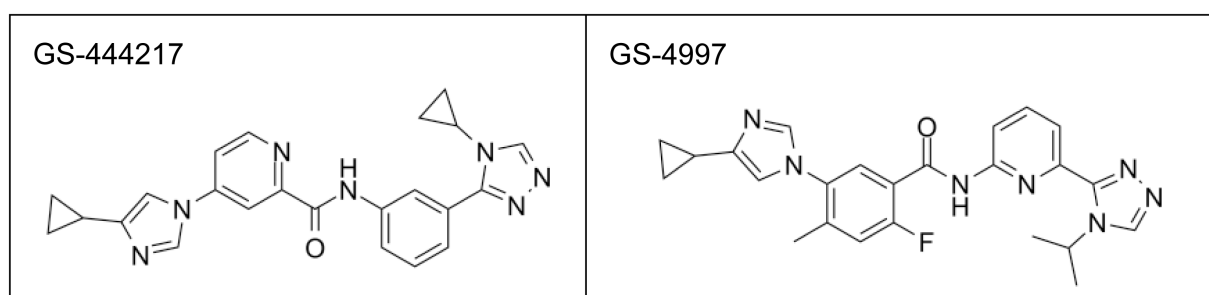


Figure 31. The chemical structure of GS444217 and GS4997.

GS444217: CAS number 1262041-49-5, Molecular formula C₂₃H₂₁N₇O and GS4997: CAS number 1448428-04-3, Molecular formula C₂₄H₂₄FN₇O as listed by PubChem (The National Center for Biotechnology Information).

GS444217 had not been previously assessed as a potential therapeutic for aminoglycoside ototoxicity. Therefore, three analyses were undertaken to validate GS444217 as a therapeutic for preventing hearing loss in aminoglycoside treated individuals. First, the effect of GS444217 on hair cell survival was tested *in vitro*. This was assessed using hair cell counts performed in C57BL/6 cochlear explants after treatment with 10 fold increasing doses of GS444217 (0-100 μ M). The next step tested the anti-apoptosis effects of GS444217 treatment, in neomycin treated C57BL/6 cochlear explants using hair cell quantification. Finally, the efficacy of aminoglycoside antibiotics when co-administered with GS444217 *in vitro* was assessed, to investigate whether GS444217 treatment could interfere with primary aminoglycoside treatments.

4.1. The effect of GS444217 on hair cells

4.1.1. GS444217 treatment does not induce hair cell death *in vitro*

To ascertain whether GS444217 was compatible with hair cell survival, cochlear explants were treated with GS444217, ranging from 0 μ M-100 μ M, for 24 hours (Figure 32A, blue line). Tenfold increasing doses of GS444217 were selected to investigate potential cytotoxicity, starting at 0.1 μ M, which is below the previously reported EC₅₀ of ~1.6 μ M and increasing to 100 μ M (Liles et al. 2018). GS444217 did not induce significant inner or outer hair cell death under any of the conditions tested (one way ANOVA; outer hair cell counts $p=0.36$, inner hair cell counts $p=0.27$). Therefore, the effect of GS444217 treatment was then tested in neomycin treated cochlear explants.

4.1.2. Outer hair cells treated with GS444217 are resistant to neomycin toxicity *in vitro*

To investigate pharmacologically-mediated ASK1 inhibition as an otoprotective strategy against aminoglycoside toxicity, cochlear explants were pre-treated for 16 hours with GS444217 or vehicle (DMSO), before being incubated with 1 mM of neomycin for 24 hours (Figure 32A, orange line). GS444217 doses ranged from 0 μ M-100 μ M.

Similar to previous observations made in Chapter 3, inner hair cells were highly resistant to neomycin treatment in this experiment, with the loss of < 1% of inner hair cells after 24 hours of 1 mM neomycin, compared to 51% of outer hair cells (Figure 32A). Overall, there was no significant difference ($p = 0.69$) in the number of inner hair cells in neomycin treated explants that had been pre-treated with either vehicle (22.7 ± 0.7) or GS444217 (23.1 ± 0.5) at the 24 hour time point.

In contrast, GS444217 treatment markedly improved outer hair cell survival. In particular, GS444217 treatments between 1 μ M and 100 μ M significantly attenuated hair cell death in neomycin-treated explants (Figure 32A). For explants pre-treated with DMSO before being exposed to 1 mM neomycin, only $51\% \pm 6\%$ Myosin VIIa-positive outer hair cells remained at the 24 hour time point (rounded average $\pm$ S.E.M). Outer hair cell survival increased to $68\% \pm 3\%$ with a pre-treatment of 0.1 μ M GS444217, and 1 μ M GS444217 treatment resulted in $92\% \pm 4\%$ outer hair cell survival. A concentration of 100 μ M GS444217 pre-treatment achieved $95\% \pm 2\%$ outer hair cell survival (Figure 32B). Due to limited tissue availability, a full time course and dose curve could not be performed using GS444217. Therefore, the longest time-frame was then tested (three days) in conjunction with a 100 μ M GS444217 dose, which had achieved the greatest hair cell survival in the previous dose response experiment (Figure 32A). In this experiment, 100 μ M GS444217 afforded significant ($p=0.029$) protection for outer hair cells, with only 16 ± 2 Myosin VIIa-positive outer hair cell survival in vehicle pre-treated explants ($n=5$), compared to 32 ± 6 in the GS444217 pre-treated explants ($n=4$), after three days of 1 mM neomycin treatment (Figure 32C). Likewise, inner hair cell survival was elevated in GS444217 pre-treated explants (14.9 ± 1 , $n = 4$) compared to DMSO pre-treated explants (10.2 ± 2 , $n = 5$) after three days of 1 mM neomycin treatment (Figure 32C). However these hair cell counts were not statistically different (0.07). Qualitatively, inner and outer hair cells in neomycin-treated explants pre-treated with 100 μ M GS444217 also appeared less condensed and disorganised (Figure 32D) after three days of 1 mM neomycin treatment.

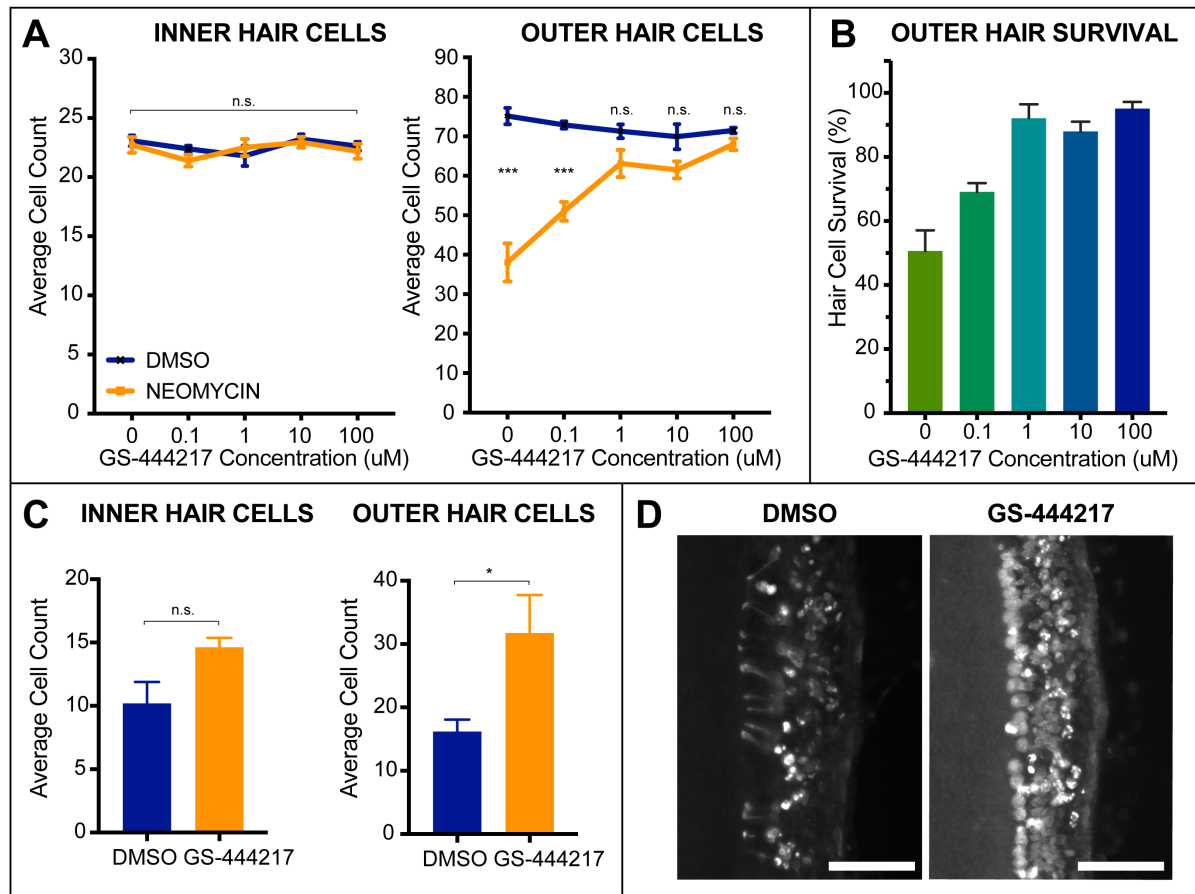


Figure 32. GS444217 protects hair cells against neomycin toxicity *in vitro*.

A) The average inner (left) and outer (right) Myosin VIIa-positive hair cell counts for explants pre-treated with GS444217 for 16 hours (0-100 μ M) before being treated with 1 mM neomycin or vehicle (24 hours). Cell counts represent the averages of two 180 μ m x 90 μ m fields of view, taken from the mid-turn region of the cochlear explant. N values for 0, 0.1, 1, 10 and 100 GS444217 respectively = 7, 9, 5, 7, 9 (DMSO) and 8, 6, 7, 9, 8 (neomycin). Explants collected at P3. **B)** Outer hair cell counts presented as percentage survival compared to average hair cell counts in the DMSO control group. **C)** The average inner (left) and outer (right) Myosin VIIa-positive hair cell counts for P3 explants pre-treated (16 hours) with either 100 μ M GS444217 or DMSO before being subjected to 1 mM neomycin treatment for three days. DMSO n = 4, GS444217 n = 5, from littermates. **D)** Representative sections from the mid-turn of C57BL/6 explants pre-treated with DMSO (left) or GS444217 (right), after being treated with 1 mM neomycin for 24 hours and stained with Myosin VIIa. Mean with S.E.M shown (* $p < 0.05$, *** $p < 0.001$, n.s = not statistically significant). Scale bars = 45 μ M.

4.1.3. GS444217 does not block MET channels

ASK1 has been well characterised as a key mediator of ROS induced JNK and P38 activation. In addition, previous studies have demonstrated that GS444217 affords protection against ROS induced cell death by inhibiting prolonged JNK or P38 phosphorylation (reviewed in chapter 1). Therefore, it was predicted that the protective effect of ASK1 inhibition observed in neomycin-exposed hair cells (Figure 32) was due to a reduction of ASK1 mediated JNK or P38 phosphorylation. However, an alternate hypothesis is that GS444217 is blocking the hair cell MET channels, thus preventing neomycin uptake and indirectly conferring protection. This outcome appears unlikely, given that the molar mass of neomycin is greater than GS444217 (614.644 g/mol versus 411.469 g/mol respectively), and that the MET channel pore is estimated to be large (1.2 nm) and non-ion-selective (Fettiplace 2017; Farris et al. 2004). However, to formally test the possibility that GS444217 is blocking the hair cell MET channels, FM 4-64x was utilised. Traditionally, fluorescent styryl pyridinium dyes are used to monitor synaptic vesicle activity in neurons. However, the MET channel, which is unique to hair cells, allows for very rapid FM dye uptake, resulting in significant and relatively selective hair cell staining. Therefore, the FM dye uptake assay is often used as an indirect indicator of MET channel function (Meyers et al. 2003; Erickson et al. 2017; Kruger et al. 2016)

Cochlear explants were treated for 16 hours with either vehicle or 100 μ M GS444217. Then, media containing 100 μ M GS444217 or DMSO and 100 μ M FM 4-64x was added to the apical surface of cochlear explants for two minutes. Explants were then washed twice in neurobasal media and fixed in 4% PFA. In this time frame, FM dyes are able to rapidly enter hair cells via the MET channel and result in extensive fluorescent staining within the cytoplasm, whereas the short incubation period means that fluorescent staining of other cell types within organ of Corti is limited to outer cell membranes (Meyers et al. 2003; Gale et al. 2001).

FM 4-64x rapidly and extensively stained hair cells in DMSO treated explants (Figure 33), suggesting that the FM 4-64x assay is a valid indicator of hair cell specific drug uptake. Moreover, there was no qualitative difference between the FM 4-64x staining of DMSO pre-treated and GS444217 pre-treated explants, suggesting that MET

channels were not blocked by GS444217. Furthermore, orthogonal imaging showed an FM 4-64x signal in the cytoplasm of hair cells in each group (Figure 33 a' and b'). As a positive control, explants were treated with the calcium chelator EDTA (100 mM). Calcium chelators eliminate hair cell tip links, subsequently disrupting MET channel function (Assad et al. 1991; Kachar et al. 2000; Tobin et al. 2019). In the EDTA-treated explants strong FM 4-64x staining was observed on the external membranes of all cell types. However, qualitatively, the hair cells did not exhibit the same bright staining when imaged using identical parameters (Figure 33. C.) Orthogonal imaging indicated that the FM 4-64x signal was reduced in the cytoplasm of EDTA-treated hair cells (Figure 33c').

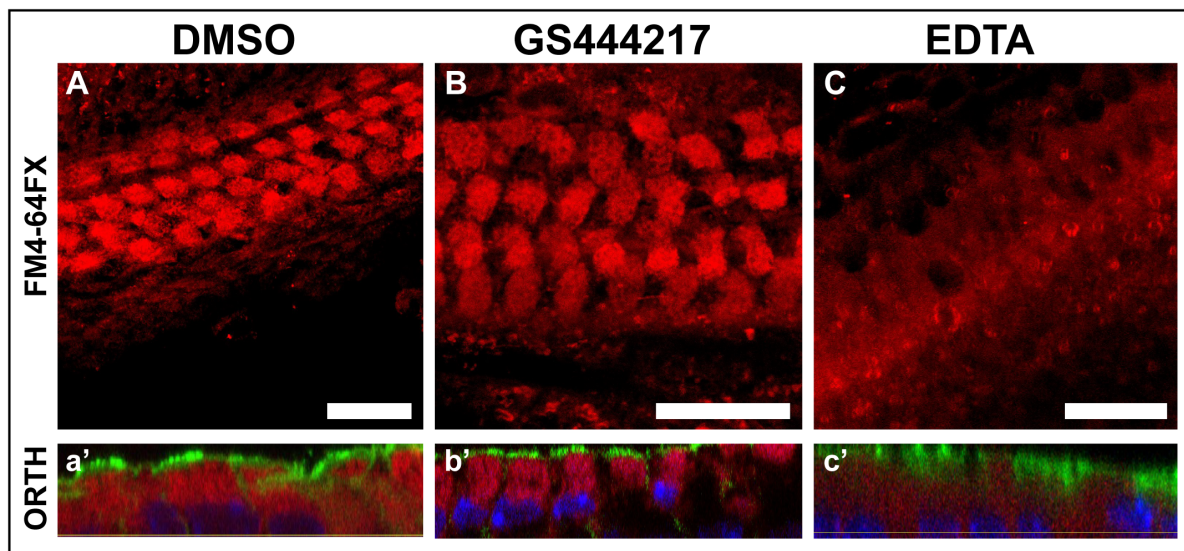


Figure 33. FM 4-64x uptake in hair cells cultured with DMSO, GS444217 and EDTA.

Confocal imaging suggested that FM 4-64x (red) rapidly and selectively entered hair cells, of DMSO (**A**) and GS444217 (**B**) treated explants. Orthogonal imaging also indicates FM 4-64x staining in the cytoplasm of DMSO (**a'**) and GS444217 (**b'**) treated explants. Conversely, FM 4-64x did not selectively stain hair cells in EDTA-treated explants (**C**), and staining in the hair cell cytoplasm was reduced in EDTA-treated explants (**c'**). Orth = orthogonal view, FM 4-64x styryl dye (red), phalloidin staining for stereocilia (green), and DAPI nuclear counterstain (blue). Scale bar represents 20 μ M. Images representative of 2-4 explants per treatment.

4.1.4. GS444217 may reduce JNK and P38 phosphorylation in neomycin-treated cochlear explants

In section 4.1.2, GS444217 treatment attenuated neomycin induced outer hair cell death. As ASK1 is an upstream regulator of prolonged P38 and JNK activation it was predicted that the protective effect of ASK1 inhibition observed in neomycin-exposed hair cells (Figure 32) was due to a reduction of ASK1 mediated JNK or P38 phosphorylation. Having demonstrated that GS444217 does not indirectly confer protection by blocking neomycin uptake through the MET channel, a combination of western blotting and confocal microscopy was used to assess p-JNK and p-P38 activity.

4.1.4.1. Western blot analysis suggests a reduction of p-P38 and p-JNK in GS444217-treated cochlear explants

Cochlear explants were pre-treated for 16 hours with vehicle or 100 μ M GS444217, before being cultured for 24 hours with 1 mM neomycin. Three explants from each treatment were then pooled for protein extraction, followed by western blot analysis. Western blotting demonstrated an apparent reduction of p-P38 in the GS444217 pre-treated cochlear explants, compared with DMSO pre-treated explants (Figure 34A). The p-JNK signal also appeared reduced in neomycin-treated cochlear explants that had been pre-treated with GS444217, compared to DMSO pre-treated explants (Figure 34B). However, quantification was not performed due to the current sample size of one.

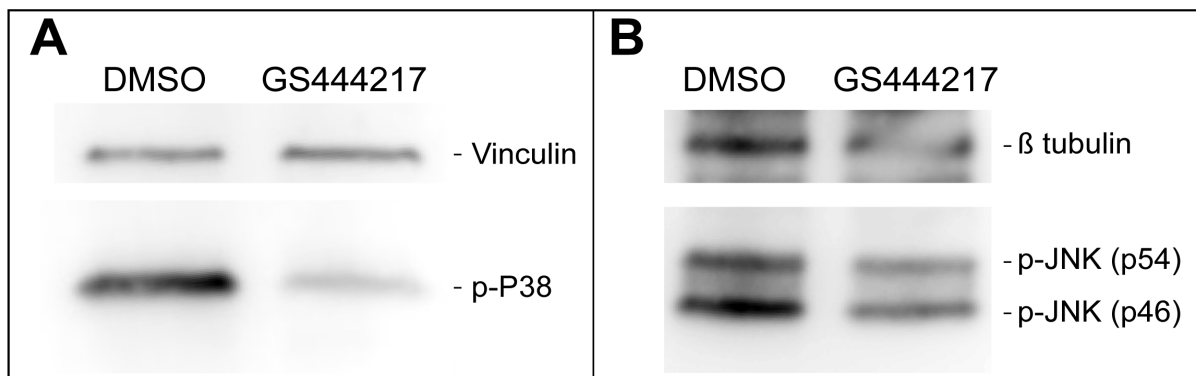


Figure 34. Western blot analysis of p-P38 and p-JNK in neomycin-treated cochlear explants.

Explants were pre-treated with 100 μ M GS444217 or DMSO for 16 hours, before being co-treated with 1 mM neomycin for 24 hours. Ten micrograms of protein was then loaded to each well for western blot analysis. **A)** Western blotting indicated a marked reduction of p-P38 in neomycin-treated cochlear explants that had been pre-treated with GS444217, compared to DMSO pre-treated explants. Vinculin was used as a loading control. **B)** Western blotting appears to show reduced p-JNK in neomycin-treated cochlear explants that had been pre-treated with GS444217, compared with DMSO pre-treated explants. However, a line of poor transfer interrupted the control band (β -tubulin). N = one blot, with three explants pooled per treatment, taken from p5 littermates. JNK 1, 2 and 3 have two splicing forms (p54 and p46), which were both identified by the p-JNK antibody utilised.

4.1.4.2. Immunohistochemistry and confocal imaging

Western blot analysis was suggestive that both p-JNK and p-P38 were reduced in neomycin-treated explants that had been pre-treated with GS444217. To further interrogate this result, immunohistochemistry was utilised to ascertain p-JNK and p-P38 localisation in neomycin-treated explants. Similar to the qualitative observations made using *Ask1*^{-/-} mice (Figure 29, Page 112), notable staining for phosphorylated JNK occurred in both DMSO pre-treated and GS444217 pre-treated explants (Figure 35). However, the p-JNK signal appeared to be much stronger and punctate in many cells of the DMSO pre-treated explants, when compared to GS444217 pre-treated explants (that were stained and imaged in the same manner). The punctate p-JNK signal was not predominant in hair cells, but visible in numerous supporting cell types.

Likewise, considerable punctate p-P38 staining was observed in both DMSO pre-treated and GS444217 pre-treated, neomycin exposed explants (Figure 36). However, the signal appeared much brighter in DMSO pre-treated explants compared to the GS444217 pre-treated explants that had been stained and imaged under identical conditions. In the DMSO pre-treated explants, bright p-P38 puncta were visible throughout the tissue. In addition, strong p-P38 staining was observed in the anatomical location where the Hensen's cells reside, and under the hair cells. This signal was difficult to localise precisely without performing further immunohistochemistry, however the stained region under the hair cells, is likely to include phalangeal or pillar cells. In the GS444217 pre-treated neomycin exposed explants, there appeared to be fewer, bright p-P38 puncta, and there was no strong staining around the Hensen cells and pillar cells, like that observed in the DMSO controls.

Interestingly, the bright p-P38 signal in DMSO controls resembled protein aggregates, which are a common artefact in immunohistochemistry. However, the primary antibody used has been validated by numerous research groups and the secondary antibody had not produced such strong artefact in previous experiments (Benkafadar et al. 2019; Iida et al. 2019; Rios Garcia et al. 2017). Furthermore, the prevalence of the bright signal was notably lower in GS444217 pre-treated neomycin exposed explants. Nevertheless, an alternate antibody was tested, and reproduced a comparable punctate pattern (Figure 37). Whilst, further experimentation will ascertain with more certainty what this signal represents, the p-P38 staining was observed to be mostly nuclear and, p-P38 staining was notably reduced in neomycin-treated explants that had been pre-treated with GS444217 when compared to DMSO pre-treated explants.

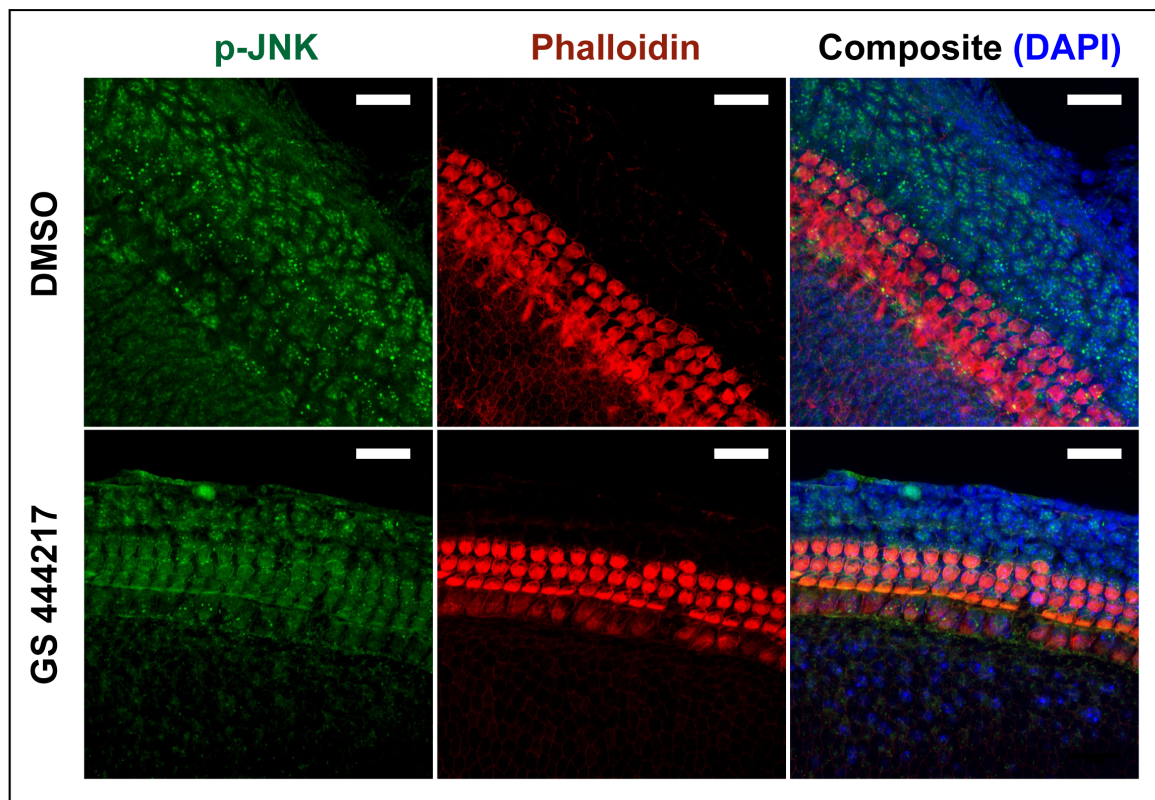


Figure 35. JNK phosphorylation in neomycin-treated explants.

Cochlear explants were pre-treated with 100 μ M GS444217 or DMSO control, before a 24 hour, 1 mM neomycin treatment. Explants were then fixed and stained for p-JNK (green), phalloidin (red) and DAPI (blue). Both GS444217 and DMSO-treated explants showed positive p-JNK staining. However, the signal was notably more punctate in the DMSO pre-treated explants when compared to the GS444217-treated explants. Scale bar = 20 μ m. Images representative of three explants per treatment group, from P3 littermates.

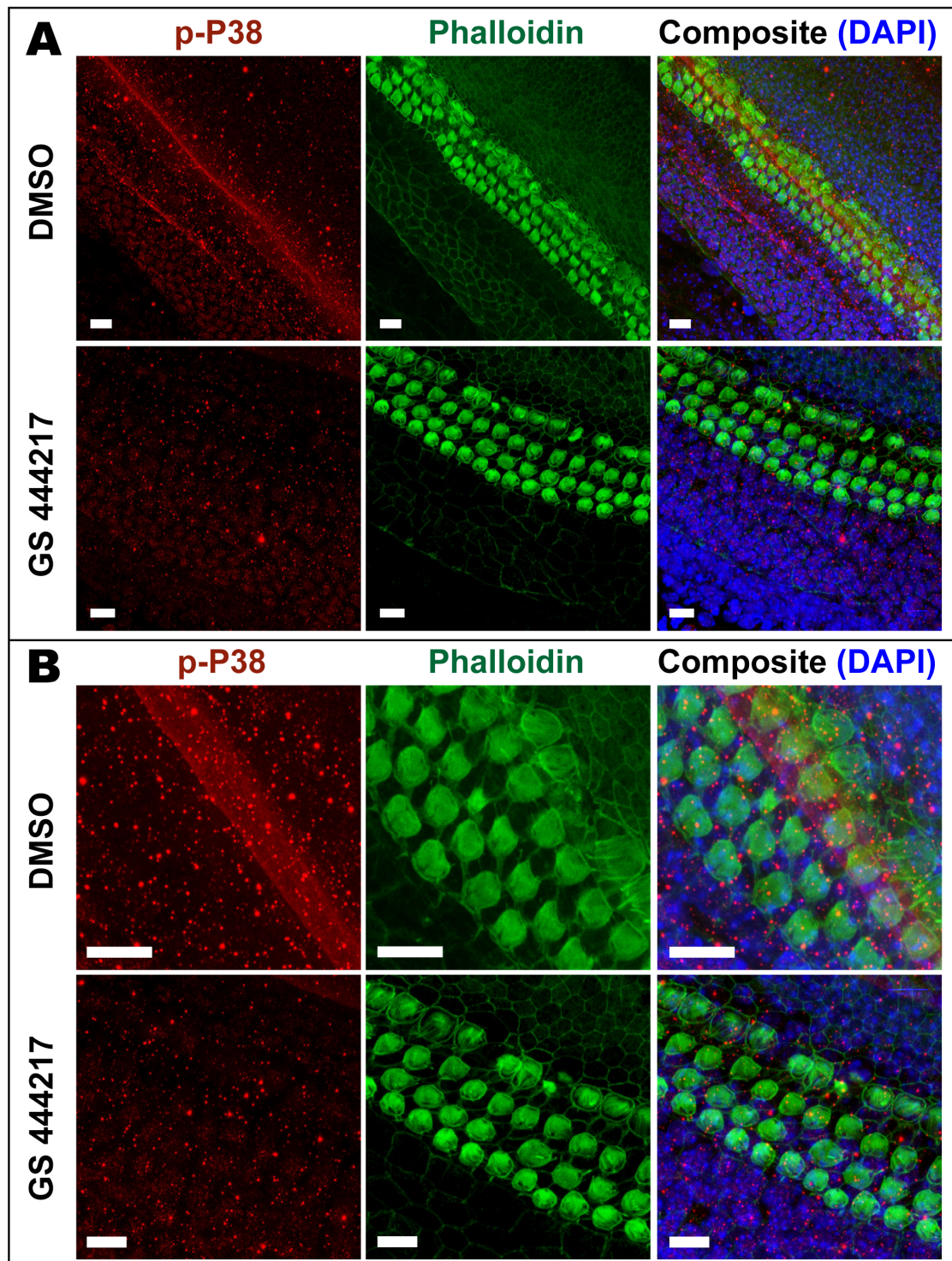


Figure 36. P38 phosphorylation in neomycin-treated explants.

A) Neomycin-treated DMSO and GS444217 explants demonstrated punctate p-P38 staining. **B)** Higher magnification imaging indicated that p-P38 staining was not specific to hair cells. Structures underlying the inner hair cells appeared to demonstrate higher signal intensity. p-P38 (red), phalloidin (green), and DAPI (blue) Images representative of six explants per treatment group, from 12 littermates. Scale bars = 10 μ m.

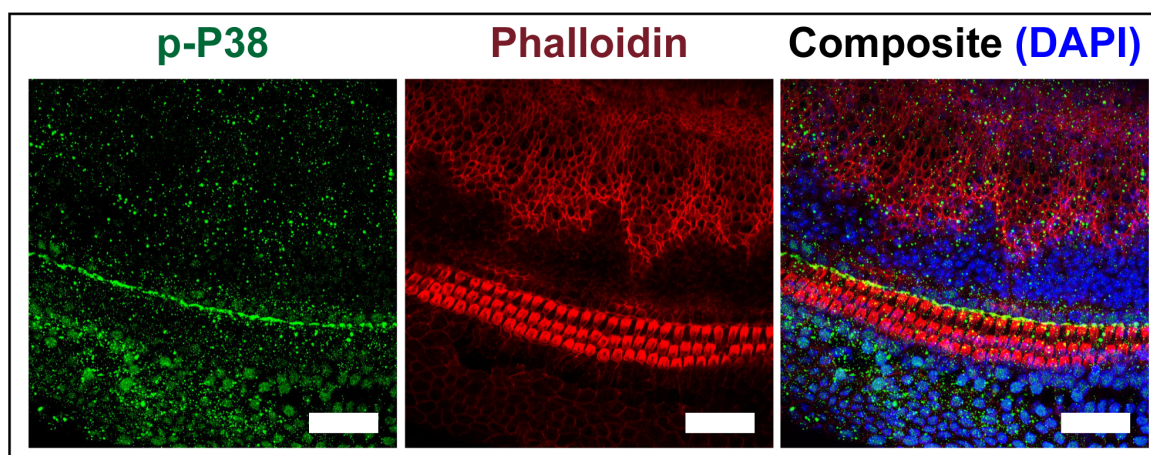


Figure 37. p-P38 immunofluorescence utilising an alternate secondary antibody.

Cochlear explants were treated with 1 mM neomycin for 24 hours before being stained for p-P38 (green), phalloidin (red) and DAPI (blue). P-P38 staining produced bright signals throughout the explants. Scale bars = 40 μ m.

4.2. GS444217 does not impair antibiotic efficacy against *Pseudomonas aeruginosa* isolates

In this chapter, the ASK1 inhibitor GS444217 has shown promise as an otoprotective compound, attenuating neomycin-induced hair cell death *in vitro*. However, it was not known whether GS444217 would impair the efficacy of aminoglycoside antibiotics. There are numerous methods available to ascertain the efficacy of antibiotics against bacteria (reviewed in (Reller et al. 2009)). However, the broth dilution test is the most commonly used reference method, due to the robust and quantitative results that can be achieved. For the broth dilution test, a serial dilution of antibiotics is performed in liquid broth, typically ranging from 0-512 μ g/ml. The broth is then inoculated with a known quantity of bacteria and incubated. The result of the test is recorded as the lowest concentration of antimicrobial agent that inhibits bacterial growth (as measured by optical density or fluorescence). This quantitative result is defined as the minimum inhibitory concentration (MIC).

Bacteria were co-cultured with increasing concentrations of GS444217 to ascertain whether GS444217 co-treatment changed aminoglycoside MICs (Figure 38 A). Resazurin salt was used to indicate cell viability; appearing blue, and weakly fluorescent when bacterial growth was inhibited. Conversely, viable bacteria metabolise resazurin into resofurin, which is a bright pink and highly fluorescent compound (Figure 38 B). Using this method, the MIC of aminoglycosides tobramycin, amikacin and neomycin were calculated for three strains of *Pseudomonas aeruginosa* (*P. aeruginosa*). Two of the *P. aeruginosa* strains (0307 and 0315) were derived from sputum samples collected from individuals with cystic fibrosis, during routine visits at the Royal Children's Hospital and one reference strain (ATTC 27853) was originally derived blood before being deposited with the American Type Culture Collection.

The broth dilution test demonstrated that each strain of *P. aeruginosa* had a unique susceptibility profile for each antibiotic (Figure 39). For example, the reference strain, ATTC 27853 had a lower MIC for all three aminoglycosides when compared to the clinical isolates (0307 and 0315). Based on clinical breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), both clinical isolates in this experiment showed intermediate resistance to tobramycin (Figure 39A) (Leclercq et al. 2013). This resistance may be indicative of previous tobramycin exposure. Although not documented (due to de-identification), it is likely that the clinically isolated strains were derived from individuals that would have received tobramycin treatments in their lifetimes. In addition, each *P. aeruginosa* strain will have a unique genetic profile, which contributes to MIC variability between strains.

Most importantly, increased GS444217 co-treatment had no effect on antibiotic efficacy. The broth dilution test showed that GS444217 concentrations ranging from 0.1 μ M to 100 μ M did not significantly change the minimum antibiotic concentration required (of tobramycin, amikacin or neomycin) to inhibit any of the *P. aeruginosa* strains (Figure 39).

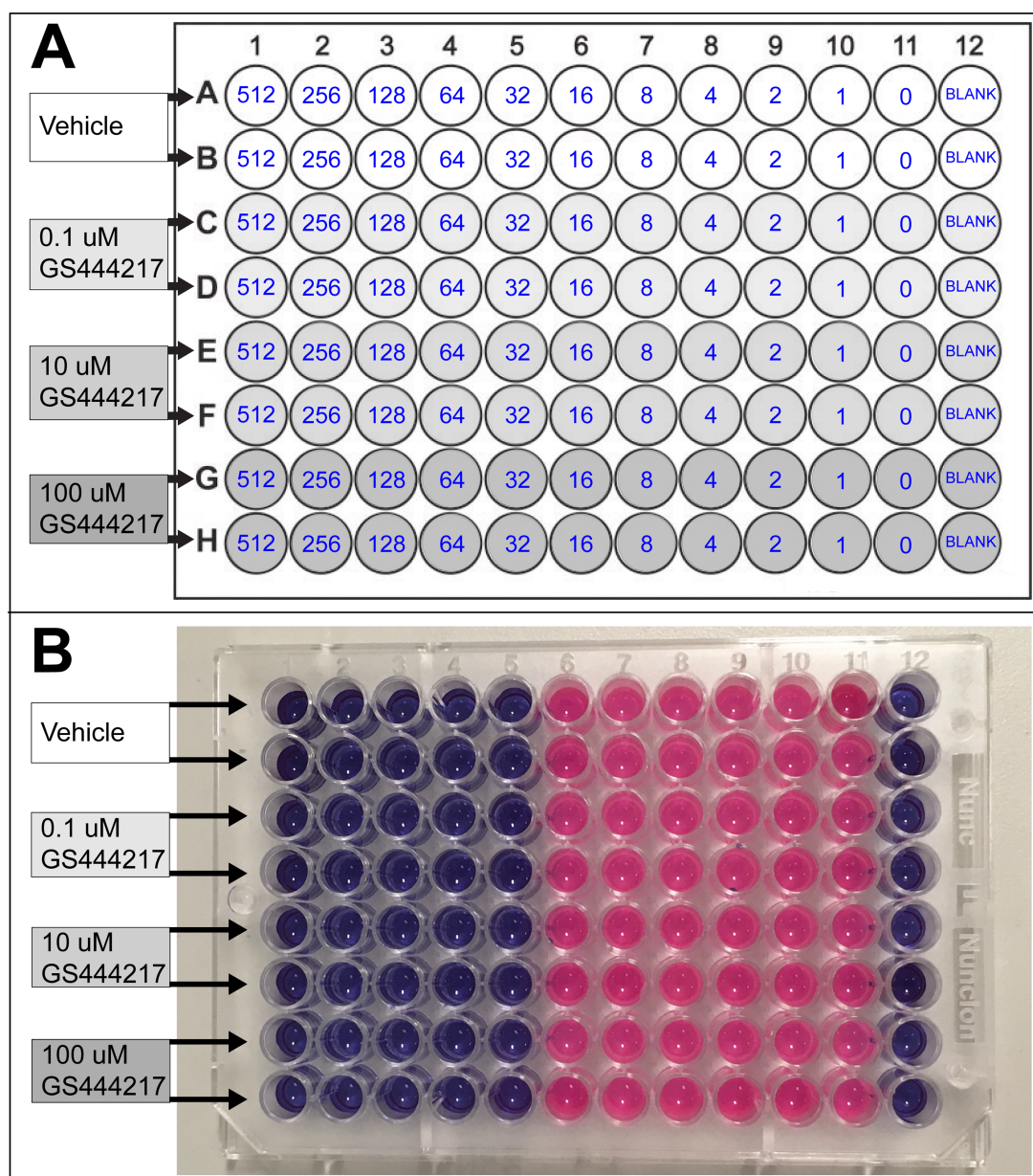


Figure 38. The broth dilution test of aminoglycoside minimum inhibitory concentrations

A) A schematic representation of the broth dilution test. Each plate contained serial aminoglycoside dilutions starting at 512 μ g/ml and decreasing to 0 μ g/ml (blue numbers). The concentration of GS444217 increased from 0-100 μ M, from the top to the bottom of the plate, with each concentration tested in duplicate (black arrows). **B)** A representative broth dilution plate demonstrating the metabolism of resazurin (blue) to resofurin (pink) that occurs when *P. aeruginosa* growth is not inhibited. For this example the aminoglycoside's MIC is 32 μ g/ml.

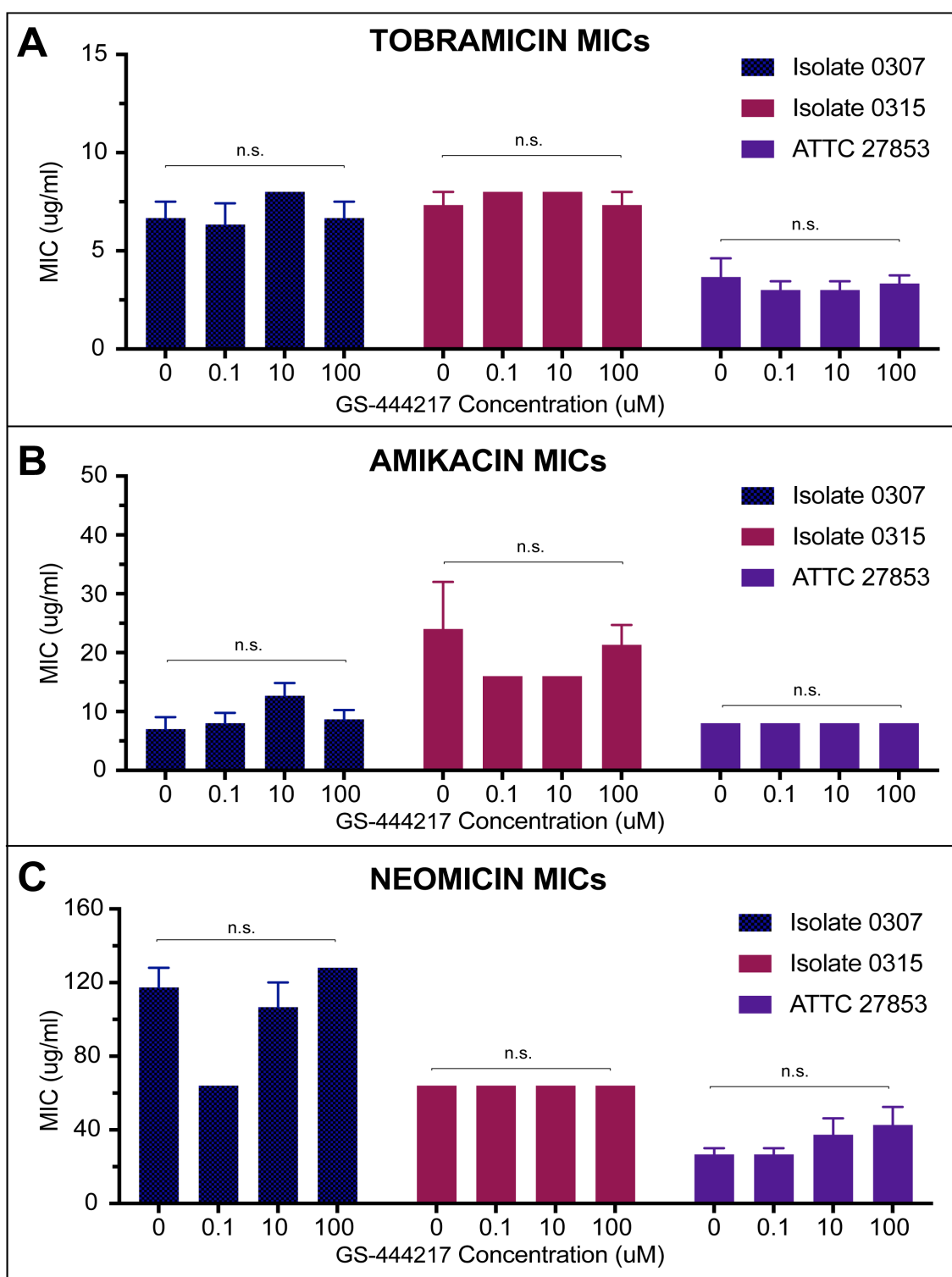


Figure 39. Minimum inhibitory concentrations (MICs) of three aminoglycosides against three *P. aeruginosa* isolates.

MIC's were calculated for **A)** tobramycin **B)** amikacin and **C)** neomycin as the average of two replicates on a broth dilution plate, n = three plates per isolate, for each antibiotic. Error bars are S.E.M (no error bars indicates that MIC's were the same for all three plates).

4.3. Discussion and future directions

In this chapter, pharmacological inhibition of ASK1 using GS444217 was investigated as an otoprotective strategy to moderate aminoglycoside-induced hair cell death. First, the potential for GS444217 toxic effects in hair cells was tested *in vitro* using cochlear explants. Qualitative and quantitative assessment of hair cell morphology and number showed that GS444217 treatment did not cause any inner or outer hair cell death (Figure 32A). Importantly, when cochlear explants were treated with 1 mM neomycin, GS444217 pre-treatment afforded significant protection against outer hair cell death (Figure 32A).

Interestingly, inner hair cell survival appeared to be better in the experiment described in this chapter, when compared to previous experiments (described on page 103). In the previous experiment, 13% of inner hair cells were lost after 24 hours of 1mM neomycin treatment. Conversely, only 1% of inner hair cells were lost in this experiment. Therefore, it is possible that the DMSO pre-treatment utilised in this chapter, is also affording protection against neomycin toxicity. For this experiment, explants were treated with DMSO, or GS444217 (suspended in DMSO), starting on the day of dissection and lasting for 16 hours before co-treatment with 1 mM neomycin commenced. In contrast, explants used in previous experiments were cultured overnight without any DMSO added before neomycin was used. This is potentially important, as DMSO is in and of itself a potent antioxidant, capable of inducing molecular changes in the cell (Sanmartin-Suarez et al. 2011; Tuncer et al. 2018). Furthermore, DMSO has been shown to increase the effects of experimental otoprotective compounds (Melki et al. 2010; Momin et al. 2011). It is therefore quite possible that the DMSO/GS444217 pre-treatment specific to this experiment improved hair cell survival. However, as the experiments utilising *Ask1*^{-/-} mice and GS444217 were performed independently of each other, a single controlled experiment is required to test this theory. It is certainly worth testing if DMSO improves the efficacy of GS444217 treatment, as this could prove useful when considering what vehicle to utilise for future *in vivo* GS444217 applications.

To explicate the protective effect of GS444217 for neomycin treated hair cells, immunohistochemistry and western blot analysis was performed. First, hair cells were

treated with FM 4-64x, to determine if the mechanism of protection afforded by the inhibitor involved preventing neomycin entry into the hair cell via MET channels. Hair cells demonstrated specific FM 4-64x staining, suggesting that FM 4-64x was able to readily enter the hair cells when GS444217 was present. Qualitatively the uptake of dye in GS444217 treated hair cells appeared equivalent to that of control cultures treated with vehicle only. Notably, FM 4-64x has one of the largest molar mass of any styryl dyes (788.75 g/mol), further highlighting the permeability of MET channels. An important consideration however, is that FM dyes are able to enter cells through two other ion channels present in hair cells (P2X₂, and TRPV₁), albeit at a much lower rate (Meyers et al. 2003; Housley et al. 1999; Lee et al. 2013). This may account for the faint signal observed in the EDTA-treated hair cells. Alternatively it is possible that the EDTA treatment resulted in incomplete tip link channel disruption. Future experimentation will validate these findings, by examining reduced FM 4-64x exposure times (i.e. <30 seconds) and incorporating the use of radiolabelled aminoglycosides, prior to *in vivo* application. Based on the data available, GS444217 does not appear to block the hair cell MET channel.

Western blot analysis was then used to investigate whether p-P38 or p-JNK levels were altered in neomycin-treated explants that had been pre-treated with GS444217. Preliminary results suggest that both p-P38 and p-JNK were reduced in GS444217 pre-treated explants, when compared to DMSO pre-treated explants. Notably, p-P38 appeared to be more reduced than p-JNK. Importantly, tissue availability has limited validation of these data with current observations limited to a sample size of one western blot. However once available, replicate blots will facilitate protein quantification, normalised to loading controls. Additional controls will also be utilised, including explants cultured with no treatment, explants treated with DMSO only and explants treated with neomycin—but no pre-treatment. These controls will be useful for elucidating the extent to which P38 and JNK phosphorylation is the result of the process of dissection and culture alone; how much p-P38 and p-JNK changes after neomycin exposure, and for further elucidation of the protective effects afforded by both DMSO and GS444217.

Immunohistochemistry was also used to examine the localisation of p-JNK and p-P38 in cochlear explants that had been pre-treated for 16 hours with either DMSO or GS444217, before being exposed to 1 mM neomycin, for 24 hours. Qualitatively, the

immunofluorescence signal of p-JNK was brighter and more punctate in DMSO pre-treated explants when compared to GS444217-treated explants. The punctate p-JNK pattern was present in most cells of the explant, including, but not limited to hair cells. Previous experiments utilising C57BL/6 explants that had been treated with neomycin for six hours showed similar results (Figure 29, page on 111). Combined, these data support the hypothesis that prolonged JNK phosphorylation is occurring in neomycin-treated cochlear explants. However, explants treated with 100 μ M GS444217 do not show the same bright p-JNK signals. It is not currently known which subcellular compartment(s) these p-JNK positive structures represent, however a mitochondrial association is possible. The translocation of p-JNK to the mitochondrial membrane is a well described aspect of apoptosis (reviewed in (Dhanasekaran & Reddy 2008). When p-JNK relocates to the mitochondrial membrane, it increases mitochondrial permeability and subsequently inhibits mitochondrial energy production. Importantly, one functional outcome of the association of p-JNK with the mitochondria is that p-JNK antagonises pro-survival Bcl2 proteins and stimulates Bid-Bax mediated release of cytochrome C (Dhanasekaran & Reddy 2008; Hanawa et al. 2008). Furthermore, the translocation of p-JNK to the mitochondria enhances ROS production by up to 80% (Chambers & LoGrasso 2011). Based on the p-JNK profile observed in neomycin-treated explants pre-treated with DMSO and GS444217, the inhibitor is potentially impacting the apoptotic role of p-JNK and therefore obstructing the normal progression of mitochondrial-mediated cell death in the neomycin-treated explants.

Staining for p-P38 was also extensive in neomycin-treated explants, but appeared notably reduced in those pre-treated with GS444217. A bright and punctate signal was present throughout the entire explant, suggestive of both nuclear and cytoplasmic localisation. This indistinct patterning of p-p38 is commonly reported as P38 has no nuclear localisation signal (Wood et al. 2009). However, nuclear translocation of p-P38 has been reported to occur subsequent to double stranded DNA breaks (Wood et al. 2009). The p-P38 signal observed in neomycin-treated cochlear explants suggests that some nuclear translocation had occurred. Explants treated with either DMSO or GS444217 demonstrated this putative nuclear translocation of p-P38, however the signal appeared to be reduced in GS444217 pre-treated explants. These observations are consistent with the hypothesis that GS444217 is reducing ASK1 mediated P38

phosphorylation and potentially inhibiting apoptosis. However, the p-P38 signal in GS444217 pre-treated explants indicates that alternate mechanisms are inducing P38 phosphorylation in neomycin-treated explants.

Collectively, confocal microscopy and western blot analyses support the concept that both p-JNK and p-P38 are driving apoptosis in the neomycin-treated cochlear explant. p-JNK or p-P38 staining was not specific to hair cells at the 24 hour time-point. Whereas, previous observations in cochlear explants treated with neomycin for six hours (Figure 29) indicated a strong, hair cell specific p-JNK response. Therefore, it is possible, that the stages of apoptosis (such as the p-JNK mitochondrial association) have already occurred in hair cells at the 24 hour time-point, due in part to their heightened neomycin uptake facilitated by MET channels. The strong p-JNK and p-P38 signals observed throughout the cochlear explant after 24 hours of 1 mM neomycin treatment may be indicative of delayed toxicity in other cells types. To further clarify this, a time course experiment highlighting the levels and localisation of p-JNK and p-P38 in the neomycin-treated explants will be performed.

The observation that GS444217 may have impacted the normal phosphorylation of both JNK and P38 in neomycin-treated explants highlights the potential of ASK1 inhibition as a strategy for preventing ototoxicity. Notably, P38 and JNK are significant mediators of cell death, not restricted to hair cells, nor restricted to the use of ototoxic drugs. Therefore, this strategy may afford auditory protection in other situations, such as cochlear surgery and acute acoustic trauma. Furthermore, the mitigation of P38 and JNK mediated apoptosis may also protect other cell types that are important for cochlear function. This is discussed in further detail, in Chapter 5.

An important pre-requisite for any otoprotective strategy is that the primary care of the individual is not compromised. For example, aminoglycosides are necessarily used in individuals with cystic fibrosis, to treat chronic lung infections. For this patient cohort, diminishing the antibiotic efficacy of aminoglycosides could ultimately lead to respiratory failure and death. For this reason, the effect of increasing concentrations of GS444217 on the efficacy of aminoglycosides against *P. aeruginosa* was tested *in vitro*. *P. aeruginosa* was used as the test organism, because it is the most common cause of infection in the lungs of individuals with cystic fibrosis. The aminoglycosides tobramycin and amikacin were selected for use in this experiment because they are the

most clinically utilised aminoglycosides for *P. aeruginosa* treatment. Neomycin was also tested as an aminoglycoside frequently used in the laboratory. The broth dilution test demonstrated that GS444217 does not increase the minimum concentration required to completely inhibit *P. aeruginosa* growth, for all three aminoglycosides tested. Importantly, this result was replicated in a standard *P. aeruginosa* reference strain and two isolate strains derived from the sputum of people with cystic fibrosis. Notably, this experiment indicated that 100 μ M GS444217 did not impair antibiotic efficacy. Whereas, previous experiments (Figure 32A) showed that a much lower concentration (1 μ M) significantly attenuated neomycin induced hair cell death *in vitro*. Combined, these results are a positive indication that ASK1 inhibition could be used to prevent aminoglycoside-induced hearing loss, without impacting primary aminoglycoside care. Further studies are now needed to confirm that GS444217 does not impair antibiotic efficacy against other common infections. For example, aminoglycosides are also used in neonates with sepsis, which is often caused by Group B streptococcus, *E. coli*, *Staphylococcus aureus* or Enterobacteriaceae infection (Muller-Pebody et al. 2011; Braye et al. 2019). For these newborns, aminoglycoside treatment is the greatest risk factor for developing permanent hearing loss (Maqbool et al. 2015; Poonual et al. 2016). Ensuring that more disease-causing organisms are susceptible to aminoglycoside therapy during GS444217 co-administration will develop the evidence base that ASK1 inhibition is a useful and safe strategy against aminoglycoside ototoxicity. Therefore, ASK1 inhibition would be able to benefit more individuals requiring lifesaving aminoglycosides that could otherwise permanently damage their hearing.

4.4. Concluding Remarks

Pharmacological inhibition of ASK1 activity using GS444217 significantly reduces neomycin-induced hair cell death *in vitro*. Furthermore, immunohistochemistry suggests that GS444217 treatment may reduce p-P38 and p-JNK activity in other supporting cells in the neomycin-treated cochlear explant. Importantly, GS444217 co-treatment does not impact antibiotic efficacy against *P. aeruginosa*. Together, this data strongly supports the use of ASK1 inhibition as a strategy for preventing aminoglycoside-induced hair cell death. Moreover, this data provides the necessary foundation, to justify further investigation in pre-clinical models examining the protection of inner ear hair cells from aminoglycoside ototoxicity.

Chapter 5. Discussion and conclusions

Over one hundred medicines have ototoxic properties. Where possible, the use of such medicines is avoided due to the potential side effects of acquired deafness, tinnitus, ataxia, nausea and vertigo. However, ototoxic medicines frequently achieve lifesaving outcomes that cannot be attained with alternate treatment strategies. Currently, no adjuvant therapy exists that mitigates ototoxic outcomes. Consequently, individuals treated with ototoxic medicines often experience a permanently reduced quality of life. To address this issue, global research efforts have been directed towards delineating the mechanisms underlying drug-induced hair cell death. For this research, a large focus has been placed on the ototoxic aminoglycosides, which, as the most commonly used antibiotics worldwide, are a significant causes of drug-induced hair cell death (Xie et al. 2011; Hermann 2007). From this research effort, increased ROS production in aminoglycoside treated hair cells has been extensively documented. One downstream consequence is MAPK activation, with JNK and P38 identified as important mediators of hair cell death. However, direct inhibition of ROS, JNK or P38 is problematic as an otoprotective strategy, because these factors are important for normal cellular and physiological function. Therefore, indirect modulation of these pathways might represent an effective alternate therapeutic option. Importantly, the upstream regulators of JNK or P38 have not been well studied in the auditory system. ASK1 was considered to be a particularly relevant upstream target, given that ASK1 inhibition can reduce the pathological consequences of oxidative stress, without impacting physiological ROS activity. In particular, ASK1 inhibition has been shown to selectively regulate the prolonged ROS induced activation of P38 and JNK, in multiple disease states, without impacting pro-survival mechanisms (reviewed in Chapter 1). Furthermore, previous work by Tao et al. identified ASK1 as the only MAP3K to show significantly increased RNA expression in hair cells after aminoglycoside treatment (Tao & Segil 2015). Prior to this study, the role of ASK1 for the development and function of the auditory pathway has not been evaluated. Moreover, the utility of ASK1 inhibition for preventing aminoglycoside induced hair-cell death had not been tested. Therefore, the broad aims of this project were to evaluate the importance of ASK1 for both the development and maintenance of normal auditory function, and as a mediator of drug-induced hair cell death. Stemming from this work, a number of important observations were made with regard to these aims.

5.1. Summary of key significant findings

5.1.1. ASK1 deficiency may cause altered nerve conduction in the auditory pathway.

This project aimed to evaluate the importance of ASK1 in the inner ear by characterising the auditory phenotype of *Ask1* knockout mice. ASK1 deficiency did not impact the development or function of the peripheral auditory system, as indicated by unremarkable cochlear histology and hearing thresholds in *Ask1*^{-/-} mice. However, the results of ABR peak analysis and ASR testing indicate that ASK1 deficiency alters central neuronal conduction. In particular, ABR peak analysis showed that auditory signal transduction is faster in *Ask1*^{-/-} mice and ASR testing indicated that *Ask1*^{-/-} mice are hypersensitive to auditory stimulation. Combined, this data suggests that *Ask1*^{-/-} mice have an auditory processing disorder. It is currently unclear whether the phenotype stems from aberrant neuronal differentiation or migration during development, or whether ASK1 has an active role in neuronal signalling. Elucidating the potential role of ASK1 in central auditory processing will be difficult, as signal integration and efferent control is not well understood (Lopez-Poveda 2018; Hironaka 2008; Steube et al. 2016; Yeomans & Frankland 1995). However, in this respect *Ask1*^{-/-} mice could now prove useful for further exploration of the mechanisms underlying auditory processing. Furthermore, ASK1 deficiency has been implicated with impaired sensory gating in individuals with schizophrenia (Morris & Pratt 2014; Jones et al. 2016), and altered dopaminergic transmission in mice (Kumakura et al. 2010). In addition, a potential role of ASK1 in neurological conditions such as multiple sclerosis and amyotrophic lateral sclerosis is now emerging (Guo et al. 2010; Fujisawa et al. 2016). Therefore, future research utilising *Ask1*^{-/-} mice is likely to be widespread and could provide vital information regarding neural circuitry and the specific role of ASK1 in neuronal function.

5.1.2. ASK1 is an important mediator of aminoglycoside-induced hair cell death

ASK1 knockout mice and the ASK1 inhibitor Gs444217 were utilised to investigate the hair cell response to neomycin toxicity *in vitro*. In *Ask1*^{-/-} cochlear explants, significantly more hair cells survived neomycin treatment. In addition, adjuvant Gs444217 treatment significantly attenuated neomycin-induced hair cell death in wild type C57BL/6 cochlear explants. Collectively, this data provides robust evidence that ASK1 plays an important role in the process of neomycin-induced hair cell death, and that ASK1 inhibition can significantly limit neomycin-induced hair cell destruction when delivered *in vitro*. This observation is particularly important, as hair cell death underlies the hearing loss and vestibular dysfunction induced by aminoglycoside treatment.

5.1.3. ASK1 appears to mediate both JNK and P38 phosphorylation in the organ of Corti.

Immunohistochemistry and western blot analysis suggested that the phosphorylation of both P38 and JNK was reduced in neomycin-treated explants that were pre-treated with Gs444217, when compared to neomycin-treated explants that were pre-treated with DMSO only. This observation supports the hypothesis that ASK1 is an upstream target able to influence multiple apoptotic pathways. Moreover, ASK1 inhibition appeared to limit JNK and P38 phosphorylation not only in the hair cells of neomycin-treated explants, but also numerous supporting cell types. This is important, as the structural integrity of the organ of Corti is vital for hair cell function and survival. For example, supporting cells provide hair cells with a rigid structural foundation in an environment subject to constant mechanical stimulation. Supporting cells also facilitate the rapid recycling of Na⁺ and K⁺ that is required for hair cell mechanotransduction (Wan et al. 2013). Moreover, the complex molecular transport systems and gap junctions between supporting cells and hair cells facilitate cochlear homeostasis, and supply hair cells with important anabolic substances, such as the neurotrophins and ATP required for ribbon synaptogenesis, Ca²⁺ specific signalling,

and hair cell metabolism (Sobkowicz et al. 2002; Wan et al. 2013; Kammen-Jolly et al. 2001; Ramirez-Camacho et al. 2006; Zwislocki et al. 1992). Therefore, the protective effect of ASK1 inhibition for aminoglycoside exposed supporting cells is likely to further enhance hair cell survival *in vivo*.

5.1.4. GS444217 does not affect antibiotic efficacy

The broth dilution test was used to show that GS444217 treatment did not impact the efficacy of the aminoglycoside antibiotics neomycin, tobramycin, and amikacin against patient-derived and reference strains of *P. aeruginosa*. This outcome is critical, given the lifesaving nature of aminoglycoside treatment in humans. Notably, previous studies have found drug interactions between putative adjuvant therapies and primary treatments, which significantly hinders the application of an otoprotective substance (Treskes et al. 1991; Wu et al. 2005; Doolittle et al. 2001). When this occurs, alternate drug delivery must be considered, such as transtympanic injections, which have been found to cause intolerable pain in some treated individuals (Riga et al. 2013). Thus, the observation that GS444217 does not affect aminoglycoside antibiotic efficacy *in vitro*, is particularly encouraging, suggesting that systemic interactions between primary treatment and an adjuvant ASK1 inhibitor therapy are unlikely. Whilst this must still be assessed *in vivo*, the safety and bioavailability of ASK1 inhibitors taken orally in clinical trials, combined with the observation that ASK1 inhibition does not affect aminoglycoside antibiotic efficacy *in vitro*, implies that ASK1 inhibition could be applied as an otoprotective strategy in the clinic.

5.1.5. GS444217 represents an opportunity for the prevention of aminoglycoside-induced ototoxic outcomes.

Collectively, data produced in this project provides valuable evidence that ASK1 inhibition may be a valid strategy for mitigating aminoglycoside-induced hearing loss in humans. Therefore, further experimentation is warranted to provide the pre-clinical evidence required to justify human clinical investigation. As ASK1 inhibition has been proven safe in a number of human clinical trials, strong *in vivo* efficacy data would provide the additional evidence base required to drive the translation of this work *from bench to bedside*.

5.2. Project limitations and future contingencies

5.2.1. Applications to the vestibular System

For this project, a number of important observations were made from *in vitro* studies utilising mouse cochlear explants. However, vestibular dysfunction is also an important aspect of ototoxicity, with hair cells of the vestibular apparatus considered to be even more susceptible to aminoglycoside toxicity than cochlear hair cells (Ishiyama et al. 2006; Handelsman 2018). The toxic effect of aminoglycosides on vestibular hair cells is thought to be under-diagnosed and reported, because affected individuals are often bedridden or unwell when symptoms first appear (Handelsman 2018; Bitner-Glindzicz et al. 2007). Nevertheless, for these individuals, permanent vestibular damage can ultimately limit their functional independence (Bitner-Glindzicz et al. 2007). It is therefore important that a putative therapeutic strategy for the prevention of ototoxicity aims to protect both the cochlear and vestibular hair cells. Fortunately, significant mechanistic similarities have been noted in cochlear and vestibular hair cell death in response to aminoglycoside treatment. In particular, Sugahara et al. 2006 and Francis et al. 2011 have demonstrated that JNK phosphorylation is a crucial step for neomycin-induced hair cell death in the mouse utricle (Sugahara et al. 2006; Francis et al. 2011). Furthermore, Kim et al, 2009 demonstrated that ROS production correlates with gentamycin-induced utricular hair cell death (Kim et al. 2009). Therefore, the available data suggests that ASK1 inhibition may be an effective, protective therapy against aminoglycoside vestibular hair cell toxicity. Replicating the findings of this project in explant preparations derived from the mouse utricle and crista would provide further evidence that ASK1 inhibition prevents ototoxicity of the vestibular system. Moreover, researchers with access to electively terminated foetal material could replicate this work in cochlear and vestibular explants, providing important efficacy data in human tissue. The ethics and utility of foetal hair cell explants is a complex issue that will not be discussed in this thesis. However, experiments utilising hair cells from foetal tissue terminated at 11-18 weeks gestation have been described previously by Lim et al. 2014.

5.2.2. Applications for other systems

In addition to protecting hair cells of the inner ear, it is possible that ASK1 inhibition could protect other susceptible tissues from aminoglycoside toxicity. For example, aminoglycoside-induced nephrotoxicity is well described and there is a growing body of evidence indicating that aminoglycosides are neurotoxic (Segal et al. 1999; Mattappalil & Mergenhagen 2014; Oishi et al. 2015; Lindsey & Townes-Anderson 2018). Similar to aminoglycoside induced ototoxicity, the nephro and neurotoxic effects of aminoglycosides have been associated with mitochondrial dysfunction and ROS production. Furthermore, ASK1 mediated P38 and JNK signalling has been implicated in ROS-mediated diseases affecting the kidney and brain (Ma et al. 2014; Tesch et al. 2015; Terada et al. 2007; Reddy & Beal 2008; Spuch et al. 2012; Kadowaki et al. 2005). Collectively, these studies suggest that ASK1 inhibition could provide protection against aminoglycoside toxicity in numerous tissues. Whilst this remains to be tested, an *in vivo* model of aminoglycoside toxicity could provide proof-of-principle evidence for both aminoglycoside ototoxicity and other forms of aminoglycoside toxicity.

5.2.3. Pursuing an *in vivo* model of aminoglycoside toxicity

In Chapter 3, the challenges of utilising the mouse to model ototoxicity *in vivo* were discussed. Overall, numerous attempts to model ototoxicity in mice have been unsuccessful, indicating an intrinsic resistance to aminoglycoside toxicity in the mouse ear. The guinea pig is considered to be a better model organism for auditory research, and ototoxic outcomes have been achieved using aminoglycosides in the guinea pig. However, given that aminoglycosides can have toxic outcomes in the kidney and central nervous system, and that ASK1 inhibition has already been proven safe in humans, the pig may be a better model for collecting the pre-clinical evidence required to justify the trial of ASK1 inhibition in aminoglycoside treated humans. The homology between humans and pigs is notable, with the pig genome much more similar to the human genome than that of rodent models (Bendixen et al. 2010). Moreover, pigs have a similar metabolic rate to humans, and are a closer match in terms of size and weight. The porcine immune system closely mimics that of humans, and other physiological similarities exist, particularly in the vital organs. As a result, pigs have been used extensively for research regarding brain function, infectious disease, reproduction,

organ transplantation, nutrition, drug pharmacokinetics, and other physiological studies of the skin, lung, liver, heart, kidney, and gut (Bendixen et al. 2010; Lunney 2007; Tang & Mayersohn 2018). Pigs are also considered a good model for auditory specific research, with an auditory range and cochlear structure that is very similar to that of humans (Heffner & Heffner 2007; Heffner & Heffner 1990; Chen et al. 2018). Unlike rodent models, the pig cochlea is mature at birth, which would facilitate the comparison of ototoxic outcomes in juvenile pigs with those frequently induced by aminoglycoside use in babies with sepsis, or children with cystic fibrosis (Guo et al. 2015). Furthermore, *in vivo* modelling utilising the pig could provide critical information regarding ASK1 inhibitor dosing and the treatment window in which ASK1 inhibition is protective. In this project 1 μ M of GS444217 significantly attenuated hair cell death caused by 1 mM neomycin treatment, however a similar trend was also achieved using only 0.1 μ M GS444217. It is difficult to predict the exact dosing regimen that will be required to achieve similar cochlear concentrations *in vivo*, however, previous studies have demonstrated excellent oral bioavailability of GS444217, achieving plasma concentrations markedly higher than 1 μ M. For example, a single oral dose of 50 mg/kg resulted in GS444217 plasma levels of 92.8 mM in mice (Budasz et al. 2018). In rats, a single oral dose of 30 mg/kg, achieved peak plasma concentrations approaching 100 μ M, although GS444217 plasma levels declined to approximately 0.1 μ M after 24 hours (Liles et al. 2018). This indicates that twice daily GS444217 dosing may be beneficial. Other ASK1 inhibitors have also been tested *in vivo*, with 30 mg/kg of GS459679 achieving plasma concentrations of approximately 6 μ M in mice, 6.5 hours after administration (Xie et al. 2015). It is also possible that the otoprotective effect of GS444217 will be stronger *in vivo*. Supporting structures such as the stria vascularis will be functioning *in situ*, providing an environment more conducive to hair cell survival. In addition, aminoglycosides are unlikely to reach the high concentrations *in vivo* (1 mM) that were utilised in this *in vitro* project. For example, a study of 113 individuals receiving amikacin over a two week period, indicated that on average, peak plasma concentrations did not exceed 17 μ M amikacin (Contreras et al. 1989). Given the metabolic similarities between pigs and humans, pigs are likely to provide significant preclinical pharmacokinetic data for investigating ASK1 inhibition as a protective adjuvant therapy.

A final consideration regarding the utility of ASK1 inhibition *in vivo*, is verifying that the inhibitor does not alter aminoglycoside antibiotic properties. The pig has been used extensively for the study of infectious microbial disease (Meurens et al. 2012), and swine models of cystic fibrosis exist (Welsh et al. 2009). Such a model would be valuable for studying the progression of *P. aeruginosa* infection, and the efficacy of aminoglycoside antibiotics when an adjuvant ASK1 inhibitor is used. Whilst this project provided *in vitro* evidence that ASK1 inhibition is protective against aminoglycoside ototoxicity, and that ASK1 inhibition does not change the antibiotic properties of aminoglycosides, recapitulating the work in a live pig model would provide the evidence required to confidently pursue the clinical application of ASK1 inhibition in aminoglycoside-treated humans. Furthermore, swine *in vivo* modelling would assist with clinical trial design, by indicating realistic outcomes that could be specified as pre-defined endpoints and noteworthy secondary outcomes.

5.2.4. Addressing alternate forms of acquired hearing loss

This project focused on aminoglycoside-induced hearing loss. However, numerous medicines are ototoxic (Table 3). In particular, platinum based chemotherapeutics remain a significant cause of ototoxic outcomes. Like aminoglycoside ototoxicity, chemotherapy-induced hair cell death is associated with redox imbalance (Jamesdaniel et al. 2016; Rybak et al. 2019). Thus, it is possible that ASK1 inhibition could attenuate the ototoxicity of platinum based chemotherapeutics, including cisplatin, carboplatin, and the analogue oxaliplatin. Furthermore, ASK1 inhibition may be protective against other platinum toxicities, including neuro, nephro and hepatotoxicity. However, this remains to be tested and caution must be exercised to ensure that ASK1 inhibition does not compromise an individuals' cancer treatment.

To date, ASK1 inhibition has not been associated with an increased cancer risk (Tesch et al. 2016). Rather, there is evidence that ASK1 inhibition can limit cancer growth. For example, ASK1 inhibition slows the growth of gastric cancer xenografts in mice (Hayakawa et al. 2012; Hayakawa et al. 2011) and ASK1 inhibition attenuates the drug resistance of cancer cells with elevated ABCG2, a driver of cancer drug resistance (Ji et al. 2019). Moreover, platelet-specific deletion of ASK1 prevents tumour metastasis in mice with established lung cancer, induced by the injection of either melanoma or Lewis lung carcinoma cells (Kamiyama et al. 2017). However, reduced ASK1 expression

has also been associated with aggressive hepatic tumours (Nakagawa et al. 2011; Jiang et al. 2016). Therefore, the effects of ASK1 inhibition need to be assessed in numerous and diverse cancer cell lines before being considered as an adjuvant therapy for chemotherapeutic ototoxicity.

Beyond drug-induced hair cell death, ROS production and subsequent JNK activation has been well characterised in animal models of both noise- and surgically-induced hair cell death. For example, JNK inhibition via local application of D-JNKI-1 into the scala tympani is able to prevent noise induced or electrode-induced hearing loss (Wang et al. 2003; Eshraghi et al. 2007; Wang et al. 2007). It is therefore possible that ASK1 inhibition could prove useful for those exposed to acoustic trauma or individuals undergoing cochlear surgery, such as cochlear implantation. For example, military personnel can be exposed to explosions, gunfire and loud aircraft (Mardassi et al. 2016). In these types of situation, the use of hearing protection can be inappropriate or impossible. As a result, the prevalence of permanent hearing loss is much greater in military personnel than the general population (Mardassi et al. 2016; Yong & Wang 2015). Given the reported production of ROS, and the subsequent activation of JNK in acoustically damaged hair cells, it is conceivable that ASK1 inhibition may mitigate noise-induced hair cell death in the acute setting.

Similarly, individuals receiving cochlear implants have a very high risk of acquiring further hearing loss. Whilst the person in need of a cochlear implant already has significant hearing impairment, the preservation of residual hearing can have a profound impact (Bas, Dinh, et al. 2012). For example, individuals with residual hearing can use a hearing aid to enhance the innate auditory signal produced by remaining hair cells, which, when combined with the cochlear implant's signal, allows for improved fine pitch perception. As a result, the individual's ability to perceive and enjoy music, and recognise complex speech is significantly enhanced (Neben et al. 2018; Gfeller et al. 2006; Gantz et al. 2005). Therefore, a strategy to limit the damage caused during and post cochlear surgery could significantly improve the auditory outcomes in patients with cochlear implants.

Given that post-operative hair cell death has been associated with ROS mediated JNK activation (Jia et al. 2013) it is likely that ASK1 inhibition would be useful in this

setting. In the guinea pig, local delivery of the JNK inhibitor AM-111 during cochlear implantation limited immediate hair cell and neuronal apoptosis (Eshraghi et al. 2013). However, ASK1 inhibition has the potential to be of greater benefit. In particular, oral ASK1 inhibitors have been proven tolerable in humans. Therefore, an individual preparing for surgery could be pre-treated with the inhibitor, which would likely achieve better cochlear distribution. Moreover, post-operative ASK1 inhibition could be maintained. This is particularly important, as chronic inflammation and the development of fibrotic tissue post cochlear implant surgery can cause delayed hearing loss and impede implant function. ASK1 has been extensively implicated in inflammatory and fibrotic responses underpinning numerous diseases (Yamaguchi et al. 2003; Terada et al. 2007; Liles et al. 2018; Schuster et al. 2017; Younossi et al. 2018; Loomba et al. 2018), thus, ASK1 inhibition is a candidate for preventing fibrotic responses to surgery. Furthermore, this project has shown that cell death in the cochlea can be mediated by both P38 and JNK, and that ASK1 inhibition can attenuate the phosphorylation of both. Therefore, ASK1 inhibition could attenuate not only the initial hair cell death associated with JNK activation, but potentially P38 mediated death of supporting cells and post-operative inflammation or fibrosis that can occur months later. Further work with animal models of cochlear implant surgery will ascertain the benefits of ASK1 inhibition in this capacity.

5.3. Future applications and implications

This project has provided fundamental evidence that ASK1 inhibition may be a useful strategy for the prevention of aminoglycoside ototoxicity. The observations within this work provide a strong foundation that can be built upon using pre-clinical *in vivo* models that may ultimately lead to the development of an adjuvant otoprotective therapy. Such a therapy would have far-reaching implications, as aminoglycoside ototoxicity is a significant health issue, not only for those directly affected, but also clinical health care providers and the economy in general. Avoiding the distressing and lifelong co-morbidities of acquired hearing loss, such as loneliness, impaired mobility, mental fatigue, cognitive decline, and increased mortality risk, is critically important for the health of an individual (Wayne & Johnsrude 2015; Lin & Ferrucci 2012; Fisher et al. 2014; Lin et al. 2011; Lin et al. 2013; Rieffe 2012). From an economic perspective, preventing aminoglycoside ototoxicity would reduce the financial burden of acquired hearing loss within the national health, education and welfare systems. In the case of

critical healthcare, the prevention of aminoglycoside ototoxicity may also improve the utility of aminoglycoside antibiotics. At present, clinicians must consider the risk of permanent hearing loss against the risk to life when prescribing an aminoglycoside treatment. However, reducing ototoxic outcomes would ease the burden experienced by clinicians during this decision making process and potentially allow for the wider application of aminoglycoside therapy.

5.4. Concluding Remarks

This project is the first to have investigated the role of ASK1 specifically within the auditory pathway of mice. Important insights were made regarding auditory signal transduction in *Ask1* knockout mice and the role of ASK1 as a regulator of P38 and JNK mediated cell death in the inner ear. Overall, ASK1 was found to be an important mediator of hair cell death, with ASK1 inhibition affording significant protection against neomycin toxicity. These observations indicate that ASK1 inhibition should be further evaluated as an otoprotective strategy for the prevention of aminoglycoside induced hearing loss. In conclusion, this project, has added to the general body of scientific knowledge regarding cell death in the inner ear and the role of ASK1 in the auditory pathway. This information will be useful for the future development of otoprotective strategies and will likely be applicable in other subfields of auditory neuroscience.

References

- Abadi, S., Khanbabaee, G., & Sheibani, K., 2016. Auditory Brainstem Response Wave Amplitude Characteristics as a Diagnostic Tool in Children with Speech Delay with Unknown Causes. *Iranian journal of medical sciences*, 41(5), pp.415–421.
- Access-Economics, 2006. Listen hear: the economic impact and cost of hearing loss in Australia.
- Adler, H. & Raphael, Y., 1996. New hair cells arise from supporting cell conversion in the acoustically damaged chick inner ear. *Neuroscience letters*, 205(1), pp.17–20.
- Aedo, C. et al., 2016. The Corticofugal Effects of Auditory Cortex Microstimulation on Auditory Nerve and Superior Olivary Complex Responses Are Mediated via Alpha-9 Nicotinic Receptor Subunit. *PLoS ONE*, 11(5), p.e0155991.
- Ahmed, R.M. et al., 2012. Gentamicin ototoxicity: a 23-year selected case series of 103 patients. *The Medical journal of Australia*, 196(11), pp.701–704.
- Aitkin, L.M., Anderson, D.J. & Brugge, J.F., 1970. Tonotopic organization and discharge characteristics of single neurons in nuclei of the lateral lemniscus of the cat. *Journal of neurophysiology*, 33(3), pp.421–440.
- Akiyoshi, M., 1978. Evaluation of ototoxicity of tobramycin in guinea pigs. *The Journal of antimicrobial chemotherapy*, 4, pp.69–72.
- Alharazneh, A. et al., 2011. Functional hair cell mechanotransducer channels are required for aminoglycoside ototoxicity. *PLoS ONE*, 6(7), p.e22347.
- Amos, L.A. et al., 2018. ASK1 inhibitor treatment suppresses p38/JNK signalling with reduced kidney inflammation and fibrosis in rat crescentic glomerulonephritis. *Journal of cellular and molecular medicine*, 22(9), pp.4522–4533.
- Andéol, G. et al., 2011. Auditory Efferents Facilitate Sound Localization in Noise in Humans. *The Journal Of Neuroscience*, 31(18), p.6759.
- Angrilli, A. et al., 1996. Startle reflex and emotion modulation impairment after a right amygdala lesion. *Brain : a journal of neurology*, 119, pp.1991–2000.
- Aran, J.M. et al., 1982. The comparative ototoxicities of gentamicin, tobramycin and dibekacin in the guinea pig. A functional and morphological cochlear and vestibular study. *Acta otolaryngologica*, 390, pp.1–30.
- Ashmore, J., 2008. Cochlear outer hair cell motility. *Physiological reviews*, 88(1), pp.173–210.
- Assad, J.A., Shepherd, G.M. & Corey, D.P., 1991. Tip-link integrity and mechanical transduction in vertebrate hair cells. *Neuron*, 7(6), pp.985–994.

- Baird, R.A. et al., 2000. Hair Cell Recovery in Mitotically Blocked Cultures of the Bullfrog Sacculle. *Proceedings of the National Academy of Sciences USA* (22), p.11722.
- Balak et al. 1990. Regenerated hair cells can originate from supporting cell progeny: Evidence from phototoxicity and laser ablation experiments in the lateral line system. *The Journal of Neuroscience*, 10(8), pp.2502-2512
- Bas, E., Dinh, C.T., et al., 2012. Conservation of hearing and protection of hair cells in cochlear implant patients' with residual hearing. *Anatomical record*, 295(11), pp.1909–1927.
- Bas, E., Van de Water, T.R., et al., 2012. Efficacy of three drugs for protecting against gentamicin-induced hair cell and hearing losses. *British Journal of Pharmacology*, 166(6), pp.1888–1904.
- Battaglia, A. et al., 2003. Involvement of ras activation in toxic hair cell damage of the mammalian cochlea. *Neuroscience*, 122(4), pp.1025–1035.
- Beck, K.D., 2013. Understanding the Causes of Reduced Startle Reactivity in Stress-Related Mental Disorders. *New Insights into Anxiety Disorders*. Ch. 6.
- Bendixen, E. et al., 2010. Advances in porcine genomics and proteomics--a toolbox for developing the pig as a model organism for molecular biomedical research. *Briefings in functional genomics*, 9(3), pp.208–219.
- Benjamini, Y., Krieger, A.M. & Yekutieli, D., 2006. Adaptive linear step-up procedures that control the false discovery rate. *Biometrika*, 93(3), pp.491–507.
- Benkafadar, N. et al., 2019. ROS-Induced Activation of DNA Damage Responses Drives Senescence-Like State in Postmitotic Cochlear Cells: Implication for Hearing Preservation. *Molecular neurobiology*, 56(8), pp.5950–5969.
- Berg, A.L., Spitzer, J.B. & Garvin, J.H., 1999. Ototoxic Impact of Cisplatin in Pediatric Oncology Patients. *Laryngoscope*, 109(11), pp.1806–1814.
- Berglund, A.M. & Ryugo, D.K., 1987. Hair cell innervation by spiral ganglion neurons in the mouse. *The Journal of comparative neurology*, 255(4), pp.560–570.
- Bermingham, N.A. et al., 1999. Math1: an essential gene for the generation of inner ear hair cells. *Science*, 284(5421), pp.1837–1841.
- Berndtsson, M. et al., 2007. Acute apoptosis by cisplatin requires induction of reactive oxygen species but is not associated with damage to nuclear DNA. *International journal of cancer*, 120(1), pp.175–180.
- Berne, R.M. et al., 2008. Berne and Levy physiology. 7th Edition, *Elsevier*.
- Betts, G. et al., 2012. Anatomy and Physiology. *OpenStax CNX*. Available at: [openstax.org/books/anatomy-and-physiology/](https://openstax.org/books/anatomy-and-physiology).
- Bitner-Glindzicz, M. et al., 2009. Prevalence of mitochondrial 1555A-->G mutation in European children. *New England Journal of Medicine*, 360(6), pp.640–642.

- Bitner-Glindzicz, M., Rahman, S. & Seemungal, B.M., 2007. Ototoxicity caused by aminoglycosides. Response: Aminoglycoside Vestibulotoxicity. *The BMJ*, 335(7624), p.784.
- Blakley, B.W., 1997. Clinical forum: a review of intratympanic therapy. *The American journal of otology*, 18(4), pp.520–531.
- Bock, G.R. et al., 1983. Effects of N-acetylcysteine on kanamycin ototoxicity in the guinea pig. *Hearing research*, 9(3), pp.255–262.
- Bodmer, D. et al., 2002. Rescue of auditory hair cells from ototoxicity by CEP-11 004, an inhibitor of the JNK signaling pathway. *Laryngorhinootologie*, 81(12), pp.853–856.
- Boi, R. et al., 2012. Hearing loss and depressive symptoms in elderly patients. *Geriatrics and gerontology international*, 12(3), pp.440–445.
- Bramhall, N.F. et al., 2014. Lgr5-positive supporting cells generate new hair cells in the postnatal cochlea. *Stem cell reports*, 2(3), pp.311–322.
- Brancho, D. et al., 2005. Role of MLK3 in the regulation of mitogen-activated protein kinase signaling cascades. *Molecular and cellular biology*, 25(9), pp.3670–3681.
- Braye, K. et al., 2019. Epidemiology of neonatal early-onset sepsis in a geographically diverse Australian health district 2006-2016. *PLoS ONE*, 14(4), p.e0214298.
- Britton, Z. & Arshad, Q., 2019. Vestibular and Multi-Sensory Influences Upon Self-Motion Perception and the Consequences for Human Behavior. *Frontiers in Neurology*, 10, p.63.
- Brownell, W.E. et al., 1985. Evoked mechanical responses of isolated cochlear outer hair cells. *Science*, 227(4683), pp.194–196.
- Brummett, R.E., Vernon, J.A. & Meikle, M.M., 1971. Ototoxicity of tobramycin in guinea pigs. *Archives of otolaryngology*, 94(1), pp.59–63.
- Bubici, C. & Papa, S., 2014. JNK signalling in cancer: in need of new, smarter therapeutic targets. *British Journal of Pharmacology*, 171(1), pp.24–37.
- Budas, G.R. et al., 2018. ASK1 Inhibition Halts Disease Progression in Preclinical Models of Pulmonary Arterial Hypertension. *American journal of respiratory and critical care medicine*, 197(3), pp.373–385.
- Burns, J.C. & Stone, J.S., 2017. Development and regeneration of vestibular hair cells in mammals. *Seminars in cell and developmental biology*, 65, pp.96–105.
- Burns, J.C. et al., 2015. Single-cell RNA-Seq resolves cellular complexity in sensory organs from the neonatal inner ear. *Nature communications*, 6, p.8557.
- Cabanas, M.J., Vazquez, D. & Modolell, J., 1978. Inhibition of ribosomal translocation by aminoglycoside antibiotics. *Biochemical and biophysical research communications*, 83(3), pp.991–997.

- Caceres, A. et al., 1984. MAP2 is localized to the dendrites of hippocampal neurons which develop in culture. *Brain Research*, 315(2), pp.314–318.
- Carpinelli, M.R. et al., 2013. Two ENU-induced alleles of Atp2b2 cause deafness in mice. *PLoS ONE*, 8(6), p.e67479.
- Cederroth, C.R., 2012. Loss of aminoglycoside sensitivity in HEI-OC1 cells? *Hearing research*, 292(1-2), pp.83–5– author response pg 86.
- Celebi, S. et al., 2013. The effect of intratympanic vitamin C administration on cisplatin-induced ototoxicity. *European Archives Of Otorhinolaryngology*, 270(4), pp.1293–1297.
- Chambers, J.W. & LoGrasso, P.V., 2011. Mitochondrial c-Jun N-terminal kinase (JNK) signaling initiates physiological changes resulting in amplification of reactive oxygen species generation. *The Journal of biological chemistry*, 286(18), pp.16052–16062.
- Chen, W. et al., 2018. Cochlear morphology in the developing inner ear of the porcine model of spontaneous deafness. *BMC Neuroscience*, 19(1), p.28.
- Chen, Y. et al., 2013. Cotransfection of Pax2 and Math1 promote in situ cochlear hair cell regeneration after neomycin insult. *Scientific reports*, 3, pp.2996–2996.
- Cheng, A.G., Cunningham, L.L. & Rubel, E.W., 2005. Mechanisms of hair cell death and protection. *Current opinion in otolaryngology and head and neck surgery*, 13(6), pp.343–348.
- Chertow, G.M. et al., 2019. Effects of Selonsertib in Patients with Diabetic Kidney Disease. *Journal of the American Society of Nephrology : JASN*, 30(10), p.1980.
- Choi, M.-J. et al., 2019. Cisplatin-Induced Ototoxicity in Rats Is Driven by RIP3-Dependent Necroptosis. *Cells*, 8(5).
- Choi, S.J. et al., 2013. Ginkgo biloba extracts protect auditory hair cells from cisplatin-induced ototoxicity by inhibiting perturbation of gap junctional intercellular communication. *Neuroscience*, 244, pp.49–61.
- Choung, Y.H. et al., 2009. Generation of highly-reactive oxygen species is closely related to hair cell damage in rat organ of Corti treated with gentamicin. *Neuroscience*, 161(1), pp.214–226.
- Ciuman, R.R., 2010. The efferent system or olivocochlear function bundle - fine regulator and protector of hearing perception. *International journal of biomedical science* 6(4), pp.276–288.
- Clerici, W.J. et al., 1996. Direct detection of ototoxicant-induced reactive oxygen species generation in cochlear explants. *Hearing research*, 98(1-2), pp.116–124.
- CMU, 2019. *Anatomy and Physiology*, Carnegie Mellon University via Lumen Learning. Available at: <https://courses.lumenlearning.com/nemcc-ap/chapter/special-senses-hearing-audition-and-balance/>.

- Coate, T.M., Scott, M.K. & Gurjar, M., 2019. Current concepts in cochlear ribbon synapse formation. *Synapse*, 73(5), pp.e22087–e22087.
- Coffin, A.B., Rubel, E.W. & Raible, D.W., 2013. Bax, Bcl2, and p53 Differentially Regulate Neomycin- and Gentamicin-Induced Hair Cell Death in the Zebrafish Lateral Line. *Journal of the Association for Research in Otolaryngology*, 14(5), pp.645–659.
- Coleman, J.K.M. et al., 2007. AM-111 protects against permanent hearing loss from impulse noise trauma. *Hearing research*, 226(1-2), pp.70–78.
- Collet, L. et al., 1990. Effect of contralateral auditory stimuli on active cochlear micro-mechanical properties in human subjects. *Hearing research*, 43(2-3), pp.251–261.
- Contreras, A.M. et al., 1989. Serial trough and peak amikacin levels in plasma as predictors of nephrotoxicity. *Antimicrobial Agents and Chemotherapy*, 33(6), pp.973–976.
- Cook, D., 2018 and 2020. CGF166 -Update on a promising new treatment for hearing loss. Available at: www.hearinglossjournal.com/cgf166-update-on-a-promising-new-treatment-for-hearing-loss/ [Accessed July 5, 2019]. Replaced by www.hearinglossjournal.com/novartis-suspends-cgf166-trials/ [Accessed April 16, 2020]
- Corwin, J.T., 1981. Postembryonic production and aging in inner ear hair cells in sharks. *The Journal of comparative neurology*, 201(4), pp.541–553.
- Corwin, J.T. & Cotanche, D.A., 1988. Regeneration of Sensory Hair Cells after Acoustic Trauma. *Science*, 240(4860), pp.1772–1774.
- Cosh, S. et al., 2018. The relationship between hearing loss in older adults and depression over 12 years: Findings from the Three-City prospective cohort study. *International journal of geriatric psychiatry*, pp.1654–1661.
- Cotanche, D.A. & Corwin, J.T., 1991. Stereociliary bundles reorient during hair cell development and regeneration in the chick cochlea. *Hearing research*, 52(2), pp.379–402.
- Cox, B.C. et al., 2014. Spontaneous hair cell regeneration in the neonatal mouse cochlea in vivo. *Development*, 141(4), pp.816–829.
- Crankshaft-Publishing, 2019. Auditory and Vestibular Systems. Available at: <http://what-when-how.com/neuroscience/auditory-and-vestibular-systems-sensory-system-part-3/> [Accessed April 5, 2019].
- Cruz, R.M., Lambert, P.R. & Rubel, E.W., 1987. Light microscopic evidence of hair cell regeneration after gentamicin toxicity in chick cochlea. *Archives of otolaryngology head and neck surgery*, 113(10), pp.1058–1062.
- Culler, E. et al., 1943. A Revised Frequency-Map of the Guinea-Pig Cochlea. *The American Journal of Psychology*, 56(4), pp.475–500.

- Cunningham, C.L. & Muller, U., 2019. Molecular Structure of the Hair Cell Mechano-electrical Transduction Complex. *Cold Spring Harbor perspectives in medicine*, 9(5).
- Cunningham, L.L., Cheng, A.G. & Rubel, E.W., 2002. Caspase activation in hair cells of the mouse utricle exposed to neomycin. *The Journal Of Neuroscience*, 22(19), pp.8532–8540.
- Dallos, P. et al., 2008. Prestin-based outer hair cell motility is necessary for mammalian cochlear amplification. *Neuron*, 58(3), pp.333–339.
- Dammeyer, P. et al., 2014. Cisplatin and oxaliplatin are toxic to cochlear outer hair cells and both target thioredoxin reductase in organ of Corti cultures. *Acta otolaryngologica*, 134(5), pp.448–454.
- Dasari, S. & Tchounwou, P.B., 2014. Cisplatin in cancer therapy: molecular mechanisms of action. *European Journal of Pharmacology*, 0, pp.364–378.
- Decker, M.W., Curzon, P. & Brioni, J.D., 1995. Influence of separate and combined septal and amygdala lesions on memory, acoustic startle, anxiety, and locomotor activity in rats. *Neurobiology of learning and memory*, 64(2), pp.156–168.
- Deloitte, 2017. *The Social and Economic Cost of Hearing Loss in Australia*.
- Denk, W. et al., 1995. Calcium imaging of single stereocilia in hair cells: localization of transduction channels at both ends of tip links. *Neuron*, 15(6), pp.1311–1321.
- Devarajan, P. et al., 2002. Cisplatin-induced apoptosis in auditory cells: role of death receptor and mitochondrial pathways. *Hearing research*, 174(1-2), pp.45–54.
- Dhanasekaran, D.N. & Reddy, E.P., 2008. JNK signaling in apoptosis. *Oncogene*, 27(48), pp.6245–6251.
- Ding, D., Stracher, A. & Salvi, R.J., 2002. Leupeptin protects cochlear and vestibular hair cells from gentamicin ototoxicity. *Hearing research*, 164(1-2), pp.115–126.
- Dinh, C.T. et al., 2015. Molecular regulation of auditory hair cell death and approaches to protect sensory receptor cells and/or stimulate repair following acoustic trauma. *Frontiers in Cellular Neuroscience*, 9, p.96.
- Dirain, C.O. et al., 2018. Evaluation of Mitoquinone for Protecting Against Amikacin-Induced Ototoxicity in Guinea Pigs. *Otology & neurotology*, 39(1), pp.111–118.
- Doolittle, N.D. et al., 2001. Delayed sodium thiosulfate as an otoprotectant against carboplatin-induced hearing loss in patients with malignant brain tumors. *Clinical cancer research* 7(3), pp.493–500.
- Doucet, J.R. & Ryugo, D.K., 2003. Axonal pathways to the lateral superior olive labeled with biotinylated dextran amine injections in the dorsal cochlear nucleus of rats. *The Journal of comparative neurology*, 461(4), pp.452–465.

- Doucet, J.R., Rose, L. & Ryugo, D.K., 2002. The cellular origin of corticofugal projections to the superior olivary complex in the rat. *Brain Research*, 925(1), pp.28–41.
- Drga, V., Plack, C.J. & Yasin, I., 2016. Frequency Tuning of the Efferent Effect on Cochlear Gain in Humans. *Advances in experimental medicine and biology*, 894, pp.477–484.
- Driscoll, S., 2018. Ototoxic Drugs Affect Hearing and Balance. healthyhearing.com.au/ototoxic-drugs-affect-hearing-balance.
- Duckert, L.G. & Rubel, E.W., 1993. Morphological correlates of functional recovery in the chicken inner ear after gentamycin treatment. *The Journal of comparative neurology*, 331(1), pp.75–96.
- Ehret, G. & Romand, R., 1997. The central auditory system. *Oxford University Press*.
- Ekdale, E.G., 2013. Comparative Anatomy of the Bony Labyrinth (Inner Ear) of Placental Mammals. *PLoS ONE*, 8(6), pp.e66624–e66624.
- Emanuel, D., Maroonroge, S. & Letowski, T., 2009. Auditory function: Physiology and function of the hearing system. pp. 307–334.
- Erickson, T. et al., 2017. Integration of Tmc1/2 into the mechanotransduction complex in zebrafish hair cells is regulated by Transmembrane O-methyltransferase (Tomt). *eLife*, 6.
- Eshraghi, A.A. et al., 2007. Blocking c-Jun-N-terminal kinase signaling can prevent hearing loss induced by both electrode insertion trauma and neomycin ototoxicity. *Hearing research*, 226(1-2), pp.168–177.
- Eshraghi, A.A. et al., 2013. Molecular mechanisms involved in cochlear implantation trauma and the protection of hearing and auditory sensory cells by inhibition of c-Jun-N-terminal kinase signaling. *Laryngoscope*, 123 pp.S1–14.
- Eybalin, M., 1993. Neurotransmitters and neuromodulators of the mammalian cochlea. *Physiological reviews*, 73(2), pp.309–373.
- Farris, H.E. et al., 2004. Probing the pore of the auditory hair cell mechanotransducer channel in turtle. *The Journal of physiology*, 558(Pt 3), pp.769–792.
- Fausti, S.A. et al., 1992. Early detection of ototoxicity using high-frequency, tone-burst-evoked auditory brainstem responses. *Journal of the American Academy of Audiology*, 3(6), pp.397–404.
- Fetoni, A.R. et al., 2014. Curcuma longa (curcumin) decreases in vivo cisplatin-induced ototoxicity through heme oxygenase-1 induction. *Otology & neurotology*, 35(5), pp.e169–77.
- Fetoni, A.R. et al., 2015. Molecular targets for anticancer redox chemotherapy and cisplatin-induced ototoxicity: the role of curcumin on pSTAT3 and Nrf-2 signalling. *British journal of cancer*, 113(10), pp.1434–1444.

- Fettiplace, R., 2017. Hair Cell Transduction, Tuning, and Synaptic Transmission in the Mammalian Cochlea. *Comprehensive Physiology*, 7(4), pp.1197–1227.
- Fisher, D. et al., 2014. Impairments in hearing and vision impact on mortality in older people: the AGES-Reykjavik Study. *Age and Ageing*, 43(1), pp.69–76.
- Fletcher, M.D., Krumbholz, K. & de Boer, J., 2016. Effect of Contralateral Medial Olivocochlear Feedback on Perceptual Estimates of Cochlear Gain and Compression. *Journal of the Association for Research in Otolaryngology*, 17(6), pp.559–575.
- Flores, E.N. et al., 2015. A non-canonical pathway from cochlea to brain signals tissue-damaging noise. *Current biology* 25(5), pp.606–612.
- Francis, S.P. et al., 2013. A novel role of cytosolic protein synthesis inhibition in aminoglycoside ototoxicity. *The Journal Of Neuroscience*, 33(7), pp.3079–3093.
- Francis, S.P. et al., 2011. Celastrol inhibits aminoglycoside-induced ototoxicity via heat shock protein 32. *Cell Death and Disease*, 2, p.e195.
- Franco, R. & Cidlowski, J.A., 2009. Apoptosis and glutathione: beyond an antioxidant. *Cell death and differentiation*, 16(10), pp.1303–1314.
- Franco, R. & Cidlowski, J.A., 2006. SLCO/OATP-like transport of glutathione in FasL-induced apoptosis: glutathione efflux is coupled to an organic anion exchange and is necessary for the progression of the execution phase of apoptosis. *The Journal of biological chemistry*, 281(40), pp.29542–29557.
- Frolenkov, G.I., 2006. Regulation of electromotility in the cochlear outer hair cell. *The Journal of physiology*, 576(Pt 1), pp.43–48.
- Froud, K.E. et al., 2015. Type II spiral ganglion afferent neurons drive medial olivocochlear reflex suppression of the cochlear amplifier. *Nature communications*, 6, p.7115.
- Fuchs, P.A. & Glowatzki, E., 2015. Synaptic studies inform the functional diversity of cochlear afferents. *Hearing research*, 330(Pt A), pp.18–25.
- Fuchs, P.A. & Lauer, A.M., 2019. Efferent Inhibition of the Cochlea. *Cold Spring Harbor perspectives in medicine*, 9(5).
- Fujisawa, T. et al., 2016. The ASK1-specific inhibitors K811 and K812 prolong survival in a mouse model of amyotrophic lateral sclerosis. *Human Molecular Genetics*, 25(2), pp.245–253.
- Galanski, M., Jakupiec, M.A. & Keppler, B.K., 2005. Update of the preclinical situation of anticancer platinum complexes: novel design strategies and innovative analytical approaches. *Current medicinal chemistry*, 12(18), pp.2075–2094.
- Gale, J.E. et al., 2001. FM1-43 dye behaves as a permeant blocker of the hair-cell mechanotransducer channel. *The Journal Of Neuroscience*, 21(18), pp.7013–7025.

- Gantz, B.J. et al., 2005. Preservation of hearing in cochlear implant surgery: advantages of combined electrical and acoustical speech processing. *Laryngoscope*, 115(5), pp.796–802.
- Gao, Z., Chen, Y. & Guan, M.X., 2017. Mitochondrial DNA mutations associated with aminoglycoside induced ototoxicity. *Journal of otology*, 12(1), pp.1–8.
- Gfeller, K.E. et al., 2006. Music perception with cochlear implants and residual hearing. *Audiology and neurotology*, pp.12–15.
- Goldberg, J.M., 2012. The vestibular system : a sixth sense. *University Press*.
- Gopel, W. et al., 2014. Mitochondrial mutation m.1555A>G as a risk factor for failed newborn hearing screening in a large cohort of preterm infants. *BMC pediatrics*, 14, p.210.
- Gopinath, B. et al., 2009. Depressive symptoms in older adults with hearing impairments: the Blue Mountains Study. *Journal of the American Geriatrics Society*, 57(7), pp.1306–1308.
- Goutman, J.D., Elgoyhen, A.B. & Gómez-Casati, M.E., 2015. Cochlear hair cells: The sound-sensing machines. *Federation of European Biochemical Societies: letters*, 589(22), pp.3354–3361.
- Groves, A.K., 2010. The Challenge of Hair Cell Regeneration. *Experimental biology and medicine*, 235(4), pp.434–446.
- Gubbels, S.P. et al., 2008. Functional auditory hair cells produced in the mammalian cochlea by in utero gene transfer. *Nature*, 455(7212), pp.537–541.
- Guinan, J 2010. Cochlear efferent innervation and function. *Current opinion in otolaryngology and head and neck surgery*, 18(5), pp.447–453.
- Guo, W. et al., 2012. Endocochlear Potential and Potassium Concentration Recording in Minipig Cochlea. *Journal of otology*, 7(2), pp.103–105.
- Guo, W. et al., 2015. Hearing and Cochlear structures in a Swine Model. *Journal of otology*, 5(2), pp.97–100.
- Guo, X. et al., 2010. Regulation of the severity of neuroinflammation and demyelination by TLR-ASK1-p38 pathway. *EMBO molecular medicine*, 2(12), pp.504–515.
- Hamidi, S. et al., 2008. Avicenna's treatise on otology in Medieval Persia. *Otology & neurotology*, 29(8), pp.1198–1203.
- Hanawa, N. et al., 2008. Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. *The Journal of biological chemistry*, 283(20), pp.13565–13577.
- Handelsman, J.A., 2018. Vestibulotoxicity: strategies for clinical diagnosis and rehabilitation. *International Journal of Audiology*, 57, pp.S99–S107.

- Harada, A. et al., 2002. MAP2 is required for dendrite elongation, PKA anchoring in dendrites, and proper PKA signal transduction. *The Journal of cell biology*, 158(3), pp.541–549.
- Hartman, B.H. et al., 2009. Hes5 expression in the postnatal and adult mouse inner ear and the drug-damaged cochlea. *Journal of the Association for Research in Otolaryngology*, 10(3), pp.321–340.
- Hashino, E. et al., 1992. Hair cell regeneration in the adult budgerigar after kanamycin ototoxicity. *Hearing research*, 59(1), pp.46–58.
- Hay-McCutcheon, M.J., Reed, P.E. & Cheimariou, S., 2018. Positive Social Interaction and Hearing Loss in Older Adults Living in Rural and Urban Communities. *Journal of speech, language, and hearing research*, 61(8), pp.2138–2145.
- Hayakawa, R. et al., 2012. Therapeutic targets in the ASK1-dependent stress signaling pathways. *Proceedings of the Japan Academy. Series B, Physical and biological sciences*, 88(8), pp.434–453.
- Hayakawa, Y. et al., 2011. Apoptosis signal-regulating kinase 1 and cyclin D1 compose a positive feedback loop contributing to tumor growth in gastric cancer. *Proceedings of the National Academy of Sciences USA*, 108(2), pp.780–785.
- Hayakawa, Y. et al., 2012. Apoptosis signal-regulating kinase-1 inhibitor as a potent therapeutic drug for the treatment of gastric cancer. *Cancer science*, 103(12), pp.2181–2185.
- Heffner, H.E. & Heffner, R.S., 2007. Hearing ranges of laboratory animals. *Journal of the American Association for Laboratory Animal Science* 46(1), pp.20–22.
- Heffner, R.S. & Heffner, H.E., 1990. Hearing in domestic pigs (*Sus scrofa*) and goats (*Capra hircus*). *Hearing research*, 48(3), pp.231–240.
- Henkel, R., Sandhu, I.S. & Agarwal, A., 2019. The excessive use of antioxidant therapy: A possible cause of male infertility? *Andrologia*, 51(1), p.e13162.
- Hermann, T., 2007. Aminoglycoside antibiotics: old drugs and new therapeutic approaches. *Cellular and molecular life sciences*, 64(14), pp.1841–1852.
- Hiel, H. et al., 1992. Cellular and subcellular localization of tritiated gentamicin in the guinea pig cochlea following combined treatment with ethacrynic acid. *Hearing research*, 57(2), pp.157–165.
- Hironaka, N., 2008. Acoustic Startle Response in the Study of Developmental Toxicity. *Congenital Anomalies*, 39(1), pp.3–12.
- Hirose, K. & Sato, E., 2011. Comparative analysis of combination kanamycin-furosemide versus kanamycin alone in the mouse cochlea. *Hearing research*, 272(1-2), pp.108–116.
- Hirose, K., Hockenbery, D.M. & Rubel, E.W., 1997. Reactive oxygen species in chick hair cells after gentamicin exposure in vitro. *Hearing research*, 104(1-2), pp.1–14.

- Hirose, K., Rutherford, M.A. & Warchol, M.E., 2017. Two cell populations participate in clearance of damaged hair cells from the sensory epithelia of the inner ear. *Hearing research*, 352, pp.70–81.
- Hoshino, A. et al., 2015. Mercury Exposure in a Riverside Amazon Population, Brazil: A Study of the Ototoxicity of Methylmercury. *International Archives of Otorhinolaryngology*, 19(2), pp.135–140.
- Housley, G.D. et al., 1999. Expression of the P2X(2) receptor subunit of the ATP-gated ion channel in the cochlea: implications for sound transduction and auditory neurotransmission. *The Journal Of Neuroscience*, 19(19), pp.8377–8388.
- Huffman, R.F. & Henson, O.W.J., 1990. The descending auditory pathway and acousticomotor systems: connections with the inferior colliculus. *Brain research. Brain research reviews*, 15(3), pp.295–323.
- Husain, K. et al., 1996. 4-methylthiobenzoic acid protection against cisplatin nephrotoxicity: antioxidant system. *Fundamental and applied toxicology*, 32(2), pp.278–284.
- Huth, M. et al., 2015. Designer aminoglycosides prevent cochlear hair cell loss and hearing loss. *The Journal of Clinical Investigation*, 125(2), pp.583–592.
- Huth, M.E., Ricci, A.J. & Cheng, A.G., 2011. Mechanisms of Aminoglycoside Ototoxicity and Targets of Hair Cell Protection. *International Journal of Otolaryngology*, 2011, p.19.
- Iida, M. et al., 2019. Src inhibition attenuates polyglutamine-mediated neuromuscular degeneration in spinal and bulbar muscular atrophy. *Nature communications*, 10(1), p.4262.
- Imamura, S.-I. & Adams, J.C., 2003. Distribution of gentamicin in the guinea pig inner ear after local or systemic application. *Journal of the Association for Research in Otolaryngology*, 4(2), pp.176–195.
- Ishiyama, G. et al., 2006. Gentamicin ototoxicity: clinical features and the effect on the human vestibulo-ocular reflex. *Acta otolaryngologica*, 126(10), pp.1057–1061.
- Izumikawa, M. et al., 2005. Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. *Nature medicine*, 11(3), pp.271–276.
- Jamesdaniel, S., Rathinam, R. & Neumann, W.L., 2016. Targeting nitrative stress for attenuating cisplatin-induced downregulation of cochlear LIM domain only 4 and ototoxicity. *Redox biology*, 10, pp.257–265.
- Janak, P.H. & Tye, K.M., 2015. From circuits to behaviour in the amygdala. *Nature*, 517(7534), pp.284–292.
- Jayakody, D. et al., 2018. Impact of Aging on the Auditory System and Related Cognitive Functions: A Narrative Review. *Frontiers in neuroscience*, 12, p.125.

- Jen, H. et al., 2019. Transcriptomic and epigenetic regulation of hair cell regeneration in the mouse utricle and its potentiation by Atoh1. *eLife*, 8, p.e44328.
- Ji, N. et al., 2019. Selonsertib (GS-4997), an ASK1 inhibitor, antagonizes multidrug resistance in ABCB1- and ABCG2-overexpressing cancer cells. *Cancer letters*, 440-441, pp.82–93.
- Jia, H. et al., 2013. Molecular and cellular mechanisms of loss of residual hearing after cochlear implantation. *Annals of Otology, Rhinology & Laryngology*, 122(1), pp.33–39.
- Jiang, C. et al., 2016. Apoptosis signal-regulating kinase 1 mediates the inhibitory effect of hepatocyte nuclear factor-4 α on hepatocellular carcinoma. *Oncotarget*, 7(19), pp.27408–27421.
- Jiang, H. et al., 2006. Caspase-independent pathways of hair cell death induced by kanamycin in vivo. *Cell death and differentiation*, 13(1), pp.20–30.
- Jiang, H., Sha, S.-H. & Schacht, J., 2005. NF-kappaB pathway protects cochlear hair cells from aminoglycoside-induced ototoxicity. *Journal of Neuroscience Research*, 79(5), pp.644–651.
- Jones, J.E. & Corwin, J.T., 1993. Replacement of lateral line sensory organs during tail regeneration in salamanders: identification of progenitor cells and analysis of leukocyte activity. *The Journal Of Neuroscience*, 13(3), pp.1022–1034.
- Jones, L.A. et al., 2016. Cognitive mechanisms associated with auditory sensory gating. *Brain and cognition*, 102, pp.33–45.
- Bosher, S.K & Warren R.L., 1968. Observations on the electrochemistry of the cochlear endolymph of the rat: a quantitative study of its electrical potential and ionic composition as determined by means of flame spectrophotometry. *Proceedings of the Royal Society of London. Series B. Biological Sciences*, 171(1023), pp.227–247.
- Kachar, B. et al., 2000. High-resolution structure of hair-cell tip links. *Proceedings of the National Academy of Sciences USA*, 97(24), pp.13336–13341.
- Kadowaki, H. et al., 2005. Amyloid beta induces neuronal cell death through ROS-mediated ASK1 activation. *Cell death and differentiation*, 12(1), pp.19–24.
- Kagan, V.E. et al., 2005. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nature Chemical Biol.* 1(4), pp.223–232.
- Kalinec, G. et al., 2016. HEI-OC1 cells as a model for investigating drug cytotoxicity. *Hearing research*, 335, pp.105–117.
- Kalinec, G. et al., 2005. Pivotal role of Harakiri in the induction and prevention of gentamicin-induced hearing loss. *Proceedings of the National Academy of Sciences USA*, 102(44), pp.16019–16024.

- Kamiyama, M. et al., 2017. ASK1 facilitates tumor metastasis through phosphorylation of an ADP receptor P2Y₁₂ in platelets. *Cell death and differentiation*, 24(12), pp.2066–2076.
- Kammen-Jolly, K. et al., 2001. Connexin 26 in human fetal development of the inner ear. *Hearing research*, 160(1-2), pp.15–21.
- Kamogashira, T., Fujimoto, C. & Yamasoba, T., 2015. Reactive Oxygen Species, Apoptosis, and Mitochondrial Dysfunction in Hearing Loss. *BioMed Research International*, 2015, p.617207.
- Kandel, E.R. et al., 2013. Principles of neural science. [electronic resource]. *McGraw-Hill's Access Medicine*.
- Karabulut, B. et al., 2019. Effect of Hyperbilirubinemia on Medial Olivocochlear System in Newborns. *The journal of international advanced otology*.
- Kaur, T. et al., 2015. Macrophage recruitment and epithelial repair following hair cell injury in the mouse utricle. *Frontiers in Cellular Neuroscience*, 9, p.150.
- Kawamoto, K. et al., 2003. Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. *The Journal Of Neuroscience*, 23(11), pp.4395–4400.
- Kelley, M.W., Talreja, D.R. & Corwin, J.T., 1995. Replacement of hair cells after laser microbeam irradiation in cultured organs of corti from embryonic and neonatal mice. *The Journal Of Neuroscience*, 15(4), pp.3013–3026.
- Kelly, J.B. et al., 1998. Projections from the superior olive and lateral lemniscus to tonotopic regions of the rat's inferior colliculus. *Hearing research*, 116(1-2), pp.43–54.
- Kelly, M.C. et al., 2012. Atoh1 directs the formation of sensory mosaics and induces cell proliferation in the postnatal mammalian cochlea in vivo. *The Journal Of Neuroscience*, 32(19), pp.6699–6710.
- Kiang, N.Y. et al., 1982. Hair-cell innervation by spiral ganglion cells in adult cats. *Science*, 217(4555), pp.175–177.
- Kim, J.-B. et al., 2009. Antioxidant and anti-apoptotic effect of melatonin on the vestibular hair cells of rat utricles. *Clinical and experimental otorhinolaryngology*, 2(1), pp.6–12.
- Kimura, A. et al., 2007. Efferent connections of the ventral auditory area in the rat cortex: implications for auditory processing related to emotion. *The European journal of neuroscience*, 25(9), pp.2819–2834.
- King, A.J., Teki, S. & Willmore, B.D.B., 2018. Recent advances in understanding the auditory cortex. *F1000Research*, 7.
- Kitasato, I. et al., 1990. Comparative ototoxicity of ribostamycin, dactimicin, dibekacin, kanamycin, amikacin, tobramycin, gentamicin, sisomicin and netilmicin in the inner ear of guinea pigs. *Chemotherapy*, 36(2), pp.155–168.

- Knight, K.R.G., Kraemer, D.F. & Neuwelt, E.A., 2005. Ototoxicity in children receiving platinum chemotherapy: underestimating a commonly occurring toxicity that may influence academic and social development. *Journal of clinical oncology*, 23(34), pp.8588–8596.
- Knudson, I.M. & Melcher, J.R., 2016. Elevated Acoustic Startle Responses in Humans: Relationship to Reduced Loudness Discomfort Level, but not Self-Report of Hyperacusis. *Journal of the Association for Research in Otolaryngology*, 17(3), pp.223–235.
- Kocyigit, I. et al., 2015. Preventing amikacin related ototoxicity with N-acetylcysteine in patients undergoing peritoneal dialysis. *European Archives of Otorhinolaryngology*, 272(10), pp.2611–2620.
- Koehler, K.R. et al., 2017. Generation of inner ear organoids containing functional hair cells from human pluripotent stem cells. *Nature biotechnology*, 35(6), pp.583–589.
- Kofler, M. et al., 2001. Influence of gender on auditory startle responses. *Brain Research*, 921(1-2), pp.206–210.
- Kopelman, J. et al., 1988. Ototoxicity of high-dose cisplatin by bolus administration in patients with advanced cancers and normal hearing. *Laryngoscope*, 98(8 Pt 1), pp.858–864.
- Kopke, R. et al., 2015. Efficacy and safety of N-acetylcysteine in prevention of noise induced hearing loss: a randomized clinical trial. *Hearing research*, 323, pp.40–50.
- Kopke, R.D. et al., 1997. Use of organotypic cultures of Corti's organ to study the protective effects of antioxidant molecules on cisplatin-induced damage of auditory hair cells. *The American journal of otology*, 18(5), pp.559–571.
- Kranzer, K. et al., 2015. A systematic review and meta-analysis of the efficacy and safety of N-acetylcysteine in preventing aminoglycoside-induced ototoxicity: implications for the treatment of multidrug-resistant TB. *Thorax*, 70(11), pp.1070–1077.
- Kruger, M. et al., 2016. Natural Bizbenzoquinoline Derivatives Protect Zebrafish Lateral Line Sensory Hair Cells from Aminoglycoside Toxicity. *Frontiers in Cellular Neuroscience*, 10, p.e22347.
- Kujawa, S.G. & Liberman, M.C., 2009. Adding insult to injury: cochlear nerve degeneration after “temporary” noise-induced hearing loss. *The Journal Of Neuroscience*, 29(45), pp.14077–14085.
- Kumakura, K. et al., 2010. Hyperactivity in novel environment with increased dopamine and impaired novelty preference in apoptosis signal-regulating kinase 1 (ASK1)-deficient mice. *Neuroscience research*, 66(3), pp.313–320.
- Kumar, A. & Barman, A., 2002. Effect of efferent-induced changes on acoustical reflex. *International Journal of Audiology*, 41(2), pp.144–147.

- Kurioka, T. et al., 2015. ERK2 mediates inner hair cell survival and decreases susceptibility to noise-induced hearing loss. *Scientific reports*, 5. p16839
- Lahne, M. & Gale, J.E., 2008. Damage-induced activation of ERK1/2 in cochlear supporting cells is a hair cell death-promoting signal that depends on extracellular ATP and calcium. *The Journal Of Neuroscience*, 28(19), pp.4918–4928.
- Laird, P.W. et al., 1991. Simplified mammalian DNA isolation procedure. *Nucleic Acids Research*, 19(15), p.4293.
- Lauer, A.M. et al., 2013. Morphological characterization of bushy cells and their inputs in the laboratory mouse (*Mus musculus*) anteroventral cochlear nucleus. *PLoS ONE*, 8(8), p.e73308.
- Le Duc, J., Fournier, P. & Hébert, S., 2016. Modulation of Prepulse Inhibition and Startle Reflex by Emotions: A Comparison between Young and Older Adults. *Frontiers in aging neuroscience*, 8, p.1.
- Leclercq, R. et al., 2013. EUCAST expert rules in antimicrobial susceptibility testing. *Clinical microbiology and infection* 19(2), pp.141–160.
- Lee, J. et al., 2004. Signaling pathway for apoptosis of vestibular hair cells of mice due to aminoglycosides. *Acta otolaryngologica*, (551), pp.69–74.
- Lee, J. et al., 2013. Different uptake of gentamicin through TRPV1 and TRPV4 channels determines cochlear hair cell vulnerability. *Experimental & molecular medicine*, 45, p.e12.
- Lee, J. et al., 2010. Epicatechin protects the auditory organ by attenuating cisplatin-induced ototoxicity through inhibition of ERK. *Toxicology Letters*, 199(3), pp.308–316.
- Leitner, M.G., Halaszovich, C.R. & Oliver, D., 2011. Aminoglycosides inhibit KCNQ4 channels in cochlear outer hair cells via depletion of phosphatidylinositol (4,5)bisphosphate. *Molecular pharmacology*, 79(1), pp.51–60.
- LeMasurier, M. & Gillespie, P.G., 2005. Hair-cell mechanotransduction and cochlear amplification. *Neuron*, 48(3), pp.403–415.
- Lenoir, M. et al., 1999. Morphological and molecular changes in the inner hair cell region of the rat cochlea after amikacin treatment. *Journal of neurocytology*, 28(10), pp.925–937.
- Li, C. et al., 2014. Hearing Impairment Associated With Depression in US Adults, National Health and Nutrition Examination Survey 2005-2010 *JAMA Otolaryngology–Head & Neck Surgery*, 140(4), pp.293–302.
- Li, Y. et al., 2011. Co-administration of cisplatin and furosemide causes rapid and massive loss of cochlear hair cells in mice. *Neurotoxicity research*, 20(4), pp.307–319.

- Liberman, M.C., 1982. Single-neuron labeling in the cat auditory nerve. *Science*, 216(4551), pp.1239–1241.
- Liberman, M.C., 2015. Video Tutorial: Cochlear Dissection. Available at: www.masseyeandear.org/research/otolaryngology/investigators/laboratories/eaton-peabody-laboratories/epl-histology-resources/video-tutorial-for-cochlear-dissection or <https://vimeo.com/144531710> [Accessed May 26, 2019].
- Liberman, M.C. et al., 2002. Prestin is required for electromotility of the outer hair cell and for the cochlear amplifier. *Nature*, 419(6904), pp.300–304.
- Liles, J.T. et al., 2018. ASK1 contributes to fibrosis and dysfunction in models of kidney disease. *The Journal of Clinical Investigation*, 128(10), pp.4485–4500.
- Lin, F.R. & Ferrucci, L., 2012. Hearing loss and falls among older adults in the United States. *Archives of internal medicine*, 172(4), pp.369–371.
- Lin, F.R. et al., 2013. Hearing loss and cognitive decline in older adults. *JAMA Internal Medicine*, 173(4), pp.293–299.
- Lin, F.R. et al., 2011. Hearing Loss and Incident Dementia. *Archives of Neurology*, 68(2), pp.214–220.
- Lindsey, A.E. & Townes-Anderson, E., 2018. Antibiotics Reduce Retinal Cell Survival In Vitro. *Neurotoxicity research*, 33(4), pp.781–789.
- Liu, C., Glowatzki, E. & Fuchs, P.A., 2015. Unmyelinated type II afferent neurons report cochlear damage. *Proceedings of the National Academy of Sciences USA*, 112(47), pp.14723–14727.
- Liu, W. et al., 1998. Caspase inhibitors prevent cisplatin-induced apoptosis of auditory sensory cells. *Neuroreport*, 9(11), pp.2609–2614.
- Loomba, R. et al., 2018. The ASK1 inhibitor selonsertib in patients with nonalcoholic steatohepatitis: A randomized, phase 2 trial. *Hepatology*, 67(2), pp.549–559.
- Lopez, C., 2013. A neuroscientific account of how vestibular disorders impair bodily self-consciousness. *Frontiers in integrative neuroscience*, 7, pp.91–91.
- Lopez-Poveda, E.A., 2018. Olivocochlear Efferents in Animals and Humans: From Anatomy to Clinical Relevance. *Frontiers in Neurology*, 9, p.197.
- Lunney, J.K., 2007. Advances in swine biomedical model genomics. *International journal of biological sciences*, 3(3), pp.179–184.
- Lush, M.E. et al., 2019. scRNA-Seq reveals distinct stem cell populations that drive hair cell regeneration after loss of Fgf and Notch signaling. *eLife*, 8.
- Ma, F.Y., Liu, J. & Nikolic-Paterson, D.J., 2009. The role of stress-activated protein kinase signaling in renal pathophysiology. *Brazilian journal of medical and biological research*, 42(1), pp.29–37.

- Ma, F.Y., Tesch, G.H. & Nikolic-Paterson, D.J., 2014. ASK1/p38 signaling in renal tubular epithelial cells promotes renal fibrosis in the mouse obstructed kidney. *The American Journal of Physiology: Renal Physiology*, 307(11), pp.F1263–73.
- Maass, J.C. et al., 2015. Changes in the regulation of the Notch signaling pathway are temporally correlated with regenerative failure in the mouse cochlea. *Frontiers in Cellular Neuroscience*, 9, p.110.
- Maass, J.C. et al., 2016. Transcriptomic Analysis of Mouse Cochlear Supporting Cell Maturation Reveals Large-Scale Changes in Notch Responsiveness Prior to the Onset of Hearing. *PLoS ONE*, 11(12), p.e0167286.
- Mackenzie, S.M., Raible, D.W. & Matsui, J.I., 2012. Proliferative Regeneration of Zebrafish Lateral Line Hair Cells after Different Ototoxic Insults. *PLoS ONE*, 7(10), pp.1–8.
- Maison, S.F., Usubuchi, H. & Liberman, M.C., 2013. Efferent Feedback Minimizes Cochlear Neuropathy from Moderate Noise Exposure. *The Journal Of Neuroscience*, 33(13), p.5542.
- Maqbool, M. et al., 2015. Screening for Hearing Impairment in High Risk Neonates: A Hospital Based Study. *Journal of clinical and diagnostic research* 9(6), pp.SC18–SC21.
- Marcotti, W., van Netten, S.M. & Kros, C.J., 2005. The aminoglycoside antibiotic dihydrostreptomycin rapidly enters mouse outer hair cells through the mechano-electrical transducer channels. *The Journal of physiology*, 567(Pt 2), pp.505–521.
- Mardassi, A. et al., 2016. Acute acoustic trauma: how to manage and how to prevent? *La Tunisie medicale*, 94(11), p.664.
- Marullo, R. et al., 2013. Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions. *PLoS ONE*, 8(11), p.e81162.
- Marx, V., 2019. A dream of single-cell proteomics. *Nature methods*.
- Matsui, J.I. et al., 2003. Caspase inhibitors promote vestibular hair cell survival and function after aminoglycoside treatment in vivo. *The Journal Of Neuroscience*, 23(14), pp.6111–6122.
- Matsui, J.I., Gale, J.E. & Warchol, M.E., 2004. Critical signaling events during the aminoglycoside-induced death of sensory hair cells in vitro. *Journal of neurobiology*, 61(2), pp.250–266.
- Matsui, J.I., Ogilvie, J.M. & Warchol, M.E., 2002. Inhibition of caspases prevents ototoxic and ongoing hair cell death. *The Journal Of Neuroscience*, 22(4), pp.1218–1227.
- Mattappalil, A. & Mergenhagen, K.A., 2014. Neurotoxicity with antimicrobials in the elderly: a review. *Clinical therapeutics*, 36(11), pp.1489–1511.e4.
- Mattei, C. et al., 2019. Generation of Vestibular Tissue-Like Organoids From Human

- Pluripotent Stem Cells Using the Rotary Cell Culture System. *Frontiers in cell and developmental biology*, 7, pp.25–25.
- McFadden, S.L. et al., 2003. M40403, a superoxide dismutase mimetic, protects cochlear hair cells from gentamicin, but not cisplatin toxicity. *Toxicology and applied pharmacology*, 186(1), pp.46–54.
- McGovern, M.M. et al., 2019. Multiple supporting cell subtypes are capable of spontaneous hair cell regeneration in the neonatal mouse cochlea. *Development*, 146(4).
- McGovern, M.M. et al., 2018. Spontaneous Hair Cell Regeneration Is Prevented by Increased Notch Signaling in Supporting Cells. *Frontiers in Cellular Neuroscience*, 12, p.120.
- Mehraei, G. et al., 2016. Auditory Brainstem Response Latency in Noise as a Marker of Cochlear Synaptopathy. *The Journal Of Neuroscience*, 36(13), pp.3755–3764.
- Melki, S.J. et al., 2010. Pharmacological protection of hearing loss in the mouse model of endolymphatic hydrops. *Laryngoscope*, 120(8), pp.1637–1645.
- Mellado Lagarde, M.M. et al., 2014. Spontaneous regeneration of cochlear supporting cells after neonatal ablation ensures hearing in the adult mouse. *Proceedings of the National Academy of Sciences USA*, 111(47), pp.16919–16924.
- Meltzer, N. & Ryugo, D., 2006. Projections from auditory cortex to cochlear nucleus: A comparative analysis of rat and mouse. *The anatomical record*. 288(4), pp.397–408.
- Mener, D.J. et al., 2013. Hearing loss and depression in older adults. *Journal of the American Geriatrics Society*, 61(9), pp.1627–1629.
- Meurens, F. et al., 2012. The pig: a model for human infectious diseases. *Trends in microbiology*, 20(1), pp.50–57.
- Meyers, J.R. et al., 2003. Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. *The Journal Of Neuroscience*, 23(10), pp.4054–4065.
- Milinkeviciute, G., Muniak, M.A. & Ryugo, D.K., 2017. Descending projections from the inferior colliculus to the dorsal cochlear nucleus are excitatory. *The Journal of comparative neurology*, 525(4), pp.773–793.
- Min-Xin, G., 2006. Mitochondrial DNA Mutations Associated with Aminoglycoside Ototoxicity. *Journal of otology*, 1(2), pp.65–75.
- Mishra, S.K. & Biswal, M., 2019. Neural Encoding of Amplitude Modulations in the Human Efferent System. *Journal of the Association for Research in Otolaryngology*, 20(4), pp.383–393.

- Moller, A.R., Jannetta, P.J. & Sekhar, L.N., 1988. Contributions from the auditory nerve to the brain-stem auditory evoked potentials (BAEPs): results of intracranial recording in man. *Electroencephalography and clinical neurophysiology*, 71(3), pp.198–211.
- Momin, S.R. et al., 2011. Hearing preservation in Guinea pigs with long-standing endolymphatic hydrops. *Otology & neurotology*, 32(9), pp.1583–1589.
- Morris, B.J. & Pratt, J.A., 2014. Novel treatment strategies for schizophrenia from improved understanding of genetic risk. *Clinical genetics*, 86(5), pp.401–411.
- Muller-Pebody, B. et al., 2011. Empirical treatment of neonatal sepsis: are the current guidelines adequate? *Archives of disease in childhood. Fetal and neonatal edition*, 96(1), pp.F4–8.
- Muniak, M.A. & Ryugo, D.K., 2014. Tonotopic organization of vertical cells in the dorsal cochlear nucleus of the CBA/J mouse. *The Journal of comparative neurology*, 522(4), pp.937–949.
- Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. *Biochemical Journal*, 417(Pt 1), pp.1–13.
- Naeem, M., Rahimnadjad, N.A. & Behnoud, F., 2010. Can aspirin protect or at least attenuate gentamicin ototoxicity in humans? *Saudi medical journal*, 31(5), pp.587–588.
- Nakagawa, H. et al., 2011. Apoptosis signal-regulating kinase 1 inhibits hepatocarcinogenesis by controlling the tumor-suppressing function of stress-activated mitogen-activated protein kinase. *Hepatology*, 54(1), pp.185–195.
- Nandalal, P. & Somashekar, R.K., 2007. Prevalence of Staphylococcus aureus and Pseudomonas aeruginosa in indoor air flora of a district hospital, Mandya, Karnataka. *Journal of environmental biology*, 28(2), pp.197–200.
- Narayanan, P. et al., 2015. Length regulation of mechanosensitive stereocilia depends on very slow actin dynamics and filament-severing proteins. *Nature communications*, 6, p.6855.
- Nayagam, B.A., Muniak, M.A. & Ryugo, D.K., 2011. The spiral ganglion: connecting the peripheral and central auditory systems. *Hearing research*, 278(1-2), pp.2–20.
- Neben, N. et al., 2018. Outcome evaluation on cochlear implant users with residual hearing. *Cochlear implants international*, 19(2), pp.88–99.
- NHMRC, 2013. *Australian code for the care and use of animals for scientific purposes*. 8 ed., Available at: www.nhmrc.gov.au/about-us/publications/australian-code-care-and-use-animals-scientific-purposes.
- Nicholas, B.D. et al., 2017. Protein Synthesis Inhibition and Activation of the c-Jun N-Terminal Kinase Are Potential Contributors to Cisplatin Ototoxicity. *Frontiers in Cellular Neuroscience*, 11, p.303.

- Nitz, A. et al., 2013. Prospective evaluation of cisplatin- and carboplatin-mediated ototoxicity in paediatric and adult soft tissue and osteosarcoma patients. *Oncology Letters*, 5(1), pp.311–315.
- Noack, V. et al., 2017. An Antioxidant Screen Identifies Candidates for Protection of Cochlear Hair Cells from Gentamicin Toxicity. *Frontiers in Cellular Neuroscience*, 11, pp.242–242.
- Obsil, T. & Obsilova, V., 2017. Structural aspects of protein kinase ASK1 regulation. *Advances in Biological Regulation*, 66, pp.31–36.
- Oesterle, E.C. et al., 2008. Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear. *Journal of the Association for Research in Otolaryngology*, 9(1), pp.65–89.
- Ogier, J.M., 2013. *Looper*. A genetic and phenotypic characterisation of a new mouse model for CHARGE syndrome. *University of Melbourne Honours Thesis*.
- Ogier, J.M. et al., 2018. An intronic mutation in Chd7 creates a cryptic splice site, causing aberrant splicing in a mouse model of CHARGE syndrome. *Scientific reports*, 8(1), p.5482.
- Ogier, J.M. et al., 2014. CHD7 deficiency in “Looper,” a new mouse model of CHARGE syndrome, results in ossicle malformation, otosclerosis and hearing impairment. *PLoS ONE*, 9(5), p.e97559.
- Ogier, J.M., Burt, R.A., et al., 2019. Organotypic Culture of Neonatal Murine Inner Ear Explants. *Frontiers in Cellular Neuroscience*, 13, p.895.
- Ogier, J.M., Lockhart, P.J. & Burt, R.A., 2019. Intravenously delivered aminoglycoside antibiotics, tobramycin and amikacin, are not ototoxic in mice. *Hearing research*, p.107870.
- Oishi, N. et al., 2015. XBP1 mitigates aminoglycoside-induced endoplasmic reticulum stress and neuronal cell death. *Cell Death and Disease* p.e1763.
- Oishi, N., Kendall, A. & Schacht, J., 2014. Metformin protects against gentamicin-induced hair cell death in vitro but not ototoxicity in vivo. *Neuroscience letters*, 583, pp.65–69.
- Ono, M. & Ito, T., 2015. Functional organization of the mammalian auditory midbrain. *The journal of physiological sciences*. 65(6), pp.499–506.
- Owens, K.N. et al., 2009. Response of mechanosensory hair cells of the zebrafish lateral line to aminoglycosides reveals distinct cell death pathways. *Hearing research*, 253(1-2), pp.32–41.
- Pannese, A., Grandjean, D. & Fröhholz, S., 2015. Subcortical processing in auditory communication. *Hearing research*, 328, pp.67–77.
- Parker, M., Brégeaud, A. & Edge, A.S.B., 2010. Primary culture and plasmid electroporation of the murine organ of Corti. *Journal of Visualized Experiments* (36).

- Peelle, J.E. et al., 2011. Hearing loss in older adults affects neural systems supporting speech comprehension. *The Journal Of Neuroscience*, 31(35), pp.12638–12643.
- Perez, P. & Bao, J., 2011. Why do hair cells and spiral ganglion neurons in the cochlea die during aging? *Aging and Disease*, 2(3), pp.231–241.
- Petitpre, C. et al., 2018. Neuronal heterogeneity and stereotyped connectivity in the auditory afferent system. *Nature communications*, 9(1), p.3691.
- Pienkowski, M., Adunka, O.F. & Lichtenhan, J.T., 2018. Editorial: New Advances in Electrocochleography for Clinical and Basic Investigation. *Frontiers in neuroscience*, 12, p.e1.
- Pirvola, U. et al., 2000. Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. *The Journal Of Neuroscience*, 20(1), pp.43–50.
- Plontke, S.K., 2015. A Three-part, Multicenter, Open Label, Single Dose Study to Assess the Safety, Tolerability, and Efficacy of Intra Labyrinthine (IL) CGF166 in Patients With Severe-to-profound Hearing Loss. 272(6), pp.1301–1303. Available at: clinicaltrials.gov/ct2/show/NCT02132130.
- Poli, E. & Angrilli, A., 2015. Greater general startle reflex is associated with greater anxiety levels: a correlational study on 111 young women. *Frontiers in behavioral neuroscience*, 9, p.10.
- Poonual, W. et al., 2016. Risk factors for hearing loss in infants under universal hearing screening program in Northern Thailand. *Journal of multidisciplinary healthcare*, 9, pp.1–5.
- Popper, A.N. & Hoxter, B., 1984. Growth of a fish ear: 1. Quantitative analysis of hair cell and ganglion cell proliferation. *Hearing research*, 15(2), pp.133–142.
- Prezant, T.R. et al., 1993. Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. *Nature Genetics*, 4(3), pp.289–294.
- Priuska, E.M. & Schacht, J., 1995. Formation of free radicals by gentamicin and iron and evidence for an iron/gentamicin complex. *Biochemical pharmacology*, 50(11), pp.1749–1752.
- Purves, D. et al. 2001a. Hair Cells and the Mechanoelectrical Transduction of Sound Waves. In *Neuroscience. 2nd edition*. Sinauer Associates. Available at: <https://www.ncbi.nlm.nih.gov/books/NBK10867/>.
- Purves, D. et al 2001b. *Neuroscience: The External Ear*, Sinauer Associates. Available at: <https://www.ncbi.nlm.nih.gov/books/NBK10908/>.
- Purves, D. et al. 2001c. *Neuroscience: The Inner Ear*, Sinauer Associates. Available at: <https://www.ncbi.nlm.nih.gov/books/NBK10946/>.
- Qin, L. et al., 2015. ROS-p53-cyclophilin-D signaling mediates salinomycin-induced glioma cell necrosis. *Journal of experimental & clinical cancer research* 34, p.57.

- Qiu, X. & Muller, U., 2018. Mechanically Gated Ion Channels in Mammalian Hair Cells. *Frontiers in Cellular Neuroscience*, 12, pp.100–100.
- Ramirez-Camacho, R. et al., 2006. Central role of supporting cells in cochlear homeostasis and pathology. *Medical hypotheses*, 67(3), pp.550–555.
- Ratjen, F., Brockhaus, F. & Angyalosi, G., 2009. Aminoglycoside therapy against *Pseudomonas aeruginosa* in cystic fibrosis: a review. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, 8(6), pp.361–369.
- Rattay, F. & Danner, S.M., 2014. Peak I of the human auditory brainstem response results from the somatic regions of type I spiral ganglion cells: evidence from computer modeling. *Hearing research*, 315(100), pp.67–79.
- Reddy, P.H. & Beal, M.F., 2008. Amyloid beta, mitochondrial dysfunction and synaptic damage: implications for cognitive decline in aging and Alzheimer's disease. *Trends in molecular medicine*, 14(2), pp.45–53.
- Reid, M.A., Flores-Otero, J. & Davis, R.L., 2004. Firing patterns of type II spiral ganglion neurons in vitro. *The Journal Of Neuroscience*, 24(3), pp.733–742.
- Reller, L.B. et al., 2009. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clinical infectious disease* 49(11), pp.1749–1755.
- Ren, H. et al., 2016. DAPT mediates atoh1 expression to induce hair cell-like cells. *American journal of translational research*, 8(2), pp.634–643.
- Ricci, A.J., Crawford, A.C. & Fettiplace, R., 2003. Tonotopic variation in the conductance of the hair cell mechanotransducer channel. *Neuron*, 40(5), pp.983–990.
- Richardson, G.M., Lannigan, J. & Macara, I.G., 2015. Does FACS perturb gene expression? *Cytometry. Part A : the journal of the International Society for Analytical Cytology*, 87(2), pp.166–175.
- Rieffe, C., 2012. Awareness and regulation of emotions in deaf children. *The British journal of developmental psychology*, 30(Pt 4), pp.477–492.
- Riga, M.G. et al., 2013. Transtympanic injections of N-acetylcysteine for the prevention of cisplatin-induced ototoxicity: a feasible method with promising efficacy. *American journal of clinical oncology*, 36(1), pp.1–6.
- Rios Garcia, M. et al., 2017. Acetyl-CoA Carboxylase 1-Dependent Protein Acetylation Controls Breast Cancer Metastasis and Recurrence. *Cell metabolism*, 26(6), pp.842–855.e5.
- Rosen, J.B. et al., 1991. A direct projection from the central nucleus of the amygdala to the acoustic startle pathway: anterograde and retrograde tracing studies. *Behavioral neuroscience*, 105(6), pp.817–825.

- Ruan, Q. et al., 2014. Topographic and quantitative evaluation of gentamicin-induced damage to peripheral innervation of mouse cochleae. *Neurotoxicology*, 40, p.86–96.
- Rybak, L.P. & Ramkumar, V., 2007. Ototoxicity. *Kidney international*, 72(8), pp.931–935.
- Rybak, L.P. et al., 2009. Cisplatin Ototoxicity and Protection: Clinical and Experimental Studies. *The Tohoku journal of experimental medicine*, 219(3), pp.177–186.
- Rybak, L.P. et al., 2007. Mechanisms of cisplatin-induced ototoxicity and prevention. *Hearing research*, 226(1-2), pp.157–167.
- Rybak, L.P., Mukherjea, D. & Ramkumar, V., 2019. Mechanisms of Cisplatin-Induced Ototoxicity and Prevention. *Seminars in hearing*, 40(2), pp.197–204.
- Rybak, L.P., Talaska, A.E. & Schacht, J., 2008. Drug-Induced Hearing Loss. *Springer Handbook of Auditory Research*, 31, pp.219–256.
- Ryugo, D.K., Haenggeli, C.-A. & Doucet, J.R., 2003. Multimodal inputs to the granule cell domain of the cochlear nucleus. *Experimental brain research*, 153(4), pp.477–485.
- Rzadzinska, A.K. et al., 2004. An actin molecular treadmill and myosins maintain stereocilia functional architecture and self-renewal. *The Journal of cell biology*, 164(6), pp.887–897.
- Salganik, R.I., 2001. The benefits and hazards of antioxidants: controlling apoptosis and other protective mechanisms in cancer patients and the human population. *Journal of the American College of Nutrition*, 20(5 Suppl), pp.464S–472S–discussion 473S–475S.
- Samarajeewa, A., Jacques, B.E. & Dabdoub, A., 2019. Therapeutic Potential of Wnt and Notch Signaling and Epigenetic Regulation in Mammalian Sensory Hair Cell Regeneration. *Molecular therapy*, 27(5), pp.904–911.
- Sanmartin-Suarez, C. et al., 2011. Antioxidant properties of dimethyl sulfoxide and its viability as a solvent in the evaluation of neuroprotective antioxidants. *Journal of pharmacological and toxicological methods*, 63(2), pp.209–215.
- Santi, P.A. et al., 1982. Kanamycin and bumetanide ototoxicity: anatomical, physiological and behavioral correlates. *Hearing research*, 7(3), pp.261–279.
- Schindelin, J. et al., 2012. Fiji: an open-source platform for biological-image analysis. *Nature methods*, 9(7), pp.676–682.
- Schuster, S. et al., 2017. Apoptosis signal-regulating kinase 1 (ASK1) inhibition reduces liver fibrosis and apoptosis in a NLRP3 mutant model of NASH. *Journal of Hepatology*, 66(1), pp.S608–S609.
- Seddon, J.A. et al., 2012. Hearing loss in patients on treatment for drug-resistant tuberculosis. *The European respiratory journal*, 40(5), pp.1277–1286.

- Segal, J.A. et al., 1999. Aminoglycoside neurotoxicity involves NMDA receptor activation. *Brain Research*, 815(2), pp.270–277.
- Selimoglu, E., 2007. Aminoglycoside-induced ototoxicity. *Current pharmaceutical design*, 13(1), pp.119–126.
- Sha, S.-H., Qiu, J.-H. & Schacht, J., 2006. Aspirin to Prevent Gentamicin-Induced Hearing Loss. *New England Journal of Medicine*, 354(17), pp.1856–1857.
- Sha, S.H. & Schacht, J., 2000. Antioxidants attenuate gentamicin-induced free radical formation in vitro and ototoxicity in vivo: D-methionine is a potential protectant. *Hearing research*, 142(1-2), pp.34–40.
- Sha, S.H. & Schacht, J., 1997. Prevention of aminoglycoside-induced hearing loss. *The Keio journal of medicine*, 46(3), pp.115–119.
- Sha, S.H. & Schacht, J., 1999a. Salicylate attenuates gentamicin-induced ototoxicity. *Laboratory investigation*, 79(7), pp.807–813.
- Sha, S.H. & Schacht, J., 1999b. Stimulation of free radical formation by aminoglycoside antibiotics. *Hearing research*, 128(1-2), pp.112–118.
- Sha, S.H. et al., 2001. Differential vulnerability of basal and apical hair cells is based on intrinsic susceptibility to free radicals. *Hearing research*, 155(1-2), pp.1–8.
- Shimizu, A. et al., 2003. Calpain and caspase inhibitors protect vestibular sensory cells from gentamicin ototoxicity. *Acta otolaryngologica*, 123(4), pp.459–465.
- Shneiderman, A. et al., 1999. Input-output relationships of the dorsal nucleus of the lateral lemniscus: possible substrate for the processing of dynamic spatial cues. *The Journal of comparative neurology*, 410(2), pp.265–276.
- Simmons, D.D. et al., 2010. Oncomodulin identifies different hair cell types in the mammalian inner ear. *The Journal of comparative neurology*, 518(18), pp.3785–3802.
- Sininger, Y.S., Abdala, C. & Cone-Wesson, B., 1997. Auditory threshold sensitivity of the human neonate as measured by the auditory brainstem response. *Hearing research*, 104(1-2), pp.27–38.
- Siveke, I. et al., 2019. A Temporal Filter for Binaural Hearing Is Dynamically Adjusted by Sound Pressure Level. *Frontiers in neural circuits*, 13, pp.8–8.
- Smith, S.B. et al., 2017. Efferent modulation of pre-neural and neural distortion products. *Hearing research*, 356, pp.25–34.
- So, H. et al., 2007. Cisplatin cytotoxicity of auditory cells requires secretions of proinflammatory cytokines via activation of ERK and NF-kappaB. *Journal of the Association for Research in Otolaryngology*, 8(3), pp.338–355.

- Sobkowicz, H.M., August, B.K. & Slapnick, S.M., 2002. Influence of neurotrophins on the synaptogenesis of inner hair cells in the deaf Bronx waltzer (bv) mouse organ of Corti in culture. *International journal of developmental neuroscience*, 20(7), pp.537–554.
- Somdas, M.A. et al., 2015. N-acetylcysteine Prevents Gentamicin Ototoxicity in a Rat Model. *The journal of international advanced otology*, 11(1), pp.12–18.
- Song, J. et al., 2013. Apoptosis signal-regulating kinase 1 (ASK1) is linked to neural stem cell differentiation after ischemic brain injury. *Experimental & molecular medicine*, 45, p.e69.
- Specht, H. et al., 2019. High-throughput single-cell proteomics quantifies the emergence of macrophage heterogeneity. *bioRxiv*, p.665307.
- Spoendlin, H., 1985. Anatomy of cochlear innervation. *American journal of otolaryngology*, 6(6), pp.453–467.
- Spoendlin, H. & Schrott, A., 1989. Analysis of the human auditory nerve. *Hearing research*, 43(1), pp.25–38.
- Spuch, C., Ortolano, S. & Navarro, C., 2012. New insights in the amyloid-Beta interaction with mitochondria. *Journal of aging research*, 2012, pp.e324968.
- Stepanyan, R.S. et al., 2011. TRPA1-mediated accumulation of aminoglycosides in mouse cochlear outer hair cells. *Journal of the Association for Research in Otolaryngology*, 12(6), pp.729–740.
- Stephenson, S.E.M. et al., 2018. Generation and characterisation of a parkin-Pacrg knockout mouse line and a Pacrg knockout mouse line. *Scientific reports*, 8(1), p.7528.
- Steube, N. et al., 2016. Dependence of the Startle Response on Temporal and Spectral Characteristics of Acoustic Modulatory Influences in Rats and Gerbils. *Frontiers in behavioral neuroscience*, 10, p.e45123.
- Stevens, S.M. et al., 2015. The Mouse Round-window Approach for Ototoxic Agent Delivery: A Rapid and Reliable Technique for Inducing Cochlear Cell Degeneration. *Journal of Visualized Experiments* (105), p.53131.
- Steyger, P.S. et al., 1997. Calbindin and parvalbumin are early markers of non-mitotically regenerating hair cells in the bullfrog vestibular otolith organs. *International journal of developmental neuroscience*, 15(4-5), pp.417–432.
- Stone, L.S., 1937. Further experimental studies of the development of lateral-line sense organs in amphibians observed in living preparations. *Journal of Comparative Neurology*, 68(1), p.83.
- Stone, L.S., 1933. The development of lateral-line sense organs in amphibians observed in living and vital-stained preparations. *Journal of Comparative Neurology*, 57(3), p.507.

- Sugahara, K., Rubel, E.W. & Cunningham, L.L., 2006. JNK signaling in neomycin-induced vestibular hair cell death. *Hearing research*, 221(1-2), pp.128–135.
- Suthakar, K. & Ryugo, D.K., 2017. Descending projections from the inferior colliculus to medial olivocochlear efferents: Mice with normal hearing, early onset hearing loss, and congenital deafness. *Hearing research*, 343, pp.34–49.
- Swerdlow, N.R. et al., 1993. Men are more inhibited than women by weak prepulses. *Biological psychiatry*, 34(4), pp.253–260.
- Swerdlow, N.R. et al., 1999. Sex differences in sensorimotor gating of the human startle reflex: all smoke? *Psychopharmacology*, 146(2), pp.228–232.
- Tang, H. & Mayersohn, M., 2018. Porcine Prediction of Pharmacokinetic Parameters in People: A Pig in a Poke? *Drug metabolism and disposition: the biological fate of chemicals*, 46(11), pp.1712–1724.
- Tang, P. & Watson, G.M., 2015. Proteomic identification of hair cell repair proteins in the model sea anemone *Nematostella vectensis*. *Hearing research*, 327, pp.245–256.
- Tao, L. & Segil, N., 2015. Early transcriptional response to aminoglycoside antibiotic suggests alternate pathways leading to apoptosis in sensory hair cells in the mouse inner ear. *Frontiers in Cellular Neuroscience*, 9, pp.190–190.
- Taylor, R. & Forge, A., 2005. Hair cell regeneration in sensory epithelia from the inner ear of a urodele amphibian. *The Journal of comparative neurology*, 484(1), pp.105–120.
- Teng, J. et al., 2001. Synergistic effects of MAP2 and MAP1B knockout in neuronal migration, dendritic outgrowth, and microtubule organization. *The Journal of cell biology*, 155(1), pp.65–76.
- Terada, Y. et al., 2007. Important role of apoptosis signal-regulating kinase 1 in ischemic acute kidney injury. *Biochemical and biophysical research communications*, 364(4), pp.1043–1049.
- Terreros, G. & Delano, P.H., 2015. Corticofugal modulation of peripheral auditory responses. *Frontiers in Systems Neuroscience*, 9, p.134.
- Tesch, G.H. et al., 2015. ASK1 Inhibitor Halts Progression of Diabetic Nephropathy in *Nos3*-Deficient Mice. *Diabetes*, 64(11), pp.3903–3913.
- Tesch, G.H., Ma, F.Y. & Nikolic-Paterson, D.J., 2016. ASK1: a new therapeutic target for kidney disease. *American Journal of Physiology: Renal Physiology*, 311(2), pp.F373–F381.
- Thomas, E.D. & Raible, D.W., 2019. Distinct progenitor populations mediate regeneration in the zebrafish lateral line. *eLife*, 8, p.e43736.

- Tilney, L.G. & Saunders, J.C., 1983. Actin filaments, stereocilia, and hair cells of the bird cochlea. I. Length, number, width, and distribution of stereocilia of each hair cell are related to the position of the hair cell on the cochlea. *The Journal of cell biology*, 96(3), pp.807–821.
- Tilney, L.G. & Tilney, M.S., 1988. The actin filament content of hair cells of the bird cochlea is nearly constant even though the length, width, and number of stereocilia vary depending on the hair cell location. *The Journal of cell biology*, 107(6 Pt 2), pp.2563–2574.
- Tilney, L.G., Tilney, M.S. & DeRosier, D.J., 1992. Actin filaments, stereocilia, and hair cells: how cells count and measure. *Annual review of cell biology*, 8, pp.257–274.
- Tirko, N.N. & Ryugo, D.K., 2012. Synaptic plasticity in the medial superior olive of hearing, deaf, and cochlear-implanted cats. *The Journal of comparative neurology*, 520(10), pp.2202–2217.
- Tobin, M. et al., 2019. Stiffness and tension gradients of the hair cell's tip-link complex in the mammalian cochlea. *Elife*. ppe43473
- Tokgoz, B. et al., 2011. Protective effect of N-acetylcysteine from drug-induced ototoxicity in uraemic patients with CAPD peritonitis. *Nephrology Dialysis Transplantation*, 26(12), pp.4073–4078.
- Tokgoz, S.A. et al., 2012. Protective effects of vitamins E, B and C and L-carnitine in the prevention of cisplatin-induced ototoxicity in rats. *The Journal of laryngology and otology*, 126(5), pp.464–469.
- Ton, C. & Parng, C., 2005. The use of zebrafish for assessing ototoxic and otoprotective agents. *Hearing research*, 208(1-2), pp.79–88.
- Tran B.H.P. et al., 1983. Gentamicin persistence in rat endolymph and perilymph after a two-day constant infusion. *Antimicrobial Agents and Chemotherapy*, 23(2), pp.344–346.
- Treskes, M. et al., 1991. The chemical reactivity of the modulating agent WR2721 (ethiofos) and its main metabolites with the antitumor agents cisplatin and carboplatin. *Biochemical pharmacology*, 42(11), pp.2125–2130.
- Tsukano, H. et al., 2017. Reconsidering Tonotopic Maps in the Auditory Cortex and Lemniscal Auditory Thalamus in Mice. *Frontiers in neural circuits*, 11, pp.14–14.
- Tucci, D.L. & Rubel, E.W., 1990. Physiologic status of regenerated hair cells in the avian inner ear following aminoglycoside ototoxicity. *Otolaryngology head and neck surgery*, 103(3), pp.443–450.
- Tuncer, S. et al., 2018. Low dose dimethyl sulfoxide driven gross molecular changes have the potential to interfere with various cellular processes. *Scientific reports*, 8(1), p.14828.
- Vago, P., Humbert, G. & Lenoir, M., 1998. Amikacin intoxication induces apoptosis and cell proliferation in rat organ of Corti. *Neuroreport*, 9(3), pp.431–436.

- Vandebona, H. et al., 2009. Prevalence of mitochondrial 1555A-->G mutation in adults of European descent. *New England Journal of Medicine*, 360(6), pp.642–644.
- Verhulst, S. et al., 2016. Individual Differences in Auditory Brainstem Response Wave Characteristics: Relations to Different Aspects of Peripheral Hearing Loss. *Trends in Hearing*, 20, p.2331216516672186.
- Villanueva, C. & Kross, R.D., 2012. Antioxidant-induced stress. *International Journal of Molecular Sciences*, 13(2), pp.2091–2109.
- Vyas, P. et al., 2019. Characterization of transgenic mouse lines for labeling type I and type II afferent neurons in the cochlea. *Scientific reports*, 9(1), p.5549.
- Wallhagen, M.I., 2010. The stigma of hearing loss. *The Gerontologist*, 50(1), pp.66–75.
- Walters, B.J. et al., 2015. Pseudo-immortalization of postnatal cochlear progenitor cells yields a scalable cell line capable of transcriptionally regulating mature hair cell genes. *Scientific reports*, 5, p.17792.
- Wan, G., Corfas, G. & Stone, J.S., 2013. Inner ear supporting cells: rethinking the silent majority. *Seminars in cell and developmental biology*, 24(5), pp.448–459.
- Wang, J. et al., 2003. A peptide inhibitor of c-Jun N-terminal kinase protects against both aminoglycoside and acoustic trauma-induced auditory hair cell death and hearing loss. *The Journal Of Neuroscience*, 23(24), pp.8596–8607.
- Wang, J. et al., 2004. Caspase inhibitors, but not c-Jun NH₂-terminal kinase inhibitor treatment, prevent cisplatin-induced hearing loss. *Cancer research*, 64(24), pp.9217–9224.
- Wang, J. et al., 2007. Inhibition of the c-Jun N-terminal kinase-mediated mitochondrial cell death pathway restores auditory function in sound-exposed animals. *Molecular pharmacology*, 71(3), pp.654–666.
- Wang, Q. & Steyger, P.S., 2009. Trafficking of systemic fluorescent gentamicin into the cochlea and hair cells. *Journal of the Association for Research in Otolaryngology*, 10(2), pp.205–219.
- Warady, B.A. et al., 1993. Aminoglycoside ototoxicity in pediatric patients receiving long-term peritoneal dialysis. *Pediatric nephrology*, 7(2), pp.178–181.
- Warrier, V. et al., 2018. Genome-wide meta-analysis of cognitive empathy: heritability, and correlates with sex, neuropsychiatric conditions and cognition. *Molecular psychiatry*, 23(6), pp.1402–1409.
- Wayne, R.V. & Johnsrude, I.S., 2015. A review of causal mechanisms underlying the link between age-related hearing loss and cognitive decline. *Ageing Research Reviews*, 23(Pt B), pp.154–166.

- Wei, X. et al., 2005. Minocycline prevents gentamicin-induced ototoxicity by inhibiting p38 MAP kinase phosphorylation and caspase 3 activation. *Neuroscience*, 131(2), pp.513–521.
- Weisz, C., Glowatzki, E. & Fuchs, P., 2009. The postsynaptic function of type II cochlear afferents. *Nature*, 461(7267), pp.1126–1129.
- Welsh, M.J. et al., 2009. Development of a porcine model of cystic fibrosis. *Transactions of the American Clinical and Climatological Association*, 120, pp.149–162.
- Wersall, J., Lundquist, P.-G. & Björkroth, B., 1969. Ototoxicity of Gentamicin. *The Journal of infectious diseases*, 119(4-5), pp.410–416.
- WHO, 2018. Addressing the rising prevalence of hearing loss. *World Health Organization*.
- WHO, 2016. Childhood Hearing Loss: Strategies for prevention and care. *World Health Organization*.
- Willott, J.F. et al., 2003. Acoustic startle and prepulse inhibition in 40 inbred strains of mice. *Behavioral neuroscience*, 117(4), pp.716–727.
- Wilson, D.H., 1997. Hearing in South Australia : disability, impairment and quality-of-life. *University of Adelaide PhD Thesis*.
- Wingfield, A. & Grossman, M., 2006. Language and the aging brain: patterns of neural compensation revealed by functional brain imaging. *Journal of neurophysiology*, 96(6), pp.2830–2839.
- Winslow, R.L. & Sachs, M.B., 1987. Effect of electrical stimulation of the crossed olivocochlear bundle on auditory nerve response to tones in noise. *Journal of neurophysiology*, 57(4), pp.1002–1021.
- Wisplinghoff, H. et al., 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical infectious diseases*. 39(3), pp.309–317.
- Wood, C.D. et al., 2009. Nuclear localization of p38 MAPK in response to DNA damage. *International journal of biological sciences*, 5(5), pp.428–437.
- Wright, M., 1947. Regeneration and degeneration experiments on lateral line nerves and sense organs in anurans. *The Journal Of Experimental Zoology*, 105(2), pp.221–257.
- Wu, C.-Y. et al., 2015. Protective role of L-ascorbic acid, N-acetylcysteine and apocynin on neomycin-induced hair cell loss in zebrafish. *Journal of applied toxicology* 35(3), pp.273–279.
- Wu, Y.J., Muldoon, L.L. & Neuwelt, E.A., 2005. The chemoprotective agent N-acetylcysteine blocks cisplatin-induced apoptosis through caspase signaling pathway. *The Journal of pharmacology and experimental therapeutics*, 312(2), pp.424–431.

- Xie, J., Talaska, A.E. & Schacht, J., 2011. New developments in aminoglycoside therapy and ototoxicity. *Hearing research*, 281(1-2), pp.28–37.
- Xie, Y. et al., 2015. Inhibitor of apoptosis signal-regulating kinase 1 protects against acetaminophen-induced liver injury. *Toxicology and applied pharmacology*, 286(1), pp.1–9.
- Yakunina, N. et al., 2019. Functional MRI evidence of the cortico-olivary efferent pathway during active auditory target processing in humans. *Hearing research*, 379, pp.1–11.
- Yamaguchi, O. et al., 2003. Targeted deletion of apoptosis signal-regulating kinase 1 attenuates left ventricular remodeling. *Proceedings of the National Academy of Sciences USA*, 100(26), pp.15883–15888.
- Yamamoto, N. et al., 2006. Inhibition of Notch/RBP-J signaling induces hair cell formation in neonate mouse cochleas. *Journal Of Molecular Medicine*, 84(1), pp.37–45.
- Yang, Q. et al., 2016. The expression of NLRX1 in C57BL/6 mice cochlear hair cells: Possible relation to aging- and neomycin-induced deafness. *Neuroscience letters*, 616, pp.138–146.
- Yasin, I., Drga, V. & Plack, C.J., 2014. Effect of human auditory efferent feedback on cochlear gain and compression. *The Journal Of Neuroscience*, 34(46), pp.15319–15326.
- Yeomans, J.S. & Frankland, P.W., 1995. The acoustic startle reflex: neurons and connections. *Brain research. Brain research reviews*, 21(3), pp.301–314.
- Ylikoski, J. et al., 2002. Blockade of c-Jun N-terminal kinase pathway attenuates gentamicin-induced cochlear and vestibular hair cell death. *Hearing research*, 163(1-2), pp.71–81.
- Yong, J.S. & Wang, D., 2015. Impact of noise on hearing in the military. *Military Medical Research*, 2, pp.6–6.
- Yoo, J. et al., 2014. Cisplatin otoprotection using transtympanic L-N-acetylcysteine: a pilot randomized study in head and neck cancer patients. *Laryngoscope*, 124(3), pp.E87–94.
- Yost, W.A., 2007. Fundamentals of hearing : an introduction. 5th Edition. Brill Publishing.
- Younossi, Z.M. et al., 2018. Improvement of hepatic fibrosis and patient-reported outcomes in non-alcoholic steatohepatitis treated with selonsertib. *Liver international* 38(10), pp.1849–1859.
- Zenner, H.P., 1986. Motile responses in outer hair cells. *Hearing research*, 22, pp.83–90.

- Zhang, K.D. & Coate, T.M., 2017. Recent advances in the development and function of type II spiral ganglion neurons in the mammalian inner ear. *Seminars in cell and developmental biology*, 65, pp.80–87.
- Zhang, X. & Gan, R.Z., 2013. Dynamic properties of human tympanic membrane based on frequency-temperature superposition. *Annals of biomedical engineering*, 41(1), pp.205–214.
- Zhao, L.-D. et al., 2011. Effects of DAPT and Atoh1 overexpression on hair cell production and hair bundle orientation in cultured Organ of Corti from neonatal rats. *PLoS ONE*, 6(10), pp.e23729–e23729.
- Zheng, J. et al., 2000. Prestin is the motor protein of cochlear outer hair cells. *Nature*, 405(6783), pp.149–155.
- Zheng, J. & Gao, W., 2000. Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. *Nature neuroscience*, 3(6), pp.580–586.
- Zwislocki, J.J. et al., 1992. Ionic coupling among cells in the organ of Corti. *Hearing research*, 57(2), pp.175–194.

Appendix 1. Protocol published during PhD candidature.

Text and images from this manuscript were used in this thesis. Full details are provided in the Preface.

Organotypic Culture of Neonatal Murine Inner Ear Explants

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The inner ear is a complex organ containing highly specialised cell types and structures that are critical for sensing sound and movement. *In vivo*, the inner ear is difficult to study due to the osseous nature of the otic capsule and its encapsulation within an intricate bony labyrinth. As such, mammalian inner ear explants are an invaluable tool for the study and manipulation of the complex intercellular connections, structures, and cell types within this specialised organ. The greatest strength of this technique is that the complete organ of Corti, or peripheral vestibular organs including hair cells, supporting cells and accompanying neurons, is maintained in its *in situ* form. The greatest weakness of *in vitro* hair cell preparations is the short time frame in which the explanted tissue remains viable. Yet, cochlear explants have proven to be an excellent experimental model for understanding the fundamental aspects of auditory biology, substantiated by their use for over 40 years. In this protocol, we present a modernised inner ear explant technique that employs organotypic cell culture inserts and serum free media. This approach decreases the likelihood of explant damage by eliminating the need for adhesive substances. Serum free media also restricts excessive cellular outgrowth and inter-experimental variability, both of which are side effects of exogenous serum addition to cell cultures. The protocol described can be applied to culture both cochlear and vestibular explants from various mammals. Example outcomes are demonstrated by immunohistochemistry, hair cell quantification, and electrophysiological recordings to validate the versatility and viability of the protocol.

Keywords: organ of Corti, peripheral vestibular organs, dissection, cochlea, hair cell culture, mouse, immunohistochemistry, inner ear

INTRODUCTION

An obstacle faced by inner ear biologists worldwide, is that hair cells from the inner ear cannot be immortalised. In the absence of immortal hair cell lines, the House Ear Institute-Organ of Corti 1 (HEI-OC1) auditory cell line has been used extensively when performing cytotoxic assays, screening protective compounds, elucidating molecular channel and receptor function and protein expression analysis (Kalinec et al., 2016; Park et al., 2016). Whilst serving an important purpose for screening assays, there are significant limitations when using HEI-OC1 for auditory research. Notably, HEI-OC1 cells do not represent a specific auditory phenotype, nor are they able to achieve the complex cell-to-cell interactions present within the highly organised organ of Corti or vestibular

sensory epithelium. Moreover, without the ability for specialised mechano-electric transduction, HEI-OC1 drug uptake patterns differ significantly from that of genuine inner ear hair cells. Drug responses vary depending on cell culture conditions and genetic drift has been problematic in the culture of HEI-OC1 (Kalinec et al., 2016). Over time, HEI-OC1 cells have developed drug resistance and have lost some key hair cell characteristics, such as prestin expression which is normally present in cochlear outer hair cells (Cederroth, 2012; Walters et al., 2015; Kalinec et al., 2016). As such, fundamental research in the field of auditory biology relies extensively on the use of mammalian inner ear explant preparations.

Ex vivo explant cultures maintain the entire organ of Corti or vestibular epithelium including the delicate inner ear hair cells, supporting cells and neurons in a three-dimensional form, akin to their *in situ* counterparts. As a result, the highly organised, dynamic microenvironment including the specialised cell types that reside within it, remain intact. Preservation of intercellular connections and complex structures provide greater insight into cell-specific stress responses than reduced preparations such as dissociated cell cultures. For example, Wu et al. (2015) were able to extensively characterise the changes in an inner ear explant preparation that were induced by tumour necrosis factor (TNF)-alpha treatment (Wu et al., 2015). In hair cells, changes in stereocilia formation, endoplasmic reticulum components and mitochondrial structures were described. Outer hair cell expulsion from the epithelium was observed as supporting cells attempted to seal the peri-cuticular plate. The chronological order with which cell types (such as pillar cells, Deiters' cells and hair cells) underwent apoptosis, the timing of cell junction degradation, and the presence of factors such as caspase 3 was also shown. This example highlights the incredible versatility and usefulness of the cochlear explant as an *ex vivo* experimental preparation in which variables can be tightly controlled (Wu et al., 2015). Other modern inner ear explant applications include studies of ototoxicity and screening of potentially otoprotective compounds (Lee et al., 2017; Perny et al., 2017), investigating cell stress effects and death pathways (Jiang et al., 2016; Nicholas et al., 2017), exploring novel regenerative strategies (Nayagam et al., 2013; Gunewardene et al., 2016), and elucidating the functions of specific proteins, channels, receptors, and synapses (Flores-Otero et al., 2007; Brugeaud et al., 2014). Much of what we now know regarding the function and structure of the inner ear, and in particular inner ear hair cells, has been established using *in vitro* inner ear explant preparations (Sobkowicz et al., 1975, 2009b; Nordemar, 1983; Russell and Richardson, 1987; Lim et al., 2011).

As a tool for auditory research, inner ear explants have been in use for over 40 years (Sobkowicz et al., 1975, 2009a,b). Consequently, countless protocols exist for the culture of inner ear explants and many iterations have remained in use for a number of years (Doetzlhofer et al., 2009; Mulvaney and Dabdoub, 2014; Haque et al., 2015; Wu et al., 2015; Nicholas et al., 2017). Herein, we present a modernised technique [adapted for *mus musculus* from *Cavia porcellus* protocols (Coleman et al., 2007; Nayagam et al., 2013)]. This protocol utilises organotypic cell culture inserts and serum free media. The protocol is

simpler for the researcher and eliminates steps that increase the likelihood of explant damage. We also provide instructions for the dissection of mouse neonatal inner ear organs. The culture method may be applied for the culture of both cochlear and vestibular explants from neonatal mice and rats (Coleman et al., 2007; Nayagam and Minter, 2012; Nayagam et al., 2013). Methods for immunohistochemistry, imaging, hair cell quantification and electrophysiological recordings are provided. Illustrative results are included to demonstrate the versatility and efficacy of this protocol.

Advantages Afforded by This Protocol

The inner ear is a complex 3D structure, where cell patterning and communication is critical for cell survival. The use of organotypic cell culture inserts protects the explant tissue sample during media changes, prevents drying of the tissue during prolonged culture, provides excellent diffusion of soluble growth factors to the tissue across the substrate and provides uniform structural support. The porous membrane allows cellular outgrowth, whilst limiting the cell layering that can occur on impermeable surfaces. The membrane can be easily cut away to facilitate transfer of the explants for physiological recordings, immunohistochemistry, and/or further analyses.

Organotypic Cell Culture Inserts Eliminate the Requirement for Adhesive Substances, Thereby Improving Explant Quality

Cell culture inserts eliminate the need for an adhesive substance, which saves time, reduces the likelihood of damage, and is simpler for the user. Conversely, commonly used adhesives such as collagen, Matrigel or Cell Tak can be particularly unforgiving for the user. For instance, unintentional contact with adhesive when orienting the explant can fix the tissue in an unusable position. Even when placed appropriately, the action of pressing an explant onto an adhesive substance is likely to cause damage and uneven structural support, which is particularly problematic for functional studies. When orienting an explant onto the cell culture insert in media, there is no risk of accidental adhesion and the explant can be easily maneuvered into position without causing damage to delicate cell types.

Serum Free Media Improves Experimental Consistency

Animal sera are highly variable and poorly defined mixtures of hormones, vitamins, lipids, transport proteins, binding factors, proliferation factors, growth factors, and other unknown components. Batches from the same supplier are highly variable. Therefore, the addition of serum impairs assay efficacy, compromises experimental consistency and increases the likelihood of pathogenic contamination (Gstraunthaler et al., 2013).

Serum components can also induce undesirable immune responses and encourage inconsistent cellular proliferation (Harrill et al., 2015). For example, in early post-natal cochlear explant preparations the addition of serum encourages proliferation of dividing cell types in the organ of Corti and supporting cell differentiation. As a result, the ultrastructure

of the four rows of hair cells is disrupted more quickly than observed in the absence of serum (Nayagam et al., 2013). Additionally, animal sera are expensive and subject to global shortages. In contrast, serum-free media contains consistent nutritional and hormonal formulations, which improves assay sensitivity and supports more homogeneous and reproducible cell assays (Harrill et al., 2015).

Limitations When Culturing Inner Ear Tissues

Inner Ear Hair Cell Survival Is Limited *In vitro*

Optimal inner ear hair cell survival in culture is considered to be <1 week, however longer culture periods have been reported in both mouse and rat explants (Flores-Otero et al., 2007; Nayagam et al., 2013). A 72 h time-point has been applied in the ototoxic assays described herein. However, survival of hair cells has been described after up to 14 days in culture (Sobkowicz et al., 1975; Nayagam et al., 2013).

Neonatal Explants Contain Immature Cell Types

In vivo, inner ear hair cells are terminally differentiated by embryonic day 14.5, however a period of quiescence may exist in the early postnatal stages. Prior to the onset of hearing at around postnatal day 12, immature hair cells are capable of spontaneous firing. Auditory ganglia continue to proliferate and embryonic protein expression may persist (Kamiya et al., 2001; Marcotti et al., 2003). Therefore, the developmental age of tissue should

be considered when evaluating responses in the inner ear explant preparation *in vitro*. For instance, cultured inner ear hair cells may retain some regenerative capacity and protein expression will vary between explants derived between P1 and P5 (Walters and Zuo, 2013; Cox et al., 2014).

Cochlear Explants Contain Resident Macrophages

Macrophage activity within the explant can activate both pro-survival and pro-death signals *in vitro*. Whilst this may complicate experimental outcomes, it is important to consider that similar macrophage activity occurs *in vivo* (reviewed in Francis and Cunningham, 2017).

The addition of serum to culture media can drastically enhance the activity of macrophages *in vitro*, creating less homogenous inflammatory populations and introducing variability to experimental outcomes (refer to advantages of serum-free culture noted above) (Homma and Yamamoto, 1990; Rey-Giraud et al., 2012).

Foreword

This protocol describes the fundamental procedures for dissecting and culturing early post-natal murine inner ear explants on organotypic cell culture membranes, rather than the glass-based cultures previously described (Parker et al., 2010; Haque et al., 2015). The protocol for culturing explants is relatively simple. However, the initial cochlear dissection is intricate and requires a high degree of skill. A great deal

TABLE 1 | Recommended reagent list for the dissection and culture of mammalian inner ear hair cells.

Reagent	Supplier	Catalogue number	Recommended storage
MEM	Life Technologies	42360-032	49 ml aliquots at −20°C
DMEM/F12/GlutaMAX	ThermoFisher	10565-018	4°C
Non-essential amino acids	Life Technologies	11140-050	500 µl aliquots at 4°C
Pen/Strep	Life Technologies	15140-148	500 µl aliquots at −20°C
Penicillin	Sigma Aldrich	P3032	Aliquots at −20°C
Neurobasal-A medium	Life Technologies	10888-022	49 ml aliquots at −20°C
N2 supplement	Life Technologies	17502-048	500 µl aliquots at −20°C
L-glutamine	Life Technologies	25030-149	500 µl aliquots at −20°C
D-glucose	Life Technologies	15023-021	75 mg aliquots at room temperature
Steriflip disposable vacuum filters	Merck	SE1M179M6	Room temperature
Millicell cell culture insert, 30 mm, hydrophilic PTFE, 0.4 µm	Merck	PICM03050	Room temperature
6 well culture plate	ThermoFisher	140675	Room temperature
4% PFA	As preferred		As preferred
Phosphate buffered saline	As preferred		As preferred
Myosin-VII antibody (Rabbit anti-mouse)	Sapphire Bioscience	PTS-25-6790-C050	−20°C
Goat anti-rabbit IgG HandL Alexa Fluor 488	Sapphire Bioscience	AB150085	−20°C
Prolong diamond anti-fade with DAPI	Life Technologies	P36962	Room temperature
Prolong gold anti-fade with DAPI	Life Technologies	P36941	Room temperature
Phalloidin stain	ThermoFisher	A12379	−20°C
PTFE printed Microscope slides	Pro Sci Tech	G35068	N/A
Coverslips	Pro Sci Tech	G417	N/A
<i>In situ</i> cell death detection kit	Sigma Aldrich	11684795910	−20°C
Triton X-100	Sigma Aldrich	X100-500ml	Room temperature
Goat Serum	Sapphire Bioscience	AB156046	500 µl aliquots at −20°C
10% neutral buffered formalin	Thermo Fisher Scientific	FNNJJ0185B	Room temperature

of practice is required to ensure dissected explants are of a quality suitable for use in experimentation. This should be considered when applying for ethics approval or preparing experiments. As an example of the diversity of data that can be acquired using organotypic explant culture, we provide electrophysiological recordings, immunohistochemistry, and quantification of cell survival. Countless alternative techniques can be applied depending on experimental questions. Scanning electron microscopy, RNA sequencing, and complex staining procedures have all been used to elucidate fundamental aspects of cellular biology within the *ex vivo* inner ear explant. However, new techniques will continue to arise, adding value to the inner ear explant as an *ex vivo* model for the study of specialised cell types contained within it.

Animal Ethics Approval

All experiments described conform to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th edition, 2013. Approval was granted by the animal ethics committees of The University of Newcastle and The Murdoch Childrens Research Institute, in project numbers A766 (MCRI) and A-2013-325 (UoN).

MATERIALS AND EQUIPMENT

To complete the dissection described in this protocol a dissecting microscope is required. A laminar flow cabinet or biohazard hood is recommended, but not essential. Recommended dissection tools are curved forceps, scalpels (#10 and #15), pointed forceps (#5 and #55), and a periosteal elevator. However, alternate tools may be used according to user preference. Common lab items are required, such as sterile Petri dishes, 70% ethanol, disposable cleaning wipes (such as kim-wipes), pipettes (0.1–1,000 µl range), and a CO₂ incubator. Wide bore 1,000 µl pipette tips are useful. A number of organotypic cell culture membranes are available—with varying pore size or membrane material. However, we routinely use Merck Millipore Millicell[®] organotypic cell culture inserts. These inserts consist of a solid plastic wall and an optically transparent, hydrophilic, polytetrafluoroethylene membrane with a pore size of 0.4 µm (Biopore[™]). Further equipment and reagents required for

inner ear explant culture, staining and mounting are listed below (**Table 1**).

PROTOCOL

General Set-Up

Note: A laminar flow cabinet minimises potential sources of airborne contamination, thus maximising the number of useful experimental preparations. If a laminar flow cabinet is not available, this protocol should be performed in a room without a thoroughfare. Remember to frequently sterilise surfaces and instruments with 70% ethanol. For wiping surfaces, Kim Wipes are recommended, as regular tissues leave small particles behind.

Thoroughly clean and sterilise the laminar flow (or work bench) before setting-up the dissection microscope. Once in place, sterilise the microscope paying particular attention to parts that will be frequently used, such as focus controls.

Make a small quantity of fresh Minimal Essential Media (MEM) solution and Neurobasal (NB) solution and/or DMEM/F12 GlutaMAX solution, as per **Table 2**. Filter sterilise each solution and place on wet ice in the laminar flow hood. Explants that were used for electrophysiological recordings of inner ear hair cells were dissected in glycerol-based Ringer's solution (Ye et al., 2006) (**Supplementary Table 1**). NB solution is used for cochlear explant culture, whereas DMEM/F12 GlutaMAX solution is used for vestibular explant culture. *Note: Ensure glucose powder is completely dissolved before filter sterilising. Solutions can be stored for 2–3 days at 4°C.*

Within the laminar flow, sterilise pipettes and dissection tools and allow them to dry. Under sterile conditions, open a 6-well culture plate and Millicell packaging. Place the cell culture inserts into the wells and add 1 ml NB solution (or DMEM/F12 GlutaMAX solution) to each well. Add the media under the membrane by holding the pipette tip against the side of the well and slowly releasing the media. Close the plate and incubate for a minimum of 30 min before commencing culture. For hair cell studies: incubate at 37°C, 5% CO₂; for neuron specific research: incubate at 37°C, 10% CO₂.

Collecting Tissue

Euthanise mouse pups (ideally aged between P0–5) by decapitation (or by alternative ethically approved methodology).

TABLE 2 | Recommended solutions required for this protocol.

MEM solution		NB solution (for cochlear preparations)		DMEM/F12 GlutaMAX solution (for vestibular preparations)	
Reagent	Quantity	Reagent	Quantity	Reagent	Quantity
Minimum essential media	49 mL	Neurobasal-A media	49 mL	DMEM/F12 Glutamax	15 mL
Non-essential amino acids	500 µL	N2 supplement	500 µL	D-(+)-glucose	42.73 mg
Penicillin/streptomycin	500 µL	L-glutamine	500 µL	Penicillin	200 µl
		D-glucose (Dextrose) powder	75 µg		

MEM solution is used in the initial dissection steps. NB solution is used for final cochlear explant dissection and culture. Alternatively, glycerol-based Ringer's solution and DMEM/F12 GlutaMAX solution are used for final vestibular explant dissection and culture, respectively. Separate reagent aliquots for each solution can be stored at –20°C to avoid contamination or freeze thawing of the main stock. Once combined, solutions can be stored for 2–3 days at 4°C.

Collect heads in a sterile Petri dish and place the closed dish in wet ice.

Dissecting the Temporal Bones

Note: Videos of the major steps in the dissection of the inner ear from the temporal bone have been published with detailed instructions for dissection of both the cochlea (Parker et al., 2010; Haque et al., 2015) and vestibular organs (Tung et al., 2013). Alternative tools may be used based on user preference and availability.

Working quickly in the laminar flow hood:

- Use curved forceps to hold the head in place and bisect the skull with a no.10 scalpel.
- Remove the brain using a gentle scooping motion with a small periosteal elevator.
- Using the scalpel remove the snout, scrape away the skin, and remove unnecessary bone.
- Place the temporal bones into a fresh, Petri dish containing ice-cold MEM.
- Repeat dissection for all remaining heads. Use multiple Petri dishes to maintain clear media for dissections (4–6 temporal bones per Petri dish).

Isolating the Inner Ear

Use the dissection microscope with low magnification. In a Petri dish containing fresh, ice-cold MEM solution use #5 forceps to remove the inner ear from the temporal bone. Place the dissected organs into ice-cold, NB solution (cochlear) or glycerol-based Ringer's solution (vestibular) and maintain on ice. Repeat for all remaining temporal bones.

Note: After 3–4 dissections, the media in the Petri dish will become cloudy and obstruct the users view. Replace with cold, fresh MEM solution as required. Likewise, replace the Petri dish if it becomes scratched.

Cochlear Dissection

Gently dissect the cochlea from the vestibular apparatus at the promontory (i.e. their junction, **Figure 1A**) and move the cochlea to a dish containing fresh, ice-cold NB solution.

Increase magnification to improve visualisation of round and oval windows. Use a small picking motion to remove the bone from the cochlea (#55 or jeweler's forceps are ideal). Once the bony cochlear wall is removed, grasp the spiral ligament/stria vascularis at the base- and unwind it from the modiolus (**Figure 1B**). Carefully secure the basal section of the cochlea with one pair of forceps whilst gently dissecting the basal organ of Corti from the peripheral processes of the auditory neurons in the modiolus.

Note: If the stria vascularis and organ of Corti are inadvertently unwound from the modiolus together, it is possible to gently separate the tissues in the NB solution.

Vestibular Dissection

Using a dissecting microscope, dissect the vestibular organs from the inner ear preparation in an ice-cold slurry of glycerol-based Ringer's solution (Lim et al., 2011). Using forceps to hold the bony cochlea, use a small picking motion to remove bone

overlying the anterior and horizontal canals and the vestibule. Remove bone surrounding the anterior and horizontal canals, so that the membranous labyrinth is unobstructed. Once the membranous labyrinth is clearly visible, use fine curved spring scissors to cut and remove the canal membrane close to the neuroepithelium of the anterior and horizontal cristae. Trim and remove the membrane overlying the utricle. Use very sharp spring scissors to cut the overlying membrane, any pulling motion will damage the delicate hair cells. Once the membrane covering the vestibular triad has been removed, continue to remove bone from around the utricle and gently trim the vestibular part of the vestibulocochlear nerve, to free the triad from the bone.

Transfer the Explants to Millicell Membranes

Cochlear Explants

Note: A video of this step is provided (Supplementary Video 1).

Using a wide bore and preferably a silicone-coated pipette to collect ~100 μ L of NB solution from the dissection dish. Once there is solution inside the pipette, the explant can be drawn into the pipette tip. Expel the explant and media onto the Millicell membrane in one movement. This will result in the explant being suspended within a small liquid bolus with a positive meniscus. Orient the explant into the correct position (**Figure 1C**). The organ of Corti should be facing upwards with four rows of hair cells visible on the peripheral edge of the explant (as *in situ*).

Note: The explant is "right side up" if both Reissner's membrane and the tectorial membrane are clearly visible (Figure 1C). If the explant is upside down, add more media to orient correctly. The explant is easy to manipulate in a bolus of media.

Once the explant is in the desired position, use forceps to carefully fold Reissner's membrane away from the hair cells toward the explant modiolus. The tectorial membrane also needs to be gently folded away from the rows of hair cells, towards the explant modiolus, so as to expose their apical stereocilia (start at the apex). Finally, using a smaller pipette, gently aspirate media from the top of the Millicell membrane, starting at the edge of the meniscus: the tectorial membrane will fold away from the hair cells and the explant will attach to the Millicell membrane (**Figure 1D**).

Note: While a little media remains, the apex and base of the explant can be pushed apart so that the explant resembles a crescent shape rather than a spiral. This makes the apex easier to image- and also limits neurons from growing over the hair cells. Be careful to remove any cochlear bone fragments that may have been transferred in the media with the explant.

Up to five explants may be cultured on each Millicell membrane, however between two to four is ideal.

Vestibular Explants

The same technique described above is used for transferring the dissected vestibular triad explant to the Millicell membrane, using a wide bore pipette, filled with DMEM/F12 GlutaMAX culture solution. Ensure the vestibular triad is the right way up. The neuroepithelium that has recently been exposed should face upward, so that the utricle, anterior and horizontal cristae are

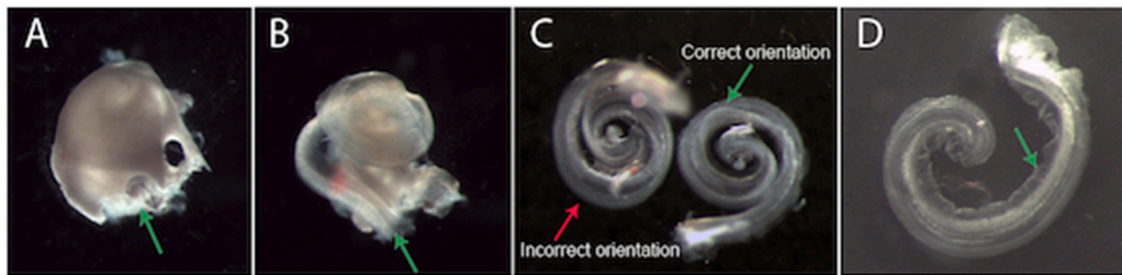


FIGURE 1 | Dissection and plating of a mouse cochlear explant. **(A)** An example cochlea that has been removed from the temporal bone (vestibular apparatus removed). It is easiest to begin removing the bony cochlear wall from the base of the cochlea, starting at either the round or oval window (green arrow, **A**). **(B)** Once the bony cochlear wall is removed, grasp the spiral ligament at the base (green arrow, **B**) and unwind it from the modiolus. The arrow is pointing at a dark line. This is a dissection “landmark” that will help ascertain a gap between the stria vascularis and neurosensory epithelium. This gap is a good place to start when prising the stria vascularis from the organ of Corti. **(C)** Two example explants in different positions. The explant on the left is incorrectly oriented with the hair cells touching the surface of the membrane. The explant on the right is in the correct position. When the explant is correctly oriented, Reissner’s membrane is clearly visible (green arrow, **C,D**). **(D)** An example explant that has adhered to the Millicell membrane. When excess NB solution is removed, the explant adheres to the Millicell membrane. At this stage, gently moving the explant into a crescent shape (as shown) is recommended. After a few hours incubation, the explant can no longer be moved. Note: Reissner’s membrane (green arrow, **D**) has been moved away from the hair cells and has adhered to the Millicell membrane.

clearly visible [see **Figure 2**, Lim et al. (2014)]. The vestibular nerve faces down, resting directly on the membrane. Typically, a triad explant from each ear may be transferred and cultured on a single membrane.

Incubation

Culture inner ear explants overnight to allow proper adhesion. Treatment can begin immediately if desired by adding to the solution underneath the membrane.

For hair cell studies: incubate at 37°C, 5% CO₂.

If this protocol is being adapted for inner ear neuron specific research: incubate at 37°C, 10% CO₂.

Commence Experimental Treatments

Aspirate the NB or DMEM/F12 GlutaMAX solution and replace with fresh media including treatment or control that has been warmed to 37°C. Replace media daily as desired.

Electrophysiology

Electrophysiological recordings can be made at any point during the culture time frame but were performed after 2 days *in vitro* in this report. Inner ear explants are transferred to a recording chamber perfused with oxygenated Liebovitz’s L15 cell culture media (ThermoFischer Scientific, Australia) with a flow rate of 2–3 bath exchanges/minute. Cells are visualised and targeted for recording using infrared differential interference contrast optics. Borosilicate electrodes (3–5 M Ω) are filled with potassium-gluconate internal solution containing (in mM); 42 Kgluconate, 98 KCl, 4 HEPES, 0.5 EGTA, 1 Mg₂ATP, 5 Na₃GTP. Electrophysiological recordings were done at room temperature using a Multiclamp 700B (Molecular Devices) amplifier, sampled at 20 kHz and filtered at 2–10 kHz and acquired using Axograph software (Molecular Devices). Series resistance was monitored throughout recordings and data was rejected if it increased by more than 20%.

Fix and Wash Explants for Immunohistochemistry

Aspirate media from each well and replace with 1 mL ice-cold 4% paraformaldehyde (PFA). A drop of fixative on top of each explant is also recommended. Fix at room temperature with very gentle rocking for 5–10 min.

Note: Over-fixation of explants will adversely affect immunocytochemistry. Do not allow explants to dry out at any point during the fixation or staining process.

Aspirate the fixative and wash each well thrice with 1 mL 1 x Phosphate Buffered Saline (PBS). A drop of PBS should also be added to the top of each explant to rinse away any residual PFA.

Note: At this point slide preparation can commence or the plate can be stored at 4°C for up to 3 days, with each well containing PBS -not fixative. For longer-term storage, use a solution of 2% sodium azide in PBS. Note however, that best results for immunohistochemistry are achieved by staining freshly fixed tissues.

Immunohistochemistry

Myosin-VIIa Inner Ear Hair Cell Stain

Prepare blocking solution (PBS + 2–10% serum relative to secondary antibody + 0.1% Triton X) and place on roller mixer. While the blocking solution mixes, label Teflon printed slides and add 20 μ L 1x PBS to each well on the slide.

Note: If slides with printed wells are not available, drawing segments on a slide using a PAP pen will suffice. Remember—do not allow explants to dry out.

Aspirate PBS from each well in the plate and use a #15 scalpel or Vannas micro surgical scissors (WPI) to cut the membrane surrounding each explant. Take care not to fold or tear the membrane whilst doing so. Using forceps, transfer the segments (membrane containing explant) to a well on the printed slide, with the explant facing up. Repeat until all explants have been placed onto slides. Aspirate PBS from each explant on the slide and add 20 μ L of blocking solution to each explant. Incubate for 30–60 min at room temperature in a humidified chamber.

Note: An empty pipette tip container containing water serves as an effective humidified chamber.

Dilute primary antibody, such as Myosin-VIIa in blocking solution (1:500 for cochlear explants 1:200 for vestibular explants, or per serial dilutions if staining using alternative antibodies). Aspirate blocking solution from explants and pipette 20 μ L of the primary antibody solution onto each culture. Incubate for 1 h at room temperature, or overnight at 4°C (cochlear explants) or room temperature (vestibular explants) in a humidified chamber.

Wash explants with 20 μ L blocking solution or 0.1 M phosphate buffered saline (PBS) for 2–10 min, aspirate and repeat with fresh blocking solution or PBS three times. To remove any tiny aggregates from the secondary antibody, briefly centrifuge (20 s at $\sim$ 12,000 g). Dilute secondary antibody 1:200–500 in blocking solution. Pipette 20 μ L of secondary antibody solution onto each explant and incubate for 2 h at room temperature (in humidified chamber) or overnight at 4°C. Wash wells with 20 μ L blocking solution or PBS for 2–10 min, aspirate and repeat with fresh blocking solution or PBS three times.

*Note: At this point there are several options: keep explants in the final rinse of blocking solution and image as a wet specimen; perform additional staining such as the *in situ* TUNEL stain (step 13) or apply further antibodies; or, mount under coverslips (step 14).*

Stereocilia Staining Using Phalloidin

Prepare a detergent solution of PBS + 0.1% Triton X and mix on a roller mixer.

While the solution mixes, prepare the explants for slide-based staining as described above. Aspirate PBS from each explant on the slide and add 20 μ L of detergent solution to each explant. After 5 min, aspirate the solution and replace with 20 μ L detergent solution containing a fluorescently labelled phalloidin (Invitrogen) diluted 1:80 as per manufacturer's instructions). Gently add the detergent/phalloidin mixture in a drop wise

manner on top of the explant. Incubate for 10 min and then wash each well thrice for 10 min with 20 μ L PBS. *Note: At this point explants can be imaged as a wet specimen or mounted onto glass slides (step 14). Incubating phalloidin on an explant for longer than 15 min will result in extensive over staining of all cell types, preventing clear visualisation of structures.*

Tunel Stain

Mix the *in situ* cell death detection kit solutions as per manufacturer instructions (Sigma Aldrich). Add 10 μ L of the solution to each explant and incubate at 37°C for 1 h (in humidified chamber). Wash wells with 20 μ L blocking solution for 2 min, aspirate and repeat with fresh blocking solution three times.

Mount Explants Under Coverslips and Seal Slides

Aspirate blocking solution and add a small drop of Prolong Gold anti-fade mounting medium (with or without DAPI) to each well. Starting at a 45° angle, gently place coverslip on slide. Do not press down or attempt to lift the coverslip once placed. Seal edges with clear nail varnish and allow to dry overnight. Fluorescence will last for a number of months, with slides stored in a lightproof slide box. However, explants should be imaged as soon as possible.

Note: When higher resolution imaging is being performed (such as stereocilia imaging), Prolong Diamond anti-fade mountant is recommended as it has a refractive index similar to immersion oil. Ensure the selected coverslip thickness is appropriate for the objective being used (most objectives are designed to be used with a #1.5 coverslip—approx. 0.17 mm).

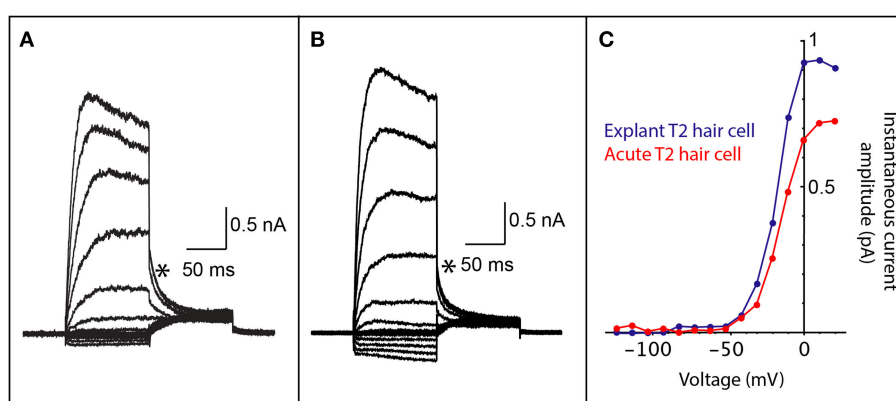


FIGURE 2 | Comparison of whole cell patch clamp recordings from both acutely dissected and explant cultured vestibular hair cells. **(A)** Voltage-activated currents from the crista of a mouse type II vestibular hair cell, prepared as a vestibular triad explant preparation that was cultured for 2 days. **(B)** Voltage-activated currents from the crista of a mouse type II vestibular hair cell from an acute vestibular triad preparation (2 h post dissection). **(C)** An IV plot of instantaneous tail current amplitudes (measured at asterisks in **A,B**) from hair cells in cultured explant preparation (blue trace) and the acute vestibular triad preparation (red trace). The voltage-activated instantaneous tail currents from the explant preparation are comparable to those obtained from an acute preparation and are consistent with recordings from type II vestibular hair cells.

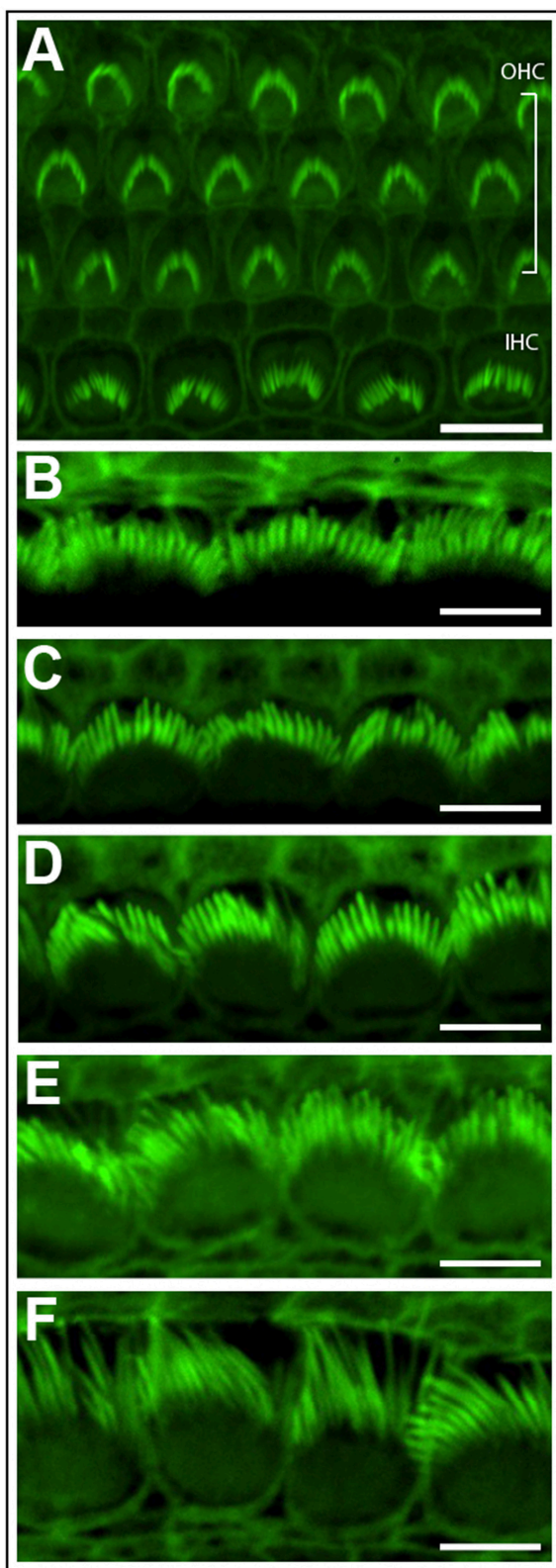


FIGURE 3 | Phalloidin stained cochlear hair cells. **(A)** One row of inner hair cells (IHC) and three rows of outer hair cells (OHC) illustrating normal polarity (Continued)

FIGURE 3 | and patterning of cochlear stereocilia bundles. Scale bar = 10 μm . **(B–F)** The length of inner hair cell stereocilia (green) increases from the basal to apical regions of the cochlea. Conversely, stereocilia width decreases from the cochlear base to apex. Images were progressively collected from the basal region of the explant **(B)** through to the mid-section **(D)** through to the apex **(F)** to demonstrate the graded change in stereocilia length and width. Scale bar = 5 μm . Tissues mounted using Prolong Diamond anti-fade reagent and coverslip #1.5.

RESULTS

Physiological Recordings From Inner Ear Hair Cells in Explant Culture

To demonstrate that cell culture with Millicell inserts maintains viable inner ear hair cells, we performed electrophysiological recordings on hair cells from the vestibular apparatus. Electrophysiological recordings are essential for determining whether cells are functionally viable following culture as explant preparations. Whole-cell patch clamp recording gives an indication of the presence and function of voltage-gated ion channels, receptor-mediated currents, intrinsic membrane properties and discharge properties (if recording from neurons), depending on the configuration used. We maintained mouse vestibular explants on Millicell membranes for 2 days in culture and then recorded voltage-activated currents (**Figure 2A**). These cell responses, which are due to K^+ channel activation, can only be recorded from cells that are viable. The profile of voltage-activated responses is consistent with recordings from type II vestibular hair cells in an acute semi-intact preparation (**Figures 2B,C**). Furthermore, the amplitude and kinetics of voltage-activated currents in mouse explants are comparable to those previously studied. All hair cell recordings in explant or acute preparation were from the anterior or horizontal cristae ampullares. These data show the viability of the explant preparation described for electrophysiological recordings. Recordings are not possible from cells that are unhealthy or dead since the cell membrane degrades preventing recording in the whole-cell patch configuration.

Immunostaining and Cell Viability Assays

Inner ear explants can be labelled using immunofluorescence techniques tailored to highlight particular proteins, structures or regions of interest within the explant- whilst attached to the Millicell membrane.

Phalloidin Staining

Phalloidin staining can highlight the stereocilia located on the apical surface of inner ear hair cells (**Figure 3**). This technique is a rapid and cost effective way to observe stereocilia polarity and patterning (**Figure 3A**), before committing to more expensive techniques such as scanning electron microscopy. Subtle changes in stereocilia morphology, such as the increase in their length from the base to apex in the cochlea can be observed. In the cochlear basal turn, the stereocilia are very short (**Figure 3B**), however stereocilia length

increases towards the cochlear apex (Garfinkle and Saunders, 1983) (**Figure 3F**). Conversely, stereocilia width gradually

decreases from the basal to apical regions of the cochlea (Sekerková et al., 2011).

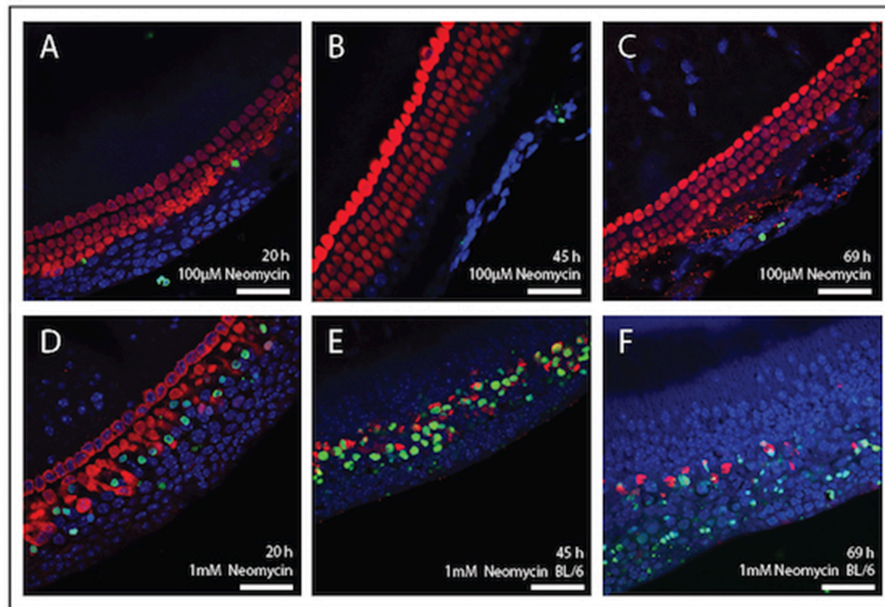


FIGURE 4 | Immunolabelling of inner ear hair cells from mouse cochlear explant preparations after 20, 45, and 69 h in culture. Cochlear explants were treated with the ototoxic drug, neomycin. Changes in cellular morphology and DNA damage indicative of apoptosis were detected using the TUNEL assay (green). DAPI (blue) was used to stain all cell nuclei and Myosin-VII (red) specifically stained surviving hair cells. **(A–C)** Explants treated with 100 μ M neomycin (20, 45, and 69 h). **(D–F)** Explants were treated with 1 mM neomycin (20, 45, and 69 h). Scale bar = 40 μ m. Tissues mounted using Prolong Gold anti-fade reagent and coverslip #1.

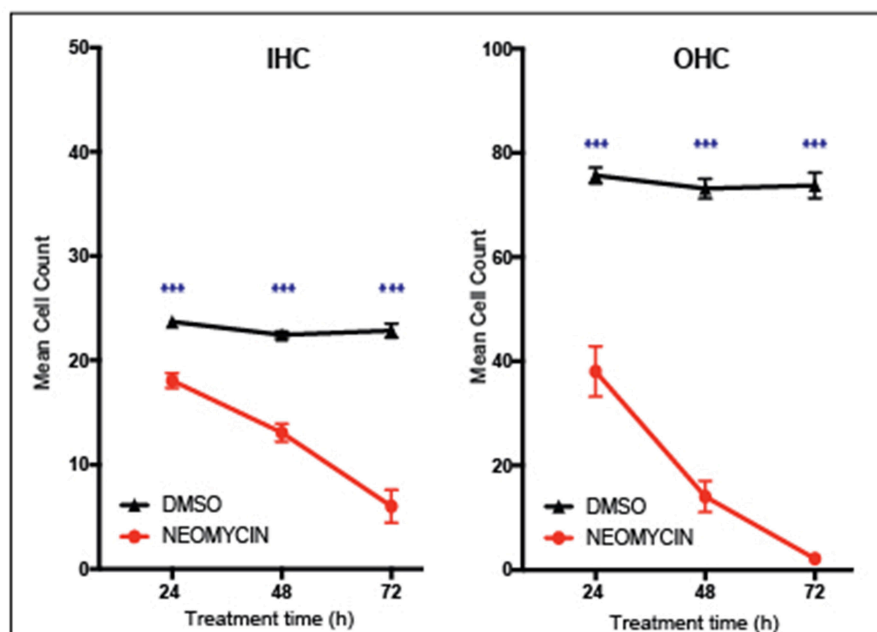


FIGURE 5 | Inner ear hair cell survival over a 72-h time course. *In vitro* hair cell counts were performed 24, 48, or 72 h after treatment with either 1 mM neomycin, or Dimethyl sulfoxide equivalent (20 μ l DMSO in 992 μ l media). Cell counts are based on the average number of Myosin VIIa-positive hair cells counted in two 180 $\times$ 90 μ m sections of the cochlear explant mid-turn. Mean with standard error of the mean (SEM) shown, $n = 6$ –9 explants per treatment, *** $p < 0.001$.

TUNEL Staining

Figure 4 shows mouse cochlear explants that have been treated with the ototoxic drug neomycin. In this example immunohistochemistry was used to visualise the cochlear hair cells and a fluorescent TUNEL stain was used to highlight cells undergoing apoptosis. Structural changes associated with cell death, such as cell condensation are visible and the TUNEL stain clearly indicates the presence of degraded DNA in apoptotic cells. Such cell death, or cell survival can then be quantified according to treatment and timeframe and is outlined below.

Quantification

Performing cell counts is an important step for quantifying cell survival under a range of experimental conditions. Cell counts can be a particularly powerful way to show the effects of a compound on hair cell viability. For example, **Figure 5** shows that the aminoglycoside antibiotic neomycin is ototoxic, destroying cochlear hair cells. After 3 days treatment with 1 mM neomycin, 98 % of outer hair cells were destroyed whereas inner hair cells were less affected with 67% destroyed after 3 days of neomycin treatment. Using this technique, it is then possible to quantify positive effects of putative otoprotective compounds. Any section of the explant can be counted, however it is important to note the hair cells in the apical region are highly resistant to ototoxicity, due to reduced drug uptake. Conversely, cells in the basal region are more susceptible to cell death and are also more likely to be damaged during dissection.

SUMMARY

We have described the fundamental procedures for dissecting, plating, and culturing early post-natal murine inner ear explants using Millicell culture inserts and serum- free conditions. The use of serum free media for cochlear and vestibular explant culture is becoming more common (Kopke et al., 1997; Cunningham et al., 2002; Amarjargal et al., 2009; Bas et al., 2014; Glueckert et al., 2015; Wu et al., 2018). And, published methods similar to this protocol further validate the efficacy of organotypic cell culture inserts when culturing cochlear or vestibular tissues (Praetorius et al., 2010; Baker and Staecker, 2012; Dammeyer et al., 2014; Defourny et al., 2015; Ma et al., 2017). In our hands, this protocol has been used to culture both cochlear and vestibular explants derived from rats and mice (Coleman et al., 2007; Nayagam et al., 2013; Gunewardene et al., 2016). We have provided examples of cell viability and function using patch-clamp electrophysiology, immunohistochemistry, and quantification. Alternative techniques can be readily applied to the described explants depending upon specific experimental questions. It is important to remember that, whilst the protocol is simple to follow, the dissections described require patience and practice. The use of Millicell culture inserts described in this protocol eliminates the need for adhesive substances whilst

still anchoring the explant. As a result, important experimental tissues are not inadvertently damaged or lost during initial adhesion or subsequent media changes. The explant can be easily maneuvered into position with minimal direct contact and the Millicell membrane provides uniform structural support during media changes and culture. Additionally, the use of serum free media restricts excessive cellular outgrowth and inter-experimental variability.

ETHICS STATEMENT

All experiments described conform to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th edition, 2013. Approval was granted by the animal ethics committees of The University of Newcastle and The Murdoch Childrens Research Institute, in project numbers A766 (MCRI) and A-2013-325 (UoN).

AUTHOR CONTRIBUTIONS

JO, RB, RL, and BN conceived the experiments. JO, BN, and RL wrote the manuscript. JO prepared all figures for publication. HD and RL performed the vestibular explant experiments and produced the diagrams presented in **Figure 2**. JO conducted all cochlear explant experiments producing the data and diagrams presented in **Figures 3–5**. Cochlear dissections for **Figure 1** images and **Supplementary Video 1** were performed by BN and edited for publication by JO. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fncel.2019.00170/full#supplementary-material>

REFERENCES

Amarjargal, N., Andreeva, N., Gross, J., Haupt, H., Fuchs, J., Szczepek, A. J., et al. (2009). Differential vulnerability of outer and inner hair cells during and after

oxygen-glucose deprivation in organotypic cultures of newborn rats. *Physiol. Res.* 58, 895–902.

Baker, K., and Staecker, H. (2012). Low dose oxidative stress induces mitochondrial damage in hair cells. *Anat. Rec.* 295, 1868–1876. doi: 10.1002/ar.22594

- Bas, E., Van De Water, T. R., Lomberas, V., Rajguru, S., Goss, G., Hare, J. M., et al. (2014). Adult human nasal mesenchymal-like stem cells restore cochlear spiral ganglion neurons after experimental lesion. *Stem Cells Dev.* 23, 502–514. doi: 10.1089/scd.2013.0274
- Brugaud, A., Tong, M., Luo, L., and Edge, A. S. (2014). Inhibition of repulsive guidance molecule, RGMA, increases afferent synapse formation with auditory hair cells. *Dev. Neurobiol.* 74, 457–466. doi: 10.1002/dneu.22136
- Cederroth, C. R. (2012). Loss of aminoglycoside sensitivity in HEI-OC1 Cells? *Hear. Res.* 292, 83–85. doi: 10.1016/j.heares.2012.08.011
- Coleman, B., Fallon, J. B., Pettingill, L. N., de Silva, M. G., and Shepherd, R. K. (2007). Auditory hair cell explant co-cultures promote the differentiation of stem cells into bipolar neurons. *Exp. Cell Res.* 313, 232–243. doi: 10.1016/j.yexcr.2006.10.010
- Cox, B. C., Chai, R., Lenoir, A., Liu, Z., Zhang, L., Nguyen, D. H., et al. (2014). Spontaneous hair cell regeneration in the neonatal mouse cochlea *in vivo*. *Development* 141, 816–829. doi: 10.1242/dev.103036
- Cunningham, L. L., Cheng, A. G., and Rubel, E. W. (2002). Caspase activation in hair cells of the mouse utricle exposed to neomycin. *J. Neurosci.* 22, 8532–8540. doi: 10.1523/JNEUROSCI.22-19-08532.2002
- Dammeyer, P., Hellberg, V., Wallin, I., Laurell, G., Shoshan, M., Ehrsson, H., et al. (2014). Cisplatin and oxaliplatin are toxic to cochlear outer hair cells and both target thioredoxin reductase in organ of corti cultures. *Acta Otolaryngol.* 134, 448–454. doi: 10.3109/00016489.2013.879740
- Defourny, J., Mateo Sánchez, S., Schoonaert, L., Robberecht, W., Davy, A., Nguyen, L. et al. (2015). Cochlear supporting cell transdifferentiation and integration into hair cell layers by inhibition of ephrin-B2 signalling. *Nat. Commun.* 6:7017. doi: 10.1038/ncomms8017
- Doetzlhofer, A., Basch, M. L., Ohyama, T., Gessler, M., Groves, A. K., and Segil, N. (2009). Hey2 regulation by FGF provides a notch-independent mechanism for maintaining pillar cell fate in the organ of corti. *Dev. Cell* 16, 58–69. doi: 10.1016/j.devcel.2008.11.008
- Flores-Otero, J., Xue, H. Z., and Davis, R. L. (2007). Reciprocal regulation of presynaptic and postsynaptic proteins in bipolar spiral ganglion neurons by neurotrophins. *J. Neurosci.* 27, 14023–14034. doi: 10.1523/JNEUROSCI.3219-07.2007
- Francis, S. P., and Cunningham, L. L. (2017). Non-autonomous cellular responses to ototoxic drug-induced stress and death. *Front. Cell. Neurosci.* 11:252. doi: 10.3389/fncel.2017.00252
- Garfinkle, T. J., and Saunders, J. C. (1983). Morphology of inner hair cell stereocilia in C57BL/6J mice as studied by scanning electron microscopy. *Otolaryngol. Head Neck Surg.* 91, 421–426. doi: 10.1177/019459988309100415
- Glueckert, R., Pritz, C. O., Roy, S., Dudas, J., and Schrott-Fischer, A. (2015). Nanoparticle mediated drug delivery of rolipram to tyrosine kinase B positive cells in the inner ear with targeting peptides and agonistic antibodies. *Front. Aging Neurosci.* 7:71. doi: 10.3389/fnagi.2015.00071
- Gstraunthaler, G., Lindl, T., and van der Valk, J. (2013). A plea to reduce or replace fetal bovine serum in cell culture media. *Cytotechnology* 65, 791–793. doi: 10.1007/s10616-013-9633-8
- Gunewardene, N., Crombie, D., Dottori, M., and Nayagam, B. A. (2016). Innervation of cochlear hair cells by human induced pluripotent stem cell-derived neurons *in vitro*. *Stem Cells Int.* 2016:1781202. doi: 10.1155/2016/1781202
- Haque, K. D., Pandey, A. K., Kelley, M. W., and Puligilla, C. (2015). Culture of embryonic mouse cochlear explants and gene transfer by electroporation. *J. Vis. Exp.* 52260. doi: 10.3791/52260
- Harrill, J. A., Robinette, B. L., Freudenrich, T. M., and Mundy, W. R. (2015). Media formulation influences chemical effects on neuronal growth and morphology. *In vitro* cellular and developmental biology. *Animal* 51, 612–629. doi: 10.1007/s11626-015-9873-3
- Homma, S., and Yamamoto, N. (1990). Activation process of macrophages after *in vitro* treatment of mouse lymphocytes with dodecylglycerol. *Clin. Exp. Immunol.* 79, 307–313.
- Jiang, P., Ray, A., Rybak, L. P., and Brenner, M. J. (2016). Role of STAT1 and oxidative stress in gentamicin-induced hair cell death in organ of corti. *Otol. Neurotol.* 37, 1449–1456. doi: 10.1097/MAO.0000000000001192
- Kalinec, G., Thein, P., Park, C., and Kalinec, F. (2016). HEI-OC1 cells as a model for investigating drug cytotoxicity. *Hear. Res.* 335, 105–117. doi: 10.1016/j.heares.2016.02.019
- Kamiya, K., Takahashi, K., Kitamura, K., Momoi, T., and Yoshikawa, Y. (2001). Mitosis and apoptosis in postnatal auditory system of the C3H/He strain. *Brain Res.* 901, 296–302. doi: 10.1016/S0006-8993(01)02300-9
- Kopke, R. D., Liu, W., Gabaizadeh, R., Jacono, A., Feghali, J., Spray, D., et al. (1997). Use of organotypic cultures of corti's organ to study the protective effects of antioxidant molecules on cisplatin-induced damage of auditory hair cells. *Am. J. Otol.* 18, 559–571.
- Lee, J. H., Oh, S. H., Kim, T. H., Go, Y. Y., and Song, J. J. (2017). Anti-apoptotic effect of dexamethasone in an ototoxicity model. *Biomater. Res.* 21:4. doi: 10.1186/s40824-017-0090-x
- Lim, R., Drury, H. R., Camp, A. J., Tadros, M. A., Callister, R. J., and Brichta, A. M. (2014). Preliminary characterization of voltage-activated whole-cell currents in developing human vestibular hair cells and calyx afferent terminals. *J. Assoc. Res. Otol.* 15, 755–766. doi: 10.1007/s10162-014-0471-y
- Lim, R., Kindig, A. E., Donne, S. W., Callister, R. J., and Brichta, A. M. (2011). Potassium accumulation between type I hair cells and calyx terminals in mouse crista. *Exp. Brain Res.* 210, 607–621. doi: 10.1007/s00221-011-2592-4.
- Ma, X., Liu, Y., Muhammad, W., Liu, D., Wang, J., Zhou, H., et al. (2017). Autophagy-related protein 12 associates with anti-apoptotic B cell lymphoma-2 to promote apoptosis in gentamicin-induced inner ear hair cell loss. *Mol. Med. Rep.* 15, 3819–3825. doi: 10.3892/mmr.2017.6458
- Marcotti, W., Johnson, S. L., Rusch, A., and Kros, C. J. (2003). Sodium and calcium currents shape action potentials in immature mouse inner hair cells. *J. Physiol.* 552 (Pt 3), 743–761. doi: 10.1113/jphysiol.2003.043612
- Mulvaney, J. F., and Dabdoub, A. (2014). Long-term time lapse imaging of mouse cochlear explants. *J. Vis. Exp.* e52101. doi: 10.3791/52101
- Nayagam, B. A., Edge, A. S., Needham, K., Hyakumura, T., Leung, J., Nayagam, D. A., et al. (2013). An *in vitro* model of developmental synaptogenesis using cocultures of human neural progenitors and cochlear explants. *Stem Cells Dev.* 22, 901–912. doi: 10.1089/scd.2012.0082
- Nayagam, B. A., and Minter, R. L. (2012). A comparison of *in vitro* treatments for directing stem cells toward a sensory neural fate. *Am. J. Otolaryngol.* 33, 37–46. doi: 10.1016/j.amjoto.2010.12.009
- Nicholas, B. D., Francis, S., Wagner, E. L., Zhang, S., and Shin, J. B. (2017). Protein synthesis inhibition and activation of the C-jun N-terminal kinase are potential contributors to cisplatin ototoxicity. *Front. Cell. Neurosci.* 11:303. doi: 10.3389/fncel.2017.00303
- Nordemar, H. (1983). Embryogenesis of the inner ear. II. The late differentiation of the mammalian crista ampullaris *in vivo* and *in vitro*. *Acta Otolaryngol.* 96, 1–8.
- Park, C., Thein, P., Kalinec, G., and Kalinec, F. (2016). HEI-OC1 cells as a model for investigating prestin function. *Hear. Res.* 335, 9–17. doi: 10.1016/j.heares.2016.02.001
- Parker, M., Brugaud, A., and Edge, A. S. (2010). Primary culture and plasmid electroporation of the murine organ of corti. *J. Vis. Exp.* 1685. doi: 10.3791/1685
- Perny, M., Solyga, M., Grandgirard, D., Roccio, M., Leib, S. L., and Senn, P. (2017). Streptococcus pneumoniae-induced ototoxicity in organ of corti explant cultures. *Hear. Res.* 350, 100–109. doi: 10.1016/j.heares.2017.04.012
- Praetorius, M., Hsu, C., Baker, K., Brough, D. E., Plinkert, P., and Staeker, H. (2010). Adenovector-mediated hair cell regeneration is affected by promoter type. *Acta Otolaryngol.* 130, 215–222. doi: 10.3109/00016480903019251
- Rey-Giraud, F., Hafner, M., and Ries, C. H. (2012). *In vitro* generation of monocyte-derived macrophages under serum-free conditions improves their tumor promoting functions. *PLoS ONE* 7:e42656. doi: 10.1371/journal.pone.0042656
- Russell, I. J., and Richardson, G. P. (1987). The morphology and physiology of hair cells in organotypic cultures of the mouse cochlea. *Hear. Res.* 31, 9–24.
- Sekerková, G., Richter, C. P., and Bartles, J. R. (2011). Roles of the espin actin-bundling proteins in the morphogenesis and stabilization of hair cell stereocilia revealed in CBA/CaJ congenic jerker mice. *PLoS Genet.* 7:e1002032–32. doi: 10.1371/journal.pgen.1002032
- Sobkowicz, H. M., Bereman, B., and Rose, J. E. (1975). Organotypic development of the organ of corti in culture. *J. Neurocytol.* 4, 543–572.
- Sobkowicz, H. M., Loftus, J. M., and Slapnick, S. M. (2009a). Tissue culture of the organ of corti: part II. *Acta Otolaryngol.* 113 (Suppl 502), 20–36. doi: 10.3109/00016489309130127

- Sobkowicz, H. M., Loftus, J. M., and Slapnick, S. M. (2009b). Tissue culture of the organ of corti: part I. *Acta Otolaryngol.* 113 (Suppl 502), 1–19. doi: 10.3109/00016489309130126
- Tung, V. W., Di Marco, S., Lim, R., Brichta, A. M., and Camp, A. J. (2013). An isolated semi-intact preparation of the mouse vestibular sensory epithelium for electrophysiology and high-resolution two-photon microscopy. *J. Vis. Exp.* e50471. doi: 10.3791/50471
- Walters, B. J., Diao, S., Zheng, F., Walters, B. J., Layman, W. S., and Zuo, J. (2015). Pseudo-immortalization of postnatal cochlear progenitor cells yields a scalable cell line capable of transcriptionally regulating mature hair cell genes. *Sci. Rep.* 5:17792. doi: 10.1038/srep17792
- Walters, B. J., and Zuo, J. (2013). Postnatal development, maturation and aging in the mouse cochlea and their effects on hair cell regeneration. *Hear. Res.* 297, 68–83.
- Wu, J., Li, W., Lin, C., Chen, Y., Cheng C., Sun, S., et al. (2018). Co-regulation of the notch and wnt signaling pathways promotes supporting cell proliferation and hair cell regeneration in mouse utricles. *Sci. Rep.* 6:29418. doi: 10.1038/srep29418
- Wu, Q., Wang, G. P., Xie, J., Guo, J. Y., and Gong, S. S. (2015). Tumor necrosis factor-alpha-induced ototoxicity in mouse cochlear organotypic culture. *PLoS ONE* 10:e0127703. doi: 10.1371/journal.pone.0127703
- Ye, J. H., Zhang, J., Xiao, C., and Kong, J. Q. (2006). Patch-clamp studies in the cns illustrate a simple new method for obtaining viable neurons in rat brain slices: glycerol replacement of NaCl protects CNS neurons. *J. Neurosci. Methods* 158, 251–259. doi: 10.1016/j.jneumeth.2006.06.006

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SCIENTIFIC REPORTS

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An intronic mutation in *Chd7* creates a cryptic splice site, causing aberrant splicing in a mouse model of CHARGE syndrome

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Alternate splicing is a critical regulator of gene expression in eukaryotes, however genetic mutations can cause erroneous splicing and disease. Most recorded splicing disorders are caused by mutations of splice donor/acceptor sites, however intronic mutations can affect splicing. Clinical exome analyses largely ignore intronic sequence, limiting the detection of mutations to within coding regions. We describe 'Trooper', a novel mouse model of CHARGE syndrome harbouring a pathogenic point mutation in *Chd7*. The mutation is 18 nucleotides upstream of exon 10 and creates a cryptic acceptor site, causing exon skipping and partial intron retention. This mutation, though detectable in exome sequence, was initially dismissed by computational filtering due to its intronic location. The *Trooper* strain exhibited many of the previously described CHARGE-like anomalies of CHD7 deficient mouse lines; including hearing impairment, vestibular hypoplasia and growth retardation. However, more common features such as facial asymmetry and circling were rarely observed. Recognition of these characteristic features prompted manual reexamination of *Chd7* sequence and subsequent validation of the intronic mutation, highlighting the importance of phenotyping alongside exome analyses. The *Trooper* mouse serves as a valuable model of atypical CHARGE syndrome and reveals a molecular mechanism that may underpin milder clinical presentation of the syndrome.

Alternate splicing is a critical mechanism of gene regulation, with both spliceosomal assembly and catalysis conserved in eukaryotic cells. Simple point mutations causing incorrect mRNA splicing were once considered to be responsible for around 15% of human genetic disease, however computational estimates now suggest that over 50% of genetic disorders are caused by aberrant splicing^{1,2}. Additionally, intronic sequence is now understood to contain critical signals such as splice donor (5'), acceptor (3') and branch sites that define exon/intron boundaries and trigger the correct removal of non-coding sequence from precursor mRNA transcripts to produce mature mRNA. Mutations within consensus sequences can result in cryptic splice site activation, exon skipping and intron retention or frameshift - which in turn affects gene expression and functionality resulting in severe disease, such as CHARGE syndrome.

CHARGE syndrome is a rare human disorder characterized by ocular Coloboma, Heart defects, choanal Atresia, Retarded growth, Genital hypoplasia and Ear anomalies. In most cases, the syndrome is caused by a point mutation within the chromo domain helicase DNA binding protein 7 gene (*CHD7*). Numerous pathogenic mutations in *CHD7* splice sites have been recorded in humans, however, their prevalence is low, which may be due to stringent criteria set in exome analysis pipelines³⁻⁵. For example, intronic mutations are predominantly considered pathogenic when present within the canonical splice donor-acceptor pair and mutations outside of this intronic region are disregarded as variants of unknown significance. However, a growing body of evidence suggests that even deep intronic mutations are able to severely disrupt gene expression⁶. Herein we describe a

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murine model of CHARGE syndrome carrying an ethylnitrosourea (ENU) mutagenesis -induced point mutation in *Chd7*. Interestingly, the pathogenic non-canonical splice mutation occurs 16 nucleotides upstream of the 3' AG splice site, however intron retention and exon skipping occurs. The *Trooper* mouse presents with a mild CHARGE-like phenotype, exhibiting a range of anomalies including growth retardation, hearing-impairment and vestibular hypoplasia. Thus, the *Trooper* mouse is a valuable model of atypical human CHARGE syndrome and the molecular mechanisms that underpin this condition.

Methods

Ethics Statement. Procedures were approved by the animal ethics committees of The Walter and Eliza Hall Institute (WEHI) and Murdoch Childrens Research Institute (MCRI) in project numbers 2011.016 and A726 respectively. All experiments conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th edition, 2013.

Mice. Colonies of mice were maintained at the WEHI and the MCRI. Mice housed at MCRI were group-housed in individually ventilated micro-isolator cages (Tecniplast, Buguggiate, VA, Italy) on a 14 hour light/10 hour dark cycle. Mice housed at WEHI were group-housed in individually ventilated micro-isolator cages (Airlaw, Smithfield, NSW, Australia) on a 12 hour light/12 hour dark cycle. Animals were provided with standard Barastoc mouse chow (Ridley AgriProducts, Melbourne, VIC, Australia) and sterilized water *ad libitum*. Environmental enrichment was provided in the form of cardboard toys and sunflower seeds.

Mutagenesis Screen. Male BALB/c mice were injected intraperitoneally with 85 mg/kg ethylnitrosourea (ENU, Sigma-Aldrich, Castle Hill, NSW, Australia) weekly for 3 weeks as previously described⁷. After a rest period of 12 weeks to recover fertility, treated males were backcrossed with untreated BALB/c females. The resulting progeny (G1) were screened using an acoustic startle response (ASR) test at 8 weeks of age. Mice with an ASR below 200 mV in response to white noise bursts of 115 dB SPL were test-mated to determine heritability of the phenotype. The *Trooper* strain was serially backcrossed to BALB/c for 15 generations prior to phenotypic analysis.

Genotyping. Genomic DNA was isolated from ear punctures as previously described⁸. Mice were genotyped for the *Chd7 Trooper* mutation using the Amplifluor SNPs HT genotyping system FAM-JOE and Amplifluor Assay Optimization Buffers (S + 25 mM MgCl) (Merck Millipore, Kilsyth, VIC, Australia) using the primers GAAGGTGACCAAGTTCATGCTGGTCAAATGACTTTAGTTCTGATTT (forward wild-type), GAAGGTGACCAAGTTCATGCTGGTCAAATGACTTTAGTTCTGATTA (forward mutant) and TCATAAGGGAGTGAGACCA (reverse).

Acoustic Startle Response. The SR-LAB plug and play ASR system (San Diego Instruments, San Diego, CA, USA) was used to measure the startle response. Testing was conducted in an illuminated environment during the light phase of the lighting cycle. A perspex restraint chamber was used and mice were acclimatised to 70 dB SPL of background white noise for one minute. Clicks were presented in a pre-programmed pseudorandom order and separated by intervals of three to eight seconds. Each mouse underwent six trials of 70, 85, 90, 95 and 100 dB SPL and 16 trials of 115 dB SPL (40 ms white noise click). The largest and smallest recording were deleted before the average startle amplitude was calculated. Prism v 6.0b software was used for data compilation (GraphPad Software Inc, La Jolla, CA, USA).

Auditory Brainstem Response. An evoked potentials workstation (Tucker Davis Technologies, Alachua, FL, USA) was used to assess the auditory brainstem response (ABR) as previously described⁹. Intraperitoneal injection of 100 mg/kg ketamine and 20 mg/kg xylazine was used and anesthetized mice were kept on a heat pad with eyes protected using REFRESH night time (Allergan, NSW, Australia). A free-field magnetic speaker (model FF1, Tucker Davis Technologies) was placed 10 cm from the left pinna. Computer-generated clicks (100 μ s duration, with a spectrum of 0–50 kHz) and 3 ms pure tone stimuli of 4, 8, 16 and 32 kHz were presented with maximum intensities of 100 dB SPL. Subdermal needle electrodes (S06666-0, Rochester Electro-Medical, Inc., Lutz, FL, USA) were positioned at the apex of the skull (+ve), the left cheek (–ve) and the left hind leg (ground). ABRs traces were calculated and analysed using the average of 512 stimuli repeats in BioSig software (Tucker Davis Technologies). The threshold was detected by visual analysis, as the lowest intensity stimulus that reproducibly evoked an ABR.

Mutation Identification

Massively Parallel Sequencing. Exome sequencing was completed by the Australian Genome Research Facility (AGRF) using the 100803_MM9_exome_rebal_2_EZ_HX1 exome capture array (Roche Nimblegen, Madison, WI, USA), TruSeq Sample Preparation Kit (Illumina, San Diego, CA, USA) and HiSeq. 2000 Sequencing System (Illumina) using DNA isolated from two N7 *Chd7*^{+/Trooper} liver samples. The Bioinformatics Team at the Australian Phenomics Facility then utilised a custom analysis pipeline to align sequence reads to the reference genome (C57BL/6 NCBI m37). Raw single nucleotide variant (SNV) calls were filtered and a list of candidate SNVs created as described¹⁰.

Data Availability. Deep-sequencing data was deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive, SRA accession: SRP126877 (<https://www.ncbi.nlm.nih.gov/sra/SRP126877>).

Linkage Mapping. A mapping cohort was produced by intercrossing BALB/c-*Chd7*^{+/Trooper} with C57BL/6 mice. At 8 weeks of age, F1 offspring were ABR tested and those with hearing impairment (click threshold >40 dB

SPL) were intercrossed, generating 336 F1N1 progeny. Progeny were ABR-tested at 8 weeks of age and euthanized for the collection of tissues. Genomic DNA was isolated from liver sections as previously described⁸. Twenty hearing-impaired F1N1 progeny (click ABR ≥ 45 dB SPL) were genotyped for 660 SNPs spaced at 5–10 Mb intervals throughout the genome using the iPLEX Gold method¹¹, the MassARRAY System Sequenom, San Diego, CA, USA) and an Autoflex MALDI-TOF mass spectrometer (Bruker, Billerica, MA, USA) at AGRF. Haplotypes were aligned in Excel version 14.3.4 (Microsoft, Redmond, WA, USA) for visual identification of shared regions of heterozygosity.

Sanger sequencing. The *Chd7 Trooper* SNV was PCR-amplified from genomic DNA and sequenced using primers GTCTTGGTCAGGGAAGCAGA and GGATATGAGCTACAGCATTTATTGAA. Capillary separation was performed at the AGRF. Seqman version 10.1 software (DNASTAR, Madison, WI, USA) was used for electropherogram sequence alignment.

Transcriptome analysis. Mice were euthanized by cervical dislocation. Brain was immediately collected, bisected and placed into RNAlater (Life Technologies) on wet ice. Following a 12 hour incubation at 4°, tissue was disrupted using a mortar and pestle. RNA was extracted using a Qiagen RNeasy miniKit and cDNA was then synthesized using the SensiFAST™ cDNA Synthesis Kit (Bioline). cDNA was amplified by PCR using primers CTCTCAGAGATTGAGGATGACCT and TTTTTCACCTGCTCTTCCG and Pfx polymerase (Invitrogen) according to manufacturers recommendations. Purified PCR products were blunt ligated into pBluescript II phagemids digested with EcoRV, and E. coli NEB10-Beta cells transformed with ligation reactions. Colonies containing inserts were identified by blue-white screening, and sequenced using the alternative reverse primer (ACAACCACGTTCAACTCCGT).

X-ray Micro-Computed Tomography (μ CT). Mice were euthanized by cervical dislocation. Cochleae were dissected from the temporal bones and stored in 4% neutral buffered formalin. μ XCT measurements were carried out using an Xradia® micro XCT200 (Carl Zeiss X-ray Microscopy, Inc.), which uses a microfocus X-ray source with a rotating sample holder and an imaging detector system. The source is a closed x-ray tube (tube voltage 40 kV, peak power 10 W). One data acquisition set consisted of 361 equiangular projections over 180 degrees providing a complete tomographic reconstruction. The exposure time was 8 seconds for each projection. The tomographic scan involved rotating the sample whilst recording transmission images on the CCD. Each projection image was corrected for the non-uniform illumination in the imaging system, determined by taking a reference image of the beam without sample. A filtered back-projection algorithm is used to obtain the 3D reconstructed image. The final three-dimensional reconstructed image size was $512 \times 512 \times 512$ voxels with the voxel size of $7.6 \mu\text{m}$ along each side and Field of View (FOV) of $(3.9 \text{ mm})^3$. Avizo-6.2 software (Mercury Computer Systems Inc., France) was used for image segmentation. Imaging, 3D modelling and evaluation were performed blinded of the genotype

Statistical Analysis. Statistical analysis was completed in Prism 6 software (GraphPad Software Inc.). Testing included two-way ANOVAs with post hoc t-tests, Wilcoxon-Mann-Whitney tests and was corrected for repeated testing using the Holm Sidak method.

Results

The *Trooper* strain emerged in an ENU mutagenesis screen that has produced numerous models of deafness^{9,12,13}. G1 founder mice were identified using the ASR test and subsequently bred to validate heritability. The *Trooper* phenotype proved heritable in an autosomal dominant fashion.

The *Trooper* Phenotype is caused by a mutation in *Chd7*. The chromosomal location of the *Chd7* mutation was first identified using meiotic mapping. A cohort of F1N1 mice were grouped as hearing impaired (threshold ≥ 45 dB SPL) or normal (hearing threshold ≤ 35 dB SPL) utilising the ABR click test. Twenty hearing impaired F1N1 mice were genotyped for 660 SNPs spaced every 5–10 Mbp throughout the genome. A shared common haplotype was observed proximally on the long arm of chromosome 4, between the centromere and rs13477553 (Fig. 1A). This localized the *Trooper* mutation to a 9Mbp region between the centromere and rs13477553.

Liver derived genomic DNA of two BALB/c-*Chd7*^{+/Trooper} N7 mice was subjected to exome enrichment and massively parallel DNA sequencing. Raw single nucleotide variant calls were filtered through a custom analysis pipeline to create a list of candidate SNVs for each mouse. However, no candidate SNVs were returned within the minimal linkage region on chromosome 4. Given that *Chd7* fell within the linkage interval and that *Trooper* mice exhibited similar phenotypic attributes to the previously described *Chd7*^{Looper} mouse⁹, the exome sequence of *Chd7* was re-inspected. Visual examination of sequence alignment data showed that a point mutation within intron 9 of *Chd7* (c.3219-18 T > A, NCBI reference sequence NM_001277149.1) had been detected, but had subsequently been filtered out by the computational pipeline. Sanger sequencing of genomic DNA from hearing impaired and normal littermates validated the SNV (Fig. 1B).

The *Trooper* mutation creates a cryptic splice site. To investigate transcriptome changes, PCR amplification of *Chd7*^{+/Trooper} complementary DNA was performed. Gel electrophoresis indicated that three transcripts were present. For validation, molecular cloning of the mutant transcripts was performed in a blue/white screen. PCR sequencing of 44 colonies revealed three different transcripts (Fig. 2A), 14 of which had exon 10 skipped, 6 retained part of intron 10 and 24 were wild type (Fig. 2B and C).

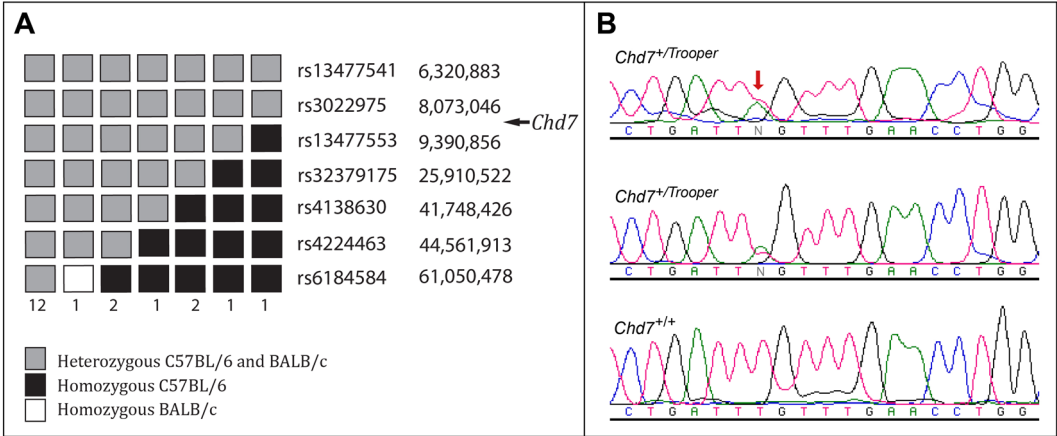


Figure 1. *Trooper* mice harbour an intronic point mutation in *Chd7*. (A) 20 F₁N₁ mice were classified as hearing impaired following click-ABR testing (threshold ≥ 45 dB SPL). SNP Haplotypes for the proximal region of chromosome 4 are illustrated, with the numbers below each haplotype representing the number of mice observed with that haplotype. The region between the centromere and rs13477553 was heterozygous in all hearing impaired mice. (B) DNA sequence electropherograms of a region of *Chd7* intron 9 in two hearing impaired *Trooper* mice and an unaffected littermate. Both affected mice were heterozygous for a c.3219-18T>A mutation in *Chd7*, which was predicted to affect splicing.

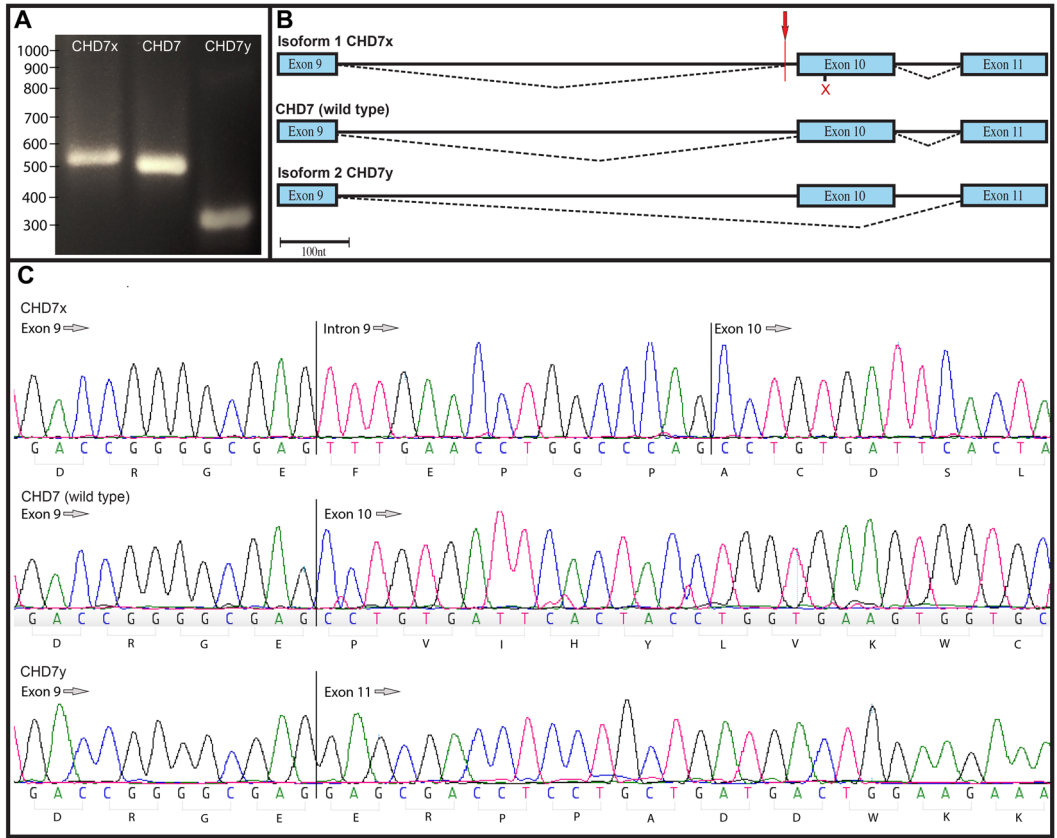


Figure 2. The *Trooper* mutation causes alternate *Chd7* transcripts. (A) An agarose gel electrophoresis illustrating the three PCR products obtained from the blue/white screen. PCR products were stained using gel red and run through 2.5% agarose for 120 minutes at 80 V. (B) A schematic representation of splicing around the *Trooper* mutation in the three amplicons. *Chd7_x* retains 16 nt of intron 9 immediately following the mutation site (red arrow), resulting in frame shift which then causes a premature stop codon in exon 10 (red x). The wild type transcript is present as mice are heterozygous for the mutation and the smallest of the transcripts, *Chd7_y*, is created by the skipping of exon 10 and remains in frame. (C) Sequence electropherograms of the three amplicons in the region of the *Chd7 Trooper* mutation.

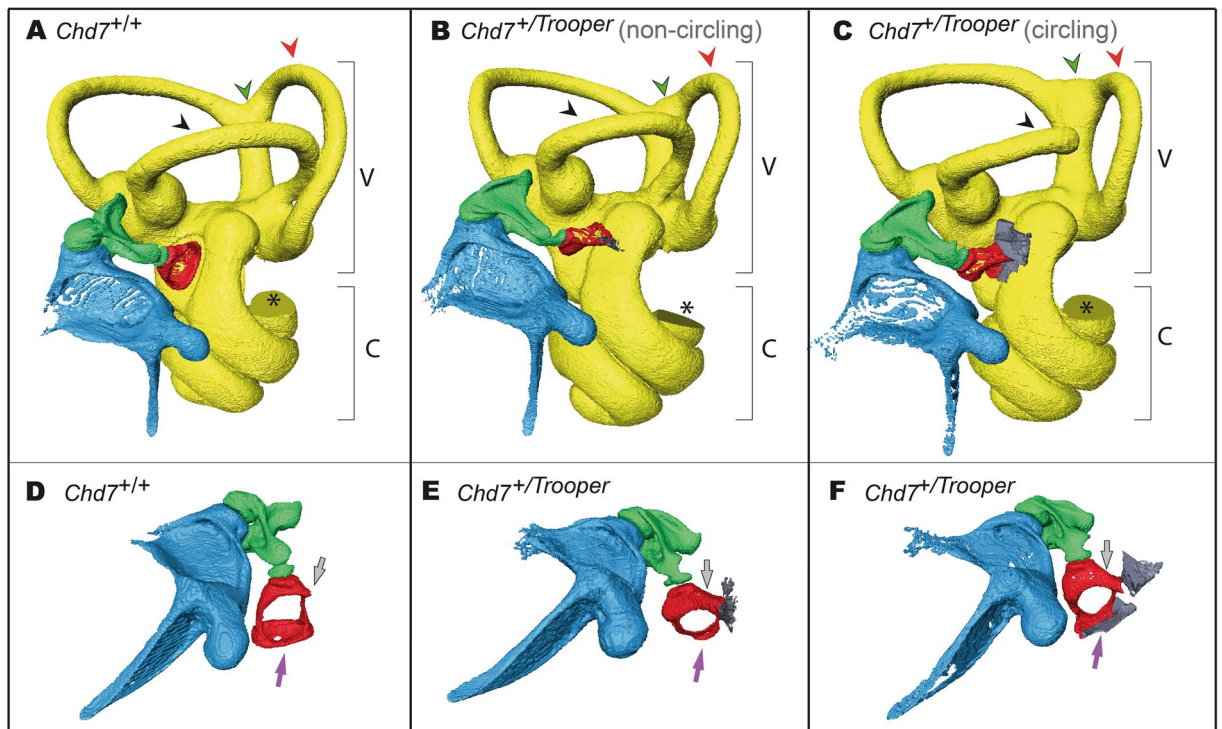


Figure 3. *Trooper* mice have ossicle and semicircular canal malformations. (A–C) X-ray μ CT images of the left middle and inner ears of $Chd7^{+/+}$ and $Chd7^{+/Trooper}$ mice including the vestibular apparatus (V) and cochlea (C). The anterior canal (red arrowhead) is hypoplastic and the intersection of the posterior and superior canals (green arrowhead) is enlarged and misshapen in both $Chd7^{+/Trooper}$ mice when compared with a $Chd7^{+/+}$ control. (B) In the non-circling $Chd7^{+/Trooper}$ mouse the lateral semicircular canal (black arrowhead) is complete but hypoplastic. (C) In the circling $Chd7^{+/Trooper}$ mouse the lateral semicircular canal (black arrowhead) is incomplete. The missing section in each cochlea (*) is an artifact of the imaging/reconstruction process. $n = 1$ $Chd7^{+/+}$ and 2 $Chd7^{+/Trooper}$ mice. (D,E) An alternate view of the above middle ears. The $Chd7^{+/Trooper}$ stapes was rounded and small with an unusual bone growth extending from the tubercle to the otic capsule (grey arrow) and the stapes footplate (purple arrow) was also deformed. In the non-circling *Trooper*, the footplate was under developed and not contacting the oval window however the more severely affected $Chd7^{+/Trooper}$ (with circling behavior) has stapedia fusion with the oval window.

***Trooper* Mice Have Middle Ear defects and Vestibular Organ Hypoplasia.** To assess *Trooper* mice for phenotypes associated with CHARGE syndrome, the middle and inner ears were imaged using μ CT. This was done on *Trooper* mice with and without circling behavior. The $Chd7^{+/Trooper}$ stapes was malformed in a small rounded shape and an unusual bone growth extended between the tubercle and otic capsule. In the non-circling mouse, μ CT imaging showed that the stapes footplate was deformed and did not contact the oval window (Fig. 3B). However, a more severely affected $Chd7^{+/Trooper}$ (with circling behavior) had the stapedia footplate fused to the oval window (Fig. 3C). μ CT also showed partial development of the lateral semicircular canal and varied levels of posterior and anterior canal hypoplasia. The intersection of the posterior and superior canals is enlarged and misshapen.

In CHARGE affected individuals, hypoplasia of the semicircular canals can cause vestibular areflexia, resulting in poor motor and speech development¹⁴. In mice, this impairment manifests in head bobbing, tilting and circling behaviors¹⁵. These traits were observed in *Trooper* mice indicating that their vestibulo-ocular reflex is impaired. However, a full vestibular evaluation was not performed.

***Trooper* mice are hearing impaired.** ABR thresholds of $Chd7^{+/Trooper}$ mice were significantly elevated between 4 and 16 kHz when compared with wild type controls (Fig. 4A). Hearing impairment was greatest at lower frequencies, with an average threshold shift of 35–40 dB SPL at 4 and 8 kHz. An average threshold shift of 20 dB was observed at 16 kHz. At 32 kHz, $Chd7^{+/Trooper}$ mice tended to have slight hearing threshold elevation, however this was not significant.

The *Trooper* mutation is homozygous lethal. $Chd7^{+/Trooper}$ mice crossed with BALB/c mice produced 277 progeny consisting of 148 $Chd7^{+/+}$ (66f, 82m) and 129 $Chd7^{+/Trooper}$ (71f, 58m). We expected a 1:1 sex and genotype ratio and no significant deviation from this distribution was observed (two tailed $P = 0.224$, using the Chi Square test). Intercrossing of $Chd7^{+/Trooper}$ mice produced 39 offspring, none of which were homozygous for the mutation (deviating significantly from the expected distribution-two tailed $P = 0.002$, using the Chi Square test). 18 mice were $Chd7^{+/+}$ (9f, 9m) and 21 were $Chd7^{+/Trooper}$ (14f, 7m), indicating that homozygotes were dying embryonically. Heterozygous mice were fertile and reasonably healthy, but displayed growth deficiency (Fig. 4B–D).

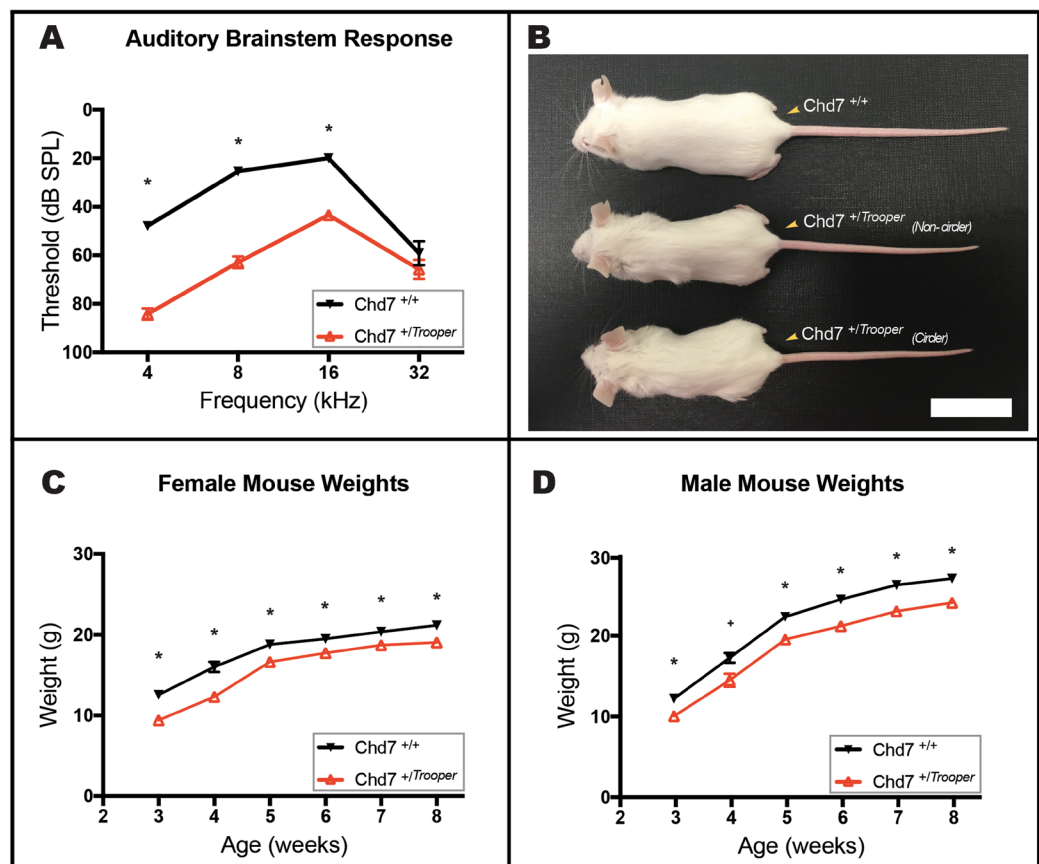


Figure 4. Trooper mice are hearing impaired and have growth deficiencies. (A) Average ABR thresholds of *Chd7*^{+/Trooper} (n = 12) and *Chd7*^{+/+} (n = 12) mice. *Chd7*^{+/Trooper} thresholds were significantly elevated at 4, 8 and 16 kHz in comparison to *Chd7*^{+/+} controls. **p* < 0.001 based on t- tests performed with the Holm-Sidak correction for multiple comparisons. Error Bars = SEM. (B) Photograph of female *Chd7*^{+/Trooper} and *Chd7*^{+/+} littermates (at 50 days of age) illustrating the reduced size and length of *Chd7*^{+/Trooper} mice. Scale Bar = 3 cm. (C,D) Average weights of male and female *Chd7*^{+/+} (n = 19 female and 20 male) and *Chd7*^{+/Trooper} (n = 26 female and 21 male) mice, from 3–8 weeks of age.

Discussion and Conclusions

The *Trooper* mutation emerged from an ENU mutagenesis screen designed to identify hearing impaired mice. Linkage mapping, exome sequencing and Sanger sequencing were used to pinpoint a mutation (c.3219-18T > A) in the *Chromodomain Helicase DNA binding 7* (*Chd7*) gene. Mutations in this gene cause a pattern of defects collectively known as CHARGE syndrome in humans. CHARGE is a complex disease, affecting multiple organs with extreme heterogeneity between individuals. Likewise, variable phenotypic penetrance is common amongst the numerous murine CHARGE models. *CHD7* deficient mouse lines are typically named after their striking circling behavior. Examples such as *Cyclone*, *Dizzy*¹⁶, *Whirligig*¹⁷ and *Looper*⁹ have marked vestibular deformity, severe eye anomalies, choanal defects, cleft palate and multiple minor CHARGE features. On the other hand, tail chasing is essentially absent from the *Trooper* strain. Choanal atresia and heart defects are unlikely given the survival rates of *Chd7*^{+/Trooper} mice post weaning, and common pathologies such as microphthalmia and blepharoconjunctivitis were rarely observed (Table 1). Additionally *Chd7*^{+/Trooper} hearing loss was less severe than in other strains (Fig. 5)⁹. Overall, the *Trooper* phenotype is particularly mild (Table 1). Like *Trooper*, the atypical CHARGE phenotype in humans lacks major diagnostic features such as heart defects and choanal atresia. However hearing loss, growth deficiency and inner ear malformations remain common¹⁸. The consistency of inner ear malformation across all forms of CHARGE syndrome highlights that structural patterning in the ear is particularly sensitive to *CHD7* expression levels. Indeed, *CHD7* knockdown inhibits neural crest cell migration into the pharyngeal arches, impacting derived structures such as the ossicle chain¹⁹. To date, functional analysis of *CHD7* has proven difficult, due to the exceptional size of the gene (185Kbp, with 42 exons, GRCh38.p7, NC_000008.11, protein ~ 336 kDa). As a result, there is no clear explanation as to why some patients present with a milder form of the disease, however it is thought that *CHD7* truncating mutations cause a more severe phenotype than missense mutations²⁰. *CHD7* deficiency does affect cellular differentiation, proliferation and migration in a dose specific manner, which may account for some of the milder CHARGE phenotypes¹⁹. Yet, a genotype-phenotype correlation does not exist. This is most clearly evident when multiple sibling pairs (including monozygotic twins) carrying identical mutations present with significant phenotypic differences⁵.

The *Trooper* mutation creates an AG marker within a cryptic splice site, that interrupts canonical splicing of *Chd7*. In eukaryotes, dinucleotides at the 5' (GT) and 3' (AG) ends of intronic sequence are essential for specifying

Phenotype	<i>Chd7</i> Trooper	<i>Trooper</i> WT	<i>Chd7</i> Looper	<i>Looper</i> WT
Circling	4% (2/52)	0% (0/46)	83% (5/6)	0% (0/10)
Hearing impairment	100% (12/12)	0% (0/12)	100% (18/18)	0% (0/22)
Semicircular Canal malformation	100% (2/2)	0% (0/1)	100% (3/3)	0% (0/1)
Blepharoconjunctivitis	12% (6/52)	0% (0/46)	69% (11/16)	0% (0/19)
Post Weaning Survival (of total colony population)	53% (52/98)	47% (46/98)	41% (68/167)	59% (99/167)

Table 1. Penetrance of *Trooper* pathology compared with *Looper* pathology. The *Trooper* phenotype is milder than previously reported *Chd7* mouse mutants.

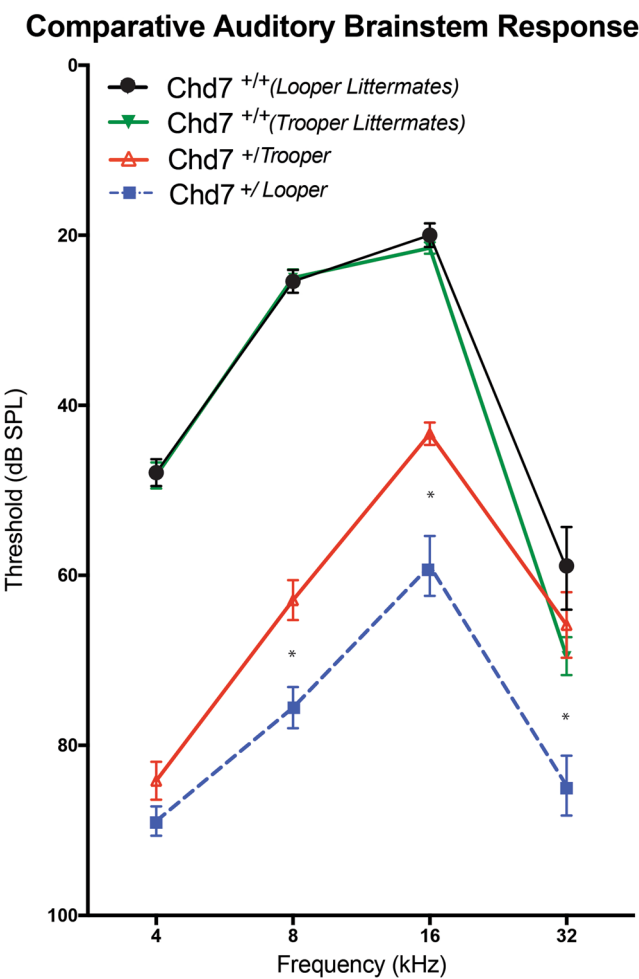


Figure 5. *Trooper* mice have a milder hearing phenotype than the previously reported *Looper* strain. Average ABR thresholds of *Chd7*^{+/+}/*Trooper*, *Chd7*^{+/+}/*Looper*, and their *Chd7*^{+/+} controls. The hearing of *Trooper* mice is on average, significantly better than *Looper* mice (**p* < 0.002 based on t- tests performed with the Holm-Sidak correction for multiple comparisons). *Chd7*^{+/+}/*Looper* and the corresponding *Chd7*^{+/+} control cohort were previously published in Ogier *et al.*⁹).

splice site positions. These markers define exon boundaries and lead to the recruitment of the spliceosome for intron removal. Multiple cryptic splice sites exist within precursor-mRNAs, however they are not used unless the authentic splice site is interrupted by mutation. In rarer cases, such as the *Trooper* mutation, intronic de novo 3' splice sites are activated by the creation of an AG marker in the polypyrimidine tract²¹. Generally, activated cryptic splice sites are within 100 nucleotides and upstream of the authentic splice site²². The *Trooper* mutation

is consistent with these observations and is preceded by a pyrimidine rich sequence, which likely initiates spliceosome assembly. Thus, the *Chd7*^{+/Trooper} mutation activates a cryptic splice site, prematurely signaling the end of intron 9. As a result, two aberrant isoforms are produced.

The isoform we termed *Chd7*_x retains 16 nucleotides from the 3' end of intron 9. This insertion causes a frame shift, leading to a premature stop codon in Exon 10. CHD7_x terminates before the helicase, BRK and SANT domains and is presumed to be non functional. The predicted truncation may induce nonsense mediated mRNA decay. However, the presence of CHD7_x cDNA in the blue/white screen shows that the product was not completely degraded prior to reverse transcription.

The second transcript, *Chd7*_y, skips Exon 10. Skipping of Exon 10 removes a large portion of the second of two chromo domains in CHD7 required for histone tail binding. However, remaining in frame, *Chd7*_y likely avoids mRNA degradation. It is certainly possible that *Chd7*_y retains some function and given the *Trooper* phenotype, a dominant negative effect is unlikely.

The atypical CHARGE pathology observed in *Chd7*^{+/Trooper} mice indicates that one of the alternate isoforms is retaining a measure of functionality, or that the spliceosome is occasionally creating enough wild type CHD7 to reach a critical threshold. As the wild type *Chd7* transcript was not significantly over represented in the blue/white screen, we predict that CHD7_y is retaining partial functionality with a single chromodomain.

Chromodomains are highly conserved, structural components of large chromatin remodeling proteins that regulate gene activity and genome organization. In a protein specific manner, chromodomains present in tandem, individually or with related 'chromo shadow domains'²³. A defining characteristic of Chromodomain Helicase DNA binding proteins is the presence of dual chromodomains. Whilst the mechanism of H3 histone binding in chromatin remodelers differs significantly between proteins containing single and dual chromodomains, the efficacy of H3 binding is comparable²⁴. Therefore CHD7_y will likely retain histone binding capabilities despite deletion of the C-terminal chromodomain. A human CHD7 splice variant (CHD7s) provides further evidence that CHD7_y may be functional without tandem chromodomains. CHD7s lacks the C-terminal chromodomain as well as the helicase/ATPase, DNA-binding, and BRK domains. CHD7s acts cooperatively with CHD7 in the nucleus and antagonizes CHD7 in the nucleoplasm²⁵. The ability of CHD7s to drive 45S rRNA gene transcription indicates that the CHD7 N-terminal chromodomain is functional in the absence of the CHD7 C-terminal chromodomain. Further analysis of the *Trooper* CHD7 isoforms will likely establish the functional importance of chromodomains within a tandem repeat.

There is mounting evidence of the significance of RNA splicing with regard to human disease, which suggests the practice of filtering out intronic variants (of unknown significance) is limiting our ability to diagnose patients. Currently, as many as 30% of CHARGE patients lack a genetic diagnosis. Some chromosomal deletions, one *SEMA3E* mutation, one *RERE* duplication and one *KMT2D* mutation have reportedly caused CHARGE like attributes^{26–28}. However *CHD7* mutations are the preponderant cause of clinically diagnosed CHARGE²⁷. We postulate that the poor genetic diagnosis rate is due to methodology in diagnostic laboratories, which tend to limit *CHD7* analysis to coding exons and dinucleotide splice sites^{3,5}. Certainly, the *Trooper* mutation would be missed with current diagnostic methods and as the 48 nt leading to exon 10 in *Chd7* is conserved between species, the mutation could be pathogenic in humans (NCBI ref seq. NG_007009.1). The sequence conservation of this region also indicates functional relevance beyond the canonical splice site.

After projects derived from the Encyclopedia of DNA Elements (ENCODE) contentiously suggested that 80% of the human genome had biochemical functions, a wealth of knowledge has been gained showing that the evolutionarily dynamic intronic sequence is biologically relevant in humans^{29–31}. In an age of rapidly evolving diagnosis via genetic testing, the *Trooper* mouse is a timely reminder that as the methodology around exome and genome sequence analysis develops, it would be wise to reconsider ways to screen for mutations in introns, particularly in sequence with homology to cryptic splice sites.

Conclusion

Trooper mice carry an intronic ENU –induced point mutation that results in alternate splicing of *Chd7*. The strain has a mild phenotypic presentation resembling atypical CHARGE syndrome including hearing impairment, hypoplasia of the semicircular canals and stapes malformation. One of the *Chd7* alternate transcripts detected in *Trooper* is predicted to be non-functional whilst the other is likely translated, with the resultant protein at least partially functional. The combined expression of CHD7 and CHD7_y in *Trooper* relative to the haploinsufficiency observed in other models such as *Looper* likely explains the milder presentation of the phenotype.

Further study of the *Trooper* *Chd7* isoforms will provide insight into the functional domains of CHD7 and clarify the dosage effects of CHD7 expression on development and function in a variety of organ systems. In addition, the identification of this mutable cryptic splice site within the intronic sequence of *Chd7* should prompt closer scrutiny of the non-coding genomic sequence of CHARGE patients for whom coding mutations of CHD7 have not been identified.

References

1. Krawczak, M., Reiss, J. & Cooper, D. N. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Human Genetics* **90**, 41–54 (1992).
2. Lopez-Bigaz, N., Audit, B., Ouzounis, C., Parra, G. & Guigo, R. Are splicing mutations the most frequent cause of hereditary disease? *FEBS Lett* **579**, 1900–1903 (2005).
3. Bartels, C. F., Scacheri, C., White, L., Scacheri, P. C. & Bale, S. Mutations in the CHD7 gene: the experience of a commercial laboratory. *Genet Test Mol Biomarkers* **14**, 881–891 (2010).
4. Kim, H.-G. *et al.* Mutations in CHD7, encoding a chromatin-remodeling protein, cause idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Am J Hum Genet* **83**, 511–519 (2008).
5. Jongmans, M. C. J. *et al.* CHARGE syndrome: the phenotypic spectrum of mutations in the CHD7 gene. *Journal of Medical Genetics* **43**, 306–314 (2006).
6. Vaz-Drago, R., Custodio, N. & Carmo-Fonseca, M. Deep intronic mutations and human disease. *Hum Genet* **136**, 1093–1111 (2017).

7. Bode, V. C. Ethylnitrosourea mutagenesis and the isolation of mutant alleles for specific genes located in the T region of mouse chromosome 17. *Genetics* **108**, 457–470 (1984).
8. Laird, P. W. *et al.* Simplified mammalian DNA isolation procedure. *Nucleic Acids Res* **19**, 4293 (1991).
9. Ogier, J. M. *et al.* CHD7 deficiency in ‘Looper’, a new mouse model of CHARGE syndrome, results in ossicle malformation, otosclerosis and hearing impairment. *PLoS One* **9**, e97559 (2014).
10. Andrews, T. D. *et al.* Massively parallel sequencing of the mouse exome to accurately identify rare, induced mutations: an immediate source for thousands of new mouse models. *Open Biol* **2**, 120061 (2012).
11. Mendisco, F. *et al.* Application of the iPLEX Gold SNP genotyping method for the analysis of Amerindian ancient DNA samples: benefits for ancient population studies. *Electrophoresis* **32**, 386–393 (2011).
12. Carpinelli, M. R., Manning, M. G., Kile, B. T. & Burt, R. A. Two ENU-induced alleles of Atp2b2 cause deafness in mice. *PLoS One* **8**, e67479 (2013).
13. Carpinelli, M. R. *et al.* A new mouse model of Canavan leukodystrophy displays hearing impairment due to central nervous system dysmyelination. *Dis Model Mech* **7**, 649–657 (2014).
14. Admiraal, R. J. & Huygen, P. L. Vestibular areflexia as a cause of delayed motor skill development in children with the CHARGE association. *Int J Pediatr Otorhinolaryngol* **39**, 205–222 (1997).
15. Vidal, P.-P., Degallaix, L., Josset, P., Gasc, J.-P. & Cullen, K. E. Postural and locomotor control in normal and vestibularly deficient mice. *J Physiol* **559**, 625–638 (2004).
16. Nolan, P. M. *et al.* A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. *Nat Genet* **25**, 440–443 (2000).
17. Hawker, K., Fuchs, H., Angelis, M. H. & Steel, K. P. Two new mouse mutants with vestibular defects that map to the highly mutable locus on chromosome 4. *Int J Audiol* **44**, 171–177 (2005).
18. Hale, C. L., Niederriter, A. N., Green, G. E. & Martin, D. M. Atypical phenotypes associated with pathogenic CHD7 variants and a proposal for broadening CHARGE syndrome clinical diagnostic criteria. *American journal of medical genetics. Part A* **170A**, 344–354 (2016).
19. Bajpai, R. *et al.* CHD7 cooperates with PBAF to control multipotent neural crest formation. *Nature* **463**, 958–962 (2010).
20. Marcos, S. *et al.* The prevalence of CHD7 missense versus truncating mutations is higher in patients with Kallmann syndrome than in typical CHARGE patients. *J Clin Endocrinol Metab* **99**, E2138–43 (2014).
21. Kralovicova, J., Christensen, M. B. & Vorechovsky, I. Biased exon/intron distribution of cryptic and de novo 3' splice sites. *Nucleic Acids Res* **33**, 4882–4898 (2005).
22. Nakai, K. & Sakamoto, H. Construction of a novel database containing aberrant splicing mutations of mammalian genes. *Gene* **141**, 171–177 (1994).
23. Aasland, R. & Stewart, A. F. The chromo shadow domain, a second chromo domain in heterochromatin-binding protein 1, HP1. *Nucleic Acids Res* **23**, 3168–3173 (1995).
24. Flanagan, J. F. *et al.* Double chromodomains cooperate to recognize the methylated histone H3 tail. *Nature* **438**, 1181–1185 (2005).
25. Kita, Y., Nishiyama, M. & Nakayama, K. I. Identification of CHD7S as a novel splicing variant of CHD7 with functions similar and antagonistic to those of the full-length CHD7L. *Genes Cells* **17**, 536–547 (2012).
26. Jordan, V. K. *et al.* Genotype-phenotype correlations in individuals with pathogenic RERE variants. *Hum Mutat* Epub ahead of print, <https://doi.org/10.1002/humu.23400> (2018).
27. Sanlaville, D. & Verloes, A. CHARGE syndrome: an update. *Eur J Hum Genet* **15**, 389–399 (2007).
28. Badalato, L. *et al.* KMT2D p.Gln3575His segregating in a family with autosomal dominant choanal atresia strengthens the Kabuki/CHARGE connection. *Am J Med Genet A* **173**, 183–189 (2017).
29. ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. *Nature* **489**, 57–74 (2012).
30. Patrushev, L. I. & Kovalenko, T. F. Functions of noncoding sequences in mammalian genomes. *Biochemistry (Mosc)* **79**, 1442–1469 (2014).
31. Rands, C. M., Meader, S., Ponting, C. P. & Lunter, G. 8.2% of the Human genome is constrained: variation in rates of turnover across functional element classes in the human lineage. *PLoS Genet* **10**, e1004525 (2014).

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Author Contributions

J.M.O. and R.A.B. wrote the manuscript. J.M.O., R.A.B., M.R.C., B.K.M., M.A.W. conceived the experiments, J.M.O., M.R.C., B.D.A. conducted the experiments. J.M.O. and B.D.A. prepared figures. All authors reviewed the manuscript.

Additional Information

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Appendix 3. Manuscript published during PhD candidature.

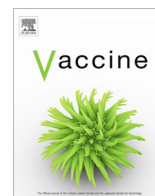
Methods used in this manuscript were also used in this thesis. Full details are provided in the Preface.



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Effect of a pneumococcal whole cell vaccine on influenza A-induced pneumococcal otitis media in infant mice



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ABSTRACT

The pneumococcus remains a common cause of otitis media (OM) despite the widespread introduction of pneumococcal conjugate vaccines. In mice, a pneumococcal whole cell vaccine (WCV) induces serotype-independent protection against pneumococcal colonisation and invasive disease via T_H17- and antibody-mediated immunity, respectively. We investigated the effect of WCV on influenza A-induced pneumococcal OM in an infant mouse model.

C57BL/6 mice were immunised subcutaneously with a single dose of WCV or adjuvant at 6 days of age, infected with pneumococci (EF3030 [serotype 19F] or PMP1106 [16F]) at 12 days of age, and given influenza A virus (A/Udorn/72/307 [H3N2], IAV) at 18 days of age to induce pneumococcal OM. Pneumococcal density in middle ear and nasopharyngeal tissues was determined 6 and 12 days post-virus. Experiments were repeated in antibody (B6.μMT^{-/-}) and CD4⁺ T-cell-deficient mice to investigate the immune responses involved.

A single dose of WCV did not prevent the development of pneumococcal OM, nor accelerate pneumococcal clearance compared with mice receiving adjuvant alone. However, WCV reduced the density of EF3030 in the middle ear at 6 days post-viral infection ($p = 0.022$), and the density of both isolates in the nasopharynx at 12 days post-viral infection (EF3030, $p = 0.035$; PMP1106, $p = 0.011$), compared with adjuvant alone. The reduction in density in the middle ear required antibodies and CD4⁺ T cells: WCV did not reduce EF3030 middle ear density in B6.μMT^{-/-} mice ($p = 0.35$) nor in wild-type mice given anti-CD4 monoclonal antibody before and after IAV inoculation ($p = 0.91$); and WCV-immunised CD4⁺ T cell-deficient GK1.5 mice had higher levels of EF3030 in the middle ear than their adjuvant-immunised counterparts ($p = 0.044$).

A single subcutaneous dose of WCV reduced pneumococcal density in the middle ears of co-infected mice in one of two strains tested, but did not prevent OM from occurring in this animal model.

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1. Introduction

Otitis media (infection of the middle ear; OM) is a common paediatric condition, and *Streptococcus pneumoniae* (pneumococcus) is

a leading cause [1,2]. OM typically arises when bacteria disseminate from the nasopharynx, where the pneumococcus resides in up to 90% of healthy children under the age of five [3], to the middle ear cavity via the Eustachian tube. Young children suffer the highest rates of OM. Pneumococcal conjugate vaccines (PCVs) induce immunity against the pneumococcal polysaccharide capsule and are highly effective against invasive pneumococcal disease [4]. However, their impact on OM has been more variable. Clinical trials evaluating the effect of the 7- and 13-valent PCVs demonstrated a 25–

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56% reduction in acute otitis media (AOM) caused by any pneumococcus, and a ~60% reduction in AOM caused by PCV-type pneumococci [5–7]. Importantly, PCVs only protect against a small subset of pneumococcal serotypes and their use has led to increases in disease caused by non-vaccine serotypes [8]. Alternative vaccines which induce protection against non-capsular pneumococcal antigens may provide enhanced protection against OM compared to PCVs.

The pneumococcal whole cell vaccine (WCV) consists of a killed, unencapsulated pneumococcal strain delivered with adjuvant [9]. WCV was developed to induce broad protection against all pneumococci at low cost. Preclinical studies have demonstrated WCV to be protective against both invasive pneumococcal disease and colonisation in mice [9,10] and have provided sufficient evidence to allow the progression of WCV into clinical trials [11]. Only one preclinical study has examined the ability of WCV to prevent pneumococcal OM. Malley et al. tested the effect of two or three doses of WCV, consisting of ethanol-killed whole cell antigen (WCA) combined with various adjuvants, given intranasally (i.n.) to adult mice before i.n. challenge with pneumococci [12]. Pneumococcal density was enumerated in nasopharyngeal and middle ear washes one week after challenge and compared to that in mice which received adjuvant alone. Each schedule of WCV (with cholera toxin adjuvant) significantly reduced the number of mice colonised with a serotype 6B isolate, and also reduced the density of nasopharyngeal colonisation [12]. Further, WCV significantly protected mice from middle ear infection with a serotype 23F isolate [12]. Despite these promising results, it is unclear whether WCV prevented the migration of pneumococci into the middle ear, or if WCV-mediated responses accelerated the clearance of pneumococci following the development of infection, as was observed for WCV-mediated protection against colonisation [13]. Moreover, the immune mechanisms involved in protection of WCV against OM have not been investigated.

Co-infection with a respiratory virus is associated with OM in young children [14,15]. We have developed an infant mouse model of pneumococcal OM [16] in which pneumococcal colonisation is induced experimentally by delivering a small dose of the bacterium to the nares of an unanaesthetised animal. Pneumococci remain localised in the upper respiratory tract for approximately five weeks [17], mimicking a carriage episode similar to that observed in young children [18,19]. Subsequent i.n. co-infection of colonised mice with influenza A virus (IAV) causes pneumococci to multiply in the nasopharynx and disseminate to the middle ear cavity, resulting in OM and hearing loss [16]. In this study, we adapted this model to investigate the effect of WCV on IAV-induced pneumococcal OM in infant mice.

2. Materials and methods

2.1. Animals

All animal experiments were approved by the Animal Ethics Committee of the University of Melbourne (1413144) and were conducted in accordance with the Prevention of Cruelty to Animals Act (1986) and the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes (2013). C57BL/6 (WT), B6.μMT^{-/-} and GK1.5 mice were bred and housed at the Biological Resources Facility (BRF), Department of Microbiology and Immunology at The Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia. Litters of mice were housed in ventilated cages with unlimited access to sterile food and water.

2.2. Bacterial and viral strains

Pneumococcal isolates EF3030 (serotype 19F) and PMP1106 (serotype 16F) were passaged in mice and recovered from the

nasopharynx before experimental use. IAV strain A/Udorn/72/307 (H3N2, A/Ud/72) was used in all experiments. Bacterial and viral infectious stocks were prepared as described previously [20].

2.3. Vaccine

WCV was prepared as described previously [21]. Whole cell antigen (WCA) consisted of pneumococcal strain RM200 (a derivative of the unencapsulated pneumococcal strain RX1E containing a non-toxic pneumolysin and deletion of the autolysin gene [22]) killed by treatment with beta-propiolactone. Each 100 μl WCV dose contained 100 μg of WCA and 2.5 μg/μl aluminium phosphate (Alhydrogel [Brenntag]) in sterile saline. In all vaccination experiments the effects of WCV were compared to a group of mice receiving the same dose of Alhydrogel in 100 μl of saline (adjuvant).

2.4. Otitis media model

Litters of 12-day-old mice were infected i.n. with $\sim 2 \times 10^3$ CFU of pneumococci in 3 μl of PBS followed by i.n. infection with $\sim 10^{2.5}$ PFU of IAV in 3 μl of PBS 6 days later. In vaccination experiments, mice were first immunised with WCV or adjuvant via subcutaneous injection to the back of the neck at 6 days of age, followed by co-infection as described above. Mice were euthanised by CO₂ inhalation at predetermined time points post-infection. Nasopharyngeal and middle ear tissue were collected for enumeration of pneumococci by culture on gentamicin horse blood agar as described previously [20]. IAV was quantified by reverse transcriptase qPCR (RTqPCR, see below). Blood samples and spleens were harvested and analysed for immune responses as described below. To obtain plasma, whole blood was collected in heparinised tubes and samples were incubated overnight at 4 °C. Red blood cells were removed by centrifugation (2400 x g for 5 min followed by a further 11,300 x g at 4 °C for 5 min). Samples were stored at -80 °C until tested. To obtain single cell suspensions of splenocytes, spleens were mechanically disassociated by passing the tissue through a 70 μm cell strainer (Falcon). Splenocytes were collected by centrifugation (350 x g at 4 °C for 5 min) and resuspended in 2 ml of TAC buffer (containing a final concentration of 17 mM Tris and 140 mM ammonium chloride) at room temperature for 10 min with infrequent inversion to lyse red blood cells. RPMI 1640 medium (Sigma-Aldrich) was added to each sample to stop the reaction and splenocytes were washed and collected by centrifugation as above and resuspended in 1 ml Dulbecco's Modified Essential Medium with Ham's F12 medium (DMEM/F12 [Gibco]).

To investigate the immune mechanisms involved in mediating WCV effects on pneumococcal OM, mice deficient in antibodies (B6.μMT^{-/-} mice [23]), CD4⁺ T cells (GK1.5 mice [24]), or CD4⁺ T cells at the time of OM (WT mice treated with GK1.5 monoclonal antibody [mAb]) were co-infected as described above with additional modifications. As previous studies have determined that antibodies are required for the development of OM in co-infected B6.μMT^{-/-} mice [25], such mice were administered serum obtained from naive WT adult mice via intraperitoneal (i.p.) injection one day before, and each day following, IAV infection (aged 17, 19–23 days-old respectively). To deplete CD4⁺ T cells at the time of OM induction, GK1.5 mAb (Walter and Eliza Hall Institute [WEHI]) was administered via i.p. injection one day before, and two days after, IAV infection (aged 17 and 20 days-old respectively). Each dose consisted of 125 μg of antibody per 10 g of body weight in a total volume of 50 μl. The mAb rat isotype IgG2b antibody (WEHI) was administered to a control group at the same concentration to control for non-specific effects of antibody injection.

2.5. IAV RTqPCR

To quantify viral loads in mouse samples, tissues were processed as described previously [20] and viral RNA was extracted using the QIAamp viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was used as a template for one-step cDNA synthesis and RTqPCR using previously published primer and probe sequences amplifying the matrix gene of IAV [26]. RTqPCR reactions were performed in duplicate using the Strata-gene MxPro 3005. Each 20 µl reaction contained 4 µl of extracted RNA, 10 µl of SensiFAST probe No-ROX one-step master mix, 0.2 µl of reverse transcriptase, 0.2 U/µl RiboSafe RNase inhibitor (all Bioline), 400 nM of each primer (Sigma-Aldrich) and 100 nM probe (Sigma-Aldrich). RTqPCR conditions were as follows: 1 cycle of 10 min at 45 °C and 1 cycle of 2 min at 95 °C followed by 40 cycles of 5 s at 95 °C and 20 s at 60 °C. To quantify the number of matrix gene copies in each sample, plasmid DNA extracted from an *E. coli* strain transformed with the 4007 bp pHW plasmid containing the matrix protein from Ud/A/72 (XLI-B pHWUd/A/72matrix) was used to generate a standard curve. IAV loads are expressed as genome copies/ml based on 1 mg of plasmid DNA being equivalent to 227.7 IAV virions (assuming one copy of the matrix gene was present in each *E. coli* plasmid).

2.6. Histology

To analyse inflammation in the middle ear cavity, haematoxylin and eosin-staining was performed [58]. Mice were euthanised and were perfused intracardially with 0.4 g/L paraformaldehyde. The middle ears were dissected and fixed for at least 1 h in 0.4 g/L paraformaldehyde (Sigma-Aldrich). Tissues were washed three times in Tris Buffered Saline (TBS), containing a final concentration of 0.05 M Tris-HCl [Sigma-Aldrich] and 9 g/L sodium chloride [Sigma-Aldrich], pH 7.6. Tissues were then incubated for seven days in decalcification buffer (100 g/L EDTA [Sigma-Aldrich] in PBS, pH 7.2–7.4) before infiltration with TBS containing 3 g/L sucrose. Decalcified tissues were embedded in a 1:1 ratio of TBS + 3 g/L sucrose and Tissue-Tek® Optimal Cutting Temperature compound (OCT [Sakura Finetek Inc]) overnight at 4 °C before being transferred to pure OCT and further incubated overnight at 4 °C. OCT was solidified using isopentane in a container with liquid nitrogen. Sections of 2 µm tissue were cut using the HM525 NX Cryostat (ThermoFisher Scientific), fixed onto glass slides and stained with haematoxylin (Sigma-Aldrich) for 2 min to identify cell nuclei, washed in distilled water, and then Scott's tap water (Sigma-Aldrich) was applied for 1 min. Sections were washed in distilled water and then counterstained with 10 g/L eosin (Sigma-Aldrich) and washed again with distilled water. Slides were dehydrated with absolute ethanol (3 × 1 min), cleared with xylene (3 × 1 min [Sigma-Aldrich]) and mounted with a coverslip. Slides were visualised using the Leica DM 1000 light microscope (Leica Microsystems) and scored blindly for inflammatory infiltrate in the middle ear cavity by an independent pathologist as described previously [16].

2.7. ELISA and ex vivo stimulation of whole blood

WCA-specific IgG was detected in plasma or in whole blood following ex vivo stimulation with vaccine antigen (WCA), or PBS as a control, using the method described by Lu et al. [9] with some modifications. 96-well ELISA plates (Maxisorp immuno plate [Nunc]) were coated overnight with 100 µg of WCA and blocked with 10 g/L BSA in PBST (PBS + 0.5 ml/L Tween20 [Sigma-Aldrich]). Samples were diluted in PBST and added to duplicate wells for 2 h at 37 °C. Anti-mouse IgG biotin-conjugated antibody (Sigma-Aldrich) was added to each well at a dilution of 1 in

5000, and plates were incubated for 1 h at 37 °C. To detect antigen-IgG complexes, wells were washed and horseradish peroxidase (HRP)-labelled Streptavidin (Streptavidin-HRP [KPL]) was added to each well at a dilution of 1 in 5000 and incubated for 1 h at room temperature. Bound streptavidin-HRP was visualised using TMB substrate (KPL) and the optical density at 450 nm was read using a CLARIOstar plate reader (BMG Labtech) or an ELx808 Absorbance reader (BioTek). A standard curve was run on each plate using a 1 in 2 dilution series of positive control sera collected from adult mice which had received two subcutaneous doses of WCV at two-week intervals, two weeks after the second dose. A 1 in 1000 dilution of the control sera was assigned a value of 1000 arbitrary units (AU), and anti-WCA IgG responses in samples were interpolated from the standard curve.

IL-17A was measured in supernatants from splenocytes stimulated as described previously [13] using the Quantikine Mouse IL-17 ELISA kit (R&D Systems Inc.) according to the manufacturer's instructions.

2.8. Flow cytometry

The depletion of CD4⁺ T cells in mice treated with GK1.5 or rat IgG2b mAb antibody was confirmed by flow cytometry. Splenocytes were prepared as described above and labelled with fluorochrome conjugated antibodies specific for cell surface markers CD4 (clone RM4.4 [BD Horizon]), CD3 (clone 17A2 [BD Horizon]) and Thy1.2 (clone 53-2.1 [eBioscience]). The splenocyte samples were analysed using an LSRII flow cytometer (BD Bioscience) according to the manufacturer's instructions. Dead cells were identified and excluded in each sample by the addition of 1 µg/10⁶ cells of propidium iodide (PI [Sigma-Aldrich]) immediately before analysis. For all samples, 1 × 10⁵ events were collected and recorded.

2.9. Statistics

Data were analysed using GraphPad Prism version 6.0 h for MacIntosh and Stata version 15.1. Experimental groups were compared using the Mann Whitney U Test (Prism). A two-tailed Fisher's exact test was used to compare differences in the number of mice which developed unilateral OM, bilateral OM or no OM (Stata). The statistical test used for each analysis is indicated in the accompanying text and figure legends. P values less than 0.05 were considered statistically significant.

3. Results

To investigate the effect of WCV immunisation on OM, we first adapted our published model [16]. Mice were colonised with pneumococci at 12 days of age and then infected with IAV at 18 days of age (Fig. 1A). Pneumococcal isolates were chosen to represent serotypes that are commonly carried (19F) or that have been increasingly detected (16F) in carriage and OM in high-risk populations following PCV introduction [27,28]. Both pneumococcal isolates tested (EF3030 and PMP1106) were able to disseminate to the middle ears post-IAV administration (Fig. 1B and C). Co-infection induced pneumococcal OM in 16 of 17 mice colonised with EF3030 (94.1%) and 17 of 18 mice colonised with PMP1106 (94.4%) at six days post-IAV. The majority of mice had cleared the bacteria from the middle ears by 14 days post-IAV infection (Fig. 1B and C). Six days after IAV-infection, most co-infected mice had bilateral pneumococcal OM, regardless of the pneumococcal isolate used (Fig. 1D and E). During clearance, the proportion of mice with bilateral OM decreased, with a resultant increase in mice with unilateral and then no OM (Fig. 1D and E), indicating that

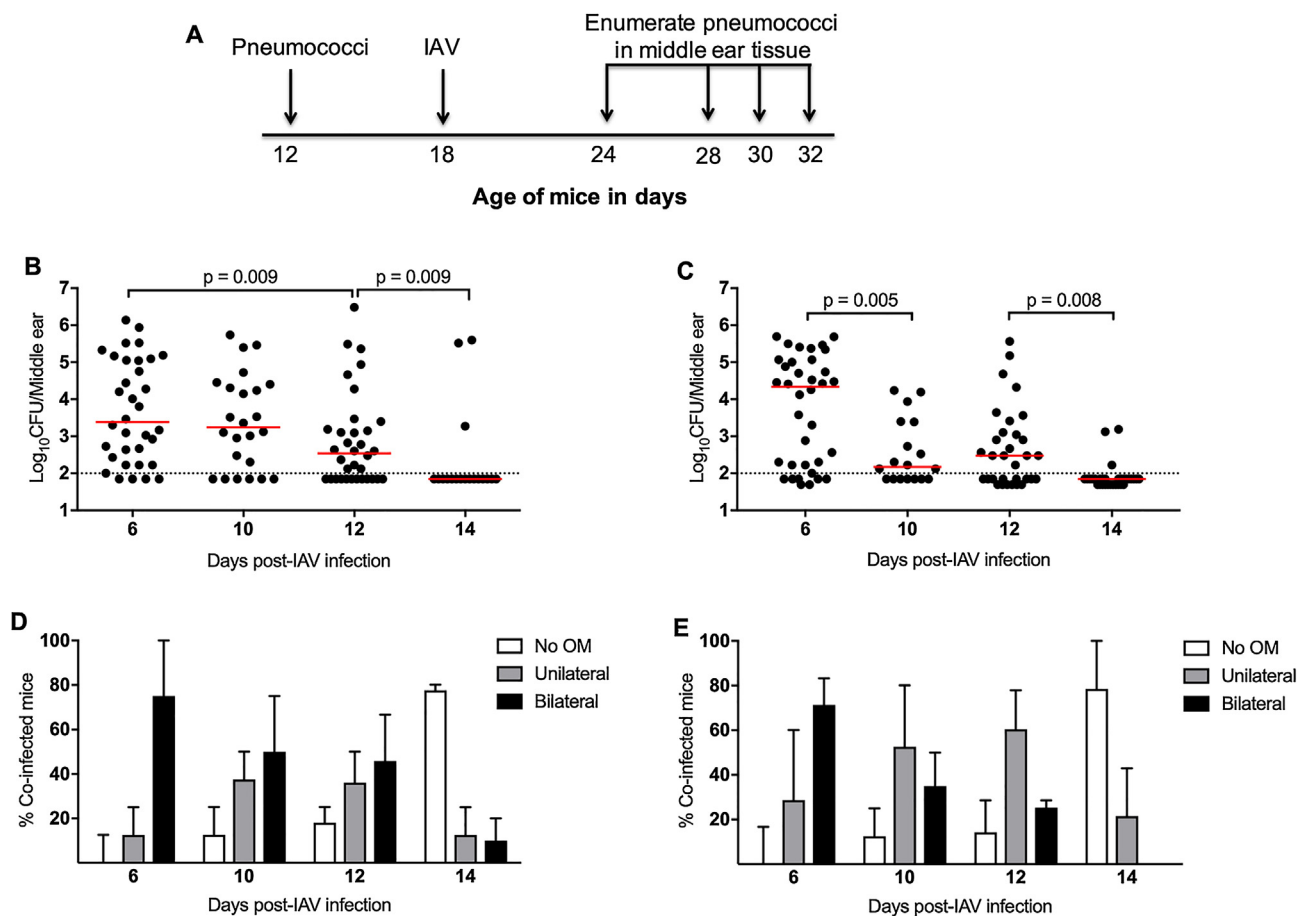


Fig. 1. Development and clearance of OM in mice co-infected with pneumococci and IAV. Mice were co-infected with pneumococci and IAV (A). At various time points post-IAV infection, pneumococcal density in the middle ear tissues was determined in mice colonised with EF3030 (B) or PMP1106 (C). Counts from each middle ear are represented as individual data points with red lines depicting the median. The dotted line represents the limit of detection (100 CFU). The proportion of co-infected mice with EF3030 (D) or PMP1106 (E) detected in neither (no OM, white bars), one (unilateral OM, grey bars) or both (bilateral OM, black bars) middle ears over time was determined. The error bars show the 95% confidence interval of the median. All graphs show data pooled from at least two independent experiments per time point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

co-infected mice clear pneumococci from each ear independently. IAV was only detected in the middle ears of 2 of 32 (EF3030) and 2 of 26 (PMP1106) co-infected mice at 6 days post-IAV and was not detected in the middle ears after this time point.

To evaluate the effect of WCV on IAV-induced pneumococcal OM at the peak of disease, infant mice were vaccinated with a single subcutaneous dose of WCV (or adjuvant) to the back of the neck at 6 days of age, and then co-infected as shown in Fig. 1A. Pneumococcal and IAV density was measured in nasopharyngeal and middle ear tissue 6 days after infection with IAV. WCV significantly reduced the density of EF3030 in the middle ears of co-infected mice (Fig. 2A, $p = 0.022$), despite the mice having comparable levels of IAV at this site (Fig. 2C, $p = 0.93$). In 4 of 21 (19%) of WCV-immunised mice, EF3030 was not detected in either ear, whereas all 18 mice treated with adjuvant had detectable EF3030 in at least one ear (Table 1). Of mice with detectable EF3030 in any ear, bilateral OM was observed in 11 of 21 (52%) of mice administered WCV compared to 13 of 18 (72%) of the adjuvant control group (Table 1). No significant differences were observed in the median density of EF3030 in the nasopharynx (Fig. 2A, $p = 0.093$), or in IAV levels in the nasopharynx or middle ears of adjuvant- and WCV-treated mice.

In contrast to results observed with EF3030, WCV did not reduce PMP1106 density in the middle ears of co-infected mice (Fig. 2B, $p = 0.47$). In 2 of 21 WCV-immunised mice PMP1106

was not detected in either ear, whereas all 18 mice immunised with adjuvant had detectable PMP1106 in at least one ear (Table 1). There were no differences in the proportion of mice displaying bilateral OM (Table 1: 11 of 18 (61%) vs 13 of 21 (62%)), in the median density of PMP1106 in the nasopharynx (Fig. 2B, $p = 0.34$), or in the proportion of mice exhibiting nasopharyngeal loads of less than 10^5 CFU between vaccine groups (4 of 21 (19%) vs 4 of 18 (22%)). Nasopharyngeal IAV loads were comparable between mice administered WCV or adjuvant and co-infected with PMP1106 (Fig. 2D, $p = 0.63$).

Interestingly (Fig. 2), mice had differing IAV loads in the nasopharynx depending on whether they were co-infected with EF3030 or PMP1106. This was the case for both adjuvant-treated mice (EF3030 median 1.88×10^7 IAV genome copies vs PMP1106 median 3.00×10^6 IAV genome copies, $p = 0.0008$, Mann Whitney U test) and WCV-treated mice (EF3030 median 7.76×10^6 IAV genome copies vs PMP1106 median 3.35×10^6 IAV genome copies, $p = 0.0169$, Mann U Whitney test).

Preliminary histological analysis revealed no significant differences in inflammation of the middle ear between WCV-immunised and adjuvant control groups for mice co-infected with EF3030 (20% vs 50%, $p = 0.69$) or PMP1106 (0% vs 10%, $p = 0.67$).

To investigate whether WCV accelerated the clearance of pneumococci from the middle ears, we compared mice given adjuvant alone with those immunised with WCV 12 days after infection

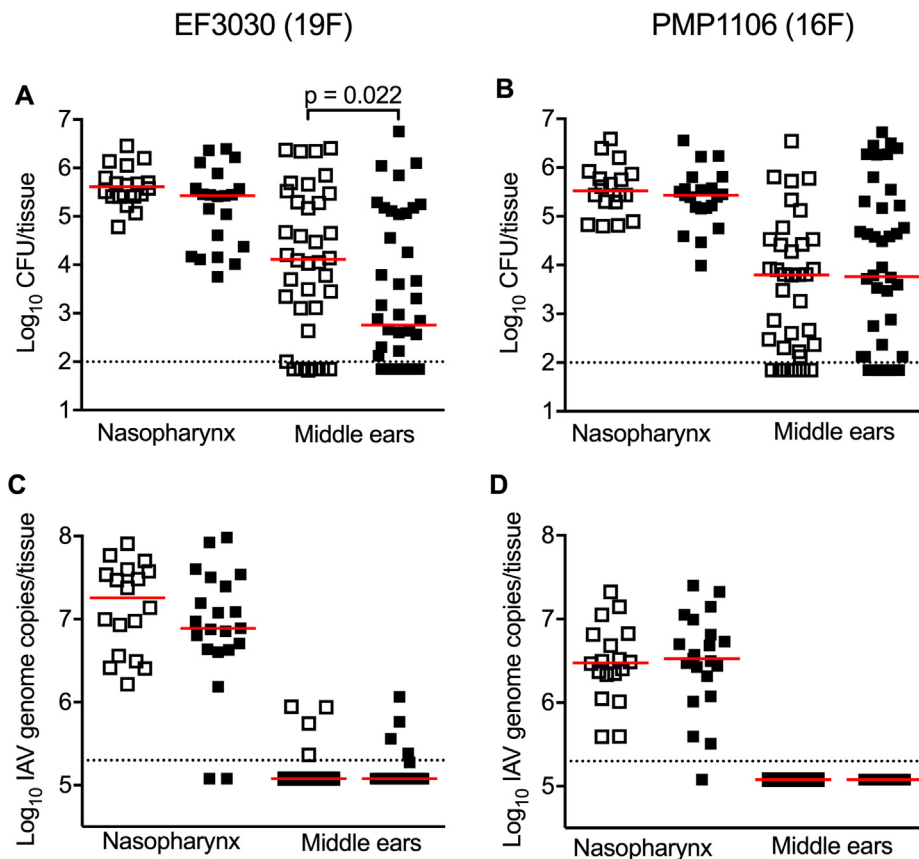


Fig. 2. Effect of WCV on pneumococcal OM six days post-IAV. Mice were administered one dose of either adjuvant (open squares) or WCV (closed squares) subcutaneously and then co-infected with pneumococci (EF3030, left panel or PMP1106, right panel) and IAV as depicted in Fig. 1A. Pneumococcal (A, B) and IAV (C, D) levels in the nasopharynx and middle ears were determined 6 days post-IAV infection. For the nasopharynx, each data point represents an individual mouse. Counts from each middle ear are represented as individual data points. Figures show data pooled from three independent experiments. Red lines show the median; the dotted line represents the limit of detection (100 CFU for Figures A and B; 1.7×10^5 IAV genome copies for Figures C and D). The Mann Whitney U Test was used to compare control (adjuvant alone) and WCV-treated mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Effect of WCV on pneumococcal OM type in mice following co-infection.

Pneumococcal isolate	Group	6 days post-IAV				12 days post-IAV			
		OM type			p ^a	OM type			p
		No OM	Unilateral	Bilateral		No OM	Unilateral	Bilateral	
EF3030	Adjuvant	0/18 (0) ^b	5/18 (28)	13/18 (72)	0.167	8/20 (40)	6/20 (30)	6/20 (30)	0.931
	WCV	4/21 (19)	6/21 (29)	11/21 (52)		11/25 (44)	6/25 (24)	8/25 (32)	
PMP1106	Adjuvant	0/18 (0)	7/18 (39)	11/18 (61)	0.569	8/21 (38)	5/21 (24)	8/21 (38)	0.846
	WCV	2/21 (10)	6/21 (29)	13/21 (62)		8/18 (44)	5/18 (28)	5/18 (28)	

^a Comparison of adjuvant vs WCV, Fisher's exact test, two-tailed.

^b Data are expressed as number of mice/total mice (%).

with IAV, and found no difference in the number of mice that developed OM (Table 1, EF3030: 14 of 25 (56%) vs 12 of 20 (60%); PMP1106: 13 of 21 (62%) vs 10 of 18 (56%)), nor the levels of pneumococci in their middle ears (Fig. 3A, $p = 0.61$; Fig. 3B, $p = 0.22$). No significant differences were observed between IAV loads in the middle ears of mice administered WCV or adjuvant (Fig. 3C, $p = 1$; Fig. 3D, $p = 0.88$) 12 days post-IAV infection. However, a significant reduction in pneumococcal density was observed in the nasopharynx of mice administered WCV, compared to mice administered adjuvant, for both pneumococcal isolates tested (Fig. 3A, $p = 0.035$; Fig. 3B, $p = 0.011$). This reduction was observed despite comparable levels of IAV in the nasopharynx among vaccine groups (Fig. 3C, $p = 0.34$; Fig. 3D, $p = 0.63$).

We next investigated the immune responses associated with the observed reduction of EF3030 density in the middle ears. Previous studies in adult mice have demonstrated roles for CD4⁺ T cells, and the cytokine IL-17A, in WCV-mediated protection against pneumococcal colonisation [13,27]. We hypothesised that, since the nasopharynx and the middle ear are both mucosal sites, CD4⁺ T cells and IL-17A would be involved in the observed WCV-mediated reduction of EF3030 density in the middle ear. We therefore measured splenic IL-17A responses in vaccinated and co-infected mice (EF3030) 6 days post-IAV infection. *In vitro* stimulation of splenocytes with WCA induced significant production of IL-17A compared to stimulation with PBS (Fig. 4A, $p < 0.001$ for both adjuvant and WCV). In WCA-stimulated samples, we observed a

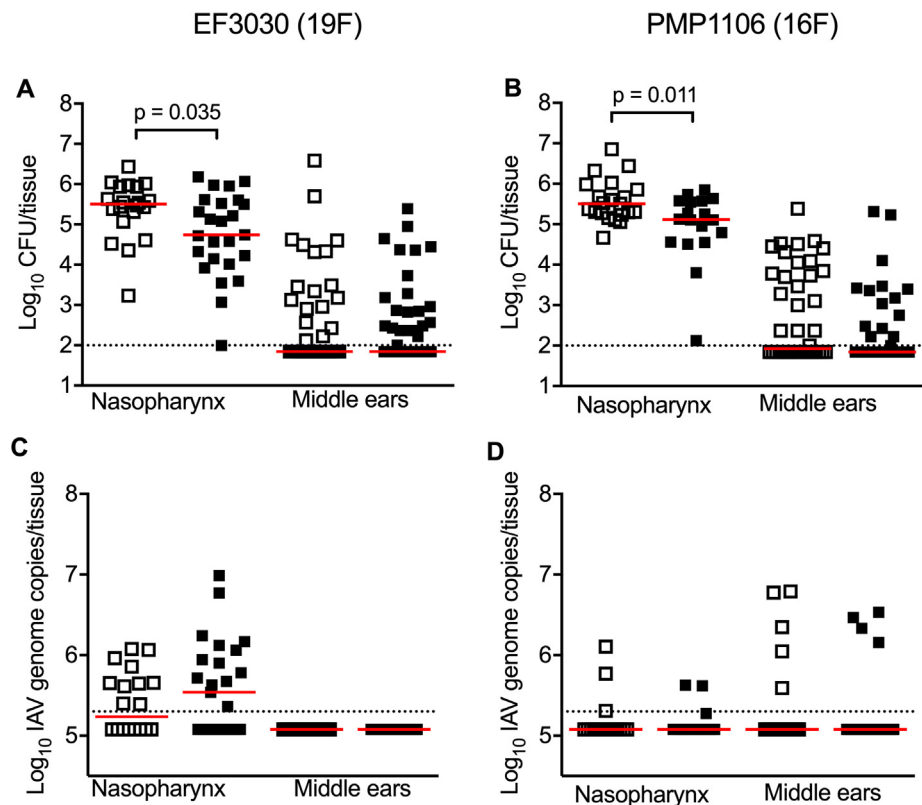


Fig. 3. Effect of WCV on pneumococcal OM 12 days post-IAV. Mice were administered one dose of either adjuvant (open squares) or WCV (closed squares) subcutaneously and then co-infected with pneumococci (EF3030, left panel or PMP1106, right panel) and IAV as depicted in Fig. 1A. Pneumococcal and IAV levels in the nasopharynx and middle ears were determined 12 days post-IAV infection for mice co-infected with EF3030 (A, C) or PMP1106 (B, D). For the nasopharynx, each data point represents an individual mouse. Counts from each middle ear are represented as individual data points. All graphs show data pooled from three independent experiments. Red lines show the median; the dotted line represents the limit of detection (100 CFU for Figures A and B; 1.7×10^5 IAV genome copies for Figures C and D). The Mann Whitney *U* Test was used to compare control (adjuvant alone) and WCV-treated mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

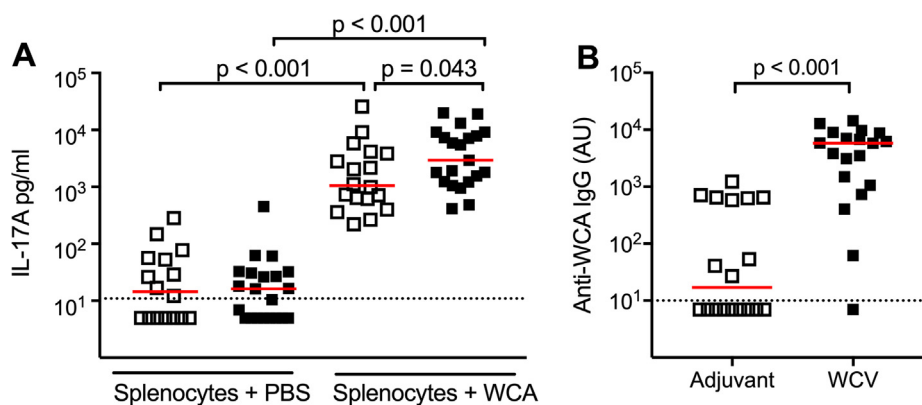


Fig. 4. IL-17A and IgG responses in vaccinated co-infected mice. Mice were administered one dose of adjuvant (open squares) or WCV (closed squares) subcutaneously and then co-infected (with EF3030 and IAV) as depicted in Fig. 1A. Blood and spleens were harvested 6 days post-IAV infection. WCA-specific IL-17A was measured by ELISA from the supernatants of splenocyte suspensions that had been stimulated with WCA or PBS for 72 h (A). Anti-WCA IgG responses were measured in plasma (B). Each data point represents an individual mouse. All graphs show data pooled from three independent experiments; red lines show the median; the dotted line represents the limit of detection: 10.9 pg/ml of IL-17A, 15.625 AU of IgG, 100 CFU. The Mann Whitney *U* Test was used to compare immune responses in control (adjuvant alone) and WCV-treated mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

small but significant increase in IL-17A responses from mice administered WCV and co-infected with EF3030, compared to the adjuvant-control mice (Fig. 4A, $p = 0.043$).

To investigate the contribution of vaccine-induced antibodies, we measured WCA-specific IgG in the plasma of vaccinated and co-infected mice. As expected, IgG responses were significantly higher in WCV-immunised mice, compared to mice administered adjuvant (Fig. 4B, $p < 0.001$).

We sought to further investigate the role of antibodies and IL-17A in the WCV-mediated reduction of EF3030 in the ears using mice deficient in antibodies and CD4^+ T cells. To determine the role of CD4^+ T cells in the WCV-induced reduction of EF3030 density in the middle ears, we used the CD4^+ T cell-deficient GK1.5 transgenic mouse strain [24]. To investigate the effect of CD4^+ T cells only at the time of OM development (i.e. following IAV infection), we depleted WT mice of CD4^+ T cells via i.p. administration of the

GK1.5 mAb before (at 17 days old) and after (at 23 days old) IAV infection. Preliminary experiments confirmed that the dosing schedules chosen effectively depleted $CD4^+$ T cells at the time of IAV infection, and during the development of IAV-mediated pneumococcal OM, in WT mice (Fig S1). To investigate the role of antibodies in the WCV-mediated reduction of EF3030 in the middle ears, we used antibody-deficient B6. μ MT $^{-/-}$ mice. Pneumococcal and IAV co-infection of B6. μ MT $^{-/-}$ mice resulted in reduced dissemination of pneumococci to the middle ears compared to co-infection of WT C57BL/6 mice, but dissemination could be increased by providing B6. μ MT $^{-/-}$ mice with a source of non-specific antibodies via administration (passive transfer) of naïve C57BL/6 mouse serum [25]. We confirmed that passive transfer of naïve mouse serum to co-infected B6. μ MT $^{-/-}$ mice enhanced

pneumococcal density in the middle ears of co-infected B6. μ MT $^{-/-}$ mice (Fig S2, $p = 0.003$) using the infection timeline described in Fig. 1A.

To determine if a single dose of WCV administered at 6 days of age could reduce EF3030 density in the middle ears of co-infected mice in the absence of $CD4^+$ T cells and antibody responses, we compared pneumococcal density in the middle ears of vaccinated and co-infected GK1.5 mice, WT mice depleted of $CD4^+$ T cells, and B6. μ MT $^{-/-}$ mice. WCV immunisation (compared with adjuvant alone) no longer reduced EF3030 density in the middle ears following co-infection in any of these immune-depleted models (Fig. 5B). No significant differences were observed between adjuvant and WCV-immunised immune-deficient mice that developed OM or the proportion of mice exhibiting bilateral OM (Table 2). In addition,

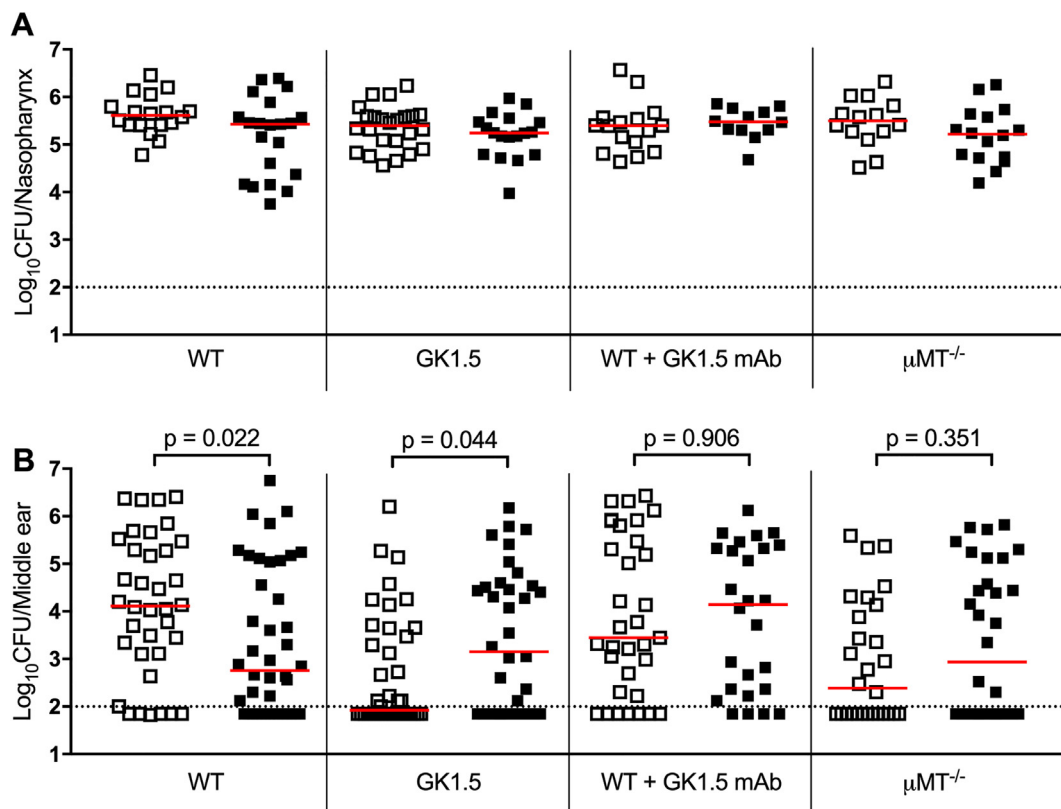


Fig. 5. Effect of $CD4^+$ T cell and antibody-depletion on the WCV-mediated impact on pneumococcal density following co-infection with EF3030. C57BL/6 (WT), GK1.5 or B6. μ MT $^{-/-}$ mice were administered one dose of adjuvant (open squares) or WCV (closed squares) subcutaneously and then co-infected (with EF3030 and IAV) as depicted in Fig. 1A. $CD4^+$ T cells were depleted in WT mice before and after IAV infection (WT + GK1.5 mAb) as described in the methods. B6. μ MT $^{-/-}$ mice (μ MT $^{-/-}$) were administered naïve serum as described in the methods. Pneumococcal density was measured in the nasopharynx (A) and middle ears (B) at 6 days post-IAV infection. Each nasopharyngeal data point represents an individual mouse. Counts from each middle ear are represented as individual data points. Red lines show the median; the dotted line represents the limit of detection (100 CFU). All graphs show data pooled from at least two independent experiments. The Mann Whitney U Test was used to compare adjuvant and WCV-treated mice in each experimental condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Effect of WCV on EF3030 OM six days post-IAV in immune-deficient mice.

Mouse strain	Group	Number of mice/total mice (%)			p^a
		No OM	Unilateral	Bilateral	
WT	Adjuvant	0/18 (0)	5/18 (28)	13/18 (72)	0.167
	WCV	4/21 (19)	6/21 (29)	11/21 (52)	
GK1.5	Adjuvant	7/20 (35)	5/20 (25)	8/20 (40)	0.386
	WCV	3/18 (17)	7/18 (39)	8/18 (44)	
WT + GK1.5 mAb	Adjuvant	2/16 (13)	2/16 (13)	12/16 (75)	0.613
	WCV	1/12 (8)	0/12 (0)	11/12 (92)	
μ MT $^{-/-}$	Adjuvant	4/14 (29)	4/14 (29)	6/14 (43)	0.904
	WCV	4/16 (19)	6/16 (38)	6/16 (38)	

^a Comparison of adjuvant vs WCV, Fisher's Exact test.

tion, no significant differences in pneumococcal density in the nasopharynx were observed between adjuvant and WCV-treated mice in each model (Fig. 5A, $p > 0.05$ for all). Taken together, these results suggest that both CD4⁺ T cells and antibodies are involved in the WCV-mediated reduction in EF3030 density observed in WT co-infected mice.

4. Discussion

Our results demonstrate that although WCV significantly reduced pneumococcal nasopharyngeal density following IAV infection, it did not prevent the spread of pneumococci from the nasopharynx to the middle ear, nor did it enhance clearance of OM in our model. WCV significantly reduced pneumococcal density in the middle ears of co-infected mice at the peak of OM in one of two strains tested. Experiments in immune-deficient/depleted mice indicate that both CD4⁺ T cells and antibodies were involved in the WCV-mediated reduction of pneumococcal isolate EF3030 density in the middle ears.

There are multiple factors that may explain why WCV was unable to prevent the development of OM in this model. These include deficiencies in the immune response of infants to vaccination [29] and pneumococcal infection [30], the inability to provide more than one immunisation prior to infection, the limited time available for mice to mount protective immunity before the induction of OM, and the magnitude of pneumococcal disease induced following co-infection, evidenced by almost 100% of co-infected mice developing pneumococcal OM (Fig. 1). Mechanistically, i.e. IAV infection induces inflammation in the nasopharynx which leads to a significant increase in pneumococcal colonisation density and duration at this site [17,31]. In addition, IAV can disperse pneumococcal nasopharyngeal biofilms to release virulent bacteria with a greater propensity to infect the middle ear [32]. To prevent the development of OM in this model, WCV-induced immunity would need to control pneumococcal colonisation density and prevent the bacteria from reaching the middle ear, or to mount a rapid immune response in the middle ear to clear disseminating pneumococci. The former scenario was observed in 4 of 21 and 2 of 21 mice administered WCV and infected with EF3030 and PMP1106, respectively, compared to none of 18 mice administered adjuvant alone (Table 1). The observed reduction of EF3030 density at 6 days post-IAV suggests that WCV temporarily achieved the latter scenario, but this was not observed for PMP1106. However, given that there was no reduction in EF3030 density in the middle ear at a later time point (Fig. 2A), our data demonstrate that WCV did not enhance the overall clearance of this isolate from the middle ears. The clinical relevance of the reduced density of EF3030 observed during peak OM is unknown, and histology did not demonstrate a reduction in inflammation in WCV-immunised mice. Hearing tests were not conducted as part of this study, but given that levels of EF3030 in the middle ears of WCV-immunised mice were comparable to those that have previously been associated with hearing impairment following co-infection in this model [16], we do not expect that the WCV-mediated reduction in density to translate to improved hearing in vaccinated mice.

Studies of pneumococcal OM in mice have demonstrated that inflammatory mediators including neutrophil-recruiting IL-17A [33] and myeloperoxidase [34], as well as TLR2 signalling and neutrophil and macrophage recruitment [35], are important in clearing pneumococci from the middle ear, while T and B cells migrate to and proliferate at this site several days after infection [36,37]. The role of antibodies in clearing pneumococcal OM remains uncertain. Clinical evidence from small cohorts suggest that poor local and systemic antibody responses to pneumococcal antigens are associated with recurrent OM and upper respiratory tract infec-

tions in children [38,39], but larger studies in high-risk populations have not confirmed this association [40]. In an experimental co-infection setting, antibodies, along with neutrophil extracellular traps, can facilitate pneumococcal multiplication in the middle ear [25]. WCV-mediated protection against pneumococcal colonisation requires IL-17A but not antibodies, whereas protection against invasive disease is antibody-mediated [9,41]. As expected, we observed significantly enhanced systemic IL-17A and antibody responses in WCV-immunised mice co-infected with EF3030 compared to mice administered adjuvant alone (Fig. 4A, D). It is possible that the WCV-mediated reduction in EF3030 middle ear density was dependent on immune responses that took place earlier than 6 days post-IAV. Alternatively, higher levels of WCV-induced antibodies and CD4⁺ T cells (the major subset responsible for IL-17A production in splenocytes following *in vitro* stimulation by WCA [13]) may have migrated to the site of infection. Although we did not measure IL-17A or antibodies in the middle ear, other studies have demonstrated successful extravasation of vaccine-specific antibodies to the middle ear following parenteral immunisation [42]. It is possible that increased IL-17A and antibody responses in the middle ear may have initially reduced EF3030 density in WCV-immunised mice. Our data demonstrating that WCV could no longer reduce EF3030 density in mice deficient of CD4⁺ T cells and antibody responses (Fig. 5B) support this hypothesis. This is also consistent with data from preclinical evaluation of other investigational pneumococcal vaccines. For example, Rosch et al. [43] demonstrated that CD4⁺ T cells at the time of vaccination were required to prevent OM following pneumococcal challenge (with or without IAV co-infection) for both a live attenuated pneumococcal vaccine (BHN97ΔftsY) delivered i.n., and for i.p. vaccination with PCV13. These authors also found that protection was associated with CD4⁺ T cell-dependent immunoglobulin isotype switching (mouse IgG2a and 2c for BHN97ΔftsY, and IgG1 for PCV13) [43]. Additionally, co-infected mice deficient in IL-17A receptor signalling had higher pneumococcal loads in the middle ears compared to co-infected WT mice following i.n. immunisation with recombinant PspA and cholera toxin [44]. A role for T_H17 responses in preventing OM is also supported by findings in a cohort of otitis-prone children in New York. The definition of stringently otitis-prone (sOP) was assigned to children experiencing three or more OM episodes within a 6- to 12-month period by Pichichero and colleagues [45], who examined OM episodes using tympanocentesis in 840 children over 10 years. Investigations of immune responses in this population demonstrated that sOP children showed reduced production of IL-17A by peripheral blood memory T cells after stimulation with heat-killed pneumococci or pneumococcal protein antigens compared to non-sOP children [46,47], suggesting that reduced CD4⁺ T cell-mediated IL-17A responses were associated with an increased risk of recurrent OM.

Antigenic differences between the pneumococcal challenge strains tested here and the WCV strain, RM200, may have accounted for the lack of protection observed in our model, and explain why WCV reduced EF3030 but not PMP1106 density in the middle ears of co-infected mice. Previous studies have investigated the ability of WCV-induced immune responses to bind to and kill different strains and serotypes of pneumococci *in vitro*. Using ELISA and flow cytometry, Moffitt et al. [21] demonstrated that WCV-specific antisera bound to a range of pneumococci (representing 12 serotypes and at least 10 MLST types, and including both invasive and carriage isolates), and 20 isogenic mutants of TIGR4 expressing different capsule polysaccharides, at significantly higher levels compared to adjuvant-specific antisera. However, the fold-increase in binding was variable between strains. Less strain variation was observed when IL-17A responses were detected following stimulation of WCV-immunised splenocytes with different pneumococcal strains (26–43-fold difference in expression [21]),

and when WCV-specific antibodies were tested for their ability to induce phagocytosis of pneumococcal strains by mouse peritoneal cells *in vitro* (12 to 39% killing [48]).

Pneumococcal strains can vary greatly at the genetic level in a way that gives rise to considerable variation in pneumococcal surface proteins, which are the targets of the WCV-response. For example, of 616 pneumococcal carriage isolates, Croucher et al. [49] identified 39 and 59 variants of pneumococcal surface proteins A (PspA) and C (PspC), respectively. Genetic comparison of RM200, EF3030 and PMP1106 could show if this is due to differences in the structure of antigens in all three strains. For example, differences in PspA may prevent or reduce WCV-induced anti-PspA from binding to PMP1106 and reducing IAV-induced OM [50].

Differences between the two pneumococcal strains may have also affected the host response to IAV in our model: mice co-infected with EF3030 had higher levels of IAV in the nasopharynx compared with mice co-infected with PMP1106. Diavatopoulos et al. observed that mice had reduced IAV loads in the presence of pneumococci [51]. More recently, Ortigoza et al. demonstrated that pneumococcal neuraminidases (NanA and NanB) reduce IAV acquisition and shedding [52]. It is possible that differences in sialidase expression by pneumococcal strain may have influenced IAV levels in our model.

Although WCV did not affect the density of nasopharyngeal colonisation 6 days post-IAV (Fig. 2A and B), it significantly reduced colonisation density at 12 days post-IAV for both pneumococcal strains tested (Fig. 3A and B). This is consistent with the broad efficacy of WCV in adult and infant mice [9,10,13,29]. The enhanced nasopharyngeal clearance observed in WCV-immunised mice may translate to protection against future episodes of pneumococcal OM following additional IAV infections. A second IAV challenge in vaccinated and co-infected mice that remain colonised with pneumococci would evaluate whether WCV protection against IAV-induced OM increased with time. High pneumococcal carriage density is associated with the development of pneumococcal disease [53–55] and transmission [56,57]. Therefore, WCV-mediated reductions in carriage may protect against pneumococcal disease and transmission.

In conclusion, this study was the first to test the effect of WCV on IAV-induced pneumococcal disease. WCV administration did not prevent the development of pneumococcal OM, nor speed up the resolution of infection. However, a strain-dependent reduction in density of pneumococci in the middle ears was observed. In addition, WCV reduced the nasopharyngeal density of both isolates tested. Overall, our data suggest that WCV may not directly impact on IAV-induced OM within an individual, but may have a population-level effect due to enhanced clearance of pneumococcal colonisation.

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Authors contributions

JM, EMD, RAB, JMO, JEKM, RRB, RM, OLW and CS participated in study design, and data analysis and interpretation. NW performed FACS analysis and JSP conducted the histological assessment of

level of inflammation in middle ear tissue sections. All other experiments were performed by JM. JM drafted the manuscript with CS and OLW. All authors participated in editing of the manuscript and approved of the final manuscript.

Conflict of interest

OLW, CS, EMD and JM are recipients of the Robert Austrian Award in Pneumococcal Vaccinology, which is funded by Pfizer but awarded by ISPPD. RM is a scientific founder, consultant, and equity owner at Affinivax, a biotechnology company based in Cambridge MA that is devoted to the development of vaccines for developing and developed countries. EKM is a member of the PATH Pneumococcal Vaccine Project wSP Scientific Advisory Board. The other authors have no conflicts of interest to declare.

Declaration of interests

The authors declare that they have no known competing financial interests.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2019.03.013>.

References

- [1] DeAntonio R, Yarzabal JP, Cruz JP, Schmidt JE, Kleijnen J. Epidemiology of otitis media in children from developing countries: a systematic review. *Int J Pediatr Otorhinolaryngol* 2016;85:65–74.
- [2] Van Dyke MK, Pirçon J-Y, Cohen R, Madhi SA, Rosenblüt A, Macias Parra M, et al. Etiology of acute otitis media in children less than 5 years of age: a pooled analysis of 10 similarly designed observational studies. *Pediatr Infect Dis J* 2017;36:274–81.
- [3] Adegbola RA, DeAntonio R, Hill PC, Roca A, Usuf E, Hoet B, et al. Carriage of *Streptococcus pneumoniae* and other respiratory bacterial pathogens in low and lower-middle income countries: a systematic review and meta-analysis. *PLoS ONE* 2014;9:e103293.
- [4] Conklin L, Loo JD, Kirk J, Fleming-Dutra KE, Deloria-Knoll M, Park DE, et al. Systematic review of the effect of pneumococcal conjugate vaccine dosing schedules on vaccine-type invasive pneumococcal disease among young children. *Pediatr Infect Dis J* 2014;33:S109–18.
- [5] Pettigrew MM, Alderson MR, Bakaletz LO, Barenkamp SJ, Hakansson AP, Mason KM, et al. Panel 6: Vaccines. *Otolaryngol Head Neck Surg* 2017;156:S76–87.
- [6] Fletcher MA, Fritzell B. Pneumococcal conjugate vaccines and otitis media: an appraisal of the clinical trials. *Int J Otolaryngol* 2012;9:312935–3015.
- [7] Tregnagh MW, Sáez-Llorens X, López P, Abate H, Smith E, Póseman A, et al. Efficacy of pneumococcal nontypable *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV) in young Latin American children: a double-blind randomized controlled trial. *PLoS Med* 2014;11:e1001657.

- [8] Weinberger DM, Malley R, Lipsitch M. Serotype replacement in disease after pneumococcal vaccination. *Lancet* 2011;378:1962–73.
- [9] Lu Y, Leite L, Goncalves VM, Dias Wde O, Liberman C, Fratelli F, et al. GMP-grade pneumococcal whole-cell vaccine injected subcutaneously protects mice from nasopharyngeal colonization and fatal aspiration-sepsis. *Vaccine* 2010;28:7468–75.
- [10] Malley R, Lipsitch M, Stack A, Saladino R, Fleisher G, Pelton S, et al. Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. *Infect Immun* 2001;69:4870–3.
- [11] Alderson MR. Status of research and development of pediatric vaccines for *Streptococcus pneumoniae*. *Vaccine* 2016;34:2959–61.
- [12] Malley R, Morse SC, Leite LCC, Areas APM, Ho PL, Kubrusly FS, et al. Multiserotype protection of mice against pneumococcal colonization of the nasopharynx and middle ear by killed nonencapsulated cells given intranasally with a nontoxic adjuvant. *Infect Immun* 2004;72:4290–2.
- [13] Lu YJ, Gross J, Bogaert D, Finn A, Bagrade L, Zhang Q, et al. Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog* 2008;4:e1000159.
- [14] Henderson FW, Collier AM, Sanyal MA, Watkins JM, Fairclough DL, Clyde WA, et al. A longitudinal study of respiratory viruses and bacteria in the etiology of acute otitis media with effusion. *N Engl J Med* 1982;306:1377–83.
- [15] Pettigrew MM, Gent JF, Pyles RB, Miller AL, Nokso-Koivisto J, Chonmaitree T. Viral-bacterial interactions and risk of acute otitis media complicating upper respiratory tract infection. *J Clin Microbiol* 2011;49:3750–5.
- [16] Short KR, Diavatopoulos DA, Thornton R, Pedersen J, Strugnell RA, Wise AK, et al. Influenza virus induces bacterial and nonbacterial otitis media. *J Infect Dis* 2011;204:1857–65.
- [17] John M. Evaluation of the probiotic *Streptococcus salivarius* for the inhibition of the pneumococcal colonisation and prevention of otitis media [PhD Thesis]. Victoria, Australia: University of Melbourne; 2014.
- [18] Hill PC, Townend J, Antonio M, Akisanya B, Ebruke C, Lahai G, et al. Transmission of *Streptococcus pneumoniae* in rural Gambian villages: a longitudinal study. *Clin Infect Dis* 2010;50:1468–76.
- [19] Turner P, Turner C, Jankhot A, Phakaudom K, Nosten F, Goldblatt D. Field evaluation of culture plus latex sweep serotyping for detection of multiple pneumococcal serotype colonisation in infants and young children. *PLoS ONE* 2013;8:e67933.
- [20] Short KR, Diavatopoulos DA, Reading PC, Brown LE, Rogers KL, Strugnell RA, et al. Using bioluminescent imaging to investigate synergism between *Streptococcus pneumoniae* and influenza A virus in infant mice. *J Vis Exp* 2011:e2357.
- [21] Moffitt KL, Yadav P, Weinberger DM, Anderson PW, Malley R. Broad antibody and T cell reactivity induced by a pneumococcal whole-cell vaccine. *Vaccine* 2012;30:4316–22.
- [22] Lu Y, Yadav P, Clements JD, Forte S, Srivastava A, Thompson CM, et al. Options for inactivation, adjuvant, and route of topical administration of a killed, unencapsulated pneumococcal whole-cell vaccine. *Clin Vaccine Immunol* 2010;17:1005–12.
- [23] Kitamura D, Roes J, Kühn R, Rajewsky K. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. *Nature* 1991;350:423–6.
- [24] Zhan Y, Corbett AJ, Brady JL, Sutherland RM, Lew AM. CD4 help-independent induction of cytotoxic CD8 cells to allogeneic P815 tumor cells is absolutely dependent on costimulation. *J Immunol* 2000;165:3612–9.
- [25] Short KR, Koeckritz-Blickwede von M, Langereis JD, Chew KY, Job ER, Armitage CW, et al. Antibodies mediate formation of neutrophil extracellular traps in the middle ear and facilitate secondary pneumococcal otitis media. *Infect Immun* 2014;82:364–70.
- [26] van Elden LJ, Nijhuis M, Schipper P, Schuurman R, van Loon AM. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. *J Clin Microbiol* 2001;39:196–200.
- [27] Mackenzie GA, Carapetis JR, Leach AJ, Morris PS. Pneumococcal vaccination and otitis media in Australian Aboriginal infants: comparison of two birth cohorts before and after introduction of vaccination. *BMC Pediatr* 2009;9:14.
- [28] Leach AJ, Wigger C, Hare K, Hampton V, Beissbarth J, Andrews R, et al. Reduced middle ear infection with non-typeable *Haemophilus influenzae*, but not *Streptococcus pneumoniae*, after transition to 10-valent pneumococcal non-typeable *H. influenzae* protein D conjugate vaccine. *BMC Pediatr* 2015;15:162.
- [29] Bogaert D, Weinberger D, Thompson C, Lipsitch M, Malley R. Impaired innate and adaptive immunity to *Streptococcus pneumoniae* and its effect on colonization in an infant mouse model. *Infect Immun* 2009;77:1613–22.
- [30] Chelvarajan L, Popa D, Liu Y, Getchell TV, Stromberg AJ, Bondada S. Molecular mechanisms underlying anti-inflammatory phenotype of neonatal splenic macrophages. *J Leukoc Biol* 2007;82:403–16.
- [31] FitzPatrick M, Royce SG, Langenbach S, McQualter J, Reading PC, Wijburg O, et al. Neonatal pneumococcal colonisation caused by influenza A infection alters lung function in adult mice. *Sci Rep* 2016;6:22751.
- [32] Marks LR, Davidson BA, Knight PR, Hakansson AP. Interkingdom signaling induces *Streptococcus pneumoniae* biofilm dispersion and transition from asymptomatic colonization to disease. *mBio* 2013;4:e00438–e513.
- [33] Wang W, Zhou A, Zhang X, Xiang Y, Huang Y, Wang L, et al. Interleukin 17A promotes pneumococcal clearance by recruiting neutrophils and inducing apoptosis through a p38 mitogen-activated protein kinase-dependent mechanism in acute otitis media. *Infect Immun* 2014;82:2368–77.
- [34] Xiang Y, Jin C, Wang W, Wang Z, Huang Y, Fan F, et al. The critical role of myeloperoxidase in *Streptococcus pneumoniae* clearance and tissue damage during mouse acute otitis media. *Innate Immun* 2016;23:296–306.
- [35] Huang Y, Wang Z, Jin C, Wang L, Zhang X, Xu W, et al. TLR2 promotes macrophage recruitment and *Streptococcus pneumoniae* clearance during mouse otitis media. *Pediatr Res* 2016;80:886–93.
- [36] Forséni M, Hansson GK, Bagger-Sjöbäck D, Hultcrantz M. Infiltration of immunocompetent cells in the middle ear during acute otitis media: a temporal study. *Am J Otol* 1999;20:152–7.
- [37] Jecker P, Pabst R, Westermann J. Proliferating macrophages, dendritic cells, natural killer cells, T and B lymphocytes in the middle ear and Eustachian tube mucosa during experimental acute otitis media in the rat. *Clin Exp Immunol* 2001;126:421–5.
- [38] Xu Q, Casey JR, Pichichero ME. Higher levels of mucosal antibody to pneumococcal vaccine candidate proteins are associated with reduced acute otitis media caused by *Streptococcus pneumoniae* in young children. *Mucosal Immunol* 2015;8:1110–7.
- [39] Estrada J, Najera M, Pounds N, Catano G, Infante AJ. Clinical and serologic response to the 23-valent polysaccharide pneumococcal vaccine in children and teens with recurrent upper respiratory tract infections and selective antibody deficiency. *Pediatr Infect Dis J* 2016;35:205–8.
- [40] Wiertsema SP, Corscadden KJ, Mowe EN, Zhang G, Vijayasekaran S, Coates HL, et al. IgG responses to pneumococcal and *Haemophilus influenzae* protein antigens are not impaired in children with a history of recurrent acute otitis media. *PLoS ONE* 2012;7:e49061.
- [41] Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, Lipsitch M. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *Proc Natl Acad Sci USA* 2005;102:4848–53.
- [42] Bakaletz LO, Holmes KA. Evidence for transduction of specific antibody into the middle ears of parenterally immunized chinchillas after an upper respiratory tract infection with adenovirus. *Clin Diagn Lab Immunol* 1997;4:223–5.
- [43] Rosch JW, Iverson AR, Humann J, Mann B, Gao G, Vogel P, et al. A live-attenuated pneumococcal vaccine elicits CD4+ T-cell dependent class switching and provides serotype independent protection against acute otitis media. *EMBO Mol Med* 2014;6:141–54.
- [44] Habets MN, van Selm S, van Opzeeland FJ, Simonetti E, Hermans PWM, de Jonge MI, et al. Role of antibodies and IL17-mediated immunity in protection against pneumococcal otitis media. *Vaccine* 2016;34:5968–74.
- [45] Pichichero ME. Ten-year study of the stringently defined otitis-prone child in Rochester, NY. *Pediatr Infect Dis J* 2016;35:1033–9.
- [46] Basha S, Kaur R, Mosmann TR, Pichichero ME. Reduced T-Helper 17 responses to *Streptococcus pneumoniae* in infection-prone children can be rescued by addition of innate cytokines. *J Infect Dis* 2017;215:1321–30.
- [47] Sharma SK, Casey JR, Pichichero ME. Reduced memory CD4+ T-cell generation in the circulation of young children may contribute to the otitis-prone condition. *J Infect Dis* 2011;204:645–53.
- [48] Campos IB, Herd M, Moffitt KL, Lu Y, Darrieux M, Malley R, et al. IL-17A and complement contribute to killing of pneumococci following immunization with a pneumococcal whole cell vaccine. *Vaccine* 2017;35:1306–15.
- [49] Croucher NJ, Campo JJ, Le TQ, Liang X, Bentley SD, Hanage WP, et al. Diverse evolutionary patterns of pneumococcal antigens identified by pangenome-wide immunological screening. *Proc Natl Acad Sci USA* 2017;114:E357–66.
- [50] Greene CJ, Marks LR, Hu JC, Redding R, Mandell L, Roche-Hakansson H, et al. Novel strategy to protect against influenza virus-induced pneumococcal disease without interfering with commensal colonization. *Infect Immun* 2016;84:1693–703.
- [51] Diavatopoulos DA, Short KR, Price JT, Wilksch JJ, Brown LE, Briles DE, et al. Influenza A virus facilitates *Streptococcus pneumoniae* transmission and disease. *FASEB J* 2010;24:1789–98.
- [52] Ortigoza MB, Blaser SB, Zafar MA, Hammond AJ, Weiser JN. An infant mouse model of influenza virus transmission demonstrates the role of virus-specific shedding, humoral immunity, and sialidase expression by colonizing *Streptococcus pneumoniae*. *mBio* 2018;9:e02359–e2361.
- [53] Smith-Vaughan H, Byun R, Nadkarni M, Jacques NA, Hunter N, Halpin S, et al. Measuring nasal bacterial load and its association with otitis media. *BMC Ear Nose Throat Disord* 2006;6:10.
- [54] Albrich WC, Madhi SA, Adrian PV, van Niekerk N, Telles JN, Ebrahim N, et al. Pneumococcal colonisation density: a new marker for disease severity in HIV-infected adults with pneumonia. *BMJ Open* 2014;4:e005953–e5963.
- [55] Khan MN, Xu Q, Pichichero ME. Protection against *Streptococcus pneumoniae* invasive pathogenesis by a protein-based vaccine is achieved by suppression of nasopharyngeal bacterial density during Influenza A virus coinfection. *Infect Immun* 2017;85:e00530–e616.
- [56] Short KR, Reading PC, Wang N, Diavatopoulos DA, Wijburg OL. Increased nasopharyngeal bacterial titers and local inflammation facilitate transmission of *Streptococcus pneumoniae*. *mBio* 2012;3:e00255–e312.
- [57] Zangari T, Wang Y, Weiser JN. *Streptococcus pneumoniae* transmission is blocked by type-specific immunity in an infant mouse model. *mBio* 2017;8:e00188–e217.
- [58] Ogier JM et al. CHD7 deficiency in ‘Looper’, a new mouse model of CHARGE syndrome, results in ossicle malformation, otosclerosis and hearing impairment. *PLoS One* 2014;9:e97559.