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REVIEW ARTICLE

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# Auckland hearing science discovery and translation in purinergic signaling and inner ear therapeutics

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**ABSTRACT**

The inner ear is a complex sensory organ responsible for hearing and balance. It is deeply embedded in the temporal bone with challenging access for diagnostic and therapeutic purposes. Stress and injury to the peripheral hearing organ (cochlea) lead to temporary or permanent sensorineural hearing loss (SNHL), which is the most common form of hearing loss resulting from cellular and molecular damage to the sensory hair cells and primary auditory neurons in the spiral ganglion. These cells cannot regenerate, and their loss leads to hearing disability. Hearing aids can amplify sound and improve residual hearing ability but cannot restore function; therefore, alternative therapies are urgently needed. The pharmacological approach to treating SNHL has been our mainstream research over the past two decades. This review describes our studies investigating the purinergic signalling system in the cochlea and its implications for inner ear therapies. Using animal models of SNHL, we have established that purinergic P1 (adenosine) and P2 (ATP) receptors can prevent or mitigate cochlear injury by reducing cochlear sensitivity to loud sound and improving the survival of sensorineural tissues. Here, we highlight our research investigating the therapeutic potential of P1 and P2 receptor agonists and antagonists in inner ear disorders.

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## Introduction

This review presents the journey of the Auditory Neuroscience Group at the University of Auckland over the last three decades in establishing the role of the purinergic signalling system in the cochlea. We will briefly describe cochlear anatomy, introduce various elements of the purinergic signalling system in the cochlea, and then describe our work on purine-based inner ear therapeutics.

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## Cochlear anatomy

The inner ear, located in the temporal bone cavity, consists of the vestibule and semicircular canals, which contain receptors for balance, and the cochlea, the peripheral organ of hearing (Raphael and Altschuler 2003). The cochlea has three fluid-filled compartments, the scala vestibuli, scala media, and scala tympani, which spiral around the central bony structure (modiolus) that contains blood vessels and the auditory nerve. The scala vestibuli and scala tympani contain perilymphatic fluid high in sodium (148 mM) and low in potassium (4.2 mM) ions (Wangemann 2002). The scala vestibuli communicates with the scala tympani through an aperture at the cochlear apex called the helicotrema (Raphael and Altschuler 2003). The central cochlear duct, or scala media, sits between the scala vestibuli and scala tympani and contains endolymph high in potassium (157 mM) and low in sodium (1.3 mM) (Wangemann 2002). This high $K^+$ content in endolymph generates the 80–100 mV endocochlear potential, which is the driving force for sensory transduction. The thin Reissner's membrane is a barrier between the scala media and scala vestibuli, and the reticular lamina of the organ of Corti separates the endolymph in scala media from the perilymph in scala tympani (Raphael and Altschuler 2003).

The organ of Corti (OoC), residing on top of the acellular basilar membrane, houses two types of mechanosensory hair cells, the inner hair cells (IHCs) and the outer hair cells (OHCs), and at least six types of non-sensory supporting cells (Raphael and Altschuler 2003). IHCs form afferent synaptic connections with the spiral ganglion neurons and transmit auditory signals to the brain. OHCs are essential for sound amplification and are controlled by a neural circuit where afferent input from inner and outer hair cells feeds back via medial olivocochlear efferents located in the brainstem auditory nuclei. Deiters' and Hensen's cells are two types of supporting cells that provide structural and metabolic support to OHCs, while pillar cells resting on the basilar membrane form the tunnel of Corti (Figure 1).

The lateral wall of the cochlea comprises stria vascularis, which is responsible for generating and maintaining the endocochlear potential, and spiral ligament, which supports the stria vascularis and cochlear fluid homeostasis. Spiral ganglion neurons located in Rosenthal's canal of the modiolus innervate the sensory hair cells and transmit auditory signals to the auditory nuclei in the brainstem (Raphael and Altschuler 2003).

## Purinergic signalling

The purinergic signalling system includes a complex framework of receptors for extracellular nucleotides such as ATP (P2 receptors) and nucleosides such as adenosine (P1 receptors) (Figure 2). The principal conduits for ATP release are integral membrane proteins known as pannexin and connexin hemichannels (Chen et al. 2015). Some P2 receptors are ligand-gated ion channels (P2X receptors), and others are G protein-coupled (P2Y receptors). The ionotropic P2X and the metabotropic P2Y receptors are widely distributed in cochlear tissues (Housley et al. 2009; Köles et al. 2019). To date, seven subtypes of the P2X family (P2X1–7) and eight subtypes of the P2Y family (P2Y1,2,4,6,11–14) have been cloned and functionally characterised (Boeynaems et al. 2005). P2 receptor signalling is regulated by a family of ecto-enzymes that hydrolyse

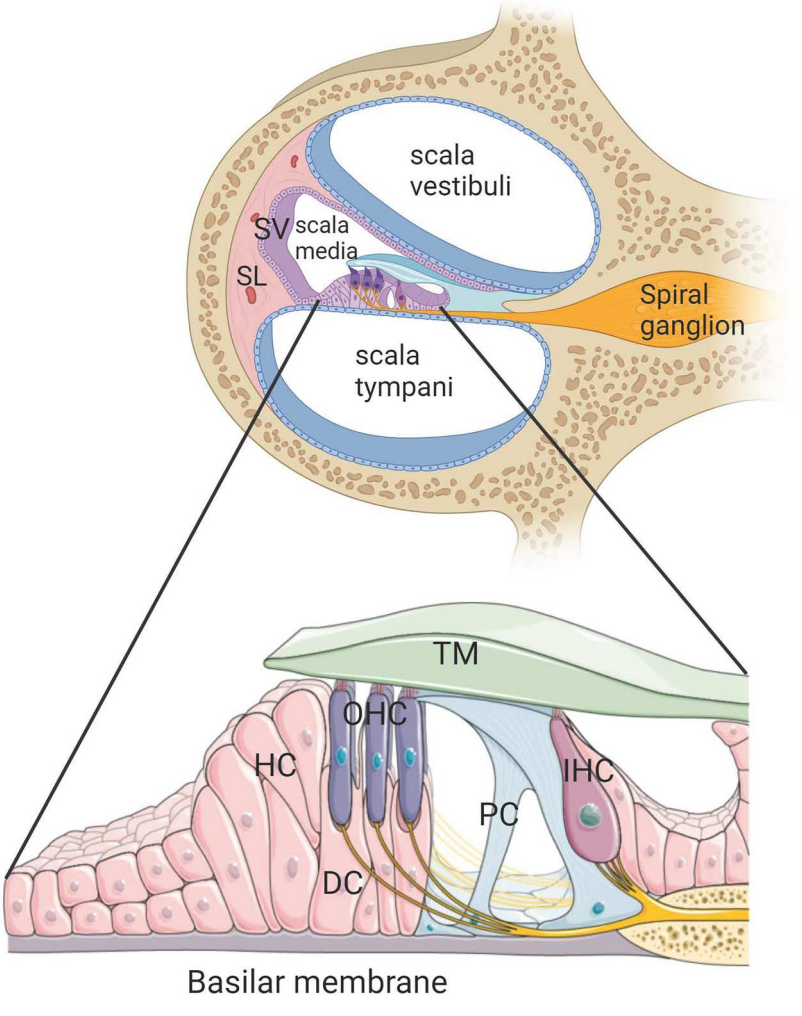


**Figure 1.** The cochlea is the peripheral organ of hearing. The cochlea comprises three fluid-filled compartments: scala vestibuli and scala tympani containing $Na^+$-rich perilymph, and scala media containing $K^+$-rich endolymph. The lateral wall of the cochlea comprises the spiral ligament (SL), which is made up of fibrocytes, and the secretory tissues of the stria vascularis (SV). The organ of Corti is the sensory organ of the cochlea sitting on the basilar membrane. It contains two types of sensory cells, inner hair cells (IHC) and outer hair cells (OHC), covered by the tectorial membrane (TM). Sensory cells are surrounded by supporting cells, such as Deiters' cells (DC), Hensen's cells (HC) and pillar cells (PC). Created with BioRender.com.

ATP to adenosine. These enzymes, known as ectonucleotidases (Robson et al. 2006; Zimmermann 2021), have extensive distribution in the rodent cochlea (Vlajkovic and Thorne 2022). Adenosine is an endogenous neuromodulator that mediates physiological actions by interacting with four cell surface-located adenosine (P1) receptors ($A_1$, $A_{2A}$, $A_{2B}$, $A_3$) (Jacobson and Gao 2006). Adenosine receptors are distributed throughout the body and activate diverse cellular signalling pathways that define their tissue-specific roles (IJzerman et al. 2022). Adenosine $A_1$ receptors also protect the cochlea from stress and injury (Vlajkovic and Thorne 2022).

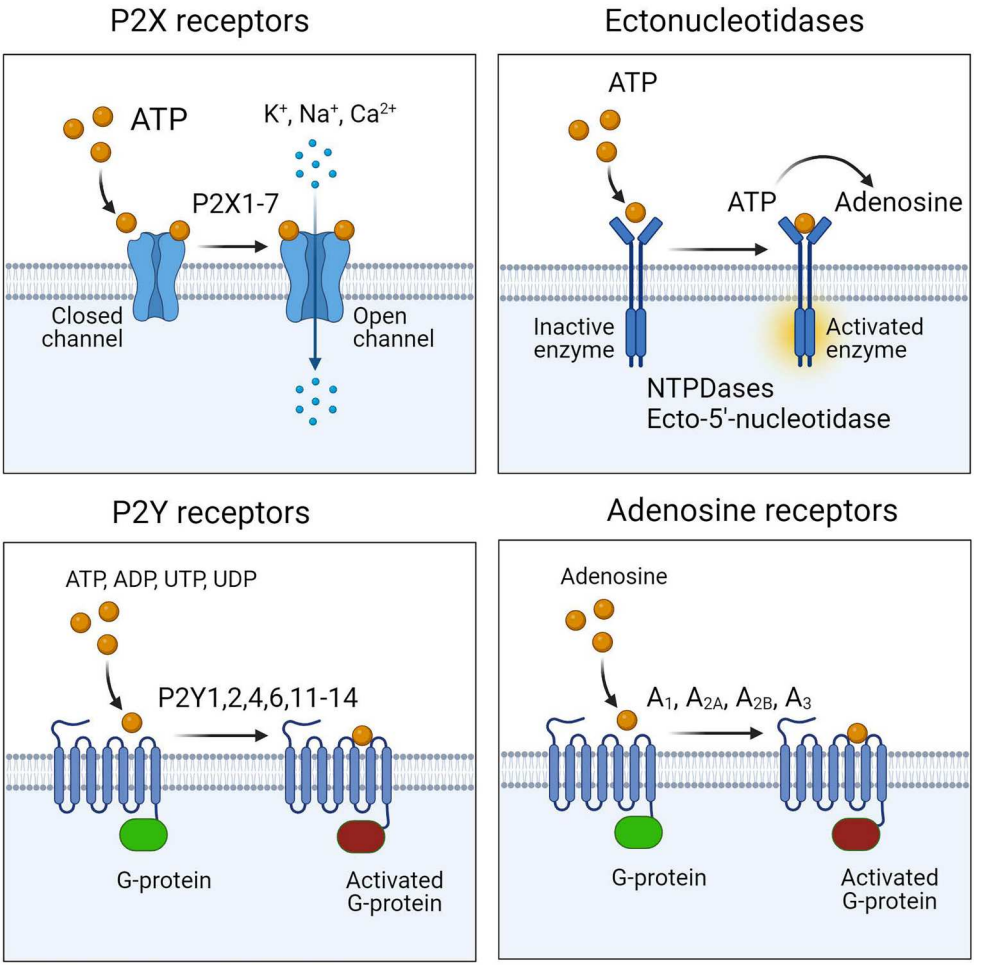


**Figure 2.** Purinergic signalling system. Extracellular ATP activates ATP-gated ion channels (P2X receptors) permeable to mono- and divalent cations and G protein-coupled receptors (P2Y receptors) activated by various nucleotides (ATP, UTP, ADP, UDP). ATP is hydrolysed to adenosine by surface-located ectonucleotidases (NTPDases and ecto-5′-nucleotidase). Adenosine activates four types of G protein-coupled adenosine receptors ($A_1$, $A_{2A}$, $A_{2B}$, $A_3$). Created with BioRender.com.

## Our journey

Our journey uncovering the complexity of the purinergic signalling system in the cochlea started at the University of Auckland Department of Physiology in the early the initial identification of $Ca^{2+}$ responses evoked by applying extracellular ATP to isolated cochlear hair cells (Ashmore and Ohmori 1990), a partnership supported by the New Zealand Medical Research Council and New Zealand Lottery Board was established in the Department of Physiology at Auckland University to investigate the physiological significance of what at the time was seen as a remarkable characteristic of these sound transducer cells in the ear. The funding enabled the early adoption of single-cell voltage-clamp electrophysiology and molecular imaging in Auckland. This research rapidly revealed, via precision delivery of ATP molecules at discrete positions along the length of guinea pig cochlear

OHCs, that the receptors for ATP were localised to the apical (transduction) site of the cells, suggesting a novel humoral functionality (Ashmore et al. 1992; Housley et al. 1992). At this stage, the field of P2 receptor neurobiology, fostered by Geoffrey Burnstock (Burnstock 1990) was challenged by the limited specificity of probes. However, applying the ATP analogue 2'-(or-3')-O-(trinitrophenyl)adenosine-5'- triphosphate (TNF-ATP), which has the unusual property of increasing its fluorescence when bound to ATP receptors, yielded remarkable insight to the cochlear ATP signalling mechanism. The TNF-ATP flashed as it was delivered to the sensory (apical) pole of the OHC, validating the electrophysiological assessment of the site of ion channel action and showing the actual location of the ATP-gated ion channels on the apical cell membrane. This prompted Mockett et al. (1994) to propose that the physiological role of ATP must be principally associated with the endolymphatic compartment of the cochlear partition (Mockett et al. 1994). It was further reinforced with additional physiological imaging of the ATP-gated ion entry, confirmed as the endolymph-facing surface (Mockett et al. 1994).

In a follow-up study, Mockett et al. (1995) used two radioligands to identify extracellular ATP binding sites in guinea pig cochlear tissue. [3H]alpha, beta-methylene-ATP, a high-affinity probe for P2X receptors, showed that the ATP-gated channels were also prominent on supporting cells in the OoC. The use of deoxyadenosine 5'-(alpha-[35S]thio)triphosphate ([35S]dATP alpha S), a high activity probe for the P2Y receptors, showed differential labelling, broadening the physiological base for purinergic signalling to the two P2 receptor types within the organ of Corti, stria vascularis, and spiral prominence region in the spiral ligament and suggesting complex extracellular purine-mediated control of cochlear function (Mockett et al. 1995).

In the same year, Muñoz et al. (1995) evaluated functional changes in the guinea pig cochlea after administering ATP into the scala media. Exogenous ATP showed a dose-dependent suppressive effect on the endocochlear potential (EP) and cochlear microphonic (CM) receptor potential generated primarily by OHCs. The response was inhibited by the non-selective P2 receptor antagonists suramin and reactive blue 2. This study provided the first evidence of P2 receptor-mediated effects of extracellular ATP on cochlear function in anaesthetised animals.

We then investigated the concentration of ATP in cochlear fluids. Muñoz et al. (1995) measured the concentration of ATP in the endolymph, perilymph, and cerebrospinal fluid (CSF) collected from anaesthetised guinea pigs using the luciferase-luciferin reaction. ATP concentrations in endolymph, perilymph and CSF were similar: $12.9 \pm 2.4$ nM, $10.5 \pm 3.9$ nM, and $16.1 \pm 5.4$ nM, respectively (Muñoz et al. 1995). This study was the first to provide evidence of free extracellular ATP in cochlear fluids.

The next intriguing question was the source of ATP in the cochlea. In the study by White et al. (1995), guinea pig cochlear tissues (the sensory epithelium and lateral wall) were incubated with the acridine derivative quinacrine, which fluoresces upon binding to ATP. This study showed that purine compounds were localised in discrete vesicles within the marginal cells of the stria vascularis, most likely serving as a source of extracellular ATP in the cochlea. ATP localisation was confirmed by cell fractionation studies to extract the vesicles from the lateral wall tissues and measure the ATP concentration in them (Muñoz et al. 2001). The same study (Muñoz et al. 2001) also demonstrated elevated ATP levels in the endolymph during noise exposure, suggesting that ATP is increasingly secreted from the vesicular stores under metabolic stress conditions.

The limitations on resolving cochlear purinergic signal transduction elements inherent to pharmacological profiles of P2X and P2Y analogues were largely resolved when the receptors were cloned, revealing the breadth of the gene families, as detailed earlier. Key to the cochlear studies was the cloning of the P2RX2 gene by the Julius lab (Brake et al. 1994). This P2X2 receptor subunit assembles as a trimer to form the cochlear ATP-gated non-selective cation channel. In cochlear tissue, we were able to identify novel splice variants of the P2RX2 mRNA transcript (Housley et al. 1995) and produce a riboprobe against the *P2rx2* mRNA transcripts for *in situ* hybridisation in rat cochlear tissues (Housley et al. 1998). This work initiated a longstanding collaboration with the Ryan lab at the University of California San Diego, with funding from the Royal Society of New Zealand Marsden Fund, Deafness Research Foundation of New Zealand, and the Maurice and Phyllis Paykel Trust. The study in the rat cochlea also revealed significant P2X2 receptor expression in the spiral ganglion neurons, supporting a role for extracellular ATP release from the IHCs alongside the primary auditory neurotransmitter glutamate (Housley and Ryan 1997; Housley et al. 1998).

Alongside the early characterisation of P2 receptor neurobiology in the inner ear, the team at Auckland investigated the physiological regulation of the P2 receptor signalling by a family of ecto-enzymes that hydrolyse ATP in the extracellular space (Vlajkovic et al. 1996). To assess ectonucleotidase activity in cochlear perilymph and endolymph, we used anaesthetised animals or an isolated guinea pig cochlea (Vlajkovic et al. 1996; Vlajkovic et al. 1998a, 1998b). The viability of the isolated guinea pig cochlea was maintained with oxygenated artificial perilymph perfused at a rate of 100 µl/min (Vlajkovic et al. 1998a, 1998b). Effluent collected from the cochlea was assayed for adenine nucleotide metabolites by reverse-phase high-performance liquid chromatography (RP-HPLC). Upon perfusion, extracellular ATP and ADP were rapidly and sequentially hydrolysed to adenosine by ecto-enzymes lining intact cochlear tissues. Our results indicate the presence of considerable ectonucleotidase activity within the perilymphatic compartment of the cochlea (Vlajkovic et al. 1998b). Similarly, extracellular ATP degradation in the cochlear endolymphatic compartment was $Ca^{2+}/Mg^{2+}$-dependent, and was not affected by inhibitors of intracellular ATPases (Vlajkovic et al. 1998a), supporting the role of NTPDases in regulating extracellular P2 receptor signalling.

After these early studies, aided by early access to antibody probes against P2X and P2Y receptors, our research focused on the characterisation of receptor expression plasticity in different environmental conditions and the role of P2 receptors in cochlear development and maintenance of hearing sensitivity. In parallel, we investigated the expression and distribution of ectonucleotidases and adenosine receptors in the cochlea and their respective roles in cochlear protection from stress and injury.

## P2x receptor diversity in the cochlea

We and others have shown that P2X receptor distribution depends on the stage of cochlear development. Our immunolocalisation studies demonstrated that the developing mammalian cochlea contains all P2X receptor subunits, whereas in the adult cochlea, there is a more limited expression profile (Housley et al. 2009; Köles et al. 2019). For example, Nikolic et al. (2001) showed that the P2X1 receptor is transiently expressed

in the otic capsule, spiral limbus, epithelial cells of the Reissner's membrane, and spiral ganglion neurons (SGN) during early postnatal development in rats but is absent after hearing onset (around postnatal day 12).

In contrast, the P2X2 receptor (P2X2R) is the predominant P2X subtype expressed in adult rat, mouse and guinea pig cochlea (King et al. 1998; Housley et al. 1999; Järlebark et al. 2000; Järlebark et al. 2002). P2X2R was immunolocalised in epithelial cells lining the cochlear partition, including the inner and outer sensory hair cells and supporting Deiters' cells in the organ of Corti and Reissner's membrane that separates endolymph in scala media from perilymph in scala vestibuli. P2X2 receptor subunit expression was also observed in cochlear type I SGN involved in afferent neurotransmission (Salih et al. 1998; Housley et al. 1999). Salih et al. demonstrated that the SGN in rat cochlea express three different isoforms of the P2X2 receptor subunit generated by alternative splicing and postulated that the variations in P2X2R isoforms might confer functional heterogeneity (Salih et al. 1998; Salih et al. 2002).

Huang et al. have shown that the $P2X_3$ receptor (P2X3R) is transiently expressed in the developing cochlea (Huang et al. 2005). P2X3R subunit expression is specifically targeted to the afferent terminals before the onset of hearing in rats and likely contributes to the neurotrophic signalling that activates auditory neurotransmission. The transient expression of these receptors and precise spatiotemporal profile suggest the role of $P2X_3R$ in synaptic pruning (Huang et al. 2005; Huang et al. 2006). This was confirmed by Greenwood et al. (2007), who demonstrated with single-cell RT–PCR that tight temporal control of the P2RX2-3 mRNA splice variant, alongside expression of the P2RX3 gene transcript, occurred at the time of consolidation of the spiral ganglion neurites to the target hair cells, inhibiting brain-derived neurotrophic factor (BDNF) – mediated neuritogenesis.

Nikolic et al. studied P2X7 receptor subunit expression in the embryonic (E14-E18 days) and postnatal (P0-adult) rat cochlea using immunohistochemistry (Nikolic et al. 2003). Strong P2X7 immunolabelling was observed in the SGN from E18 to adult. P2X7 immunolabelling was observed in the inner and outer hair cells over a limited period during development, from birth to P6. The P2X7 immunolocalisation in the olivocochlear efferent fibres innervating the sensory hair cells implicates extracellular ATP in establishing auditory neurotransmission (Nikolic et al. 2003).

The localisation of the P2X receptors to the apical surface of the hair cells supports an ATP-mediated modulation of $K^+$ current across the cochlear partition. P2X receptors could regulate hearing sensitivity by controlling the EP as a driving force for mechanoelectrical transduction in sensory hair cells (Housley et al. 1998). The study by Muñoz et al. (1999b) supported the evidence that the extracellular ATP introduced into the endolymphatic compartment of the guinea pig cochlea suppresses cochlear function, EP in particular, via P2X receptors. Quantitation of the ATP-gated (P2X2R) currents in guinea pig hair cells isolated from different regions of the OoC showed remarkable sound frequency-related regulation of channel expression, with a significant progressive increase in the currents (number of channels) in OHCs isolated from progressively more basal (higher frequency encoding) regions of the cochlea (Raybould and Housley 1997). This is required to regulate the tonotopic gradient in standing basolateral $K^+$ conductance of these cells, which would functionally impact the OHC-mediated 'cochlear amplifier'. Supporting this postulate, IHCs have similar ATP-gated conductances irrespective of which region of the OoC the cells are derived (Raybould et al. 2001).

Overall, these studies strongly link purinergic regulation of sound transduction, modulation of cochlear micromechanics by hair cells, supporting cells, and auditory neurotransmission (Housley et al. 1999; Housley et al. 2002; Thorne et al. 2002). Extracellular ATP may also have a role in maintaining cochlear electrochemical homeostasis, regulating hearing sensitivity and vascular tone (Muñoz et al. 1999a; Housley 2000; Housley and Thorne 2000). ATP can be classified as a putative auditory neurotransmitter based on the P2X receptor localisation at the afferent synapses of the inner hair cells and functional verification of ATP-gated currents in SGN in situ. In addition, ATP release from the transient structure in the developing cochlea, known as Kölliker's organ, is involved in developing sound-independent spontaneous activity in the auditory system before the onset of hearing (Dayaratne et al. 2014, 2015).

## P2x receptors and cochlear adaptive response to noise

Wang et al. (2003) demonstrated for the first time, using riboprobe in situ hybridisation, quantitative immunofluorescence, and patch-clamp, that $P2X_2$ receptor gene expression in the cochlea was adaptive, as sustained loud sound promoted $P2X_2$ receptor upregulation in cochlear regions regulating hearing sensitivity and auditory neurotransmission, with average ATP-gated currents 3-fold greater in OHCs from noise-exposed rats. Our subsequent studies showed that $P2X_2$ receptor upregulation is critical for cochlear protection from loud sound.

Thorne et al. (2004) demonstrated that introducing ATP into the scala media of the guinea pig cochlea decreases the EP and the cochlear partition resistance (CoPR) of the endolymphatic compartment, suggesting a novel ATP-mediated regulatory mechanism that can reduce hearing sensitivity. The follow-up studies have directly demonstrated that ATP-activated $P2X_2$ receptors in the cochlear partition provide a $K^+$ shunt conductance away from the endolymphatic compartment, which reduces the driving force for sound transduction and, consequently, hearing sensitivity (Housley et al. 2013; Morton-Jones et al. 2015). $K^+$ shunt conductance contributes to protective hearing adaptation with sustained elevated sound levels that likely prevent permanent hearing loss. In a critical study, Housley et al. (2013) demonstrated that mice with genetic deletion of the *P2rx2* gene had hair cells lacking any ATP-gated currents, and these mice failed to exhibit temporary threshold shift (TTS) observed in wildtype mice after exposure to sustained moderate noise levels (85 dB SPL). Instead, the mice lacking the P2X2 receptor showed increased vulnerability to sustained loud sounds at higher noise levels due to the lack of adaptive response to loud sounds. This promoted the novel concept that a component of the reversible hearing loss with elevated noise was 'purinergic hearing adaption'. This intrinsic humoral physiological homeostatic regulation was confirmed to dynamically regulate cochlear amplifier sensitivity (Cederholm et al. 2019), which confers otoprotection in noise alongside the dynamic efferent medial olivocochlear system (Housley et al. 2020). These studies extended to a broad international collaboration, where accelerated noise- and age-related hearing loss in the *P2rx2* knockout mice was shown to parallel progressive hearing loss due to a point mutation of the *P2RX2* gene in two Chinese families diagnosed with DFNA41 autosomal dominant progressive non-syndromic hearing loss. This presents before children reach their teenage years (Yan et al. 2013), and the rate of hearing loss is exacerbated when members of these families live in noisy environments.

Together, these studies highlight the critical role of P2X2R in hearing protection from loud sounds in animals and humans. Interestingly, the adaptive response of the cochlea to noise may be reduced in older animals, which could increase their susceptibility to noise-induced cochlear injury and hearing loss (Telang et al. 2010). However, the strength of the P2X2R–mediated purinergic otoprotection in individuals depends not only on the degree to which the ATP-gated ion channels are present on the hair cells and other epithelial cells lining the cochlear partition in individuals across their lifetime but also upon variations of associated signalling complex elements, particularly regarding the ATP-release pathways and the ectonucleotidase-mediated signal termination.

## P2y receptor localisation in the cochlea

Huang et al. (2010) evaluated the distribution of five P2Y receptors, P2Y1, P2Y2, P2Y4, P2Y6, and P2Y12, during cochlear development using immunohistochemistry. The P2Y receptors were present in the cells lining the cochlear partition from the late embryonic period. P2Y2 and P2Y4 receptors were associated with the greater epithelial ridge, a transient cell population that disappears during postnatal cochlear maturation (Huang et al. 2010). P2Y receptors (P2Y2, P2Y4, P2Y6, and P2Y12) were detected in the SGN at birth except for P2Y1, which was expressed later in the postnatal age, and the same expression pattern was retained until adulthood. The extensive distribution of purinergic P2Y receptors suggests their multiple roles in cochlear development, maintaining cochlear homeostasis, and regulating sound transduction and neurotransmission (Housley et al. 2009).

## Ectonucleotidases in the cochlea

Ectonucleotidases are surface-located enzymes that hydrolyse extracellular nucleotides to their respective nucleosides, terminating P2 receptor signalling in mammalian tissues (Robson et al. 2006) (Figure 2). We have characterised the ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) family of ectonucleotidases in the mammalian cochlea and shown that the enzymes from this family (NTPDase1-8) are expressed in the adult rat cochlea with distribution in the vasculature, sensory, and neural tissues (Vlajkovic et al. 2009).

NTPDase1 and NTPDase2 are the principal NTPDases in the rat and mouse cochlea (Vlajkovic et al. 2002a, 2002b). Both NTPDases are distributed in the apical and synaptic regions of the sensory hair cells. NTPDase1 was also immunolocalised in the cochlear vasculature and spiral ganglion neurons, whereas NTPDase2 immunoreactivity was detected in the stria vascularis (Vlajkovic et al. 2002a, 2002b). These two enzymes likely regulate extracellular ATP signalling that controls cochlear blood flow, sound transduction, and cochlear neurotransmission (Vlajkovic et al. 2002a, 2002b).

NTPDase3 also regulates ATP signalling associated with auditory neurotransmission (Vlajkovic et al. 2006). At elevated sound levels, NTPDase3 immunolabeling is increased in the synaptic regions of the inner and outer hair cells, suggesting the potential neuroprotective nature of this ectonucleotidase (Vlajkovic et al. 2006).

NTPDase 5 and 6 are intracellular members of the NTPDase family that can be released in a soluble form. Changes in the expression pattern of both enzymes were observed in the developing and adult rat cochlea (O'Keeffe et al. 2010a, 2010b). In the

adult rat cochlea, NTPDase5 upregulation with loud sound suggests its role in cochlear response to noise stress (O'Keeffe et al. 2010b). NTPDase6 immunolocalisation in the sensory hair cells of the developing cochlea and vestibular end organ suggests its role in hair cell bundle development in both sensory organs (O'Keeffe et al. 2012).

We have also demonstrated that alternative splicing in the NTPDase family contributes to the diversity of purinergic signalling, and may explain differences in kinetic and biochemical properties of ecto-enzymes in the cochlea (Vlajkovic et al. 1999; Wang et al. 2005). Our studies suggest that P2X receptor activation stimulates the insertion of NTPDase2 from intracellular stores into the plasma membrane, which may reflect a regulatory mechanism preventing excessive stimulation and desensitisation of P2 receptors (Vlajkovic et al. 2007).

Stimuli such as noise or hypoxia could induce the excessive release of ATP into the cochlear fluid spaces (Muñoz et al. 2001). Membrane-bound NTPDases 1, 2, and 3 appear essential for regulating extracellular nucleotide concentrations and P2 receptor signalling in the cochlea in adverse environmental conditions. In the rat cochlea exposed to traumatic noise (110 dB SPL), we observed increased expression of NTPDase1-3 and increased ATPase activities in the cochlea (Vlajkovic et al. 2004; Vlajkovic et al. 2006). This likely represents a homeostatic response of cochlear tissues to regulate ATP signalling during noise exposure and thus protect the cochlea (Vlajkovic et al. 2004).

## Adenosine receptor signalling in the cochlea

Adenosine is a purinergic signalling molecule that modulates cellular activity in the brain and peripheral organs via four G protein-coupled receptors ($A_1$, $A_{2A}$, $A_{2B}$, $A_3$) (IJzerman et al. 2022) (Figure 2). Adenosine is also a constitutive cell metabolite with an established role in tissue protection. We have shown that adenosine concentrations in cochlear fluids are regulated through selective adenosine uptake by nucleoside transporters (Vlajkovic et al. 2009). Two equilibrative (ENT1 and ENT2) and two concentrative (CNT1 and CNT2) adenosine transporters are expressed in the rat cochlea (Khan et al. 2007). Khan et al. (2007) demonstrated that bi-directional nucleoside transport in the cochlea regulates adenosine concentrations in cochlear fluid spaces and supports adenosine recycling.

Vlajkovic, Abi, et al. (2007) demonstrated a differential distribution of adenosine receptors in the mammalian cochlea. The specific tissue distribution of adenosine receptors in the vasculature, sensory, and neural tissues implies their respective roles in regulating cochlear blood flow, sensory transduction, and auditory neurotransmission (Vlajkovic et al. 2009).

Adenosine receptors also regulate the cochlear response to stress and injury (Vlajkovic et al. 2009; Vlajkovic and Thorne 2022). Activation of adenosine $A_1$ receptors, in particular, can reduce oxidative stress and inflammation in the cochlea and thus prevent cochlear injury (Vlajkovic et al. 2009; Vlajkovic and Thorne 2022). $A_{2A}$ receptors can also modify cochlear function in health and disease (Vlajkovic and Thorne 2022). The balance between $A_1$ and $A_{2A}$ receptors appears critical for cochlear response to oxidative stress, an underlying mechanism of noise-induced, age-related, and drug-induced hearing loss. Our studies on mice with global deletion of $A_1$ or $A_{2A}$ receptors

demonstrated the distinct roles of these receptors in cochlear physiology and response to injury (Vlajkovic et al. 2017). In $A_1R$ knockout mice, we observed early-onset high-frequency hearing loss aggravated by noise exposure (Vlajkovic et al. 2017). In contrast, the $A_{2A}R$ deletion improved the survival of sensorineural tissues in the cochlea after exposure to traumatic noise. The $A_{2A}R$ knockout mice demonstrated better preservation of OHC and afferent synapses and minimal loss of SGN after noise exposure. This study suggests that the loss of $A_1R$ increases susceptibility to cochlear injury and hearing loss, whilst the absence of $A_{2A}R$ increases cochlear resistance to acoustic trauma (Vlajkovic et al. 2017).

## Adenosine $A_1$ receptors prevent sensorineural hearing loss

The adenosine $A_1$ receptors are the primary mediators of cytoprotection in the cochlea by inhibiting oxidative stress, inflammation and apoptotic pathways (Vlajkovic and Thorne 2022). Wong et al. (2010) first showed that post-exposure treatment with $A_1R$ agonists in rats acutely exposed to traumatic noise can mitigate noise-induced hearing loss (NIHL). $A_1R$ agonists (adenosine, 2-chloro-N-cyclopentyladenosine, or CCPA) were applied locally to the round window membrane of the cochlea 6 h after noise exposure. The treatment reduced auditory brainstem response threshold shifts, oxidative stress markers in the cochlea, and hair cell loss (Wong et al. 2010).

Later studies (Vlajkovic et al. 2010; Vlajkovic et al. 2014; Chang et al. 2017) demonstrated the extraordinary potential of a selective $A_1R$ agonist, adenosine amine congener (ADAC), for the systemic post-exposure treatment of NIHL in rats. We have demonstrated the dose-dependent rescue effect of ADAC on noise-induced cochlear injury and established the time window for treatment (Vlajkovic et al. 2014). ADAC was most effective in the first 24 h after noise exposure at doses 50–200 μg/kg administered as five consecutive daily intraperitoneal injections. Hearing acuity was assessed using auditory brainstem responses (ABR), and ADAC provided up to 21 dB protection averaged across 8–28 kHz frequencies. Delayed treatment 48 h after noise exposure provided slightly lower but still clinically significant improvement in ABR thresholds (>10 dB) (Vlajkovic et al. 2014). We also observed increased survival of sensory hair cells and reduced oxidative stress markers in ADAC-treated cochlea (Vlajkovic et al. 2010). These data show that ADAC mitigates NIHL in a dose- and time-dependent manner, strongly supporting ADAC development as a potential clinical treatment for acute NIHL.

ADAC can also protect the cochlea from ototoxic injury caused by anti-cancer drugs and aminoglycoside antibiotics (Gunewardene et al. 2013; Lin et al. 2019). Intraperitoneal administration of ADAC significantly reduced cisplatin-induced threshold shifts in rats, protected against cisplatin-induced hair cell loss, and reduced apoptosis in marginal cells of the stria vascularis (Gunewardene et al. 2013). Using an organotypic tissue culture model of the neonatal mouse cochlea, Lin et al. (2019) investigated the effect of ADAC and adenosine on sensory hair cell survival after exposure to the ototoxic aminoglycoside neomycin. The activation of adenosine receptors by ADAC and adenosine conferred partial protection from neomycin ototoxicity. These studies suggest that the $A_1R$ contributes significantly to cochlear protection from ototoxic drugs and mitigates drug-induced hearing loss.

## Adenosine $A_{2A}$ receptors and hearing loss

The $A_{2A}$ receptors also play a significant role in cochlear response to injury but, interestingly, in the opposite direction to the $A_1R$. We have previously demonstrated that the $A_{2A}R$ knockout mice show better preservation of cochlear afferent synapses and spiral ganglion neurons than wildtype mice after acoustic overexposure (Vlajkovic et al. 2017). Consistent with this, Han et al. (2019) showed that $A_{2A}R$ inhibition can mitigate excitotoxic injury caused by NMDA and kainic acid in a rat organotypic tissue culture of the cochlea. The administration of istradefylline (an FDA-approved $A_{2A}R$ antagonist) along with NMDA and kainic acid prevented deafferentation of the inner hair cells (Han et al. 2019). This study proved that $A_{2A}R$ inhibition has a neuroprotective role in the cochlea, promoting cochlear recovery from excitotoxic injury.

Age-related hearing loss (ARHL) is the most common sensory disorder in older people, and yet, the treatment options are limited to medical devices such as hearing aids and cochlear implants. We have demonstrated that $A_{2A}$ receptor targeting is a novel strategy for the preventative treatment of ARHL. In a study by Shin et al. (2021), C57BL/6 mice showing early onset hearing loss were given weekly istradefylline injections from 6 to 12 months of age, and auditory function was assessed periodically by ABR. Weekly injections of istradefylline attenuated ABR threshold shifts by approximately 20 dB at mid to high frequencies (16–32 kHz) and improved hair cell survival in the cochlea (Shin et al. 2021). This study presents the first evidence for the rescue potential of istradefylline in ARHL.

## Regulators of adenosine receptor signalling and hearing loss prevention

In addition to the activation/inhibition of adenosine receptors, the inhibition of adenosine metabolism can also change hearing outcomes in animal models of ARHL and NIHL. Vlajkovic et al. (2011) demonstrated that inhibition of adenosine kinase (Adk), the principal enzyme in adenosine metabolism that regulates intracellular and extracellular adenosine concentrations, could provide partial protection from ARHL in a mouse model of accelerated hearing loss. A selective Adk inhibitor ABT-702 was administered to mice systemically twice a week for six months. At nine months, mice treated with ABT-702 exhibited reduced ABR threshold shifts and improved survival of sensory hair cells relative to non-treated mice. The study was the first evidence that ARHL could be mitigated or delayed by enhancing endogenous adenosine receptor signalling in the cochlea (Vlajkovic et al. 2011).

A study by Fok et al. (2020) demonstrated that enhancing endogenous $A_1R$ signalling in the cochlea by inhibiting the Regulator of G protein signalling 4 (RGS4) can greatly improve hearing outcomes in rats exposed to traumatic noise. RGS4 inhibits signal transduction pathways initiated by $A_1R$. In this study, adult rats were exposed to high-level noise (110 dB SPL, 8–16 kHz) for 2 h. 24 or 48 h after noise exposure, a small molecule RGS4 inhibitor CCG-4986 was delivered intratympanically in a Poloxamer-407 gel formulation for sustained drug release. Intratympanic administration of CCG-4986 24 h after noise exposure attenuated noise-induced permanent hearing loss by up to 32 dB. The later drug administration (48 h) was also effective, improving auditory thresholds by up to 19 dB. For perspective, 3 dB in the log scale halves hearing loss. The functional

improvement was associated with improved survival of sensorineural tissues and afferent synapses in the cochlea. This study shows that intratympanic administration of an RGS4 inhibitor can rescue cochlear injury and hearing loss induced by acoustic overexposure, representing a potentially novel paradigm for treating noise-induced SNHL.

## Conclusions

Developing inner ear therapies targeting purine receptors has been a long scientific journey that began with basic research characterising P1 and P2 receptors in the cochlea. Purinergic signalling is a complex system of extracellular receptors for ATP and adenosine, pannexin and connexin hemichannels for ATP release, ecto-enzymes regulating ATP concentrations in cochlear fluids, and adenosine transporters. Our studies have shown that P2X and P2Y receptors are essential for cochlear development, regulation of electrochemical homeostasis, auditory neurotransmission, and adaptation to elevated sound levels. Ectonucleotidases are crucial in regulating P2 receptor signalling in the cochlea in physiological and pathophysiological conditions. We and others have also identified adenosine receptor signalling as a critical element of the cochlear response

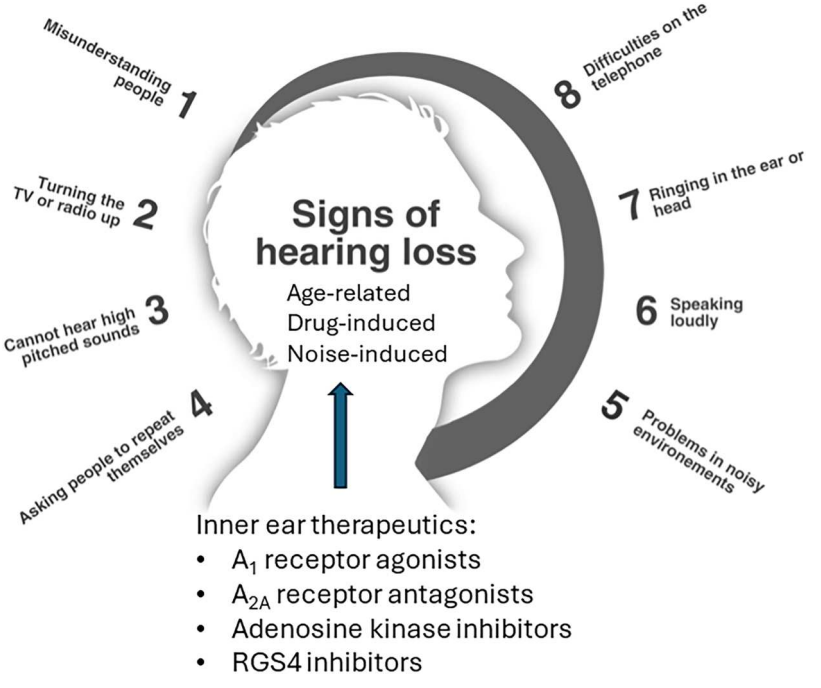


**Figure 3.** Adenosine receptors as inner ear therapeutics. The most common forms of sensorineural hearing loss (SNHL) are age-related, drug-induced, and noise-induced hearing loss. The rescue effect of adenosine receptors has been demonstrated in preclinical studies using animal models of SNHL. These studies showed that the balance of $A_1$ and $A_{2A}$ receptors is critical for cochlear response to stress and injury. Pharmacological interventions include the intratympanic or systemic administration of $A_1$ receptor agonists, $A_{2A}$ receptor antagonists, adenosine kinase (Adk) inhibitors, or RGS4 (Regulator of G protein signalling 4) inhibitors. Local intratympanic injections are the preferred delivery route as they preclude off-target effects associated with systemic administration.

to environmental stressors. The balanced activity of adenosine $A_1$ and $A_{2A}$ receptors regulates cochlear neurotransmission, blood flow, and reactions to stress and injury. Using animal models of SNHL (noise-, drug- and age-related), we have established that pharmacological targeting or adenosine receptors and adenosine metabolic pathways can rescue the cochlea from injury and improve the survival of sensorineural tissues (Figure 3). Substantial progress made in the last three decades presented in this review is a reflection of the Auckland Auditory Neuroscience Group legacy of discovery and translational research, built through innovation and collaboration, that has helped establish the field of purinergic signalling in the inner ear and laid the foundations for the development of adenosine receptor-based therapies for different forms of sensorineural hearing loss.

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

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