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The use of animal models to study cell transplantation in neuropathic hearing loss

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Abstract

Auditory neuropathy (AN) is a form of sensorineural deafness specifically affecting the conduction of the nerve impulse from the cochlear hair cells to the auditory centres of the brain. As such, the condition is a potential clinical target for 'cell replacement therapy', in which a functioning auditory nerve is regenerated by transplanting an appropriated neural progenitor.

In this review, we survey the current literature and examine possible experimental models for this condition, with particular reference to their compatibility as suitable hosts for transplantation. The use of exogenous neurotoxic agents such as ouabain or β-bungarotoxin is discussed, as are ageing and noise-induced synaptopathy models. Lesioning of the nerve by mechanical damage during surgery and the neuropathy resulting from infectious diseases may be very relevant clinically, and we discuss whether there are good models for these situations. We also address genetic models for AN, examining whether the phenotypes truly model the clinical situation in their human counterpart syndromes - we use the example of the hyperbilirubinaemic Gunn rat as a particular instance in this regard.

1. Introduction

The death of the spiral ganglion neuron is a sombre affair. Damage to the auditory nerve may happen for a number of reasons and can occur as the cause or consequence of hair cell loss - in either case, the neural lesion is permanent and can be devastating for the patient. In the situation of profound hair cell loss, the therapeutic option of cochlear implantation is open to some patients, but this is reliant on there being a degree of neural reserve within the cochlea, so for some this is not a suitable option. The ability to restock the auditory nerve with a stem cell/progenitor transplant and restore functionality in patients with auditory neuropathy (AN) would be of significant benefit for both those with a pure neural lesion and those potential cochlear implant (CI) recipients.

Morphologically, spiral ganglion neurons (SGNs) are bipolar, with a peripheral process innervating the inner hair cells (IHCs) and a central projection relaying the acoustic stimulus to the cochlear nucleus in the brainstem. Consequently, any transplanted cells must possess the intrinsic capacity to 'rewire' in both directions, in a host environment which can support this regeneration.

1.1 Scope

The scope of this review will be restricted to the discussion of potential cell therapies for the neural aspect of sensorineural hearing loss (SNHL). Although significant progress has been made in the *in vitro* production of hair cells (Koehler et al., 2017; Li et al., 2003), there still remain several major technical hurdles to overcome, as current protocols are starkly inefficient at generating numbers adequate for meaningful hearing restoration. And while it can be argued that highly differentiated hair cells are not necessary for cell replacement - given that the progenitor population is the one to be transplanted - the approaches to cell delivery into the scala media and successful integration of the exogenous cells into the epithelium are as yet in their infancy, with limited success. It will be a future biological challenge to have cells home and orient correctly within the exquisitely regimented array of the organ of Corti.

1.2 Sensorineural pathology in the human ear

Auditory neuropathy (AN) can be described as a complex of conditions affecting neural output between the cochlea and the brain, specifically when hair cell function is apparently normal (Starr and Rance, 2015). For example in the audiology clinic, a patient may have a normal distortion product otoacoustic emission (DPOAE) response, but abnormal auditory brainstem response (ABR) wave patterns. Determining the site of the underlying dysfunction, for instance whether it lies within the SGNs and damage to their bipolar processes, is key to understanding the aetiology of the hearing deficit (Moser and Starr, 2016; Rance and Starr, 2015; Santarelli, 2010). Within AN, a subset of disorders may be caused by synaptopathy - a degradation of the specialised ribbon synapse at the level of the IHC. The faithful transmission of the signal between the hair cell and the auditory centres is necessary for the spatial and temporal integration of incoming sounds; degradation of this information may be causative factors for age-related and noise-induced hearing loss (Moser and Starr, 2016)

Detailed and systematic histopathological studies of the damage induced by various causes to the human cochlea in general, and to the spiral ganglion in particular, are still scarce. However, a few key papers have explored the underlying pathology in human ears developed as a consequence of ageing, some genetic conditions and exposure to ototoxic drugs (briefly discussed in Sagers et al. (2017). A comprehensive study was performed by Makary et al. (2011) reporting the decline of the spiral ganglion population in the human cochlea induced by ageing. They counted neurons in 100 cochleae from 100 individuals from a broad age range and cases were selected to include only those with a normal complement of inner or outer hair cells. Neuronal counts declined from a mean of 33679 in the first decade of life, to 22,444 in the tenth decade. This represents approximately a third of the normal neuronal complement being lost thorough primary degeneration, i.e. not following the loss of hair cells. Sagers et al. (2017) described the analysis of 30 ears from 23 patients, and showed that primary neuronal degeneration produced by several different pathologies correlated with the elevation of auditory thresholds and, more importantly, poor word recognition. Based on their data, the study developed a model that predicts that mean hearing thresholds are increased by 6.0dB for each 10% of neurons lost.

Progress in the transplantation field has been steady, with a few groups showing cell integration and functional restoration in ‘deafened’ animal models - but are these models a good facsimile of the clinical situation? It is likely that an initial good model for transplantation should have a near total loss of the host’s SGNs, so that any restored function or innervation can be attributed with confidence to the transplanted cells. However, given the partial loss seen in some human conditions, it will be good in the future to develop a subtler approach with a graded deficit, which might replicate some of the clinical situations more accurately.

This review will cover some of the animal models available currently, with an analysis of which are, in our opinion, the most appropriate for these studies, at this stage.

2. Ototoxic lesions of the auditory nerve

2.1 β-Bungarotoxin

β-bungarotoxin is a potent neurotoxin isolated from the venom of the krait family of snakes. Its activity is manifold, and it can paralyse the neuromuscular junction by transiently increasing and then blocking acetylcholine release. It is also lethal in the central nervous system – *in vitro* studies

have elucidated how it kills hippocampal neurons by the binding to and internalisation of voltage-gated potassium channels, leading to an increase in intracellular calcium ions and reactive oxygen species generation, resulting in apoptosis (Herkert et al., 2001; Shakhman et al., 2003).

As an ototoxic agent, it may have application through its more selective actions on neurons rather than hair cells – in the developing chick embryo, application of the toxin gave rise to a loss of afferent and efferent nerve fibres in the basilar papilla and saccular macula and a degeneration in the neurons of the acoustic-vestibular ganglion, in the context of developmentally normal hair cells (Hirokawa, 1977). In mammalian studies, β-bungarotoxin has been use to de-innervate mouse cochlea explants *in vitro* (Corrales et al., 2006) and to de-afferent the rat cochlea *in vivo* prior to stem cell transplants (Jiao et al., 2014; Palmgren et al., 2010; 2012). However, direct application of β-bungarotoxin to the rat round window niche using gelfoam, led to substantial increments of the ABR thresholds with no immediately evident spiral ganglion loss. A significant death of SGNs was only detectable 3 weeks post intervention. This time window between intervention and cellular damage would suggest that the effect is indirect and, although hair cells are preserved, other structures could have been affected such as the stria vascularis. This may limit the use of this model for a targeted reparative strategy to the spiral ganglion

Furthermore, when we modified these protocols and applied them to the gerbil, mixed results were achieved. Local application of β-bungarotoxin to the gerbil round window membrane (RWM) on a gelfoam plug showed no evidence of threshold shift, when concentrations comparable to those used in previously published work were used. At a higher concentration though, a substantial raising of the ABR threshold to a click stimulus could be attained within a few days of treatment, with a concomitant loss of Type I SGNs found by immunofluorescence after 5 weeks (Fig.1F, H, N, P) compared with the untreated condition (Fig.1B, D, J, L). Both the Type II SGNs (Fig. 1E, H) and the hair cells (Fig. 1M, P) were unaffected by the treatment. However, in order to achieve this neural loss, the toxin concentration had to be increased to a level whereby unpalatable 'off-target' effects such as hind-limb paresis and urinary incontinence were evident, prompting the termination of these experiments for welfare reasons. The potential for intra- and extra-cochlear off-target effects would lead to it being a less than ideal candidate for use as a neuropathy-inducing agent, particularly since it was no more effective than ouabain (see section 2.5, below).

2.2 Pyridoxine

Pyridoxine, or Vitamin B6 in common parlance, has been associated with the onset of toxic sensory neuropathy in patients consuming high daily doses (van Hunsel et al., 2018), and has been employed as a neuropathological agent in animal models (Umapathi and Chaudhry, (2005) and references therein). There are limited reports of pyridoxine's use as a specific agent to target auditory neurons. Hong et al. (2009) showed that daily systemic injections of the compound at 175, 350 or 700mg/kg gave a dose-dependent threshold shift of up to 60dB for both click and 8kHz tone stimuli. Interestingly, the onset of the deficit was around 5 weeks after the initiation of the treatment and gradually worsened until the experiments ceased at 10 weeks; the histology of the auditory nerve showed atrophy of the nerve fibres and thinning of their myelin sheaths. Notably, off-target effects such as the ataxia seen in rat pyridoxine models (Perry et al., 2004) were absent in these mice, save for a mild effect on the tail flick latency. There may also be an outer hair cell (OHC) loss associated with this pathology, shown by a loss of otoacoustic emission (OAE) signals arising 8 weeks after the onset of treatment – however this loss could be ameliorated by the neuroprotective effects of trigonellone or freeze-dried coffee, implying that the hair cell loss may arise as a secondary consequence of SGN deterioration rather than a primary insult by the pyridoxine (Hong et al., 2009). Further work is required to shade in the details of these intriguing findings - as such, this may prove

a good model for a slow and chronic neuropathy, as opposed to the rapid and catastrophic neural loss found with other agents.

2.3 Sodium salicylate

Transient SNHL and tinnitus have been well characterised as side effects of high doses of sodium salicylate, the active ingredient in aspirin. There is a breadth of data suggesting that salicylate has pleotropic effects on the hearing system – it is posited that salicylate-induced upregulation of the electromotility protein prestin in the OHCs may lead to their overactivity. The consequent imbalance with IHC firing may lead then lead to the perception of tinnitus, which itself may be exacerbated by salicylate's central effects on GABAergic and serotonergic activity in auditory areas of the brain (Sheppard et al., 2014). There is, however, conflicting evidence from *in vitro* and *in vivo* studies to suggest that chronic salicylate exposure may lead to a selective and permanent loss of SGNs via superoxide-mediated apoptosis. In organotypic cultures from young rat pups, Deng et al. (2013) saw a salicylate-mediated SGN loss which spared the adjacent hair cells and support cells; their *in vivo* experiments showed that chronic exposure led to a decrease in the compound action potential (CAP) measured at the round window (RW) (Sheppard et al., 2014). However, the I/O function produced indicated that the OHCs were functioning normally and the anomaly lay with the discharge from damaged SGNs. Conversely in the guinea pig, Feng and co-workers (2011a; 2011b) saw an upregulation of caspase-mediated apoptosis in both hair cells and SGNs after chronic salicylate administration.

So, although salicylate intoxication of the cochlea may represent a physiological model in terms of a 'real world' situation, it may not give a 'clean' neuropathy model. Thus, it may be difficult to interpret the results of a neural cell transplant in the potential context of concomitant hair cell damage and altered cortical activity.

2.4 Glutamatergic excitotoxicity

Excessive stimulation of the afferent dendrites by glutamate (or some of its analogues such as kainic acid) can lead to the loss of SGNs and may be the pathological process bringing about the damage caused by cochlear ischaemia, age related hearing loss with underlying vascular atrophy (Pujol et al., 1990) and even sepsis (Schmutzhard et al., 2013). The excitotoxic loss is worst in SGNs with low spontaneous discharge rates ('low-SR' fibres) and high sound thresholds (Furman et al., 2013) and so could lead to impaired speech comprehension, which may be particularly relevant in patients presenting with so-called 'hidden hearing loss' (HHL) caused by cochlear synaptopathy.

For many years, signalling at the afferent post-synaptic density remained opaque. The most established view is that afferent neurotransmission between the IHCs and the SGNs is predominantly mediated by AMPA receptors (Takago and Oshima-Takago, 2018). However, using a combination of RT-PCR and immunohistochemistry, Peppi et al. (2012) suggested that the 5 kainate receptor subunits (GluK1-5) were localised to the post-synaptic density of the afferent fibres, where they were coexpressed with AMPA receptors - this receptor mix may be underlying the transmission of the signal from the IHCs. This localisation was further refined by Fujikawa et al. (2014), who showed that GluK2 and GluK5 were present at the post-synaptic side of the afferent cleft, with, intriguingly, GluK2 also being present on the IHC, suggesting that the recycling of glutamate may be occurring on both sides of the synapse.

Overstimulation of the cochlea with kainic acid has been used as a model for glutamatergic damage. Although at lower doses, the loss of neural responses has been found to be reversible in the guinea pig (Sakai et al., 2008) and chinchilla (Zheng et al., 1997; Zheng et al., 1999), an increased

dose has been shown to lead to a 34% loss in Type I SGNs in the rat (Juiz et al., 1989). Using the different components of the ABR wave complex as a metric for the health of different aspects of auditory neural transmission, Henry and Abrams (2018) found that kainate intoxication caused a persistent reduction in wave i amplitude in the budgerigar, implying a selective damage or synaptopathy had occurred at the level of the auditory nerve. The differing susceptibility of the SGNs may reflect current thinking as to the heterogeneity of the sensory afferent population (Petitpre et al., 2018; Shrestha et al., 2018). There may be species-differences in the susceptibility of the hair cells to excitotoxic damage. For instance, in the rat, the hair cells were unscathed by the kainate insult (Juiz et al., 1989) but in the gerbil, the glutamate agonist AMPA was found to induce IHC death at high concentrations (Hyodo et al., 2009).

There is potential in the use of kainic acid or other glutamatergic agonists as a method of inducing AN - if the dose can be titrated correctly, it may give a clean loss of neurons, or more intriguingly, the loss of a subset of neurons, which may have implications for the treatment of cochlear synaptopathy-based HHL.

2.5 Ouabain

Ouabain is derived from *Stropanthus gratus*, a member of the liana family, whose leaves and seeds have been used for both traditional medicine and arrow poison in many African countries. Latterly, it has been adopted in Western medicine as an agent for the treatment of hypotension and cardiac issues (Whayne, 2018). It acts by inhibiting the α subunit of the Na^+, K^+-ATPase pump (NKA); NKA is a ubiquitous heterotrimeric enzyme consisting of catalytic α and β subunits and a regulatory γ subunit which work together to move sodium and potassium ions across cell membranes against their concentration gradient using ATP as an energy source. The different α subunits (ATP1A1-4) have varying sensitivities to ouabain, with α3 being more strongly inhibited than α2, and α1 being the least affected by the drug. ATP1A3 is particularly abundant in neurons, particularly throughout the peripheral processes and somata of the Type I SGNs (Delprat et al., 2007; McLean et al., 2009), whereas ATP1A2 is expressed by glia and ATP1A1 has a broad distribution. The topical application of ouabain to the RWM can be used as a tool to induce cell death in the spiral ganglion neurons without damaging the organ of Corti - this is advantageous as a model for cellular transplantation, as it preserves the putative trophic support given by the hair cells and support cells to the grafted progenitors.

The use of ouabain as a neurotoxic agent has been widespread in the field. Early work demonstrated the effects of ouabain on the endocochlear potential by direct infusion of the drug into the perilymph of the guinea pig (Konishi and Mendelsohn, 1970) or application at the RW in the chinchilla (Rybak et al., 1984), brought about by its actions on the stria vascularis. However, these effects were transient and almost completely absent in the gerbil (Schmiedt et al., 2002). Ouabain's use as neuropathy-inducing agent was therefore pioneered in the gerbil (Lang et al., 2005; Matsuoka et al., 2007; Schmiedt et al., 2002) where it has been shown to induce apoptosis of Type I SGNs within a few hours post-application, leading to robust obliteration of this cell type by 4-8d post-treatment. This provides an ideal backdrop for cell transplantation studies (Chen et al., 2012; Matsuoka et al., 2007). The mechanism of cell death being induced has been a matter of debate, with the term 'necroptosis' being coined. Cultured cortical neurons begin by swelling transiently in response to ouabain treatment, similar to neurons undergoing an excitotoxic lesion (see section 2.4), followed by intracellular Ca^{2+} build-up and a K^+ efflux which triggers cell shrinkage characteristic of necrosis and caspase activation leading to apoptosis (Xiao et al., 2002); these processes are thought

to occur concurrently within single cells, leading to a 'hybrid death' phenotype. *In vivo*, this process may be triggered by ouabain-mediated upregulation of Receptor Interacting Protein 3 (Rip3), a necroptosis pathway activator - Wang et al. (2014) showed that the ~50dB ABR threshold shift induced in the rat by 10mM ouabain could be prevented by the concurrent application of apoptosis and necroptosis inhibitors. This may have implications clinically for acute ototoxic incidents, with there being scope for the prevention of cell death with such drugs. Intriguingly, Type II SGNs are spared and do not die after ouabain treatment, and there remains debate as to why this should be the case. A simple explanation would be that they do not express ATP1A3 (McLean et al., 2009) and so are relatively ouabain resistant, due to their expression of a less-sensitive subunit. However, their perdurance even after exposure to high concentrations of the drug (Chen et al., 2012; Lang et al., 2005) suggests that they may be more efficient at invoking protective mechanisms - the toxic influx of Ca^{2+} caused by ouabain might be being buffered by the higher levels of calcineurin present in the Type II SGNs compared to the Type I (Lang et al., 2005), and so the necroptosis pathways remain untriggered.

Indeed, the use of low doses (10-100µM) in the gerbil gives a gradation of fibre loss which may be a relevant clinical model for synaptopathy-based HHL, wherein a patient's pure tone audiogram may be normal but speech becomes unintelligible to them in a noisy environment. Bourien (2014) and Huet and co-workers (2018) demonstrated in the gerbil that a 33µM dose of ouabain led to the loss of low-SR nerve fibres which did not affect the CAP threshold or amplitude, but which could lead to a reduced discriminatory ability in noisy circumstances. Increasing this dose to 66µM again did not alter the CAP threshold, but did reduce its amplitude due to the loss of medium- and high-SR fibres and a loss of synchrony in the response onset. Similar work in the mouse (Parthasarathy and Kujawa, 2018; Yuan et al., 2014) set out to titrate the ouabain treatment to give differing degrees of deafferentation and look at the effects on synaptic density and auditory responses. A loss of presynaptic ribbons and a decrease in the post-synaptic density at the IHCs was found, alongside tantalising evidence of neural plasticity - abnormal giant terminals were also seen in aberrant locations on the IHC, indicative of a neural remodelling which has failed to associate correctly with the hair cell ribbon synapses. Concomitantly, a raising of ABR thresholds was found, but only once a substantial number (>80%) of synapses had been lost. Thus, by using ouabain at these lower doses, there may be scope for uncovering the subtleties of the progressive loss of synapses, fibres and cell bodies which may be causative for the AN phenotype.

The concept of 'central compensation' can also be addressed with ouabain treatment. In some intricate and elegant experiments, Chambers and co-workers (2016a; 2016b) have demonstrated the plasticity of central processing after an auditory lesion. In mice treated with 1mM ouabain, the ABR and acoustic startle responses were both eliminated, but a behavioural paradigm for tone detection still gave a positive response, even in animals who had lost >90% of their synapses. Incredibly, activity in the auditory centres of the brain was maintained: in the inferior colliculus and the auditory cortex, activity recovers during the month following the lesion, and in some cases is more robust than in control animals. An explanation for this finding could be that the basic features of sound, encoded by variations in spike rate, can be reinstated and that the central gain on signals from the peripheral afferents can be increased to compensate for erosion of this input. These higher-level, hyperexcitable circuits might amplify signals which are too weak to be picked up in the form of an ABR in the brainstem. Thus, ouabain might provide a good model for the over-reaction of central systems thought to underlie the tinnitus and hyperacusis comorbidities sometimes found with AN.

It is important to consider the condition of the cochlea post-ouabain, particularly if the onward goal is cell transplantation. The inflammatory response post-ouabain in the mouse has been carefully characterised, revealing a recruitment of macrophages after a 3mM treatment (Brown et al., 2017) which are seen to engulf the dying SGNs and an upregulation of haematopoietic stem cell homing factors (Noble et al., 2018). There also must be consideration of the effect on the resident glial population - a 200µM dose *in vitro* (Xiao et al., 2002) does not affect glia. It has been reported that 3mM ouabain can trigger proliferation of the resident Schwann cell population in the mouse cochlea with an increase in $BrdU^{+}/Sox10^{+}$ cells. There is also an upregulation of neurogenesis markers such as GFAP and nestin in these cells, suggesting that the glia of the injured nerve may possess neural stem cell-like properties (2011; 2015). There may be, however, some discrepancies over this result - Zhang et al. (2017) applied this same 3mM dose in the same mouse strain and saw a loss of S100+ Schwann cells; and in our lab, we see a loss of these cells in both mouse and gerbil with a 5mM dosing regimen (Mallick et al., manuscript in preparation).

Despite its widespread usage, there has been a wide variation in the literature as to the dosage and treatment regimens - 'application at the RWM' seems to have a range of interpretations - and so, there are somewhat mixed results, which may also reflect anatomical differences between species, alongside differential drug sensitivities. In the guinea pig, some workers have shown that comparatively little damage was done to the SGNs after RW application in spite of spiral ligament, stria vascularis and OHC damage being present (Hamada and Kimura, 1999), whereas others have observed diverse and unpredictable responses with no damage to the stria and variable SGN damage (Schomann et al., 2018). Results from ouabain treatment in the rat suggest that its toxic phenotype may lie somewhere between that of the gerbil and the guinea pig - a basal to apical gradient in the severity of SGN loss was found, with a substantial loss of both IHCs and OHCs (Fu et al., 2012). Suggestions have been made to explain these disparities, with a likely explanation being the underlying anatomy of the species in question. For example, the modiolus of the gerbil cochlea is closer to the RWM than that of the guinea pig or rat (Chamberlain, 1977; Schmiedt et al., 2002) and so there may be more direct access for the drug to intoxicate the neurons. Additionally, there may be an effect between different experimenters - numerous, short applications of the drug may give rise to a more severe and consistent toxic phenotype than a single incubation, since the steepness of the concentration gradient is repeatedly replenished.

The clinical relevance of ouabain application as a model for human hearing loss conditions may extend beyond selective fibre loss and synaptopathies. There have been reports of patients with mutations in ATP1A3 presenting with CAPOS syndrome (cerebellar ataxia, areflexia, pes cavus, optic atrophy and SNHL) (Demos et al., 2014; Han et al., 2017). More recently, the hearing loss has been revealed as a form of AN, with normal OAEs but abnormal ABRs indicative of a neural dyssynchrony (Tranebjaerg et al., 2018), suggesting that ouabain application may be a good model for the deafness aspect of this rare condition.

3. Mechanical lesions of the auditory nerve

In chemically-lesioned AN models (e.g. by the application of ouabain), the primary insult occurs at the level of the soma or the peripheral processes of the spiral ganglion fibres. But in surgical situations, for instance when a patient undergoes the excision of an acoustic neuroma via a retro-sigmoidal approach, the VIIIth nerve can be damaged within its more central portion, beyond the internal auditory meatus. Earlier studies by Spoendlin and Suter (1975; 1976) described some intriguing results after resection of the auditory nerve in the cat and the guinea pig. He observed a

gradual loss of the Type I fibres which began in the basal turn and progressed over the course of several months; after two years however, the majority of SGNs had been lost and only the Type II neurons perdured - their resilience, also observed after ouabain damage to the peripheral processes, is surely worthy of note. This cut lesion model also showed evidence of neural regeneration - Spoendlin described the presence of 'giant' fibre endings packed with mitochondria which form some form of synapse at the IHC, and remarkably this phenomenon endured for nearly two years post-lesion. Alas, the data trail for this potential recovery from such central-process lesions went cold for over two decades until Sekiya, Ito and co-workers published a series of papers elucidating a method for generating an alternative auditory nerve injury model to the central axonal processes. By gently crushing the nerve at the level of the internal auditory meatus in the rat, they could induce a progressive, apoptotic loss of Type I SGNs whereby the extent of damage could be titrated according to the speed and depth of compression (Sekiya et al., 2000; Sekiya et al., 2003). Again, the extraordinary resistance of the Type II neurons to this damage was noted - even after 8 months, they remained intact, as did the hair cells (Matsumoto et al., 2008). However, there was a reduction in cell numbers centrally in the posterior ventral cochlear nucleus and the dorsal cochlear nucleus (Sekiya et al., 2009) implying an anterograde degeneration progressing centrally as well as the retrograde losses in the cochlea itself. Critically however, this lesioning spared the internal auditory artery - a key factor in this model, since loss of blood supply to the cochlea is known to result in complete fibrosis of the organ within a couple of weeks (Sekiya et al., 2000).

The response of the glia to the crush injury is a key feature of this model which may lend it clinical relevance. In patients undergoing surgery to remove tumours from this area, there is often a delayed hearing loss observed - on waking, their hearing seems to have been preserved, only to degrade over the subsequent couple of months. This degeneration may correlate with the amount of mechanical trauma the auditory nerve receives during the course of the surgery (Sekiya et al., 2011). In the rat model, after 8 weeks a dense, gliotic 'scar' had formed, spanning the lesion and extending beyond the Obersteiner-Redlich zone into the peripheral cochlear nerve. However, the overgrowth of these GFAP- positive, astrocytic fibres has the potential to be a blessing in disguise. Although this scar tissue may squeeze the life from any remaining auditory nerve fibres (Sekiya et al., 2011) and is generally considered to inhibit the growth of neural fibres, it could be induced to act as a scaffold for a cell transplant. It may have the potential to bridge any gaps and give a route along which cells can migrate into the periphery of the cochlea (Sekiya et al., 2006; Sekiya et al., 2015). The crush lesion also recruits a large influx of macrophages at the compression site and it was found that treatment of injured animals with methylprednisolone could reduce this inflammatory response and protect the SGNs (Sekiya et al., 2001). Thus, steroid treatment may provide an effective therapeutic avenue for hearing preservation in patients undergoing, for instance, vestibular Schwannoma surgery.

4. Ischaemia

Ischaemia induces swelling of the afferent dendrites beneath the IHCs, in a mechanism thought to be mediated by glutamate (see section 2.4 above, regarding the similarities of this with excitotoxic damage). A loss of blood flow to the cochlea rapidly starves its cells of ATP, and this energy depletion leads to a drop in the endocochlear potential (Tabuchi et al., 2010). This causes glutamate to build up in the synaptic cleft and accrue in the perilymph as it is no longer taken up correctly by cells, via transporters such as GLAST, with the effect of a loss of ionic homeostasis and water and ion influx into the dendritic processes of the afferents. However, as catastrophic as this may seem at initial examination, the synapse is remarkably plastic and, if the episode of anoxia is

brief (5 minutes or shorter), then there is repair of dendrite and the hearing loss is transient. If there is a prolonged period of starvation though, the damage becomes irreversible, leading to cell blebbing and eventual death. This may require some finessing to be used as a cell transplantation model, as there is evidence of a hair cell loss in conjunction with SGN losses after an ischaemic incident, as found in the guinea pig by Lin and coworkers (Lin et al., 2010). Although in the gerbil, there is contradictory evidence regarding whether the damage occurs mostly at the level of the hair cells rather than the SGNs after transient ligation of the vertebral arteries (Okada et al., 2013; Taniguchi et al., 2002; Watanabe et al., 2009; Yoshida et al., 2007) - the outcome may depend on the duration of the anoxia.

5. Metabolic causes of neuropathy

5.1 Diabetes

An under-reported but impactful consequence of Type 1 and Type 2 diabetes mellitus is the increased prevalence of hearing loss amongst patients (Bainbridge et al., 2008) and, with the global increase in diabetes and pre-diabetic conditions, this is likely to become a significant contributing factor in adding to the deafness burden world-wide. The aetiology of this hearing loss is currently unclear, with speculation based on other, hyperglycaemic consequences that the microvasculature of the cochlea may be affected, with consequences for the stria vascularis and maintenance of the endolymph. Chronically-raised blood glucose levels also leads to oxidative stress, microglial activation and neuropathy (Helzner and Contrera, 2016) and there are strong indications of degeneration in the peripheral auditory fibres (Bainbridge et al., 2008). ABR testing in Type 1 patients revealed changes in the latency of waves i, iii and iv (Mujica-Mota et al. (2018) and references therein); and, in the limited histology data from diabetic patients, degenerative changes in the SGNs have been observed, amongst other pathologies (Makishima and Tanaka, 1971). Animal models for these conditions are known to have hearing pathologies as part of their phenotypic spectrum - for instance, the Zucker Diabetic Fatty rat has a moderate increase in ABR thresholds (Meyer zum Gottesberge et al., 2015) - and there is evidence for AN in the mouse model of Type 1 diabetes (Hong and Kang, 2008). The condition can be induced by a single injection of streptozotocin (STZ) and animals show aberrant ABR responses when tested 4-10 weeks later, with raised click thresholds and altered inter-peak latencies, with the severity of the delay being directly related to the initial dose of STZ (Hong and Kang, 2008; 2014; Hong et al., 2008). Thus, this model may be useful for examining AN with differing severity levels; information is, as yet, lacking regarding the histology of the cochlea in these animals and it will be key for taking this important model forward.

5.2 Neonatal jaundice

Neonatal jaundice is a risk factor for damage within the auditory system. It arises due to elevated levels of bilirubin in the bloodstream, for example because of the high levels of red blood cell turnover in the newborn, and leads to a characteristic yellowing of the skin. Although common, particularly in pre-term infants, it is usually self-limiting, requiring minimal intervention for successful resolution. However, in cases with an early and rapid onset (less than 24 hours *post partum*) or with a prolonged duration (2-3 weeks of symptoms), this severe jaundice may reflect an underlying metabolic issue and become pathological, resulting in kernicterus (bilirubin encephalopathy) - babies are left with lasting and often severe neurological damage, not least of which is an auditory phenotype (Ramachandran, 2016). Lesions are thought to occur in the cochlear nucleus, the superior olive and the lateral lemniscus amongst other components of the central

auditory processing pathway - Perlman et al. (1983) showed a variety of disruptions to the ABR waves measured from jaundiced babies. There is some controversial evidence for the existence of peripheral lesions, with reports of high frequency hearing loss and 'word aphasia', which could be indicative of a cochlear synaptopathy condition (Shapiro and Nakamura, 2001)**.** Despite the therapeutic options available, neonatal jaundice remains a leading cause of neurodevelopmental disorders, particularly in developing nations, with the financial and societal costs that accompany the care of neurodisability (Amin et al., 2017).

Modelling neonatal hyperbilirubinaemia in animals has been approached in a number of ways. The injection of bilirubin alone is adequate to induce mild auditory damage in the guinea pig (Ye et al., 2012) and rat (Gokdogan et al., 2016). However, in order to model the more severe damage seen in affected children, accompanying the bilirubin with sulphonamide-based compounds such as sulfadimethoxine ('sulfa') has been an effective strategy in mice (Schiavon et al., 2018), rats (Karplus et al., 1988) and Rhesus monkeys (Ahlfors et al., 1986). These sulphonamides outcompete bilirubin for serum albumin binding sites, causing it to be displaced and to leach out into tissues where it causes damage by inducing neuroinflammation and triggering intracellular stress mechanisms (Schiavon et al., 2018). In addition, hyperbilirubinaemia may occur as a secondary consequence of haemolytic disease in the newborn - the impaired recycling of red blood cells causes both iron and bilirubin levels to rise, with associated neurotoxic consequences. To model this condition, the drug phenylhydrazine (PHZ) has been used to exacerbate haemolysis in young rats (Li et al., 2014; Mejia et al., 2008). This causes an increase in circulating bilirubin levels and thereby induces a transient auditory impairment in young rats (Li et al., 2014) with a longer-lasting loss of Neurofilament-200 immunoreactivity in the SGNs. Coupled with the apparent sparing of the hair cells, this model warrants further investigation as a potential candidate for neural progenitor transplant. As might be expected, the PHZ-induced damage can be exacerbated by co-injection with sulfa (Amini et al., 2017).

The Gunn rat (Gunn, 1938) is a spontaneously-arising animal model for Crigler-Najjar type 1 syndrome, a recessive familial metabolic disorder in which a deficiency in the uridine-diphosphate-glucuronosyl transferase A1 (UDP-GT1A1) enzyme prevents the conjugation of bilirubin to glucuronide in the liver. Consequently, bilirubin cannot be excreted as normal in the bile and accumulates at toxic levels in the bloodstream – the lipid solubility of this unconjugated bilirubin facilitates its transfer across the blood-brain barrier and its subsequent deposition in brain tissue, causing kernicterus. Rats carrying the jj genotype are phenotypically similar to affected human patients, displaying a pronounced jaundice (Fig 2A, B) - pups can be identified with 100% confidence on the basis of their skin colouration prior to confirmation by genotyping. They may also display neurological deficits such as ataxia – rats display a pronounced 'wobble' when walking (Chaniary et al. (2009) and our unpublished observations), cerebellar hypoplasia (Fig. 2C and Conlee and Shapiro (1997)) and mild hearing loss. A similar phenotype is seen when the UGT1 locus is targeted in the mouse (Bortolussi et al., 2012; Nguyen et al., 2008; Ronzitti et al., 2016).

However, the Gunn mutation in isolation may not give rise to an adequate auditory dysfunction as a result of the bilirubin-induced neurotoxicity – for instance, mild aberrations in the latency of certain components of the ABR wave complex were found (Shapiro and Hecox, 1988; Uziel et al., 1983), whereas other workers found no deficit in the jj mutants (Levi et al., 1981). There was also evidence of normal ECoch recordings and an intact organ of Corti (Uziel et al., 1983). Consequently, it has become routine to enhance the phenotype either by inducing haemolysis to increase circulating bilirubin with phenylhydrazine (Rice and Shapiro, 2008) or, more commonly, by injecting affected animals with sulphonamide-based compounds such as sulfasoxisole (Blanc and

Johnson, 1959) or 'sulfa' (Schutta and Johnson, 1969), which cause the circulating bilirubin to leach out into the tissues. Sulfa injections produce a more severe form of the ABR latency irregularities seen in untreated jj animals, ranging from interwave latency anomalies through to an absence of waves i to iv (Shapiro, 1993; Shapiro and Hecox, 1988; Shapiro and Conlee, 1991) and for many years, these effects were assumed to be a problem intrinsic to central auditory processing deficits – sulfa injections were seen to result in significant reductions in cell size in the medial nucleus of the trapezoid body (MNTB), alongside a reduction in the volume of the cochlear nucleus which was directly correlated to the amplitudes of the different wave components of the ABRs in those animals (Conlee and Shapiro, 1991; Shapiro and Conlee, 1991). Evidence of a failure of synaptic transmission at the glutamatergic Calyx of Held terminals in the MNTB was demonstrated in some very elegant experiments where degeneration of the excitatory presynapse was shown by multi-photon imaging (Haustein et al., 2010). However, hints at a peripheral lesion in the cochlea itself or in the auditory nerve were shown by the diminution of wave i in the ABRs of sulfa treated animals (Shapiro and Conlee, 1991) and evidence of damage to the SGNs was shown by Shaia et al. (2005). Three days after being injected with sulfa, the SGNs appeared shrunken and demyelinated compared to saline-injected controls, accompanied by axonal degeneration in the central portion of the nerve but in the context of good preservation of both inner and OHCs. There is a preferential loss of large-diameter axons, which may underlie the temporal dyssynchrony observed in both these animals and in patients diagnosed with AN.

This animal would, in theory, represent an ideal candidate for a neuropathy/transplantation model, since it loses the neuronal reserve but spares the mechanotransduction machinery. The Gunn rat has already been used as a transplant model for other aspects of its pathology. Animals have undergone liver transplants with hepatocytes derived from human neonatal livers (Tolosa et al., 2015) or induced hepatocytes derived from reprogrammed human skin fibroblasts (Chen et al., 2015), and in both reports, long term correction of the enzymatic defect and a lowering of serum bilirubin levels was achieved. There have also been shorter-term studies looking at the potential of human neural progenitor transplants to repair the kernicterus-induced damage in the brains of jj animals, with the intriguing observation that the raised levels of bilirubin may actually act as an anti-inflammatory and immunosuppressive agent, protecting the transplanted cells from the host's immune system (Yang et al., 2018; Yang et al., 2017).

We have directed our attention to the use of the Gunn rat as a host for human otic neural progenitor (hONP) transplants, as a parallel model to the ouabain paradigm we currently employ. However, despite our best efforts, the results in our hands were disappointing. We took two approaches for the development of a neuropathy model - namely, the investigation of the phenotype in older animals to see if a long-term neurotoxicity would arise from a lifetime of high serum bilirubin levels and a repeat of the sulfa administration experiments in young pups to assess the resultant auditory phenotype (Fig. 3). The results on both accounts were unexpected. Firstly, an aged cohort of Jj (n=7) and jj rats (n=10) ranging from 6 to 18 months of age were tested in our standard ABR paradigm. There was no shift in ABR threshold for the click (Jj - 28dB SPl, jj - 27.6dB SPl; Fig. 3A) or pure tone (Fig. 3C) protocols. There was no reduction in the amplitude of wave ii-iii in the click ABR complex (Fig. 3B) at any of the measured sound intensities, or in any of the individual peak latencies (data not shown) for the jj animals compared with their Jj siblings, implying that neural conduction along the auditory pathway is occurring normally. When serum bilirubin levels were measured using the ultramicromethod (Walters and Gerarde, 1970), the jj animals were found to have an average serum bilirubin level of 8.15mg/dl (n=11 animals, range of 3.21 to 13.14mg/dl) compared to 0.21mg/dl in their Jj relatives (n=7 animals, range of 0.01 to 0.4mg/dl), confirming a

lifelong bilirubinaemia. Injecting jj pups with sulfadimethoxine at early ages had no effect on the auditory system when ABRs were measured 4-6 weeks later - auditory thresholds were not significantly different in sulfa-treated animals compared with saline-treated sibs at the click (saline - 20.8dB SPl, sulfa - 23.2dB SPl; Fig. 3D) or tone (Fig. 3F) levels, with no change in wave ii-iii amplitude (Fig. 3E) or peak latencies (data not shown). Both injected cohorts were hyperbilirubinaemic, with levels of 5.9mg/dl in saline treated jj animals (n=9, range 4.66mg/dl to 8.96mg/dl). In neither the aged jj cohort nor the sulfa-injected jj animals did we see a significant reduction of spiral ganglion neurons - when we compare this with the rapid and catastrophic neural loss we achieve with ouabain (Chen et al., 2012), it becomes apparent that, currently, the Gunn rat is not an appropriate model for ONP transplantation.

Notwithstanding the apparent lack of a neuronal phenotype, the Gunn rat proved difficult as a consistent model for neuropathy and transplantation in our experience. There was a marked difference in the life outcomes for jj mutant pups. Mutant homozygotes broadly fell into two populations, being either nominally healthy (notwithstanding their tendency to being underweight compared with siblings, see Gunn (1944) for the first description of this), or moribund - this is in marked contrast to the six-point clinical rating scale described by Spencer et al. (2002). Pups, particularly from larger litters would fail to thrive and enter a vicious downward spiral from around P10 to weaning age, at around 3-4 weeks (also described by Graham et al. (1980). They would become irritable, hyperactive (Stanford et al., 2015) and hypervocal (Lenhardt, 1982) followed by a descent into lethargy. A lack of feeding, either due a failure of the pups to navigate to the mother or by active maternal neglect (Cahalan and Graham, 1978), would result in dehydration which is thought to enhance transfer of bilirubin across the blood-brain-barrier (Bratlid et al., 1983).Despite the best attempts at supportive therapies with fluid replacement, nutritional supplementation and the use of activated charcoal or calcium phosphate chews to reduce serum bilirubin levels (Davis et al., 1983; Van Der Veere et al., 1996) there was an inexorable slide into further neural deterioration and animals would require euthanasia. Our experience with the colony mirrored that described by both Gunn (1944) and Johnson et al. (1959), in which a substantial proportion of jj pups were lost, whilst the survivors remained remarkably unscathed. This bimodal phenotypic distribution may underline the importance of the contribution of the genetic background to the expressivity of the condition, similar to the results seen by Stobie et al. (1991), who saw a four-fold increase in jj pup mortality when the mutation was carried on the ACI genetic background as opposed to the RHA background. Indeed, these observations mirror those of Bortolussi et al. (2014) who saw a marked difference in survival of $UGT1^{-/-}$ mouse pups depending on the background strain harbouring the mutation. After a couple of generations in our facility, it was discovered that our Gunn colony was not an inbred line, as initially thought, with the appearance of albino progeny from Agouti 'hooded' parents arising from outcrossing of the colony at their previous institution - the effect of modifiers within the genetic background should not be taken for granted and may well have been influencing the Gunn phenotype.

An unexpected consequence of the Gunn phenotype was also revealed in animals surviving in the longer term. At the age of 6-9 months and beyond, animals developed haematuria, with post mortem examination revealing scarring and cystic lesions on the kidneys. There is little evidence of this in the Gunn rat literature - Martinez-Maldonado et al. (1969) and Call and Tisher (1975) both address a urinary concentrating defect which may be due to crystalline bilirubin affecting kidney function; and bilirubin-induced nephropathy was described by Odell et al. (1967) – but it is clearly a problem in terms of keeping animals for a longer procedural duration, particularly since transplanted animals would require immunosuppression using drugs which may cause additional nephrotoxicity (Whiting and Simpson, 1983).

6. Genetic models

Having a genetically inducible, 'clean' model of AN, in which the hearing loss is purely at the level of the neurons without the added complication of hair cell involvement, would be a boon for hearing researchers. It is known that human patients carrying mutations in genes expressed in the SGNs or auditory nerve have worse outcomes after cochlear implantation. For example, pathogenic variants of TMPRSS3, a gene of unknown function, lead to a profound deafness with normal cochlear microphonics but reduced auditory nerve activity and therefore poorer results in implanted patients (Shearer et al., 2018).

A few reports exist describing mouse mutants, in which the criteria of SGN degeneration/loss without apparent effects on the hair cells are fulfilled. The saposinB knock-out mouse models a slow and progressive demyelination and ultimate loss of the SGNs, triggered by a primary loss of the satellite cells cosseting the sensory afferent fibres, whilst the efferent system and hair cells are preserved intact (Akil et al., 2015). The deafness in these animals becomes apparent from 6 months old, which is roughly equivalent to 30 years of age in humans; thus these mice may effectively mirror the inexorable hearing loss experienced by many of us with advancing age - although, to be used as a neuroprogenitor transplant host, it may require the grafting of a bi-potent cell population which have the capacity to differentiate as both SGNs and Schwann cells.

Demyelination of the auditory nerve is thought to contribute to AN, particularly where neural dyssynchrony may be occurring (Long et al., 2018). To this end, there have been reports of mouse models which may model this problem. In the *Trembler*^{J} mouse, peripheral demyelination of the auditory nerve is predicted to underlie the latency and wave i amplitude anomalies seen in the ABR traces; a similar but less severe phenotype was seen in mice harbouring a conditional disruption of the Schwann cells (Zhou et al., 1995a; Zhou et al., 1995b). A targeted and specific reduction of the SGNs with diphtheria toxin (Pan et al., 2017) did not alter ABR thresholds in the recipient mice, but dramatically reduced the amplitude of the CAP response. Alongside a significant drop in the numbers of SGNs post-treatment, there was also evidence of degenerative pathology in terms of the thickness of the myelin sheath, albeit with the caveat that this may have occurred as a secondary consequence of the neural intoxication.

Intriguingly, there is evidence that demyelination may be causative for some forms of HHL (Kohrman et al., 2019). In a mouse model where the cochlear Schwann cells were genetically ablated, the resulting transient loss in myelination of the SGNs resulted in a permanent drop in the amplitude of wave i of the ABR complex at suprathreshold sound intensities, in the absence of any synaptic loss (Wan and Corfas, 2017). Rather, persistent damage was observed at the first heminodes of the SGNs, implying that the propagation of the neural response was being affected. This may underlie the anomalous hearing phenotypes found in patients with demyelinating neuropathy conditions such as Guillain-Barré syndrome or Charcot-Marie-Tooth disease (Kohrman et al., 2019; Rance and Starr, 2015).

There is also scope for the further characterisation of the auditory neural phenotype in mouse models of diseases known to cause neuropathy in patients. For example, the mitochondrial disease Friedrich's ataxia causes SNHL in many patients, with dyssynchrony in AN firing patterns (Santarelli et al., 2015): mouse models of this condition are known to recapitulate many of the disease symptoms, but the physiology or histology of the auditory system has not yet been described (Chandran et al., 2017). As a caveat to relying too heavily on extrapolation between

species however, in the dominant mitochondrial condition optic atrophy 1 (Opa1), human patients have no activity in the auditory nerve in spite of apparently normal hair cell function (Huang et al., 2009), whereas the mouse model shows normal hearing (Davies et al., 2007).

On the other hand, some genetic models of AN, whilst being invaluable for their contribution to our knowledge of exactly how the cochlea functions, may not be currently suitable as models for cell transplantation. For example, the otoferlin mouse mutants model the autosomal recessive human DFNB9 condition, in which a profound, prelingual deafness with the hallmarks of AN arise (Pangrsic et al., 2010; Roux et al., 2006). However, the lesion affects the replenishment of vesicles at the IHC ribbon synapse; consequently, the deafness phenotype would not necessarily be improved by a neural progenitor transplant replacing the SGNs. In the case of bone remodelling disorders such as Paget's disease, there is often an accompanying hearing loss (Amilibia Cabeza et al., 2018). A mouse knockout for the osteoprotegerin protein models such a condition (Kao et al., 2013), and there is clear evidence for a substantial degeneration of the SGNs in these animals which does not affect the hair cells, so this could be, in theory, a target for transplantation - however, the confounding effects of the conductive loss also found in this model could hinder the physiological assessment of any recovery.

Menière's disease (MD) is a complex and debilitating disorder characterised by endolymphatic hydrops and, in many cases, a fluctuating hearing loss. There is a wealth of literature and much debate in the field regarding the underlying pathology and this is largely beyond the scope of this review. However, the Phex mouse mutant may be a useful adjunct to the canon of MD research (Semaan et al., 2013). A mutation in the X-linked phosphate regulating gene (Phex) produces, amongst other bone-related abnormalities, endolymphatic hydrops. Moreover, there is a progressive loss of SGNs in an apical to basal gradient, which recapitulates the degenerative pattern seen in patients; the hair cells are preserved until advanced stages and it would be interesting to see if they could be preserved with an early progenitor transplant.

7. Noise-Induced Hearing Loss (NIHL)

A scourge of modern-day living is the constant bombardment of noise, at levels of exposure never previously experienced in human history - traffic, machinery, the ubiquitous 'ear bud' piping music into the external auditory meatus - all of these add up to contribute to increasing levels of hearing impairment, increasingly amongst younger adults. Permanent damage due to noise-induced hearing loss (NIHL) is brought about by pathological levels of sound which blast through the hair cells, ripping away the stereociliary bundle. However, a more insidious, pervasive damage is thought to underlie the concept of the 'temporary threshold shift' - noise which causes a steep increase in ABR threshold, which rapidly and subsequently returns to normal. The landmark study by Kujawa and Liberman (2009) laid the groundwork for the creation of animal models of NIHL, with careful titration of the noise 'dose' in order to maximally damage the ANFs without harming the hair cells, save for a little damage to the OHCs in the 'hook' region at the extreme base of the cochlea. The work demonstrated that although ABR thresholds remained normal, there was a reduction in wave i amplitude, specifically in the same tonotopic regions corresponding to the toxic noise frequencies. Examination of the synapses showed a degradation of the contacts between the IHCs and the ANFs; in subsequent studies, refinements in staining techniques showed that both the pre- (using CtBP2) and post-synaptic (using Glu2/3 or PSD-95) densities were affected and for a comprehensive overview of the work in this field, the reader is pointed to the review of Hickox and Whitton (Hickox et al., 2017). The synaptic loss then leads to a slow degeneration of the SGNs, caused by a gradual

retraction of the afferent fibres and death of the cell bodies, possibly due to a loss of trophic support from the organ of Corti. Indeed, in mice where the ErbB-neuregulin signalling axis has been disrupted by the expression of a dominant negative ErbB receptor in the supporting cells of the organ of Corti, the SGNs loss their trophic support and die back, due to an apparent drop in neurotrophin (NT-3) expression, demonstrating the critical feedback and support mechanisms the SGNs rely upon for their health (Stankovic et al., 2004).

Close examination of the electrophysiological aspects of the synaptopathic phenotype using single unit recordings uncovered again a particular vulnerability to damage in the low-SR fibres in the guinea pig (Furman et al., 2013) and mouse (Liberman et al., 2015), somewhat akin to their peculiar sensitivity to ouabain (see section 2.5). The loss of these high-threshold fibres has been proposed to underlie the phenomenon of cochlear synaptopathy described above. When levels of ambient noise increase, the hypothesis is that the low-SR fibres are pressed into action on account of their large dynamic range and their relative insensitivity to noise masking. However, there is considerable recent controversy regarding the relevance of these animal models. In a cohort of human patients with known 'speech in noise' deficits, there was no correlation with (albeit self-reported) noise exposure. Moreover, no anomalies were found during electrophysiological testing - ABR wave i amplitude was normal (if anything, with a trend to be slightly higher than in 'normal' hearing controls) and there was no alteration in the wave i-v ratio (Guest et al., 2018). There is currently little evidence in humans that low-SR fibres have high response thresholds or that synaptopathy leads to perceptual consequences. There may be so many other confounding effects in human sound processing that while animal models are of use for understanding the cellular and biochemical mechanisms of sound intoxication, they may be limited for deeper perceptual interpretation.

There is an intriguing finding in animals exposed to low level noise (LLN), equivalent in human terms to working in a relatively noisy, but not superficially 'pathogenic', environment. This degree of noise exposure is enough to give temporary shifts in ABR threshold, which return to normal when the LLN ceases with apparently no damage to the SGNs or hair cells. However, Frye et al. (2018) showed that there was an infiltration of macrophages which took two months to return to normal; it is tempting to speculate what harm this could lead to in the longer term, with repeated exposure. Indeed, an increase in inflammatory mediators by glia and the neurons themselves, alongside leukocyte recruitment post-noise exposure could represent an amenable target for therapeutic intervention to prevent noise-induced damage (Arslan et al. (2017); Fuentes-Santamaria et al. (2017); Tan et al. (2013) and references therein).

8. Ageing

One of the seemingly inevitable consequences of the march of time is our decreased ability to hear that clock ticking. By the age of 65, half of us will suffer from some degree of age-related hearing loss (ARHL), and with the advances in public health and medicine which have so dramatically increased our lifespan, we are still trying to understand how to improve this aspect of our healthspan.

For many years, the dogma seemed to be that the cumulative daily insults we expose our hair cells to would ultimately lead to their dysfunction and death, with the subsequent die-back of the SGNs as they lost support from the organ of Corti. There is a change in thought about this direct pathway though, based on information from different animal models and also the recognition of cochlear synaptopathy-mediated HHL in older adults (see section 7, above), where hair cell function is clinically normal but neural responses are affected under certain acoustic conditions. There may exist a selection of differing pathologies resulting from independent degeneration of the hair cells,

SGNs or stria vascularis; there is also some evidence that degeneration of the fibrocytes of the spiral limbus may be the primary insult resulting in neural loss, particularly in the apical region of the cochlea (Ohlemiller and Gagnon, 2004). The value of an animal neuropathy model for ageing must lie in its ability to recapitulate the pathology seen in human patients. As explained in the introduction, this becomes a Gordian knot in hearing research, due to the paucity of post-mortem human samples and the conflicting range of lesions which have been documented in these tissues. Counts from cochlear samples from the over-60s showed a 30-40% loss of OHCs, but with a greater than 60% loss of SGNs in the majority of cases - meanwhile, the IHCs were mostly intact (Wu et al., 2018). This SGN loss is slower than in most comparable animal models, and there is also the phenomenon of 'neuritic presbycusis', in which the peripheral fibres of the SGNs are lost, but the cell bodies survive. This could be a therapeutic window of opportunity for repair – work by Suzuki et al. (2016) has shown in the noise-induced model that it may be possible to regrow these peripheral fibres and re-establish synapsis by the judicious application of neurotrophic factors such as NT3 or BDNF. At the very least, the protection afforded by these factors may stall any further damage and allow for greater success in CI patients (Landry et al., 2011; 2013).

The ageing human neuropathy has a parallel with the ouabain neuropathy model (see section 2.5 above) - in both the ageing human and the intoxicated animal cochlea, the Type II SGNs remain steadfast and persist. Perhaps herein lies the secret to neural survival; if we could install the Type II afferents' protective mechanisms across the afferent population, we may be to stop neural degeneration in its tracks. Current advances in genomics have shed light on the different 'flavours' of SGNs and so a deeper analysis of these datasets may shed light on these key differences (Petitpre et al., 2018; Shrestha et al., 2018).

Whereas a model for an age-related pure neuropathy still requires work, there is a substantial body of work on the synaptopathy aspect of ageing, and its relationship with noise exposure. Experiments with mice experiencing noise at young ages showed a deterioration of neural responses and SGN degeneration in old age compared to noise-naïve animals of the same age (Kujawa and Liberman, 2006), implying that the sublethal changes introduced by this early noise exposure made the cochlea more vulnerable to age-related damage. A single dose of synaptopathic noise early in life could accelerate the loss of synapses in older age, spreading out along to the cochlea to encompass even regions which were not initially covered by the pathological noise frequency band (Fernandez et al., 2015; Kujawa and Liberman, 2015). There is also a tendency to lose the low-SR synaptic contacts as a result of noise exposure, so maybe carefully-titred noise lesions will be an appropriate facsimile for the human condition, where temporal degradation of the neural signal and a diminishing of hearing acuity could be a sequela of low-SR loss (Kujawa and Liberman, 2015; Parthasarathy and Kujawa, 2018).

Modelling the synaptic loss in the ageing mouse using a machine learning approach may give us an invaluable tool to take to the clinic. By populating their model with wave i ABR amplitudes and DPOAE responses, Bramhall et al. (2018) were able to predict the number of remaining synapses in mice aged between 1 month and over 2 years to an astonishing degree of accuracy, confirming their computational results with immunofluorescence for synaptic, hair cell and afferent fibre markers. Using this model in a clinical setting could be a revolutionary tool - being able to accurately predict the benefit a patient would receive from a given prosthesis would save the heartache of crushed hopes and be of significant gain from a healthcare economics perspective.

There are genetic conditions whereby the clinical manifestations of hearing loss appear to mimic ARHL but on a more accelerated timescale - in patients carrying mitochondrial (mtDNA) mutations, the phenotypic spectrum of the resulting syndrome can often encompass progressive

deafness similar to that seen in ARHL. By creating a 'mutator' mouse strain with a progressive accumulation of mtDNA lesions, Niu et al. (2007) demonstrated how the resultant mutations resulted in a progressive hearing loss with an apoptotic degeneration of both peripheral and central auditory neurons which spared the IHCs, alongside a loss of neural density centrally in the cochlear nucleus. When comparing the ABR profiles of the 10 month old 'mutator' mice with 2 year old wild-types of the same strain, there was a similar and profound raising of the hearing threshold, the suggestion being that the mutator mice were showing an accelerated presbyacusis phenotype. This mouse strain could be of great benefit to the field of ARHL because it may develop the relevant phenotypes without the attendant financial and ethical considerations of maintaining an ageing mouse colony.

Despite many years of research, the mechanisms underlying ARHL, largely remain opaque. There are, however, some interesting hints that the symbiotic relationship between the SGNs and their glia (Wang et al., 2009). Mice were generated carrying a conditional knock-out of two of the fibroblast growth factor receptors (FGFR1 and FGFR2), specifically affecting the peripheral Schwann cells and central oligodendrocytes of the auditory nerve. These animals displayed a late-onset and progressive loss of hearing, the underlying pathology of which was a 'pure' neuropathy: animals had a significant (ca. 50%) loss of SGNs across all three cochlear turns without any losses in the hair cells or mutant glia. The authors proposed a breakdown in the reciprocal signalling arrangement between the SGNs and the glia: in the normal situation, they hypothesised that FGF secreted by said neurons activates FGF-responsive pathways in the glia, which respond by secreting the neurotrophins the SGNs need for support. Consequently, there could be a protective role for FGF in neuropathy models, both in ageing and acute situations.

ARHL is likely to be a multifactorial problem, with a number of contributing features such as ageing, genetics, and environmental influences e.g. exposure to chemicals or noise pathologies.

9. Infectious diseases

9.1 Viruses

Many viral diseases are known to result sensorineural deafness, either by a direct cytotoxic effect on cochlear tissues, or by secondary damage caused by inflammation. For some viral infections e.g. herpes simplex virus, the damage is thought to occur in the stria vascularis or the organ of Corti and so discussion of these conditions is outwith the scope of this review.

Advances in public health have led to a reduced incidence in these viral causes of deafness, particularly in developed nations - for instance, mass vaccination for measles, mumps and rubella in the U.K. has almost eliminated post-infection deafness for the former two diseases and greatly reduced cases of congenital rubella syndrome in infants. In some cases, anti-viral therapies have helped with the outcomes for infected patients - for example, treatment with ganciclovir can halt the progression of cytomegalovirus (CMV)-induced SNHL (Cohen et al., 2014). However, CMV still remains an unfortunate burden when untreated or in its asymptomatic form: it has a high incidence, occurring in 0.2-0.5% of live births, of whom 10% will go on to develop hearing loss, accounting overall for a quarter of childhood deafness cases (Usami et al., 2017). The virus causes a wide variety of hearing loss phenotypes, which may be profound, asymmetric, and, in some cases, progressive with a delayed onset which may occur up to 16 years of age (Riga et al., 2018). As may be expected, there is debate regarding the underlying pathology, with an understandably limited amount of post-mortem tissue available for examination; there is evidence of damage to the stria vascularis and

supporting cells with an infiltration of inflammatory cells around both infected and non-infected cells (Teissier et al., 2011).

Modelling CMV infection *in vivo* has proved difficult, with conflicting opinions as to the optimal method of infection (intra-cerebral or intra-peritoneal?) and differing patterns of damage seen in the mouse and guinea pig (Bradford et al., 2015; Carraro et al., 2017; Li et al., 2015; Schraff et al., 2007; Tian et al., 2015; Wang et al., 2013; White et al., 2006). Perhaps of use as an AN model would be the neonatal intra-peritoneal injection model used by Bradford et al. (2015) - 6 weeks post-infection, infected mice had raised ABR thresholds, in some instances with unilateral and/or fluctuating hearing loss, thus mirroring the phenotypic spectrum of human patients. Histological examination showed infection, followed by a substantial loss, of the SGNs, accompanied by an infiltration of CD3+ cells and an upregulation of proinflammatory cytokines. Indeed, a prolonged inflammatory insult with high levels of IL-6, TNFα, ROS and the activation of the 'inflammasome' (Schachtele et al., 2011; Zhuang et al., 2018) may bring about the demise of the SGNs as a secondary insult rather than the primary viral infection itself, and this may be comparable to the macrophage infiltration found in the spiral ganglion region after ouabain intoxication (Brown et al., 2017).

Lassa fever is a haemorrhagic virus with over 500,000 cases diagnosed annually, mainly in West Africa. When considering that one third of surviving patients develop SNHL during the latter stages of their infection or during convalescence (Mateer et al., 2018), there is a considerable long-term healthcare and socio-economic burden from this neglected disease. A lack of biopsy or post-mortem specimens entails that currently, the aetiology of the deafness is as yet unclassified. Developing a mouse model for this disease has proved challenging - somewhat unsurprisingly, there is a very high attrition rate in animals when they are infected with a virulent human strain (Yun et al., 2015). However, when a milder variant is used, the animals develop SNHL 1-2 months after the initial infection - similar to the human situation - with degeneration of the SGNs which spares the vast majority of the hair cells. Notwithstanding the regulatory difficulties of working with human pathogens, there is scope for exciting research with this model, as it may fulfil the criteria for being a 'pure' AN model - it would be interesting to observe the CAP and ABR measurements over time and in the presence of ribavirin, the anti-viral therapy for Lassa infections. There is again an influx of CD3+ lymphocytes into the auditory nerve tissue, which may be a unifying feature when considering models of auditory nerve damage - perhaps one is intrinsic to the other.

9.2 Bacterial infections

Although recurrent bacterial infections in the middle ear cleft (known as 'glue ear' or otitis media) can result in deafness, this is usually a conductive loss brought about by fusion of the middle ear bones - rarely, there may be damage to the auditory nerve accompanying this, giving a 'mixed' hearing loss. Nevertheless, there are bacterial diseases which can result in AN. Hearing loss is one of the most common complications of bacterial meningitis, with nearly 10% of patients being left with some degree of deafness due to hair cell or auditory nerve damage from the bacteria themselves, the exotoxins they produce, or the immune response mounted to the infection (Perny et al., 2016). Henceforth, an animal model for this infection is important for the understanding of the underlying pathological mechanisms. In an infant rat model of *Streptococcus pneumoniae* infection, a dose-dependent hearing loss was observed - the higher the initial bacterial inoculum, the greater the shift in hearing thresholds, with the focus of damage being in the basal turn. This was also reflected in the degree of SGN loss, which was more substantial in high frequency rather than low frequency regions. The OHCs were severely affected too, but whilst the IHCs remained unscathed superficially, an

investigation of the remaining afferent synapses revealed a significant loss of presynaptic ribbons. Consequently, this model may be good for recapitulating the post-meningitic sensory deficits, and be of use for investigating drug-based interventions to prevent such damage. Interestingly, there was a positive correlation between the increase in threshold with the level of TNFα in the cerebrospinal fluid - still more evidence that the immune system may play both the role of superhero and supervillain in the cochlea. At the moment, however, this model has some limitations for its use in neural cell transplantation studies. The severe damage suffered by the hair cells (primarily the OHCs) would be a barrier to measure functional recovery. Moreover, the health of the central pathway has not yet been properly explored; it is likely that it would also be compromised given the administration of the bacterial inoculum into the *cisterna magna*.

10. Concluding remarks

In summary, the causes leading to the loss of SGNs in humans and experimental animals are multiple, and the underlying physiopathogenic mechanisms are better understood in some conditions than in others. However, and as mentioned in the introduction, an ideal model to study cell replacement of the cochlear neurons by cell transplantation should have a phenotype that is robust, reproducible and, ideally, affects mostly the SGNs preserving, at least partially, other cell types.

We believe that the ouabain model has been reasonably well characterised and offers an appropriate framework from which to develop a cell-based strategy. In our own experience, it is far more reproducible and robust than other models we have explored, such as the β-bungarotoxin application or the Gunn rat. Reproducibility and the limited ability to accurately control the extent of the damage are still the limitations of the subtler, graded models. Future studies will, hopefully, develop even better model systems that could be used to further advance cell therapy technologies in the ear.

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867

868 **Figure legends**

869

870 Fig. 1 – Effects of 0.5μM β-bungarotoxin application to RWM, 5 weeks post-treatment. There is a loss
871 of Type I SGNs in the treated (E-H; M-P) vs. the untreated ear (A-D, I-L), as shown by the drastic
872 reduction in βIII-TUBULIN positive cells (green cells in F, N; compared to B, J). There is preservation
873 of Type II neurons shown by the perdurance of PERIPHERIN positive cells (arrowheads, A, E). The
874 continued presence of MYOSIN7A in the treated ear (M) compared with the untreated (I) shows that
875 both inner (*) and outer ([) hair cells are unharmed by the treatment (I, M).

876

877 Fig. 2 - Gunn rat phenotypes. Gunn rat pups are obvious by d7 due to their yellow colouration (A, jj)
878 compared to their pink heterozygous siblings (A, Jj). Adults retain a yellow tinge to their ears, paws
879 and tail (B - 'Archie' - 18 month old homozygous male), and have a smaller cerebellum (cb) (jj vs. Jj in
880 C). Some animals also develop kidney lesions (D) - the organ becomes misshapen and may have
881 urine-filled cysts (arrow, D). Genotyping the animals is simple and based on a RFLP - the Gunn
882 mutant allele loses an MvaI site. PCR products from genomic DNA covering this region are digested
883 and the resulting fragments are visualised by standard agarose gel electrophoresis (E), allowing Jj
884 hets and jj homs to be identified. Differences in serum bilirubin levels (F) are obvious in all jj animals;
885 quantification of this in pre- and post-weaning (ca.22-28d) animals shows this to be highly
886 statistically significant (one-way ANOVA, $p<0.0001$). Mean bilirubin levels were 0.81mg/dl for
887 heterozygous pups (n=12), compared to 17.34mg/dl for their homozygous siblings (n=20); and
888 0.23mg/dl for adult heterozygotes (n=31) vs. 10.54mg/dl for adult homozygotes (n=26).

889

890 Fig. 3 - Comparison of auditory responses in aged het (Jj; age range 8-18 months, n=7) vs. hom (jj;
891 age range 6-18 months, n=10) animals (A-C) and hom pups injected with saline (n=9) or sulfa (n=9)
892 and assayed 4-6 weeks post exposure (D-F). There is no significant difference in ABR click threshold
893 in aged jj mutant animals compared to Jj hets (A), nor is there any alteration in the wave ii-iii
894 complex amplitude (B). Pure tone thresholds are statistically similar at all tested frequencies (C; 2-
895 32kHz). Similarly, sulfa injections have no effect in the longer term - click thresholds (D), wave ii-iii
896 complex amplitude (E) and pure tone thresholds (F) are all unaffected in jj animals treated with sulfa
897 compared to those injected with saline.

898

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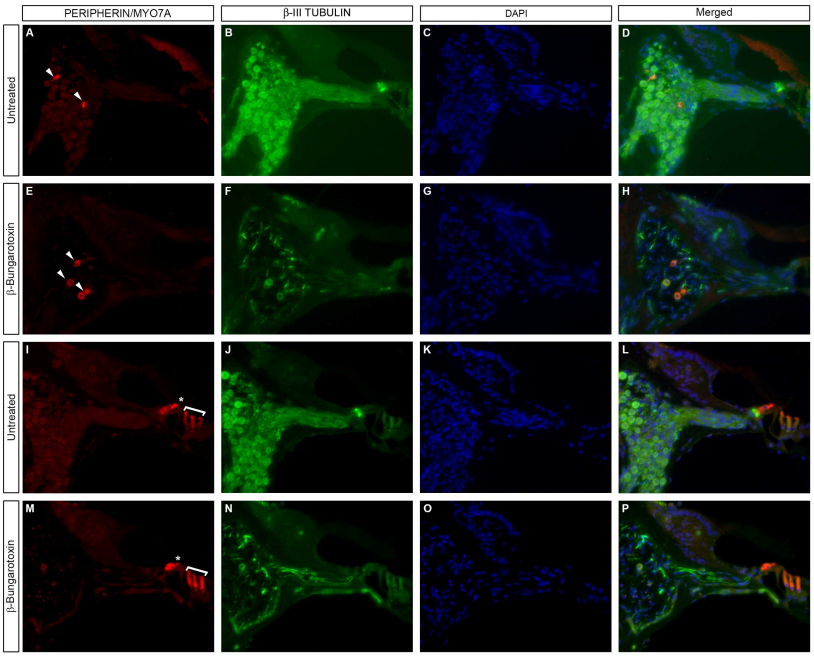

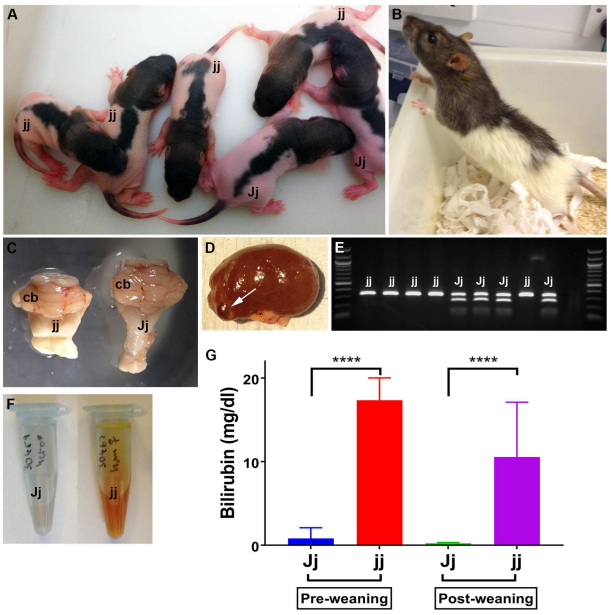

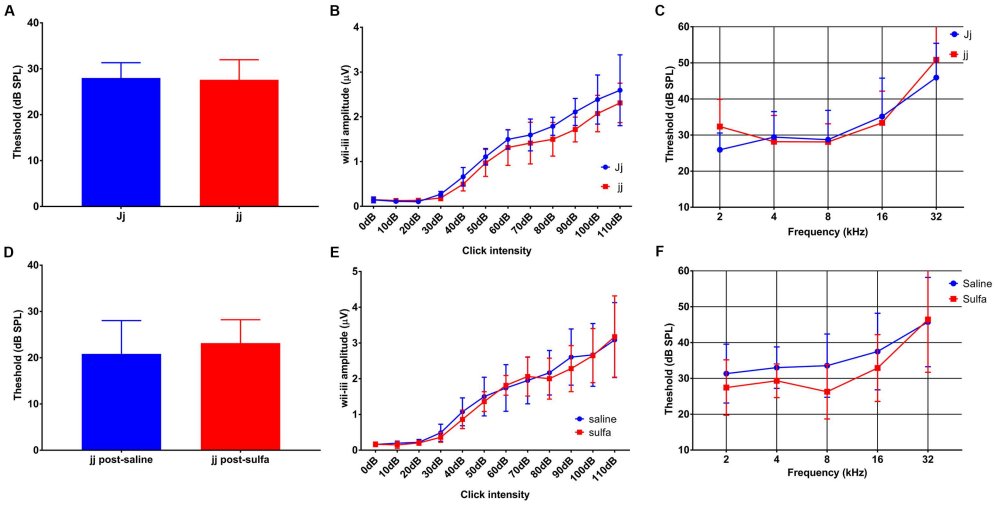

Highlights

- This article reviews the current available animal models that could potentially be used to study stem cell transplantation to treat auditory neuropathy
- We discuss a range of etiologies and species, covering ototoxic and metabolic damage, mechanical trauma and ischemia, infectious agents, genetics, noise-induced damage and aging.
- A critical assessment is provided of our current understanding of the pathogenesis behind the models, their relevance to human clinical conditions and their potential application for cell therapy studies.