Published in final edited form as:

Hear Res. 2015 November ; 329: 1–10. doi:10.1016/j.heares.2015.04.008.

No longer falling on deaf ears: mechanisms of degeneration and regeneration of cochlear ribbon synapses

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Abstract

Cochlear ribbon synapses are required for the rapid and precise neural transmission of acoustic signals from inner hair cells to the spiral ganglion neurons. Emerging evidence suggests that damage to these synapses represents an important form of cochlear neuropathy that might be highly prevalent in sensorineural hearing loss. In this review, we discuss our current knowledge on how ribbon synapses are damaged by noise and during aging, as well as potential strategies to promote ribbon synapse regeneration for hearing restoration.

1. Introduction

In the mammalian cochlea sound is detected and processed by the sensory hair cells (HCs). Outer hair cells (OHCs) are responsible for the amplification of acoustic signals while inner hair cells (IHCs) transduce the sound-induced mechanical signals into synaptic transmission to the peripheral processes of spiral ganglion neurons (SGNs). Depending on species and cochlear location, each IHC is innervated by 5–30 type I SGN fibers (Bohne et al., 1982; Kujawa et al., 2009; Liberman et al., 1990; Meyer et al., 2009). Neurotransmission between IHCs and type I SGNs is mediated by the ribbon synapses, specialized synaptic structures that allow fast and sustained neurotransmitter release from pre-synaptic vesicles (Safieddine et al., 2012). The functional characteristics of ribbon synapse are critical for reliable and precise encoding of acoustic information with temporal acuity and fidelity (Parsons et al., 2003). OHCs also form ribbon synapses with type II SGNs, but the density of these synapses (1-2 per OHC) is much lower than that of IHCs and the functions of these synapses remain poorly understood (Knirsch et al., 2007; Weisz et al., 2009; Weisz et al., 2014; Weisz et al., 2012).

Formation and maturation of cochlear ribbon synapses is a multi-step process involving synaptic pruning and refinement [reviewed in (Yu et al., 2014)]. Multiple factors and cellular activities have been reported to influence the development and maintenance of IHC

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ribbon synapses, including neurotrophins (Wan et al., 2014; Zuccotti et al., 2012), thrombospondins (Mendus et al., 2014), hormonal signaling (Graham et al., 2011; Sendin et al., 2007) and Gata3-mafb and Foxo3 transcriptional networks (Gilels et al., 2013; Yu et al., 2013).

Alterations in ribbon synapse number or function, result in abnormal synaptic transmission between IHCs and the SGNs, a phenomenon referred to as cochlear synaptopathy (Furman et al., 2013; Moser et al., 2013; Sergeyenko et al., 2013). Cochlear synaptopathy is responsible for several forms of genetic deafness in human. For example, disruption of synaptic transmission by mutations of the vesicular glutamate transporter VGLUT3 underlies DFNA25, an autosomal-dominant form of progressive deafness (Ruel et al., 2008). Mutations in the calcium sensor otoferlin interrupt replenishment and fusion of the presynaptic vesicles and cause DFNB9, a prelingual autosomal-recessive form of deafness (Yasunaga et al., 1999). In addition to genetic causes, recent studies have unveiled a previously underappreciated role for ribbon synapse degeneration in noise-induced (Furman et al., 2013; Kujawa et al., 2009; Lin et al., 2011; Maison et al., 2013; Puel et al., 1998; Wan et al., 2014) and age-related hearing loss (Liberman et al., 2014; Sergeyenko et al., 2013; Stamatakis et al., 2006). It has been proposed that this acquired cochlear synaptopathy is primarily caused by glutamate excitotoxicity (Chen et al., 2004; Puel et al., 1998; Puel et al., 1994; Ruel et al., 2007) and underlies loss of hearing acuity in noisy environments (Furman et al., 2013; Kujawa et al., 2009; Sergeyenko et al., 2013).

Several recent articles have reviewed in depth aspects of cochlear ribbon synapse biology, including their formation, structure and function (Glowatzki et al., 2008; Matthews et al., 2010; Moser et al., 2006; Nouvian et al., 2006; Safieddine et al., 2012; Sterling et al., 2005; Yu et al., 2014). Here we will discuss current knowledge on how ribbon synapses are damaged by injury and normal aging, and the consequence of this damage to auditory function. We will also discuss potential candidate molecules and pathways that may promote regeneration of these synapses to restore normal hearing.

2. IHC ribbon synapses: degeneration

Noise-induced and age-related hearing loss are major forms of sensorineural hearing loss. Both types of hearing loss are associated with multiple risk factors, including genetic predispositions, poor diet and chronic diseases such as diabetes and hypertension (Daniel, 2007; Lee, 2013). Degeneration of hair cells and sensory neurons were thought to be the cause of these disorders (Liberman et al., 1984; Robertson, 1982). However, emerging evidence indicates that degeneration of the ribbon synapses might be a major underlying cause of hearing impairment, particularly when difficulty in hearing and speech perception in noisy environments is the chief complaint (Alvord, 1983; Dubno et al., 1984; Gordon-Salant et al., 1993; Kujala et al., 2004; Kumar et al., 2012; Ruggles et al., 2012; Snell et al., 2000; Stone et al., 2008; Walton, 2010).

2.1. Cochlear synaptopathy in noise-induced hearing loss

Exposure to loud noise can cause elevated hearing thresholds that may recover within a few weeks or become irreversible, depending on the duration and intensity of the trauma (Clark,

1991). Permanent threshold shifts (PTS) result from degeneration of the cochlear mechanosensory hair cells or damage of the hair bundles, usually caused by chronic exposure to loud noise or acute exposure to high intensity noise (Clark, 1991; Dolan et al., 1975; Liberman et al., 1984). In contrast, a more “benign” level of noise exposure can induce temporary threshold shifts (TTS) that do not involve hair cell or stereocilia degeneration (Wang et al., 2002). For example, in rodent models, certain noise overexposures produce moderate threshold shifts that recover completely within 10-14 days after exposure (Kujawa et al., 2009). Although the pathology of TTS appears less severe than PTS, recent studies have identified TTS as a serious risk for permanent damage to the cochlea and irreversible hearing loss. For example, a single dose of noise exposure causing TTS results in significant loss of SGNs within 1-2 years in mice or guinea pigs (Kujawa et al., 2009; Lin et al., 2011). It has been proposed that repeated exposure to TTS sound levels has a cumulative effect that directly leads to PTS and irreversible hearing loss (Melnick, 1991). Indeed, mice exposed to three doses of TTS noise levels with 2-week intervals developed PTS and high frequency hearing loss (Wang et al., 2012). However, compared to the damage caused by high intensity occupational noise, the danger of this “benign” noise exposure is greatly underappreciated, partly because these sound levels are often not at all disturbing, if not enjoyable, e.g., during recreational activities.

Noise-induced TTS has been suggested to involve multiple pathological processes, including metabolic fatigue of cochlear cells (Canlon et al., 1983), impairment of cochlear blood flow (Nakai et al., 1988), production of cochlear reactive oxygen species (Cassandro et al., 2003) and reversible structural changes in the organ of Corti (Nordmann et al., 2000). A significant component of acute cochlear responses to noise overexposure is the swelling of SGN terminals at IHC ribbon synapses (Robertson, 1983; Spoendlin, 1971), suggestive of noise-induced glutamate excitotoxicity (Chen et al., 2004). Consistent with this hypothesis, OHC ribbon synapses, whose postsynaptic afferent terminals do not express AMPA type receptors (Matsubara et al., 1996) and presynaptic sites have much lower rate of glutamate release (Weisz et al., 2009; Weisz et al., 2014; Weisz et al., 2012), are not damaged by TTS noise exposure (Kujawa et al., 2009). TTS noise has been also reported to increase the volume of cytoplasmic vacuoles in presynaptic IHC compartments, presumably caused by buildup of recycled synaptic membranes that are not efficiently reconstituted to functional synaptic vesicles (Mulroy et al., 1990). These events are accompanied by dramatically decreased ribbon synaptic densities within 2-24 h post-exposure (Kujawa et al., 2009; Wan et al., 2014) and also dramatic reduction in the number of synaptic vesicles, size and packing density of the synaptic bodies (Henry et al., 1995). Importantly, despite a complete reversal of auditory brain-stem response (ABR) thresholds at 2 weeks post-exposure, the loss of ribbon synapses appears to be permanent (Kujawa et al., 2009; Lin et al., 2011; Wan et al., 2014). Accordingly, the neural response amplitudes (ABR peak 1 amplitudes), which depend on both neural discharge rate and synchronization, are also irreversibly reduced after TTS noise exposure. Thus, this “benign” level of noise is sufficient to cause permanent damage to hair cell synaptic transmission (**Fig. 1**).

Noise exposure is also a known risk factor for tinnitus (a self-perceived “ringing” sound in the ear without external acoustic stimuli) and hyperacusis (sound hyper-sensitivity) (Anari et

al., 1999; Auerbach et al., 2014; Hickox et al., 2014; Knipper et al., 2013). It is thought that both hyperacusis and tinnitus are the result of hyperactivity in the central auditory circuit initiated by lack of peripheral sensory input (Auerbach et al., 2014; Eggermont, 2013). Many patients with tinnitus and hyperacusis have normal hearing thresholds (Coelho et al., 2007; Norena et al., 2003; Roberts et al., 2008; Weisz et al., 2006) but reduced suprathreshold ABR wave 1 amplitudes (Gu et al., 2012; Schaette et al., 2011), suggestive of cochlear neuropathy not detectable by audiograms. In recent years, a number of studies have revealed a previously unrecognized link between synaptic degeneration and noise-induced tinnitus (Ruttiger et al., 2013; Singer et al., 2013). Mice with noise-induced cochlear synaptopathy also suffer from hyperacusis, as measured by enhanced acoustic startle response and prepulse inhibition (Hickox et al., 2014). Together, it has been proposed that although the central neural plasticity is essential for the long-lasting consequence of tinnitus and hyperacusis, primary cochlear synaptopathy plays an important role in triggering the over-adaptive responses of the central auditory pathway (Knipper et al., 2013).

2.2. Cochlear synaptopathy in age-related hearing loss

Age-related hearing loss (or presbycusis) is one the most common health conditions to affect the elderly. Loss of hearing with aging is associated with progressive degeneration of hair cells (Bartolome et al., 2001; Bartolome et al., 2002; Bohne et al., 1990; Ingham et al., 1999; Keithley et al., 1982) and spiral ganglion neurons (Bartolome et al., 2001; Bartolome et al., 2002; Cohen et al., 1990; Keithley et al., 1989). However, it has been noticed that in some aged human cochleae, there is minimal loss of hair cells and spiral ganglion neurons but substantial deficits of the sensory fibers in the osseous spiral lamina (Chen et al., 2006; Nadol, 1979; Pauler et al., 1986; Spoendlin et al., 1988; Spoendlin et al., 1990), suggesting that damage to the afferent nerve terminals and their associated ribbon synapses could be the primary defects in some patients with age-related hearing loss. Studies in aging rodent have also documented age-related loss of afferent synapses without loss of sensory hair cells and neuronal bodies (Liberman et al., 2014; Sergeyenko et al., 2013; Stamatakis et al., 2006). This primary synaptic degeneration is progressive, with 30% reduction in middle age and 50% reduction near the end of life, compared to juveniles (Liberman et al., 2014; Sergeyenko et al., 2013). Similar to the observation in noise-induced hearing loss, the changes in density of ribbon synapses correlate with the reductions in the amplitudes of auditory responses (Liberman et al., 2014; Sergeyenko et al., 2013). Although the molecular basis of age-related synaptic degeneration is not known, it is possible that a life-time of moderate sound exposure producing repeated glutamate excitotoxicity has a cumulative effect on synaptopathy (Maison et al., 2013). Consistent with this notion, swelling/enlargement of afferent terminals is also observed in aged mouse cochlea (Stamatakis et al., 2006), similar to that produced by noise overexposure (Robertson, 1983; Spoendlin, 1971) and ischemic exposures (Pujol et al., 1993).

2.3. Synapses of low-spontaneous rate fibers are selectively damaged by noise and aging

Ribbon synapses on single inner hair cells are formed by innervation from multiple cochlear afferent fibers that differ in their spontaneous firing rates (SR), thresholds to acoustic stimulation and central projections (Borg et al., 1988; Liberman, 1978; Liberman, 1991; Schmiedt, 1989; Taberner et al., 2005; Tsuji et al., 1997). High-SR fibers (SR > 20 spikes/s)

are activated by lower sound pressure levels and saturate rapidly, whereas low-SR fibers (SR < 20 spikes/s) respond to higher sound pressure levels but show little or no saturation (Liberman, 1978; Winter et al., 1990). Neurophysiological and morphological studies indicate that the low-SR fibers, which have high threshold and small afferent terminals, are more susceptible to damage by both noise exposure (Furman et al., 2013; Liberman, 1978) and aging (Schmiedt et al., 1996; Stamatakis et al., 2006). A recent study showed that selective ablation of low-SR (< 18 spikes/s) neurons by low dose of ouabain has no effect on compound action potential (CAP) threshold but reduced CAP amplitudes (Bourien et al., 2014), further supporting the notion that ribbon synapses of low-SR fibers are the primary targets of noise-induced and age-related cochlear neuropathy (**Fig. 1**). The preferential loss of ribbon synapses from low-SR fibers has little or no effect on ABR thresholds because low-SR fibers are selectively activated at high sound pressure levels. However, loss of these ribbon synapses results in significant reduction in suprathreshold ABR wave 1 amplitudes because both high- and low-SR fibers contribute to the summed activities of auditory nerves (Kujawa et al., 2009; Lin et al., 2011; Sergeyenkov et al., 2013; Wan et al., 2014).

Single-fiber intracellular labeling experiments indicate that low-SR fibers tend to innervate the modiolar side of the IHCs while high-SR fibers concentrate in the pillar side of IHCs (Liberman, 1982; Tsuji et al., 1997). Compared to high-SR fibers, the low-SR fibers have smaller nerve terminals, lower mitochondria content and form afferent synapses with larger ribbon size and smaller GluA2 receptor patches (Liberman et al., 2011; Liberman, 1980a; Liberman, 1980b; Liberman et al., 1990). These morphological features undergo dynamic changes in injured cochlea (Furman et al., 2013; Stamatakis et al., 2006; Yin et al., 2014). For example, the modiolar-pillar spatial segregation of ribbon size and GluA2 patch size diminishes after noise exposure and olivocochlear efferent lesion (Furman et al., 2013; Yin et al., 2014), demonstrating dynamic redistribution of the surviving ribbon synapses and nerve terminals. Why low-SR fibers are preferentially damaged by noise and aging is unclear, but it has been speculated that lower expression of the glutamate-aspartate transporter Glast in the modiolar side of the IHC (Furness et al., 2003) may result in lower capacity for clearance of excess glutamate, leading to higher excitotoxic potential at low-SR nerve terminals. It has also been suggested that low-SR nerve terminals have fewer mitochondria, leading to lower “calcium buffering” capacity, thus resulting in higher risk for glutamate excitotoxicity (Pivovarov et al., 2010).

2.4. Functional significance of cochlear ribbon synapse degeneration

Based on the contribution of low-SR fibers to acoustic responses, significant loss of their ribbon synapses are expected to reduce the dynamic range of sound detection, disrupt encoding of timing and intensity cues at high sound pressure levels (Frisina et al., 1996; Zeng et al., 1991), and reduce the ability to phase lock and amplitude modulate (Joris et al., 1994). Because the low-SR fibers are more resistant to masking by continuous background noises (Costalupes et al., 1984), this primary cochlear synaptopathy should result in distorted representation of sound signals in the presence of a background noise (Reiss et al., 2011; Silkes et al., 1991; Young et al., 1986). This encoding deficit may have significant contribution to hearing difficulties in noisy environments, a.k.a. the cocktail party effect, a common complaint from elderly people (Dubno et al., 1984; Gordon-Salant et al., 1993;

Ruggles et al., 2012; Snell et al., 2000; Walton, 2010) and patients with a history of noise exposure even when their audiogram thresholds are normal (Alvord, 1983; Kujala et al., 2004; Kumar et al., 2012; Stone et al., 2008).

Loss of ribbon synapses after noise exposure is followed by a slow and progressive degeneration of SGN bodies and axons that may only appear months to years after noise exposure (Kujawa et al., 2009; Lin et al., 2011). Similarly, age-dependent neuronal loss parallels the ribbon synapse loss, but with several months delay (Sergeyenko et al., 2013). The delayed onset of SGN cell death might reflect the slow loss of access to neurotrophic factors derived from the sensory epithelia as afferent terminals retract (Sugawara et al., 2007; Wiechers et al., 1999; Zhang et al., 2002). A recent study in aged human cochlea has also shown a significant age-dependent degeneration of SGNs, with ~30% neuronal loss at 95 years (Makary et al., 2011). Although corresponding data on ribbon synapse density is lacking, it is conceivable that the degree of synaptic loss is significantly greater than 30% in these aged human ears. Evidence from animal models indicates that loss of ribbon synapses and afferent terminals has a long lasting effect on the survival of the afferent neurons which goes beyond an early onset of synaptic excitotoxicity (**Fig. 1**).

In other parts of the nervous systems, synaptic dysfunction has long been considered a major contributor to disease, including in stroke (Aarts et al., 2003b; Little et al., 1974), epilepsy (Seal et al., 2008; Tanaka et al., 1997) and neurodegenerative disorders such as Huntington's (Li et al., 2003), Alzheimer's (Forero et al., 2006) and neuromuscular diseases (Plomp et al., 2009). Glutamate excitotoxicity is considered an important mechanism for synaptopathy and neuropathy in many of these brain diseases, particularly stroke and epilepsy [reviewed in (Aarts et al., 2003a; Mehta et al., 2013)]. In the central nervous system, glutamate excitotoxicity results in dendrosomal swelling and dendritic spine loss and a delayed neuronal degeneration (Ikonomidou et al., 1989; Olney, 1969). These pathological processes resemble those observed in the cochlea after excitotoxic or noise insults, with acute swelling and loss of cochlear afferent nerve terminals and progressive degeneration of SGN cell bodies (Kujawa et al., 2009; Lin et al., 2011; Puel et al., 1998; Puel et al., 1994; Pujol et al., 1993). The similarities between the features and mechanisms of synaptopathy in the cochlea and other parts of the nervous systems suggest that these synaptic dysfunctions may also share common therapeutic targets.

3. IHC ribbon synapses: regeneration

In vitro and *in vivo* studies suggest that cochlear ribbon synapses have intrinsic but very limited regenerative capacity (Chen et al., 2012; Kujawa et al., 2009; Lin et al., 2011; Liu et al., 2013; Liu et al., 2014; Maison et al., 2013; Martinez-Monedero et al., 2006; Matsumoto et al., 2008; Shi et al., 2013b; Tong et al., 2013; Wan et al., 2014). Incomplete repair of these structures after insult or during aging could result in cumulative loss of sensory input, leading to hearing difficulty in noisy environment and a delayed onset of permanent hearing loss. Thus, regenerating the ribbon synapses represents a key strategy for hearing restoration due to noise exposure, ischemia and aging.

3.1. Limited spontaneous regeneration of cochlear ribbon synapses

Any regenerative medicine approach targeting sensorineural hearing loss caused by SGN or hair cell death will not only require restoration of the lost cells but also of their functional synaptic connections. Successful regeneration of functional cochlear synapses requires concerted efforts in re-innervation of IHCs with SGN terminals, re-formation of both pre-synaptic ribbons and post-synaptic densities, re-establishment of neurotransmission and sound-evoked neural responses. Additionally, for complete restoration of auditory function, the lost synapses need to be regenerated with normal density, correct subcellular localization and specific spontaneous firing properties.

Efforts have been made to replenish lost SGNs and these studies give insights into how new SGNs might innervate hair cells spontaneously (Chen et al., 2012; Martinez-Monedero et al., 2006; Matsumoto et al., 2008; Tong et al., 2013). A study based on co-culturing denervated organ of Corti with dissociated neonatal SGNs showed that SGN fibers could innervate organ of Corti and localize the synaptic protein Synapsin adjacent to the contact sites with hair cells (Martinez-Monedero et al., 2006) and form afferent like synapses labeled with juxtaposed PSD95 and CtBP2 (Tong et al., 2013). Another study showed that, when cultured with denervated organ of Corti, ES cell-derived neurons can also innervate hair cells and form contacts immunopositive for the synaptic proteins Synapsin I and Synaptophysin, but the putative synapses observed in these studies were significantly less than the normal densities of SGN-IHC ribbon synapses (Matsumoto et al., 2008). In addition, although some synaptic markers were found at site of hair cell and ES-derived neuron contacts, synaptic ribbons were absent (Matsumoto et al., 2008). Therefore, whether these few re-formed synaptic contacts are functional ribbon synapses remains to be determined.

A recent study reported the partial restoration of auditory function in ouabain-deafened adult gebrils with transplanted ES cell-derived otic progenitors (Chen et al., 2012). The transplanted otic progenitors replenished a population of SGNs that express afferent terminal markers GluA2 and NKA α 3 at the basal poles of IHCs. Although the densities of reformed afferent synapses were not quantitatively examined, it is likely that the synaptic regeneration was limited as indicated by the incomplete restoration of ABR amplitudes (Chen et al., 2012). Limited synapse regeneration was also observed in animals with noise-induced (Furman et al., 2013; Kujawa et al., 2009; Lin et al., 2011; Maison et al., 2013; Shi et al., 2013b; Wan et al., 2014) and ototoxicity-mediated cochlear synaptopathy (Liu et al., 2013; Liu et al., 2014), which correlate with incomplete restoration of ABR amplitudes. Thus, it appears that limited intrinsic capacity for ribbon synapse regeneration presents a significant barrier to hearing recovery after SGN injury.

3.2. Candidate molecules to promote ribbon synapse regeneration

In the central nervous system, many factors and signaling pathways have been shown to promote axonal regeneration and synapse reformation after injury or neurodegeneration (Deyst et al., 1995; Duan et al., 2005; Gordon-Weeks et al., 2014; Spejo et al., 2014; Xu et al., 2012). These “synaptotrophic” factors are important for re-innervation of nerve fibers and to re-establish functional connections between the axonal terminals and the target

presynaptic terminals (Cline et al., 2008). Here we review molecules and pathways implicated in the resprouting of the cochlear afferent nerve and/or regeneration of the ribbon synapses that may serve as therapeutic targets for hearing loss due to cochlear synaptopathy.

3.2.1. Neurotrophins as “synaptotrophins” in the inner ear—Members of the neurotrophin family (NGF, BDNF, NT-3, NT-4/5) have well-established roles in promoting synaptogenesis and regeneration of the central synapses (Alto et al., 2009; Deng et al., 2013; Marler et al., 2008; Park et al., 2013). BDNF and NT-3 and their cognate receptors TrkB and TrkC are expressed in the cochlea before and after birth (Ramekers et al., 2012). During embryonic development, BDNF/TrkB and NT-3/TrkC are critical for sensory neuron survival and the initial establishment of neuronal projections to sensory epithelia in the vestibule and cochlea, respectively [reviewed in (Fritzsche et al., 2004)].

Recent studies have shed new light into the roles of these neurotrophins in the formation of inner ear ribbon synapses. For example, neonatal conditional knockout of BDNF or NT-3 in inner ear supporting cells results in specific loss of vestibular or cochlear ribbon synapses respectively (Gomez-Casati et al., 2010; Wan et al., 2014). Correspondingly, loss of supporting-cell BDNF expression causes vestibular dysfunction while NT-3 loss causes hearing loss (Gomez-Casati et al., 2010; Wan et al., 2014). Interestingly, in these studies loss of supporting cell-derived NT-3 or BDNF did not affect the survival of sensory hair cells or neurons, suggesting that supporting cell-derived neurotrophins are not essential for the survival of adult inner ear sensory neurons. However, since survival times in these studies were relatively short (3 months), the possibility that neuronal loss could take place at later times was not assessed. The preservation of neurons in the absence of supporting cell-derived neurotrophins might also be a response to other endogenous trophic factors. For example, both insulin-like growth factor-1 (IGF1) and macrophage migration inhibitory factor (MIF) are expressed in the postnatal cochlea and loss of IGF1 or MIF results in SGN cell death or altered innervation (Bank et al., 2012; Camarero et al., 2001).

A role of endogenous NT-3 in cochlear synaptogenesis has been also suggested by experiments using cultured neonatal cochlear tissues and excitotoxicity (Wang et al., 2011). In this experimental paradigm, synaptic contacts between SGNs and IHCs reform, and this process is inhibited by NT-3 neutralizing antibodies, but not by those raised against BDNF (Wang et al., 2011). These studies indicate that endogenous NT-3, most likely produced by supporting cells, regulates the formation of IHC ribbon synapses.

In view of the roles of BDNF and NT-3 in synaptogenesis, it is conceivable that these neurotrophins can assist in synaptic repair after injury when added exogenously. Indeed, a combination of BDNF and NT-3 promotes re-formation of putative ribbon synapses between dissociated SGNs and deafferented organ of Corti (Tong et al., 2013). Interestingly, when added separately, both BDNF and NT-3 promote SGN re-innervation and post-synaptic marker expression in explant culture after excitotoxic drug treatment (Wang et al., 2011). Most significantly, overexpression studies in animals indicate that NT-3 might be more potent than BDNF for ribbon synapse regeneration *in vivo*; i.e. conditional over-expression of NT-3 but not of BDNF promotes regeneration of ribbon synapses after noise exposure (Wan et al., 2014). What could be the reason for the difference between the *in vitro* and *in*

vivo experiments? A potential explanation is the differences in the concentrations of the neurotrophins presented in each case. In animal studies, both BDNF and NT-3 were over-expressed by only 1.5-2 fold over the normal endogenous levels, while the tissue culture experiments involved doses well above saturation (3.6 nM) for both receptor activation and neuronal survival in cell-based assays (Ip et al., 1993; Wang et al., 2008). Considering the observation that TrkB is expressed at higher level than TrkC in adult rodent SGNs (Ylikoski et al., 1993), it is conceivable that NT-3/TrkC signaling is much more potent in promoting synapse regeneration than BDNF/TrkB, and that a much higher dose of BDNF may be required for synapse regeneration *in vivo*. It has been reported that BDNF and NT-3 may modulate each other's signaling via their common co-receptor p75NTR (Ivanisevic et al., 2003; Zaccaro et al., 2001). As TrkB, TrkC and p75NTR are all expressed on SGNs (Ramekers et al., 2012), another possibility is that high concentration of exogenous BDNF used in the explant culture studies modulates NT-3 signaling indirectly through p75NTR to promote synaptogenesis.

Although exogenous BDNF does not improve synaptic regeneration after noise exposure delivery during the day (Wan et al., 2014), it may promote synapse regeneration when the mice are noise exposed during the night. Recent study by Meltser et al. suggests that BDNF/TrkB signaling is critical for gating the circadian auditory clock to sound. TTS noise for mice during the day significantly elevated BDNF expression levels in the cochlea; however, the same noise during the night failed to induce BDNF expression and resulted in PTS and dramatic ribbon synapse loss (Meltser et al., 2014). How ribbon synapse loss resulted in PTS in this study is unknown. An interesting hypothesis is that BDNF may be required for the maintenance of high-SR fibers, and loss of BDNF sensitizes these high-SR fibers to noise resulting in threshold shifts. Interestingly, BDNF agonist had no effects on threshold shifts after day noise exposure but it protected against night noise trauma and partially prevented synaptic loss. Therefore, BDNF, either alone or in combination with NT-3, has particular therapeutic potential for hearing loss induced by noise during active circadian phase.

The potential for neurotrophins as treatments for hearing loss go beyond synapse regeneration, as these molecules have shown strong pro-survival and neuritogenic activities on SGNs as well (Ramekers et al., 2012). This has therefore implication for enhancing SGN transplants (Chen et al., 2012; Shi et al., 2013a) and cochlear implants (Budenz et al., 2012; Pinyon et al., 2014).

3.2.2. Is glutamate synaptotrophic in injured cochlea?—Glutamate released from presynaptic vesicles regulates synaptogenesis of retinal ganglion neurons, cortical and hippocampal neurons (McAllister, 2007; Sabo et al., 2006; Tashiro et al., 2003; Wong et al., 2001). While glutamate excitotoxicity has been implicated in the pathogenesis of hearing loss caused by noise, ischemia and aging, it appears that continued glutamatergic synaptic transmission is required for regeneration of the ribbon synapse after initial trauma. Dissociated SGNs were found to form fewer synaptic contacts with deafferented organ of Corti from Vglut3 knockout mice than those from wild types (Tong et al., 2013), suggesting that failure of glutamate release from hair cells impairs synaptic regeneration *in vitro*.

Although it is well-established that glutamate excitotoxicity causes swelling and degeneration of IHC post-synaptic terminals in multiple animal models, whether glutamate also plays a role in synaptic regeneration *in vivo* remains controversial. For example, perilymphatic perfusion of the glutamate agonist AMPA causes excitotoxic damage to synapses followed by spontaneous synaptic recovery. However, co-treatment with the NMDA antagonist D-AP5 delays the functional and structural recovery, suggestive of a synaptotrophic role for glutamate that is mediated by NMDA receptors (d'Aldin et al., 1997; Puel et al., 1997). The synaptotrophic role of NMDA activation has been reported in other neuronal populations as well. For example, chronic treatment of cortical or retinal neurons with a subtoxic dose of glutamate or NMDA protects against excitotoxic trauma through a mechanism involving CREB activation and neurotrophin synthesis (Chuang et al., 1992; Raval et al., 2003; Rocha et al., 1999). In contrast, another study reported that NMDA blockade by MK-801 that reduces synaptic swelling and prevents loss of auditory thresholds after acoustic trauma, suggesting that NMDA function is excitotoxic (Duan et al., 2000). Furthermore, local applications of NMDA antagonists was also reported to prevent noise-induced tinnitus (Guitton et al., 2007; Guitton et al., 2003) and a recent clinical trial using NMDA receptor antagonist AM-101 also demonstrated that NMDA blockade improves patient reported outcomes, such as tinnitus loudness and sleep difficulties (van de Heyning et al., 2014). A potential reason for the discrepancies mentioned above could be the different stimulation paradigms, i.e. AMPA agonist vs noise exposure. Noise exposure results in synaptic release of excessive glutamate, which over-activates both AMPA and NMDA receptors and both of which contribute to excitotoxicity and hearing loss (Duan et al., 2006; Jager et al., 2000; Oestreicher et al., 2002; Oestreicher et al., 1999). However, when excitotoxicity is induced by intratympanic perfusion of AMPA, there should be no over-activation of NMDA receptors. Basal level of NMDA receptor activation by low amount of spontaneously released glutamate at the synaptic cleft (Cousillas et al., 1988) may be critical for synaptic regeneration after AMPA-mediated excitotoxicity. Studies examining the temporal changes in glutamate concentrations, AMPA/NMDA receptor activation and expression patterns after excitotoxic injury will be an important step to address the synaptotrophic roles of glutamate.

Other molecules and neuronal circuits appear to influence synapse regeneration or protect against synaptic degeneration. For example, guidance molecules play important roles in axonal and synaptic regeneration in the central nervous system (Kyoto et al., 2007; Skaper, 2012), and thus are interesting targets for therapy against cochlear synaptopathy. This has been illustrated in a study showing that antibodies against the inhibitory guidance molecule RGMa promote SGN reinnervation and re-formation of synaptic contacts with deafferented organ of Corti (Brugaud et al., 2014). Recent studies show that cochlear efferent signals are also critical for maintenance of the afferent ribbon synapse (Liberman et al., 2014; Maison et al., 2013). De-efferentation of MOC/LOC neurons exacerbates both noise-induced (Maison et al., 2013) and age-related (Liberman et al., 2014) loss of ribbon synapses and hearing sensitivity. Thus, an alternative strategy for synapse regeneration might be to modulate or amplify the efferent negative feedback (eg. by applying acetylcholine, GABA or dopamine), particularly when the excitotoxic insults are still present.

4. Conclusions and outlook

In recent years, there has been growing appreciation of the importance of cochlear synaptopathy in sensorineural hearing loss (Kujawa et al., 2009; Lin et al., 2011; Liu et al., 2013; Sergeyenko et al., 2013; Wan et al., 2014), hyperacusis (Hickox et al., 2014; Knipper et al., 2013) and tinnitus (Knipper et al., 2013; Rüttiger et al., 2013; Singer et al., 2013). Damage to the highly specialized ribbon synapses seems to underlie a form of auditory neuropathy that is characterized by fully recoverable ABR threshold shifts, decreased ABR response amplitudes, reduced low-SR fiber terminals and a delayed onset of sensory neuron cell death (**Fig. 1**). The immediate consequence of this synaptopathy is also referred to as the “hidden” hearing loss, which may underlie hearing difficulties in noisy environments. Over the course of months to years after ribbon synapse degeneration, loss of SGN cell bodies becomes apparent (**Fig. 1**). Therefore, timely intervention for auditory synaptopathy caused by excitotoxic insults is critical for long term maintenance of the auditory neurons.

A number of factors that exhibit putative synaptotrophic activity after excitotoxicity-mediated loss of ribbon synapses, e.g. neurotrophins and glutamate, are also important for synaptogenesis during development (Gomez-Casati et al., 2010; Tashiro et al., 2003; Wan et al., 2014). Therefore, molecules and pathways regulating endogenous formation and maturation of ribbon synapses, such as thrombospondins (Mendus et al., 2014), thyroid hormone (Sendin et al., 2007) and Ephrin-A5/EphA4 (Defourny et al., 2013), might also have the ability to induce or modulate synaptic regeneration after injury.

In vitro studies using explant cochlear cultures and dissociated neurons have provided valuable information on factors that might have the ability to promote afferent re-innervation and reformation of synaptic contacts *in vivo* (Brugaud et al., 2014; Martinez-Monedero et al., 2006; Tong et al., 2013; Wang et al., 2011). However, due to technical barriers, these studies are performed on cochlear tissues from neonatal animals, in which the afferent terminals are developing and undergoing significant structural changes and whose ribbon synapses are still immature (Huang et al., 2012; Safieddine et al., 2012; Sobkowicz et al., 1986; Yu et al., 2014). It will be therefore important to test these synaptotrophic molecules *in vivo* in adult injured cochlea.

The neurotrophins NT-3 and BDNF are among the most promising therapeutic candidates for treating cochlear neuropathy (**Fig. 1**) (Budenz et al., 2012; Ramekers et al., 2012). It has been well established that both BDNF and NT-3 can promote survival of SGNs in rodent cochlea injured by ototoxins (Bowers et al., 2002; Chen et al., 2001; McGuinness et al., 2005), noise exposure (Duan et al., 2000; Zhai et al., 2011) or infection (Demel et al., 2011; Li et al., 2005). Recent studies showing the synaptotrophic roles of BDNF (Meltser et al., 2014) and NT-3 (Wan et al., 2014) in noise-injured cochlea further underscore the beneficial effects of these neurotrophins. Many interesting questions have arisen from these findings. TrkB and TrkC are expressed by SGNs but not hair cells in postnatal cochlea. BDNF and NT-3 therefore exert their activity on SGNs to modulate post-synaptic terminals. However, pre-synaptic ribbons on the inner hair cells are also lost after trauma and reform and juxtapose to post-synaptic markers (Kujawa et al., 2009; Lin et al., 2011; Meltser et al., 2014; Shi et al., 2013b; Wan et al., 2014). How the pre-synaptic ribbons are regenerated by

neurotrophin signaling remains to be determined. To form a functional ribbon synapse, the afferent nerve fibers must re-innervate the hair cells and re-differentiate into post-synaptic terminals with the correct structural and biochemical properties. Studies elucidating how neurotrophin signaling regulates these structural and biochemical specializations will reveal the molecular mechanisms of synaptic regeneration and suggest novel therapeutic targets for cochlear synaptopathy.

Acknowledgments

The authors thank Dr. Horacio U. Saragovi for comments and Dr. Elissa H. Patterson for critical reading of the manuscript. The work was supported by National Institute on Deafness and Other Communication Disorders Grant R01 DC 004820 (to GC) and the Hearing Health Foundation (to GW).

List of Abbreviations

HC	hair cell
IHC	inner hair cell
OHC	outer hair cell
SGN	spiral ganglion neuron
PTS	permanent threshold shifts
TTS	temporary threshold shifts
ABR	auditory brainstem response
CAP	compound action potential
SR	spontaneous firing rate
NT-3	neurotrophin-3
BDNF	brain-derived neurotrophic factor

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Highlights

- Cochlear ribbon synapses are required for rapid and reliable neurotransmission
- Degeneration of ribbon synapses is a common pathology in sensorineural hearing loss
- Strategies promoting regeneration of ribbon synapses are discussed

Fig. 1.

An overview of cochlear synaptopathy. In a healthy cochlea, ribbon synapses are formed on IHCs by innervation of low-SR (primarily at the modiolar side) and high-SR (primarily at the pillar side) SGN nerve fibers. Noise exposure (top grey arrow) preferentially damages the low-SR ribbon synapses within hours after exposure (acute synaptopathy). During normal aging (bottom grey arrow), these low-SR ribbon synapses are also preferentially lost, albeit in a much slower rate (progressive synaptopathy). This synapse loss is followed by nerve terminal retraction and delayed neuronal loss that occurs months or years later. SGNs degeneration might be the consequence of lack of trophic support normally provided by the sensory epithelia. Neurotrophins NT-3 and BDNF have potent effects on promoting long term survival of SGNs after injury by ototoxins, noise or infection. NT-3, BDNF and possibly glutamate (Glu) signaling also promotes regeneration of the ribbon synapses after noise exposure. Whether NT-3, BDNF or Glu promotes synaptic regeneration during age-related cochlear synaptopathy remains to be determined. IHC, inner hair cell; SGN, spiral ganglion neuron; IPhC, inner phalangeal cell; IBC, inner border cell; BC, border cell; IPC, inner pillar cell; NT-3, neurotrophin-3; BDNF, brain-derived neurotrophic factor; Glu, glutamate; IGF1, insulin-like growth factor-1; MIF, migration inhibitory factor.