Efferent synapses return to inner hair cells in the aging cochlea

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Abstract

Efferent innervation of the cochlea undergoes extensive modification early in development, but it is unclear if efferent synapses are modified by age, hearing loss, or both. Structural alterations in the cochlea affecting information transfer from the auditory periphery to the brain may contribute to age-related hearing deficits. We investigated changes to efferent innervation in the vicinity of inner hair cells (IHCs) in young and old C57BL/6 mice using transmission electron microscopy to reveal increased efferent innervation of IHCs in older animals. Efferent contacts on IHCs contained focal presynaptic accumulations of small vesicles. Synaptic vesicle size and shape were heterogeneous. Postsynaptic cisterns were occasionally observed. Increased IHC efferent innervation was associated with a smaller number of afferent synapses per IHC, increased outer hair cell loss, and elevated auditory brainstem response thresholds. Efferent axons also formed synapses on afferent dendrites but with a reduced prevalence in older animals. Age-related reduction of afferent activity may engage signaling pathways that support the return to an immature state of efferent innervation of the cochlea.

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Keywords: Cochlear innervation; Age-related hearing loss; Olivocochlear; Efferent; Neural plasticity

1. Introduction

It is estimated that 25% of people aged 65–75 years and 70%–80% of people older than age 75 suffer from hearing loss associated with aging (Lin et al., 2011; Sprinzl and Riechelmann, 2010). Cumulative structural alterations in the cochlea affecting information transfer from the auditory periphery to the brain may contribute to age-related hearing deficits. Changes in the proportion of low spontaneous rate auditory nerve fibers observed with age (Schmiedt et al., 1996) may be linked to afferent synapse alterations at the inner hair cell (IHC) including loss of afferent terminals; enlarged synaptic terminals, mitochondria, postsynaptic densities (PSDs), and synaptic bodies; flattened PSDs; increased prevalence of PSDs associated with multiple or missing synaptic bodies; and increased synaptic vesicle density (Stamataki et al., 2006).

Changes in efferent feedback to the cochlea also may occur during age-related hearing deficits. The mammalian olivocochlear efferent system consists of 2 distinct pathways: a medial olivocochlear (MOC) component that projects bilaterally from the superior olivary complex to the outer hair cells (OHCs) and a lateral olivocochlear (LOC) component that projects ipsilaterally from the superior olivary complex to the auditory nerve dendrites contacting IHCs (summarized in Fig. 1; recently reviewed by Brown, 2011). These projection patterns reflect the mature innervation of adult animals. Activation of the MOC component causes hyperpolarization of OHCs by acetylcholine release (Blanchet et al., 1996; Evans, 1996), resulting in modulation of the cochlear response (Russell and Murugasu, 1997). The resulting suppression of the cochlear response modulates the auditory nerve’s dynamic range (e.g., Dolan and Nuttall, 1988; Galambos, 1956; Kawase and Liberman, 1993; Kawase et al., 1993; Liberman and Brown, 1986; Winslow and Sachs, 1987), enhances the representation of transient signals in noise (Dolan and Nuttall, 1988; Kawase and Liberman, 1993; Winslow and
Sachs, 1987), protects the auditory system from acoustic trauma (e.g., Kujawa and Liberman, 1997; Lauer and May 2011; Maison et al., 2002; Rajan, 2000). Deficient MOC function has been reported in older human listeners and mice (Jacobson et al., 2003; Zettel et al., 2007). The functional role of the LOC system is poorly understood, but it appears to modify afferent activity through various neurotransmitter systems and may protect the ear from acoustic overexposure and/or balance the sensitivity of the 2 ears to sensory stimulation (Darrow et al., 2006, 2007; Le Prell et al., 2003, 2005).

Efferent innervation of the cochlea undergoes extensive modification early in development (Simmons et al., 2011), including the loss of efferent contacts from IHCs near the onset of hearing. It is not known if these changes are permanent, or if they vary with acoustic damage, aging, or both. C57BL/6J mice show age-related loss of MOC terminals contacting OHCs (Fu et al., 2010), but virtually nothing is known about age-related changes to efferent innervation in the IHC area. In the present study, we investigated changes to efferent innervation in the vicinity of IHCs in aged mice.

2. Methods

2.1. Subjects

Adult female C57BL/6J (C57) mice were studied at 2–3 months of age (n = 3) and 8–11 months of age (n = 3). The efferent innervation of the 22 kHz region of the cochlea was examined for a total of 10 cells from young animals and 8 cells from old animals using transmission electron microscopy. A total of 3 axosomatic efferent synapses and 348 axodendritic efferent synapses were observed in young animals, and 35 axosomatic efferent synapses and 60 axodendritic synapses were observed in old animals. Auditory brainstem response (ABR) thresholds and age-related alterations to afferent synapses were previously quantified in the same mice (Stamataki et al., 2006), and the number of afferent synapses per IHC reported here are from this dataset. Behavioral hearing deficits and patterns of hair cell loss were also characterized in the older mice (Francis et al., 2003; Prosen et al., 2003). In Table 1 we summarize the pure tone thresholds measured by Stamataki et al. (2006) by averaging across the frequencies tested. This average provides a single summary measure of hearing status, similar to what is often reported by audiologists testing human listeners.

No outer or middle ear pathology was observed in any of the subjects at the time of tissue harvest. All procedures were conducted in accordance with protocols approved by

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age, mo (wk)</th>
<th>Pure tone average (dB SPL)</th>
<th>Cochlear location studied (% related to base)</th>
<th>OHC loss, %</th>
<th>Average axosomatic efferent synapses per IHC, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young 1</td>
<td>2 (9)</td>
<td>NA</td>
<td>40</td>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td>Young 2</td>
<td>3 (12)</td>
<td>22.5</td>
<td>48</td>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td>Young 3</td>
<td>3 (13)</td>
<td>35.2</td>
<td>47</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Old 1</td>
<td>8 (35)</td>
<td>71.5</td>
<td>45</td>
<td>40.5</td>
<td>1.33</td>
</tr>
<tr>
<td>Old 2</td>
<td>8 (35)</td>
<td>82.4</td>
<td>47</td>
<td>70.1</td>
<td>3.3</td>
</tr>
<tr>
<td>Old 3</td>
<td>11.5 (46)</td>
<td>91.85</td>
<td>47</td>
<td>100</td>
<td>7</td>
</tr>
</tbody>
</table>

Age, hearing thresholds, cochlear location, and % outer hair cell (OHC) loss from Stamataki et al. (2006).

Key: IHC, inner hair cell; NA, not applicable; SPL, sound pressure level.
the Johns Hopkins University Animal Care and Use Committee.

2.2. Tissue processing

The tissue processing methods have been described in detail elsewhere (Francis et al., 2003, 2004; Stamatakis et al., 2006). In brief, intracochlear perfusion of fixatives was performed in deeply anesthetized animals (intraperitoneal injections of 100 mg/kg ketamine and 20 mg/kg xylazine in 14% ethanol). A small incision was made behind the pinna and soft tissue was removed to visualize the bulla. The ear canal was separated from the bulla, the malleus and incus removed, and the bulla chipped away using small rongeurs to expose the cochlea. A small hole was made in the cochlear apex, the stapes footplate was removed from the oval window, and a solution containing 1% OsO4 and 1% potassium ferracyanide [FeK3(CN)6] was gently perfused through the cochlea for 3–5 minutes. The animal was decapitated, and cochlear perfusion was resumed through both the oval and round windows for 5–10 minutes. The heads were postfixed for approximately 45 minutes in the same fixative prior to removal of the temporal bones. The cochlea were dissected from the head and decalcified with 0.1 M ethylenediaminetetraacetic acid (EDTA) with 1% glutaraldehyde. Decalcified cochleae were then dehydrated in graded alcohols and propylene oxide and embedded in araldite. Cochleae were sectioned at 40 –50 mm thickness parallel to the modiolus and mounted between sheets of Aclar (Honeywell, Baltimore, MD, USA). The entire cochlear spiral was reconstructed from light microscope photographs using NIH Image (rsbweb.nih.gov/nih-image/about.html), Voxblast (Vaytech, Inc., Faurfield, IA, USA), and Neurolucida (Microbrightfield, Inc., Essex, VT, USA) software. Average section thickness (74.2 ± 17.2 nm) was calculated for each block of tissue using the minimal folds method on every 10th section (Falia and Harris, 2001). Three-dimensional reconstructions depicting efferent and afferent terminals on IHCs were generated using Reconstruct software (Falia, 2005).

Axosomatic innervation was compared between age groups. Nerve fibers containing a focal accumulation of synaptic vesicles in the immediate presynaptic region and the absence of presynaptic bodies in the adjacent hair cell were classified as efferent contacts. The efferent identity of the nerve was further confirmed by the presence of multiple efferent synapses on afferent dendrites and other IHCs when possible. Identification of postsynaptic cisterns on the inner hair cell side opposite the vesicle cloud was also confirmatory of efferent synapses; however, depending on the plane of section, cisterns were not always visible. These classification criteria were based on previous studies of cochlear efferent innervation (Liberman, 1980; Shnerson et al., 1982; Spoendlin, 1985). The number of axosomatic efferent nerve synapses was quantified by complete 3-D reconstruction of 2–4 inner hair cells from each animal.

Axodendritic efferent innervation was also evaluated. All dendrites associated with afferent synapses on IHCs were followed in alternate sections to the habenula perforata. Axodendritic efferent contacts were identified by the presynaptic aggregation of vesicles often associated with increased density of the postsynaptic membrane.

Group differences were evaluated using 2-tailed Student t tests for quantitative data or χ2 tests for categorical data. The tests include the Bonferroni correction that calculates probabilities when tests are run multiple times. Effects were considered to be statistically significant when the p value was <0.05.

3. Results

3.1. Ultrastructure of efferent nerve endings in the IHC area in young and old animals

Axodendritic efferent synapses were common in young animals. While efferent terminals occasionally approached the IHC in young animals, axosomatic synapses were rare. In contrast, both axosomatic and axodendritic efferent synapses were observed in older animals. Fig. 2A shows a section through the basal (synaptic) pole of an IHC of an older animal that is contacted by 4 axosomatic efferent terminals. Sections through the basal pole of IHCs from older animals demonstrated irregular shaped profiles and contained numerous vacuoles as in this example, possibly signifying deterioration of the cell (Ruel et al., 2007; Stamatakis et al., 2006). Efferent terminals were occasionally observed closer to the apical (cuticular) pole of the IHCs,
but the majority was found around the basal (synaptic) pole. Fig. 2B shows a section through an IHC from a younger animal near the basal pole of the cell. An efferent fiber contacts the IHC but does not form a synapse. A second efferent fiber forms an axodendritic synapse with a large afferent terminal characterized by the presence of a prototypical ribbon synapse.

High magnification images (Fig. 3A–C, E) revealed axoso-matic efferent terminals with diffuse, heterogeneous vesiculation and frequent clouds of small vesicles in the cytoplasm (arrows) in older animals. Synaptic vesicle accumulations were associated with electron dense material in the presynaptic area. Dense core vesicles were occasionally observed. Post-

3.2. Increased axosomatic efferent innervation of inner hair cells in older animals

Older animals showed an increase in the number of axosomatic efferent synapses per IHC compared with young animals (Fig. 5A). The number of axosomatic efferents varied considerably in the older animals, such that 0 or 1 axosomatic synapse was observed on some cells, whereas others had 5 or more. Young animals typically had 0 to 1 axosomatic efferents. In general, animals with fewer than 12 afferent synapses had more axosomatic efferent terminals. However, the occurrence of more than 12 afferent synapses was typically associated with a greater number of efferent synapses per
Fig. 3. (A–C) High magnification images of axosomatic efferent terminals in older animals (8–11 months old). Focal accumulations of vesicles associated with fuzzy electron dense material in the presynaptic area and synaptic cleft are visible (arrows). Postsynaptic cisternae are visible in some sections (asterisk). IHC, inner hair cells; v, vacuoles. (D) Axosomatic efferent terminal contacting an IHC of a young animal (3 months old). This terminal does not form a synapse. (E) Four sections through an axosomatic efferent terminal forming synapses with 2 IHCs in an older animal (11 months old). Small vesicles accumulate in the presynaptic area (arrows). The presynaptic area and the synaptic cleft display electron dense material. Postsynaptic cisternae are visible at both synapses (asterisks). Scale bars = 500 nm; magnification in A and B: ×40,000; C and D, ×30,000; E, ×20,000.
dendrite. The relationship between number of afferent synapses and number of axodendritic synapses was not as clearly defined by the cutoff of 12 afferent synapses observed for axosomatic synapses, but in general fewer axodendritic synapses occurred in cells with fewer than 12 afferent synapses.

3.3. Relationship to age, hearing status, and histological characteristics of cochlear region

Table 1 summarizes subject age, hearing status, and histological characteristics of the cochleae. All but the axosomatic efferent data are from Stamataki et al. (2006). Pure tone averages were calculated by averaging the auditory brainstem response thresholds previously measured for 8, 16, and 32 kHz tones (Stamataki et al., 2006). In cases where no threshold was available due to the lack of response, a value of 100 dB was substituted in the calculation. Greater outer hair cell loss and higher pure tone thresholds were associated with more axosomatic efferent synapses per hair cell. The animal with the greatest proportion of OHC loss and the highest pure tone average had the most axosomatic efferent synapses per IHC (Old3). This animal was also 11 weeks older than the other 2 animals.

3.4. Distribution of axosomatic efferents in older animals

Fig. 6A shows 2 views of a reconstructed IHC with efferent (red) and afferent (blue) terminals from an old animal (top) and a young animal (bottom). Efferent terminals in older animals tended to occupy the low frequency side of the IHCs (toward the apex of the cochlea, which encodes low frequency signals). The radial position of each synapse was measured as an angle relative to a reference line passing through the center of the IHC and perpendicular to the line of pillar cells and the bony center, or modiolus, of the cochlea. Synapses with angles of 0° to 180° were located on the low frequency side of the IHC, whereas synapses with angles >180° were located on the high frequency side (toward the base). The distribution of axosomatic efferent synapses in older animals visualized in 3 dimensions (Fig. 6B) shows that the majority of axosomatic efferent synapses occurred on the low frequency side ($\chi^2 = 6.818; p = 0.009$). Axosomatic efferents were equally distributed across the pillar and modiolar sides of the IHC ($\chi^2 = 0.030; p = 0.8618$). The distribution of axosomatic terminals in young animals was not plotted because very few were observed across all IHCs. The distribution of afferent synapses is replotted from Stamataki et al. (2006) to reiterate the finding that the remaining afferent synapses in older animals also occur on the low frequency side of the IHCs.

4. Discussion

We have shown that efferent innervation of mouse IHCs increases with age-related hearing loss. Transient efferent contacts with IHCs occurring early in development have been known to exist for some time in rodents, but these contacts are lost from the IHC by the time of hearing onset (Katz et al., 2004; Simmons et al., 2011). Here we provide evidence for increased IHC-associated efferents in aging adult animals.

The observed age-related change in efferent innervation of IHCs in hearing-impaired mice may signal a form of adaptive neural plasticity in response to the partial deafferentation described by Stamataki et al. (2006). Though the majority of studies of peripheral neural plasticity after deafferentation concern acute or experimentally-induced injury
to the sensory organ, compensatory reorganization of retinal neurons in response to naturally occurring age-related macular degeneration has been reported in the visual system (Sullivan et al., 2007).

4.1. Age-related changes in axosomatic efferent terminals: return to early developmental stages?

Axosomatic efferent innervation of IHCs was increased in older C57 mice and displayed morphological features similar to those of young developing C57 mice. Shnerson and colleagues (1982) found efferent fibers directly contacting IHCs from postnatal days 3–9. These efferent terminals contained focal accumulations of small round vesicles, a fuzzy postsynaptic area, and the occasional postsynaptic cistern. Some of these efferents also formed synapses with auditory nerve dendrites. By postnatal day 12, however, the majority of efferents contacted auditory nerve dendrites. A transient return to an immature-like state of IHC innervation has also been described after application of a high dose of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; Ruel et al., 2007).

The axosomatic efferents observed in old C57 mice could indicate a return of efferent cholinergic inhibition of IHCs that occurs transiently during postnatal development (Glowatzki and Fuchs, 2000). Medial olivocochlear efferent terminals leave the OHCs of aging C57 mice (Fu et al., 2010) and thus may migrate back to the IHC region. Plasticity of acetylcholine receptors (AChRs) in the auditory system has been documented. For example, mice that lack β2 AChRs experience large losses of spiral ganglion neurons (Bao et al., 2005). An age-related loss of β2 AChRs in C57 mice may therefore be associated with spiral ganglion neuron degeneration, highlighting the trophic influence of cochlear efferent innervation in the settings of both development and injury. Age-related changes in the expression of other AChRs that may affect synapse maintenance and localization have not been described in the cochlea, but numerous changes in expression of nicotinic AChRs have been noted in the aging brain (e.g., Ferrari et al., 1999; Hellström-Lindahl and Court, 2000).

Sobkowicz and colleagues (Sobkowicz and Slapnick 1994; Sobkowicz et al., 1997, 2002) also identified axosomatic efferent terminals in the cochlear apex and mid-turn of developing mice of the outbred ICR strain. As in aging C57 mice, efferents in young ICR mice occasion-

Fig. 5. On average, older mice had more axosomatic efferent synapses (A) and more axosomatic efferent synapses per inner hair cell (IHC) were associated with fewer afferent synapses (B). The presence of fewer than 12 afferent synapses was typically associated with 5 or more axosomatic efferent synapses per IHC in older mice. Young mice always had more than 12 afferent synapses and only 1 or no axosomatic efferent synapses. Older mice had fewer efferent synapses per dendrite (C) and fewer efferent synapses per dendrite were associated with fewer afferent synapses per IHC (D). Afferent synapse data were from Stamataki et al. (2006).
ally formed synapses with 2 IHCs, termed “inter-receptor synapses.” GABAergic signaling is known to produce neurotrophic effects (Lauder, 1993) and has been suggested to play a role in the maintenance of hair cells and auditory nerve fibers (Maison et al., 2006; Ruel et al., 2001). The reappearance of axosomatic efferent terminals in old C57 mice may reflect a compensatory sprouting of GABAergic terminals to promote survival of the IHCs. However, to date, intracellular recording from immature IHCs has documented cholinergic, but not GABAergic synaptic inputs (Glowatzki and Fuchs, 2000; Gómez-Casati et al., 2009; Goutman et al., 2005; Johnson et al., 2011; Katz et al., 2004; Kong et al., 2008; Taranda et al., 2009; Wersinger et al., 2010). Confirmation of the functionality of efferent terminal subtypes contacting IHCs of older animals will require technically challenging immunolabeling and physiological studies.

4.2. Distribution of axosomatic efferent synapses

Axosomatic efferent synapses were located primarily on the low frequency side of the IHCs in older mice, congruent with the location of surviving afferent synapses reported by Stamatakì et al. (2006). This distribution of new axosomatic

Fig. 6. (A) Three-dimensional reconstructions of efferent (red) and afferent (blue) terminals contacting an inner hair cell (IHC) of an older animal (top) and a young animal (bottom) viewed from the base of the hair cell and from the low frequency side. (B) Polar plots revealed that efferent synapses occurred primarily on the low frequency side of the IHCs in older animals, similar to the distribution of remaining afferent terminals (adapted from Stamatakì et al., 2006). There was no difference in the distribution of efferent synapses on the pillar versus modiolar sides.
contacts is not consistent with either a random or opportunistic process based on filling newly available space on the cell. Rather, it suggests a purposeful colocalization of residual afferent and newly established efferent innervation on the low-frequency side of the cell. Afferent innervation on the low-frequency side of the IHC is distinguished by a higher proportion of larger terminals, which are more likely to be spared than smaller terminals on the high-frequency surface (Stamatakis et al., 2006). The preservation of these endings in the face of glutamate excitotoxicity or other insults during the aging process may result from their intrinsic capacity to adapt and/or trophic support from efferent innervation.

The migration of efferent axons to the IHC could be part of a reversal of normal development with recapitulation of the neonatal condition accompanied by morphological changes at the afferent synapse. A reversal of afferent morphologic maturity for example, is demonstrated in the higher incidence of both absent and multiple synaptic bodies, as well as flattened postsynaptic densities (Stamatakis et al., 2006). The constellation of changes in hair cell innervation does not in itself demonstrate causal relations. However, the preferential colocation of afferent and efferent contacts on the “low frequency” side of IHCs suggests the possibility of communication between these neurons, perhaps to impact the survival of remaining afferent dendrites innervating IHCs in the aging or injured cochlea.

4.3. Functional implications of age-related return of IHC efferent innervation

Age-related degeneration of afferent synapses in C57 mice is thought to be a response to chronic excitotoxic insult due to erratic, fluctuating glutamate release from IHCs with abnormal tip link structure (Stamatakis et al., 2006). Increased direct signaling between efferent fibers and IHCs in aging mice could promote survival of remaining afferent synapses by boosting antieexcitotoxic mechanisms. Excitotoxic damage to auditory nerve terminals resulting from excessive glutamate release occurs when dopaminergic lateral efferent inhibition is blocked (Ruel et al., 2001). Enhanced efferent inhibition resulting from a proliferation of axosomatic efferent synapses in aging C57 mice could reduce glutamate release, thereby lessening damage to the auditory nerve terminals.

Increased efferent regulation of the IHCs in aging mice also could affect spontaneous activity in the auditory nerve. Lesions and pharmacological blockade of olivocochlear neurons alter auditory nerve spontaneous firing rate (SR), tuning, and dynamic range under certain conditions (Liberman, 1990; Ruel et al., 2001; Walsh et al., 1998; Zheng et al., 1999). The proportion of low SR auditory nerve fibers decreases with age (Schmiedt et al., 1996) and hearing loss (Liberman and Dodds, 1984; Ryugo et al., 1998). Loss of low SR fibers may disrupt encoding of timing and intensity cues at high sound levels (Frisina et al., 1996; Zeng et al., 1991) and representation of signals in noise (Reiss et al., 2011; Silkes and Geisler, 1991; Young and Barta, 1986). These encoding deficits may contribute to distortion of speech representation and reduced directional hearing commonly reported in older listeners. Enhanced efferent input to the IHCs of aging C57 mice may be a compensatory response to changes in the distribution of auditory nerve fiber SR in an effort to preserve the heterogeneity that is essential for normal encoding of acoustic cues.

4.4. Future directions/implications

This study reports an increased number of efferent synapses on IHCs of aging, hearing-impaired mice. Electrophysiological recordings will be needed to ascertain whether these are functional synapses, a nontrivial effort given the difficulty of maintaining microdissected cochlear tissue. This question takes added significance from recent reports that the earliest cholinergic responses of neonatal IHCs are excitatory, because efferent-associated small conductance calcium-gated potassium channels (known as SK channels) that render the synapse inhibitory appear 1 day later during development (Roux et al., 2011). Thus, a closely related question is whether aged IHCs upregulate the SK channels that also diminish after the onset of hearing (Katz et al., 2004). Immunohistology and/or in situ hybridization for SK channels, as well as the α9 and α10 subunits of the hair cell acetylcholine receptor, will further define the molecular mechanisms involved in the regulation of synapses on aged IHCs. Studies are also needed to determine if similar neuroplasticity occurs in other cochlear frequency regions and at other time points throughout the course of aging.

Disclosure statement

The authors disclose no conflicts of interest.

All procedures were conducted in accordance with protocols approved by the Johns Hopkins University Animal Care and Use Committee.

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