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SYMPOSIUM REVIEW

A 'calcium capacitor' shapes cholinergic inhibition of cochlear hair cells

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Abstract Efferent cholinergic neurons project from the brainstem to inhibit sensory hair cells of the vertebrate inner ear. This inhibitory synapse combines the activity of an unusual class of ionotropic cholinergic receptor with that of nearby calcium-dependent potassium channels to shunt and hyperpolarize the hair cell. Postsynaptic calcium signalling is constrained by a thin near-membrane cistern that is co-extensive with the efferent terminal contacts. The postsynaptic cistern may play an essential role in calcium homeostasis, serving as sink or source, depending on ongoing activity and the degree of buffer saturation. Release of calcium from postsynaptic stores leads to a process of retrograde facilitation via the synthesis of nitric oxide in the hair cell. Activity-dependent synaptic modification may contribute to changes in hair cell innervation that occur during development, and in the aged or damaged cochlea.

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Introduction and background

Mammalian cochlear hair cells are inhibited by cholinergic neurons projecting from the superior olivary complex in the brainstem (Guinan, 2006). These olivocochlear efferents are activated by sound (Robertson, 1984; Brown, 1989), providing negative feedback that suppresses the response of cochlear afferent neurons (Galambos, 1956; Wiederhold & Kiang, 1970; Winslow & Sachs, 1987). Medial olivocochlear efferents inhibit outer hair cells (OHCs) to improve signal detection in background noise (May & McQuone, 1995; Hienz *et al.* 1998) and can provide protection from acoustic trauma (Rajan & Johnstone, 1988). In addition to these roles in the mature cochlea, cholinergic efferents temporarily inhibit inner hair cells during the first 2 postnatal weeks in rodents (Glowatzki & Fuchs, 2000; Marcotti *et al.* 2004), possibly to shape spontaneous afferent activity prior to the onset of hearing (Johnson *et al.* 2013; Sendin *et al.* 2014). Intriguingly, this immature innervation pattern reappears in a mouse model of age-related hearing loss (Lauer *et al.* 2012). Thus, efferent feedback regulates hair cell function moment-by-moment to improve acoustic detection, over the course of days as protection from acoustic trauma, and in as yet undefined ways early and late in life. This

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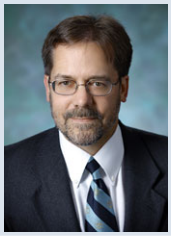

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multiplicity of roles and timescales implies modulatory mechanisms that extend beyond changes in membrane potential and conductance. Efferent transmission to hair cells is strongly activity dependent (Goutman *et al.* 2005; Ballestero *et al.* 2011) resulting in part from a nitric oxide-mediated process of retrograde facilitation (Kong *et al.* 2012). Studies of postsynaptic calcium signalling and the elaborate synaptic ultrastructure provide a starting point for investigating shorter-term plasticity and longer-term rearrangements during development and ageing.

Inhibitory efferent innervation is common to hair cells in all vertebrates (Manley & Koppl, 1998; Simmons, 2002a). In auditory hair cells of reptiles (turtles; Art *et al.* 1984), birds (chickens; Fuchs & Murrow, 1992a) and mammals (rodents; Erostegui *et al.* 1994; Blanchet *et al.* 1996; Evans, 1996b; Nenov *et al.* 1996) inhibition is produced by acetylcholine (ACh) opening ionotropic ACh receptors, leading to activation of calcium-dependent potassium channels. Cellular studies have increasingly turned to efferent inhibition of the mammalian cochlea with the obvious motivation to understand the human condition. But equally, the specificity of innervation, its plasticity and modulation during development, and the diversity of functional roles for cochlear efferents make them intrinsically interesting. A cross-section of the mature mammalian organ of Corti illustrates the specificity of these connections (Fig. 1).

In the mature cochlea the cholinergic medial olivocochlear efferents (originating in the medial olivary complex) contact OHCs (Guinan, 2006). Lateral olivocochlear efferents (lateral olivary complex origin) contact the dendrites of type I afferents beneath inner hair cells to release ACh, and possibly GABA, dopamine and peptide neurotransmitters as well (Ruel *et al.* 2007). During cochlear maturation, prior to the onset of hearing, inner hair cells are inhibited temporarily by efferent synaptic contacts (Glowatzki & Fuchs, 2000) similar in action to those found later on OHCs (Oliver *et al.* 2000; Lioudyno *et al.* 2004), and thought to be mediated by the same pool of medial olivocochlear neurons (Simmons, 2002b).

Galambos first showed that electrical stimulation of efferent axons suppressed the VIIIth nerve compound action potential evoked by sound (Galambos, 1956). Subsequent single unit recordings confirmed and extended those observations (Wiederhold & Kiang, 1970; Winslow & Sachs, 1987). The first intracellular recordings of hair cell inhibition were obtained from fish lateral line hair cells (Flock & Russell, 1973, 1976). Subsequent studies in the turtle inner ear provided detailed evidence for the effect of efferent inhibition on acoustic receptor potentials (Art *et al.* 1982, 1984, 1985). Voltage-clamp recording from chicken hair cells helped define the ionic mechanisms and unusual pharmacology of the hair cell's acetylcholine receptor (AChR; Shigemoto & Ohmori, 1991; Fuchs & Murrow, 1992a,*b*). In all vertebrate hair cells examined to date, two types of ion channel mediate the effects of acetylcholine (Fig. 2). The mammalian hair cell's AChR includes α9 and α10 subunits that form a non-selective cation channel with ionic conductance and pharmacology identical to that of the native hair cell AChR (Elgoyhen *et al.* 1994, 2001). Calcium influx activates nearby small conductance (SK) potassium channels (Erostegui *et al.* 1994; Blanchet *et al.* 1996; Evans, 1996a; Nenov *et al.* 1996;

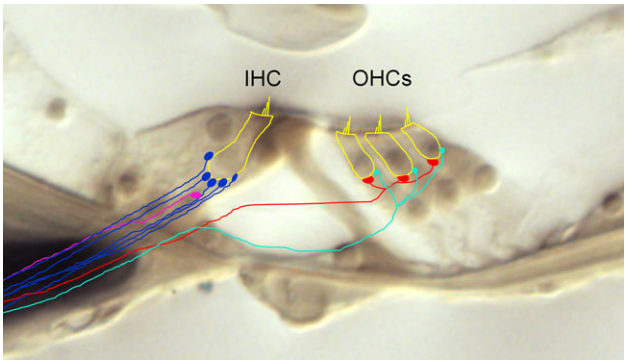

Figure 1. Cross-section of the organ of Corti (3-week-old rat cochlea – unstained)
Hair cells are outlined in yellow, stereocilia and innervation schematized. Type I (blue) and type II (turquoise) afferents contact inner and outer hair cells, respectively. Type II afferents travel hundreds of micrometres toward the cochlea base before contacting hair cells (not shown). Medial (red) and lateral (fuchsia) efferents contact outer hair cells and the dendrites of type I afferents, respectively. Only a single lateral efferent is shown for clarity; in reality a rich plexus of endings is formed beneath each inner hair cell.

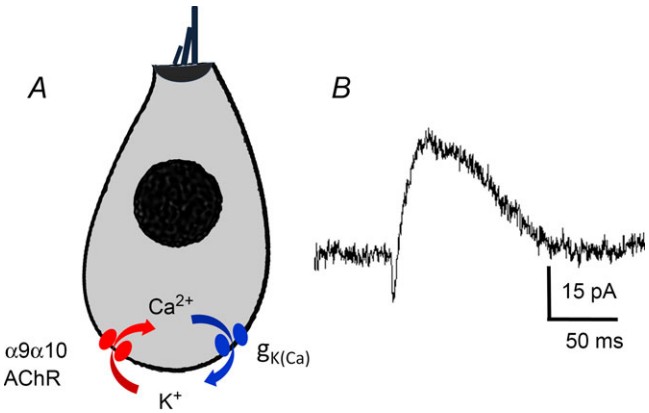

Figure 2. Two-channel mechanism of hair cell inhibition
A, the ionotropic α9α10-containing AChR allows calcium entry that opens nearby calcium-activated potassium channels. *B*, spontaneous synaptic current recorded from rat outer hair cell (membrane potential clamped at –60 mV). Biphasic waveform results from sequential gating of channels as shown in *A* (unpublished recording by M. Lioudyno).

Dulon *et al.* 1998; Yuhas & Fuchs, 1999; Matthews *et al.* 2005) and large conductance BK channels in some hair cells (Kong *et al.* 2005, 2007; Wersinger *et al.* 2010).

The cistern as a calcium capacitor

Cholinergic inhibition of hair cells relies on a rise in postsynaptic calcium to activate calcium-dependent potassium channels. This unusual method of inhibition no doubt involves the near-membrane postsynaptic cistern that is co-extensive with the presynaptic efferent terminal (Fig. 3; Smith & Sjostrand, 1961; Saito, 1980; Fuchs *et al.* 2014), an association also described for cholinergic ('C', cisternal) synapses in spinal cranial motor neurons (Yamamoto *et al.* 1991; Nagy *et al.* 1993). Both in hair cells and motor neurons the synaptic cistern has been proposed to serve as a calcium store, akin to the sarcoplasmic reticulum that supports contraction in muscle, raising the possibility that cholinergic inhibition occurs through some combination of calcium influx and release from internal stores. The participation of a calcium store is supported by the effects of ryanodine and other store-active agents (Sridhar *et al.* 1997; Evans *et al.* 2000; Lioudyno *et al.* 2004). On the other hand, the voltage dependence of ACh-evoked SK current (Martin & Fuchs, 1992) and the brief time course of efferent synaptic potentials (Oliver *et al.* 2000) are best explained as resulting from calcium influx alone.

Detailed examination of cisternal structure suggests a possible synthesis of these viewpoints. Multiple efferent terminals contact single OHCs (Fig. 3; Murthy *et al.* 2009) to cover several square micrometres of cell surface. The synaptic cistern is co-extensive with the efferent terminals (Fig. 4), lying only 14 nm from the postsynaptic membrane (Fuchs *et al.* 2014) thereby defining a restricted diffusion space where influx through AChRs can raise calcium to high levels. Thus, for brief exposures to ACh the cistern need only serve as a sink, or fixed buffer (Fig. 5*A*) to absorb calcium and enable the rapid decay of 'quantal' potassium currents (Glowatzki & Fuchs, 2000; Oliver *et al.* 2000; Katz *et al.* 2004; Ballestero *et al.* 2011). On the other hand, should the cistern become calcium-loaded by sustained influx, uptake would slow, so that SK channel gating would be prolonged, calcium-induced calcium release might occur, and additional calcium-dependent processes could be triggered (Fig. 5*B*), as suggested by previous measurements with fluorescent calcium indicators (Evans *et al.* 2000). Thus, one might view the cistern as analogous to a capacitor, providing a path for current flow (calcium uptake) until fully charged, at which point current (calcium) flows elsewhere in the circuit (hair cell). In this model, endoplasmic calcium-induced channels (e.g. ryanodine receptors) allow calcium flow into, or out of the store, depending on driving force. Calcium uptake via ATP-dependent pumps also should be involved.

The effects of calcium store drugs such as ryanodine can be interpreted in light of this model. Such treatments should especially alter postsynaptic currents when the synaptic cistern is calcium-loaded and able to serve as a store. Thus, drugs affecting calcium uptake (inhibitors of endoplasmic calcium pumps such cyclopiazonic acid and thapsigargin) and release (ryanodine and cyclic adenosine phosphoribose (cADPR)) altered the effect of prolonged

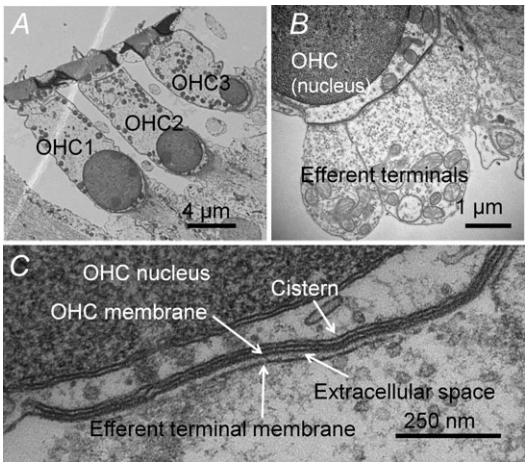

Figure 3. Efferent synapses on mouse outer hair cells
A, cochlear cross-section showing OHCs from three rows (OHC1–3). *B*, higher power view of multiple efferent terminals on one wild-type OHC. *C*, high magnification showing parallel membranes that demarcate the synaptic cistern in apposition to an efferent terminal (reproduced from Fuchs *et al.* 2014, with permission).

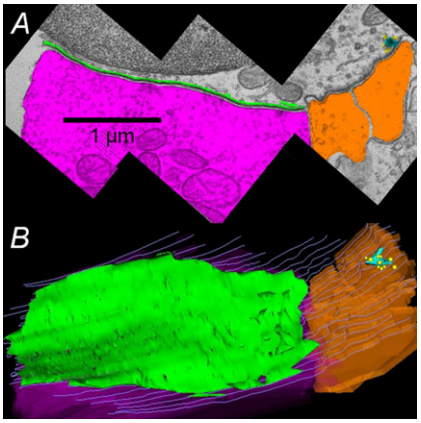

Figure 4. Demarcation and reconstruction of an efferent synapse on a mouse OHC
A, single cross-section with the efferent terminal in magenta, the synaptic cistern in green and afferent boutons in umber. *B*, *Z*-axis projection (tilted forward ~30 deg from the plane of section) from a serial reconstruction of 29 sections including that in *A* (same scale). Same colour scheme as in *A* with hair cell membrane shown in grey lines. A presynaptic ribbon (turquoise) and associated vesicles (yellow) face the afferent bouton (reproduced from Fuchs *et al.* 2014, with permission).

cholinergic activation *in vivo* or in excised tissue, presumably because the synaptic cistern was loaded by calcium influx through AChRs (Sridhar *et al.* 1997; Evans *et al.* 2000; Lioudyno *et al.* 2004) or via voltage-gated channels clustered at nearby ribbon synapses (Fig. 4*B*; Frank *et al.* 2009; Meyer *et al.* 2009).

This model also predicts that responses to the brief release of ACh should be relatively *in*sensitive to drugs like ryanodine. With some caveats this prediction is partially upheld. The first caveat is that synaptic currents evoked by high potassium or electrical stimulation of efferents *were* altered in amplitude by ryanodine (Lioudyno *et al.* 2004; Kong *et al.* 2012). However, this may be due to changes in efferent release probability by a process of calcium-dependent retrograde facilitation (see section 'NO-dependent retrograde facilitation' below). The time course of synaptic currents may be more revealing since this was unaffected by ryanodine at −80 mV (inward potassium currents in 40 mM external potassium; Lioudyno *et al.* 2004). However, the second caveat is that synaptic waveform was significantly prolonged when studied at −40 mV (Kong *et al.* 2012). This difference may result from calcium loading via voltage-gated influx at −40 mV, but absent at −80 mV. These observations call for further study of the voltage dependence of calcium store activity in hair cells, as has been described for smooth muscle (Wu *et al.* 2002). Voltage-gated calcium influx best activates SK channels in turtle hair cells during inhibition of endoplasmic calcium pumps (Tucker & Fettiplace, 1996) supporting their presence in the synaptic cistern. However, neither ryanodine receptors nor SERCA pumps have been immunolocalized to hair cell synaptic cisterns, thus their positions in the model are arbitrary (Fig. 5), although calcium-induced calcium release channels probably do face the plasma membrane, by analogy with sarcoplasmic reticulum (Franzini-Armstrong & Protasi, 1997).

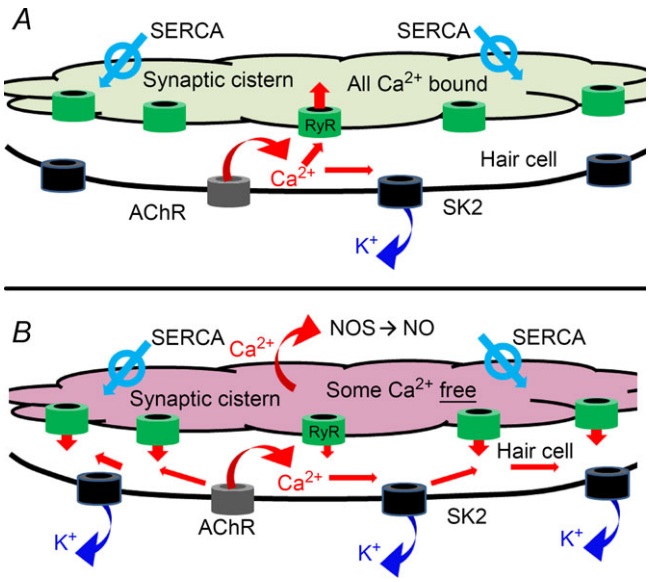

Figure 5. The cistern as a calcium capacitor
A, Under normal operating conditions (low levels of efferent or hair cell activity) calcium influx through hair cell AChRs (grey) is rapidly absorbed and bound by the synaptic cistern – perhaps through calcium-gated calcium channels such as the ryanodine receptor (green channels). *B*, overloading of the cistern (e.g. from prolonged efferent activity, or by voltage-gated calcium influx at nearby ribbons, pumped into the cistern by sarco(endo)plasmic reticulum calcium-ATPase (SERCA)) slows the uptake of efferent calcium and leads to calcium-induced calcium release that amplifies and extends activation of SK channels (black channels). The spread of calcium is also thought to stimulate synthesis of nitric oxide (NO) to drive retrograde facilitation.

Plasticity of efferent transmission

Electrical stimulation of the efferent axons in excised cochlear coils has been used to quantify transmitter release and aspects of synaptic plasticity. The mammalian cochlea provides two opportunities for such experiments: on inner hair cells before the onset of hearing, and on outer hair cells in the mature cochlea. At the inner hair cell contact the resting quantum content was about 1, but with repetitive stimulation rose 2- to 3-fold (Goutman *et al.* 2005). During prolonged trains of stimulation the summed, facilitated IPSPs could completely prevent action potential firing in these immature inner hair cells. Efferent synaptic transmission onto older OHCs had a smaller resting quantum content and facilitation was more prominent (Ballestero *et al.* 2011). Efferent transmitter release onto inner hair cells is supported by a combination of P/Q- and N-type voltage-gated calcium channels (VGCCs; Zorrilla de San Martin *et al.* 2010). In addition, L-type VGCCs and associated BK potassium channels in the efferent terminal act as negative modulators of transmitter release, presumably by abbreviating the presynaptic action potential. Activation of metabotropic GABA receptors ($GABA_{B(1a,2)}$Rs) down-regulates the amount of ACh released at the efferent synapse by inhibiting P/Q-type VGCCs (Wedemeyer *et al.* 2013). These are mechanisms of negative feedback, suppressing efferent transmission. The following section describes positive feedback in the form of retrograde facilitation, driven by calcium-dependent production of nitric oxide (NO) in the hair cell. This effect depends on calcium signals associated with the synaptic cistern.

NO-dependent retrograde facilitation

Cholinergic efferent contacts are found on IHCs prior to the onset of hearing at postnatal day 12–14. Efferent release of ACh onto such young IHCs evokes outward SK-mediated currents (inhibitory

postsynaptic currents, IPSCs) at −40 mV. When the membrane-impermeant ryanodine receptor agonist, cyclic adenosine phosphoribose (cADPR) was included in the intracellular (pipette) solution, evoked IPSCs were longer-lasting (Kong *et al.* 2012), supporting the hypothesis that ryanodine receptors are present on the synaptic cistern; their enhanced gating by cADPR prolonged SK channel activity. In addition, and unexpectedly, evoked and spontaneous IPSCs occurred more frequently, as though presynaptic release efficacy had increased. This was confirmed by measuring efferent quantum content during low frequency (1 Hz) evoked release. Treatment with cADPR or ryanodine (1 μM) increased quantum content 5- to 9-fold. Efferent quantum content could also be increased significantly by voltage-gated calcium influx into the hair cell (depolarizing steps interleaved with efferent stimulation). Thus, increases in postsynaptic calcium by voltage-gated influx or enhanced release from internal stores led to an increase in presynaptic quantum content – presumably by way of a retrograde extracellular messenger. Exposure to the nitric oxide (NO) donor 3–morpholinosydnonimine (SIN–1; 100–250 μM) increased the amplitude and frequency of spontaneous IPSCs, nominating NO as a messenger for retrograde facilitation. Exposure to the NO scavenger 2–(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) effectively prevented the increase in quantum content normally produced by cADPR or hair cell depolarization.

Inner and outer hair cells can produce NO in response to ATP-evoked calcium influx (Shen *et al.* 2005, 2006), and nitric oxide synthase immunoreactivity has been described throughout cochlear epithelia, including hair cells, and afferent and efferent nerve endings (Heinrich *et al.* 1997; Riemann & Reuss, 1999). NO stimulates guanylate cyclase to produce cyclic GMP, leading to cGMP-dependent phosphorylation of vesicular release proteins and effects on calcium regulation (Meffert *et al.* 1996; Garthwaite, 2008). Given the variety of voltage-gated channels present in the efferent terminal (Zorrilla de San Martin *et al.* 2010) one possibility is that NO alters channel gating through direct nitrosylation (Bredt & Snyder, 1994). BK channel activity has been observed directly in efferent terminals (Wangemann & Takeuchi, 1993); it will be of interest to examine modulation by NO.

Developmental plasticity of efferent innervation

During postnatal maturation of the cochlea, cholinergic efferents inhibit inner hair cells directly (Glowatzki & Fuchs, 2000), perhaps to modulate ongoing spontaneous generation of action potentials (Johnson *et al.* 2011, 2013; Sendin *et al.* 2014) that drive activity in associated afferent neurons (Tritsch *et al.* 2007; Tritsch & Bergles, 2010). These efferent contacts on inner hair cells are temporary, appearing near the day of birth, and have disappeared by the onset of hearing at about postnatal days 12–14 in mice and rats (Fig. 6; Katz *et al.* 2004; Roux *et al.* 2011). The synaptic mechanism is identical to that found later on OHCs, mediated by $\alpha9\alpha10$-containing AChRs and associated SK calcium-dependent potassium channels. It is not yet known what mechanisms direct these changes in innervation, but some hints are provided by observations in transgenic mouse models. For example, efferent synapses remain intact and functional up to 4 weeks after birth on IHCs of calcium channel null mice (Brandt *et al.* 2003), suggesting some interdependence between developmental changes in hair cell excitability and innervation.

One consequence of presbycusis (age-related hearing loss) in humans and in mouse models is the loss of afferent contacts onto inner hair cells (Nadol, 1979; Pauler *et al.* 1986; Spoendlin & Schrott, 1990; Chen *et al.* 2006; Stamataki *et al.* 2006). IHCs also lose afferent synaptic contacts after acoustic trauma (Kujawa & Liberman, 2009) or ototoxic insult (Schmiedt *et al.* 2002). Efferent synaptic contacts return to aged inner hair cells (Lauer *et al.* 2012) in the C57Bl6 mouse that is a model for age-related hearing loss (Fig. 6). Presumptive efferent contacts also have been observed on IHCs following afferent denervation by ouabain (Ruel *et al.* 2007; Yuan *et al.* 2013). The factors governing synaptic rearrangements during development, ageing and after trauma are unknown. Certainly there will be a role for growth factors and guidance molecules (Wang & Green, 2011; Brugeaud *et al.* 2013). Another possibility is activity-dependent governance of efferent synaptic morphology (Murthy *et al.* 2009) and perhaps competition between afferent and efferent neurites for synaptic territory on the hair cell. Such competition is

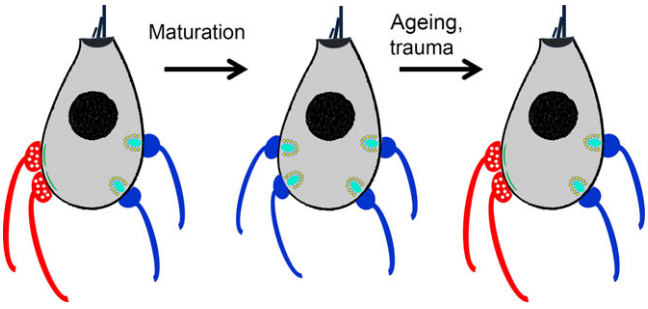

Figure 6. Efferent re-arrangements on inner hair cells
Inner hair cells of the cochlea are temporarily innervated by efferent neurons (red) prior to the onset of hearing (postnatal day 12–14 in rats and mice). Efferent contacts may reappear under conditions that reduce afferent (blue) innervation: age-related hearing loss, acoustic trauma (?), ototoxic damage (?). Thin green lines at efferent contacts represent postsynaptic cisterns. Ribbons (turquoise) surrounded by vesicles (yellow) face afferent boutons.

suggested by the more extensive afferent innervation of OHCs in SK2-null mice that lose efferent contacts (Fuchs *et al.* 2014).

Speculation

Efferent neurons that inhibit cochlear hair cells exhibit several forms of synaptic plasticity. It is not clear yet what larger role negative feedback plays (Zorrilla de San Martin *et al.* 2010; Wedemeyer *et al.* 2013). However, short-term facilitation (Goutman *et al.* 2005; Ballestero *et al.* 2011) is required to raise the initially low quantum content, and is consistent with the higher frequency firing required for inhibition *in vivo* (Gifford & Guinan, 1987). Retrograde facilitation via calcium-dependent NO production is particularly interesting in the context of the developmental and trauma-related changes described above. The synaptic cistern, in addition to its role in regulating on-going synaptic calcium signals, could link global hair cell activity to innervation (Fig. 7). Several questions come to mind. What is the functional capacity of efferent synapses on aged or damaged IHCs? Do they recapitulate the cholinergic inhibition seen during development, or take on a novel role? Is the establishment or maintenance of returning efferent synapses activity dependent or NO dependent? Do efferent and afferent synapses compete for territory on the IHC? Answers to these questions will help to identify targets for modulation of synaptic changes wrought by cochlear damage.

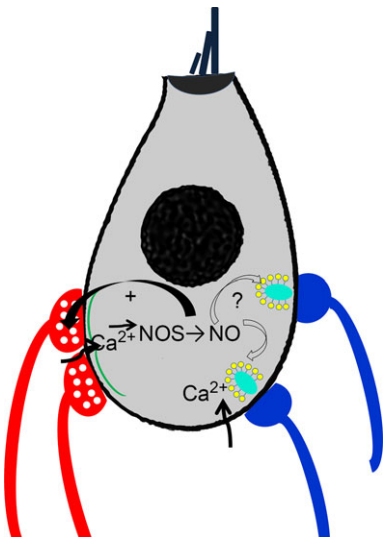

Figure 7. Nitric oxide (NO) dependent plasticity
Prior to the onset of hearing, and following cochlear damage, efferent re-innervation of inner hair cells may be modulated by NO-dependent feedback. This can be driven by calcium influx at efferent or afferent specializations. It will be of interest to determine whether NO can also alter afferent synaptic function.

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Additional information

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