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Hair Cell Afferent Synapses

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Abstract

This review will cover advances in the study of hair cell afferent synaptic function occurring between 2005 and 2008. During this time capacitance measurements of vesicular fusion have continued to be refined, optical methods have added insights regarding vesicle trafficking, and paired intracellular recordings have established the transfer function of the afferent synapse at high resolution. Further, genes have been identified with forms of deafness known as auditory neuropathy, and their role in afferent signaling explored in mouse models. With these advances, our view of the hair cell afferent synapse has continued to be refined, and surprising properties have been revealed that emphasize the unique role of this structure in neural function.

Introduction

Hair cells are the mechanosensors of the inner ear, converting the mechanical energy of sound or head movements into receptor potentials for transmission to afferent neurons whose action potentials carry that information to the central nervous system. That neurotransmission occurs across specialized synaptic structures, named synaptic ribbons after their resemblance to those found in retinal photoreceptors and bipolar cells [1–3]. Like retinal synapses, hair cell ribbon synapses are said to be ‘tonic’ in the sense that they release neurotransmitter continuously in the absence of frank stimulation, and do so without generating action potentials presynaptically. Synaptic transmission is driven by graded changes in hair cell membrane potential, but expressed in the postsynaptic afferent neuron as a rate code of action potential frequency. The coding of sound and head motion by single afferent fibers has been studied extensively [4] and provides a rich context in which to probe hair cell synaptic function. In this review we will focus on the inner hair cell to Type I afferent synapse of the mammalian cochlea, with some comparative examples. Less is known about transmission from outer hair cells to Type II afferents. However recent studies have shown that outer hair cells possess the functional attributes of transmitter release: voltage-gated calcium channels [5,6] and voltage-dependent capacitance changes [7]. Future effort here promises to provide intriguing insights since a majority of these contacts occur without ribbons in the outer hair cells [8], and glutamate receptors, if present, appear to be different from those at inner hair cell contacts [9].

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Voltage gated calcium channels

The hair cell's receptor potentials drive transmitter release through the activation of voltage-gated calcium channels. In cochlear hair cells these are encoded by the CaV1.3 ($\alpha 1D$) gene [10], a member of the dihydropyridine-sensitive, L-type channel class related to those of cardiac and skeletal muscle. In keeping with the frequency-coding demands on hair cells, hair cell voltage-gated calcium currents are very rapidly-gating (time constant of activation on the order of 300 μsec at room temperature), and show little inactivation in the adult [11,12]. Since the identified alpha subunit is subject to marked calcium-dependent inactivation (CDI) when expressed in other cells it has been suggested that calmodulin-like calcium binding proteins that interrupt CDI may be expressed in hair cells to make the channels non-inactivating [13,14]. Another possibility is that some hair cells express CaV1.3 splice variants from which the CDI domain has been eliminated [15]. What is certain is that the hair cell's calcium channels must have significant open probability at the resting membrane potential, since spontaneous activity of afferent neurons results from ongoing transmitter release driven by dihydropyridine-sensitive channel gating [16].

Calcium buffering

Hair cells are subject to ongoing calcium influx at both their ciliary and synaptic poles. Since over-accumulation can be toxic, hair cells must spend energy to buffer, sequester or extrude calcium. More particularly, afferent synaptic function requires calcium transients that rise and fall rapidly in order to encode the temporal content of sounds, including phase-locking to several kHz. Estimates of diffusible calcium buffers in hair cells have been based on a method of comparison whereby a calcium-dependent process (e.g. activation of calcium-gated potassium channels) is carried out with various experimentally-applied cytoplasmic calcium buffers [17]. These results can then be compared to that process observed with the native cytoplasmic calcium buffer (preserved by the use of perforated-patch recording methods). From such measurements in various hair cell types the diffusible cytoplasmic buffer was found to be equivalent to 1 to 2 mM BAPTA a 'rapid' buffer with on rates of $500 \mu\text{M}^{-1}\text{s}^{-1}$ and an equilibrium binding affinity of 0.192 μM [18]. Similar estimates have been derived from a detailed Monte Carlo model of interactions between calcium and buffer molecules, arriving at 1.2 mM 'fast' buffer for the frog saccular hair cell [19]. Furthermore, this modeling showed that the affinity, binding rate and diffusion constant of calbindin- D_{28k} best fit the native buffer. A third approach to identifying hair cell calcium buffers comes from quantitative immunogold labeling [20]. By comparison to standards it was determined that inner hair cells in the rat cochlea have ~0.5 mM calcium binding sites, predominantly as parvalbumin- α . The identity, properties and concentration of cytoplasmic buffer are of considerable importance since these are a major determinant of the size and spread of calcium signals responsible for triggering vesicular fusion.

Molecular components of transmitter release

Hair cells express some, but not all components of the so-called 'SNARE' complex that mediates calcium-dependent vesicular fusion in presynaptic terminals. In particular, hair cells don't have synaptotagmin I or II [21], perhaps substituted with the novel 'deafness gene' product, otoferlin. Mutations in human otoferlin result in nonsyndromic auditory neuropathy (DFNB9) [22], deafness resulting from deficits downstream of cochlear mechano-transduction. Like synaptotagmin, otoferlin interacts with core members of the SNARE complex, SNAP25 and syntaxin I, in a calcium dependent manner [23]. It is hypothesized that otoferlin could be the calcium sensor for exocytosis in hair cells. Inner hair cells of otoferlin knockout mice have greatly reduced transmitter release (measured by membrane capacitance) as do younger outer hair cells [7]. However, other studies have

shown that transmitter release can still occur in the absence of otoferlin [24], and vestibular function seemed unimpaired in *otof*^{-/-} mice [23,25,26].

In addition to otoferlin, a variety of other gene products have been identified with auditory neuropathy, or otherwise implicated in cochlear signaling [27]. Among these, genetic knockout of the ribbon-associated protein, bassoon, reduces the number of anchored ribbons and is associated with a drop in calcium current magnitude, as well as a selective deficit in the readily releasable pool of vesicles [28]. Other insights have arisen from the observation that thyroid hormone (TH) activity is required for normal hearing and for synaptic maturation of hair cells. The role of TH in guiding inner hair cell development has been studied in detail in athyroid (*pax8*^{-/-}) mice and hypothyroid rats [24,29]. In these deaf animals the normal developmental improvement in efficiency of synaptic transmission does not occur, and *pax8*^{-/-} mice have significantly less SNAP25, synaptobrevin 1 and bassoon mRNA at postnatal day 14–17 than do wildtype littermates [29].

The synaptic transfer function

In addition to molecular identification of constituents of the ribbon synapse, there has been considerable insight gained into its physiology, especially the quantitative relationship between the hair cell's membrane potential and transmitter release onto the postsynaptic afferent. The fundamental calcium sensitivity of vesicular fusion has been measured as a change in membrane capacitance produced in two ways: by variation of calcium influx as a function of driving force, rather than channel gating, e.g., [30], and by flash photolysis of photo-labile calcium buffers [31]. By both measures the fusion process rises as the fourth to fifth power of calcium, much as observed in other synapses. More recently this relationship has been re-examined using postsynaptic recording of evoked synaptic currents, which confirm the higher-order relationship to calcium [32]. Thus, the fundamental calcium dependence of vesicular fusion at the hair cell ribbon appears similar to that found elsewhere, a calcium sensor with an affinity of 10 – 100 μM [31], binding 4–5 calcium ions to undergo a conformational change that triggers vesicular release of neurotransmitter.

What appears somewhat more unusual is that vesicular release over the physiological voltage range between the resting potential and -20 mV (within which auditory coding occurs) rises in linear proportion to the increase in calcium current, whether measured as an increase in capacitance [30,33–35] or via recordings of postsynaptic currents [32,36]. The interpretation is that within this voltage range, the calcium sensor is saturated by calcium influx when a nearby channel opens (eliminating binding as a consideration), so that the probability of vesicular release depends only on the probability of calcium channel gating. When considered in combination with the effects of altered cytoplasmic buffers, the linear dependence on calcium influx has been interpreted as support for a so-called 'nanodomain' model of vesicular fusion [37] whereby a single voltage-gated calcium channel opens to trigger release of a single nearby vesicle. Thus, the progressive recruitment of open calcium channels (larger macroscopic calcium current) causes a progressive increase in release, as each new open channel brings with it a fixed unit of vesicular release. This may not apply during early postnatal development or in the apical low frequency range of the adult gerbil cochlea, where a calcium dependence of release with a power relation of ~ 4 and ~ 2 has been found [35].

The striking architecture of the ribbon synapse has been carefully detailed by serial section electron microscopy [38], enabling physically realistic models of ribbon function. Combined with functional measures of calcium influx and buffering, one such model demonstrates that the ribbon concentrates vesicles at the active zone to minimize latency and to improve the precision of postsynaptic spike timing [39].

Vesicle recycling

Measurements of capacitance in hair cells can reveal both the addition, and subtraction of vesicular membrane as exocytosis and endocytosis proceed [40,41]. After sustained depolarization of frog saccular hair cells, synaptic vesicles decline in number, and large endocytic cisterns arise, which might be a source of synaptic vesicles [42]. Whatever the mechanism, such slow endocytosis (τ 7–14 s) is certainly inadequate to support the hair cell's rapid, continuous vesicular release, suggesting that other methods of recycling must exist. Measurements of vesicular traffic in hair cells also have been made using a fluorescent dye, FM1-43, to mark synaptic vesicles [43]. Dye-labeled vesicles formed into ~ 20 clusters along the baso-lateral surface of the inner hair cell [43], similar to the number of ribbons obtained with EM [44]. Sustained depolarization of the hair cell caused a 30% loss of dye from such clusters, corresponding to a release rate of $1.4 \text{ vesicles ms}^{-1} \text{ ribbon}^{-1}$. In contrast to endocytic rates observed with capacitance, the clusters regained fluorescence (presumably the addition of vesicles) with a time constant of 87 ms, corresponding to a rate of $1.1 \text{ vesicle ms}^{-1} \text{ ribbon}^{-1}$. Dye-loaded vesicles originated at the apical surface of the hair cell (where dye exposure occurred) and spread through the cytoplasm, suggesting that ribbons reload from an inexhaustible pool of preformed vesicles. In this model one role for the ribbon is as a 'vesicle trap', concentrating synaptic vesicles into a release-ready pool to sustain continuous, rapid synaptic transmission.

Multivesicular release

A surprising aspect of ribbon transmission is that multiple vesicles can be released at once, even without a change in membrane potential. Initial evidence for multivesicular release was found at the inner hair cell afferent synapse in the postnatal rat cochlea [45]. In this preparation the auditory nerve fiber receives input from just one afferent terminal, usually contacting a single inner hair cell ribbon synapse. Patch clamp recordings from the afferent terminal showed AMPA-receptor-mediated EPSC (excitatory postsynaptic current) amplitude distributions that were highly skewed and ranged from 20 to 800 pA with a peak at about 40 pA, suggested to be the quantal size. Interval analysis suggested temporal clustering of EPSCs and about 30 % of the EPSCs exhibited marked inflections during their rise and decay times, as though made up of smaller subunits. Remarkably, 70 % of the EPSCs, even with the largest amplitudes, showed fast rise times and exponential decay times with no inflections. This implies that the largest EPSCs resulted from the tightly coordinate release of up to 20 vesicles, assuming linear summation. The average EPSC at these postnatal synapses would include 3–6 vesicles. Neef *et. al.* [46] used the variance of capacitance recordings in mouse inner hair cells to estimate the apparent size of elementary fusion events and compared this value with the capacitance of single vesicles based on diameter measurements from electron micrographs. From these approximations the authors concluded that coordinate release of vesicles occurs in postnatal and mature mouse inner hair cells.

Multivesicular release also occurs at saturating and non-saturating retinal ribbon synapses made by rods, rod bipolar cells and ON cone bipolar cells; serving different purposes at these different synapses (for review see [47]). In summary, multivesicular release has been suggested for ribbon synapses in different cell types, sensory systems and species and therefore may be a general mechanism by which ribbon synapses operate. It remains to be seen whether multivesicular release provides distinct functions in these varied settings, and whether, therefore, detailed mechanisms might vary as well.

Further evidence for multivesicular release at the hair cell ribbon synapse would be provided if the multivesicular events could be broken down into single quantal events, for example,

by reducing calcium. For retinal ribbon synapses, lowering extracellular calcium provides smaller synaptic currents on average [48,49]. Surprisingly, at the rat hair cell afferent synapse a change in calcium influx or cytoplasmic buffering changed only the probability of release, and the average EPSC amplitude was unaffected [32].

Thus, the mechanism for multivesicular release at ribbon synapses is still unresolved. One possibility is that a group of vesicles docked at the plasma membrane could be released synchronously. Such a process would depend on very rapid movement of vesicles to refill such sites. An alternative is that a form of compound fusion might occur [50,51] as has been shown in secretory cells [52]. In this scenario, vesicles tethered to the ribbon fuse with each other and a docked vesicle, either before or after calcium influx triggers release of the docked vesicle. If such a process of vesicular fusion produces large EPSCs at rat inner hair cell synapses, the number of vesicles fusing must be independent of calcium, perhaps favoring the 'pre-calcium' fusion sequence. Recently, Matthews & Sterling [53] used electron microscopy to show that after repetitive stimulation of exocytosis, large vesicles had formed close to the plasma membrane in retinal bipolar cells. Some of these cisternal structures were attached to ribbons by filaments, and some opened to the extracellular medium. The authors suggest that these cisternae might represent synaptic vesicles fused by compound exocytosis. Ultimately, optical methods may be required to determine the mechanisms of multivesicular release at the hair cell synapse [54].

Hair cell vesicular glutamate transporters

Early in the process of preparing glutamatergic synaptic vesicles for release, vesicular glutamate transporters (VGLUTs) fill vesicles with neurotransmitter. There are three VGLUT isoforms in the mammalian CNS, VGLUT1-3 [55]. Three recent studies using forward and reverse genetic approaches concluded that VGLUT3 is the vesicular glutamate transporter required for hair cell afferent transmission. Mice lacking VGLUT3 were profoundly deaf [56,57], and a mutagenesis screen turned up a zebrafish larval mutant, *asteroid* with severe auditory and vestibular deficits [58]. The *asteroid* mutation was localized to the VGLUT3 gene and intentional disruption of this gene replicated the *asteroid* phenotype. VGLUT3 but not VGLUT1 or VGLUT2 mRNA and protein, were specifically localized to cochlear inner hair cells in the organ of Corti [56,57]. In the zebrafish VGLUT3 and VGLUT1 were localized to hair cells of the ear and lateral line [58]. In VGLUT3 deficient animals, hair cells seem to function normally: zebrafish hair cells have normal receptor potentials [58] and mouse inner hair cells exhibit calcium and potassium currents and exocytosis of vesicles similar to wildtype animals [56,57]. However afferent activity is abolished in both model systems, suggesting that VGLUT3 is necessary for filling hair cell synaptic vesicles with glutamate. Two families have also been identified with autosomal-dominant nonsyndromic DFNA25 deafness and the gene SLC17A8, most likely responsible for this deficit, encodes VGLUT3 [57].

The function of VGLUT1 remains under question. Antibodies to VGLUT1 label guinea pig inner hair cells [59], although mice lacking VGLUT1 do not appear to have hearing deficits [56]. On the contrary, zebrafish hair cells express VGLUT1 and VGLUT3 and the loss of either protein results in deafness and balance defects [58]. However, VGLUT1 cannot rescue the phenotype of VGLUT3 mutant zebrafish, and vice-versa.

Postsynaptic glutamate receptors

It is well established that AMPA receptors mediate fast synaptic transmission at the hair cell afferent synapse [36,45,60]. Chen *et al.* [61] have provided evidence that synaptic strength might be modulated by AMPA receptor cycling at the afferent fiber postsynaptic density. They showed that acoustic trauma in mice caused a reduction of surface AMPA (GluR2)

receptors in auditory nerve fibers. Recovery of acoustic thresholds and surface AMPA receptors followed a similar time course. Cochlear infusion of AMPA receptor blockers prevented noise-induced loss of surface AMPA receptors. Interestingly, infusion of NMDA receptor blockers caused a smaller shift in acoustic threshold after noise exposure and less reduction in surface AMPA receptors, suggesting that NMDA receptors at the afferent synapse might be involved in modulating surface AMPA receptors.

Using immuno-fluorescence and immuno-electron microscopy, Ruel *et al.* [62] located NMDA receptors at the inner hair cell afferent synapse in the rat and guinea pig cochlea (NR1, NR2A/B). Glutamate did not activate NMDA receptors in spiral ganglion neurons; however, when co-applied with salicylate or arachidonic acid, an NMDA response was enabled. Similarly, the discharge rate of single auditory nerve fibers increased when salicylate was applied, and this effect was reversed by NMDA receptor blockers. This study suggests that salicylate increases arachidonate levels and thereby activates NMDA responses that modulate afferent fiber activity. This work is particularly relevant in the context of tinnitus research as salicylate-induced tinnitus provides an important experimental model [63].

Conclusions

Afferent (ribbon) synapses of hair cells have to meet a number of functional challenges, including sustained (tonic) activity and rapid temporal coding. To do so the hair cell's calcium channels don't fully inactivate, thus supporting tonic release, and potent buffering mechanisms rapidly modulate free calcium. Through as yet unknown mechanisms, a virtually unlimited supply of vesicles is marshalled for release at the ribbon synapse, presumably from a large, freely-mobile cytoplasmic pool. Perhaps as a consequence of this voluminous re-supply, multivesicular release appears to be a common feature of ribbon function. Multivesicular release might provide different advantages for different synapses, and is certain to shape information transfer at the solitary synapse between the inner hair cell and an afferent partner.

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