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The Efferent Medial Olivocochlear-Hair Cell Synapse

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Abstract

Amplification of incoming sounds in the inner ear is modulated by an efferent pathway which travels back from the brain all the way to the cochlea. The medial olivocochlear system makes synaptic contacts with hair cells, where the neurotransmitter acetylcholine is released. Synaptic transmission is mediated by a unique nicotinic cholinergic receptor composed of α9 and α10 subunits, which is highly Ca^{2+} permeable and is coupled to a Ca^{2+}-activated SK potassium channel. Thus, hyperpolarization of hair cells follows efferent fiber activation. In this work we review the literature that has enlightened our knowledge concerning the intimacies of this synapse.

Keywords

hair cells; nicotinic receptors; alpha9alpha10 nicotinic receptors; synaptic plasticity; prestin; cochlea; SK channels

1. Introduction

Sensory systems respond to stimulus from the surrounding world and use specialized receptor cells at the periphery to translate those stimuli into electrical signals that neurons can interpret. Further processing of sensory stimuli by the central nervous system generates a representation of the outer world called a percept. Sound detection begins when sound waves strike the eardrum, which transmits that physical stimulus to the organ of Corti within the cochlea, the sensory epithelium of the mammalian inner ear. The mechanoreceptor cells of the organ of Corti then transform this mechanical input into electrical signals that are sent

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We provide a review of the properties of the efferent olivocochlear-hair cell synapse
Activation of efferent fibers leads to hyperpolarization of hair cells
This is brought about by the activation of an atypical nicotinic receptor
This receptor is highly calcium permeable
Calcium activates a calcium-dependant SK potassium channel

to the central nervous system by the auditory nerve (Hudspeth, 1997). Different to vision, touch and the chemical senses, sound transduction is directly modulated right at the periphery by efferent fibers (olivocochlear, OC) that travel in reverse, from the brain back to the inner ear (Guinan, 1996). The present work reviews data which has helped advance our understanding of how the efferent-hair cell synapse operates.

2. Hair cells of the cochlea

Hair cells of the inner ear are very few, when compared to the millions of photoreceptors of the retina: approximately 16,000 sensory hair cells in the human cochlea. In addition, mammalian hair cells do not regenerate after damage, thus the importance of protecting the inner ear from insults such us exposure to loud sound (Lim, 1986; Brigande and Heller, 2009), which leads to pathologies such as hearing loss and tinnitus (Eggermont and Roberts, 2004; Elgoyhen and Langguth, 2010). Hair cells are organized in a tonotopic fashion (arranged by frequency sensitivity): those sensitive to high frequency sound are at the basal end nearer to the tympanic middle ear and those sensitive to low frequency are at the apical end of the coiled cochlea (Hudspeth, 1997). Hair cells have a high degree of specialization, with the apical pole carrying the hair bundle specialized for mechanotransduction and the basal pole highly specialized for the release of neurotransmitter. In mammals, a further degree of specialization and division of labor is attained by the presence of two types of hair cells, arranged in rows along the organ of Corti. Inner hair cells (IHCs), of which there are approximately 3,500 in each human cochlea, are the primary receptor cells and are innervated by dendrites of the auditory nerve. Outer hair cells (OHCs), approximately 11,000 in each human cochlea, are arranged in three rows and are involved in sound amplification and fine tuning of the basilar membrane (Hudspeth, 1997). They have a much less pronounced afferent innervation, but are the target of an efferent neural pathway, the medial OC (MOC) fibers, that make direct contact at the base of the OHCs (Rasmussen, 1946; Guinan et al., 1983; Warr, 1992; Guinan, 1996). IHCs are also the target of a descending pathway, the lateral OC pathway, but in this case the efferent axons form a synapse on the postsynaptic (afferent) terminal and will not be discussed further here.

3. Outer hair cells and amplification

When sound reaches the cochlea, it produces mechanical vibrations. These are sensed and transduced into an electrical response by motion of the hair bundles of hair cells and activation of the mechanically-gated ion channels. In addition, the hair cells perform work and deliver energy to the system, thus increasing the magnitude of their mechanical input. This amplification of the stimulus constitutes a positive feedback that enhances the sensitivity of hearing (Dallos, 2008; Hudspeth, 2008).

In mammals, OHCs are the principal players providing the feedback underlying cochlear amplification. Two alternative mechanisms for amplification have been described: an old one, also shared by non-mammalian vertebrates, where amplification results from a nonlinearity in the transduction mechanism itself (Chan and Hudspeth, 2005; Jia and He, 2005; Kennedy et al., 2005) and a newer one in which the hair cell receptor potential drives a novel motile process within the lateral membrane of the OHC soma (Brownell et al., 1985; Dallos, 2008). In the latter case, a process known as somatic electromotility (Dallos, 2008), hyperpolarization causes the cell to expand along its longitudinal axis and depolarization causes it to contract. Somatic electromotility of OHCs, as the basis for cochlear amplification, is a mammalian novelty and is mediated by the motor-protein prestin (Zheng et al., 2000) a member of the solute carrier anion-transport family 26 (*SLC26*) (Mount and Romero, 2004; Franchini and Elgoyhen, 2006). Although prestin orthologues exist in non-mammalian vertebrates (Weber et al., 2003; Franchini and Elgoyhen, 2006; Albert et al.,

2007; Elgoyhen and Franchini, 2011; Tan et al., 2011), an evolutionary analysis has shown that only mammalian prestin shows strong signatures of positive selection, most likely underlying the acquisition of amino acid substitutions to account for the motor function (Franchini and Elgoyhen, 2006; Schaechinger and Oliver, 2007; Elgoyhen and Franchini, 2011; Tan et al., 2011). The contribution of sterocilia-vs somatic-based mechanisms for amplification (or the interaction of both processes) in mammals is still a matter of debate.

4. Efferent innervation of the mammalian cochlea

While OHC respond to auditory stimulation and modulate the micromechanics of the cochlear partition independent of central nervous system control, they are targets of efferent or centrifugal fibers which originate in the brain (Guinan, 1996). Olivocochlear efferent neurons permit the central nervous system to control the way that sounds are processed in the auditory periphery. Lateral OC efferents originate from small neurons in or around the lateral superior olivary nucleus and project predominately to the IHC area of the ipsilateral cochlea. They make synaptic contacts on the radial dendrites of Type I auditory afferents postsynaptic to the IHCs. MOC efferents originate from larger neurons located ventral, medial and anterior to the medial superior olivary nucleus and project mostly contralaterally to make synaptic contacts directly onto OHCs (Rasmussen, 1946; Warr, 1975; Warr, 1992). In addition, before the onset of hearing, OC efferents make functional transient direct synaptic contacts with IHCs (Glowatzki and Fuchs, 2000; Katz et al., 2004).

Efferent inhibition can be activated by sound presented to the contralateral ear (Kujawa et al., 1994). However, most studies of efferent inhibition have been performed by electrical stimulation of efferent axons and measurement of effects in the cochlea (Guinan, 1996). Medial efferents are myelinated, whereas lateral efferents are not. Myelinated fibers have a lower threshold for extracellular current stimulation than do unmyelinated fibers. Moreover, MOC axons travel nearer to the floor of the fourth ventricle where stimulating electrodes are usually placed. Taken together, these observations imply that electrical stimulation activates medial but not lateral efferents. Thus, most efferent effects that have been described so far are attributed to the MOC system (Guinan, 1996).

Electrical stimulation in the floor of the fourth ventricle activates contra- and ipsilateral axons of the MOC efferents to reduce the amplitude of the compound action potential (‘N1’) produced by a brief acoustic stimulus (Galambos, 1956), especially at low sound levels. Moreover, basilar membrane motion is diminished by efferent activity (Murugasu and Russell, 1996; Russell and Murugasu, 1997). These effects most likely result from an inhibition of the motor function of OHCs, which is required for sensitive IHCs responses, thus indicating that MOC activity reduces amplification. Efferent inhibition also affects the cochlear tuning mechanism. Thus, efferent activity suppresses the response of a single auditory nerve fiber such that a louder tone is required to produce a threshold response (Wiederhold and Kiang, 1970; Gifford and Guinan, 1987). This threshold shift is maximal at the fiber’s characteristic frequency, but smaller for frequencies above and below the characteristic frequency. This results in a broader tuning curve and therefore a diminished frequency selectivity of the afferent neuron.

The ultimate effect and functional role/s of MOC activity on audition is still a matter of active research. This include, the control of the dynamic range of hearing (Guinan, 1996), improvement of signal detection in background noise (Dolan and Nuttall, 1988; Winslow and Sachs, 1988; Kawase et al., 1993), mediating selective attention (Oatman, 1976; Delano et al., 2007), and protection from acoustic injury (Liberman, 1991; Rajan, 2000; Taranda et al., 2009b).

5. Neurotransmitters at the MOC-hair cell synapse and hair cell responses

Acetylcholine (ACh) is the main neurotransmitter released at the MOC-OHC synapse and for which a clear hair cell response has been described (Housley and Ashmore, 1991; Fuchs and Murrow, 1992b; Fuchs and Murrow, 1992a; Fuchs, 1996). The first hints of the cholinergic nature of the cochlear efferents were provided by the histochemical reaction for acetylcholinesterase labeled processes in the intact cochlea, which disappear in surgically deefferented cochleas (Churchill et al., 1956; Schuknecht et al., 1959). Extensive biochemical and immunohistochemical studies have further supported the hypothesis that ACh is the main neurotransmitter of the MOC system (Eybalin, 1993). At the electron microscopic level, choline acetyltransferase-like immuno-labeled patches were shown to correspond to large axosomatic synapses on the OHCs (Eybalin and Pujol, 1987).

Antibodies made directly against either gamma amino butyric acid (GABA) or its synthesizing enzyme glutamate decarboxylase, show immunoreactivity in cell bodies located in the superior olivary complex and in terminals located below hair cells, suggesting GABA as a second neurotransmitter of the efferent system (Eybalin, 1993). Fibers forming large axosomatic synapses with the OHCs would belong to the medial efferent system (Eybalin and Altschuler, 1990; Maison et al., 2003a). ACh and GABA might be colocalized in the same neurons. Thus, immunoelectron microscopy studies provided strong evidence for choline acetyltransferase and glutamate decarboxilase colocalization in efferent terminals on OHCs throughout the rat cochlea (Dannhof et al., 1991). A study in mice suggests the complete congruence of GABAergic and cholinergic markers in the OHC area (Maison et al., 2003a). The role of efferent gabaergic neurons to OHCs is mostly unknown.

Based largely on chemical neuroanatomical studies, the neuropeptide calcitonin gene-related peptide (CGRP) has been proposed as a neurotransmitter or neuromodulator in the auditory system (Kuriyama et al., 1990). CGRP-containing terminals have been identified in radial afferents beneath IHCs and medial efferent synapses with OHCs (Tohyama et al., 1989; Kuriyama et al., 1990; Cabanillas and Luebke, 2002; Maison et al., 2003a). However, the function of this peptide on efferent function remains for the most part unknown.

Efferent innervation of hair cells is not exclusive to mammals. In fact, it is as old as hair cells themselves (Manley and Koppl, 1998; Simmons, 2002). The first electrophysiological recordings of hair cell responses to efferent activation came from non-mammalian vertebrates. The advent of the *ex vivo* organ of Corti preparation (Glowatzki and Fuchs, 2000; Oliver et al., 2000) has further shown that the overall mechanisms of hair cell inhibition have been conserved among vertebrates. Intracellular recordings performed in hair cells of the fish lateral line show that efferent activity causes hyperpolarizing inhibitory post-syna ptic potentials (IPSPs), which are sensitive to cholinergic antagonists (Flock and Russell, 1973), thus indicating the cholinergic nature of efferents responses. Subsequent studies have shown similar IPSPs and/or responses to direct application of ACh in hair cells of frogs (Ashmore and Russell, 1983; Sugai et al., 1992), reptiles (Art et al., 1984), birds (Shigemoto and Ohmori, 1991; Fuchs and Murrow, 1992b) and mammals (Housley and Ashmore, 1991; Glowatzki and Fuchs, 2000; Oliver et al., 2000; Katz et al., 2004; Lioudyno et al., 2004; Gomez-Casati et al., 2005; Goutman et al., 2005). These consist of a long-lasting hyperpolarization which is preceded by a brief depolarization. As discussed in the following sections, hyperpolarization is the result of the influx of cations (Na^+ and Ca^{2+}) through α9α10 nicotinic cholinergic receptors (nAChRs) and the subsequent activation of a calcium-sensitive SK2 potassium channel (Housley and Ashmore, 1991; Fuchs and Murrow, 1992b; Fuchs and Murrow, 1992a; Elgoyhen et al., 1994; Fuchs, 1996; Nenov et al., 1996b; Dulon et al., 1998; Oliver et al., 2000; Elgoyhen et al., 2001).

6. The α9α10 nAChR of hair cells

Throughout the nervous system ACh exerts its effects through two pharmacologically, structurally, and genetically distinct receptor types, namely the muscarinic and the nicotinic receptors (Caulfield and Birdsall, 1998; Lukas et al., 1999). Metabotropic muscarinic receptors are linked to second messenger systems through the activation of G proteins, while the ionotropic nicotinic receptors are ligand-gated ion channels. Although the cholinergic nature of the MOC was known since the late ´50s (Churchill et al., 1956; Schuknecht et al., 1959), the structure of the cholinergic receptor mediating synaptic transmission at hair cells remained unknown for almost four decades. Early electrophysiological recordings and calcium imaging experiments performed in the chicken hair cells showed that the applicati on of ACh hyperpolarized the hair cells and also increased the internal Ca^{2+} concentration for several minutes (Shigemoto and Ohmori, 1991). The hyperpolarization was attributed to the activation of calcium-dependent potassium channels in response to the release of Ca^{2+} from intracellular stores, due to the activation of muscarinic cholinergic receptors (Shigemoto and Ohmori, 1991). Based on the pharmacological properties of ACh-evoked hyperpolarizing currents in OHCs of the guinea pig cochlea, the participation of muscarinic receptors in the activation of potassium channels was also proposed (Kakehata et al., 1993). On the contrary, an additional study performed in isolated guinea pig OHCs concluded that ACh promoted Ca^{2+} influx from the extracellular space and subsequently activated a Ca^{2+}-dependent K^+ current, through the activation of nicotinic receptors (Housley and Ashmore, 1991). These results were further supported by additional pharmacological experiments performed in guinea pig OHCs (Erostegui et al., 1994). Moreover, similar conclusions were obtained by (Fuchs and Murrow, 1992b; Fuchs and Murrow, 1992a), who performed tight-seal recordings in chicken hair cells during brief (50-100 ms) applications of ACh at a membrane potential of −40 mV. In this case, ACh evoked a small inward current followed within milliseconds by a much larger and longer lasting outward K^+ current. The ACh-evoked K^+ current depended on Ca^{2+} in the external saline and could be prevented when the cell was dialyzed with the rapid Ca^{2+} buffer BAPTA (Fuchs and Murrow, 1992a). In addition, based on its pharmacological profile, a novel nicotinic cholinergic receptor present at hair cells was proposed (Fuchs and Murrow, 1992b). The conundrum of the nature of the cholinergic receptor of hair cells was mainly based on its baroque pharmacological profile, since it is neither activated by muscarine nor nicotine, it is blocked by nicotine and it is blocked by the nicotinic antagonists curare and α-bungarotoxin, the muscarinic antagonist atropine, the glycinergic antagonist strychnine and the GABAergic antagonist bicuculline (Fuchs, 1996).

The puzzle was solved with the use of molecular biological techniques and the cloning of a novel nAChR subunit from a rat olfactory epithelium cDNA library, α9 (Elgoyhen et al., 1994). The primary structure of this new protein clearly indicated that it belonged to the nicotinic family of cholinergic receptor subunits (Figure 1). These are members of the “Cys-loop” family of neurotransmitter-gated ion channels that also includes $GABA_A$, $GABA_C$, glycine and 5-hydroxytryptamine-3 ($5HT_3$) receptors, as well as some invertebrate anionic glutamate and histamine receptors (Karlin, 2002). Receptors belonging to this family are formed by five homologous subunits assembled around a central ion-conducting pore. Each subunit contains, in its ligand-binding, amino-terminal half, two (presumably disulfide-linked) cysteine residues separated by 13 other residues, thus giving this family the name of “Cys-loop” receptors. Four transmembrane regions span the membrane and transmembrane region two lines the pore of the channel. A big intracellular loop hangs between transmembrane regions three and four, and the carboxi-terminal region is extracellular. Several nicotinic receptor subunits have already been identified. The nicotinic receptor at the neuromuscular junction mediates fast synaptic transmission and has a $(\alpha 1)_2\beta 1\gamma\delta$ stoichiometry (Karlin and Akabas, 1995). Ten genes that encode neuronal nicotinic subunits

have been cloned in the vertebrate central or peripheral nervous system: α2–α8 and β2–β4 (Boulter et al., 1986; Boulter et al., 1987; Deneris et al., 1988; Wada et al., 1988; Duvoisin et al., 1989; Couturier et al., 1990; Schoepfer et al., 1990; Le Novere et al., 2002). In heterologous expression systems, the neuronal subunits α2, α3, α4, and α6 lead to the assembly of functional nicotinic receptors in combination with either β2 or β 4. They have a pentameric structure that includes two α and three β subunits (but alternate stoichiometries have been described) (Moroni and Bermudez, 2006). The α7 and α8 subunits form part of a different group within the neuronal nAChR, because they can assemble into functional receptors in the absence of any other subunit and α7 receptors account for the α-bungarotoxin-binding sites in the central nervous system (Couturier et al., 1990).

Although α9 belongs to the nAChR family, it is a distant member based on homology at the amino acid level (Elgoyhen et al., 1994). It clearly forms an evolutionary early divergent branch within the nAChR family, being closest to the ancestor that gave rise to the family (Le Novere and Changeux, 1995; Tsunoyama and Gojobori, 1998; Le Novere et al., 2002; Dent, 2006). This is in agreement with the fact that it has shared pharmacological properties with $GABA_A$, glycine and serotonin type 3 receptors (Rothlin et al., 1999; Rothlin et al., 2003). Thus, when expressed in *Xenopus laevis* oocytes, α9 forms homomeric (Elgoyhen et al., 1994), calcium-permeable (Katz et al., 2000), ACh-gated channels with the following pharmacological properties, which are largely indistinguishable from those reported for the native hair cell cholinergic receptor: it is not activated but blocked by nicotine and muscarine, by the nicotinic antagonist curare and by the neuronal nAChR antagonist α-bungarotoxin (in a reversible manner, different to the blockade of neuronal receptors), the muscarinic antagonist atropine, the glycinergic antagonist strychnine, the GABAergic antagonist bicuculline and the serotonin type 3 receptor antagonists ICS 205-930 and ondansetron (Elgoyhen et al., 1994; Rothlin et al., 1999; Verbitsky et al., 2000; Rothlin et al., 2003). Modulation of α9-containing receptors by opioid compounds (Lioudyno et al., 2000; Lioudyno et al., 2002), ryanodine (Zorrilla de San Martin et al., 2007), ototoxic drugs such as quinine (Ballestero et al., 2005) and aminoglycoside antibiotics (Rothlin et al., 2000) and neramexane, a drug under investigation for the treatment of tinnitus (Plazas et al., 2007; Suckfull et al., 2011), has been also reported. Moreover, some α-conotoxins which are valuable tools to differentiate nicotinic receptors, have high affinity for α9–containing receptors (McIntosh et al., 2005; Ellison et al., 2006; Nevin et al., 2007; McIntosh et al., 2009). Although originally identified in the olfactory epithelium, the similar pharmacological profile of recombinant α9 and hair cell cholinergic receptors, clearly indicates that α9 is a component of the receptor. Moreover, a combination of *in situ* hybridization and reverse transcription-polymerase chain reaction (RT-PCR) experiments has confirmed α9 transcripts in cochlear and vestibular hair cells of several vertebrate species (Elgoyhen et al., 1994; Glowatzki et al., 1995; Hiel et al., 1996; Morley et al., 1998; Simmons and Morley, 1998; Lustig et al., 1999; Hiel et al., 2000).

Although the pharmacological properties of recombinant α9 receptors clearly recapitulate those of the native hair cell receptor, the current-voltage relationship, the Ca^{2+} sensitivity, and the desensitization properties of homomeric α9 receptors do not match those seen in isolated hair cells (Blanchet et al., 1996; Dulon and Lenoir, 1996; McNiven et al., 1996). The cloning of the α10 nAChR from a rat cochlear cDNA library and the expression of both α9 and α10 in *Xenopus laevis* oocytes has demonstrated that the α9α10 receptor recapitulates the pharmacological and biophysical properties of hair cell receptors (Elgoyhen et al., 2001). It is now accepted that the hair cell cholinergic receptor that mediates synaptic transmission between efferent olivocochlear fibers and hair cells of the cochlea, is formed by both α9 and α10 subunits (Elgoyhen et al., 2001; Lustig et al., 2001; Sgard et al., 2002) (Figure 2). Using a reporter mutation approach, a pentameric structure with a $(\alpha9)_2(\alpha10)_3$ stoichiometry has been proposed (Plazas et al., 2005) (Figure 1).

Since cholinergic efferent feedback to hair cells is a common feature among all vertebrates (Simmons, 2002), one would expect that the evolutionary history of the genes coding for the α9 and the α10 subunits would look similar along all vertebrate lineages. A phylogenetic analysis of α9 and α10 subunits across vertebrates has provided surprising results (Franchini and Elgoyhen, 2006). These indicate that in mammals the genes coding for α10 subunits (*CHRNA10*) display a different evolutionary history. Thus, although *CHRNA9* has been under strong purifying selection in all vertebrates, *CHRNA10* has been under positive selection pressure only in the mammalian lineage (Franchini and Elgoyhen, 2006; Elgoyhen and Franchini, 2011). These data have indicated a possible scenario for the evolution of these nicotinic receptor subunits: after a duplication event that created the *CHRNA9* and *CHRNA10* genes, they co-existed without much functional diversion. At some point, in the lineage leading to mammals, amino acid changes started to accumulate rapidly producing *CHRNA10* to diverge from *CHRNA9*. This might suggest that mammalian α9α10 nAChRs acquired a novel function or new properties which evolved in conjunction with the specialization of mammalian hearing. Co-varying with the evolutionary history of *CHRNA10* is prestin, the protein responsible for somatic electromotility of mammalian OHCs, which has also been under positive selection pressure only in mammals (Franchini and Elgoyhen, 2006). Thus, it is tempting to speculate that *CHRNA10* has evolved to give the mammalian auditory system feedback control of prestin-driven somatic electromotility, a capacity that is not required in non-mammalian species (Elgoyhen and Franchini, 2011).

7. Ca^{2+}-activated K^+ currents and calcium stores

It is an accepted notion that hyperpolarizat ion of hair cells following activation of the nicotinic receptor is brought about by the activation of a K^+ current due to the increase of intracellular Ca^{2+} (Housley and Ashmore, 1991; Fuchs and Murrow, 1992a; Blanchet et al., 1996; Yuhas and Fuchs, 1999; Glowatzki and Fuchs, 2000; Oliver et al., 2000; Katz et al., 2004; Gomez-Casati et al., 2005). Ca^{2+} increase following ACh application has been demonstrated by calcium imaging (Shigemoto and Ohmori, 1991; Blanchet et al., 1996) and by the use of intracellular calcium buffering (Fuchs and Murrow, 1992a; Oliver et al., 2000). Moreover, the “bell shaped” current-voltage relation of ACh responses, which is maximal around −40 mV and disappears at more positive potentials, following the reduction of the driving force for Ca^{2+} influx (Evans, 1996; Nenov et al., 1996a; Glowatzki and Fuchs, 2000; Gomez-Casati et al., 2005), is typical of Ca^{2+}-activated K^+ currents that rely on calcium influx (Berkefeld et al., 2010). BK channels were initially proposed as underlying the K^+ current (Shigemoto and Ohmori, 1991). However, this current is insensitive to cesium block, thus precluding the participation of BK channels (Yuhas and Fuchs, 1999). The use of pharmacological tools has further demonstrated that the K^+ channel belongs to the small conductance, Ca^{2+}-activated SK family (Doi and Ohmori, 1993; Nenov et al., 1996b; Yuhas and Fuchs, 1999; Glowatzki and Fuchs, 2000; Oliver et al., 2000). Moreover, by *in situ* hybridization (Dulon et al., 1998) and immunohistochemistry (Oliver et al., 2000), the SK2 nature of the channel has been established. BK channels might still play a role in high frequency OHCs of the basal cochlea (Wersinger et al., 2011). The activation of the SK component requires extracellular Ca^{2+} (Housley and Ashmore, 1991; Fuchs and Murrow, 1992a; Yuhas and Fuchs, 1999), probably indicating influx of Ca^{2+} through the α9α10 nAChR (Figure 2). This correlates with the high Ca^{2+} permeability reported for recombinant α9α10 (Weisstaub et al., 2002) and native hair cell receptors (Gomez-Casati et al., 2005), PCa/PNa ~ 9, which resembles that shown for ligand-gated ion channels with the highest Ca^{2+} permeability, such as α7 nAChRs (Bertrand et al., 1993; Séguéla et al., 1993), N-methyl-D-aspartic acid glutamate receptors (Mayer and Westbrook, 1987; Burnashev et al., 1992) and cyclic nucleotide-gated channels (Dzeja et al., 1999).

Intracellular Ca^{2+} stores have also been proposed as the source of this cation for SK activation (Shigemoto and Ohmori, 1990; Shigemoto and Ohmori, 1991; Kakehata et al., 1993; Yoshida et al., 1994; Evans, 1996; Lioudyno et al., 2004). In particular, ryanodine receptor expression in hair cells of the rat cochlea has been reported and ryanodine and other store-active compounds alter the K^+ currents evoked in hair cells by ACh (Lioudyno et al., 2004). Thus, it has been proposed that an adjoining synaptoplasmic cistern present in hair cells acts as a tightly coupled calcium store to serve calcium-induced calcium release, similar to that produced by ryanodine receptors of the sarcoplamsic recticulum in striated muscles (Lioudyno et al., 2004)(Figure 2). This is also based on the physical presence of a synaptic cistern in hair cells, a near-membrane (within 20 nm) endoplasmic reticulum that is co-extensive with the efferent synaptic contact (Gulley and Reese, 1977; Hirokawa, 1978; Saito, 1983).

8. Synaptic responses and synaptic plasticity at the mammalian efferent synapse

The establishment of the *ex-vivo* organ of Corti preparation (Glowatzki and Fuchs, 2000; Oliver et al., 2000) has enabled an in depth description of the properties of the efferent-hair cell synapses. Inner and outer hair cells, supporting cells and both afferent and efferent synaptic contacts remain functional for several hours after excision of the cochlear turns from mice or rats at different postnatal stages (from day 0 to 21). Therefore, using this preparation it has been possible to study both the pre- and postsynaptic components and the synaptic mechanisms at functioning mammalian OC-hair cells synapses.

Using this cochlear preparation, sIPSCs have been observed in neonatal IHCs (Glowatzki and Fuchs, 2000; Katz et al., 2004; Marcotti et al., 2004a; Gomez-Casati et al., 2005; Goutman et al., 2005; Zorrilla de San Martin et al., 2010) and in OHCs after the onset of hearing (P21) (Oliver et al., 2000; Lioudyno et al., 2004) (Figure 3). These sIPSCs are due to the spontaneous release of ACh from the efferent synaptic terminals acting on α9α10 nACRs which allow the influx of Ca^{2+} into the hair cell thus promoting the subsequent activation of SK2 potassium channels (Dulon et al., 1998; Glowatzki and Fuchs, 2000; Oliver et al., 2000; Elgoyhen et al., 2001; Katz et al., 2004). Consistently, in both types of hair cells, synaptic currents are biphasic (fast inward current followed by a slower outward component) at membrane potentials between E_K and 0 mV (Figure 3). Negative to E_K, the ACh activated current is inward, as both currents through the nAChR and the SK channel flow in the same direction. The kinetically-dominant outward SK component has a decay time constant of 30 to 50 ms (at room temperature), while inward current through the nAChR (isolated by using the fast calcium chelator BAPTA) decays approximately three to five-fold faster (Glowatzki and Fuchs, 2000; Oliver et al., 2000; Katz et al., 2004; Gomez-Casati et al., 2005). The resting potential of both IHCs and OHCs is positive to E_K, therefore the functional significance of efferent activity is to hyperpolarize the hair cells.

The random timing of spontaneous events, however, has precluded the assessment of efferent release mechanics and plasticity. Therefore, further studies of both the efficacy of hair cell inhibition and the presynaptic molecules and mechanisms involved, required the ability to electrically evoke release from the efferent endings. This was first accomplished by Goutman *et al.* (2005) who studied the characteristics of electrically evoked ACh release at the neonatal transient rat MOC-IHC synapse. Electrical stimulation of the efferent axons to produce evoked release at the MOC-OHC synapse has proven more difficult perhaps due to damage of these fibers upon crossing the tunnel of Corti. Notwithstanding, very recently, Ballestero, Elgoyhen and Katz (unpublished observations) have been able to study the characteristics of synaptic transmission at the MOC-OHC synapse by electrically stimulating the efferent fibers in cochlear preparations from mice at postnatal ages 11-13. At

the transient MOC-IHC (Goutman et al., 2005) the kinetics, voltage-dependence and pharmacology of electrically evoked responses resemble those of spontaneous synaptic currents (Glowatzki and Fuchs, 2000; Oliver et al., 2000; Katz et al., 2004; Gomez-Casati et al., 2005), further supporting the notion that the inhibitory sign of the MOC-hair cell cholinergic synapses are due to Ca^{2+} entry through the α9α10 nAChR and the subsequent gating of calcium-activated SK2 potassium channels.

When studying the mechanics of synaptic transmission, Goutman *et al.* (2005) showed that transmitter release at the rat MOC-IHC synapse is of quantal nature and that low frequency (0.25- 1 Hz) electrical stimulation evokes IPSCs with a mean probability of occurrence of 0.35 and mean amplitudes of −20 pA at −90 mV, corresponding to a quantum content of about 1. However, with repeated stimulation at high frequencies (40 Hz), IPSCs become larger and more often. When the efferent fibers are stimulated by trains of 10 successive shocks, IPSCs reach more than −100 pA in amplitude due to summation and facilitation. Moreover, the frequency of calcium action potentials in these neonatal IHCs, which promote the release of glutamate before the onset of hearing (Beutner and Moser, 2001; Marcotti et al., 2004b), is reduced by electrical stimulation of the efferent fibers, provided that the stimulation frequency is above 2 Hz (Goutman et al., 2005). These observations indicate that efferent inhibition only is effective when occurring repetitively, and at sufficiently high frequencies so that facilitation of transmitter release can occur (Goutman et al., 2005).

At the presynaptic level, some important questions relevant for understanding the mechanics of synaptic transmission are starting to be solved, namely the types of ion channels that support and/or regulate the release of ACh and the sensitivity of the process of release to extracellular Ca^{2+}. These issues are being studied in the mouse MOC-IHC synapse before the onset of hearing (postnatal day 12 in altricial rodents) by recording IPSCs in the whole-cell configuration while electrically stimulating the MOC efferent axons (Zorrilla de San Martin et al., 2010). An important question is whether other neurotransmitters and neuromodulators like GABA and CGRP, which have been reported to be present at the MOC cholinergic efferent synaptic terminals (Eybalin, 1993; Cabanillas and Luebke, 2002; Maison et al., 2003a), modulate ACh responses in cohlear hair cells. The involvement of the gabaergergic system in the regulation of MOC-hair cell synapses is being studied, using the above mentioned approach, at the transient MOC-IHC synapse in cochlear preparations from wild-type and $GABA_B$ knock-out mice by Wedemeyer, Elgoyhen and Katz (unpublished observations).

In a recent work Zorrilla de San Martin *et al.* (2010) showed that the quantum content of transmitter release at the mouse MOC-IHC synapse is low, around 1 (at 1Hz stimulation frequency), similar to that described for the same synapse in neonatal rats (Goutman et al., 2005). In addition, they have investigated the sensitivity of the release process to Ca^{2+}, based on the known high non-linearity of the relationship between external Ca^{2+} and the amount of transmitter released (Dodge and Rahamimoff, 1967). They found that the best fit to the power relation between external Ca^{2+} and quantum content ($m = K[Ca^{2+}]o^n$, (Dodge and Rahamimoff, 1967)) has a coefficient of about 2.6 which suggests the cooperative involvement of at least two Ca^{2+} molecules in those described at other fast synapses triggering the release of each vesicle of ACh. This value falls in the lower range of (Dodge and Rahamimoff, 1967; Mintz et al., 1995; Borst and Sakmann, 1996) and may reflect differences in the release machinery and/or the types of voltage gated calcium channels (VGCCs) supporting release at this synapse. At the MOC-IHC synapse, transmitter release is supported by both P/Q and N-type VGCCs (Zorrilla de San Martin et al., 2010). This is consistent with that shown for many mammalian synapses, at which both P/Q and N-type VGCCs mediate synaptic transmission (Reid et al., 2003; Fedchyshyn and Wang, 2005). Moreover, L-type VGCC functionally coupled to the activation of BK channels negatively

regulate ACh release at the MOC-IHC synapse (Zorrilla de San Martin et al., 2010). Thus, the following scenario has been proposed (Zorrilla de San Martin et al., 2010). Depolarization from an incoming action potential activates P/Q-, N-, and L-type VGCCs. Influx of Ca^{2+} via P/Q- and N-type VGCCs closely associated with the release machinery would support release. In addition, influx of Ca^{2+} via L-type VGCCs (functionally coupled with BK channels) and possibly farther away from the release machinery (Urbano et al., 2001; Flink and Atchison, 2003), together with membrane depolarization, would activate BK channels (Figure 2). As reported for other systems (Storm 1987, Marcantoni 2007), activation of BK channels would accelerate repolarization and reduce transmitter release.

This transient MOC-IHC synapse is functional before the onset of hearing (Glowatzki and Fuchs, 2000; Katz et al., 2004) and its activity can prevent spontaneous Ca^{2+}-action potentials present in neonatal (Goutman et al., 2005). These Ca^{2+} action potentials (Glowatzki and Fuchs, 2000; Tritsch et al., 2007) trigger glutamate release at the first synapse of the auditory system (Beutner and Moser, 2001; Glowatzki and Fuchs, 2002) and is thought to be critical for the establishment and refinement of synaptic connections in the auditory system (Tritsch et al., 2007; Tritsch and Bergles, 2010; Tritsch et al., 2010). Therefore, this negative feedback loop made up by L-type VGCCs coupled to the activation of BK channels reduces ACh release from the efferent terminals and may thus be relevant for achieving the patterned activity at the first auditory synapse that likely contributes to the correct establishment of synapses throughout the auditory pathway.

9. Lessons from genetically modified mice

The generation of different genetically modified mouse models has enabled further understanding of the function of key players at the MOC efferent-hair cell synapse. These include those that target modifications in the genes encoding the α9 and α10 nAChRs, SK2, $GABA_A$ receptor subunits and CGRP.

The analysis of mice carrying a null mutation for *CHRNA9* have provided a clear demonstration that this subunit is a main component of the native OHC cholinergic receptor (Vetter et al., 1999). These mice fail to show suppression of cochlear responses (measured by distortion product otoacoustic emissions and compound action potentials) during efferent fiber electrical stimulation at the floor of the fourth ventricle. These results further demonstrate the key role α9 receptors play in mediating the known effects of the olivocochlear system. Moreover, these null mutant mice have aberrant efferent innervation, being OHCs innervated by one large terminal, instead of multiple smaller terminals as in wild types. This suggests that α9-containing nAChRs play some role in normal synaptic formation and establishment (Vetter et al., 1999; Murthy et al., 2009b). Behavioral studies on this α9 knockout mouse model have shown no decrease in tone detection and intensity discrimination in continuous background noise, suggesting that central efferent pathways work in combination with the peripheral olivocochlear system to enhance hearing in noise (May et al., 2002). Further studies have shown no changes in cochlear sensitivity, based on compound action potential thresholds, and OHC electromotility in the α9 knockouts (He et al., 2004). *CHRNA10* null mutant mice have shown that, while functional homomeric α9 channels are present in OHCs, they are insufficient to drive normal olivocochlear efferent inhibition to the cochlea, demonstrating that the α10 subunit is also an essential component of the hair cell nAChR (Vetter et al., 2007).

The generation of a mouse model that overexpresses the α9 subunit and an α9 knockin with enhanced MOC activity, have suggested the participation of the α9α10 nAChRs in protecting the inner ear from damage produced by overly loud sounds (Maison et al., 2002; Taranda et al., 2009b). This adds to previous literature showing the involvement of the MOC

system in protection from acoustic injury (Liberman, 1991; Reiter and Liberman, 1995; Maison and Liberman, 2000; Rajan, 2000). The α9 overexpressor mice show significantly reduced acoustic injury from exposures causing either temporary or permanent threshold shifts, without changing pre-exposure cochlear sensitivity to low- or moderate-level sound (Maison et al., 2002). The α9 knockin mice has a threonine for leucine amino acid substitution at position L9′ (L9′T) of the second transmembrane domain of the protein and was built based on the described properties of recombinant receptors assembled from mutant receptors (Plazas et al., 2002). L9′T receptors have an enhanced apparent affinity for ACh, slower desensitization kinetics and spontaneous openings in the absence of the agonist, thus rendering receptors with a gain-of-function. Likewise, L9′T hair cells have an enhanced apparent affinity for ACh and slower desensitization kinetics in the presence ACh, which is translated into very prolonged IPSPs (Taranda et al., 2009b). Consistent with these effects, in mutant mice shock-evoked MOC activation produces both enhanced and prolonged cochlear suppression *in vivo*. Moreover, L9′T knockin mice have attenuated sound-induced permanent acoustic injury, again indicating the importance of the MOC efferents in cochlear protection. The fact that a line of mice which overexpresses SK2, thus leading to enhanced MOC activity, lack protection from acoustic injury suggests that efferent-mediated cochlear protection is mediated by other downstream effects of ACh-mediated Ca^{2+} entry, different from those involving SK2-mediated hyperpolarization (Maison et al., 2007).

By using SK2 knockout mice (Bond et al., 2004), this subtype of SK channel has been shown to be solely responsible for encoding the calcium-activated potassium channel in cochlear hair cells (Johnson et al., 2007; Kong et al., 2008). Moreover, the expression of SK2 channels seems necessary for the expression of functional ACh responses, since in SK2 knockout mice, OHCs are completely insensitive to exogenous ACh, implying absent or otherwise dysfunctional nAChRs. Likewise, spontaneous cholinergic synaptic currents are not seen in OHCs from these mice. In addition, neither efferent synaptic currents nor responses to exogenous ACh are present in neonatal IHCs in the SK2-knockout mice. The fact that cholinergic responses are completely absent in hair cells from these SK2 null mice, even though the amount of α9 and α10 mRNA, as evaluated by quantitative RT-PCR does not differ from those in wild-type animals (Kong et al., 2008; Murthy et al., 2009a), strongly suggests that SK2 is fundamentally required for the assembly, trafficking, and/or anchorage of the nAChR macromolecular synaptic complex to the membrane. This is further supported by results derived from the analysis of a line of mice that constitutively express the α10 subunit (Taranda et al., 2009a). Using this same SK2 knockout animal model, SK2 channels have been shown to be necessary for the long-term survival of MOC fibers and synapses (Murthy et al., 2009a).

Mice carrying deletions of $GABA_A$ receptor subunits have provided some evidence towards the role of the GABAergic efferent innervation to hair cells. Rather than underlying an independent action on OHC motility, GABA at the OHC/efferent synapse might modulate cholinergic effects. Thus, a reduction in electrically-evoked efferent suppression in β2 nulls has been reported. However, histological analysis revealed a reduction in density of OHC efferents, suggesting a GABAergic role in the maintenance of the efferent innervation (Maison et al., 2006). In the case of CGRP, mice carrying a null mutation of the gene coding for αCGRP have suggested a postsynaptic effect on cochlear afferent neurons via release of the neuropeptide from lateral OC terminals rather than an effect on the MOC system (Maison et al., 2003b).

10. Conclusions

A long way has been traveled since the first description of ACh as the neurotransmitter at the MOC-hair cell synapse, to the establishment of the double signature of the synaptic

currents, the discovery of the molecular structure of the nAChR, the analysis of synaptic plasticity and the generation of genetically modified mice. These basic research findings might be leading the way to new therapeutics of inner ear disorders such as noise-induced hearing loss and tinnitus (Elgoyhen et al., 2009).

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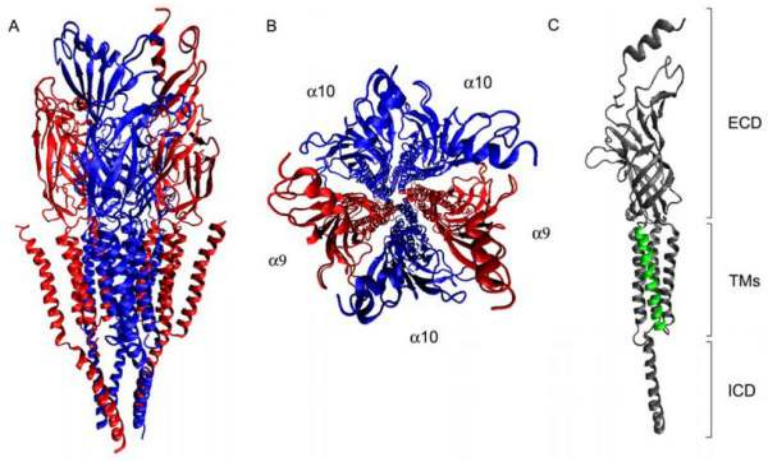

Figure 1.

Structure of a nAChR. A. Side view indicating the extracellular domain (ECD), membrane spanning regions (TMs) and only a short piece of the intracellular domain (ICD). B. View from the top indicating a putative arrangement of subunits according to the $\alpha 9_2 \alpha 10_3$ stoichiometry Plazas et al (2005). C. Structure of a monomer, in green transmembrane region 2. Adapted from: PDB ID: 2BG9.PDB N. Unwin (2005) Refined structure of the nicotinic acetylcholine receptor at 4A resolution J.Mol.Biol. 346: 967. The image was made with VMD software support. VMD is developed with NIH support by the Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign. http://www.ks.uiuc.edu/Research/vmd/.

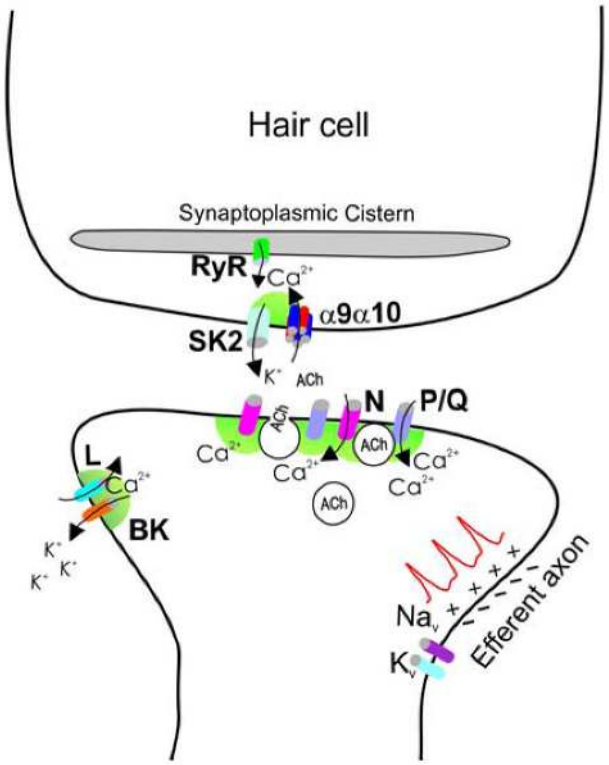

Figure 2.
Schematics of the MOC-hair cell synapse, indicating its pre- and post-synaptic components.

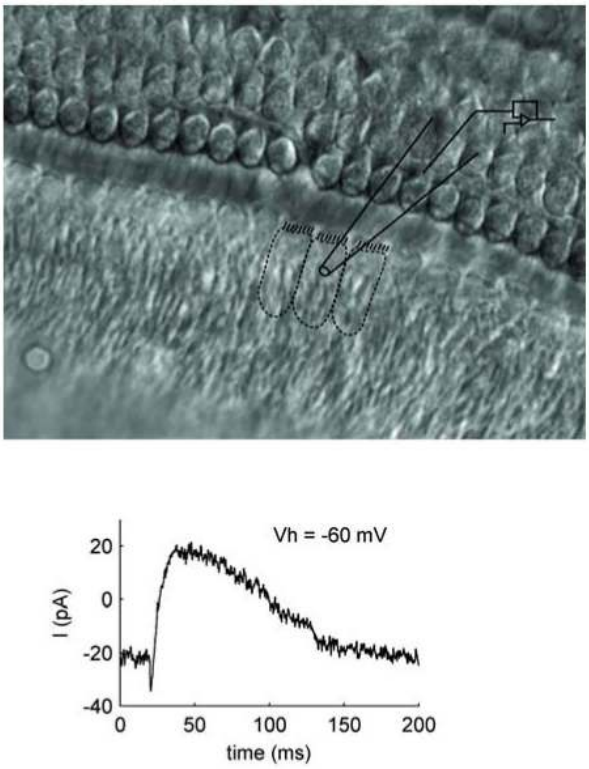

Figure 3.
Spontaneous inhibitory postsyanptic curents recorded in an OHC at −60 mV. Not the biphasic nature of responses: a rapid depolarization is followed by a prolonged hyperpolarization. Courtesy of Jimena Ballestero