α9α10 acetylcholine receptors: structure and functions

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The α9 and α10 nicotinic acetylcholine receptor (nAChR) subunits assemble as pentameric complexes with a central ligand-gated cation-selective pore. However, these subunits do not assemble with other nAChR subunits. The general topology of each subunit is similar to that of other ligand-gated, Cys-loop ion channels. Although the sequence and genomic organization of the α9 and α10 subunits determined their classification as nicotinic subunits, α9α10 nAChRs exhibit peculiar pharmacological properties that are not common to other nicotinic or muscarinic receptors. α9α10 nAChRs are expressed in diverse tissues, including auditory sensory hair cells, afferent neurons in the vestibular sensory organs, neurons of the dorsal root ganglion, skin, and bronchial epithelia. Furthermore, it has become apparent that nAChRs containing α9 and α10 subunits play essential roles in various physiological processes. For example, α9 is required for the normal development of the efferent synapses in the inner ear, and after the onset of hearing α9α10 nAChRs serve as the unique source of extracellular calcium for Ca2+-activated potassium channels in mature auditory hair cells. The development of drugs that selectively target α9/α10 receptors promises to be of therapeutic benefit since these receptors have distinct physiologic functions. They hold promise as pharmacological targets to treat complex auditory and vestibular pathologies, neuropathic pain, and pathologies of the skin and bronchia.

Keywords: α9α10 nicotinic acetylcholine receptors; cochlea; efferent; hair cell; nicotinic; vestibular

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Introduction

The nicotinic acetylcholine receptors (nAChRs) are members of the superfamily of ligand-gated, Cys-loop ion channels. Like the muscle nAChR, prototype of this superfamily, neuronal nAChRs consist of a complex of five homologous subunits lining a central cation-permeable pore. Neuronal nAChRs are pentameric receptors formed by only one α subunit type (homopentamers) or by a combination of different α and β subunits (heteropentamers). Seven α subunits (α2-α7, α9, and α10) and three β subunits (β2-β4) have been cloned and studied extensively in mammals [1, 2], nAChRs were initially thought to be expressed exclusively in neurons and neuronal tissues, however, it has recently become clear that many of them are also expressed and play critical roles in other tissues.

This review focuses on recent findings related to the structure and function of α9α10 nAChRs. α9 and α10 are the most recently cloned members of the nAChR family. The discoveries that the α9 and shortly after the α10 nAChR subunit were expressed in sensory hair cells were exciting hallmarks of hearing and vestibular research [3, 4].
There are two types of hair cells in the mammalian auditory sensory epithelia. The inner hair cells, arranged in a single row along the cochlea spiral, contact all the afferent fibers and are the primary transducers of sound. The second type is the outer hair cells, which are arranged as three rows. The outer hair cells are capable of shortening and lengthening their shape and have been proposed to work as small springs modifying the movement of the basilar membrane. All hair cells have apical stereocilia that harbors the transduction machinery able to convert mechanical stimuli into the neural signals which are sent to the brain. This transduction machinery is exquisitely sensitive; responsive to displacements as small as one nm [reviewed in [5]]. However, in addition, auditory perception is directly modulated by a sound-evoked feedback mechanism via the olivocochlear efferent system which contributes to frequency selectivity and protects the cochlea from damage due to excessive noise. Based on where the neurons are located, there are lateral and medial components to this efferent system. The medial olivocochlear system neurons originate in the medial portion of the superior olivary complex and project into the cochlea to make synapses at the base of outer hair cells [6, 7].

A major neurotransmitter at these synapses is acetylcholine (ACh) [reviewed in [9]]. In electrophysiological studies of isolated outer hair cells, native nAChRs displayed intriguing nicotinic but also muscarinic pharmacological properties [9-12]. Following ACh application to isolated outer hair cells, outward K+ currents were recorded using the whole cell voltage and current clamp configurations. These ACh-induced currents were demonstrated to be dependent on extracellular Ca²⁺. Under the same conditions, outward currents were also induced by suberyldicholine, carbachol, and dimethylphenylpiperazinium, when these agents were tested at high concentrations, however, nicotine, cytisine, and muscarine did not produce any effect. The response to ACh was blocked by low concentrations of classical nicotinic antagonists as well as non-classical nicotinic and muscarinic antagonists. For example, strychnine, curare, biccuculline, α-bungarotoxin, and trimethaphan blocked the response at low concentrations, whereas higher concentrations of atropine, 4-DAMP, AF-DX 116, pirenzepine muscarinic antagonists were required to block the response. Based on these pharmacological studies, the receptor expressed in outer hair cells was thought to be nicotinic [11, 13]. This was confirmed when the α9 and α10 subunits were cloned and their amino acid sequences demonstrated that they belonged to the Cys-loop ligand-gated ion channel superfamily and not to the G-protein coupled receptor superfamily [3, 4]. Interestingly, the α9 and α10 subunits seem to define a different subfamily of nAChRs. The alignment of the deduced amino acid sequences for the rat subunits α1-α7 and α9, and the chicken subunit α8 shows that the α9 subunit shares about 36 to 39% of the amino acid with any of these other nAChR subunits [4]. However, 57% overall amino acid sequence identity is shared between the α9 and α10 subunits, indicating that they are more closely related to each other than to the muscle nAChRs, to the α7-α8, or to the α2-α6 subfamilies [3, 14]. Additionally, the α9 and α10 subunits have the same genomic organization. For instance, the position of their introns as related to the coding sequence is identical, but differs to that found for all others nAChRs. In all previously cloned nAChRs, the boundaries of the first four exons are conserved but in the α9 and α10 subunits, exons III and IV are continuous [3, 4, 15].

**Structure of α9α10 nicotinic acetylcholine receptors**

nAChRs containing the α9 and α10 subunits are assemblies of five subunits, each with the general topological organization common to the nAChR family. This organization includes an extracellular N-terminal domain of about 200 amino acids containing the signature Cys-loop as part of the ligand binding site observed in nAChRs [16, 17]. This domain is then followed by three transmembrane domains (TM1-TM3), a cytoplasmic domain, and a fourth transmembrane domain (TM4), connecting to the extracellular carboxyl-terminal domain [17].

Intensive research has provided a great deal of information regarding the amino acids and the specific structure of the N-terminal extracellular domain which contains the binding sites for ACh and other agonists and antagonists in muscle and neuronal nAChRs [17-19]. The alignment of the protein sequences of the α9 subunit with other previously cloned nAChR subunits demonstrated that in this N-terminal extracellular domain about 25% of the amino acids are conserved [4]. In contrast, when comparing just the α9 and α10 subunits, 67% of the amino acid sequence of this domain is identical [3, 4]. The significant sequence divergence of this domain in α9α10 nAChRs with respect to other nAChRs is expected to be a major factor underlying the distinct pharmacological profile of α9α10 nAChRs. However, the molecular basis for these differences remains to be investigated.

The transmembrane domains are the most highly conserved regions of the functional domains in the nAChR. Cysteine substitution followed by accessibility studies indicated early on that the side chains of amino acids in TM2 line the pore [20]. Amino acids in TM2 have been demonstrated to play important roles on gating and on the ion selectivity in other ligand-gated ion channels, including different nAChRs.
1 increased the potency $\alpha_1$ or $\alpha_r$ with two of the 504 components, confirming the presence of three $\alpha_1$ and $\alpha_9$ subunits. As expected EC values reflect the incorporation of either one or two $\alpha_9V13T$ mutated subunits in the nAChR complex. [23]. In the same manner, coexpression of the $\alpha10V13T$ mutant with the wild-type $\alpha10$ and $\alpha9$ subunits resulted in dose-response curve to ACh with 4 components, confirming the presence of three $\alpha10$ subunits in the $\alpha9\alpha10$ nAChR pentamer. [23].

Similar experiments were performed using a second mutation at position 17, including $\alpha9I17T$ and $\alpha10M17T$. These mutations also significantly enhanced the nAChR’s sensitivity to ACh. The results from these experiments involving the hydrophobic change at position 17’ to a Thr, also confirmed that the receptor expressed had two $\alpha9$ and three $\alpha10$ subunits. [23, Plazas et al., 23] demonstrated that this stoichiometry was preferred and additionally fixed, independently of the availability of $\alpha9$ or $\alpha10$ subunits, since increasing the ratio of $V13T\alpha9$ to wild-type $\alpha10$ subunits from 1:1 to 4:1 or increasing the ratio of $V13T\alpha10$ to wild-type $\alpha9$ subunits from 1:1 to 4:1 increased the potency for ACh. Thus, within this range of cRNA available, $\alpha9\alpha10$ nAChRs preferentially assemble in a stoichiometry of two $\alpha9$ and three $\alpha10$ subunits. [23].

Importance of the cytoplasmic domain in clustering and functional interactions of nAChRs

The cytoplasmic domain between TM3 and TM4 is a key molecular determinant for the selective subcellular targeting of specific nAChRs subtypes. It is clear that different nAChR subtypes co-exist in various cells, and that they may be clustered into or specifically excluded from discrete domains in those cells or even within individual synapses. For example, the $\alpha3$ subunits are found clustered exclusively at postsynaptic sites in neurons of the ciliary ganglion. Studies of the distinct localization of chimeric receptors in which the cytoplasmic domain sequence had been substituted to targeted to extrasynaptic regions. These studies demonstrated that the cytoplasmic domain was sufficient to direct the localization of the $\alpha3$ subunit to specific sub-synaptic domains in chicken ciliary ganglion neurons. [24, 25]. In contrast, the $\alpha7$ subunits, which are expressed at comparable levels in the same neurons, are specifically targeted to extrasynaptic regions. [25]. The cytoplasmic domain of the $\alpha4$ subunit interacts with the chaperone protein 14-3-3eta and this interaction regulates the association of the
α4 subunit with scaffolding proteins and ultimately determines its sub-cellular localization [26]. Most of the sequence divergence found among nAChR subunits is found in this domain, reflecting the domain’s critical role in targeting specific subcellular regions and also in the interaction with other proteins. The best amino acid alignment of the cytoplasmic domains from the α7, α9, α10, and α3 subunits from human (H), mouse (M), chicken (C), and rat (R) (Fig 1), demonstrates that there is significant sequence conservation across species within each subtype, but only five amino acids are conserved across the different subtypes, namely P333, W335, L431, I431 and A431, in the Hα9 sequence (white letter on black background; Fig 1). A recently published alignment of 57 α9 sequences also demonstrated a high conservation of these five amino acids. The residues P333, W335 and I431 are conserved in all sequences, L is conserved in 40 sequences, while the remaining 17 sequences have I instead in this position, and only three sequences have A432 substituted by V, S, or T [27]. It is not known if there is functional significance for the high conservation of those five amino acids amongst the nAChR subtypes. However, in the midst of the sequence divergence of the cytoplasmic domain amongst subtypes the conservation of these five amino acids is notable.

Yeast-two hybrid screening is a molecular biology approach that has been used extensively to study protein-protein associations. To discover proteins that interact with the α9α10 nAChR in auditory hair cells, Akil et al. [28] used this approach and the cytoplasmic domains of α9 and α10 as ‘baits’. The cytoplasmic domains of the α9 and α10 subunits were selected as baits because these domains have been shown to determine the localization of other members of the nAChR family and because they are expected to have sites of association with other proteins. Using the cytoplasmic domain of α9 as bait, no interacting proteins were identified. Using the cytoplasmic domain of α10, Akil et al. [28] identified a protein, prosaposin, which had not been described in the cochlea [28, 29]. Prosaposin is a precursor of four glycoprotein activators of lysosomal hydrolases (saposins A, B, C and D). Systematic deletion analysis and yeast growth assays revealed that the prosaposin site for binding to α10 was localized to a 40 amino acid region in the carboxy termini, corresponding to a domain in saposin D [29]. This domain of saposin D had not been previously found to interact with other proteins.

Immunofluorescence using an antibody that recognizes both prosaposin and its cleaved product, saposin D, revealed labeling throughout the immature cochlear sensory epithelia in very young mice (one and five days old). In 10 days old and older mice, the labeling was detected in the inner hair cells, inner pillar cells, Deiter cells (supporting cells underneath the outer hair cells), in the basal pole of the outer hair cells, and in efferent synaptic contacts below the basal region of the outer hair cells. Because association between the α10 subunit and prosaposin would require the coexpression in the outer hair cells, and immunofluorescence had failed to reveal prosaposin expression in these cells, the expression of prosaposin was further investigated in microdissected cells by RT-PCR. The results of these experiments agreed with the immunofluorescence results, where prosaposin mRNA was more abundant in supporting cells, but the results also confirmed that prosaposin mRNA was present in the outer hair cells and thus may serve as a binding partner to the α10 subunit, as indicated by the yeast-two hybrid assays. Intense prosaposin label was observed in the cytoplasm of the Scarpa’s ganglion neurons and around the nucleus of the vestibular hair cells of the ampulla and utricle. Less intense label was apparent in the saccular hair cells [30].

Prosaposin knockout mice exhibited normal hearing thresholds only when very young mice were used. In 22 days old mice, significant rapidly progressing hearing loss was evident in the homozygotes mice lacking prosaposin, while the wild-type and heterozygous prosaposin-expressing littermates retained normal hearing. By one month of age, the knockout mice were deaf. This was accompanied by abnormalities in the organ of Corti innervation, and hypertrophy in the inner hair cell and the Deiter cells regions. Labeling for neuronal markers neurofilament and synaptophysin specifically for efferent endings was reduced in the immature organ of Corti when compared to the immunofluorescence labeled observed in prosaposin expressing littermates. However, after the onset of hearing, the labeling of both of these markers was more intense and concentrated abnormally in the inner hair cell and Deiter cell regions, where cellular hyperplasia was observed. In mice lacking prosaposin, loss of outer hair cells in the apex of the cochlea was also prevalent by one month of age [29].

Because α9α10 nAChRs are expressed in the vestibular sensory organs, the effects of prosaposin deletion in vestibular function was evaluated. Using four tests, the air righting reflex, contact righting reflex, swimming test, and circling/rearing frequency test, which are routinely used to evaluate vestibular function in mice, measurements of vestibular function demonstrated significantly impairment in prosaposin knockout mice [30]. Consequently, it is clear that prosaposin, or one of its mature cleaved products, saposins A–D, is very important for normal hearing and also vestibular functions. Prosaposin, or one of its mature cleaved products, saposins A–D is essential for the maintenance of innervation within the mammalian organ of Corti, and is specifically required for normal development of the efferent connections. Prosaposin
Ablation has specific effects on efferent functions in the auditory and vestibular epithelia, and this functional impairment may be due to lack of direct interaction with α10 nAChR subunit, as indicated by the Y2H results [29].

Prosaposin has been shown to function as a neurotrophic factor and also may bind to G₀-coupled receptors. A possible association of α9α10 nAChRs with G-proteins through prosaposin and the functional implications of such interactions warrant further research given the atypical pharmacology of these nAChRs in hair cells, which have been demonstrated to exhibit mixed muscarinic and nicotinic properties [23]. Indeed, substantial evidence points to involvement of GTP-binding proteins in the ACh-induced response of the outer hair cells [20, 61-63]. ACh-induced currents of isolated outer hair cells were significantly reduced when G₀-protein inhibitors were dialyzed through the patch pipette [20]. Specifically, Gα2 immunoreactivity was detected in the hair cells of the mouse cochlea [62]. A Gα2 cDNA, similar to the mouse macrophage Gα2, was cloned from a mouse cochlea cDNA library, and a second cDNA clone, had also been obtained from the auditory sensory epithelia (GenBank accession #s AK157998, S71213).

**Functions of α9α10 nicotinic acetylcholine receptors**

The expression of α9 and α10 subunits has been demonstrated in various tissues and diverse cell types. In most cases, however, their functions are the subject of intense investigation and still unclear. Initial in situ hybridization studies demonstrated the expression of the α9 subunit in E16 rat embryonic pituitary, nasal epithelium, tongue, sternohyoid muscle, adult rat pituitary, and organ of Corti [4]. In the rat, the expression of both α9 and α10 mRNAs was demonstrated in neurons of the dorsal root ganglia using in situ hybridization. Additionally, immunoreactivity experiments demonstrated α10 expression at the protein level in the dorsal root ganglia, and the coexpression of the α9 and α10 subunits was further confirmed by RT-PCR and western blotting in this ganglia [31]. Indeed, α9α10 nAChRs were detected in all neurons of the dorsal root ganglia, not only in the nociceptor neurons. Thus, the authors concluded that α9α10 nAChRs are likely to fulfill a general function in this ganglia not necessarily related to nociception [31].

It has been additionally proposed that in epidermal, basal and follicular cell layers from the skin, ACh is involved in the transition of differentiating keratinocytes from the stratum granulosum to the stratum corneum [32, 33]. Both in vitro an in vivo models of inactivation of α9 nAChR-induced signaling using antagonists or RNA interference of keratinocyte as well as experiments in knockout mice delayed wound healing, and reduced colony scattering and...
cell outgrowth from the colony. The α9 nAChR was shown to influence the gene expression of adhesion molecules, suggesting that α9 nAChR signaling regulates the assembly and disassembly of adhesion complexes, both of the cell-extracellular matrix and cell-cell types, through transcriptional, translational and posttranslational mechanisms involving structural and effector molecules important in cell shape, cytoplasm motility, and adhesive properties of keratinocytes [34].

PCR, western blot and immunohistochemical studies demonstrated the expression of the α9 and α10 subunits in populations of T-lymphocytes, however, no response to ACh was detected from any of these cells [35]. It is also known that most of the nAChRs containing α9 and α10 subunits are expressed in human and mouse bronchial epithelia [36]. The function and the mechanisms of action of these receptors in these cells remain to be elucidated.

Contrary to this, in the auditory and vestibular sensory organs it is clear that α9/α10-containing nAChRs are crucial as efferent targets for the feedback modulation of the excitability of the hair cells. α9 mRNA has been detected using in situ hybridization in the chick basilar papilla [37] and in auditory hair cells from rats and guinea pigs [4, 38, 39]. The α10 subunit has been cloned from a rat cochlea cDNA library, and transcripts were detected using in situ hybridization in auditory and vestibular hair cells in the rat [3, 40]. Efferent cholinergic fibers from the brainstem enter the inner ear forming synapses on the basolateral pole of sensory hair cells and modulating their excitability. This modulation is essential to our sense of hearing since it sharpens frequency selectivity and contributes to protection from excessive noise [6, 40]. The molecular basis of this modulation has been the topic of extensive controversy in the last few years. In contrast to all other nAChRs which support excitatory neurotransmission in auditory hair cells, agonist-evoked nAChR channel opening results in Ca\(^{2+}\) influx and brings about cellular hyperpolarization. Figure 2 provides a diagram of the major conductances involved in the process of nAChR activation. Physiologically, this hyperpolarization of the hair cells results in a reduction of the movement of the basilar membrane thus, dampening hearing sensitivity [6]. The α9 and α10 subunits are key molecular constituents mediating efferent effects on the hair cells [41, 42]. Recent studies utilizing α9/α10 knockout mice and mice lacking expression of SK2, a K\(^{+}\) channel that is functionally associated with α9/α10 nAChRs, provided additional evidence for the essential role of these receptors in the development and modulation of the hair cell’s efferent synaptic function [24, 25]. Their co-localization – or clustering – is critical for the influx of Ca\(^{2+}\) and immediate gating of a Ca\(^{2+}\)-dependent K\(^{+}\) conductance [43].

Calcium-activated K\(^{+}\) channels are present in all neurons and mediate the after hyperpolarization following action potentials. They are involved in many physiological processes including neurosecretion and smooth muscle tone, as well as modulating the action potential shape, and spike frequency adaptation. There are three main subfamilies of K(Ca\(^{2+}\)) channels based on their electrophysiological, pharmacological phenotypes, and molecular properties. The large-conductance Ca\(^{2+}\)- and voltage-activated K\(^{+}\) channels (BK) are sensitive to block by charybdotoxin (CTX) and iberiotoxin, the intermediate-conductance KCa channels are inhibited by CTX and clotrimazole, and the small conductance KCa channels (SK) are sensitive to apamin, scyllatoxin, and dequalinium chloride.

In rat organ of Corti preparations, presynaptic depolarizations were shown to induce an inhibitory hyperpolarization of the outer hair cells [43]. This response was blocked by 100 nM strychnine, an α9 nAChR blocker, and by 1 μM dequalinium, an SK channel blocker [43, 44]. Thus, nAChRs from the outer hair cells are thought to be heterooligomers composed of α9 and α10 subunits [3, 28]. Although there is significant sequence homology between the α9 and α10 subunits, and the α9 nAChR forms homomeric channels when expressed alone in Xenopus oocytes [4], when the α10 RNA is injected by itself no homomeric ACh-activated channels were expressed. However, co-injection of the α9 and α10 subunits resulted in the expression of ACh-sensitive channels with 100-fold larger current amplitude and thus significantly different from α9 homomeric receptors. The α9 subunit was then suggested to be essential in the trafficking of the α10 subunits to the plasma membrane [45]. Additionally, when the α9 and α10 where co-expressed, the properties of the receptors resembled the native hair cell receptors, indicating a direct interaction between the subunits. Other lines of evidence also confirmed the interaction between these subunits. For example, in immunoprecipitation experiments of cochlear protein extracts using α9 and α10 specific antibodies it was demonstrated that each subunit may precipitate the other one [46]. Additionally, when the extracts were prepared from transgenic mice engineered to overexpress the α9 subunit, larger quantities of the α9 and also of α10 subunits were recovered in the immunoprecipitated complexes compared to extracts from wild-type mice [43]. Noticeable, in mice, there is a broad variability of the α9 expression levels in the hair cells and this was found to be correlated with the strength of the protection from noise damage provided by the efferent system [47]. This direct correlation between α9 protein levels and the strength of protection from acoustic damage conferred by the efferent system supported a direct central role for α9 in the efferent system and has been recently...
confirmed by studies of knockin mice (discussed below) [47].

The immature sensory hair cells produce spontaneous electrical activity and the final differentiation of the mature inner hair cells seems to require specific patterns of spontaneous action potential during a critical period. The α9 is required for the development of inner hair cell synapses and specifically for the Ca\(^{2+}\)-induced vesicle fusion at the inner hair cell synaptic machinery.

The α9α10 nAChR exhibits marked differences in Ca\(^{2+}\) permeability across species. Mammalian recombinant and native α9α10 nAChRs have relative Ca\(^{2+}\) to monovalent cation permeability ratios close to or higher than 10\(^{14, 50-52}\). In contrast to this, the relative Ca\(^{2+}\) to monovalent cation permeability ratio for the chicken recombinant α9α10 nAChR is much lower, a ratio of 2, which is similar to other nAChRs that do not contain the α9α10 subunits (50). The authors conclude that the evolutionary processes that were responsible for the increased Ca\(^{2+}\) permeability of mammalian α9α10 nAChRs were specific to the mammalian lineage and happened concomitant with other adaptations that conferred high frequency hearing to mammals [27, 48-50].

**Insights from studies in transgenic mice**

Following the characterization of site-directed mutant nAChRs expressed in Xenopus oocytes, and the confirmation of the critical role of TM2 amino acids in the gating process at α9α10 nAChRs, the construction and study of the corresponding knockin mice was invaluable to advance our knowledge of the native receptor. The substitution of Leu to Thr at position 9’ in the TM2 from the α9 L9T when coexpressed with the α10 subunit in Xenopus oocytes displayed decreased EC\(_{50}\) and decreased rate of desensitization to ACh [21]. The introduction of this mutation in the mouse produced wt/L9T heterozygous as well as L9T/L9T double mutants which had no differences in life span, seemed to age and reproduce normally, had no notable behavioral phenotype, and the cochlear was normal. Additionally, the mRNA level of α9 L9T mutant nAChRs was similar to that for the α9 in wild-type mice, and there were no differences in the expression levels of α10 or SK2 either. It was also demonstrated that the capacitance and the voltage-dependent K\(^{+}\) currents of the auditory hair cells were not affected by the mutation. This comprehensive evaluation of the phenotypes in the knockin homo- and hetero-zygous mice provides confidence that the effects of the L9T mutation on hearing physiology are due to the direct role of α9 on ACh neurotransmission in the cochlear hair cells and to the altered channel properties, and not to secondary effects or artifacts of the mutation.

ACh-induced currents recorded from immature inner hair cells of L9T/wt and L9T/L9T mice displayed higher sensitivity to ACh and decreased desensitization rates compared to the ones recorded from wild-type mice, resembling the characteristics of the reconstituted L9T receptor in Xenopus oocytes [21, 53]. In immature inner hair cells following neurotransmitter release, spontaneous inhibitory postsynaptic currents (IPSC) reflect opening of the nAChR and subsequent gating of the associated Ca\(^{2+}\)-activated K\(^{+}\) channels. These currents in L9T mutant mice were more than double in magnitude, when compared with currents in wild-type mice. Additionally, in these cells the spontaneous IPSC, as well as IPSC evoked by 40 mM K\(^{+}\) lasted much longer. The activation of K\(^{+}\) currents concomitant to AChR activity was not affected by the mutation. Although electrophysiological studies in mature outer hair cells are more technically challenging that in immature inner hair cells, the effects of the L9T mutation were also studied on the mature outer hair cells. The ACh-evoked response in outer hair cells from mice carrying the L9T mutation was longer-lasting and more sensitive to ACh than this response in outer hair cells from wild type mice. In general, all the properties confirmed that outer hair cells expressed the same L9T-containing nAChR as the one expressed in the immature inner hair cells.

Interestingly, the hearing thresholds were 5-15 dB higher in both, the double mutant as well as the heterozygous when compared to the wild-type mice. This mutation strengthened cochlear suppression in vivo demonstrating the central role of α9 nAChRs in the medial efferent system. The study of this mutation also demonstrated that this system protects from damage elicited by excessive noise [53].

**Phenotype of α9 knockout mice (α9-/-)**

Mice lacking α9 expression were viable, had no noticeable abnormal phenotype and their cholinergic olivocochlear bundle was normal [42]. Since the α9α10 nAChR was thought to play a major role in efferent neurotransmission, various aspects of inner ear function and specifically of the efferent pathway were studied in the α9 knockout mice. For example, measurements of cochlear compound action potentials report the activity of cochlear afferent fibers and thus reflect the function of the inner, outer hair cells, and afferent neurons. All α9 knockout mice had normal compound action potential thresholds. Distortion product otoacoustic emissions (DPOAEs) are emissions produced by the inner ear when it is challenged with two tones. These emissions specifically reflect the function of the outer hair cells. There were no differences in the DPOAEs in α9 knockout mice when compared to wild-type mice. Thus, the lack of the α9 subunit affects neither the normal inner and outer hair cells, nor
afferent neuron functions. However, functional deficits specific to the efferent system were revealed. In wild-type, α9-expressing mice, the activation of the efferent fibers either electrically or acoustically reduces the amplitude of the compound action potential and of the DPOAEs, whereas in the α9 knockout mice, this activation had no effect either on the compound action potential or on the DPOAEs [42, 54]. This confirmed that nAChRs containing the α9 subunit play an essential role in the efferent feedback mechanism.

In addition to the lack of functional efferent effect due to the lack of the α9 subunit, studying α9 knockout mice also revealed a reduced and abnormal innervation of the organ of Corti. In the organ of Corti, the innervation of the outer and inner hair cell areas corresponds to the projections of lateral and medial components of the olivocochlear efferent bundle. In wild-type mice, presynaptic efferent terminals contact the basal pole of the outer hair cells as clusters of up to five terminals per cell, and there is also a profuse innervation consisting of smaller terminals contacting inner hair cells or neurons of the spiral ganglia under the inner hair cell region [42, 55]. The effect of the lack of α9 expression on the integrity of these terminals was studied using two different markers of the presynaptic sites, synaptophysin and vesicular acetylcholine transporter. Immunostaining with both of these markers demonstrated that in α9 knockout mice there were a lesser number of presynaptic terminals in the outer hair cells and that they were larger in size, and mostly single, instead of in clusters, as they are normally found in α9-expressing mice. Additionally, the innervation of the inner hair cell area seemed to lack the terminals closely associated with the inner hair cells. Thus, the lack of hair cell response due to the α9 deletion has an effect on the maturation of the efferent fibers and less (non-productive) contacts are established. Thus, the α9 deletion precluded the normal development of the efferent fibers in the organ of Corti of the knockout mice [42, 54]. Vetter et al. [42] proposed mechanistic hypotheses to explain how does the α9 deletion may bring about this effect.

**Phenotype of α10 knockout mice (α10-/-)**

As it was the case for the α9 subunit, Vetter et al. [56] demonstrated that the α10 subunits are also required for the expression of functional nAChRs in auditory hair cells and for normal development of the efferent fibers in the organ of Corti. Although it had been previously shown that α9 nAChRs form functional homomeric receptors when expressed alone in Xenopus oocytes [3, 4], and that the α10 deletion had no effect in the α9 transcript levels, no currents were recorded in response to ACh applications in inner hair cells from mice lacking α10 expression. There was no response even at high concentrations (i.e., 100 µM or 1 mM) of ACh in hair cells from α10 knockout mice, while in hair cells from the wild-type and heterozygous littermates, α10-expressing mice, inward currents in the range of 200-500 pA were routinely recorded under the same conditions [56].

All α10 knockout mice had normal baseline hearing, and normal DPOAEs. However, the efferent innervation of the organ of Corti seemed to be disorganized, and in general resembled the abnormal innervation in the organ of Corti of the α9 knockout mice [42, 56]. The α10 mouse had abnormal efferent innervation in the sense that there were less number of presynaptic terminals in the outer hair cells and on average the terminals were larger in size, and mostly single, instead of in clusters, as they are normally found in the α10-expressing mice. Functionally, the α10 deletion in vivo demonstrated that α10 was essential for the efferent effects on hearing function [56].

**Potential role of α9α10 nAChRs as therapeutic targets**

The potential for α9α10 nAChRs to be used as therapeutic targets is being intensively explored. α9α10 nAChRs seem to hold promise as attractive pharmacological targets partly because they have not been found assembled with other nicotinic subunits, and because of their distinct pharmacological profile when compared to that for other nAChRs. For example, various nAChRs bind α-conotoxins (α-CTX’s) with high selectivity. These are small disulfide rich peptides found in the venom of marine snails, and there is a vast diversity of them since there are around 700 species of these snails [57]. Notably, α9α10 nAChRs displayed a 260-fold higher affinity than α7 nAChRs for a recently discovered α-CTX, α-CTX PeIA. α-CTX PeIA thus may be useful to discriminate physiological processes mediated through α9α10 or α7 nAChRs in tissues where both receptors may be expressed [58]. Additionally, the α-CTX’s RgIA and Vc1.1 selectively block α9α10 nAChRs but no other nAChRs [59]. These α-CTX’s are effective painkillers, and also seem to accelerate functional recovery from nerve injury [57, 59, 60], but also reduce acute pain and produced a sustained analgesic effect in the chronic constriction nerve injury model of neuropathic pain in rats [61]. Additionally, because of their unique roles in the development and modulation of the sensory end organs of hearing and balance, the potential of α9α10 nAChRs as pharmacological targets for specific pathologies of the auditory and vestibular systems is being explored [reviewed recently [62]]. There also seems to be a niche for α9α10 nAChRs as therapeutic targets for pathologies of the skin, bronchia and even in cancers; however, the understanding of the specific roles of α9α10 nAChRs in these tissues is still
being elucidated.

Summary

α9α10 nAChRs define the most phylogenetically distant subfamily of nAChRs. Notably, in contrast to all other cholinergic neurotransmission which serve excitatory functions, cholinergic neurotransmission mediated by α9α10 nAChRs is inhibitory. α9α10 nAChRs have a much higher selectivity for Ca<sup>2+</sup> and serve as Ca<sup>2+</sup> source for the activation of K<sup>+</sup> channels and in non-neuronal cells control the expression of genes related to cell adhesion and likely important in metastatic processes. The functional associations between α9α10 nAChRs, K<sup>+</sup> channels, and other effectors are likely dependent in their close colocalization and on protein-protein interactions yet to be discovered. Partly because of the crucial role of Ca<sup>2+</sup>, at the heart of the control of a myriad of cellular processes, these functional associations are going to be the focus of exciting research and fundamental findings in the near future.

Conflicting interests

The authors have declared that no conflict of interests exist.

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Abbreviations

NACHRs: nicotinic acetylcholine receptors; Ach: acetylcholine; TM: transmembrane domain; CTX: charybdotoxin; IPSC: inhibitory postsynaptic currents; DPOAEs: Distortion product otoacoustic emissions.

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