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Roles for Gap-Junctions in Cochlear Amplification and Micromechanics Exposed by a Conexin 30 Mutation

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Abstract. Accelerated age-related-hearing-loss disrupts high-frequency hearing in inbred CD-1 mice. The p.Ala88Val (A88V) mutation in the gene coding for the gap-junction protein connexin30 (Cx30) protects the cochlear basal turn of adult CD-1Cx30$^{A88V/A88V}$ mice from degeneration and rescues hearing. Here we report the passive compliance of the cochlear partition and active frequency tuning of the basilar membrane are enhanced in the cochleae of CD-1Cx30$^{A88V/A88V}$ compared to CBA/J mice with sensitive high-frequency hearing, suggesting gap-junctions contribute to passive cochlear mechanics and energy distribution in the active cochlea. Surprisingly, the endocochlear potential that drives mechanoelectrical transduction currents in outer hair cells (OHCs) and hence cochlear amplification is greatly reduced in CD-1Cx30$^{A88V/A88V}$ mice. Yet, the saturating amplitudes of cochlear microphonic potentials in CD-1Cx30$^{A88V/A88V}$ and CBA/J mice are comparable. Although not conclusive, these results are compatible with the proposal that OHC transmembrane potentials, determined mainly by potentials extracellular to the OHCs, drive somatic electromotility.

[***All figures referred to in this paper are available at*** https://www.nature.com/articles/ncomms14530.pdf]

INTRODUCTION

Many cell types in the cochlea, including type 1 fibrocytes, cells within the basal cell region of the stria vascularis (SV), and supporting cells of the organ of Corti (OC), but not inner or outer (OHC) hair cells, are coupled together by intercellular gap junctions. The gap junctions are formed by two interacting hemichannels (connexons), on neighbouring cells. Each connexon consists of six connexin protein subunits, to permit the bidirectional flow of ions and signalling molecules. The hemichannels of type 1 fibrocytes of the spiral ligament, are formed of co-localized Cx26 and Cx30[1], deletions or mutations of which are responsible for most genetically based hearing loss[2]. Based on a specific mutation of Cx30, we have evidence to support roles for connexins in the passive and mechanical properties of the cochlea. We also have direct evidence that a mutation of Cx30 rescues hearing in a mouse strain with early onset hearing loss (ARHL). This study throws light on the identity of the control voltage for voltage-dependent motility and amplification in the cochlea.

High-frequency hearing in the CD-1 mouse deteriorates progressively from about 3 weeks in age[1]. Pathological changes in cochlear fibrocytes precede other presbycusic changes associated with age-related hearing loss (ARHL) in the CD-1 mouse[3]. Mutations of Cx30, including A88V[4], are the basis for Clouston syndrome (OMIM #129500), an autosomal dominant genetic disorder characterized by alopecia, nail dystrophies, palmoplantar hyperkeratosis, and

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sometimes hearing loss. The CD-1Cx30$^{A88V/A88V}$ mouse model carrying the p.Ala88Val (A88V in NP_001010937.1) point mutation of Cx30 generated by Bosen et al.[4],primarily to analyse the skin phenotype, surprisingly led to rescue of the high frequency hearing-loss expressed in the CD-1 background strain[4]. We confirmed this finding[5] and discovered that the passive compliance of the cochlear partition and active frequency tuning of the basilar membrane are enhanced in the cochleae of CD-1Cx30$^{A88V/A88V}$ compared to CBA/J mice with sensitive high-frequency hearing. We suggest gap junctions contribute to passive cochlear mechanics and energy distribution in the active cochlea. We also found that the endocochlear potential (EP) that drives mechanoelectrical transduction currents in outer hair cells, and hence cochlear amplification, is greatly reduced in CD-1Cx30$^{A88V/A88V}$ mice. Surprisingly, the saturating amplitudes of cochlear microphonic potentials (CM) in CD-1Cx30$^{A88V/A88V}$ and CBA/J mice are comparable. Although not conclusive, these results are compatible with the original proposal by Dallos and Evans[6] that transmembrane potentials, determined mainly by extracellular potentials, drive somatic electromotility of OHCs.

METHODS

Homozygous Cx30^{A88V} mice from a colony generated and supplied to us by Bosen et al[4]. formed the basis for a new colony of Cx30^{A88V} mice maintained under quiet conditions in our facility. All experiments were performed with littermates, male and female, of >96.9% CD-1 background (> 5 back crosses to the CD-1 background). CBA/J mice were obtained from Envigo.com. UK. All mice used in this study were kept under standard housing conditions with a 12 h/12 h dark-light cycle and with food and water ad libitum. Genotyping was performed according to the protocol provided by Bosen et al.[13]. All procedures involving animals were performed in accordance with UK Home Office regulations with approval from the local ethics committee. All methods have been described fully[5]

RESULTS

Cx30 is located similarly in CD-1Cx30$^{A88V/A88V}$ and CBA/J mice. According to the histology and Cx30 immunohistochemistry, the OC is structurally intact in all turns of the cochleae of CBA/J (n=4) and CD-1Cx30$^{A88V/A88V}$ (n=7). In contrast, the basal, high-frequency turn of CD-1Cx30$^{WT/WT}$ (n=7) mice is degenerated, with total loss of OHCs. In intact turns of the cochlea (CBA/J and CD-1Cx30$^{A88V/A88V}$), Cx30 is localized in the membranes of Deiters' cells (DCs) and outer pillar cells (OPCs) in the OC and in basal cells of the SV and spiral ligament. [see Fig. 1, https://www.nature.com/articles/ncomms14530.pdf].

CD-1Cx30$^{A88V/A88V}$ mice generate high-frequency DPOAEs. Within the sensitivity range of the high-frequency sound system used in our measurements, distortion product otoacoustic emission (DPOAE) threshold audiograms recorded from CD-1Cx30$^{A88V/A88V}$, CD-1Cx30$^{WT/WT}$, CBA/J mice are similar for frequencies below 20 kHz. Above 20 kHz, the audiograms of the CD-1Cx30$^{WT/WT}$ become less sensitive with increasing frequency. DPOAE audiograms of CD-1Cx30$^{A88V/A88V}$ and CBA/J mice are similar and reveal that OHC mediated mechanical sensitivities of the OCs of CD-1Cx30$^{A88V/A88V}$ and CBA/J mice extend at least to the 70 kHz frequency range and hence into the basal turn of cochlea. [see Fig. 2, https://www.nature.com/articles/ncomms14530.pdf]

Reduced EP in CD-1Cx30$^{A88V/A88V}$ and Cx30$^{A88V/WT}$ mice. EP was measured in the scala media by advancing the micropipettes through the OC. The EP, expressed as mean ± standard deviation measured from CD-1Cx30$^{WT/WT}$ mice was +112.8 mV ± 1.2 mV, n = 9, not significantly different from that measured from CBA/J mice of a similar age (+114.7 mV ± 2.9 mV, n = 4; p=0.11, unpaired two-tailed t-test). In contrast, EP was greatly reduced to +88.4 mV ± 2.0 mV in CD-1Cx30$^{A88V/WT}$ mice (n = 8) and to only +71.3 mV ± 2.8 mV (n = 12) in CD-1Cx30$^{A88V/A88V}$ littermates (p<0.0001 for CD-1Cx30$^{WT/WT}$ compared to CD-1Cx30$^{A88V/WT}$ or CD-1Cx30$^{A88V/A88V}$ mice; p=0.0016 for CD-1Cx30$^{A88V/WT}$ vs CD-1Cx30$^{A88V/A88V}$, unpaired t-test). Hence, a higher expression of mutated Cx30 A88V protein subunits appears to entail a greater reduction in EP.

CM produced by CD-1Cx30$^{A88V/A88V}$ but not CD-1Cx30$^{WT/WT}$ mice. Consistent with our histological findings, we recorded CM from the OC and round window (RW), only from CD-1Cx30$^{A88V/A88V}$ mice. CM was not detectable in CD-1Cx30$^{WT/WT}$, and CD-1Cx30$^{A88V/WT}$ littermates. Significantly, the peak-to-peak magnitude of the CM recorded from the extracellular spaces close to the OHCs and from the RW of normal-hearing CBA/J mice, and CD-1Cx30$^{A88V/A88V}$ mice are very similar for stimulus levels above 75 dB SPL. However, the amplitude of CM measured from CD-1Cx30$^{A88V/A88V}$ mice for any given stimulus level below ~ 60 dB SPL, is only 45% of that recorded from CBA/J mice. [see Fig. 3, https://www.nature.com/articles/ncomms14530.pdf]

Sharp sensitive BM tuning in CD-1Cx30$^{A88V/A88V}$ mice. The beam of a laser diode self-mixing interferometer was focused through the RW membrane onto locations coincident with outer pillar cells – row 1 OHCs of the basal

turn basilar membrane (BM) from its attachment to the spiral lamina. In this 50 kHz – 56 kHz region of the BM, magnitude and phase of BM displacement was measured in response to pure tones. BM displacement threshold frequency tuning curves (0.2 nm criteria) were measured from the cochleae of five CD-1Cx30$^{WT/WT}$ mice, five CD-1Cx30 $^{A88V/WT}$ mice, eight CD-1Cx30$^{A88V/A88V}$ mice, five CD-1 as controls for the background of the CD-1Cx30$^{A88V/A88V}$ mice, and four CBA/J mice as examples of mice with excellent hearing and without early onset ARHL. BM displacement threshold frequency tuning curves of CD-1Cx30$^{A88V/WT}$ and CD-1Cx30$^{WT/WT\ mice}$ are similar to those of CD-1 mice with broad, insensitive minima in the 45 kHz – 55 kHz range. *Post mortem*, responses are mostly unchanged (Figures 4B, C). Thus, in support of the immunohistochemistry and CM measurements, it appears there are no functional OHCs in the basal turn of CD-1Cx30$^{WT/WT}$ and CD-1Cx30$^{A88V/WT}$ littermates and CD-1 strain mice.

Peak thresholds of BM tuning curves measured from CD-1Cx30$^{A88V/A88V}$ mice (22.7± 5.8 dB SPL, n =8) were not significantly different from the thresholds measured in CBA/J mice (24.8 ± 3.7 dB SPL, n = 4, p=0.78, two-tailed unpaired t-test). In contrast, the bandwidths of the tuning curves measured from CD-1Cx30$^{A88V/A88V}$ mice were significantly narrower than those of WT mice: the Q_{10dB} value (characteristic frequency / bandwidth 10 dB from tip) of CD-1Cx30$^{A88V/A88V}$ was 17.4 ± 3.1 (mean ± s.d.) compared with 8.7 ± 4.3 for CBA/J mice (p = 0.0023, two-tailed unpaired t-test). The high and low frequency slopes of BM tuning curves, measured from the tip, to 20 dB above the tip, from CD-1Cx30$^{A88V/A88V}$ mice were 147 ± 8 dB.octave^{-1} and 322 ± 15 dB.octave^{-1} respectively which is significantly steeper than in CBA/J mice of 99 ± 6 dB.octave^{-1} and 187 ± 11 dB.octave^{-1} (p < 0.0001 for high and low frequency slopes, two-tailed unpaired t-test). $Q_{10\ dB}$ of CD-1Cx30$^{A88V/A88V}$ mice was correlated (r = -0.975) with the sensitivity at the tip of the threshold tuning curve; the more sensitive the preparation, the sharper the tuning. In line with our interpretation that the sharp amplified tip of the threshold curves for CD-1Cx30$^{A88V/A88V}$ mice derives from active processes, the sensitivity of *post-mortem* BM tuning curves of CD-1Cx30$^{A88V/A88V}$ mice resembled those of CD-1Cx30$^{WT/WT}$ mice.

BM response phase as functions of stimulus frequency (relative to that of the malleus) was measured from a CD-1Cx30$^{A88V/A88V}$ and CBA/J mice with a common CF at 70 dB SPL. The phase-frequency relationships of the CD-1Cx30$^{A88V/A88V}$ and CBA/J mice are similar in the low frequency tail region. However, for frequencies in the range of 45-55 kHz, the phase-frequency relations of the CD-1Cx30$^{A88V/A88V}$ mouse are steeper than those of the CBA/J mouse, which may indicate that gap-junctions contribute to energy distribution in the active cochlea resulting in the observed sharper frequency tuning of CD-1Cx30$^{A88V/A88V}$ mice. [see Fig. 4, https://www.nature.com/articles/ncomms14530.pdf]

Enhanced passive BM mechanics in CD-1Cx30$^{A88V/A88V}$ mice. BM displacement responses in the low frequency tails of threshold frequency tuning curves are dominated by stiffness of the cochlear partition at a given cochlear location (e.g.[41]). Thresholds of the tails between 15 and 40 KHz were significantly more sensitive in CD-1Cx30$^{A88V/A88V}$ mice than in CBA/J mice by 11.0 ± 0.8 dB SPL (n = 5). No significant difference could be observed at 10 kHz, which we attribute to the large noise floor, which made measurements difficult. We were unable to detect a significant difference in the phase of BM displacement in the tails of the low frequency tuning curves in the 10kHz – 45 kHz region (expressed as mean ± standard deviation, n = 5). The sensitivities of the low-frequency tails of CBA/J, CD-1 and CD-1Cx30$^{WT/WT}$ mice are similar, while the sensitivities of the low-frequency tails of tuning curves from CD-1Cx30$^{A88V/WT}$ mice are more variable and less sensitive than those of CD-1Cx30$^{A88V/A88V}$ mice by 3.2 ± 1.6 dB SPL. It is likely that the gap-junctions contribute to the passive stiffness of the cochlear partition because increased sensitivity of the low-frequency tail in CD-1Cx30$^{A88V/A88V}$ mice and, hence, decreased mechanical stiffness of the cochlear partition persisted *post mortem*. [see Fig. 4, https://www.nature.com/articles/ncomms14530.pdf]

DISCUSSION

If, as generally accepted, MET current flow is controlled by EP in series with the hair cell resting potential[7,8], it is remarkable that DPOAE audiograms and BM sensitivity in the basal turn of CD-1Cx30$^{A88V/A88V}$ mice are similar to those of CBA/J and other WT mice with excellent hearing[8-11]. Indeed, reduced EP, the driving force for MET, should reduce the current flow through the OHC hair bundles in CD-1Cx30$^{A88V/A88V}$ mice to 73% of the CBA/J mouse values, [($EP_{Cx30A88V}$ 71.3 mV + -E_{OHC}) / ($EP_{CBA/J}$ 114.7 mV + -E_{OHC}); E_{OHC} = -50 mV[12]]. Nonetheless, the maximal magnitudes of CM potentials in CD-1Cx30$^{A88V/A88V}$ mice are like those of CBA/J mice. We have no good reason to assume changes in the number or function of OHCs involved in CM generation under these stimulus conditions. Thus, the finding of a preserved CM in spite of reduced transduction currents would indicate an increased electrical impedance of the cochlear partition in the mutant mice[12]. Our hypothesis remains tentative until it is discovered how exactly the conductance properties of gap junctions expressing mutated Cx30 A88V connexins in the cochlea are changed and how this affects the electrical impedance of the cochlear partition in CD-1Cx30$^{A88V/A88V}$ and CD-1Cx30$^{A88V/WT}$ mice.

As EP is reduced, the MET currents in individual OHC of CD-1Cx30$^{A88V/A88V}$ mutants must be reduced not only at high but also at low stimulus intensities. To explain the preserved cochlear sensitivity, we suggest the predominant factor controlling OHC electromotility is not a change in the OHC intracellular potential resulting from the changing current flux through the OHC MET conductance[12]. Instead, our data support the proposal that voltage-dependent amplification is controlled by the OHC transmembrane potential changes which are due predominantly to changes in the OC potentials extracellular to the OHCs[13,14]. In this sense, the extracellular potentials in vicinity of the OHCs provide "a floating ground" for the OHC transmembrane potential. These potentials[14] are generated by the flow of sound-induced MET currents along their return pathways through the electrical impedance of the cochlear partition[13,15], which we tentatively propose is increased in CD-1Cx30$^{A88V/A88V}$ mice. Control of somatic motility by extracellular OC potentials would also enable the bandwidth of cochlear amplification to be limited only by that of the voltage-dependent motility itself[16].

We tentatively propose that a common factor may be responsible for the enhanced BM frequency tuning of CD-1Cx30$^{A88V/A88V}$ mice compared with CBA/J and other sensitive wild type mice[9-11] and for the reduced CM magnitude in response to low-intensity low-frequency tones. This proposed factor is a decrease in mechanical coupling within cochlear partition due to the Cx30 A88V mutation. A similar change in the longitudinal mechanical properties of elements of the cochlear partition has previously been shown to sharpen the mechanical tuning of the cochlea in Tectb$^{-/-}$ mice where the number of OHCs contributing towards amplification at a given cochlear location is reduced compared with that in control mice[17]. A role in the passive mechanics of the cochlear partition has so far been detected only for the Cx A88V mutation. A conditional knock-out of Cx26 from pillar and Deiters' cells, for example, causes a frequency dependent change in the passive, but not the dynamic mechanical properties of the cochlear partition[18].

Our *in vivo* data describing the effects of the A88V mutation of Cx30 and Cx26 cKO provides indirect evidence for new potential roles for gap-junctions in sensory processing in the cochlea. Further *in vivo* and *in vitro* measurements are required to understand how Cx26 and Cx30 influence the electrical and mechanosensitive properties of cochlear gap junctions and how this alters the complex electrical environment of OHCs, thereby enabling OHCs to contribute fully in their sensory-motor role to the sensitivity of the cochlea. Moreover, how do gap-junctions contribute to the static and dynamic mechanical properties of the cochlear partition and how does the A88V mutation of Cx30 rescue hearing in a mouse line that normally expresses accelerated ARHL.

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