**Introduction**

Voltage-activated Ca\(^{2+}\) channels are multimeric complexes of 3–4 subunits, the pore-forming Ca\(\alpha_1\) subunit and the auxiliary subunits Ca\(\beta\), Ca\(\alpha_2\delta\) and sometimes also Ca\(\gamma\)\(^{1-3}\). The Ca\(\alpha_1\) subunit is encoded by 10 different genes giving rise to CaV1.1–CaV1.4; CaV2.1–CaV2.3; CaV3.1–CaV3.3; the Ca\(\beta\) subunit is encoded by 4 different genes, giving rise to CaV\(\beta_1\)–CaV\(\beta_4\); as is the Ca\(\alpha_2\delta\) subunit.\(^{2,3}\) Even when neglecting the occurrence of the Ca\(\gamma\) subunit there is a wealth of combinations of particular Ca\(\alpha_1\), Ca\(\beta\) and Ca\(\alpha_2\delta\) subunits. When co-expressed with Ca\(\alpha_1\) subunits in heterologous systems, Ca\(\beta\) subunits increase current density and change various biophysical properties, such as voltage-dependence of activation and inactivation (reviewed in refs. 3–5). The view that Ca\(\beta\) subunits solely have a trafficking and chaperone function for the non-covalently bound Ca\(\alpha_{1}\) subunits has recently
changed into that of a multifunctional regulatory protein. In cells, however, express multiple CaVα subunits have preferences for distinct CaVβ subunits such as CaV1.1 for CaVβ1 in skeletal muscle, CaV1.2 for CaVβ2 in cardiac muscle, CaV2.1 for CaVβ4 in cerebellum, and CaV2.2 for CaVβ3 in brain. Many excitable cells, however, express multiple CaVα and CaVβ subunits, and the question arises if there are cell-specific combinations of particular CaVα and CaVβ subunits.

Ca2+ channels are required for transmitter release in inner and outer hair cells (HC). Before the onset of hearing around postnatal day 12 (P12) in most rodent species, inner hair cells (IHC) go through a phase of spontaneous spiking activity, in which Ca2+ channels not only provide the Ca2+ influx for exocytosis but are also responsible for IHC depolarization and for modulating gene expression. In contrast, mature IHCs need Ca2+ currents for triggering exocytosis rather than shaping the receptor potential.

In both IHCs and outer hair cells (OHC), about 90% of the Ca2+ current flows through the CaV1.3 CaVα subunit. Mammalian CaV1.3-mediated hair cell currents have rather untypical L-type channel properties such as negative activation, lack of inactivation, very rapid activation and deactivation, and low sensitivity to dihydropyridine block. There is the possibility that this particular CaVα subunit specifically co-assembles with only one type of CaVβ subunits in HCs similar to the combination of CaV1.1/CaVβ1 in skeletal and CaV1.2/CaVβ2 cardiac muscle. However, although CaVα currents in IHCs and OHCs are carried by the same CaVα subunit, biophysical properties such as the voltage-dependence of activation, the degree of inactivation, and the sensitivity to Bay K8644 differ between both HC types. In addition, the voltage-dependence of activation shifts during development for both IHCs and OHCs. These differences could be caused by different combinations of the CaV1.3 subunit with specific CaVβ subunits, which may change during postnatal HC differentiation. 

Because of the high abundance of CaV1.3 subunits, inner and outer HCs potentially provide a well-suited system to test if there is a preferred combination of a particular CaVβ subunit with this CaVα subunit. So far, little is known about CaVβ expression in hair cells. Green et al. detected mRNA for CaVβ1, 3 and 4, but not for CaVβ2 in total cochlear tissue of hearing (P18) mice. In chick cochlear hair cells, a short splice form of CaVβ4 was identified that serves as a transcription factor and regulatory protein, but nothing is known about the expression and role of CaVβ4 in mammalian HCs. The aim of the present study was to test if the CaV1.3 subunits co-assemble with only one particular CaVβ subunit, or if there are specific combinations of CaVβ subunits that could account for differential Ca2+/Ba2+ current properties observed in inner and outer hair cells and during development.

**Results**

Expression of CaVβ1-4 in the cochlea. To test if the predominant pore-forming CaVα subunit in inner and outer HCs, CaV1.3, specifically co-assembles with a particular CaVβ subunit, we analyzed CaVβ mRNA in the organ of Corti and in selectively isolated inner and outer hair cells. mRNA for all four CaVβ subunits was detected in the organ of Corti of pre-hearing (P4) and hearing (P18) NMRI mice, shown for P18 (Fig. 1A). CaVβ1 PCR amplified three transcripts of ~400, ~550 and ~700 bp length, suggesting the presence of the splice variants CaVβ1a and CaVβ1b, which is in accordance with Green et al. The occurrence of CaVβ3- and CaVβ4-specific transcripts in the organ of Corti (Fig. 1A and B) confirms this earlier study. However, the persistent presence of CaVβ2 contrasts Green et al. who could not demonstrate CaVβ2 mRNA in total cochlea. To identify possible differences of CaVβ1-4 expression in inner and outer hair cells before and after the onset of hearing, PCR was performed on cDNA synthesized from IHCs and OHCs, which were selectively collected with glass pipettes under microscope control (see Methods). Care was taken to prevent cross-contamination between OHCs and IHCs. Due to the small amount of cDNA per PCR reaction (~5–15 IHCs or OHCs, respectively), a nested PCR approach was necessary to amplify CaVβ subunits, similar to RT-PCR conditions for the CaV1.3 subunit. CaVβ2 and CaVβ3 mRNA could be consistently detected in both OHCs and IHCs at immature and mature ages, shown for P4 (Fig. 1C and D). In addition, CaVβ1 and CaVβ4 mRNA was amplified in P4 OHCs (Fig. 1C). In IHCs, however, detection of CaVβ1 and CaVβ4 mRNA was inconsistent—in part of the PCR reactions specific transcripts could be detected, in others not.

Using immunohistochemistry, antibodies against all four CaVβ subunits were tested in cochlear cryosections of NMRI mice and rats. Immunostaining, though qualitatively similar in mouse and rat cryosections, gave better signals in rat specimen. As staining was sensitive to decalcification required for sectioning cochleae >P10, results are shown here for rat cochlea at...
P8-P10 (Fig. 2), the age of maximum $I_{\text{on}}$ in IHCs. Ca$_{\beta}$1 staining was found under the base of OHCs, most likely in efferent boutons (Eff), and in a pearl-chain-like fashion representing the outer spiral bundle or afferent fibers type II (OSB; Fig. 2A). In the IHC, it was detected along the basolateral membrane and below, in the inner spiral bundle (ISB; Fig. 2A). Spiral ganglion (SG) neurons showed a punctate Ca$_{\beta}$1 staining (Fig. 2B). Ca$_{\beta}$2 staining was present in a dot-like manner at the very base of OHCs and along the entire basolateral membrane of IHCs (Fig. 2C) where Ca$_{\beta}$1,3 channels are localized.$^{13}$ SG neurons showed membrane-bound, dot-like Ca$_{\beta}$2 immunoreactivity (Fig. 2D). Ca$_{\beta}$3 antibodies diffusely stained bouton-like structures under the OHCs (most likely efferent terminals) and the OSB and weakly the basolateral pole of the IHC and the ISB (Fig. 2E). SG neurons showed membrane-bound, dot-like Ca$_{\beta}$3 immunoreactivity (Fig. 2F). Ca$_{\beta}$4 staining was found in a dot-like manner at the base of OHCs and along the basolateral membrane of IHCs (Fig. 2G), similar to the staining pattern for Ca$_{\beta}$1,3.$^{13}$ SG neurons showed a more diffuse staining for Ca$_{\beta}$4 (Fig. 2H).

Ca$_{\beta}$1,3 channels have been localized to the basolateral pole of IHCs and the very basal pole of OHCs.$^{13,27}$ Considering that at the age for which Ca$_{\beta}$1 immunohistochemical data are presented, P8-P10, inner and outer HCs and type I fibers are innervated by different types of efferent fibers from the superior olivary complex (medial olivocochlear efferents [MOC] onto immature IHCs and maturing OHCs, lateral olivocochlear efferents onto type I fibers),$^{28}$ voltage-activated Ca$^{2+}$ channels including Ca$_{\beta}$1 subunits must be expected at the axon terminals contacting IHCs/OHCs. As the contact sites of the efferent fibers are found in close co-localization to afferent contact sites,$^{29}$ it is impossible to unequivocally discriminate between Ca$_{\beta}$1 staining of the efferent boutons contacting the hair cells and Ca$_{\beta}$1 staining at IHC/OHC presynaptic sites.

In summary, mRNA and protein of all four Ca$_{\beta}$ subunits were detected in the organ of Corti, but a clear allocation to IHCs or OHCs is impossible. We therefore analyzed hearing function and HC Ca$^{2+}$ currents using Ca$_{\beta}$-deficient mice. As Ca$_{\beta}$1 is the predominant Ca$_{\beta}$ subunit in skeletal muscle and hence essential for breathing,$^{3}$ and Ca$_{\beta}$2 the predominant Ca$_{\beta}$ subunit in cardiac muscle,$^{3,25}$ deletion of these subunits in mice leads to death after birth or during embryonic development, respectively. For these reasons we concentrated on a mouse model with a targeted deletion of CACNB3, Ca$_{\beta}$3,$^{30}$ and one with a disrupted CACNB4 gene due to a spontaneous 4 bp insertion, the so-called letargic mouse,$^{31}$ Ca$_{\beta}$4lh/lh.

**Hearing function in Ca$_{\beta}$3$^{-/-}$ and Ca$_{\beta}$4lh/lh mice.** To test if the lack of either Ca$_{\beta}$3 or Ca$_{\beta}$4 impairs the signal transmission between IHCs, auditory nerve and brainstem nuclei, auditory brainstem responses (ABR) were recorded in Ca$_{\beta}$3$^{-/-}$ and Ca$_{\beta}$4lh/lh mice and wild-type control mice at 3–4 weeks of age. Click-evoked ABR thresholds for both Ca$_{\beta}$3$^{-/-}$ and Ca$_{\beta}$4lh/lh mice were indistinguishable from their controls (Fig. 3A). Frequency-specific pure tone thresholds (Fig. 3B and C) documented unimpaired transmission of the acoustic signal up the auditory pathway in Ca$_{\beta}$3$^{-/-}$ (Fig. 3B) and Ca$_{\beta}$4lh/lh mice (Fig. 3C). These results indicate that neither Ca$_{\beta}$3 nor Ca$_{\beta}$4 are essential for synaptic transmission at the IHC and at the next 1–2 afferent synapses in the auditory
pathway. Hearing performance was further tested by measuring distortion product otoacoustic emissions (DPOAE). DPOAEs provide information about the electromotility of outer hair cells. Though electromotility itself does not require voltage-activated Ca\(^{2+}\) channels, OHC activity is modulated by the intracellular Ca\(^{2+}\) level and by inhibitory efferent fibers that use Ca\(^{2+}\) channels for transmitter release. Deletion of Ca\(_{V}^{3}\) subunits point to a difference in the Ca\(_{V}^{3}\) channel currents in mouse hair cells.

**Effect of deletion of the Ca\(_{V}^{3}\) or Ca\(_{V}^{4}\) subunit on the Ba\(^{2+}\) current amplitude in mouse hair cells.** ABR thresholds are a relatively gross measure for hearing assessment and therefore only indirectly depict details about the IHC receptor potential and underlying currents. To test if Ca\(_{V}^{3}\) or Ca\(_{V}^{4}\) are essential for generation of IHC Ca\(^{2+}\) currents, we recorded Ca\(^{2+}\) channel currents using 10 mM Ba\(^{2+}\) as a charge carrier (I\(_{Ba}\)) in both, neonatal and mature IHCs of Ca\(_{V}^{3}\)-/- and Ca\(_{V}^{4}\) mice. Ca\(^{2+}\) currents were also measured in OHCs aged P1-P4, since I\(_{Ba}\) rapidly declines thereafter.

Currents were evoked by step depolarizations from -80 mV to -78 mV (mature IHCs) for 8 ms (Fig. 4). Representative I\(_{Ba}\) traces of wild-type (black traces) and mutant IHCs (gray traces) of the neonatal (Fig. 4A) and mature Ca\(_{V}^{3}\) (Fig. 4D) and the neonatal (Fig. 4G) and mature Ca\(_{V}^{4}\) group (Fig. 4K) at three selected voltages are shown. In IHCs of all genotypes, depolarization produced fast activating and deactivating inward currents. I-V relations were determined for all cells by averaging I\(_{Ba}\) in the last millisecond of the depolarizing step. Averaged I-V relations (mean ± SEM) are depicted in Figure 4B, E, H and L. Deletion of Ca\(_{V}^{3}\) had no effect on average peak current size, neither at neonatal (Fig. 4B and C) nor at mature (Fig. 4E and F) age. The deletion of the Ca\(_{V}^{4}\) subunit shifted the neonatal I-V curve by 7 mV to negative voltages (Fig. 4H; for voltage-dependent activation parameters see below), but did not change average peak I\(_{Ba}\) amplitudes (Fig. 4I). In contrast, average peak I\(_{Ba}\) of mature Ca\(_{V}^{4}\) IHCs was smaller compared with the WT (Fig. 4L and M; p = 0.049).

Neonatal OHCs of either Ca\(_{V}^{3}\) or control group, respectively, showed qualitatively similar voltage-activated Ba\(^{2+}\) currents as IHCs but with smaller amplitudes (Fig. 5A and D). Average OHC peak I\(_{Ba}\) of Ca\(_{V}^{3}\) mice was significantly diminished in comparison to Ca\(_{V}^{3}\) mice (Fig. 5B and C, p = 0.0004). In contrast, I-V curves (Fig. 5E) and average peak I\(_{Ba}\) values of Ca\(_{V}^{4}\) IHCs were not different.

Taken together, deletion of the Ca\(_{V}^{3}\) or Ca\(_{V}^{4}\) subunit partially affected I\(_{Ba}\) amplitude depending on HC type and age. Deletion of Ca\(_{V}^{3}\) markedly reduced I\(_{Ba}\) amplitude in OHCs but not IHCs. By contrast, absence of Ca\(_{V}^{4}\) had no effect on the I\(_{Ba}\) amplitude in OHCs and neonatal IHCs, but on I\(_{Ba}\) in mature IHCs. These results indicate a contribution of both Ca\(_{V}^{3}\) and Ca\(_{V}^{4}\) to the voltage-activated Ca\(^{2+}\)/Ba\(^{2+}\) currents in inner and outer HCs and point to a difference in the Ca\(_{V}\) subunit composition not only between inner and outer HCs, but also with respect to different developmental stages of the IHCs.
Effect of deletion of the Ca\(\beta_3\) or Ca\(\beta_4\) subunit on cell size and \(I_{\text{Ba}}\) activation properties. To assess if cell growth was affected by deletion of Ca\(\beta_3\) or Ca\(\beta_4\), membrane capacitances, \(C_m\), were evaluated (Table 1). Average \(C_m\) values of neonatal Ca\(\beta_3^{\text{wt}}\) and Ca\(\beta_3^{-/-}\) IHCs were not different. In contrast, capacitances of neonatal Ca\(\beta_4^{\text{lh/lh}}\) IHCs were smaller than in Ca\(\beta_4^{\text{wt}}\) (Table 1, \(p = 0.0029\)). Interestingly, neonatal Ca\(\beta_4^{\text{wt}}\) IHCs were already quite large, though not significantly larger than Ca\(\beta_3^{\text{wt}}\) IHCs, and did not further grow upon maturation (Table 1). After the onset of hearing, no difference in \(C_m\) could be noted between all groups. In neonatal OHCs, deletion of either Ca\(\beta_3\) or Ca\(\beta_4\) had no effect on cell capacitance. However, a strain-
IHCs was unaffected, but it was slightly shifted to positive values in OHCs compared with CaVβ3wt (Table 1, p = 0.0499). In neonatal IHCs of CaVβ4lh/lh mice, Vh was shifted significantly to more negative values (Table 1, p = 0.0042), which was also evident from the I-V curves (Fig. 4H). This difference in IHC Vh vanished with age. Vh of OHCs was not affected by functional deletion of CaVβ4 (Table 1). The lack of either CaVβ3 or CaVβ4 did not cause a change of the slope factor of I Ba activation in any dependent difference was noted in Cm of CaVβ3wt and CaVβ4wt OHCs (Table 1, p = 0.011).

To analyze the voltage-dependence of I Ba activation, I-V curves were fitted with a product of a second order Boltzmann function and the Goldman-Hodgkin-Katz equation for description of the driving force for Ba2+ (Methods, Equation 1). The voltages of half-maximum activation (Vh) were averaged for each HC and age group (Table 1). In CaVβ3wt mice, Vh of neonatal and mature IHCs was unaffected, but it was slightly shifted to positive values in OHCs compared with CaVβ3wt (Table 1, p = 0.0499). In neonatal IHCs of CaVβ4lh/lh mice, Vh was shifted significantly to more negative values (Table 1, p = 0.0042), which was also evident from the I-V curves (Fig. 4H). This difference in IHC Vh vanished with age. Vh of OHCs was not affected by functional deletion of CaVβ4 (Table 1). The lack of either CaVβ3 or CaVβ4 did not cause a change of the slope factor of I Ba activation in any

Table 1. Hair cell membrane capacitances and I Ba activation parameters were partially affected by deletion of CaVβ3 or CaVβ4 subunits

|                | CaVβ3 | CaVβ3-/- | CaVβ3 | CaVβ3-/- | CaVβ3 | CaVβ3-/- |
|----------------|-------|----------|--------|----------|--------|----------|
|                | (P4-P7) | (P22)    | (P1-P3) | (P22)    | (P1-P3) |          |
| Cm (pF)        | 7.0 ± 1.1 | 6.8 ± 1.5 | 9.6 ± 2.7 | 9.0 ± 2.6 | 5.2 ± 0.6 | 4.9 ± 0.8 |
| Vh (mV)        | -22.6 ± 5.0 | -23.6 ± 4.8 | -13.3 ± 4.0 | -10.5 ± 0.6 | -17.1 ± 1.5 | -15.6 ± 3.7 |
| k (mV)         | 12.7 ± 1.0 | 13.0 ± 1.3 | 10.1 ± 1.6 | 11.2 ± 0.5 | 8.0 ± 0.1  | 8.8 ± 1.2  |

Membrane capacitances and voltage-dependent activation parameters of I Ba in neonatal and mature IHCs and neonatal OHCs of CaVβ3wt, CaVβ3-/-, CaVβ4wt and CaVβ4lh/lh mice. Voltages of half-maximum activation of I Ba, Vh, and the slope factor k were obtained from fitting I-V curves (Methods, Eq. 1). All values are given as mean ± SD, numbers of cells are indicated above each column.
of the HC groups studied apart from the mature CaVβ3+ IHCs (p = 0.046).

**Activation and inactivation kinetics of I_{Ba} in IHCs and OHCs of CaVβ3+ and CaVβ4+/−/mice.** Since CaVβ subunits may influence the activation kinetics of Ca2+ channels, we fitted I_{Ba} activation kinetics according to Equation 2 (see Methods) for IHCs and OHCs. Averaged activation time constants as a function of voltage are shown in **Figure 6.** Surprisingly, Ca2+ channels activated significantly faster in neonatal IHCs of both CaVβ3+ (Fig. 6A, p = 0.0153) and CaVβ4+/−/mice (Fig. 6C, p < 0.0001) compared with their controls. Additionally, activation time constants gave different functions of voltage for neonatal IHCs of CaVβ4+/− and CaVβ4+/−/mice (p < 0.0001). Comparison of I_{Ba} activation time constants of mature IHCs revealed no significant changes when lacking the CaVβ3 (Fig. 6B) or CaVβ4 subunit (Fig. 6D), respectively. In OHCs, deletion of either subunit affected I_{Ba} activation kinetics. In the CaVβ3 group, activation time constants gave different functions of voltage for the two genotypes (Fig. 6E, two-way interaction, p = 0.0091). When CaVβ4 was absent, OHC I_{Ba} activated consistently slower than in WT animals (Fig. 6F, p = 0.013).

To conclude, activation of the Ca2+ channels was accelerated in neonatal but not in mature IHCs due to the lack of either CaVβ subunit investigated, which suggests the presence of both CaVβ3 and CaVβ4 in neonatal IHCs and a change in the subunit composition during development. Furthermore, both CaVβ3 and CaVβ4 seem to contribute to I_{Ba} of neonatal OHCs. A surprising finding was that deletion of the CaVβ4 subunit had opposite effects on I_{Ba} in neonatal IHCs—where activation kinetics was accelerated—and on I_{Ba} in neonatal OHCs—where activation was slowed—compared with WT animals.

Voltage-activated Ca2+ channels are subject to voltage-dependent (VDI) and Ca2+-dependent inactivation. With Ba2+ as the charge carrier we assessed VDI over long depolarizations (400 ms). **Figure 7A–D** shows example I_{Ba} traces for mature IHCs and neonatal OHCs. In all HCs, inactivation was quantified at V = V_{max} + 10 mV. This voltage was chosen because at V_{max} where inactivation was usually determined, I_{Ba} of neonatal IHCs of both CaVβ3+ and CaVβ4+ mice showed a slowly activating component masking any inactivation (Fig. 7A, inset). This late activating I_{Ba} component was exclusively present in IHCs of the CaVβ3 background and vanished with age. At V = V_{max} + 10 mV, I_{Ba} inactivation over-ruled the slowly activating component.

Mature IHCs of each group (Fig. 7A and B) showed weak I_{Ba} inactivation in response to sustained depolarization, as did neonatal OHCs (Fig. 7C and D). When the degree of inactivation 300 ms after peak I_{Ba} (% of peak value) was calculated it became apparent that deletion of the CaVβ3 or CaVβ4 subunit had no effect on VDI in neonatal (Fig. 7E, left) and mature IHCs (Fig. 7E, middle). However, comparison of I_{Ba} inactivation of neonatal CaVβ3+ and CaVβ4+ IHCs revealed a significant difference (Fig. 7E, left), which was most likely caused by the slowly activating component in the CaVβ3 group (Fig. 7A, inset). I_{Ba} inactivation of CaVβ3−/− IHCs increased significantly with maturation (Fig. 7E, left and middle). In neonatal OHCs of CaVβ3+ mice, the degree of I_{Ba} inactivation was significantly elevated compared

![Figure 6. Activation time constants of Ba2+ currents in hair cells are affected by deletion of Ca3, and Ca4 subunits. (A–F) Voltage-dependent I_{Ba} activation time constants, act obtained by fitting the currents by a Hodgkin-Huxley model with two gating particles (Eq. 2, 3) in neonatal and mature IHCs and neonatal OHCs of CaV3wt, CaV3−/−, CaV4wt, and CaV4−/− mice. (A) I_{Ba} activation was faster in neonatal CaV3−/− IHCs (n = 3) compared with CaV3+ IHCs (n = 3). (B) No difference was noted between activation time constants of mature CaV3+ IHCs (n = 3) and CaV3−/− IHCs (n = 4). (C) I_{Ba} activation was faster in neonatal CaV4−/− IHCs (n = 6) compared with CaV4+ IHCs (n = 8). (D) Again, this difference in activation time constants was no longer present in mature CaV4+ IHCs (n = 7) versus CaV4−/− IHCs (n = 7). (E) Activation time constants of I_{Ba} in neonatal CaV3−/− (n = 4) and CaV3−/− OHCs (n = 4–5) were different functions of voltage. (F) I_{Ba} activation was slower in neonatal CaV4−/− OHCs (n = 6) compared with CaV4+ OHCs (n = 5–6) over the whole voltage range. Activation time constants are given as mean ± SEM; *p < 0.05 for differences in the size of act; §p < 0.05 for differences in voltage dependence of act.
in chick hair cells. The immunostaining pattern observed for transport was either caused by the presence of these two mRNAs or by small contamination with mRNA coding for presynaptic proteins has been described.

Variable detection of CaVβ subunits in neonatal and mature IHCs (Table 2) were significantly changed in IHCs, because capacitance, Vh, and k were rather subtle. The current amplitude was significantly reduced in neonatal CaVβ3-/- OHCs and mature CaVβ4lh/lh IHCs, a consequence expected from the lack of a CaVβ subunit without compensatory mechanisms. As we found significant effects on Iba properties in both mouse models we conclude that both CaVβ3 and CaVβ4 normally contribute to CaV1.3 channel complexes in neonatal inner and outer HCs. One of the effects in neonatal IHCs of either mouse line was that lack of CaVβ3 and CaVβ4 speeded up Iba activation over the whole voltage range, making it unlikely that either CaVβ3 or CaVβ4 is the dominant CaVβ subunit in wild-type IHCs. Strikingly, OHCs revealed an opposite consequence of CaVβ4 deletion, a reduction of Iba activation speed. One could interpret this as differential compensatory mechanisms (‘subunit reshuffling’) working in neonatal inner and outer hair cells. The reduced Iba amplitude in CaVβ3-/- OHCs suggests that lack of CaVβ3 was less well compensated in neonatal OHCs.

A contribution of CaVβ3 and CaVβ4 to gating properties of Iba in addition to activation kinetics is further inferred by a difference in Vh in CaVβ3-/- OHCs and in young CaVβ4lh/lh IHCs and in k in mature CaVβ3-/- IHCs. Moreover, the degree of Iba inactivation
was increased in Ca\(\beta 3\) \textsuperscript{−/−} OHCs. In expression systems, addition of Ca\(\beta 1\),3,4 subunits to a pore forming Ca\(\alpha\) subunit always speeded up \(I_{\text{in}}\) inactivation, whereas only Ca\(\beta 2\)a was able to slow down inactivation.\textsuperscript{35,36} In this context it is interesting to note that synaptic transmission between photoreceptor and bipolar cells in mouse retina requires the presence of Ca\(\beta 2\) subunits.\textsuperscript{3} Ca\(\textsuperscript{2+}\) channels at ribbon synapses in mammalian photoreceptors are slowly-inactivating and employ the Ca\(\alpha 1.4\) \textsuperscript{α} subunit,\textsuperscript{1,2,4,5} which has the highest sequence homology to Ca\(1.3\).\textsuperscript{2,3} The similarities between synaptic transmission in HCs and photoreceptors (sustained release using ribbon synapses and slowly inactivating \(L\)-type Ca\(\textsuperscript{2+}\) channels) suggest a role of the Ca\(\beta 2\) subunit also for inner and outer HCs. Experiments using mice with hair-cell specific deletion of Ca\(\beta 2\) are needed to clarify this point.

Unexpectedly, we found a significant difference between neonatal IHCs of the two wild-type strains Ca\(\beta 3\) and Ca\(\beta 4\) regarding the degree of \(I_{\text{in}}\) inactivation, which was caused by a slowly activating \(I_{\text{in}}\) component for voltages \(< V_{\text{ma}}\) in Ca\(\beta 3\) IHCs (cf. Fig. 7A, inset). In addition, OHC capacitances were different in the two wild-type lines, pointing to certain variability in properties of mouse hair cells from different wild type strains. This might be due to the fact that Ca\(\beta 4\) mice derived from heterozygous Ca\(\beta 4\)\textsuperscript{+/-} breedings are still genetically heterogeneous with the 129 SvJ strain (here named Ca\(\beta 3\)) that we used as controls for Ca\(\beta 3\)\textsuperscript{−/−}.

To summarize, a differential mixture of Ca\(\beta\) subunits, including at least Ca\(\beta 2\), Ca\(\beta 3\) and Ca\(\beta 4\), is present in inner and neonatal outer HCs. Viable mouse models with a hair-cell specific deletion of Ca\(\beta 1\) and Ca\(\beta 2\) will help to better understand the differences and developmental modulations of Ca\(\textsuperscript{2+}\) currents in inner and outer HCs.

**Methods**

**Animals.** NMRI mice and Wistar rats (both Charles River, Sulzfeld, Germany) were used for RT-PCR and histohemistry. Homozygous mice with a targeted deletion of the CACNB3 gene,\textsuperscript{30} Ca\(\beta 3\)\textsuperscript{−/−}, were backcrossed for 10 generations in a 129/SvJ background; 129/SvJ wild-type mice served as controls (Ca\(\beta 3\)). Lethargic mice bearing a 4 bp insertion in the \textit{CACNB4} gene which leads to a frameshift and non-functional Ca\(\beta 4\) protein\textsuperscript{41} were backcrossed in a 129/SvJ background. Heterozygous breeding yields Ca\(\beta 4\)\textsuperscript{+/-} mice and Ca\(\beta 4\)\textsuperscript{−/-} littermate controls. For electrophysiology we used exclusively +/- animals as wild-type controls, for hearing measurements both +/- and +/-, which may be justified as only homozygous animals show the lethargic phenotype.\textsuperscript{31} Animal experiments were approved and complied with all protocol requirements at the University of Tübingen. All efforts were made to minimize the number of animals used and their suffering. Animals were killed by decapitation and cochleae were removed according to national ethical guidelines.

**RT-PCR.** Organs of Corti were dissected from NMRI mice at P4-P5 and P18-P20 and cDNA was synthesized as described.\textsuperscript{20} Mouse cortex or heart served as control tissues. For IHC/OHC-specific analysis of Ca\(\beta 1\)-4 expression, organs of Corti were dissected and fixed on a coverslip. After Hensen's and Claudius' cells had been removed with cleaning pipettes, 30–60 OHCs were harvested with pipettes of 10 μm tip opening under fast flow of Tris-Cl solution (see below). Subsequently, pillar cells were removed and about 30 IHCs were harvested with their adjacent supporting cells (inner phalangeal and border cells). For cDNA first strand synthesis, see ref. 20. For detection of Ca\(\beta\) subunits in IHCs/OHCs, a nested PCR approach was used as the amount of cDNA for a single PCR reaction corresponded to a small number of hair cells (5–15), only. PCR analysis was performed using 1–6 μl cDNA, 1–2 μl forward and reverse primer at 10 pmol/μl each, 1 puReTaq Ready-to-go PCR bead (GE Healthcare, Munich, Germany), or 12.5 μl MasterMix-S (Peqlab, Erlangen, Germany), with the reaction volume adjusted to 25 μl with dH\(\text{O} \), through 35 cycles (30’’ at 94°C; 30’’ at 58–65°C; 40’’ at 72°C). The cDNA was purified by glycogen precipitation\textsuperscript{44} as this step improved the detectability of PCR transcripts. The following external primer pairs spanning at least one exon-intron boundary and internal primers were used (5’–3’):

- Ca\(\beta 1\)\textsubscript{for}, GCC CAA GGA CTT CCT ACA CAT CAA GG, Ca\(\beta 1\)\textsubscript{rev}, GGA ACG CGT GTT GGGA GTC CTC AAT G, Ca\(\beta 1\)\textsubscript{for_nested}, CTG GTG AGA GAA GGG TGC GAG, Ca\(\beta 1\)\textsubscript{rev_nested}, GTT TTT GCT GGG GTT GTC GAC, yielding a 417 bp transcript for Ca\(\beta 1\)a, and a 551 bp transcript for Ca\(\beta 1\)b; Ca\(\beta 2\)\textsubscript{for}, CTT GCC GACTG CAT CGG GCA TAC TG, Ca\(\beta 2\)\textsubscript{rev}, CAG CTA CCC AAA CAG CTT TGG AAT TGT TCA GG, Ca\(\beta 2\)\textsubscript{for_nested}, CAC CCT CCC AGC GTA CCC CCA CGG ACG AGA GGA CAT CAA ATA CAA AHA GAC, yielding a 704 bp transcript; Ca\(\beta 3\)\textsubscript{for}, CTG TGG CAT TGG CTG TCA GGA CGA CCT AA, Ca\(\beta 3\)\textsubscript{rev}, CTC ACT GGC TCC CAT CAT CGT CCT TG, Ca\(\beta 3\)\textsubscript{for_nested}, TGG GTT CTC GGA TGA GGA GT, Ca\(\beta 3\)\textsubscript{rev_nested}, CTG ACA CCA TCC ACC ACC CGC CA, yielding a 622 bp transcript; Ca\(\beta 4\)\textsubscript{for}, GTG CCC TCG AAG ATG TGC CC, Ca\(\beta 4\)\textsubscript{rev}, AGG CTA CCC AAA CAG CTT TCT ACT CGT ACT CTG, Ca\(\beta 4\)\textsubscript{for_nested}, GAC TGG TAA AAG AGG GCT GTG AGA TTG, Ca\(\beta 4\)\textsubscript{rev_nested}, GAT TGG TCT GGG ATG AGA TAA AAG GGT GAC TGG CCT ACC CGC CA, yielding a 872 bp transcript. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH)\textsuperscript{45} was performed in parallel as a positive control in IHC/OHC-specific PCR with primers: GAPDH\textsubscript{for}, AGG ACA GCA CCT GGT CCT ACC A, GAPDH\textsubscript{rev}, AGG ACA GCA CCT GGT CCT ACC A, yielding a 417 bp transcript.

**Immunocytochemistry.** Cochleae of NMRI mice and rats (P8–P20) were fixed with 2% paraformaldehyde for 2 hrs, decalified with Rapid Bone Decalcifier (Fisher Scientific, Houston, TX) if older than P10, freeze-substituted and embedded as described.\textsuperscript{46,47} Cryosections were stained with custom-made rabbit antibodies against Ca\(\beta 1\)-4,\textsuperscript{48,49} and with secondary Cy3-conjugated antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Immunostainings using different tissues of wild-type and Ca\(\beta 3\)\textsuperscript{−/−} and Ca\(\beta 4\)\textsuperscript{−/−} mice confirmed specificity of the antibodies against Ca\(\beta 3\),\textsuperscript{48} and Ca\(\beta 4\) (Flockerzi V,
unpublished data). Specimens were embedded with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) to stain cell nuclei (blue) and viewed using an Olympus AX70 microscope equipped with epifluorescence illumination. Staining was detected in at least three specimens of ≥3 animals.

**Auditory evoked brainstem responses and otoacoustic emissions.** Auditory brainstem responses (ABR) to click and pure tone auditory stimuli and the cubic 2*f1-f2 distortion product (DP) of the otoacoustic emissions (DPOAE) for f2 = 1.24*f1 and L2 = L1-10 dB were recorded in anesthetized mice aged 3–4 weeks as described.\(^{47,50}\) In short, ABR thresholds were determined through Ca\(^{2+}\) channel currents. I\(\text{Ba}\) of OHCs and IHCs <P\(\text{12}\) were measured using the current elicited by a -10 mV voltage pre-pulse from the holding potential preceding every voltage for half-maximum activation, and k is the slope factor of the Boltzmann function. This description uses the Goldman-Hodgkin-Katz equation for describing the driving force for Ba\(^{2+}\) ions.\(^{53}\) The exponent 2 was found to fit the data best (reviewed in ref. 52).

### Data analysis

Fits of I-V curves were performed assuming the Ca\(^{2+}\) channel current can be described by a Hodgkin-Huxley model with two gating particles.\(^{52}\) 1-V curves of Ba\(^{2+}\) currents obtained by step depolarizations were fitted according to equation:

\[
I = P_{\text{max}} \frac{zFV}{RT} \exp\left(\frac{\nu - V}{k}\right)
\]

where I is the I\(\text{Ba}\) at the time the I-V was calculated; \(P_{\text{max}}\) the maximum permeability; \(v = zF/(RT)\), with z being 2, \(\nu\) the Faraday constant, R the universal gas constant, T the absolute temperature, V the membrane potential. \([\text{Ba}]_o, [\text{Ba}]_i\) denote the intra- and extracellular Ba\(^{2+}\) concentration, respectively; \(V_h\) is the voltage for half-maximum activation, and k is the slope factor of the Boltzmann function.

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References

1. Ertel EA, Campbell KP, Harpold MM, Hofmann F, Mori Y, Perez-Reyes E, et al. Nomenclature of voltage-gated calcium channels. Neuron 2000; 25:533-5.

2. Carreras WA. Structure and regulation of voltage-gated Ca\(^{2+}\) channels. Annu Rev Cell Dev Biol 2000; 16:521-55.

3. Arikakh J, Campbell KP. Auxiliary subunits: essential components of the voltage-gated calcium channel complex. Curr Opin Neurol 2003; 16:298-307.

4. Dolphin AC. Beta subunits of voltage-gated calcium channels. J Bioenerg Biomembr 2003; 35:599-620.

5. Streissnig J, Koschak A. Exploring the function and pharmacotherapeutic potential of voltage-gated Ca\(^{2+}\) channels with gene knockout models. Channels (Austin) 2008; 2:233-51.

6. Hidalgo P, Neely A. Multiplicity of protein interactions and functions of the voltage-gated calcium channel beta-subunit. Cell Calcium 2007; 42:389-96.

7. He LL, Zhang Y, Chen YH, Yamada Y, Yang J. Functional modularity of the beta2 subunit of voltage-gated Ca\(^{2+}\) channels. Biophys J 2007; 93:834-45.

8. Gregg RG, Messing A, Strube C, Beurg M, Moss R, et al. Absence of the alpha subunit (cchb1) of the skeletal muscle dihydropyridine receptor alters expression of the beta1 subunit and eliminates excitation-contraction coupling. Proc Natl Acad Sci USA 1996; 93:13961-6.

9. Ball SL, Powers PA, Shin HS, Morgans CW, Peachey NS, Gregg RG. Role of the beta(2) subunit of voltage-dependent calcium channels in the retinal outer plexiform layer. Invest Ophthalmol Vis Sci 2002; 43:1595-603.

10. Ludwig A, Flockher V, Hofmann E. Regional expression and cellular localization of the alpha1 and beta subunits of high voltage-activated calcium channels in rat brain. J Neurosci 1997; 17:1359-49.

11. Vance CL, Begg CM, Lee WL, Haase H, Copeland RD, McEnery MW. Differential expression and association of calcium channel alpha1B and beta subunits during rat brain ontogenesis. J Biol Chem 1998; 273:14495-502.

12. Brandt A, Streissnig J, Moser T. Ca3,3 channels are essential for development and presynaptic activity of cochlear inner hair cells. J Neurosci 2003; 23:10832-40.

13. Knirsch M, Brandt N, Braig C, Kuhn S, Bova M, T rost C, Ludwig A, et al. Pain perception in mice lacking the BK channel alpha, beta1 or beta4 subunits. Proc Natl Acad Sci USA 2003; 100:307-12.

14. Brandt A, Khmisch D, Moser T. Few Ca3,3 channels regulate the exocytosis of a synaptic vesicle at the hair cell ribbon synapse. J Neurosci 2005; 25:11577-85.

15. Simonds DD. Development of the inner ear sensory system across vertebrate species. J Neurobiol 2002; 53:228-50.

16. Pujol R, Lavigne-Rebillard M, Lenoir M. Development of sensory and neural structures in the mammalian cochlea. In: Rubel E, Popper A, Fay R, eds. Development of sensory and neural structures in the mammalian cochlea. Sunderland, MA: Sinauer Associates 1991.

17. Berggren PO, Yang SN, Murakami M, Efamov AM, Uhles S, Kohler M, et al. Removal of Ca\(^{2+}\) channel beta3 subunit enhances Ca\(^{2+}\) oscillation frequency and insulin exocytosis. Cell 2004; 119:273-84.

18. Rehmann E, Diederich M, Tenzer H, et al. Cardiac L-type calcium channel subunits in the developing and adult heart. Circ Res 2002; 90:626-33.

19. Fischmann B, Deterding U, Blin N, et al. Two classes of outer hair cells along the peripheral and central auditory systems. J Neurophysiol 2004; 92:2633-41.

20. Lipcombe D, Heilman TD, Xu W. L-type calcium channels: the low down. J Neurophysiol 2004; 92:2633-41.

21. Trumpp A, Zeitler D, van der Geer P, et al. The presynaptic function of voltage-gated calcium channels. Neuron 2003; 39:1595-603.

22. Pytte M, Bandlow C, Guseva L, Koppach I, Rohbock K, et al. Thyroid hormone affects Schwann cell and oligodendrocyte gene expression at the glial transition zone of the VIIIth nerve prior to cochlea function. Development 1998; 125:3709-18.

23. Sun Y, T. Macleish PR. Ionic conductances of monkey solitary cone inner segments. J Neurophysiol 1994; 71:656-65.

24. Morgans CW. Localization of the alpha1F calcium channel subunit in the rat retina. Invest Ophthalmol Vis Sci 2001; 42:2441-8.

25. Miller GM, Zhang X, Brown P, et al. Improved quantitative real-time RT-PCR for expression profiling of individual cells. Nucleic Acids Res 2002; 30:89.

26. Barber RD, Harmer DW, Coleman RA, Clark BJ. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physical Genomics 2005; 21:389-95.

27. Knipper M, Bandlow C, Guseva L, Koppach I, Rohbock K, et al. Thyroid hormone deficiency before the onset of hearing causes irreversible damage to peripheral and central auditory systems. J Neurophysiol 2000; 83:3101-12.

28. Berggren PO, Yang SN, Murakami M, Efamov AM, Uhles S, Kohler M, et al. Removal of Ca\(^{2+}\) channel beta3 subunit enhances Ca\(^{2+}\) oscillation frequency and insulin exocytosis. Cell 2004; 119:273-84.

29. Rehmann E, Diederich M, Tenzer H, et al. Cardiac L-type calcium channel subunits in the developing and adult heart. Circ Res 2002; 90:626-33.