Cav3.2 T-Type Calcium Channels Are Physiologically Mandatory for the Auditory System

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Abstract—Voltage-gated Ca2+ channels (VGCCs) play key roles in auditory perception and information processing within the inner ear and brainstem. Pharmacological inhibition of low voltage-activated (LVA) T-type Ca2+ channels is related to both age- and noise induced hearing loss in experimental animals and may represent a promising approach to the treatment of auditory impairment of various etiologies. Within the LVA Ca2+ channel subgroup, Cav3.2 is the most prominently expressed T-type channel entity in the cochlea and auditory brainstem. Thus, we performed a complete gender specific click and tone burst based auditory brainstem response (ABR) analysis of Cav3.2+/- and Cav3.2-/- mice, including i.a. temporal progression in hearing loss, amplitude growth function and wave latency analysis as well as a cochlear qPCR based evaluation of other VGCCs transcripts. Our results, based on a self-programmed automated wavelet approach, demonstrate that both heterozygous and Cav3.2 null mutant mice exhibit age-dependent increases in hearing thresholds at 5 months of age. In addition, complex alterations in W1,4v amplitudes and latencies were detected that were not attributable to alterations in the expression of other VGCCs in the auditory tract. Our results clearly demonstrate the important physiological role of Cav3.2 VGCCs in the spatiotemporal organization of auditory processing in young adult mice and suggest potential pharmacological targets for interventions in the future. © 2019 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Key words: auditory brainstem response, calcium channel, hair cells, spiral ganglion neuron, T-type, sensorineural hearing loss.

INTRODUCTION

Loss of hair cells (HCs) and spiral ganglion neurons (SGN) is the major cause of age-related hearing loss (ARHL), i.e., presbycusis which normally affects high frequencies first (Schacht and Hawkins, 2005) and displays a prevalence of > 50% in populations > 75 years of age (Gates and Mills, 2005). Calcium dyshomeostasis was proven to be of central relevance for age- and noise-related impairment of neuroacoustic function (Bao et al., 2005; Buchholz et al., 2007) with different types of voltage-gated Ca2+ channels (VGCCs) being expressed in inner and outer hair cells (OHCs and IHC, respectively), SGNs, the cochlear nucleus, the trapezoid body, the lateral superior olive (LSO) and...
further ascending components (Lopez et al., 2003; Lee et al., 2007; Shen et al., 2007; Nie et al., 2008; Simms and Zamponi, 2014; Zamponi, 2016, 2017). Based on electrophysiological and pharmacological properties, the pore-forming Ca$_{\text{v}}$-$\alpha_1$ subunits of VGCCs complexes are subdivided into high voltage-activated (HVA), i.e., Ca$_{\text{v}}$1.1–1.4 L-type and Ca$_{\text{v}}$2.1–2.3 Non-L-type Ca$^{2+}$ channels, and low voltage-activated (LVA) Ca$_{\text{v}}$3.1–3.3 T-type Ca$^{2+}$ channels. The Ca$_{\text{v}}$1.3 VGCC was the first to be correlated with auditory dysfunction, as ablation of Ca$_{\text{v}}$1.3 in mice results in degeneration of IHCs and OHCs with subsequent deafness (Platzer et al., 2000; Glueckert et al., 2003). In addition, Ca$_{\text{v}}$1.3 was shown to be involved in the activity-dependent development of the auditory brainstem, evidenced by Ca$_{\text{v}}$1.3 deficient mice exhibiting a dramatic volume reduction in all auditory brainstem centers that occurred even before hearing onset (Hirtz et al., 2011). Thus, VGCC dysfunction can cause severe functional and developmental alterations in the peripheral auditory tract due to a highly complex and fine-tuned system of balanced Ca$^{2+}$ influx that regulates spatiotemporal auditory processing.

Based on in vitro and in vivo findings on HC physiology, Ca$_{\text{v}}$1.3 VGCCs were originally considered potential candidates for the etiopathogenesis of ARHL and noise-induced hearing loss (NIHL) as well as targets for their prevention and therapy. Interestingly, age-related increases in brainstem-evoked response audiometry (BERA) thresholds turned out to be associated with a gradual decrease of Ca$_{\text{v}}$1.3 expression in IHC, OHC and the stria vascularis supporting a potential role for Ca$_{\text{v}}$1.3 in ARHL (Chen et al., 2013). Initially, it was suggested that only L-type VGCCs are involved in acoustic injury of the cochlea, as L-type blockers, e.g., diltiazem, verapamil and dihydropyridines (DHPs), but not T-type blockers such as mibefradil and flunarizine, seemed to decrease HC loss (Mills et al., 1999; Uemaetomari et al., 2009).

However, T-type VGCCs also exhibit complex spatiotemporal expression patterns in the auditory system, e.g., HCs (Inagaki et al., 2007), the cochlear nucleus (Kim and Trussell, 2007), the superior paraolivary nucleus (SPON) (Felix et al., 2011), and the LSO (Adam et al., 2001). Early immunohistochemical studies in 2 month old C57Bl/6 mice suggested weak Ca$_{\text{v}}$3.1 and Ca$_{\text{v}}$3.3, but no Ca$_{\text{v}}$3.2 VGCC expression in the organ of Corti. SGNs, however, exhibit dominant Ca$_{\text{v}}$3.2 with only moderate Ca$_{\text{v}}$3.1 and Ca$_{\text{v}}$3.3 expression (Shen et al., 2007). Whole cochlear qPCR in the same setting revealed highest transcript levels for Ca$_{\text{v}}$3.2 exceeding those for Ca$_{\text{v}}$3.1 and Ca$_{\text{v}}$3.3 by 2-fold and 100-fold, respectively (Shen et al., 2007). Additionally, no gender specific differences in expression levels were detected. Further quantification of T-type Ca$^{2+}$ channels in the cochlea of young 6–8 wk old C57Bl/6J mice again revealed transcripts of all three T-type Ca$^{2+}$ channels with Ca$_{\text{v}}$3.2 exhibiting the lowest expression (Yu et al., 2016). Lei et al. (2011) analyzed all three Ca$_{\text{v}}$3 T-type channel transcript levels in C57Bl/6J cochlea at 2, 4 and 8 months of age in which Ca$_{\text{v}}$3.2 levels clearly predominated and increased with age (Lei et al., 2011). This tendency was later confirmed for SGNs in C57Bl/6 mice aged 6–44 wk in which Ca$_{\text{v}}$3.2 transcripts also predominated (Yu et al., 2015). Thus Ca$_{\text{v}}$3 Ca$^{2+}$ channels, particularly Ca$_{\text{v}}$3.2, display a complex developmental, i.e., spatiotemporal expression pattern within the cochlea.

Importantly, it was shown in 9–11 month old mice that ablation of Ca$_{\text{v}}$3.2 results in a significant delay of age-related loss of cochlear function and preservation of SGNs, further stressing a potential role for Ca$_{\text{v}}$3.2 in ARHL (Lei et al., 2011). Consequently, Ca$_{\text{v}}$2.2 channel blockers and antiepileptic drugs (AEDs) known to inhibit T-type Ca$^{2+}$ channels, e.g., trimethadione, ethosuximide, and flunarizine, were shown to preserve SGNs during aging (Mills et al., 1999; So et al., 2005; Shen et al., 2007; Lei et al., 2011). This prophylactic and therapeutic effect of AEDs that block T-type VGCCs was later also confirmed for NIHL (Bao et al., 2013; Yu et al., 2016) and it was suggested that multi-drug-multi-target therapeutic approaches including block of T-type VGCCs e.g., via ethosuximide (Gomora et al., 2001) and zonisamide (Matar et al., 2009) in combination with dexamethasone, could be a feasible approach for NIHL treatment as well (Bao et al., 2013). Notably, the positive effects of HC preservation and hearing threshold consolidation in ARHL and NIHL models seem to be age-dependent. Yu et al. (2016) reported that C57Bl/6 mice aged 24–26 wk did not experience any beneficial impact in hearing threshold at 8, 16, or 32 kHz upon mibefradil and benidipine administration. Even more important, histomorphological deterioration was detected in IHCs, e.g. stereocilia of IHCs were disorganized and sparse upon mibefradil administration. In addition, OHC loss was found upon benidipine application.

Given the potential role of Ca$_{\text{v}}$3.2 in ARHL and NIHL, we performed complete gender specific auditory profiling of young adult Ca$_{\text{v}}$3.2$^{+/+}$ and Ca$_{\text{v}}$3.2$^{-/-}$ mice, including click and tone burst related threshold characterization, amplitude growth function and latency analysis, as well as qPCR of cochlear VGCC transcripts, to unravel the physiological role of Ca$_{\text{v}}$3.2 VGCCs in the cochlea and the ascending auditory tract. Our results demonstrate for the first time that Ca$_{\text{v}}$3.2 VGCCs are of tremendous functional importance for spatiotemporal auditory processing in different areas of the auditory system and that pharmacological interference needs careful consideration based on both beneficial and adverse age-related effects.

**EXPERIMENTAL PROCEDURES**

**Experimental animals**

Controls, heterozygous and homozygous Ca$_{\text{v}}$3.2 deficient mice were generated from cryopreserved heterozygous embryos obtained via the Mutant Mouse Resource & Research Centers (MMRRC, supported by NIH). For further details, see MMRCC stock number 9979, strain name: B6.129-Cacna1f$^{\text{tm1Kcam}}$/Mmmh, strain of origin: C57BL/6 × 129, strain genetic background: C57BL/6 (Chen et al., 2003). Wild type littermates were used as controls.
Fifty-five animals were included in this study: 23 Ca,3.2+/+ mice (12 ♀, mean body weight: 24.09 g ± 0.41 g; 11 ♂, mean body weight: 32.82 g ± 0.58 g), 15 Ca,3.2+/- mice (8 ♀, mean body weight: 23.50 g ± 0.41 g; 7 ♂, mean body weight: 33.11 g ± 0.81 g) and 17 Ca,3.2-/- mice (8 ♀, mean body weight: 22.10 g ± 0.43 g; 9 ♂, mean body weight: 29.09 g ± 0.75 g). Gender-specific auditory brainstem response (ABR) audiometry was performed in mice aged 140.67 ± 0.38 days (~ 20 wk). All mice were housed in groups of 2–5 in clear Macrolon cages type II with ad libitum access to drinking water and standard food pellets. Using ventilated cabinets (Model 9AV125PYN, Techniplast, Germany; UniProtect, Zoonlab, Germany) as a noise-protected environment, mice were maintained at a temperature of 21 ± 2°C, 50–60 % relative humidity, and on a conventional 12 h light/dark cycle with a light onset at 5:00 a.m. Animals were strictly adapted to this circadian pattern for 14 days preceding subsequent experimentation.

All animal procedures were performed according to the guidelines of the German Council on Animal Care, and all protocols were approved by the local institutional and national committee on animal care (LANUV, Germany). The authors further certify that all animal experimentation was performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised 1996 or the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of 24 November 1986 (86/609/EEC) and September 22nd, 2010 (2010/63/EU). Specific effort was made to minimize the number of animals used and their suffering.

Genotyping

Ca,3.2 mutant mice were genotyped by PCR based on the protocol of the KAPA Mouse genotyping kit (Sigma Aldrich, Germany). The following primers were used: WT-forward: 5′-CAT CTC AGG GCC TGT CCA GCA C-3′, WT-reverse/KO-forward: 5′-ATT CAA GGG CTG CCA CAG GGT A-3′, WT-reverse/ KO-reverse: 5′-GCT AAA GCG CAT GCT CCA GAC TG -3′ (see also Chen et al., 2003). PCRs were performed using a C1000 thermal cycler (BioRad, Germany) with initial denaturation (95°C – 1 min), followed by 35 cycles (denaturation 95°C – 15 sec, annealing 61°C – 15 sec, extension 72°C – 1 min) and final extension (72°C – 10 min). Subsequently, PCR products were separated via agarose gel electrophoresis and detected by ChemiDoc Touch (BioRad, Germany) (Fig. 1).

ABR recording procedure

Prior to ABR recordings, animals were anesthetized using intraperitoneal (i.p.) injection of ketamine (100 mg/kg body weight, Ketanest® S, 25 mg/ml Pfizer, Germany) and xylazine (10 mg/kg body weight, Rompun® 2%, Bayer, Germany) and placed inside a sound attenuating cubicle (ENV-018V, Med Association, Inc., USA) lined with an acoustic foam. The entire cubicle was covered with a custom made meshed metal Faraday cage (stainless steel, 2 mm thickness, 1 cm mesh size) to shield ABR recordings from external electrical interference and to protect from noise. Following anesthesia, animals were placed on a homeothermic heating blanket (ThermoLux®, Witte + Sutor, Murrhardt, Germany) inside the attenuating cubicle to maintain core body temperature. Eyes were covered with an eye ointment (5 % Dexpantenol, Bepanthen®, Bayer Vital GmbH, Germany) to protect against corneal desiccation.

For recording of monaural bioelectrical auditory potential, subdermal stainless steel electrodes (27GA 12 mm, Rochester Electro-Medical, USA) were inserted at the vertex, axial to the pinnae (positive (+) electrode) and ventrolateral to the right pinna (negative (-) electrode) (Fig. 2). The ground electrode was positioned at the hip of the animal. Impedance measurements of all electrodes were performed prior to each recording to verify proper electrode positioning / conductivity and were set < 5 kΩ. All ABR recordings were performed under free field conditions using a single loudspeaker (MF1 Multi-Function Speaker, Tucker-Davis Technologies, TDT, USA) placed 10 cm opposite the rostrum of the animals (loudspeaker leading edge perpendicular to the mouse interaural axis). Stimulus protocols for click and tone bursts were programmed using SigGenRZ software (Tucker-Davis Technologies, TDT, USA). The resultant bioelectrical signals recorded from the subdermal electrodes were transferred to a head stage (RA4LI, Tucker-Davis Technologies, TDT, USA) and further forwarded to the preamplifier (RA4PA, Tucker-Davis Technologies, TDT, USA) with 20-fold amplification. Acoustic stimulus presentation, equipment, e.g., loudspeaker control, ABR acquisition, processing, averaging and data management, were further coordinated using the RZ6 Multi I/O Processor system and BioSigRZ software (both Tucker-Davis Technologies, TDT, USA).

ABR data acquisition was performed at a sampling rate of 24.4 kHz, and signals were bandpass filtered (high pass 300 Hz, low pass 5 kHz) using a 6-pole Butterworth filter. Individual ABR data acquisition time was 25 ms, starting...
with a 5 ms baseline period prior to the individual acoustic stimulus onset (pre ABR baseline) and exceeding the 10 ms ABR section by another 10 ms baseline (post ABR stimulus onset (pre ABR baseline) and exceeding the 10 ms baseline period prior to the individual acoustic stimulus, 0.1 ms duration; tone burst, 4.5 ms duration). Ten ms after stimulus onset, another 10 ms baseline (post-stimulus TW) was recorded. Both baseline recordings (TW₄ + TW₂) were used to calculate the SD of baseline noise. The hearing threshold was reached whenever an individual ABR wave amplitude (labelled by Roman numerals I–IV) exceeded fourfold the SD of baseline noise. For wave amplitude and wave latency comparison, a “Mexican hat” based wavelet analysis was performed that automatically characterized negative peaks (blue-yellow striped lines) and positive peaks (red-grey striped lines) within the recorded ABR, thereby determining the temporal frames of W₅₋₄. Green crosses indicate maximum ABR wave amplitudes. Note that these maxima represent nominal values and do not indicate approximated values based on the “Mexican hat” based wavelet approach.

To analyze and identify frequency specific hearing thresholds in all genotypes, the frequency range 1–42 kHz were calibrated each day prior to recording using a microphone (378C01, PCB Piezotronics, NY, USA) connected to a preamplifier (480C02, PCB Piezotronics) and the RZ6 Multi I/O Processor system (Tucker-Davis Technologies, TDT, USA). The microphone was positioned inside the sound attenuating cubicle to mimic the experimental murine ear and connected to an oscilloscope (DPO3012, Tektronix, USA) to monitor and confirm the spectrum of sound stimuli using online Fast Fourier Transformation (FFT).

**Fig. 2. ABR analysis and ABR electrode position.** A) An individual ABR recorded at 65 dB SPL. An initial pre-stimulus 5 ms baseline (pre-stimulus TW₁) was followed by a transient acoustic stimulus (e.g., click, 100 μs duration; tone burst, 4.5 ms duration). Ten ms after stimulus onset, another 10 ms baseline (post-stimulus TW₂) was recorded. Both baseline recordings (TW₄ + TW₂) were used to calculate the SD of baseline noise. The hearing threshold was reached whenever an individual ABR wave amplitude (labelled by Roman numerals I–IV) exceeded fourfold the SD of baseline noise. For wave amplitude and wave latency comparison, a “Mexican hat” based wavelet analysis was performed that automatically characterized negative peaks (blue-yellow striped lines) and positive peaks (red-grey striped lines) within the recorded ABR, thereby determining the temporal frames of W₅₋₄. Green crosses indicate maximum ABR wave amplitudes. Note that these maxima represent nominal values and do not indicate approximated values based on the “Mexican hat” based wavelet approach. B) For monaural based ABR recordings, subdermal stainless steel electrodes were used. The reference electrode was positioned at the left hip, the positive electrode (+) was placed at the vertex (axial the pinnae) and the negative electrode (-) was located ventrolateral of the right pinna.

**ABR analysis – general aspects and software**

To avoid potential inconsistencies in ABR threshold determination by visual inspection/estimation, we performed automated threshold detection based on earlier publications (Bogaerts et al., 2009; Probst et al., 2013; Alvarado et al., 2014). For data processing and analysis, “R” software (The R Foundation, version 3.2.1, R Core Team 2015) was used with additional packages, including “reshape2” (version 1.4.1), “ggplot2” (version 1.0.1), “data.table” (version 1.9.4), “gdata” (version 2.13.3), “pastecs” (version 1.3.18), “waveslim” (version 1.7.5) and “MassSpecWavelet” (version 1.30.0; (Du et al., 2006). Wavelet analysis was performed using the “MassSpecWavelet” package (Du et al., 2006).

**Analysis of hearing thresholds**

To determine the click and tone burst derived thresholds of ABR recordings, three distinct time windows (TWs) were set to calculate the signal to noise ratio (SNR), i.e., TW₁ (pre stimulus, 0–5 ms), TW₂ (stimulus related, 5–15 ms) and TW₃ (post stimulus, 15–25 ms). Noise standard deviation of the baseline was calculated within two distinct TWs, i.e., TW₁ and TW₃ where no auditory evoked potentials (AEP) were observed (Fig. 2A). For each SPL measurement within an ABR record setting both mean and standard deviation were calculated for pooled TW₁ and TW₃ data. Subsequently, all recording samples were reset individually by the corresponding calculated mean. The threshold of hearing (TH) was determined by the lowest SPL (dB) where at least one wave amplitude (A) value (Wᵢ₋₄) in the ABR response time window (TW₂) exceeded fourfold of the previously calculated standard deviation (T[SP]dB; Aᵢ₋₄TW₂ > 4 × SD(TW₁,TW₃)). If no ABR wave (I–IV) was detected for frequency threshold
analysis at maximum SPL, a nominal threshold level of 100 dB was assigned.

**ABR wave amplitude and wave latency analysis**

To determine the temporal collocation of all positive (p) waves (peaks, see intercept points of red-grey lines with ABR trace) as well as negative (n) waves (pits, see intercept points of blue-orange lines with ABR trace, Fig. 2A), a "Mexican hat" wavelet based analysis was conducted which uses a default wavelet by the continuous wavelet transform (CWT)-based pattern-matching algorithm (Du et al., 2006). Mathematically, the CWT is represented as (Daubechies, 1992), see also (Valderrama et al., 2014):

\[ C(a, b) = \int_{-\infty}^{\infty} s(t) \psi_{a,b}(t) dt \]

\[ = \frac{1}{\sqrt{a}} \left( \frac{t-b}{a} \right)^{-\alpha}, a \in R^+ - \{0\}, b \in R, \]

where \( s(t) \) is the signal, \( a \) is the scale, \( b \) is the translation, \( \psi(t) \) is the mother wavelet, \( \psi_{a,b}(t) \) is the scaled and translated wavelet and \( C \) is the 2D matrix of wavelet coefficients.

In an initial step, a 65 dB measurement of each ABR run was used to identify the best scale parameters for each wave to be passed to the CWT, resulting in three classes: scale parameters 0.5–4 for all n-waves, 0.5–6 for all p-waves and 0.5–12 for wave IV, as this was the broadest wave within the samples. All classes proved to reliably detect the correct temporal collocation of waves I–IV within all 65 dB measurements.

To determine ABR waves I–IV in the accurate temporal sequence at 65 dB SPL, p-peaks (Fig. 2A, red-grey lines) and n-peaks (pits, Fig. 2A, yellow-blue lines) were identified in a fixed progression using relative positions of previously identified peaks to limit the TW of subsequent scans. Once all nine peaks had been characterized at 65 dB (Fig. 2A), these values were used as references (starting points) for all 65 dB measurements.

qPCR was performed in Ca\(_{3.2}\)\(^{++}\), Ca\(_{3.2}\)\(^{-/-}\) and Ca\(_{3.2}\)\(^{-/-}\)mice to identify potential alterations in cochlear transcript levels of other VGCCs, i.e., HVA L-type Ca\(_{1.2}\) and Ca\(_{1.3}\), HVA Non L-type Ca\(_{2.3}\), LVA T-type Ca\(_{3.1}\) and Ca\(_{3.3}\), that are known to be expressed within the cochlea and the auditory tract. For each genotype, the following gender specific subgroups were used for analysis: Ca\(_{3.2}\)\(^{++}\): \(♂\), \(n = 8, 21.27 \pm 0.38 \text{ wk}\); \(♀\), \(n = 8, 21.23 \pm 0.41 \text{ wk}\); Ca\(_{3.2}\)\(^{-/-}\): \(♂\), \(n = 8, 21.32 \pm 0.40 \text{ wk}\); \(♀\), \(n = 8, 21.04 \pm 0.31 \text{ wk}\); Ca\(_{3.2}\)\(^{-/-}\): \(♂\), \(n = 8, 21.41 \pm 0.21 \text{ wk}\); and \(♀\), \(n = 8, 20.98 \pm 0.21 \text{ wk}\). Note that experimental animals for cochlear qPCR analysis were not involved in ABR experiments to eliminate potential confounding factors.

Both cochleae of each individual animal were dissected in an RNase free environment (RNAlater® stabilization reagent, Qiagen, Germany) and snap-frozen in liquid nitrogen. Total RNA from both mouse cochleae was extracted using DirectZol™ RNA Micro Kit (Zymo Research, Freiburg i.Br., Germany) and snap-frozen in liquid nitrogen. Total RNA was evaluated using Nanodrop standard procedures (Nanodrop™ 1000, Thermo Fisher Scientific, Germany). Quality and quantity of total RNA was evaluated using Nanodrop standard procedures (Nanodrop™ 1000, Thermo Fisher Scientific, Germany). cDNA synthesis was performed via a two-step RT-PCR approach using both random hexamer and anchored-oligo(dT)\(_{12}\)\(_{18}\) primers with 250 ng total cochlear RNA from each animal for the final 50 μl first-strand cDNA mix (Transcriptor First-Strand cDNA synthesis Kit, Roche, Germany). cDNA (2 μl) served as a template for qPCR (see below), and signal detection was based on SYBR Green I Master (Roche, Germany). qPCR experiments were carried out using a LightCycler 480 System (Roche, Germany) with the following protocol (per cycle) being applied for all primer pairs (Table 1): 95°C (10 min, pre-incubation step); 95°C (10 s, denaturation step); 60°C (20 s, annealing step); 72°C (30 s, extension step). Forty total cycles were performed.

Every cochlea sample was tested in triplicate, and two negative controls were added in duplicate, (no template; no RT) for the qPCR 96-well-plate (Roche, Germany). Furthermore, to avoid inter-run variations and ensure statistical comparability among the plates, cochlea cDNA derived from C57Bl6/J mice served as a positive control and calibrator cDNA and was also used in triplicate on every plate. Amplification specificity was verified by melting curve analysis (LightCycler480 System Software, Roche). Deionized, nuclease-free water (no cDNA) and total RNA samples (without RT) were used as controls, and HPRT was utilized.
Table 1. Sequence of primer pairs used for qPCR (OriGene Technologies (a); Weiergräber et al., 2005 (b)).

| Gene   | Protein | Forward sequence (5′–3′) | Reverse sequence (5′–3′) |
|--------|---------|--------------------------|--------------------------|
| Cacna1c | Ca,1.2 α1 | GTTCTCATCCTGCTCAAACCC | GAGCTTCAGGATCTCCACCTG |
| Cacna1d | Ca,1.3 α1 | CTACGCTGCAACAGATGAAGCC | TACGGGACACAGACTGTCGA |
| Cacna1e | Ca,2.3 α1 | ATGACAGAGGCTACCAAGGAAGA | GACTGGTCTCCTATCTGTTT |
| Cacna1g | Ca,3.1 α1 | GACCATGTCCTCGTCTCATCA | TTTCAGCAAGAAGACTCGGT |
| Cacna1i | Ca,3.3 α1 | GTCTTCACCAAGATGGACGACC | ACTTCGCAAGCTCAGGTTT |
| Hprt   | Hprt    | GCTGTTGAAGAAGGTCTCT    | CAGAGGACTGAACTGGA |

as an internal reference gene. Ct-values (cycle threshold) were calculated using the LightCycler 480 System software (Roche).

Individual primer efficiency, analysis and qPCR statistics were performed using qBase + qPCR analysis software (Biogazelle, Gent, Belgium) which is based on a delta-Cq quantification model with PCR efficiency correction, reference gene normalization and inter-run calibration (Hellemans et al., 2007). All results were determined as CNRQ (Calibrated Normalized Relative Quantity) and statistically analyzed using the Mann-Whitney test.

**Statistical analysis**

All results in this study are presented as group means ± SEM using GraphPad Prism 6 software (V6.07 GraphPad Software, Inc., USA). Both genders were analyzed separately. Significant differences were compared using an ordinary one-way ANOVA for click-evoked hearing threshold analysis (Fig. 8) and latency analysis for each single wave (Fig. 12) followed by Tukey’s multiple comparisons test. Two-way repeated measure ANOVA followed by Tukey’s adjustment for multiple comparisons was performed to evaluate differences in tone burst evoked hearing thresholds (Fig. 10) and to calculate differences in amplitude growth function (Fig. 11). qPCR results were statistically analyzed using the Mann-Whitney test. Statistical significance was determined using α-level = .05 and P-values defined as *P < .05; **P < .01; ***P < .001; and ****P < .0001. Note that asterisks indicate significant differences between controls and transgenic animals (Ca,2.3+/+ and Ca,3.2+/+) whereas +/− icons represent significant differences between Ca,3.2+/+ and Ca,3.2−/−.

**RESULTS**

**Developmental alterations in Ca,3.2 transgenic mice**

The α1H knockout (Ca,3.2−/−) mouse model exhibits cardiac pathology (Chen et al., 2003), which is of particular significance as cardiovascular disease can promote hearing loss (Gates et al., 1993). Given the partially controversial results on the Ca,3,2 null mutant phenotype, we first unraveled potential developmental alterations in body weight in female and male controls (♀, n = 12; ♂, n = 11), Ca,2.3+/− (♀, n = 8; ♂, n = 7) and Ca,2.3−/− (♀, n = 8; ♂, n = 9) mice aged 140.67 ± 0.38 days. In females, a significant decrease in body weight was observed for Ca,3.2−/− mice compared to controls (Fig. 3). In males, Ca,3.2−/− mice exhibited a significant weight reduction compared to Ca,3.2+/+ mice and Ca,3.2+/− mice (Fig. 3A). In heterozygous mice, which do not display cardiac pathology (Chen et al., 2003), no alterations were observed in body weight compared to controls at 20 wk of age. Long-time body weight monitoring revealed reduced weight in female Ca,3.2+/− and Ca,3.2−/− mice with increased age (> 25 wk, Fig. 3B), and the same held true for male Ca,3.2 null mutants (Fig. 3C).

**Click and tone burst evoked ABRs in control, Ca,3.2+/− and Ca,3.2−/− mice**

To elucidate the role of Ca,3.2 VGCCs in auditory processing, we performed click and tone burst evoked ABR recordings to evaluate hearing threshold differences, amplitude growth function and latency comparison in Ca,3.2+/− mice (controls) and Ca,3.2+/− and Ca,2.3 null mutant mice (Ca,3.2−/−). Specific emphasis was placed on the evaluation of both genders, as gender-specific differences in auditory parameters and ARHL have been reported in both human (Pearson et al., 1995; Murphy and Gates, 1997) and mouse (Henry, 2004; Ison et al., 2007). ABRs to free field click (0.1 ms) and tone burst (1–42 kHz in 6 kHz steps, 4.5 ms in total with a 1.5 ms ramp time) acoustic stimuli were recorded using subdermal electrodes (for electrode positioning see Experimental Procedures). Note that vertex positive potentials are plotted as upward deflections as depicted in representative click-evoked recordings for female Ca,3.2+/+ (Fig. 4A), Ca,3.2+/− (Fig. 4B) and Ca,3.2−/− mice (Fig. 4C). Representative ABR recordings in females suggests increased click evoked ABR thresholds and reduced amplitude growth function (for details see below). Similarly, representative ABR recordings in males suggested increased click evoked ABR thresholds and reduced amplitudes in Ca,3.2+/− (Fig. 5C) compared to control (Fig. 5A) and heterozygous Ca,3.2+/− mice (Fig. 5B, for details see below). Representative tone burst evoked ABRs are depicted in Fig. 6A-C for females and in Fig. 7A-C for males. Note that representative ABR recordings suggest frequency specific hearing loss in the higher frequency range and reduced amplitude in both Ca,3.2+/− (Figs. 6B, 7B) and Ca,3.2−/− mice (Figs. 6C, 7C).

**Click related hearing thresholds in controls, Ca,3.2+/− and Ca,3.2−/− mice**

To evaluate the effect of Ca,3.2 allelic loss on general hearing performance, we analyzed click evoked ABRs for different SPLs (0–90 dB) in all three genotypes aged 140.67 ± 0.3 dB days. Using our automated ABR threshold detection system, a significant difference in hearing threshold among the
genotypes was detected. Cav3.2-/- mice exhibited a significant increase of hearing threshold compared to Cav3.2+/+ controls (Fig. 8A, B). Note that no gender specific alterations were observed in hearing thresholds within individual genotypes (Cav3.2+/+, Cav3.2+/- and Cav3.2-/-) at the age of 20 wk.

At the age of 40 wk (280.72 ± 0.60 days), hearing thresholds significantly increased in Cav3.2-/- mice compared to controls in both genders (Fig. 9). In males, the hearing threshold of α1H null mutant mice also exceeded the threshold of Cav3.2+/- mice (Fig. 9). Note that no gender specific differences within the three individual genotypes (Cav3.2+/+, Cav3.2+/- and Cav3.2-/-) were detected at the age of 40 wk.

The effect of aging on progressive hearing loss, i.e., increased hearing threshold, was analyzed in animals at 20 and 40 wk of age. In females, controls and Cav3.2+/+ mice exhibited a significant increase in hearing threshold between 20 and 40 wk (P < 0.01), whereas Cav3.2-/- mice displayed a trend (P = .079). In males, no significant increase in hearing threshold was detected for controls or heterozygous mice from 20 to 40 wk of age (P = .136 and P = .057, respectively). In male Cav3.2-/- mice however, a clear age-related increase in hearing loss was observed (P < .0001) (Figs. 8, 9).

Tone burst related hearing thresholds in controls, Cav3.2+/+ and Cav3.2-/- mice
To determine potential alterations in ABR threshold levels evoked by different tone burst frequencies (1–42 kHz, Fig. 10 A, B), we performed repeated two-way ANOVA followed by Tukey’s multiple comparisons test. Significant interaction was observed for males regarding GT and stimulus frequency, whereas no significant interaction could be confirmed in female mice. Highly significant effects were observed for a GT effect and tested frequency on threshold levels. Multiple comparisons revealed significant differences for various stimulus frequencies within the frequency range of 6–36 kHz in both Cav3.2-/- female and male mice (Fig. 10A, B).

The percentage of animals with detectable hearing thresholds for the individual frequencies is displayed in Fig. 10C, D. Unpaired two-way ANOVA revealed a significant interaction between GT and frequency for male mice, no significant interaction for female mice and high significant effects of GT and tested frequency. Cav3.2-/- mice from both genders exhibited significantly reduced hearing ability for lower (1 kHz for female mice) and higher frequencies (females: 30–42 kHz; males: 24–42 kHz) compared to Cav3.2+/+ and Cav3.2+/- animals based on Tukey’s multiple comparisons test (Fig. 10C, D).

Click-evoked ABR amplitude growth function analysis
In response to moderate to high-intensity clicks there may occur up to seven ABR peaks (WI–WVII) that are related to the following neuroanatomical structures: WI, auditory nerve (distal portion, within the inner ear); WII, cochlear nucleus (proximal portion of the auditory nerve, brainstem

Fig. 3. Body weight in Cav3.2+/+, Cav3.2+/- and Cav3.2-/- mice. A) Body weight of Cav3.2+/+ (♀, n = 12; ♂, n = 11), Cav3.2+/- (♀, n = 8; ♂, n = 7) and Cav3.2-/- (♀, n = 8; ♂, n = 9) mice was evaluated at age 140.67 ± 0.38 days. A significant decrease in body weight was observed for female Cav3.2-/- mice compared to controls (22.10 ± 0.43 g vs. 24.09 ± 0.41 g, P < .01). Male Cav3.2+/- mice displayed a significantly reduced body weight compared to both Cav3.2+/+ (29.09 ± 0.75 g vs. 32.82 ± 0.58 g, P < .01) and Cav3.2-/- animals (29.09 ± 0.75 g vs. 33.11 ± 0.81 g, P < .01). In heterozygous mice no alterations were observed compared to controls. B, C) Body weight development was monitored for controls (.), Cav3.2+/+ (■), and Cav3.2-/- mice (○) from 5 to 50 wk of age. Control values were used to generate a fitted curve (black line) with the 95% confidence interval in grey. Note that in females, a significant reduction in body weight was observed in Cav3.2+/- and Cav3.2-/- mice (B). The same held true for male Cav3.2+/- mice (C). Ordinary one-way ANOVA followed by Tukey’s multiple comparisons test was used to perform statistical analysis. The results are presented as the mean ± SEM.
termination); \( W_{III} \), superior olivary complex (SOC); \( W_{IV} \), lateral leminiscus (LL); \( W_{V} \), termination of the lateral leminiscus (LL) within the inferior colliculus (IC) on the contralateral side (Knipper et al., 2013). It should be noted that waves II–V are likely to have more than one anatomical structure of the ascending auditory pathway contributing to them.

In 51.0% of all click evoked ABR recordings, we identified five distinct positive waves at an SPL of 65 dB. In 25.4% of all recordings, we detected six distinct waves, and in 23.6% of all recordings, the number of identified distinct waves was limited to four within the first 10 ms at 65 dB SPL. Based on these findings, we focused our analysis on \( W_{I-IV} \).

**Fig. 4.** Click based ABRs in female Cav3.2 transgenic mice. Representative ABRs obtained from Cav3.2\(^{+/+}\) (A), Cav3.2\(^{+/-}\) (B) and Cav3.2\(^{-/-}\) (C) female mice (aged 140.67 ± 0.38 days) upon click stimulation (increasing SPL (dB) from 0–90 dB with 5 dB SPL steps). Each stimulus entity was presented 300 times at 20 Hz and averaged. The red line indicates the onset of the acoustic stimulus. Note that the ABR wave amplitudes are reduced in female Cav3.2\(^{-/-}\) mice (y-axis scaling has been adapted accordingly in (C)).

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**Fig. 5.** Click based ABRs in male Cav3.2 transgenic mice. Representative ABRs obtained from Cav3.2\(^{+/+}\) (A), Cav3.2\(^{+/-}\) (B) and Cav3.2\(^{-/-}\) (C) male mice (aged 140.67 ± 0.38 days) upon click stimulation (increasing SPL (dB) from 0–90 dB with 5 dB SPL steps). Each stimulus entity was presented 300 times at 20 Hz and averaged. The red line indicates the onset of the acoustic stimulus. Note that the ABR wave amplitudes are reduced in male Cav3.2\(^{-/-}\) mice (y-axis scaling has been adapted accordingly in (C)).
Identification of WI-IV was based on their latencies, i.e., WI appeared 1.588 ± 0.025 ms and 1.527 ± 0.032 ms after the acoustic stimulus in females and males, respectively; WII after 2.407 ± 0.045 ms in females and 2.389 ± 0.032 ms in males; WIII after 3.256 ± 0.056 ms in females and 3.197 ± 0.035 ms in males and WIV after 4.419 ± 0.079 ms in females and 4.331 ± 0.049 ms in males at 65 dB SPL in Cav3.2+/+ control animals (also see Fig. 12). The ABR amplitude growth function was analyzed for WI-IV (Fig. 11).

Based on the nonexistence or rare appearance of деflects (waves) for low SPL (0–25 dB), wavelet analysis detected no, or only limited, confirmed accordance of waves. For higher SPL (40–90 dB), wavelet analysis detected almost all waves (WI-IV) in experimental animals.

For WI, regular two-way ANOVA revealed a significant interaction between GT and SPL, a significant effect of SPL and no significant GT effect on amplitude growth in either genders. Multiple comparisons identified a significant delay in amplitude increase with higher SPL values (40–75 dB) for both female and male Cav3.2+/− compared to Cav3.2+/+ and Cav3.2+/- mice (Fig. 11A, B). With higher SPL levels (75–90 dB), WI amplitude in Cav3.2 null mutant mice reached similar amplitude levels as observed in Cav3.2+/+ and Cav3.2+/- mice. SPL-dependent increase in WI amplitude is delayed in Cav3.2−/− mice, reaching control levels at high SPL >80 dB. These findings could be related to the predominant expression of Cav3.2 in IHCs, OHCs and the SGN.

WII analysis revealed significance for an interaction between GT and SPL, and no significant GT effect was observed in amplitude growth for female or male mice.

Cav3.2+/- female and male mice displayed significant delays in amplitude growth, revealed by Tukey’s multiple comparisons test within the SPL range of 40–60 dB (Fig. 11C, D). As amplitude levels of Cav3.2+/+ and Cav3.2+/- in female and male mice
dropped with higher SPL (80–90 dB), Cav3.2−/− amplitude levels of both genders became significantly higher. In Cav3.2−/− mice, reduced WII amplitude indicates potential functional and/or structural impairment of the cochlear nucleus, which is in line with reports of robust cochlear Cav3.2 expression. With higher SPL, this effect is likely to be compensated, and amplitudes remain high.

For WIII, we confirmed a significant interaction between GT and SPL for Cav3.2 in male mice, no significant interaction in Cav3.2 female mice, a significant effect of SPL in both females and males, and no GT effect on amplitude growth. Tukey’s multiple comparisons test revealed no significant differences for Cav3.2−/− female mice compared to Cav3.2+/- and Cav3.2+/-, but significantly higher amplitudes were observed for Cav3.2−/− female mice for SPL 85–90 dB compared to Cav3.2+/- (Fig. 11E, F). Cav3.2−/− males exhibited decreased amplitude at intermediate (55 dB) and higher (85–90 dB) SPL values. Although T-type VGCCs are supposed to be expressed in the SOC underlying WIII, only minor alterations in WIII amplitude were detected in Ca3.2 transgenic mice for both genders. Analysis of amplitude growth for WIV revealed a significant interaction between GT and SPL in both genders, a significant GT effect for male mice, and a significant SPL effect in males and females. Both Cav3.2−/− female and male mice displayed significantly delayed amplitude growth and reduced WIV amplitude levels compared to Cav3.2+/- and Cav3.2+/- mice (40–65 dB, Fig. 11G, H). Although no detailed information on Cav3.2 expression in the lateral leminiscus (LL) underlying WIV is available, the delay observed in WIV amplitude growth points to a functional role for Cav3.2 in LL auditory processing. Statistics were performed using two-way RM ANOVA and Tukey’s multiple comparisons test for all waves and genders.

Click-evoked ABR wave latency analysis
To investigate the role of Cav3.2 VGCCs on temporal auditory processing within the inner ear and brainstem, we analyzed absolute click evoked wave latencies by measuring

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**Fig. 8.** Increased click-evoked ABR hearing thresholds in female and male Ca3.2−/− animals aged 20 wk. Click evoked audiometric hearing threshold of female (A) and male (B) Ca3.2−/− (♀, n = 12; ♂, n = 13), Ca3.2+/- (♀, n = 10; ♂, n = 9) and Ca3.2−/− (♀, n = 10; ♂, n = 9) mice aged 140.67 ± 0.38 days. One-way ANOVA followed by Tukey’s multiple comparisons test revealed a significant difference in hearing thresholds between the genotypes (females, F2, 29 = 23.10, P < .0001; males, F2, 28 = 33.23, P = < .0001, ordinary one-way ANOVA). Cav3.2−/− mice exhibited significantly increased hearing thresholds compared to Ca3.2+/- controls: ♀, 56.5 ± 2.6 dB vs. 40.0 ± 0.9 dB, P < .0001; ♂, 57.2 ± 1.5 dB vs. 38.8 ± 1.8 dB, P < .0001. The same held true in comparison to Ca3.2+/- mice (♀, 56.5 ± 2.6 dB vs. 42.0 ± 1.7 dB, P < .0001; ♂, 57.2 ± 1.5 dB vs. 39.4 ± 1.9 dB, P < .0001). Data are plotted as the mean ± SEM.

**Fig. 9.** Increased click-evoked ABR hearing thresholds in female and male Ca3.2−/− animals aged 40 wk. Click evoked hearing thresholds for female (A) and male (B) Ca3.2−/− (♀, n = 5; ♂, n = 8), Ca3.2+/- (♀, n = 4; ♂, n = 5) and Ca3.2−/− (♀, n = 4; ♂, n = 3) animals 280.72 ± 0.60 days old. One-Way ANOVA followed by Tukey’s multiple comparisons test confirmed significantly increased hearing thresholds for Cav3.2−/− mice compared to controls in both genders: ♀, 71.3 ± 10.5 dB vs. 45.0 ± 1.6 dB, P < .05; ♂, 88.3 ± 4.2 dB vs. 45.6 ± 4.6 dB, P < .01. In males, the hearing threshold of α1H null mutants also exceeded the threshold of Ca3.2+/- mice: 88.3 ± 4.2 dB vs. 54.0 ± 6.6 dB, P < .01. Data are plotted as the mean ± SEM. Note that there is an overall increase in hearing threshold compared to study groups aged 140.67 ± 0.38 days.
the processing time of each ABR wave (W<sub>1</sub>–W<sub>IV</sub>). First, this analysis was performed again at 65 dB SPL because resultant ABRs exhibited best fit using the automated complex "Mexican hat" based wavelet approach.

Statistical analysis revealed that W<sub>1</sub> latency was increased in female Cav3.2-/- mice compared to controls (Fig. 12A). In males, W<sub>1</sub> latency was increased in Cav3.2-/- mice compared to controls and Cav3.2+/- mice (Fig. 12B).

For W<sub>II</sub>, female Cav3.2+/- and Cav3.2-/- mice displayed significantly increased latency compared to controls (Fig. 12C). In males, Cav3.2+/- mice exhibited significantly increased latency compared to controls and Cav3.2-/- mice (Fig. 12D).

No differences were observed in males for W<sub>III</sub> latency (Fig. 12F). In females, only Cav3.2+/- displayed increased W<sub>III</sub> latency compared to controls, but no difference was detected for Cav3.2-/- mice (Fig. 12E).

Finally, W<sub>IV</sub> latency analysis revealed an increase in female Cav3.2+/- mice compared to controls (Fig. 12G) and an increase in male Cav3.2-/- mice compared to controls and Cav3.2+/- mice (Fig. 12H). Interestingly, gender specific differences were observed in heterozygous mice with female Cav3.2+/- mice exhibiting significantly higher latencies for W<sub>II</sub>, W<sub>III</sub>, W<sub>IV</sub> and W<sub>IV</sub> than male Cav3.2+/- animals (P < 0.01). The reasons for these differences remain to be determined.

Finally, interwave-interval (IWI) analysis (i.e., interpeak latency (IPL) analysis) revealed a significant increase in WI-IV latency in female Cav3.2+/- mice compared to controls but not in female Cav3.2-/- mice (Fig. 13A). No alterations in IWI ΔW<sub>1-IV</sub> were observed in male transgenic animals (Fig. 13B). Moreover, gender specific IWI differences were observed for ΔW<sub>1-IV</sub> in Cav3.2+/- mice.
In a second approach, latency analysis was performed at comparable sensation levels (20 dB above individual hearing thresholds). Under these conditions, no changes were observed in males from any of the three genotypes. In females, significant changes in WII, WIII and WIV latencies between controls and Cav3.2−/− mice persisted. We also observed a reduction in Wl latency in Cav3.2−/− mice compared to Cav3.2+/− mice (not shown).

**Cochlear VGCC transcript levels in Ca,3.2 transgenic mice**

Various VGCCs are expressed in the murine cochlea, including HVA L-type Ca,1.2 and Ca,1.3 channels, HVA Non-L-type Ca,2.3 channels and LVA T-type Ca,3.1–Ca,3.3 channels. qPCR was performed to assess for potential compensatory changes in transcript levels for these VGCCs. A gender specific analysis in Ca,3.2+/− and Ca,3.2−/− mice revealed that the above mentioned VGCCs exhibited no transcriptional changes that could account for the observed differences in click and tone burst related hearing thresholds, Wl/lv amplitude growth function or Wl/lv latencies (Figs. 14, 15, Table 2). In females, a statistical trend (P = .083) was detected for Ca,3.1 transcripts in Ca,3.2−/− compared to controls (Fig. 14D). In males, a statistical trend (P = .083) was observed for Ca,2.3 transcripts in Ca,3.2−/− mice compared to controls (Fig. 15C). Finally, a statistical trend (P = .065) was also observed for Ca,2.3 transcripts between Ca,3.2−/− males and females.

**DISCUSSION**

The pathophysiological implications of Ca,3.2 VGCCs were previously reported to include alteration of mechanoreception (Wang and Lewin, 2011) and pain response (Choi et al., 2007; Tsubota et al., 2018), age-induced endothelial dysfunction (Thuesen et al., 2018), epilepsy (Becker et al., 2007; Zamponi et al., 2010), retinal dysfunction (Hamby et al., 2015), (sensory) neuronal hyperexcitability (Jacus et al., 2012; Voisin et al., 2016; Zhang et al., 2018), elevated anxiety, impaired memory and reduced sensitivity to psychostimulants (Gangarossa et al., 2014). In the auditory trace fear conditioning task, Ca,3.2−/− mice performed normally but were impaired in context-cued trace fear conditioning (Chen et al., 2012). In addition, our longitudinal body weight analysis (Fig. 3B, C) indicates a complex developmental impairment, particularly in Ca,3.2−/− mice, which could be related to the cardiovascular phenotype, consisting of constitutively constricted coronary arterioles and focal myocardial fibrosis in Ca,3.2 deficient animals (Chen et al., 2003; Mizuta et al., 2010). Of note, established cardiovascular disease and individual or combined cardiovascular disease risk factors are associated with hearing loss (Erkan et al., 2015; Tan et al., 2018), although there are also studies that do not support this view (Haremza et al., 2017).

In our study, we performed gender specific analysis of both Ca,3.2−/− mice, which do not display a cardiac phenotype, and Ca,3.2−/− null mutant mice. Notably, the transgenic animals used in this study are from a C57Bl/6 background, which is important as their cochlea mimics typical characteristics of the aging human inner ear and can thus serve as a model of ARHL (presbycusis) (Ohlemiller, 2006). Age-related increases in ABR thresholds in the C57Bl/6 strain become exacerbated approximately 8–9 months of age, predominately above 12 kHz (Spngier et al., 1997; Ison et al., 2007). As experimental animals were only 5 months of age in this study, our results represent the physiological implications of Ca,3.2 in hearing physiology rather than its implications in hearing loss. Our study demonstrates that audiometric click ABR thresholds are increased in Ca,3.2−/− mice from both genders compared to controls and Ca,3.2−/− mice at 20 wk and 40 wk of age (Figs. 8, 9). No significant changes were observed for heterozygous mice compared to controls. Similar results were observed for tone burst derived ABR thresholds in both genders at the age of 20 wk (Fig. 10A, B). Lei et al. (2011) previously reported that they did not detect any significant ABR threshold shifts in Ca,3.2−/− mice from 9–11 months of age compared to controls, suggesting a preserving effect of Ca,3.2 ablation on age-related increases in ABR thresholds. This is in contrast to the increased click and tone burst related ABR thresholds in Ca,3.2−/− mice, which we observe at an earlier developmental stage (20 wk) in both genders.

Of note, Ca,3 VGCCs exhibit a complex developmental expression pattern within the cochlea (Lei et al., 2011; Yu et al., 2015, 2016). Although previous studies indicated that T-type Ca2+ channels can also be detected in various other

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**Fig. 11.** Click-evoked ABR amplitude growth function analysis of Wave I–IV in female (left) and male (right) Ca,3.2 transgenic mice and controls.Wave I–IV amplitude (μV) plotted as a function of increasing SPL (dB) for click-evoked ABR wave analysis in Ca,3.2−/− (♀, n = 12; ♂, n = 11; black line represents the approximate control curve, including the 95% confidence interval in grey), Ca,3.2+/− (♀, n = 8; ♂, n = 7; ○) and Ca,3.2−/− animals (♀, n = 7; ♂, n = 9; ■) aged 140.67 ± 0.38 days. Both Ca,3.2−/− female and male mice exhibit significantly delayed increases in amplitude growth across increasing SPL for wave I (A, B), wave II (C, D), and wave IV (G, H) compared to Ca,3.2−/− and Ca,3.2−/− mice. (E, F) For wave III, only Ca,3.2−/− male mice displayed significantly delayed amplitude growth across increasing SPL compared to female Ca,3.2−/− animals. For Wl, regular two-way ANOVA also revealed a significant interaction in GT and SPL (female: F20, 240 = 4.924, P < .0001; male F22, 264 = 8.767, P < .0001), a significant effect of SPL (female: F10, 240 = 108.6, P < .0001; male: F11, 264 = 112.3, P < .0001) and no significant GT effect on amplitude growth. Wl analysis displayed significance for GT and SPL interaction (female: F22, 253 = 9.629, P < .0001; male: F22, 264 = 11.01, P < .0001), SPL effect (female: F11, 253 = 21.90, P < .0001; male: F11, 264 = 10.67, P < .0001) but no significant GT effect on amplitude growth for either female or male mice. For Wll, we confirmed a significant GT and SPL interaction for Ca,3.2 male mice (F22, 264 = 3.656, P < .0001), no significant interaction for Ca,3.2 female mice, a significant effect of SPL (female: F11, 253 = 17.91, P < .0001; male: F11, 264 = 33.25, P < .0001) and no GT effect on amplitude growth. Finally, analysis of amplitude growth for WIV showed a significant interaction between GT and SPL (female: F22, 264 = 6.699, P < .0001; male: F22, 264 = 11.05, P < .0001), a significant GT effect for male mice (F22, 264 = 3.937, P = .0332), and a significant SPL effect (female: F11, 264 = 29.67, P < .0001; male F11, 264 = 20.17, P < .0001). Data are presented as the mean ± SEM.
A  Females
  Wave I

B  Males
  Wave I

C  Wave II

D  Wave II

E  Wave III

F  Wave III

G  Wave IV

H  Wave IV
structures of the auditory tract, detailed information about their spatiotemporal expression pattern is still lacking. In 2 month old C57BL/6 mice, Ca3.1 and Ca3.3 VGCCs are only weakly expressed in the organ of Corti, i.e., Cav3.1 in OHCs, IHCs, pillar cells, Deiters cells, and OHCs and Ca3.3 in OHCs and Deiters cells. Interestingly, no immunoreaction was detected for Ca3.2 (Shen et al., 2007). In contrast, Ca3.2 expression was dominant in the SGNs, Cav3.2 in OHCs and Deiters cells. Interestingly, no immunoreaction was detected for Cav3.2-/- (3.529 ± 0.121 ms (n = 8) vs. 3.256 ± 0.056 ms (n = 12), P < .01). Data are depicted as the mean ± SEM.

Overall, Ca3.2 VGCCs exhibit complex species specific spatiotemporal expression patterns in the auditory tract, particularly within the cochlea. Clearly, Ca3.2 expression continuously increases with age, and particularly at later stages (9–11 months), Ca3.2 exerts devastating effects on SGN viability, which manifests upon Ca3.2 ablation (Lei et al., 2011). However, at earlier developmental stages, e.g., 20 wk of age, our data suggest an alternative, i.e., physiological, role of Ca3.2 VGCCs that is distinct from its pathophysiological implications in ARHL. Indeed, the complex alterations in hearing thresholds (Figs. 8, 10), Wiv amplitude growth function (Fig. 11) and Wiv latencies (Fig. 12) in Ca3.2-/- and Ca3.2+/+ mice suggest important functional implications in the HCs / SGN, NC, SOC and the LL, indicating that Ca3.2 is a prerequisite for precise auditory information processing in young adult C57Bl/6 mice. Of note, latency analysis under comparable sensation levels revealed minor changes that may indicate a more prominent role of Ca3.2 VGCCs within the cochlea. In addition, it has to be considered that C57BL/6 mice, in contrast to CBA animals, display cadherin 23 related ARHL (Johnson et al., 2017). Thus, we cannot exclude that the genetic modification of Ca3.2 in our mouse lines potentially interferes with cadherin 23 mutation in these animals, potentially influencing observed results (Kane et al., 2012; Johnson et al., 2017).

Little information is currently available regarding expression of Ca3.2 in the ascending auditory tract, which creates challenges in attributing specific wave amplitude and wave latency alterations to specific electrophysiological alterations at the cellular level. LVA Ca2+ channels have been identified in Cartwheel cells of the dorsal cochlear nucleus. Cartwheel cells can fire both simple and complex action potential patterns, and early complex spike firing patterns and afterdepolarisations in these cells requires T-type and R-type Ca2+ channels but also BK and SK channels (Kim and Trussell, 2007). In the LSO, the firing rate of principal neurons is a linear function of interaural sound intensity differences. This type of linear response has been hypothesized to result from integration of excitatory
ipsilateral and inhibitory contralateral inputs. A previous study (Adam et al., 2001) demonstrated that this complex spike activity in rat LSO was based on T-type Ca\(^{2+}\) currents, subthreshold Na\(^{+}\) currents and hyperpolarization-activated Ih conductance being sensitive to Ni\(^{2+}\), tetrodotoxin (TTX) and Cs\(^{+}\), respectively (Adam et al., 2001). Of note, Cav1.3 VGCCs, which are known to be relevant for auditory function in IHCs, share basic electrophysiological properties similar to those of LVA channels, such as rapid activation kinetics (Koschak et al., 2001; Xu and Lipscombe, 2001; Zampini et al., 2010). Thus, given a RMP of ~60 mV in these cells (Marcotti et al., 2003), both Ca,1.3 and Ca,3.2 may support tonic transmitter release at rest and effectively couple increased sound intensities with higher rates of transmitter release. Mechanistically, Ca,3.2 might contribute to these processes by involvement in the complex spatiotemporal interdependence of intracellular Ca\(^{2+}\) levels and Ca\(^{2+}\) activated K\(^{+}\) currents (particularly via BK) in HCs as has been previously shown for Ca,1.3 as well (Joiner and Lee, 2015).

Despite these physiological implications, Ca\(^{2+}\) influx and Ca\(^{2+}\) signaling have been correlated with age-related and noise-induced neuronal cell death in HCs and SGN for some time. Consequently, T-type Ca\(^{2+}\) channel blockers were thought to be otoprotective in the setting of acquired hearing loss. Early studies suggested that trimethadione, ethosuximide and flunarizine were effective in protecting SGN and NIHL (So et al., 2005; Shen et al., 2007; Lei et al., 2011). Bao et al. (2013) confirmed this beneficial effect in NIHL for ethosuximide (Gomora et al., 2001) and zonisamide (Matar et al., 2009) for cochlear HCs and SGN. Accordingly, beneficial effects of T-type blockers on ABR threshold and SGN were also confirmed for ARHL using trimethadione, ethosuximide (Lei et al., 2011) and zonisamide (Yu et al., 2015). Importantly, the pharmaceutical drugs investigated have a multi-target character and do not exhibit selective T-type Ca\(^{2+}\) channel blocker characteristics. In contrast, in the aforementioned studies using 9–11 month old mice, T-type blockers preserved ABR thresholds and prevented excessive HC and SGN loss, Yu et al. (2016) reported the inverse in younger mice aged 24–26 wk. In a tone burst ABR setup, administration of mibefradil and benidipine did not have any beneficial impact on hearing thresholds at 8 Hz, 16 Hz and 32 kHz (despite at 24 kHz). Importantly, morphological changes were detected in IHCs and OHCs exhibiting disorganized and sparse stereocilia upon mibefradil administration and cell loss upon benidipine administration, respectively (Yu et al., 2016).

Together with our findings, these observations clearly indicate that Ca,3.2 VGCCs exert profound physiological effects in young adults that are tremendously important for
regular auditory processing within the ascending auditory pathway. However, within settings of acquired sensorineural hearing loss related to age or noise, increased Cav3.2 expression levels are likely to exert devastating effects on HC and SGN viability. Thus, we propose two pharmacotherapeutic constellations that need to be clearly differentiated: 1. late-stage treatment of ARHL and acoustic injury related NIHL in which animals/patients benefit from T-type blocker administration, and 2. early-stage application of T-type antagonists primarily related to epilepsy treatment in infants, children and young adults. In the latter group, application of T-type blockers was reportedly exerted potential negative effects on hearing up to transient/permanent hearing loss (Hori et al., 2003; Yeap et al., 2014; Hamed, 2017) (see also EMA side effect database, FDA adverse events reporting system (FAERS)). This is in full agreement with our findings that Cav3.2 is mandatory for functional and developmental integrity in the auditory system in adult mice.

**PERSPECTIVES**

Ca3.2 T-type Ca2+ channels are expressed in HCs and SGNs and T-type blockers have recently been suggested as effective for the prevention and treatment of ARHL and NIHL. Our studies demonstrate that Ca3.2 Ca2+ channels mediate important physiological functions for proper auditory information processing in the cochlea and ascending auditory tract in young adult mice. This is in contrast to the suggested otoprotective function of T-type blockers during older age. Consequently, the young adult stage could represent a vulnerable phase in which T-type blockers might exert deleterious effects on the peripheral auditory system that impact potential pharmaceutical T-type blocker treatment of ARHL and NIHL.

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**CONFLICT OF INTEREST**

The authors declare that this research was performed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Table 2. VGCC fold changes in cochleae from female (A) and male (B) controls (WT), Cav3.2+/- (HT) and Cav3.2-/- (KO) mice. Quantification results from 8 animals from each every genotype were normalized to a calibrator (positive control; cochlea from C57Bl6 animals) and fold changes (FC) were compared using Mann Whitney test in qBase plus software.

A)

| VGCC Cav-α1 | HT/WT FC | P value | KO/WT FC | P value | HT/KO FC | P value |
|------------|----------|---------|----------|---------|----------|---------|
| Cav1.3     | 1.050    | 0.798   | 1.056    | 0.721   | -1.006   | 0.878   |
| Cav1.2     | -1.064   | 0.382   | 1.087    | 0.796   | -1.196   | 0.505   |
| Cav2.3     | 1.076    | 0.959   | -1.188   | 0.574   | 1.270    | 0.574   |
| Cav3.1     | 1.264    | 0.505   | 1.323    | 0.083   | -1.047   | 0.574   |
| Cav3.3     | 1.095    | 0.798   | 1.040    | 0.878   | 1.053    | 0.959   |

B)

| VGCC Cav-α1 | HT/WT FC | P value | KO/WT FC | P value | HT/KO FC | P value |
|------------|----------|---------|----------|---------|----------|---------|
| Cav1.3     | -1.002   | 0.798   | 1.044    | 0.505   | -1.046   | 0.798   |
| Cav1.2     | 1.060    | 0.721   | 1.036    | 0.505   | 1.096    | 0.645   |
| Cav2.3     | 1.290    | 0.442   | 1.417    | 0.083   | -1.099   | 0.798   |
| Cav3.1     | -1.016   | 0.959   | 1.125    | 0.442   | -1.136   | 0.505   |
| Cav3.3     | 1.472    | 0.505   | 1.298    | 0.721   | 1.134    | 0.959   |

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