Research Article, to be submitted to The Journal of Neurophysiology

Repertoire of high voltage–activated calcium channels in lateral superior olive: functional analysis in wild-type, Ca\textsubscript{v}1.3\textsuperscript{−/−}, and Ca\textsubscript{v}1.2DHP\textsuperscript{−/−} mice

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Running head: Calcium channels in mouse LSO neurons

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Keywords: auditory brainstem; L-type Ca\textsuperscript{2+} channels; superior olivary complex (SOC); development; dihydropyridine
ABSTRACT

Ca\textsubscript{v}1.3 α subunits of high voltage-activated calcium channels (HVACCs) are essential for Ca\textsuperscript{2+} influx and transmitter release in cochlear inner hair cells and thereby for signal transmission into the central auditory pathway. Their absence leads to deafness and to striking structural changes in the auditory brainstem, particularly in the lateral superior olive (LSO). Here, we analyzed the contribution of various types of HVACCs to the total calcium current (I\textsubscript{Ca}) in developing mouse LSO neurons in order to address several questions: do LSO neurons express functional Ca\textsubscript{v}1.3 channels? What other types of HVACCs are expressed? Are there developmental changes? Do LSO neurons of Ca\textsubscript{v}1.3\textsuperscript{−/−} mice show any compensatory responses, namely up-regulation of other HVACCs? Our electrophysiological and pharmacological results show the presence of functional Ca\textsubscript{v}1.3 and Ca\textsubscript{v}1.2 channels at both postnatal day 4 and 12. Aside from these L-type channels, LSO neurons also express functional P/Q-, N-, and, most likely, R-type channels. The relative contribution of the four different subtypes to I\textsubscript{Ca} appears to be 45%/29%/22%/4% at P12. The physiological results are flanked and extended by quantitative RT-PCR data. Altogether, LSO neurons display a broad repertoire of HVACC subtypes. Genetic ablation of Ca\textsubscript{v}1.3 resulted in functional reorganization of some other HVACCs, yet did not restore normal I\textsubscript{Ca} properties. Together, our results suggest that several types HVACCs are of functional relevance for the developing LSO. Whether on-site loss of Ca\textsubscript{v}1.3, i.e., in LSO neurons, contributes to the recently described malformation of the LSO, needs to be determined by employing tissue-specific Ca\textsubscript{v}1.3\textsuperscript{−/−} animals.
INTRODUCTION

Voltage-gated Ca\(^{2+}\) channels are involved in a multitude of processes, including excitability, exocytosis, metabolism, and gene expression (Catterall 2000; Dolphin 2006; Hofmann et al. 1999; Lacinova 2005). They are divided into two subfamilies, namely low-voltage-activated and high-voltage activated Ca\(^{2+}\) channels (HVACCs). The latter usually require depolarization to about -20 mV to open (Carbone and Lux 1987; Fox et al. 1987; Randall and Tsien 1995) and comprise L-, N-, P/Q-, and R-type channels (Tsien et al. 1988). P/Q- and N-type HVACCs are sensitive to \(\omega\)-agatoxin IVA (Mintz et al. 1992) and \(\omega\)-conotoxin GVIA, respectively (Boland et al. 1994; McCleskey et al. 1987), whereas R-type channels are resistant to those blockers as well as to dihydropyridines (DHPs; Randall and Tsien 1995). L-type channels are highly sensitive to DHPs (Fox et al. 1987) and are further subdivided on the basis of four different pore-forming \(\alpha_1\) subunits named Ca\(_{\text{v}1.1}\)–Ca\(_{\text{v}1.4}\) (Catterall et al. 2005; Ertel et al. 2000; Striessnig and Koschak 2008). Ca\(_{\text{v}1.2}\) and Ca\(_{\text{v}1.3}\) subunits coexist in many tissues, including CNS neurons (Hell et al. 1993). In the mouse brain, Ca\(_{\text{v}1.3}\) accounts for 11% of L-type Ca\(^{2+}\) channels (Sinnegger-Brauns et al. 2009). Ca\(_{\text{v}1.3}\) channels are unusual in that they are activated at more negative voltages than Ca\(_{\text{v}1.2}\) channels (-46 mV vs. -32 mV, Koschak et al. 2001; -55 mV vs. -30 to -25 mV, Lipscombe et al. 2004), thus allowing long-lasting Ca\(^{2+}\) influx in response to relatively weak depolarizing pulses, which may be of developmental significance.

In the peripheral auditory system of mammals, Ca\(_{\text{v}1.3}\) channel function is crucially important for transmitter vesicle fusion and subsequent glutamate release from cochlear inner hair cells (IHCs) onto auditory nerve fibers (Brandt et al. 2003; Platzer et al. 2000). Moreover, Ca\(^{2+}\) influx through Ca\(_{\text{v}1.3}\) channels is necessary for generating spontaneous action potentials in immature IHCs (Tritsch et al. 2010; Tritsch and Bergles 2010). Deletion of Ca\(_{\text{v}1.3}\) channels leads to deafness (Baig et al. 2011; Brandt et al. 2003; Platzer et al. 2000), and due to the missing glutamate release from IHCs, cochlea-driven activation of subsequent auditory brainstem regions (spontaneous or sound-driven) is completely abolished (Fig. 1A). This abolishment also drastically affects the superior olivary complex (SOC), which comprises a group of interrelated nuclei whose neurons play essential roles in sound localization. Remarkably, SOC nuclei of Ca\(_{\text{v}1.3}\)\(^{-/-}\) mice, particularly the lateral superior olive (LSO), display severe morphological and physiological defects (Hirtz et al. 2011). These defects may be
caused by the loss of Ca\textsubscript{v}1.3 channels in the IHCs and thus by lacking peripheral input. Alternatively, and not mutually exclusive, on-site loss of the channels in LSO neurons themselves may cause the defects. Interestingly, L-type channel-mediated Ca\textsuperscript{2+} influx is essential for the proper development of SOC connectivity and contributes to preserve the inhibitory projection between the medial nucleus of the trapezoid body (MNTB) and the LSO, as demonstrated in organotypic slice cultures (Lohmann et al. 1998). The nature of the L-type channels, however, was not specified further, and as yet, there is no comprehensive electrophysiological characterization of Ca\textsubscript{v}1.3 channels and other HVACCs in LSO neurons.

The present study predominantly aimed at the identification of functional Ca\textsubscript{v}1.3 channels in LSO neurons of wild-type (WT) mice. Our analysis also covered other HVACCs in order to determine their contribution to the total Ca\textsuperscript{2+} current (\textit{I}_\text{Ca}). In addition, we assessed whether LSO neurons of Ca\textsubscript{v}1.3\textsuperscript{-/-} mice show any compensatory responses, i.e., up-regulation of other HVACCs. As subtype-selective blockers for Ca\textsubscript{v}1.3 and Ca\textsubscript{v}1.2 channels do not exist (Striessnig and Koschak 2008; Zuccotti et al. 2011), the relative contribution of each isoform to L-type channel-mediated function cannot be assessed directly. To circumvent this problem, we employed Ca\textsubscript{v}1.2 dihydropyridine-insensitive (Ca\textsubscript{v}1.2DHP\textsuperscript{-/-}) mice, in which a point mutation in the DHP binding site removes the high affinity for DHP L-type Ca\textsuperscript{2+} channel blockers (Sinnegger-Brauns et al. 2004). By analyzing both neonatal and juvenile animals, we finally addressed a putative role of individual HVACCs during early development.
MATERIALS AND METHODS

Animals
C57BL/6N WT, Ca\(v1.3^{-/-}\), and Ca\(v1.2\)DHP\(^{-/-}\) mice of both genders were used. Both mutant strains were bred on a C57BL6/N background. To assess developmental aspects, two age groups were analyzed: postnatal day (P) 4 ± 1 (the day of birth was taken as P0) and P12 ± 1 (hereafter abbreviated as P4 and P12). Animals were housed in our animal facility under standard conditions with a 12:12 h light-dark cycle. Water and food were freely available. The study was conducted in accordance with the NIH guidelines for use and care of laboratory animals. All protocols were in compliance with the German Animal Protection Law and approved by the regional animal care and use committees.

Preparation of acute brainstem slices
Mice were decapitated and their brains were rapidly removed and dissected in ice-cold preparation solution containing (in mM) 25 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 2.5 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 260 D-glucose, 2 sodium pyruvate, 3 myo-inositol, and 1 kynurenic acid. Therein, the brainstem containing the LSO was isolated and cut into 270-µm-thick coronal slices (3-5 slices per animal) with a vibratome (VT-1000S, Leica, Wetzlar, Germany). Slices were transferred into a storing solution containing (in mM) 125 NaCl, 25 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 2.5 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 10 D-glucose, 2 sodium pyruvate, 3 myo-inositol, 0.4 ascorbic acid, incubated at 37 °C for 1 hour, and thereafter maintained at room temperature until further usage. Solutions were continuously bubbled with a 95% O\(_2\) / 5% CO\(_2\) gas mixture to oxygenate the tissue and to achieve a pH of 7.4.

Electrophysiology
Slices were submerged in a recording chamber continuously perfused with O\(_2\)/CO\(_2\)-gassed extracellular recording solution composed of (in mM) 110 NaCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 5 CsCl, 35 TEA [tetraethylammonium]-Cl, 15 4-aminopyridine, 10 D-glucose, 10 HEPES; pH 7.4. Solutions were applied by a gravity-fed perfusion system at a rate of approximately 2 ml/min. The recording chamber was mounted on an upright microscope (Eclipse E600FN, Nikon, Tokyo, Japan) equipped with a Nikon Fluor 60x DIC H water immersion objective and
infrared differential interference contrast optics. Slices were illuminated with near-infrared light, and LSO neurons were visualized using an infrared-sensitive video camera system (CCD camera CS405-01, Hamamatsu, Herrsching, Germany). LSO neurons were identified by their location, orientation, size, and spindle-shaped somata. Measurements were performed only on morphologically intact neurons. Patch pipettes were pulled from borosilicate glass capillaries (GB150F-8P, Science Products, Hofheim, Germany) using a horizontal puller (P-2000, Sutter Instrument Co., Novato, USA) and had resistances of 2.5 - 4.5 MΩ when filled with the intracellular solution containing (in mM) 130 CsCl, 0.1 CaCl₂, 2 MgCl₂, 10 EGTA, 10 HEPES, 10 TEA-Cl, 0.3 Na₂GTP, 2 Na₂ATP, pH was adjusted to 7.2 with CsOH (osmolarity 290-300 mOsm). Liquid junction potentials were calculated using the program JPCalc by Barry (1994); as they amounted to -5.3 mV, they were not corrected. All experiments were performed in the tight-seal whole-cell configuration of the patch-clamp technique. Recordings were conducted with an Axopatch-1D amplifier (Molecular Devices, Union City, CA, USA). Data were sampled at 5 kHz, low-pass filtered at 10 kHz, and analyzed using pClamp and ClampFit 8.2.0.235 (Axon Instruments, Union City, USA) and Origin software (Origin Lab, Northampton, USA). Capacitive current transients were electronically compensated online and the whole cell capacitance was estimated from this compensation. Raw currents were corrected online for linear leak currents. Throughout the experiments, the series resistance was monitored and ranged between 6-20 MΩ. It was routinely compensated by 60-80%. If it exceeded 20 MΩ, recordings were excluded from further analysis. Current–voltage (I-V) relations were determined using a series of 100 ms depolarizing pulses applied at a frequency of 8 Hz which started from a holding potential of -70 mV and stepped to +70 mV (+40 mV in P12 Cd²⁺ and nifedipine experiments) with increments of 10 mV (cf. Fig. 1C). To compare the activation of currents, data were fitted with a Boltzmann equation of the form $I(V) = \frac{(V-V_{rev}) \cdot g_{max}}{1 + \exp((V-V_{1/2})/dV)}$ where $I(V)$ is the amplitude of the current activated by the depolarizing pulse to the membrane voltage $V$, $V_{rev}$ is the reversal potential, $g_{max}$ is the maximal slope conductance, $V_{1/2}$ is the voltage at which there is half-maximal activation and $dV$ is the activation slope factor. Current traces depicted in Figures 2-8 represent graphical averages obtained from three single traces. Blocker-sensitive current traces are illustrated as the graphical difference between currents obtained under control conditions and in the presence of the drug. For each neuron, the maximal amplitude that occurred in all current traces was determined and used for further
quantitative analysis. In the following, these amplitudes will be called ‘peak amplitudes’. Because considerable variability in whole cell capacitance occurred with age, peak currents were normalized to whole cell capacitances (peak current density of $I_{Ca}$, [pA/pF]) when developmental comparisons were made (cf. Fig. 9).

Pharmacological compounds

All chemicals were purchased from Sigma-Aldrich (Munich, Germany) unless stated otherwise. To block voltage-activated sodium currents, all experiments were carried out in the presence of 0.5 µM tetrodotoxin (Ascent Scientific, Bristol, UK). $K^+$ channel-mediated currents were minimized by the combination of external/internal TEA and internal Cs. In some experiments, $Cd^{2+}$ (50 µM CdCl$_2$) was added to inhibit $Ca^{2+}$ currents. HVACCs were isolated and analyzed using specific blockers added to the extracellular recording solution. Nifedipine was purchased from RBI Research Biochemicals International (Natick, USA) and used for blocking L-type channels. Isradipine and $\omega$-conotoxin GVIA were purchased from Ascent scientific and used for blocking L-type and N-type channels, respectively. All blockers were prepared as stock solutions dissolved in ethanol (nifedipine and isradipine at 10 mM) or distilled water ($\omega$-agatoxin IVA at 100 µM and $\omega$-conotoxin GVIA at 329 µM) and stored at -20 °C. Stock solutions were diluted to their final concentrations (nifedipine 20 µM, isradipine 3 µM, $\omega$-agatoxin IVA 600 nM, $\omega$-conotoxin 2 µM) in extracellular recording solution and applied via a custom-made application pipette placed in the recording chamber close to the slice.

Biocytin fills

To correlate structural and functional properties of the recorded cells biocytin was sporadically added to the intracellular solution at a final concentration of 0.5%. Typically, only one neuron was filled per slice. During the whole-cell recordings, biocytin diffused into the cell soma and the cellular processes. After detaching the pipette from the soma, slices were maintained in the recording chamber for 30 min to optimize the quality of the labeling. Thereafter, they were transferred into 4% buffered paraformaldehyde and fixed overnight. They were then rinsed and incubated for 40 min with 1% $H_2O_2$ and 0.5% Triton X-100 in PBS to inhibit endogenous peroxidases and increase permeability. Following another rinsing step, slices were incubated for 1 h in blocker solution (3% bovine serum albumin, 10% goat serum,
0.3% Triton in Tris-buffered saline, pH 7.4) with NeutrAvidin-horseradish peroxidase conjugate (1:1,000, Molecular Probes, Eugene, USA). After rinsing in 0.5% Triton/PBS, the diaminobenzidine/H$_2$O$_2$ reaction was performed using Fast diaminobenzidine tablets. The reaction product was intensified with nickel ammonium sulfate (NiSO$_4$(NH$_4$)$_2$SO$_4$*6H$_2$O). Finally, slices were dehydrated, mounted on gelatine-coated slides, and enclosed with coverslips. Biocytin-DAB-labeled LSO neurons were analyzed and photographed on an Axioskop 2 (Zeiss, Jena, Germany) with 5x and 40x objectives. The cell morphology was reconstructed using Cell F software (Olympus, Hamburg, Germany), Corel Photopaint X4 and Corel Draw X4 software (Corel, Unterschleißheim, Germany).

**RNA extraction, reverse transcription, and quantitative PCR**

Total RNA was isolated from LSOs of WT and Ca$_v$1.3$^{-/}$ mice aged P12. LSOs were dissected from acute brainstem slices and prepared as described for electrophysiological experiments. The isolated LSOs were stored in RNAlater (Ambion, Darmstadt, Germany) at -20 °C. Due to the small sample amount, eight LSO excisions were pooled for each sample from a minimum of three animals. Total RNA isolation was performed using RNeasy Lipid Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The amount of isolated RNA was measured with a NanoDrop D-1000 UV/Vis-spectrophotometer (Peqlab, Erlangen, Germany). The quality of isolated RNA was assessed using a 2100 Bioanalyzer (Agilent, Böblingen, Germany). The isolated total RNA was used to prepare cDNA. For each sample, at least 600 ng of total RNA was reverse-transcribed using random hexamers (MBI Fermentas, St. Leon-Rot, Germany). Quantitative PCR was performed with Taqman Gene Expression assays (Applied Biosystems) as published previously (Sinnerger-Brauns et al. 2009). Assay GenBank accession numbers were as follows: Ca$_v$1.2 $\alpha_1$, Mm00437917_m1; Ca$_v$1.3-42a (recognizing the short C-terminal Ca$_v$1.3 splice variant), custom-designed (forward primer, GGAAGTACCCTGCGAA GAACAC; reverse primer, CTCAGGCAGAGAACTCTAAAGCAT; probe, TTGCCC TACAGATGCTTG); Ca$_v$1.3-49 (recognizing full-length transcripts), Mm 01209927_g1; Ca$_v$2.1, Ca$_v$2.2, Mm01284736_m1; Ca$_v$2.3 Mm04944444_m1; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Mm99999915_g1. Differences in gene expression between WT and Ca$_v$1.3$^{-/}$ mice were determined with $\Delta\Delta$Ct analysis using GAPDH as the reference gene. Slopes of relative standard curves were obtained for calculation by using dilutions of whole-brain cDNA (0.0005-50 ng RNA equivalents) as templates. Because of the insignificant
variations in the slope values, a mean slope value (-3.1) could be used for ΔΔCt calculations.

All experimental data points were obtained as triplicates.

Statistics

Box-and-whisker plots depict the mean value of the peak amplitudes (black or white square), the median (horizontal line), the 25th and 75th percentile as well as whiskers from the 5th to the 95th percentile. Data from individual neurons are depicted by filled black circles. To assess for statistical significance (Winstat, R. Fitch Software, Bad Krozingen, Germany), all data sets were checked for Gaussian distribution, and outliers (more than four times standard deviation above/below the mean) were excluded. Values in bar charts are presented as mean ± standard error. Usually, a paired or an unpaired two-tailed Student’s t-test was performed. Mann-Whitney U test was used to compare differences when data did not follow a normal distribution (explicitly mentioned in the text). Significance values were as follows: p < 0.05 *, p < 0.01 **, p < 0.001 ***.
RESULTS

Identification and functional characterization of LSO neurons

Experiments were performed on LSO neurons in brainstem slices of P12 and P4 WT, Ca\textsubscript{v}1.3\textsuperscript{−/−}, and Ca\textsubscript{v}1.2 DHP\textsuperscript{−/−} mice. Photomicrographs of P12 slices from WT and Ca\textsubscript{v}1.3\textsuperscript{−/−} mice with outlined LSO nuclei and detailed views of biocytin-labeled neurons are shown in Fig. 1B. The obvious difference in size and shape between a WT LSO and a Ca\textsubscript{v}1.3\textsuperscript{−/−} LSO was previously reported (Hirtz et al. 2011). Whole-cell patch-clamp recordings of membrane currents in LSO neurons were performed using an adequate activation protocol (Fig. 1C, inset) with external and internal solutions that effectively isolated Ca\textsuperscript{2+} channel currents (cf. Methods). Inward currents were activated at membrane potentials of about -50 mV, reached their peak amplitudes at -10 mV to 0 mV, and lasted until the end of the voltage step (Fig. 1C). Upon bath application of Cd\textsuperscript{2+}, a non-specific Ca\textsuperscript{2+} channel blocker (Hagiwara and Byerly 1981), the peak amplitude of $I_{Ca}$ decreased significantly from -861.9 ± 210.1 pA to -172.8 ± 46.6 pA in WT (81.8 ± 1.5%; n = 9; p = 0.003) and from -465.7 ± 35.5 pA to -76.9 ± 10.3 pA in Ca\textsubscript{v}1.3\textsuperscript{−/−} (84.2 ± 1.8%; n = 18; p = 5.1E-10), indicating that the majority of current was carried by Ca\textsuperscript{2+} ions in both genotypes (Fig. 1D). Because T-type channels are activated by weak depolarization and are transient, in contrast to HVACCs (Catterall et al. 2005), and because Cd\textsuperscript{2+} ions reduce L-type and N-type currents significantly more efficiently than T-type currents (Fox et al. 1987), the observed currents in LSO neurons were likely mediated predominantly by HVACCs. To further distinguish between various HVACC subtypes, specific channel blockers were used in the following: the DHPs nifedipine and isradipine for L-type, the spider toxin $\omega$-agatoxin IVA for P/Q-type, and the snail toxin $\omega$-conotoxin GVIA for N-type channels (Catterall et al. 2005).

$I_{Ca}$ mediated by L-type channels in P12 mice

In the brain, L-type currents are mediated by two different channel isoforms, namely Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 (Sinnegger-Brauns et al. 2009). Unfortunately, specific blockers of L-type channels, capable of distinguishing between the two isoforms, do not exist, generating a major obstacle (Striessnig et al. 2006). However, one can circumvent this problem by employing the two mouse models Ca\textsubscript{v}1.3\textsuperscript{−/−} and Ca\textsubscript{v}1.2DHP\textsuperscript{−/−}. In the latter strain, a targeted point mutation in the Ca\textsubscript{v}1.2 $\alpha_1$ gene leads to normal expression of functional Ca\textsubscript{v}1.2
channels, yet removes the high sensitivity of these channels to DHPs, such that DHP effects lost in these mice can be attributed to Cav1.2. Reduction of Ca\textsuperscript{2+} currents by application of DHPs in Cav1.2DHP\textsuperscript{-/-} neurons can, therefore, be attributed to a blockade of Cav1.3 channels. To assess the contribution of Cav1.2 and Cav1.3 to $I_{Ca}$, we tested their sensitivity to nifedipine, the prototype of the DHP class of Ca\textsuperscript{2+} channel antagonists. We observed a clear reduction of the inward current amplitudes in WT, Cav1.3\textsuperscript{-/-}, and Cav1.2DHP\textsuperscript{-/-} neurons (Fig. 2A-F).

In WT mice, the reduction of the $I_{Ca}$ peak amplitude by nifedipine ranged from 24-73%, with a mean value of 45.0 ± 4.9% ($n = 11$; Fig. 2G,H). It was statistically significant ($p = 1.6E-06$), demonstrating the efficacy of the blocker. In Cav1.2DHP\textsuperscript{-/-} mice, the reduction of $I_{Ca}$ was 4-47%, with a mean of 30.1 ± 3.8% ($n = 10$, $p = 9E-04$, Fig. 2G,H), implying that 30% of $I_{Ca}$ is carried through Cav1.3 channels in WT P12 LSO neurons. Consequently, about 15% of $I_{Ca}$ is mediated through Cav1.2 channels. In Cav1.3\textsuperscript{-/-} mice, nifedipine reduced $I_{Ca}$ by 2-45%, with a mean value of 20.2 ± 3.8% ($n = 12$, $p = 3E-04$, Fig. 2G,H). Moreover, for both mutants, the blockade was significantly lower ($p = 6E-04$ and $p = 0.028$) than in WT neurons (Fig. 2G,H).

Together, these results indicate that about 45% of $I_{Ca}$ in WT P12 LSO neurons is carried by L-type channels, of which 30% contribute to the Cav1.3 subunits and the remaining 15% to the Cav1.2 subunits.

To corroborate the nifedipine results, a second set of experiments was performed in which nifedipine was replaced by isradipine (3 \textmu M), another highly specific DHP antagonist (Koschak et al. 2001; Striessnig et al. 1998). Again, isradipine was applied to WT, Cav1.3\textsuperscript{-/-}, and Cav1.2DHP\textsuperscript{-/-} neurons and led to reduced $I_{Ca}$ in all cases (Fig. 3A-C). The blocking efficiency concerning the peak amplitudes ranged from 30-46% in WT, from 13-25% in Cav1.3\textsuperscript{-/-}, and from 21-34% in Cav1.2DHP\textsuperscript{-/-} neurons (Fig. 3D). In all three cases, the blocking efficiency was statistically significant, with mean values amounting to 39.3 ± 1.8% ($n = 8$; $p = 6E-04$) in WT, 22.2 ± 1.9% ($n = 6$, $p = 0.0049$) in Cav1.3\textsuperscript{-/-} and 29.6 ± 2.2% ($n = 6$, $p = 4.4E-05$) in Cav1.2DHP\textsuperscript{-/-} neurons (Fig. 3D). Furthermore, the blockade was significantly higher in WT neurons compared to Cav1.3\textsuperscript{-/-} ($p = 3E-05$) and Cav1.2DHP\textsuperscript{-/-} neurons ($p = 0.0042$) (Fig. 3E).

The isradipine-based results indicate a contribution of about 40% by L-type channels to $I_{Ca}$ in WT P12 LSO neurons, similar to the situation found for nifedipine. Of these 40%, about 30% appear to be contributed by Cav1.3 subunits and the remaining 10% by Cav1.2 subunits, again in accordance with the nifedipine-based observations. Comparing both drugs, the
reduction of \( I_{Ca} \) peak amplitudes by isradipine did not differ significantly from that seen under nifedipine (WT, \( p = 0.26; \text{Ca}_{v1.3}^{-/-}, p = 0.65; \text{Ca}_{v1.2DHP}^{-/-}, p = 0.93 \)), confirming that both substances, at the concentrations used, indeed specifically block L-type channels in LSO neurons and with equal efficiency.

Fitting the I-V relationships with a Boltzmann equation yielded significantly different half activation voltages (\( V_{1/2} \)) for WT and \( \text{Ca}_{v1.3}^{-/-} \) LSO neurons (WT: -24.1 ± 1.0 mV, \( n = 28 \); \( \text{Ca}_{v1.3}^{-/-} \): -19.7 ± 0.9 mV, \( n = 35 \); \( p = 2E-03 \)). The shift towards more positive values is in accordance with the fact that \( \text{Ca}_{v1.3} \) subunits are activated at more negative voltages than \( \text{Ca}_{v1.2} \) channels. As expected, the \( V_{1/2} \) value for \( \text{Ca}_{v1.2DHP}^{-/-} \) neurons (-21.9 ± 1.8 mV; \( n = 15 \)) was not different from WT (\( p = 0.27 \)). These results provide further evidence for the coexistence of functional \( \text{Ca}_{v1.3} \) and \( \text{Ca}_{v1.2} \) channels in LSO neurons. Moreover, they corroborate the idea that \( \text{Ca}_{v1.2DHP}^{-/-} \) mice are a valid model to pharmacologically isolate \( \text{Ca}_{v1.3} \) from the other L-type channel subtype.

\( I_{Ca} \) mediated by P/Q- and N-type channels in P12 mice

The finding that 55-60% of the \( I_{Ca} \) amplitude remained unblocked by the DHP antagonists nifedipine and isradipine in P12 WT LSO neurons led us to suggest that these cells possess other types of functional HVACCs aside from L-type channels, e.g. P/Q-type and/or N-type channels. To tackle this suggestion, we performed a next series of experiments and applied the specific P/Q-type antagonist \( \omega \)-agatoxin IVA (600 nM) or the specific N-type antagonist \( \omega \)-conotoxin GVIA (2 \( \mu \)M). \( \omega \)-agatoxin IVA reduced the \( I_{Ca} \) in both WT (Fig. 4A,B) and \( \text{Ca}_{v1.3}^{-/-} \) neurons (Fig. 4C,D). The amount of blockade ranged from 6-51% in WT (\( n = 9 \)) and from 8-34% in \( \text{Ca}_{v1.3}^{-/-} \) neurons (\( n = 9 \); Fig. 5E,F). On average, peak amplitudes were significantly reduced by 28.6 ± 4.3% (WT; \( p = 0.0016 \)) and 22.8 ± 2.7% (\( \text{Ca}_{v1.3}^{-/-} \); \( p = 3E-04 \)). The blockade by \( \omega \)-agatoxin IVA did not differ in WT vs. \( \text{Ca}_{v1.3}^{-/-} \) neurons (\( p = 0.27 \)), indicating that P/Q channels do not compensate for \( \text{Ca}_{v1.3} \) deficiency. However, this issue will be addressed more thoroughly below.

\( \omega \)-conotoxin GVIA also resulted in reduced \( I_{Ca} \) in both genotypes (Fig. 5A-D). The effect ranged from 3-41% in WT and 12-35% in \( \text{Ca}_{v1.3}^{-/-} \) neurons, with mean values of 21.7 ± 3.4% (\( n = 10 \); \( p = 5E-04 \)) and 25.5 ± 2.3% (\( n = 10 \); \( p = 4E-06 \)), respectively (Fig. 5E,F). Again, there was no significant difference between WT and \( \text{Ca}_{v1.3}^{-/-} \) neurons (\( p = 0.36 \), Fig. 5F).

Taken together, these results demonstrate a considerable contribution of P/Q-type and N-
type channels to $I_{\text{Ca}}$ in P12 LSO neurons. Moreover, both types contribute to a similar amount at P12 (WT: 29% P/Q-type, 22% N-type; $\text{Ca}_{\alpha 1.3^{-/-}}$: 23% P/Q-type, 26% N-type).

$I_{\text{Ca}}$ mediated by L-type channels in P4 mice

Ca$_{\alpha 1.3}$ channels are already functional in IHCs of neonatal mice, many days before hearing onset (at P12) and at times when spontaneous activity in the cochlea drives the synaptic activity in the subsequent auditory pathway (Tritsch et al. 2010). Spontaneous neuronal activity patterns appear to be crucial for neuron survival and the generation of orderly connections in the auditory system (Friauf and Lohmann 1999; Kandler et al. 2009; Kandler and Gillespie 2005). To determine whether the repertoire of HVACCs undergoes developmental changes in the LSO, we monitored HVACC-mediated currents in LSO neurons of P4 mice. Already at this early age, Ca$^{2+}$ currents were activated. Application of nifedipine caused a considerable and statistically significant reduction of the $I_{\text{Ca}}$ peak amplitude in all three genotypes (Fig. 6A-F). The reduction ranged from 10-58% in WT, from 3-39% in Ca$_{\alpha 1.3^{-/-}}$, and from 22-39% in Ca$_{\alpha 1.2\text{DHP}^{-/-}}$ (Fig. 6G). The corresponding mean values were 36.4 ± 5.1% ($n = 10$; $p = 0.010$), 19.9 ± 3.7% ($n = 9$; $p = 0.010$), and 29.8 ± 2.0% ($n = 11$; $p = 2.7\times10^{-5}$; Fig. 6H). Significance was achieved between WT and Ca$_{\alpha 1.3^{-/-}}$ ($p = 0.020$), yet not between WT and Ca$_{\alpha 1.2\text{DHP}^{-/-}}$ ($p = 0.22$). Together, these results show that P4 LSO neurons harbor functional L-type channels, which appear to contribute by 36% to total $I_{\text{Ca}}$. Moreover, they indicate that, like at P12, the relative contribution of Ca$_{\alpha 1.3}$ channels to $I_{\text{Ca}}$ is higher than that of Ca$_{\alpha 1.2}$ channels (30% vs. 6%).

$I_{\text{Ca}}$ mediated by P/Q- and N-type channels in P4 mice

To assess the contribution of P/Q- and N-type Ca$^{2+}$ channels in P4 LSO neurons, the same approach was chosen as with P12 neurons, i.e., $I_{\text{Ca}}$ was recorded under control conditions and in the presence of the P/Q-channel inhibitor $\omega$-agatoxin IVA or the N-channel inhibitor $\omega$-conotoxin GVIA. Application of $\omega$-agatoxin IVA significantly reduced $I_{\text{Ca}}$ in both genotypes (Fig. 7A-D). The blockade ranged from 14-52% in WT mice and from 5-50% in Ca$_{\alpha 1.3^{-/-}}$ mice with mean values of 27.7 ± 3.3% ($n = 11$; $p = 0.0022$) in WT and 22.0 ± 4.4% ($n = 10$; $p = 0.0049$) in Ca$_{\alpha 1.3^{-/-}}$ (Fig. 8E,F). The difference between genotypes was not statistically significant ($p = 0.30$).
Application of ω-conotoxin GVIA also reduced the peak amplitudes (Fig. 8A-D). Blocking efficiency ranges from 14-59% in WT and from 13-41% in Ca\(_{v1.3}^{-/-}\) (Fig. 8E). On average, peak amplitudes were reduced by 29.2 ± 4.0% (n = 12; p = 4.4E-05) in WT and by 25.8 ± 3.8% (n = 9; p = 0.0022) in Ca\(_{v1.3}^{-/-}\) (Fig. 8F), with no significant difference between genotypes (p = 0.56). These data show that, aside from L-type channels, P4 LSO neurons also possess functional P/Q- and N-type channels with considerable and similar contribution (WT: 28% P/Q-type, 29% N-type; Ca\(_{v1.3}^{-/-}\): 22% P/Q-type, 26% N-type). As in the case of P12 WT, the contribution is less than that of L-type channels.

Comparison between P4 and P12

We next compared the P4 data with those obtained at P12 with the aim to assess possible developmental changes. The comparison revealed that nifedipine blocked \(I_{\text{Ca}}\) peak amplitudes in P12 WT neurons in a similar way as at P4 (Fig. 9A and Table 1; p = 0.24). This implies no major, if any, developmental changes in L-type channels. Concerning ω-agatoxin IVA and ω-conotoxin GVIA blockade, there were also no significant differences between ages in WT neurons (Fig. 9A and Table 1; p = 0.87; p = 0.18). Together, these data provide confirmatory evidence for the assumption that the repertoire of HVACCs does not change in LSO neurons between neonatal and juvenile mice. In Ca\(_{v1.3}^{-/-}\) mice, there was virtually no difference in all three pharmacological treatments (Fig. 9A and Table 1; p = 0.96; p = 0.89, p = 0.95), indicating that the loss of Ca\(_{v1.3}\) channels does not result in age-related changes of the fractions of L-, P/Q-, and N-type channels.

Concerning the absolute current amplitudes and their development, a significant increase occurred with age in WT neurons (P4: -555.5 ± 59.8 pA; n = 25; P12: -915.8 ± 47.9 pA; n = 34; p = 1.4E-05). Likewise, absolute current amplitudes increased with age in Ca\(_{v1.2}\)DHP-/- neurons (P4: -481.5 ± 39.0 pA; n = 11; P12: -883.4 ± 100.3 pA; n = 16; p = 0.0014). In contrast, no such developmental increase was observed in Ca\(_{v1.3}^{-/-}\) neurons (P4: -667.6 ± 48.9 pA; n = 21; P12: -655.9 ± 58.6 pA; n = 27; p = 0.88), implying a disturbed development of Ca\(^{2+}\) channel function upon loss of the Ca\(_{v1.3}\) subtype. This was also reflected by the observation that the peak amplitude of P4 WT did not differ from that of P4 Ca\(_{v1.3}^{-/-}\) (p = 0.16), whereas it was significantly lower in P12 Ca\(_{v1.3}^{-/-}\) neurons than in the P12 WT counterparts (p = 9.8E-04). As expected, there was no difference between the peak amplitudes of WT and Ca\(_{v1.2}\)DHP-/- neurons at P4 and P12 (p = 0.31 and p = 0.74).
with this, a reevaluation of the peak amplitudes (at -10 mV) from the samples depicted in Fig. 2B,F also demonstrated no difference between WT and Ca\textsubscript{v}1.2DHP\textsuperscript{-/-} neurons at P12 (WT: -862.7 ± 96.5 pA; n = 10; Ca\textsubscript{v}1.2DHP\textsuperscript{-/-}: -675.7 ± 85.5 pA; n = 9; p = 0.17).

In a final series of electrophysiological experiments, we analyzed the development of whole cell capacitance, which is an indirect measure for the complexity of the soma-dendritic morphology. Capacitance values did not differ between the three genotypes, neither at P12 (WT: 20.0 ± 1.3 pF, n = 35; Ca\textsubscript{v}1.3\textsuperscript{-/-}: 19.2 ± 0.7 pF, n = 36; Ca\textsubscript{v}1.2DHP\textsuperscript{-/-}: 19.8 ± 0.9 pF, n = 16; WT vs. Ca\textsubscript{v}1.3\textsuperscript{-/-}: p = 0.56; WT vs. Ca\textsubscript{v}1.2DHP\textsuperscript{-/-}: p = 0.91; Ca\textsubscript{v}1.3\textsuperscript{-/-} vs. Ca\textsubscript{v}1.2DHP\textsuperscript{-/-}: p = 0.58) nor at P4 (WT: 28.9 ± 2.5 pF, n = 23; Ca\textsubscript{v}1.3\textsuperscript{-/-}: 27.3 ± 2.3 pF, n = 20; Ca\textsubscript{v}1.2DHP\textsuperscript{-/-}: 26.7 ± 3.4 pF, n = 11; WT vs. Ca\textsubscript{v}1.3\textsuperscript{-/-}: p = 0.65; WT vs. Ca\textsubscript{v}1.2DHP\textsuperscript{-/-}: p = 0.58, Mann-Whitney U test; Ca\textsubscript{v}1.3\textsuperscript{-/-} vs. Ca\textsubscript{v}1.2DHP\textsuperscript{-/-}: p = 0.92, Mann-Whitney U test). However, considering the development of the whole cell capacitance from P4 to P12, a significant decrease occurred in WT neurons (p = 0.0033), in Ca\textsubscript{v}1.3\textsuperscript{-/-} neurons (p = 0.0026), and in Ca\textsubscript{v}1.2DHP\textsuperscript{-/-} neurons (p = 0.0043, Mann-Whitney U test). This decrease can be explained by membrane reduction through dendritic pruning. These data are consistent with recent morphological data, which have demonstrated unchanged complexities of dendritic arbors between WT neurons and those neurons (2/3 of normal) which remain in the LSO of Ca\textsubscript{v}1.3\textsuperscript{-/-} knockout mice (Hirtz et al. 2011).

Values for the peak current density of I\textsubscript{Ca} ranged from -10.7 to -41.1 pA/pF in P4 WT neurons and from -21.3 to -113.1 pA/pF in P12 WT neurons (Fig. 9B). The corresponding data for Ca\textsubscript{v}1.3\textsuperscript{-/-} neurons were -12.0 to -45.7 pA/pF at P4 and -20.7 to -83.2 pA/pF at P12 (Fig. 9B). From these values, a significant developmental increase became evident in both WT (P4: -20.0 ± 1.6 pA/pF, n = 24; P12: -51.7 ± 3.8 pA/pF, n = 35, p = 2.8E-09, Mann-Whitney U test; Fig. 9C) and Ca\textsubscript{v}1.3\textsuperscript{-/-} neurons (P4: -26.0 ± 1.9 pA/pF, n = 21; P12: -37.6 ± 3.1 pA/pF, n = 28, p = 0.0026, Mann-Whitney U test; Fig. 9C), similar to the findings in the I\textsubscript{Ca} amplitude analyses. At P4, the current density was significantly lower in WT than in Ca\textsubscript{v}1.3\textsuperscript{-/-} (p = 0.021), whereas the opposite finding was obtained at P12 (p = 0.0052, Mann-Whitney U test; Fig. 9C). At present, we do not have a straightforward explanation for this surprising result.

R-type channels and compensatory changes in the proportion of HVACC subtypes

When adding up the percentages of blockade obtained by nifedipine, ω-agatoxin IVA, and ω-conotoxin GVIA in WT and Ca\textsubscript{v}1.3\textsuperscript{-/-} LSO neurons at both P4 and P12, the sum was less than
100% in each of the four cases, indicating the presence of another HVACC channel subtype aside from L-, P/Q-, and N-type channels (Fig. 10). This subtype most likely comprises R-type channels, and the contribution amounted to 7% in P4 WT, 4% in P12 WT, 32% in P4 Ca\(_{v1.3}^{-/-}\), and 31% in P12 Ca\(_{v1.3}^{-/-}\).

The lack of age-related increases of I\(_{Ca}\) in Ca\(_{v1.3}^{-/-}\) LSO neurons between P4 and P12 demonstrates a disturbed development, but it cannot provide information about changes in the relative contribution of various HVACC subtypes. In order to address this issue, we calculated the relative contribution of the four groups of Ca\(^{2+}\) channels that contributed to I\(_{Ca}\) in WT neurons after the fraction attributed to Ca\(_{v1.3}\) subunits was subtracted. These corrected values (‘WT corr.’ in Fig. 10) can thus be directly compared with those obtained in Ca\(_{v1.3}^{-/-}\) neurons. It became obvious that Ca\(_{v1.2}\) and R-type channels appear to contribute to I\(_{Ca}\) to a larger extent in P4 Ca\(_{v1.3}^{-/-}\) neurons than they do in WT neurons. In contrast, the contribution of P/Q- and N-type channels is considerably reduced in Ca\(_{v1.3}^{-/-}\) neurons. At P12, however, the relative contribution of Ca\(_{v1.2}\) and N-type channels appears to be similar in both genotypes, whereas P/Q-type channels seem to be down- and R-type channels up-regulated. These results are indicative of a reorganization of functional HVACCs in the absence of Ca\(_{v1.3}\) subunits and also of ongoing changes with age. Nevertheless, an increase of I\(_{Ca}\) is obviously not achieved through these changes.

qRT-PCR analysis of HVACC expression in WT and Ca\(_{v1.3}^{-/-}\) LSO neurons

The results obtained in our electrophysiological analyses demonstrated the contribution of at least three different subtypes of HVACCs to I\(_{Ca}\) in LSO neurons (L-, N-, P/Q-type) and also indicated the presence of R-type channels. In addition, we found evidence that Ca\(_{v1.2}\) does not compensate for the absence of the Ca\(_{v1.3}\) subunit in P12 Ca\(_{v1.3}^{-/-}\) mice. To assess whether these data are paralleled at the transcriptional level, we examined the mRNA expression of individual HVACC \(\alpha_1\) subunits in the LSO of P12 WT and Ca\(_{v1.3}^{-/-}\) animals. By means of quantitative RT-PCR, we found that Ca\(_{v1.2}\) contributes to 49 ± 2.4% (\(n = 3\)) of all L-type transcripts and Ca\(_{v1.3}\) to 51%, with the majority (40 ± 2.1%) being attributed to the long splice variant Ca\(_{v1.3}-42\) and a minor part (11 ± 0.3%) to the short slice variant Ca\(_{v1.3}-42a\) (Fig. 11A). The presence of both variants is in accordance with recent data obtained in various brain regions (Singh et al. 2008). We also quantified the relative abundance of the transcripts for P/Q-, N-, and R-type channels and compared them with the data obtained for...
the L-type transcripts (assessment of GAPDH transcripts served as efficiency reference). We found lower levels for the three L-type transcripts than for each of the three non-L-type transcripts (Ca_{2.1}, Ca_{2.2}, Ca_{2.3}; Fig. 11B). Additionally, we tested for changes of subunit expression in Ca_{1.3}^{-/-} mice via a GAPDH-normalized \Delta\Delta Ct analysis (Marcantoni et al. 2010). As illustrated in Fig. 11C, the ratio of Ca_{2.1} mRNA expression was not different between WT and Ca_{1.3}^{-/-} mice (WT/KO: 1.03 ± 0.1, p = 0.98; n = 3), but a significant increase was evident for Ca_{1.2} (WT/KO: 1.47 ± 0.58, p = 0.016), Ca_{2.2} (WT/KO: 1.73 ± 0.06, p = 1.7E-05), and Ca_{2.3} (WT/KO: 2.04 ± 0.15, p = 3.9E-04). These gene expression results indicate that the absence of Ca_{1.3} channels may be compensated in P12 LSO neurons by a higher expression of Ca_{1.2} L-type channels as well as N-type and R-type channels. The results are partially in accordance with those described in the electrophysiological/pharmacological experiments.
DISCUSSION

This study employed electrophysiological, pharmacological, and gene expression analyses of high voltage-activated calcium channels in LSO neurons. Juvenile and neonatal WT, Ca\textsubscript{v}1.3\textsuperscript{−/−}, and Ca\textsubscript{v}1.2DHP\textsuperscript{−/−} mice were employed. The six major results can be summarized as follows:

First, LSO neurons express functional L-, P/Q-, N-, and - most likely - also R-type channels. These results are corroborated by demonstrating gene expression for five $\alpha$ subunits (L-type: $\alpha_{1C}$ and $\alpha_{1D}$; N-type: $\alpha_{1B}$; P/Q-type: $\alpha_{1A}$; R-type: $\alpha_{1E}$). Moreover, at least two splice variants encoding for Ca\textsubscript{v}1.3 are expressed and equally contribute to the L-type component. Second, both L-type channels, Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3, are functional. Third, at P12 about 45% of the Ca\textsuperscript{2+} current is mediated by L-type, 29% by P/Q-type, 22% by N-type and 4% most likely by R-type channels. Fourth, Ca\textsubscript{v}1.3 channels contribute more effectively to the L-type-mediated portion of $I_{Ca}$ than Ca\textsubscript{v}1.2 subunits. Fifth, the WT P12 profile of HVACCs is similar to that at P4. Finally, the loss of the $\alpha_{1D}$ subunit in Ca\textsubscript{v}1.3\textsuperscript{−/−} mice appears to be accompanied by changes in the contribution of other functional HVACCs, but these changes cannot reestablish normal Ca\textsuperscript{2+} current amplitudes.

Identification and functional characterization of LSO neurons

Based on their morphological characteristics, three distinct types were identified in C57BL/6 mice (Olo and Schwartz 1979) with spindle-shaped bipolar cells being the predominant type. In the present study, we chose LSO neurons according to their oval, spindle-shaped somata and their central location in the LSO. As we used a cesium-based intracellular solution, we could not use criteria like firing pattern, resting membrane potential, K\textsuperscript{+}-mediated outward currents, or hyperpolarization-activated inward currents for cell identification. Nevertheless, our whole cell capacitance values are in very good accord with data from mouse and rat principal LSO neurons (Barnes-Davies et al. 2004; Dodson et al. 2002; Leao et al. 2006; Sterenborg et al. 2010). We therefore conclude that our recordings originated from principal cells and thus from a homogenous population. Indeed, sporadic biocytin fills and subsequent morphological analysis confirmed this conclusion.

The unchanged whole cell capacitance values of LSO neurons between WT and Ca\textsubscript{v}1.3\textsuperscript{−/−} neurons do not conflict with our previous finding that the soma-dendritic field of bipolar LSO neurons was 81% larger in Ca\textsubscript{v}1.3\textsuperscript{−/−} animals (Hirtz et al. 2011, Fig. 7G). The size of the soma-
dendritic field is a measure of the extent of dendritic arbors (Elston and Rosa 1997), yet it does not correlate with the dendritic complexity. The complexity is rather illustrated by the number of dendritic segments, i.e., all branches of low and high order (Duan et al. 2002; Jacobs et al. 1993). Hirtz et al. recently assessed the complexity by the concentric shell method (Sholl 1953) and found no changes between WT and Ca$_{V1.3}^{-/-}$ bipolar LSO neurons (Hirtz et al. 2011, Fig. 7E), providing morphological evidence for unchanged whole cell capacitance.

Contribution of different types of HVACCs to $I_{Ca}$

Pharmacological studies on rodent bushy cells of the anteroventral CN (Doughty et al. 1998), neurons in the inferior colliculus (N’Gouemo and Morad 2003), octopus cells of the posteroventral CN (Bal and Oertel 2007), MSO and MNTB neurons (Barnes-Davies et al. 2001; Leao et al. 2004) consistently identified multiple components in generating the HVA currents and a significant contribution of L-type channels, albeit with considerable variation in the percentages. Like us, the authors concluded that the current that remained in the presence of the blocker cocktail was mediated by R-type channels. The reasons for the specific expression patterns of one or the other channel type in each particular nucleus are enigmatic. Due to the distinct structure, biophysical characteristics, and regulation patterns, a repertoire of HVACC subtypes provides a flexible array of Ca$^{2+}$ entry pathways, enabling fine-tuned responses to changes in membrane potential. In particular, the potential for diversity in modulation (via phosphorylation, cAMP-dependent protein kinase pathways, G-proteins; literature cited in Catterall 2000) might explain the seemingly redundant expression of L-, P/Q-, N- and R-type calcium channels.

Pharmacological considerations

Quantitative studies of $I_{Ca}$ amplitudes may be compromised by some pharmacological constraints. First, drugs may not have high channel specificity. At the concentration used here, $\omega$-agatoxin IVA effectively inhibits current through P/Q-type, but not through N- or L-type channels (literature cited in McDonough et al. 2002). Concerning $\omega$-conotoxin GVIA, its high specificity and potency on N-type channels has been consistently reported (Ichida et al. 2005; McCleskey et al. 1987; McDonough et al. 2002; Nielsen et al. 2000).
experiments, we did not apply the blockers sequentially and in combination. If redundancy of blocker specificity is a serious problem, the summed contribution of all Ca\(^{2+}\) channel currents blocked by the drugs should exceed 100\%, as observed in the IC (N’Gouemo and Morad 2003). However, in none of our experiments this was the case. Second, Ca\(_{v}1.3\) and Ca\(_{v}1.2\) may not be equally inhibitable in LSO neurons by nifedipine and isradipine. In fact, Ca\(_{v}1.3\) channels have been reported less sensitive to these drugs than Ca\(_{v}1.2\) channels (Helton et al. 2005; Lipscombe et al. 2004; Xu and Lipscombe 2001). However, the similarities between our pharmacological results and those gained with the two mouse mutant strains suggest that this concern is negligible at the concentrations used.

**Developmental aspects**

Both at P4 and P12, all HVACC types contributed to \(I_{\text{Ca}}\) in WT LSO neurons, but none of the channel types displayed considerable age-dependent changes (cf. Fig. 10 and Table 1). By contrast, an increase of the P/Q-type fraction and a concomitant decrease of the R-type fraction has been reported in the rat IC (N’Gouemo and Morad 2003; N’Gouemo and Rittenhouse 2000). Likewise, recordings from presynaptic calyces of Held terminals showed that N-type-mediated and R-type-mediated currents disappear after P10, whereas the P/Q-type fraction increases and becomes predominant (Iwasaki and Takahashi 1998). It should be noted that the authors identified no L-type fraction during the whole developmental period examined, consistent with the idea that these channels are predominantly located postsynaptically in the soma-dendritic plasma membrane.

**HVACCs in Ca\(_{v}1.3^{-}\) neurons**

Our electrophysiological data revealed that Ca\(_{v}1.3\) and Ca\(_{v}1.2\) channels appear to contribute to the L-type mediated current amplitudes with a ratio of 2:1 at P12 (30:15\% of total \(I_{\text{Ca}}\); cf. Fig. 10). Upon ablation of the Ca\(_{v}1.3\) gene, there is no obvious change in Ca\(_{v}1.2\) or N-type channels, but P/Q- and R-type channels appear to be down- and up-regulated, respectively. In contrast, our qRT-PCR results revealed increased expression levels for Ca\(_{v}1.2\) as well as N-type mRNA in Ca\(_{v}1.3^{-}\) neurons and unchanged levels of P/Q-type transcripts (cf. Fig. 11C). This indicates that the deficiency of Ca\(_{v}1.3\) channels in LSO neurons is compensated by other HVACCs at the transcriptional level.
Compensatory changes in the HVACC repertoire were observed in Purkinje cells of tottering mice, which possess a recessive mutation of the $\alpha_{1A}$ (P/Q-type) subunit. Although $\alpha_{1C}$ (Ca$_{v1.2}$) mRNA expression is significantly up-regulated (Campbell and Hess 1999), this does not prevent the tottering phenotype and ataxia; rather it may cause them. A similar form of compensation has been described in embryonic cardiomyocytes, where the deletion of the Ca$_{v1.2}$ gene results in increased expression of genes encoding for Ca$_{v1.1}$, Ca$_{v1.3}$, and Ca$_{v3.1}$, at both the mRNA and protein level (Xu et al. 2003). As shown by RT-PCR, the up-regulation was most pronounced for Ca$_{v1.3}$ (4-fold). Nevertheless, as Ca$_{v1.2}^{-/-}$ mice die before embryonic day 14 (Seisenberger et al. 2000), this implies that the substitution does not fully restore function. This is in line with our seemingly paradoxical findings presented here that, although Ca$_{v}$ channel genes are up-regulated in Ca$_{v1.3}^{-/-}$ mice, this does not result in restoration of normal $I_{Ca}$ amplitudes. Our finding that $I_{Ca}$ amplitudes in Ca$_{v1.3}^{-/-}$ mice do not increase with age, although they do so in wild-type animals, provides further evidence for a disturbed development of functional Ca$^{2+}$ channels in these animals.

An open question concerns the contribution of R-type channels to the total $I_{Ca}$ in LSO neurons. In unison with many other groups (Bal and Oertel 2007; Barnes-Davies et al. 2001; N’Gouemo and Morad 2003), we reason that the remnant current left after pharmacological blockade with nifedipine, $\omega$-agatoxin IVA, and $\omega$-conotoxin GIVA was mediated by this channel type. It will be interesting to identify functional R-type channels in the LSO, e.g. with a pharmacological inhibitor from tarantula venom (Bloodgood and Sabatini 2007; Newcomb et al. 1998; Plotkin et al. 2011). Of further interest is whether they are up-regulated in Ca$_{v1.3}^{-/-}$ neurons, as implied by our electrophysiological and qRT-PCR results (cf. Figs. 10, 11C).

With regard to a quantitative description of gene expression, previous studies compared mRNA levels with protein levels and concluded that the correlation is poor (de Sousa et al. 2009; Maier et al. 2009). It appears that the abundance of proteins is predominantly controlled at the level of translation (Schwanhausser et al. 2011). We therefore suppose that different HVACCs can compensate for each other in mRNA expression but may not be able to functionally substitute the lack of one specific channel subtype. Future studies will be required to provide more insight into how HVACC expression and function are regulated.
Conclusions

In summary, our study demonstrates the presence of virtually all subtypes of HVACCs (L-, P/Q-, N- and most likely R-type) in mouse LSO neurons at both juvenile and neonatal stages. In Ca\(^{\text{v}1.3}\)/- mice, the LSO is strikingly malformed and comprises about one third fewer neurons (Hirtz et al. 2011). As these mice lack cochlea-driven activity, their central auditory system is thus deprived from peripheral input. Therefore, the LSO defects can be transsynaptic, i.e., down-stream from cochlear dysfunction, as previously reported in cochlear ablation studies (Born and Rubel 1985; Sanes et al. 1992). However, the presence of functional Ca\(^{\text{v}1.3}\) channels in LSO neurons, as reported here, offers an alternative scenario, namely that on-site loss of the channels in the LSO neurons themselves contributes significantly to the malformation. Generation and analysis of tissue-specific Ca\(^{\text{v}1.3}\)/- animals is under way to distinguish between cochlear (peripheral) and on-site (central) effects, including the relative importance of each (Nothwang et al. 2011). Likewise, morphometric analyses of the LSO in other knockout models with cochlear dysfunction, such as prosaposin\(^{-}\)/ mice (Akil et al. 2006), otoferlin otof mice (Longo-Guess et al. 2007), and dn/dn mice (Youssoufian et al. 2008), will likely contribute to this open issue.
ACKNOWLEDGEMENTS

We thank Drs. Jutta Engel and Marlies Knipper for initial support. We are grateful to Jennifer Winkelhoff and Tina Kehrwald for excellent technical assistance. Helpful comments on this manuscript by Drs. Jan Hirtz and Hans Gerd Nothwang are appreciated. Current address of Dr. Bohumila Jurkovičová-Tarabová: Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlarska 5, Bratislava 83334, Slovak Republic. Current address of Dr. Antonella Pirone: Department of Neuroscience, Tufts University School of Medicine, Boston MA 02111, USA.

GRANTS

This work was supported by the Marie Curie Research Training Network “CavNET”, Contract MRTN-CT-2006-035367, the Deutsche Forschungsgemeinschaft (FR 1784/11-1) and the Austrian Science Fund (P20670).

AUTHOR CONTRIBUTIONS

Author contributions: E.F. and B.J.-T. designed research; B.J.-T. and A.P. performed experiments; B.J.-T., D.G., A.P., M.S.-B., and E.F. analyzed data; D.G., E.F., B.J.-T., and J.S. wrote the manuscript.
FIGURE LEGENDS

FIG. 1. The cochlea and auditory brainstem in WT and Ca\textsubscript{v}1.3\textsuperscript{-/-} mice.

A: Semi-schematic diagram of cochlear inner hair cells (IHC) and lower auditory brainstem regions, depicting the ascending pathways in wild-type (WT) (left) and Ca\textsubscript{v}1.3 deficient (Ca\textsubscript{v}1.3\textsuperscript{-/-}) mice (right). In WT, glutamate is released from IHCs onto the distal terminals of spiral ganglion neurons in the auditory nerve. This release crucially depends on an influx of Ca\textsuperscript{2+} ions through Ca\textsubscript{v}1.3 channels. Action potentials are conducted via the auditory nerve fibers into the cochlear nuclear complex (CN) and further transmitted into the superior olivary complex (SOC, highlighted in gray), which includes the lateral superior olive (LSO). In Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, glutamate release from IHCs and signal transmission is abolished (indicated by X). B: Two biocytin-filled LSO neurons in slices from WT and Ca\textsubscript{v}1.3\textsuperscript{-/-} mice at P12. The malformation (reduced size and loss of U shape) of the Ca\textsubscript{v}1.3\textsuperscript{-/-} LSO (outlined by dashed lines) is evident (Hirtz et al, 2011). Insets: Photomicrographs of the two LSO neurons at higher magnification. C: Representative current recordings on LSO neuron in WT and in Ca\textsubscript{v}1.3\textsuperscript{-/-} mice, evoked by depolarization steps to various test potentials from a holding potential of -70 mV, each performed once (inset). Recordings were obtained under control conditions and with Cd\textsuperscript{2+} in the bath solution. D: Maximal peak amplitude of WT and Ca\textsubscript{v}1.3\textsuperscript{-/-} neurons at P12 in control solution and in the presence of Cd\textsuperscript{2+} (50 µM).

FIG. 2. L-type channel-mediated currents at P12, as identified by nifedipine.

A,C,E: Representative examples of current traces at the test potential at which the peak amplitude was maximal. Recordings were performed on WT (A), Ca\textsubscript{v}1.3\textsuperscript{-/-} (C), and Ca\textsubscript{v}1.2DHP\textsuperscript{-/-} LSO neurons (E) in control solution and in the presence of nifedipine (20 µM). Corresponding nifedipine-sensitive currents were obtained by subtracting the current in the presence from that in the absence of the blocker. B,D,F: Current-voltage (I-V) relationships of the peak amplitude recorded in control solution and in the presence of nifedipine. G,H: Box plot and bar diagram, depicting the percentage of block of the peak amplitudes by nifedipine. In Ca\textsubscript{v}1.2DHP\textsuperscript{-/-} bar the proportion attributed to be mediated by Ca\textsubscript{v}1.2 channels is indicated by dotted surrounding. Bar diagram illustrates mean values ± standard error of the mean (SE) in this and all subsequent figures. Likewise, numbers in bars depict numbers of neurons analyzed.
FIG. 3. L-type channel-mediated currents at P12, as identified by isradipine.

A-C: Representative examples of current traces at the test potential at which the peak amplitude was maximal. Recordings were performed on WT (A), Ca$_{1.3}^{\pm}$ (B), and Ca$_{1.2}$DHP$^{-}$ LSO neurons (C) in control solution and in the presence of isradipine (3 µM). Corresponding isradipine-sensitive currents were obtained by subtracting the current in the presence from that in the absence of the blocker. D,E: Box plot and bar diagram, depicting the percentage of block by isradipine.

FIG. 4. P/Q-type channel-mediated currents at P12, as identified by ω-agatoxin IVA.

A,C: Representative examples of current traces at the test potential at which the peak amplitude was maximal. Recordings were performed on WT (A) and Ca$_{1.3}^{\pm}$ (C) in control solution and in the presence of ω-agatoxin IVA (600 nM). Corresponding ω-agatoxin IVA-sensitive currents were obtained by subtracting the current in the presence from that in the absence of the blocker. B,D: I-V relationships of the peak amplitude recorded in control solution and in the presence of ω-agatoxin IVA.

FIG. 5. N-type channel-mediated currents at P12, as identified by ω-conotoxin GVIA.

A,C: Representative examples of current traces at the test potential at which the peak amplitude was maximal. Recordings were performed on WT (A) and Ca$_{1.3}^{\pm}$ (C) in control solution and in the presence of ω-conotoxin GVIA (2 µM). Corresponding ω-conotoxin GVIA-sensitive currents were obtained by subtracting the current in the presence from that in the absence of the blocker. B,D: I-V relationships of the peak amplitude recorded in control solution and in the presence of ω-conotoxin GVIA. E,F: Box plot and bar diagram, depicting the percentage of block by ω-agatoxin IVA and ω-conotoxin GVIA, respectively.

FIG. 6. L-type channel-mediated currents at P4, as identified by nifedipine.

A,C,E: Representative examples of current traces at the test potential at which the peak amplitude was maximal. Recordings were performed on WT (A), Ca$_{1.3}^{\pm}$ (C), and Ca$_{1.2}$DHP$^{-}$ LSO neurons (E) in control solution and in the presence of nifedipine (20 µM). Corresponding nifedipine-sensitive currents were obtained by subtracting the current in the presence from that in the absence of the blocker. B,D,F: I-V relationships of the peak amplitude.
amplitude recorded in control solution and in the presence of nifedipine. G,H: Box plot and bar diagram, depicting the percentage of block by nifedipine.

FIG. 7. P/Q-type channel-mediated currents at P4, as identified by ω-agatoxin IVA. A,C: Representative examples of current traces at the test potential at which the peak amplitude was maximal. Recordings were performed on WT (A) and Ca\textsubscript{1.3}\textsuperscript{-/-} (C) in control solution and in the presence of ω-agatoxin IVA (600 nM). Corresponding ω-agatoxin IVA-sensitive currents were obtained by subtracting the current in the presence from that in the absence of the blocker. B,D: I-V relationships of the peak amplitude recorded in control solution and in the presence of ω-agatoxin IVA.

FIG. 8. N-type channel-mediated currents at P4, as identified by ω-conotoxin GVIA. A,C: Representative examples of current traces at the test potential at which the peak amplitude was maximal. Recordings were performed on WT (A) and Ca\textsubscript{1.3}\textsuperscript{-/-} (C) in control solution and in the presence of ω-conotoxin GVIA (2 µM). Corresponding ω-conotoxin GVIA-sensitive currents were obtained by subtracting the current in the presence from that in the absence of the blocker. B,D: I-V relationships of the peak amplitude recorded in control solution and in the presence of ω-conotoxin GVIA. E,F: Box plot and bar diagram, depicting the percentage of block by ω-agatoxin IV and ω-conotoxin GVIA, respectively.

FIG. 9. Age-related analysis of changes between P4 and P12 in WT and Ca\textsubscript{1.3}\textsuperscript{-/-} LSO neurons. A: Fraction of L-, P/Q-, and N-type HVACCs to total I\textsubscript{Ca}. B,C: Box plots and bar diagrams, depicting the I\textsubscript{Ca} density at P4 and P12.

FIG. 10. Relative contribution of the four groups of HVACCs to total I\textsubscript{Ca} at P4 and P12. In order to enable direct comparison of WT and Ca\textsubscript{1.3}\textsuperscript{-/-} data, the relative contribution of the Ca\textsubscript{1.2} subunit as well of the P/Q-, N-, and R-types in WT was calculated by mathematical subtraction of the fraction attributed to Ca\textsubscript{1.3} subunits (WT corr. = WT corrected). Notice the mean values for the absolute I\textsubscript{Ca} in all four cohorts studied (P4 WT, P4 Ca\textsubscript{1.3}\textsuperscript{-/-}, P12 WT, P12 Ca\textsubscript{1.3}\textsuperscript{-/-}).

FIG. 11. Quantitative RT-PCR assay of mRNA expression for HVACC α\textsubscript{1} subunits at P12.
A: Quantitative comparison of three different L-type transcripts, including two splice variants of Ca$_v$1.3. Relative abundance is depicted as the percentage of the total copy numbers of all three L-type subunit transcripts in each experiment ($n = 3$). B: Similar to panel A, but for six HVACC transcripts, using GAPDH as reference gene. C: Expression of Ca$_v$ subunits in Ca$_v$1.3$^{-/-}$ mice relative to WT, compared by $\Delta\Delta$Ct analysis ($n = 3$).


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Figure 1

A) COCHLEA

WT

Control 2+/Ca1.3

2+/Ca1.3

WT

Control

2+/Ca1.3

B) AUDITORY BRAINSTEM

WT

25 μm

dorsal

dorsal

25 μm

lateral

50 μm

Ca1.3+

C) WT

Control

Ca1.3+

Control

Cd2+ 50 μM

Cd2+ 50 μM

D) Peak amplitude (pA)

control  Cd2+ 50 μM  control  Cd2+ 50 μM

WT

Ca1.3+
Figure 2

A
WT
Nifedipine 20 μM
Control
Nifedipine-sensitive

B

C
Ca,1.3
Nifedipine 20 μM
Control
Nifedipine-sensitive

D

E
Ca,1.2DHP
Nifedipine 20 μM
Control
Nifedipine-sensitive

F

G

H

Block by Nifedipine (%)

** WT  Ca,1.3  Ca,1.2DHP**
Figure 3

(A) WT
Isradipine-sensitive

(B) Ca_{v,1.3}^{+}
Isradipine-sensitive

(C) Ca_{v,1.2DHP}^{+}
Isradipine-sensitive

(D) Block by Isradipine (%)

(E) Block by Isradipine (%)
Figure 4

A: WT

- agatoxin IVA 600 nM
- Control
- agatoxin IVA-sensitive

B: Control
- agatoxin IVA
- agatoxin IVA-sensitive

C: Ca_{1.3}^{+}

- agatoxin IVA 600 nM
- agatoxin IVA-sensitive

D: Control
- agatoxin IVA
- agatoxin IVA-sensitive
Figure 6

A

WT

Nifedipine 20 μM

Nifedipine-sensitive

B

Nifedipine-sensitive

-60 -40 -20 0 20 40 60

V (mV)

-600

-500

-400

-300

-200

-100

I (pA)

Control

Nifedipine

C

Ca,1.3⁺

Nifedipine 20 μM

Nifedipine-sensitive

D

Nifedipine-sensitive

-60 -40 -20 0 20 40 60

V (mV)

-600

-500

-400

-300

-200

-100

I (pA)

Control

Nifedipine

E

Ca,1.2DHP⁺

Nifedipine 20 μM

Nifedipine-sensitive

F

Nifedipine-sensitive

-60 -40 -20 0 20 40 60

V (mV)

-600

-500

-400

-300

-200

-100

I (pA)

Control

Nifedipine

G

Block by Nifedipine (%)

WT Ca,1.3⁺ Ca,1.2DHP⁺

H

Block by Nifedipine (%)

20 40 60

WT Ca,1.3⁺ Ca,1.2DHP⁺

n.s.

*
Figure 8

A

WT

100 pA

20 ms

ω-conotoxin GVIA 2 μM

Control

ω-conotoxin GVIA-sensitive

B

V (mV)

-60 -40 -20 0 20 40 60

I (pA)

-600 -500 -400 -300 -200 -100

Control

ω-conotoxin GVIA

ω-conotoxin GVIA-sensitive

C

Ca,1.3−

ω-conotoxin GVIA 2 μM

Control

ω-conotoxin GVIA-sensitive

D

V (mV)

-60 -40 -20 0 20 40 60

I (pA)

-600 -500 -400 -300 -200 -100

Control

ω-conotoxin GVIA

ω-conotoxin GVIA-sensitive

E

Block of LCa (%) vs. Ca,1.3−

0 20 40 60

ω-agatoxin IVA ω-conotoxin GVIA

F

Block of LCa (%) vs. Ca,1.3−

0 20 40 60

ω-agatoxin IVA ω-conotoxin GVIA

WT Ca,1.3−

n.s.

n.s.

11 10 12 9
Figure 9

A

Block of I_Ca (\%) CaICa density (pA/pF)

WT

\(-/-\) Ca 1.3

\(\text{n.s.}\)

B

\(\text{ICa density (pA/pF)}\)

WT

\text{P4 P12}

\(-/-\) Ca 1.3

\text{P4 P12}

\(\text{n.s.}\)

C

\(\text{I_Ca density (pA/pF)}\)

WT

\text{P4 P12}

\(-/-\) Ca 1.3

\text{P4 P12}

\text{**}\)

\text{n.s.}\)
Figure 10

The figure shows the contribution to inward currents (I_inward) from different calcium channel subtypes in WT and P12 genotypes. The data is presented for two panels, P4 and P12, each showing contributions from WT, WT corr., and Ca,1.3 (21) genotypes.

- **P4 Panel:**
  - WT (25): 7% L, 29% N, 28% P/Q, 26% Ca,1.2
  - WT corr.: 10% L, 42% N, 40% P/Q, 20% Ca,1.2
  - Ca,1.3 (21): 32% L, 22% N, 22% P/Q, 20% Ca,1.2

- **P12 Panel:**
  - WT (34): 4% L, 29% N, 26% P/Q, 26% Ca,1.2
  - WT corr.: 6% L, 31% N, 41% P/Q, 26% Ca,1.2
  - Ca,1.3 (27): 31% L, 22% N, 23% P/Q, 20% Ca,1.2

The currents are measured in pA, with values of 655 pA and 555 pA for WT and GT, respectively.
Figure 11

A. L-type % copies

B. Ratio Ca/GapDH

C. Ca1.3/WT ratio

Legend:
- L
- P/Q
- N
- R