L-type CaV1.2 deletion in the cochlea but not in the brainstem reduces noise vulnerability: implication for CaV1.2-mediated control of cochlear BDNF expression

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INTRODUCTION

Activity-dependent gene transcription is functionally relevant for animals to acquire information and adapt to the environment. One major ion involved in transducing neuronal activity and regulating transcription is calcium (Ca2+). Ca2+ influx through voltage-gated Ca2+ channels (VGGCs) serves as a key transducer coupling changes in cell surface membrane potential with local intracellular Ca2+ pathways. Among the L-type VGCCs (L-VGCCs), CaV1.2 is also expressed. Due to lethality of constitutive CaV1.2 knockout mice, the function of this ion channel as well as its putative relationship to BDNF in the auditory system is entirely elusive. We recently described that BDNF plays a differential role for inner hair cell (IHC) vesicles release in normal and traumatized condition. To elucidate a presumptive role of CaV1.2 during this process, two tissue-specific conditional mouse lines were generated. To distinguish the impact of CaV1.2 on the cochlea from that on feedback loops from higher auditory centers CaV1.2 was deleted, in one mouse line, under the Pax2 promoter (CaV1.2Pax2) leading to a deletion in the spiral ganglion neurons, dorsal cochlear nucleus, and inferior colliculus. In the second mouse line, the Egr2 promoter was used for deleting CaV1.2 (CaV1.2Egr2) in auditory brainstem nuclei. In both mouse lines, normal hearing threshold and equal number of IHC release sites were observed. We found a slight reduction of auditory brainstem response wave I amplitudes in the CaV1.2Pax2 mice, but not in the CaV1.2Egr2 mice. After noise exposure, CaV1.2Pax2 mice had less-pronounced hearing loss that correlated with maintenance of ribbons in IHCs and less reduced activity in auditory nerve fibers, as well as in higher brain centers at supra-threshold sound stimulation. As reduced cochlear BDNF mRNA levels were found in CaV1.2P Pax2 mice, we suggest that a CaV1.2-dependent step may participate in triggering part of the beneficial and deteriorating effects of cochlear BDNF in intact systems and during noise exposure through a pathway that is independent of CaV1.2 function in efferent circuits.

Voltage-gated L-type Ca2+ channels (L-VGCCs) like CaV1.2 are assumed to play a crucial role for controlling release of trophic peptides including brain-derived neurotrophic factor (BDNF). In the inner ear of the adult mouse, besides the well-described L-VGCC CaV1.3, CaV1.2 is also expressed. Due to lethality of constitutive CaV1.2 knock-out mice, the role of this ion channel as well as its putative relationship to BDNF in the auditory system is entirely elusive. We recently described that BDNF plays a differential role for inner hair cell (IHC) vesicles release in normal and traumatized condition. To elucidate a presumptive role of CaV1.2 during this process, two tissue-specific conditional mouse lines were generated. To distinguish the impact of CaV1.2 on the cochlea from that on feedback loops from higher auditory centers CaV1.2 was deleted, in one mouse line, under the Pax2 promoter (CaV1.2Pax2) leading to a deletion in the spiral ganglion neurons, dorsal cochlear nucleus, and inferior colliculus. In the second mouse line, the Egr2 promoter was used for deleting CaV1.2 (CaV1.2Egr2) in auditory brainstem nuclei. In both mouse lines, normal hearing threshold and equal number of IHC release sites were observed. We found a slight reduction of auditory brainstem response wave I amplitudes in the CaV1.2Pax2 mice, but not in the CaV1.2Egr2 mice. After noise exposure, CaV1.2Pax2 mice had less-pronounced hearing loss that correlated with maintenance of ribbons in IHCs and less reduced activity in auditory nerve fibers, as well as in higher brain centers at supra-threshold sound stimulation. As reduced cochlear BDNF mRNA levels were found in CaV1.2Pax2 mice, we suggest that a CaV1.2-dependent step may participate in triggering part of the beneficial and deteriorating effects of cochlear BDNF in intact systems and during noise exposure through a pathway that is independent of CaV1.2 function in efferent circuits.

Keywords: L-VGCCs, CaV1.2, inner ear, SOC, ABR, BDNF

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ORIGINAL RESEARCH ARTICLE
published: 09 August 2013
doi: 10.3389/fnmol.2013.00200

Frontiers in Molecular Neuroscience www.frontiersin.org August 2013 | Volume 6 | Article 20 | 1
elements such as CaRf1 in cochlear neurons (Singer et al., 2008). It is also found that BDNF is up-regulated following auditory trauma (Tan et al., 2007). These observations may be related to the contrasting roles of BDNF previously described in the intact or injured cochlea (Zuccotti et al., 2012).

On the one side, BDNF plays a crucial role to upgrade complexity of the inner hair cell (IHC) synapse, including maintenance of mature IHC number of synaptic ribbons, electron-dense presynaptic specializations that tether synaptic vesicles for exocytosis at the active zone (Yoo, 2009; Meier et al., 2006; Schnitz, 2009) in mice (Zuccotti et al., 2012) and zebrafish (Mo and Nicolson, 2011). On the other side, BDNF is harmful when acoustic overstimulation damages the mature system (Zuccotti et al., 2012). As IHC ribbons are crucial for precision of sound processing (Buran et al., 2010) and IHC ribbon loss and deafferentation after acoustic trauma is discussed in the context of age-dependent hearing loss, hyperacusis, and tinnitus (Kujawa and Liberman, 2009; Lin et al., 2010) and IHC ribbon loss and deafferentation after acoustic trauma is discussed in the context of age-dependent hearing loss, hyperacusis, and tinnitus (Kujawa and Liberman, 2009; Lin et al., 2010). These observations may be related to the contrasting roles of BDNF previously described in the intact or injured cochlea (Zuccotti et al., 2012).

To elucidate to what extent CaV1.2 might participate in the described roles of BDNF in the healthy and injured cochlea, we analyzed CaV1.2 function following conditional deletion of CaV1.2 in the auditory system. This approach is essential as mice globally lacking CaV1.2 die in utero before day 15 post-coitum (Seisenberger et al., 2000). We conditionally inactivated CaV1.2 in the auditory system using the same Cre transgenic mouse line as used for deletion of BDNF (Zuccotti et al., 2012). As feedback loops from higher auditory centers, known as the corticofugal pathway (Feliciano and Potashner, 1995), have been shown to modulate directly efferent feedback along the ascending auditory pathway as well as the IHC in a frequency-specific way (Xiao and Suga, 2002), we studied mice with an inactivation of CaV1.2 in the superior olivary complex (SOC) that represent the second central auditory processing center in the ascending auditory pathway, using the Cre expression under the Egr2 promoter (Voiculescu et al., 2000; Sathesh et al., 2012).

Both CaV1.2<sup>2fl</sup> and CaV1.2<sup>2fl</sup> conditional knock-out (KO) mice were viable and thus could be analyzed for hearing capability, sound processing along the ascending auditory pathway, and sensitivity to noise exposure. We demonstrate here that CaV1.2 is neither required in the cochlea nor in the SOC for normal IHC or outer hair cell (OHC) function, thus suggesting that BDNF function in normal IHC physiology (Zuccotti et al., 2012) does not depend on CaV1.2. In contrast, loss of CaV1.2 in the cochlea, but not in the SOC, partially mimics the described harmful BDNF effect during acoustic trauma, a feature that could be linked to reduced BDNF mRNA levels in cochlear tissue of CaV1.2<sup>Pax2</sup> mice. The results are discussed in the context of a role of L-type Ca<sup>2+</sup> channels for BDNF release during cochlear injury.

MATERIALS AND METHODS
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acoustic stimulus (i.e., the speaker remained turned off). In noise-exposed mice, the degree of the ABR threshold shift was measured more than 7 days after noise exposure, when noise-induced permanent threshold shift (PTS; Liberman, 1980; Salvi et al., 1986) have settled and a recovery from damage is no longer expected. The sham-exposed animals have completely normal hearing.

**Tissue Preparation**

For immunohistochemistry, cochleae were isolated, fixed by immersion in 2% paraformaldehyde, 1.25 mM sucrose in 100 mM phosphate-buffered saline, pH 7.4, for 2 h, and then decalcified for 45 min in RDO rapid decalcifier (Apex Engineering Products Corporation, Aurora, IL, USA) as previously described (Küpper et al., 1999; Zuccotti et al., 2012; Singer et al., 2013), cryosectioned at 10 μm, mounted on SuperFrost® plus microscope slides at −20°C.

For RNA and proteins isolation, cochleae and different brain regions were dissected with small forceps and immediately frozen in liquid nitrogen and stored at −80°C until use.

**RNA Isolation, cDNA Synthesis, and Real-Time PCR**

For real-time polymerase chain reaction (PCR) analysis, RNA from mouse cochlea was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. After reverse transcription using Sensiscript RT kit (Qiagen), real-time PCR was performed using the iCycler iQ detection system (Bio-Rad, Munich, Germany) following the manufacturer’s instructions. After reverse transcription using Sensiscript RT kit (Qiagen), real-time PCR was performed using the iCycler iQ detection system (Bio-Rad, Munich, Germany) following the manufacturer’s instructions. The mRNA isolation was performed using the Oligotex Direct DNA Mini Kit (Qiagen). The mRNA was loaded onto a denaturing 0.8% agarose formaldehyde gel and transferred onto a nylon membrane (Roche, Mannheim, Germany). The membrane was blocked for 30 min at 65°C with hybridization buffer, digoxigenin Easy Hybridization Buffer (Roche), and hybridized overnight at 65°C with riboprobes for BDNF and cyclophilin. After washing, a 1-h blocking step was performed. The membrane was incubated with anti-Dig-AP (Roche, 1:20,000). The mRNA was detected with CSP-Star ready-to-use (Roche) and exposed to X-ray films.

**Western Blot**

Western blot with the antibody CNC1, which is directed against Loop II/III of α1.2, the central Cav1.2 subunit, was performed as previously described (Helf et al., 1993; Davare et al., 1999). Briefly, tissue samples were extracted with 3x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 90°C for 5 min before SDS-PAGE in 7.5% polyacrylamide gels, transfer to polyvinylidene difluoride (PVDF) membranes, blocking with 10% milk powder and detection with CNC1 and horseradish peroxidase-coupled protein A. Tubulin with an anti-tubulin monoclonal mouse antibody (Santa Cruz sc-5286, Dallas, TX, USA) was detected in parallel at the lower part of the blot to ensure equal loading when indicated.

**Data Analysis**

**ABR Waveform Amplitude Analysis**

The ABR waveforms were analyzed for consecutive amplitude deflections (waves), each wave consisting of a starting negative (n) peak and the following positive (p) peak. Peak amplitudes of ABR waves I and III were extracted in the present study and defined as follows: wave I: In (0.9–2 ms), wave III: IIIp − IIIm (3.3–4.1 ms). A customized program was used for the extraction of ABR peaks based on the definitions given above. ABR peak-to-peak, or wave amplitude growth functions were constructed for individual ears based on the extracted peaks for increasing stimulus levels. All ABR wave amplitude growth functions were calculated for increasing stimulus levels with reference to the ABR thresholds (from −20 to a maximum of 75 dB above threshold before noise exposure and from −20 to a maximum of 55 dB above threshold after noise exposure). For illustration purpose, ABR wave amplitude growth functions as shown in Figures 3A, B, D, E and 6A, B were first linearly interpolated to the resolution of 1 data point/dB and then smoothed by a moving zero-phase Gaussian filter with a window length of 9 data points. The ABR

For double labeling studies, both antibodies were simultaneously incubated for identical time periods. Sections were viewed, as previously described (Zamponi et al., 2010), using an Olympus BX61 microscope equipped with epifluorescence illumination. Images were acquired using an Olympus XM10 CCD monochrome camera and analyzed with cellSens Dimension software (OISIS GmbH, Munster, Germany). To increase spatial resolution, slices were imaged over a distance of 15 μm within an image-stack along the z-axis (z-stack) followed by 3-dimensional deconvolution using cellSens dimension built-in algorithm.

**Northern Blot**

The mRNA isolation was performed using the Oligoex Direct mRNA Mini Kit (Qiagen). The mRNA was loaded onto a denaturing 0.8% agarose formaldehyde gel and transferred onto a nylon membrane (Roche, Mannheim, Germany). The membrane was blocked for 30 min at 65°C with hybridization buffer, digoxigenin Easy Hybridization Buffer (Roche), and hybridized overnight at 65°C with riboprobes for BDNF and cyclophilin. After washing, a 1-h blocking step was performed. The membrane was incubated with anti-Dig-AP (Roche, 1:20,000). The mRNA was detected with CSP-Star ready-to-use (Roche) and exposed to X-ray films.

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waves shown in the inset of the figures were smoothed by a moving zero-phase Gaussian filter with a window length of 5 data points (0.5 ms).

**Statistical analysis**

Unless otherwise stated, all data were presented as group mean with SD or with standard error of the mean (SEM). Differences of the means were compared for statistical significance either by Student’s t-test, one-way, or two-way ANOVA tests. The ANOVA tests were followed by multiple t-tests with Bonferroni-Holm’s adjustment of α levels. Statistical significance was tested at the level α < 0.05, and resulting p values are reported in the legends. *p < 0.05, **p < 0.01, ***p < 0.001; n.s., not significant.

**RESULTS**

**CONDITIONAL DELETION OF CaV1.2 DRIVEN BY THE Pax2 AND Egr2 PROMOTORS**

To circumvent embryonic lethality of mice globally lacking CaV1.2, CaV1.2loxP/loxP (or floxed) mice (Zuccotti et al., 2004) were either mated to Pax2-Cre mice (Ohyama and Groves, 2004) or to Egr2-Cre mice (Voicusulescu et al., 2000).

As predicted for the usage of Cre under control of the Pax2 promoter (Ohyama and Groves, 2004; Zuccotti et al., 2012), CaV1.2loxP/loxP KO mice exhibited a deletion of CaV1.2 in the cochlea, brainstem, and cerebellum, but not in the auditory cortex. This was detected by Western blot analysis (Figure 1A, n = 10 cochleae (5 mice), 2 brainstems (2 mice), 2 cerebella (2 mice), and 2 auditory cortex (2 mice); experiments done in triplicate). The deletion of CaV1.2 in spiral ganglion neurons (SGNs; Warr and Guinan, 1979) was confirmed by immunohistochemistry (Figure 1B, CaV1.2, red). No CaV1.2 staining was observed in CaV1.2loxP/loxP KO animals in comparison to control littermates (Figure 1B, red).

Usage of Cre under the control of the Egr2 promoter leads to a knock-out of CaV1.2 in the SOC forming part of the auditory brainstem (Voicusulescu et al., 2000; Rosengauer et al., 2012; Satheesh et al., 2012). This was confirmed by Western blot of the SOC (n = 3 control mice, 3 KO mice; experiments done in triplicate) where no CaV1.2 expression was detected (Figure 1C).

**HEARING FUNCTION IN CaV1.2 MOUSE LINES**

To test whether the lack of CaV1.2 in the cochlea or brainstem affects hearing function at adult stages, ABR and DPOAE in 2- to 4-month-old mice were measured.

For the CaV1.2loxP/loxP mice, no significant difference was observed compared to controls for click-evoked ABR thresholds (Figure 2A, CaV1.2loxP/loxP control: 15.6 ± 2.97 dB SPL, CaV1.2loxP/loxP KO: 16.2 ± 2.71 dB SPL, n = 16 ears, 8 mice each, two-sided Student’s t-test: p = 0.556) or tone-burst evoked ABR thresholds (Figure 2B, CaV1.2loxP/loxP control: n = 7-8 ears, 7-8 mice, CaV1.2loxP/loxP KO: n = 6-8 ears, 6-8 mice, two-way ANOVA, p = 0.899). Similar results were obtained for the CaV1.2loxP/loxP mouse line for click-evoked stimulation (Figure 2C, CaV1.2loxP/loxP control: 12.2 ± 2.20, n = 16 ears, 8 mice, CaV1.2loxP/loxP KO: 14.7 ± 4.28, n = 18 ears, 9 mice, two-sided Student’s t-test: p = 0.204) and tone-burst evoked ABR thresholds (Figure 2D, CaV1.2loxP/loxP control: n = 12-13 ears, 8-9 mice; CaV1.2loxP/loxP KO: n = 13 ears, 9 mice, two-way ANOVA, p = 0.132).

Since OHC electromotility codetermines the sound-evoked neural potentials at threshold (El-Badry and McFadden, 2007), DPOAE were measured as an objective indicator of OHC function. The DPOAE thresholds (Figures 2G, H) and the input/output (I/O) function of emission amplitudes evoked by stimulus frequency of 11.3 kHz (Figures 2D, H), the frequency showing the best hearing sensitivity for the mice, were found to be similar between control and CaV1.2loxP/loxP KO mice (CaV1.2loxP/loxP control: n = 4 ears, 4 mice; CaV1.2loxP/loxP KO: n = 4 ears, 4 mice, two-way ANOVA, p = 0.452) and CaV1.2loxP/loxP KO (CaV1.2loxP/loxP control: n = 14-15 ears, 8 mice; CaV1.2loxP/loxP KO: n = 16-18 ears, 9 mice), indicating that loss of CaV1.2 did not influence mobility of OHCs.

The click-evoked ABR waveform amplitudes are expected to change proportionally to the size of discharge rates and number of synchronously firing auditory nerve (AN) fibers (Johnson and Kiang, 1976). We, therefore, analyzed ABR waveform (Figures 3A, D),...
reflecting the summed activity of the AN fibers, and ABR wave III (Figures 3B,E), corresponding to the SOC (Melcher et al., 1996), in the two CaV1.2 mutant mouse lines where CaV1.2 is missing either in the cochlea or in parts of the auditory brainstem (Figure 1). In CaV1.2Pax2 mice, but not in CaV1.2Egr2 mice, a stronger decline of ABR wave I was observed beginning at 50 dB above threshold (Figure 3A, CaV1.2Pax2 control: n = 3–6 ears, 3–6 mice; CaV1.2Egr2 KO: n = 5–9 ears, 3 mice; two-way ANOVA, \( p = 0.233 \)). No Bonferroni–Holm’s adjustment for multiple testing; Figure 3D).

**FIGURE 2 | Continued**

No deterioration of ABR thresholds and OHC function in CaV1.2Pax2 KO and CaV1.2Egr2 KO mice. (A) Mean ± SD click-evoked ABR threshold for CaV1.2Pax2 KO (red horizontal dash, single ear thresholds as white circles) and CaV1.2Egr2 KO mice (green horizontal dash, single ear thresholds as white squares). Thresholds were not significantly different (two-sided Student’s \( t \)-test: \( p = 0.556 \)) between KO mice (18.2 ± 2.71, n = 16 ears, 8 mice) and control (19.0 ± 2.35, n = 16 ears, 8 mice). (B) Mean ± SD tone-burst-evoked ABR (F-ABR) thresholds for CaV1.2Pax2 KO mice (red) and controls (white). Thresholds were not significantly different (two-way ANOVA, \( p = 0.890 \)) between KO mice (n = 6–8 ears, 6–8 mice) and controls (n = 7–8 ears, 7–8 mice). (C) Mean ± SD DPOAE thresholds for CaV1.2Pax2 KO mice (red) and controls (white). Thresholds were not significantly different (two-way ANOVA, \( p = 0.452 \)) between KO mice (n = 4 ears, 4 mice) and controls (n = 4 ears, 4 mice). (D) Mean ± SD DPOAE amplitude (I/O function evoked by stimulus f1 = 113 kHz for CaV1.2Pax2 KO mice (red) and controls (white) showing no significant difference (two-way ANOVA: \( p = 0.132 \)).

**FIGURE 2 | Continued**

Synaptic ribbons are known to be an accurate metric of the I/O function evoked by stimulus f2 (2.7 kHz). The number of synaptic ribbons in drive postsynaptic AN fibers by a large releasable transmitter pool afferent innervation of IHCs (Kujawa and Liberman, 2009) that reflects the summed activity of the AN fibers, and ABR wave III reflecting the summed activity of the AN fibers, and ABR wave III (Figures 3B,E), corresponding to the SOC (Melcher et al., 1996), in the two CaV1.2 mutant mouse lines where CaV1.2 is missing either in the cochlea or in parts of the auditory brainstem (Figure 1). In CaV1.2Pax2 mice, but not in CaV1.2Egr2 mice, a stronger decline of ABR wave I was observed beginning at 50 dB above threshold (Figure 3A, CaV1.2Pax2 control: n = 3–6 ears, 3–6 mice; CaV1.2Egr2 KO: n = 5–9 ears, 3 mice; two-way ANOVA, \( p = 0.233 \)). No Bonferroni–Holm’s adjustment for multiple testing; Figure 3D).

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No deterioration of ABR thresholds and OHC function in CaV1.2Pax2 KO and CaV1.2Egr2 KO mice. (A) Mean ± SD click-evoked ABR threshold for CaV1.2Pax2 KO (red horizontal dash, single ear thresholds as white circles) and CaV1.2Egr2 KO mice (green horizontal dash, single ear thresholds as white squares). Thresholds were not significantly different (two-sided Student’s \( t \)-test: \( p = 0.556 \)) between KO mice (18.2 ± 2.71, n = 16 ears, 8 mice) and control (19.0 ± 2.35, n = 16 ears, 8 mice). (B) Mean ± SD tone-burst-evoked ABR (F-ABR) thresholds for CaV1.2Pax2 KO mice (red) and controls (white). Thresholds were not significantly different (two-way ANOVA, \( p = 0.890 \)) between KO mice (n = 6–8 ears, 6–8 mice) and controls (n = 7–8 ears, 7–8 mice). (C) Mean ± SD DPOAE thresholds for CaV1.2Pax2 KO mice (red) and controls (white). Thresholds were not significantly different (two-way ANOVA, \( p = 0.452 \)) between KO mice (n = 4 ears, 4 mice) and controls (n = 4 ears, 4 mice). (D) Mean ± SD DPOAE amplitude (I/O function evoked by stimulus f1 = 113 kHz for CaV1.2Pax2 KO mice (red) and controls (white) showing no significant difference (two-way ANOVA: \( p = 0.132 \)).
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FIGURE 3 | Reduction of ABR wave I amplitudes and unaltered ABR wave III amplitudes in CaV1.2Pax2 KO mice. (A) Mean ± SEM click-evoked ABR wave I amplitude growth function for CaV1.2Pax2 control (control, black line, and gray area) and CaV1.2Pax2 KO mice (KO, red squares). ABR wave I amplitudes were significantly different (two-way ANOVA, \( p = 0.048 \)) between KO (\( n = 5–9 \) ears, 3–6 mice) and controls (\( n = 3–6 \) ears, 3–6 mice), particularly at 65 dB above threshold (post hoc one-sided Student's t-test: \( p = 0.049 \), no Bonferroni–Holm's adjustment for multiple testing). The black and red waveforms in the inset depict representative ABR traces measured from one individual ear of a CaV1.2Pax2 control and a KO mouse, respectively, evoked at the level of 65 dB above the hearing threshold. The leading negative (n) and following positive (p) peak corresponded to wave I and are indicated by upward and a downward pointing arrow head associated with symbols “In” and “Ip” respectively. (B) Mean ± SEM click-evoked ABR wave III amplitude growth function for controls and CaV1.2Pax2 KO mice. ABR wave III amplitudes were not significantly different (two-way ANOVA, \( p = 0.639 \)) between KO (\( n = 2–5 \) ears, 2–4 mice) and controls (\( n = 3 \) ears, 2 mice). The leading negative (n) and following positive (p) peak corresponded to wave III and are indicated by arrow heads and marked “IIIn” and “IIIp” respectively. (C) Mean synaptic ribbon number ± SD are not significantly different (two-way ANOVA, \( p = 0.260 \)) between control and CaV1.2Pax2 KO mice in apical, medial, and midbasal cochlear turns (control: \( n = 4 \) cochleae, 3 mice; KO: \( n = 3 \) cochleae, 3 mice). (D) Mean ± SEM click-evoked ABR wave I amplitude growth function for CaV1.2Egr2 control (CaV1.2 Egr2 control, black and gray area) and CaV1.2Egr2 KO mice (green squares). ABR wave I amplitudes were not significantly different (two-way ANOVA, \( p = 0.784 \)) between KO (\( n = 7–11 \) ears, 4–8 mice) and controls (\( n = 12–13 \) ears, 7 mice). The black and green waveforms in the inset depict representative ABR traces measured from one individual ear of a CaV1.2Egr2 control mouse and KO mouse, respectively, evoked at the level of 65 dB above the hearing threshold. (E) Mean ± SEM click-evoked ABR wave III amplitude growth function for CaV1.2Egr2 KO mice and controls. ABR wave III amplitudes were not significantly different (two-way ANOVA, \( p = 0.281 \)) between KO (\( n = 9–12 \) ears, 5–7 mice) and controls (\( n = 10–11 \) ears, 6 mice). (F) Mean ± SD IHC ribbon counts for CaV1.2Pax2 control (control, white bars) and CaV1.2Egr2 KO mice (KO, green bars) in apical, medial, and midbasal cochlear turns. No significant difference (two-way ANOVA, \( p = 0.446 \)) between the number of ribbons in control and CaV1.2Egr2 KO mice (control: \( n = 3 \) cochleae, 3 mice; KO: \( n = 3 \) cochleae, 3 mice).

ACOUSTIC TRAUMA EFFECT ON HEARING FUNCTION IN CONDITIONAL CaV1.2 MUTANTS

We studied a possible role of CaV1.2 during noise damage by comparing ABR thresholds between control and CaV1.2Pax2 KO and CaV1.2Egr2 KO mice after exposing them for 1 hr to broadband noise (4–16 kHz) of 120 dB SPL. Hearing thresholds were analyzed by ABR to click stimulus 7–11 days after noise exposure (Figure 4).

All noise-exposed animals (controls and KOs) showed significant ABR threshold losses for click stimuli (Figures 4A,B, Tables 1 and 2).

However, ABR thresholds to click stimuli were significantly lower in noise-exposed CaV1.2Pax2 KO mice in comparison to noise-exposed controls after 7 days (Figure 4A, Table 1, comparison between genotypes at 7 days post noise). On the
induction of trauma.

controls a key event for threshold loss already shortly after the observed between CaV1.2Pax2 control and KO mice 7–11 days after in the brainstem, protects against noise.

day after exposure (loss appeared instantaneously and was observed already at the first

thresholds observed in CaV1.2Pax2 mutants were accompanied ing; ABR wave III amplitudes in CaV1.2Pax2 KO mice (Figure 6B, CaV1.2Pax2 control: n = 3–10 ears, 3–6 mice; CaV1.2Pax2 KO: n = 3–7 ears, 3–4 mice; two-way ANOVA, p = 0.013) were less reduced when compared to the controls at high stimulation levels of sound intensity (>30 dB above thresholds).

The changes in the ABR wave I amplitude and the hearing thresholds observed in CaV1.2Pax2 mutants were accompanied by an altered number of synaptic ribbons in the three cochlear regions (apical, medial, and midbasal). Ribbon numbers in noise

Table 1 | Auditory brainstem response thresholds for CaV1.2Pax2 mouse line evoked by click stimuli.

| CaV1.2Pax2 | Mean ± SD (n) | Significance |
|------------|--------------|--------------|
| control | 16.2 ± 2.71 (16) | *** |
| KO | 16.2 ± 2.71 (16) | *** |
| Significance | n.s. | *** |

Mean ± SD (no. of ears/no. of mice); ***p < 0.001 (two-sided t-test); n.s., not significant.

parallel evoked ABR wave amplitude growth functions were compared between control and CaV1.2Pax2 KO 7 days after noise exposure for latencies corresponding to the AN (wave I)

Table 2 | Auditory brainstem response thresholds for CaV1.2Egr2 mouse line evoked by click stimuli.

| CaV1.2Egr2 | Mean ± SD (n) | Significance |
|------------|--------------|--------------|
| control | 13.2 ± 2.00 (16) | *** |
| KO | 14.7 ± 4.28 (18) | *** |
| Significance | n.s. | n.s. |

Mean ± SD (no. of ears/no. of mice); ***p < 0.001 (two-sided t-test); n.s., not significant.

contrary, no differences were observed between control and CaV1.2Egr2 KO mice. Reduced vulnerability of click–ABR threshold loss appeared instantaneously and was observed already at the first day after exposure (Figure 5), indicating that CaV1.2 deletion may control a key event for threshold loss already shortly after the induction of trauma.

These data suggest that CaV1.2 deletion in the cochlea, but not in the brainstem, protects against noise.

Since significant differences in hearing thresholds were observed between CaV1.2Pax2 control and KO mice 7–11 days after noise exposure, click-evoked ABR wave amplitude growth functions were compared between control and CaV1.2Pax2 KO 7 days after noise exposure for latencies corresponding to the AN (wave I)

and SOC (wave III; Melcher et al., 1996). After noise exposure, both ABR wave I (Figure 6A, CaV1.2Pax2 control: n = 5–11 ears, 5–8 mice; CaV1.2Pax2 KO: n = 6–13 ears, 5–8 mice; two-way ANOVA, p = 0.004, post hoc one-sided Student’s t-test: p = 0.033 at 35 dB above threshold, Bonferroni–Holm’s adjustment for multiple testing; p = 0.026 at 40 dB above threshold, no Bonferroni–Holm’s adjustment for multiple testing) and ABR wave III amplitudes in CaV1.2Pax2 KO mice (Figure 6B, CaV1.2Pax2 control: n = 3–10 ears, 3–6 mice; CaV1.2Pax2 KO: n = 3–7 ears, 3–4 mice; two-way ANOVA, p = 0.013) were less reduced when compared to the controls at high stimulation levels of sound intensity (>30 dB above thresholds).

The changes in the ABR wave I amplitude and the hearing thresholds observed in CaV1.2Pax2 mutants were accompanied by an altered number of synaptic ribbons in the three cochlear regions (apical, medial, and midbasal). Ribbon numbers in noise
We recently showed that lack of BDNF hampers IHC synapse physiology and hearing function while it protects against noise-induced hearing loss (NIHL; Zuccotti et al., 2012). In the present study, we provide evidence that part of this crucial BDNF expression may be controlled by the L-VGCC CaV1.2.

CaV1.2 is expressed in SGN and efferent synapses (Waka et al., 2003) that originate in the olivocochlear system in the brainstem and terminate axo-dendritically on afferent type I fibers and axo-somatically on OHCs in the cochlea (White and Warr, 1983). The expression pattern of CaV1.2 is mostly non-overlapping with that of another L-VGCC, Cav1.3, that has been shown to play an essential role for hearing, since its deletion results in deafness (Platzer et al., 2008).

CaV1.2 knock-out mice are lethal at birth and thus, the role of Cav1.2 in hearing function is elusive. Cav1.2 is assumed to participate in altering synapse efficacy through transcriptional control of BDNF (Zuccotti et al., 2012).

According to West et al. (2001), CaV1.2 influx through Cav1.2 activates a CaV1.2-responsive binding protein, CaRF, that acts as a re-acting element on the BDNF exon IV promoter to control BDNF transcription after, e.g., stimulation by kainate. In this study, we analyzed mice with a conditional deletion of Cav1.2 in the cochlea, with Cre recombination controlled by the Pax2 promoter as recently described for the conditional BDNF KO mice in Zuccotti et al. (2012). We, moreover, compared the effect of deletion of Cav1.2 in the SOC using mice with Cre recombination controlled by Egfr promoter (CaV1.2 conditional KO mice).

DISCUSSION

We recently showed that lack of BDNF hampers IHC synapse physiology and hearing function while it protects against noise-induced hearing loss (NIHL; Zuccotti et al., 2012). In the present study, we provide evidence that part of this crucial BDNF expression may be controlled by the L-VGCC CaV1.2.

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CaV1.2Pax2 KO and CaV1.2Egr2 KO mice do not display significant changes of thresholds and OHC function. This implies that CaV1.2 expression in the cochlea and SOC might not be essential for maintenance of hearing thresholds and OHC function. Furthermore, we analyzed the ABR wave amplitudes, which reflect the number of synchronously firing neurons in distinct structures and nuclei along the ascending auditory pathway (Birkard and Don, 2007). Wave I amplitudes, corresponding to the neural activity from the AN fibers (Johnson and Kiang, 1976), of CaV1.2Pax2 KO mice were found significantly reduced starting at 50 dB above threshold compared to the control mice. In contrast, wave III amplitudes, corresponding to the neural activity from the cochlear nucleus (CN) and the SOC in the lower brainstem (Melcher et al., 1996), of the CaV1.2Pax2 KO mice are unchanged. Also wave I and III amplitudes of CaV1.2Pax2 KO mice appeared normal.

This may indicate that, similar to the role of BDNF (Zuccotti et al., 2012), lack of CaV1.2 in the cochlea hampers the function of the AN, but the phenotype appears to be less pronounced.

Per contrast to what has been described for the BDNF conditional KO mice (Zuccotti et al., 2012), we could not find significant differences in the number of ribbons in CaV1.2Pax2 KO mice. Ribbon structure in IHC maintains a large ready releasable pool of neurotransmitter, which is essential for neuronal survival after IHC damage (Zilberstein et al., 2012). These supporting cell types have also been shown to express BDNF (Sobkowicz et al., 2002; Zuccotti et al., 2012). Around 7–11 days after noise exposure, NIHL and a PTS were observed in both CaV1.2Pax2 KO and CaV1.2Egr2 KO mouse lines. However, the ABR threshold loss was significantly less pronounced in the CaV1.2Pax2 KO mouse mutants than in the CaV1.2Egr2 KO mice. This indicates that deletion of CaV1.2 might reduce the vulnerability to acoustic trauma. A reduced vulnerability to acoustic trauma was also observed recently upon deletion of BDNF under the same Pax2 promoter as used here for CaV1.2 deletion (Zuccotti et al., 2012). A first hint that the phenotype of CaV1.2Pax2 KO and BDNFKO mice after acoustic trauma may be causally related is yielded by the observation that BDNF mRNA is partially reduced in the cochlea of CaV1.2Pax2 KO mice. The loss of BDNF mRNA in CaV1.2Pax2 KO cochlea mainly affected the short BDNF transcripts. Short and long BDNF transcripts result from alternative 3' end processing of the BDNF transcripts at 3' untranslated regions (3'UTRs). Distinct RNA sequences in these BDNF 3'UTRs are reported to differentially regulate neuronal responses at high intensities were found to be reduced. Thus, we hypothesize that CaV1.2 in the cochlea might contribute to the neural responses of low SR AN fibers. Future studies may test this hypothesis.

It has been reported that prolonged noise exposure induces various types of hearing damage in rodents, including loss of threshold and disturbance of neural activity along the ascending auditory pathway (Kujawa and Liberman, 2009; Zuccotti et al., 2012; Rüttiger et al., 2013; Singer et al., 2013). Recent studies suggested that the trigger of the loss of SGN in the cochlea, after an acoustic overstimulation, does not originate from IHCs but rather from IHC supporting cells. In particular, inner border and inner phalangeal cells have been proposed to be essential for neuronal survival after IHC damage (Zilberstein et al., 2012). These supporting cell types have also been shown to express BDNF (Sobkowicz et al., 2002; Zuccotti et al., 2012). Around 7–11 days after noise exposure, NIHL and a PTS were observed in both CaV1.2Pax2 KO and CaV1.2Egr2 KO mouse lines. However, the ABR threshold loss was significantly less pronounced in the CaV1.2Pax2 KO mouse mutants than in the CaV1.2Egr2 KO mice. This indicates that deletion of CaV1.2 might reduce the vulnerability to acoustic trauma. A reduced vulnerability to acoustic trauma was also observed recently upon deletion of BDNF under the same Pax2 promoter as used here for CaV1.2 deletion (Zuccotti et al., 2012). A first hint that the phenotype of CaV1.2Pax2 KO and BDNFKO mice after acoustic trauma may be causally related is yielded by the observation that BDNF mRNA is partially reduced in the cochlea of CaV1.2Pax2 KO mice. The loss of BDNF mRNA in CaV1.2Pax2 KO cochlea mainly affected the short BDNF transcripts. Short and long BDNF transcripts result from alternative 3' end processing of the BDNF transcripts at 3' untranslated regions (3'UTRs). Distinct RNA sequences in these BDNF 3'UTRs are reported to differentially regulate neuronal

**FIGURE 7** Reduced expression of BDNF in the cochlea of CaV1.2Pax2 KO mice. (A) Real-time PCR at BDNF exon IX mRNA in the cochlea of control (white bar) and CaV1.2Pax2 KO mice (red bar), expressed as a percentage of the control set to 100%. A significant reduction of the mRNA level in the KO mice (CaV1.2Pax2 KO n = 11 mice; CaV1.2Pax2 KO n = 10 mice, two-sided Student’s t-test; p = 0.006). (B) Northern blot of control and CaV1.2Pax2 KO mice cochlea showing the two BDNF isoforms at 1.8 and 4.4 kb and cyclophilin as housekeeping gene at 0.8 kb. (C) Quantification of the Northern blot shows a significant reduction of the 1.8 kb BDNF mRNA isoform, but not the 4.4 kb isoform in the cochlea of CaV1.2Pax2 KO mice (CaV1.2Pax2 control, white bars: n = 12 mice; CaV1.2Pax2 KO, red bars: n = 11 mice, two-sided Student’s t-test; p = 0.193 for 4.4 kb and p = 0.043 for 1.8 kb). Data are expressed as a percentage of control set to 100%.
activity changes through altered BDNF stability and translation of these BDNF mRNA isoforms (Lau et al., 2010). While we are far from understanding the role of different BDNF transcripts in the cochlea, we may conclude that CaV1.2 short BDNF transcripts. A selected function of CaV1.2 for short BDNF transcripts during its action on destabilization of IHC/afferent contacts during acoustic trauma. This suggests that CaV1.2 short BDNF transcripts may drive destabilization of IHC/afferent contacts in the intact system, may be tested in future studies. It has to be taken into consideration that the deletion of CaV1.2 resulted in only 20–40% reduction of BDNF transcripts in the cochlea. This may explain a milder protecting phenotype than that found in the BDNF<sup>-/-</sup> KO mice. However, it cannot be ruled out that the protection against the noise-induced damage, when CaV1.2 is deleted in the cochlea, is the result of selective impact on only a subpopulation of cells or the AN fibers.

In conclusion we show that CaV1.2 deletion in the cochlea affects mainly AN fiber type with high-response thresholds. Moreover, the current data demonstrate that the deletion of CaV1.2 resulted in only 20–40% reduction of BDNF transcripts in the cochlea. We acknowledge support by Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tübingen University.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 June 2013; accepted: 20 July 2013; published online: 09 August 2013.

Citation: Zuccotti A, Lee SC, Campanelli D, Singer W, Satheesh SV, Patriarchi T, Giddey H-S, Köpschall I, Rohbock K, Nothwang HG, Hu J, Hell JW, Schimmang T, Rüttiger L and Knipper M (2013) L-type CaV 1.2 deletion in the cochlea but not in the brainstem reduces noise vulnerability: implication for CaV 1.2-mediated control of cochlear BDNF expression. Front. Mol. Neurosci. 6:20 doi: 10.3389/fnmol.2013.00020

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