Retrocochlear function of the peripheral deafness gene Cacna1d

Somisetty V. Satheesh1,†, Katrin Kunert3,†, Lukas Rüttiger4, Annalisa Zuccotti4, Kai Schönig3, Eckhard Frauf5, Marlies Knipper4, Dusan Bartsch3,‡ and Hans Gerd Nothwang1,2,*,†

1Department of Neurogenetics and 2Center for Neuroscience, Carl von Ossietzky University Oldenburg, 26111 Oldenburg, Germany, 3Department of Molecular Biology, Central Institute of Mental Health and Medical Faculty Mannheim, Heidelberg University, 68159 Mannheim, Germany, 4Department of Otolaryngology, Hearing Research Centre Tübingen (THRC), Molecular Physiology of Hearing, University of Tübingen, Elfriede Aulhorn Str. 5, 72076 Tübingen, Germany and 5Department of Biology, Animal Physiology Group, University of Kaiserslautern, POB 3049, D-67663 Kaiserslautern, Germany

Received April 12, 2012; Revised and Accepted May 30, 2012

Hearing impairment represents the most common sensory deficit in humans. Genetic mutations contribute significantly to this disorder. Here, we assessed the role of the peripheral deafness gene Cacna1d, encoding the L-type channel Ca∗1.3, in downstream processing of acoustic information. To this end, we generated a mouse conditional Cacna1d-eGFPflex allele. Upon pairing with Egr2::Cre mice, Ca∗1.3 was ablated in the auditory brainstem, leaving the inner ear intact. Structural assessment of the superior olivary complex (SOC), an essential auditory brainstem center, revealed a dramatic volume reduction (43–47%) of major nuclei in young adult Egr2::Cre;Cacna1d-eGFPflex mice. This volume decline was mainly caused by a reduced cell number (decline by 46–56%). Abnormal formation of the lateral superior olive was already present at P4, demonstrating an essential perinatal role of Ca∗1.3 in the SOC. Measurements of auditory brainstem responses demonstrated a decreased amplitude in the auditory nerve between 50 and 75 dB stimulation in Egr2::Cre;Cacna1d-eGFPflex knockout mice and increased amplitudes in central auditory processing centers. Immunohistochemical studies linked the amplitude changes in the central auditory system to reduced expression of Kv1.2. No changes were observed for Kv1.1, KCC2, a determinant of inhibitory neurotransmission, and choline acetyltransferase, a marker of efferent olivocochlear neurons. Together, these analyses identify a crucial retrocochlear role of Ca∗1.3 and demonstrate that mutations in deafness genes can affect sensory cells and neurons alike. As a corollary, hearing aids have to address central auditory processing deficits as well.

INTRODUCTION

Hearing disorders are the most common sensory deficit in humans and represent a heavy social and economic burden to individuals and societies alike (1). About 1–2 newborns in 1000 and more than half of the elderly experience hearing loss (2). It is estimated that half of all childhood deafness is due to hereditary causes (2,3). Genetic analyses have identified mutations in more than 50 genes, which have been linked to hearing impairment. Most mutations were shown to disrupt cochlear function (2,4,5). However, the precise pathogenic mechanisms are far from being fully understood (2,6).

In many cases of hearing impairment, hearing devices such as cochlear implants are prescribed to achieve well-functioning hearing. However, the benefit from these devices is often low (5,7). One reason for the poor outcome might be that perception of acoustic information requires accurate assembly and proper function throughout the auditory system. Yet, in the central

†The authors contributed equally to the manuscript.
‡Shared senior authorship.

*To whom correspondence should be addressed at: Department of Neurogenetics, Carl von Ossietzky University Oldenburg, 26111 Oldenburg, Germany. Tel: +49 4417983932; Fax: +49 4417985649; Email: hans.g.nothwang@uni-oldenburg.de

#The Author 2012. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
auditory system, the function of genes associated with hearing impairment has rarely been addressed (8,9).

One of the essential genes in inner ear cells is Cacna1d, which encodes the pore-forming α1D subunit (Ca$_{a,1.3}$) of L-type voltage-dependent calcium channels (LTCCs) (10). Ca$_{a,1.3}$ channels are essential for neurotransmitter release from inner hair cells onto auditory nerve fibers (11). In humans, cosegregation of a mutation in Cacna1d with deafness was reported in two families (12). In efforts to dissect the role of cochlea-driven activity for the development of central auditory structures, Cacna1d$^{-/-}$ mice with ubiquitous ablation of Ca$_{a,1.3}$ were recently analyzed in the auditory brainstem as well. In the superior olivary complex (SOC), an important auditory structure involved in sound localization (13,14), larger NMDA-receptor mediated excitatory postsynaptic currents were reported in P14–17-old Cacna1d$^{-/-}$ mice (15). A further study demonstrated reduced volumes of its major nuclei, changes in biophysical properties and altered gene expression (16). However, both studies did not address whether these anomalies were due to the lack of peripheral activity, as initially proposed (15). Based on the functional expression of Ca$_{a,1.3}$ in the SOC (16,17) as well as the abnormal auditory brainstem structures in Cacna1d$^{-/-}$ mice not observed in other deaf mice models, an important on-site role of Ca$_{a,1.3}$ in the SOC was suggested as an alternative (16). Since essential retrocochlear functions of peripheral deafness genes will cause a pleiotropic phenotype in the auditory system, thereby affecting auditory rehabilitation, we set out to test the hypothesis of an important function of Ca$_{a,1.3}$ in the central auditory system. We therefore generated a mouse with a targeted ablation of the gene in the central auditory brainstem, thereby affecting auditory rehabilitation, we set out to test the hypothesis of an important function of Ca$_{a,1.3}$ in the central auditory system. We therefore generated a mouse with a targeted ablation of the gene in the central auditory brainstem to circumvent peripheral deafness. This approach revealed an important retrocochlear role of Cav1.3. Furthermore, it established Cacna1d-eGFPflex mice as a valuable mouse model not only to investigate central auditory processing disorders (CAPD) but also to study other physiological functions of the Ca$_{a,1.3}$ calcium channel.

RESULTS

Generation and analyses of Cacna1d-eGFPflex, EIIa::Cre;Cacna1d-eGFPflex and Egr2::Cre;Cacna1d-eGFPflex mouse lines

To study the potential on-site role of Ca$_{a,1.3}$ in the SOC, we generated mice with a conditional Cacna1d allele for spatially restricted gene ablation. To easily monitor recombination events on the single cell level, we used the Cre/loxP-based FLEX system [Flip excision (18)], in which the ablation of Cacna1d is coupled to the expression of the reporter gene eGFP under the control of the Cacna1d promoter (Fig. 1A). To test for functionality, the targeting construct was transfected in HeLa cells under control of the constitutively active Caggs promoter. Cotransfection with Cre-recombinase resulted in expression of eGFP, whereas no signal was obtained in the absence of Cre-recombinase (Fig. 1B and C).

Next, we wanted to analyze whether the transgene allele faithfully recapitulates the expression of the endogenous Cacna1d allele. We therefore crossed Cacna1d-eGFPflex with Ella::Cre deleter mice. The adenovirus Ella promoter is already active in the ovum at the time of fertilization which leads to Cre-recombinase when the embryo is still at the one-cell zygote stage (19,20). Thus, all cells of the developing animal will have the inverted DNA sequence and the eGFP expression driven by the Cacna1d regulatory elements is germine transmissible in the absence of Cre expression. Diaminobenzidine (DAB) immunohistochemistry with antibodies against eGFP revealed a strong staining in several brain regions, demonstrating in vivo functional expression of eGFP. In a sagittal section through the entire brain, moderate to strong staining was observed in the cerebral cortex, the olfactory bulb, the superior colliculus and the adjacent optic tract, and the cerebellum (Fig. 2A). Further analyses revealed moderate staining in the periventricular nucleus in the thalamus (Fig. 2B), as well as in various brainstem areas, such as the locus coeruleus, the facial nucleus and the cuneate nucleus (Fig. 2C). Additionally, staining was observed in the hippocampus, in the subventricular zone and in the amygdala (data not shown). Transgenic littermates with no expression of Cre-recombinase showed no staining for eGFP (Fig. 2D).

Altogether, the expression pattern of eGFP closely matched the previously reported presence of Cacna1d in the cerebral cortex (21), the cerebellum (22), the olfactory bulb (23–25), the paraventricular nucleus (26) and brainstem structures (27). Given the lack of reliable antibodies against Ca$_{a,1.3}$, the Cacna1d-eGFPflex mouse will hence be an important reporter line to analyze Ca$_{a,1.3}$ expression.

To investigate the on-site role of Ca$_{a,1.3}$ in the auditory brainstem, we paired Cacna1d-eGFPflex animals with Egr2::Cre mice. This Cre driver mouse line mediates recombination in rhombomeres 3 and 5 of the embryonic neural tube, allowing genetic manipulation of neuronal populations in the auditory brainstem such as the lateral superior olive (LSO) and medial nucleus of the trapezoid body (MNTB) (Fig. 3C and D) (28–31). Importantly, the cochlea is spared from Cre-mediated recombination (Fig. 3A and B). To analyze the extent of Cre expression in the auditory brainstem in this Cre driver line, we also analyzed the inferior colliculus, the principal midbrain structure of the auditory pathway. Like in the cochlea, no X-gal staining was observed (Fig. 3E and F). Hence, recombination in Egr2::Cre mice is confined to auditory structures in the hindbrain.

Deletion of Ca$_{a,1.3}$ in Egr2::Cre;Cacna1d-eGFPflex animals (Cacna1d$^{floxed}$ in the following) was detected via immunohistochemistry against eGFP. To avoid overlapping signals from eGFP, an Alexa-Fluor 594-labeled secondary antibody was used, which gives rise to red fluorescence. Indeed, eGFP was detected in the LSO and MNTB of Cacna1d$^{floxed}$ both at P4 and P25 (Fig. 4B, D, F, H). In contrast, no specific labeling was observed in auditory structures where Cre-recombinase is not expressed such as the inferior colliculus (Fig. 4J and L) and in littermates lacking Cre-recombinase (Fig. 4A, C, E, G, I, K). Since eGFP faithfully mirrors expression of Cacna1d (Fig. 2), these data demonstrate expression of Cacna1d throughout the SOC during postnatal development. Indeed, RT–PCR analyses using two different primer pairs confirmed expression of Cacna1d in wild-type SOC tissue both at P4 and P25 (Fig. 4M). These data are in excellent agreement with electrophysiological analysis of P4 and P12 LSO neurons (17) and Ca$^{2+}$ imaging using Fura-2 in P3 LSO neurons (16), which
both demonstrated functional Ca\textsubscript{v}1.3 in the SOC. They also confirm a previous immunohistochemical study, which identified Cav1.3 in the adult mouse SOC (32).

**Immunohistochemical analyses of the SOC in Cacna1d\textsuperscript{kko} animals**

To examine the integrity of the SOC, we performed immunohistochemistry for Vglut1, a marker for excitatory inputs onto SOC neurons (33) using P4 and P25 coronal brainstem sections (Fig. 5A–D). Interestingly, the LSO of young-adult Cacna1d\textsuperscript{kko} mice lacked the U shape present in wild-type animals (Fig. 5A and B). Instead, a more round shape was found. Analyses of Nissl-stained sections revealed that its volume was reduced by 43% (wild-type: 0.0253 $\pm$ 0.0029 mm\textsuperscript{3}; Cacna1d\textsuperscript{kko}: 0.0143 $\pm$ 0.0031 mm\textsuperscript{3}, $P < 0.001$) (Fig. 5E). Likewise, the MNTB volume was reduced by 47% (wild-type: 0.0439 $\pm$ 0.0045 mm\textsuperscript{3}; Cacna1d\textsuperscript{kko}: 0.0231 $\pm$ 0.0029 mm\textsuperscript{3}, $P < 0.001$) (Fig. 5G). In contrast, there was no significant difference in the volume of the LSO or MNTB between Cacna1d\textsuperscript{fl/fl} (control) and heterozygous knockout animals (Fig. 5E and G). Cell counts in Nissl-stained sections revealed that the smaller volumes in both nuclei were due to a reduced number of neurons [LSO, wild-type: 1511 $\pm$ 153, Cacna1d\textsuperscript{kko}: 694 $\pm$ 27 (46%), $P < 0.001$; MNTB, wild-type: 4095 $\pm$ 594, Cacna1d\textsuperscript{kko}: 2310 $\pm$ 260 (56%), $P < 0.001$] (Fig. 5F and H). These changes on the anatomical and cellular level were similar to those observed in Cacna1d\textsuperscript{2/2} animals (16).

Vglut1 immunohistochemistry in P4 animals demonstrated a malformed LSO already at this neonatal stage (Fig. 5C and D). Altogether, these data indicate an essential on-site role of Ca\textsubscript{v}1.3 for proper development of the SOC.

**Auditory brainstem responses in Cacna1d\textsuperscript{kko} animals**

We next assessed the functional consequences of loss of Ca\textsubscript{v}1.3 for auditory physiology by analyzing auditory brainstem responses (ABR) and distortion product otoacoustic emission (DPOAE). ABR thresholds for pure tone, click and noise burst stimuli were similar for control and Cacna1d\textsuperscript{kko}
animals (Fig. 6A), demonstrating normal function of inner hair cells in Cacna1d\textsuperscript{cko} mice, which is in contrast to the ubiquitous Cacna1d knockout mice (11). DPOAE thresholds were only slightly (<10 dB), but significantly increased in the middle hearing range (8 and 11.3 kHz, \( P = 0.05 \), Student’s \( t \)-test), whereas thresholds for other frequencies were similar to those of control animals (Fig. 6B). The amplitude of the DPOAE at 50 dB SPL \( f_2 \) stimulation level was also slightly, but significantly reduced for Cacna1d\textsuperscript{cko} mice (open bars; \( P = 0.012 \), one-sided \( t \)-test) when compared with Cacna1d\textsuperscript{fl/fl} (closed bar) (Fig. 6C). These data indicate almost normal function of outer hair cells. Next, ABR waves were analyzed. Wave I reflects the summed activity of the auditory nerve, while later waves arise from synchronous neural activity in the auditory brainstem. Wave II is dominated by the response of globular bushy cells in the cochlear nucleus complex (CNC). Wave III is assumed to originate mainly in the CNC and SOC, and the lateral lemniscus and inferior colliculus

Figure 2. Expression of eGFP in the brain of EIIa::Cre;Cacna1d\textsuperscript{-eGFP\textsuperscript{flex}} mice. DAB-immunohistochemistry using antibodies against the reporter protein eGFP. (A) Overview of a sagittal brain section with staining in the olfactory bulb, the cerebral cortex, the optic tract, the superior colliculus and the cerebellum. (B) Coronal section at the level of the thalamus, revealing staining of the paraventricular nucleus. (C) Sagittal brainstem section with staining in the locus coeruleus, the facial nucleus and the cuneate nucleus. (D) Sagittal brain section of a single transgenic Cacna1d-GFP\textsuperscript{flex} littermate, showing no staining. At least two brains were analyzed per area. 3 V, third ventricle; 7n, facial nucleus; Cb, cerebellum; Co, cortex; Cu, cuneate nucleus; LC, locus coeruleus; OB, olfactory bulb; OT, optic tract; PVN, paraventricular nucleus; SC, superior colliculus.
contribute to wave-IV (34,35). Deletion of Cav1.3 resulted in altered ABR waves (Fig. 6D), particularly at high stimulation amplitudes (Fig. 6F). Amplitudes of waves II and III were increased at stimulation levels above 60 dB in Cacna1dcko compared with Cacna1dfl/fl animals (Fig. 6F). In contrast, wave I amplitudes were slightly decreased between 50 and 75 dB in Cacna1dcko (Fig. 6F). This argues against the inner hair cell and spiral ganglion output signal strength as the origin of the higher ABR amplitudes. These data indicate a higher excitability or synchronicity in the CNC and SOC of Cacna1dcko mice. Taken together, these physiological data reveal that Cav1.3 is essential for proper function of the SOC.

Immunohistochemical analyses of Kv1 channels and KCC2

Recently, higher ABR amplitudes were reported in Kcna12/2 mice lacking the potassium channel K1.1 (36). Furthermore, in ubiquitous Cacna1d2/2 animals, an increase in the firing rate of LSO neurons was linked to reduced expression of K1.2 (16). To analyze whether the higher ABR amplitudes in waves II and III of Cacna1d2/2 animals are associated with down-regulation of these two channels, immunohistochemical analysis was performed. Both in the LSO and MNTB, we observed strong K1.1 immunoreactivity in Cacna1d2/2 animals, which was indistinguishable from control animals (Fig. 7A–D). In contrast, immunohistochemistry for K1.2 revealed a decreased expression in the LSO and MNTB of Cacna1d2/2 animals (Fig. 7E–H). Especially, the intense punctuate staining observed in the processes of control animals was diminished in Cacna1d2/2 animals (Fig. 7E–H, high magnification inserts).

Impaired inhibition, which plays an important role in the auditory brainstem (37), might also contribute to the observed increase in ABR amplitudes. The action of GABA and glycine, the two major inhibitory neurotransmitters in the brain, is determined by the activity of the K\(^{+}\)-Cl\(^{−}\) cotransporter KCC2 (38–40). Of note, LTCCs were shown to influence KCC2 expression (41,42). We therefore performed immunohistochemistry for KCC2. Immunolabeling was strong throughout the SOC, including the LSO and MNTB, and no difference in labeling intensity or pattern was visible between control and Cacna1d2/2 animals (Fig. 8A–D). These data demonstrate that the loss of Cav1.3 affects K1.2 but not K1.1 and KCC2 expression. The diminished K1.2
expression might contribute to the increased ABR amplitudes of waves II and III in Cacna1d^ko animals.

Immunohistochemical analyses of the olivocochlear bundle

In contrast to the increased amplitudes of waves II and III, wave I amplitudes were slightly decreased between 50 and 75 dB despite normal ABR thresholds. One explanation is altered function of the olivocochlear neurons, an efferent feedback system, which regulates cochlear activity (43). Importantly, the somata of olivocochlear neurons reside within two groups of the SOC, the LSO and the ventral nucleus of the trapezoid body (VNTB) (44). To investigate whether these neurons were affected in Cacna1d^ko mice, we performed immunohistochemistry against ChAT, a marker of these neurons in rodents (44). ChAT-positive neurons were found in the LSO and VNTB and the pattern was indistinguishable between control animals and Cacna1d^ko mice (Fig. 8E and F). These data suggest that the olivocochlear system is not affected in Cacna1d^ko mice. However, our data do not exclude that molecular changes within these neurons cause altered feedback to the cochlea.

DISCUSSION

Here, we show that targeted ablation of Ca_\text{v}1.3 in the SOC results in abnormalities on the anatomical, molecular and physiological levels. These changes are not reflecting a secondary effect due to the loss of cochlea-driven activity, as ABR thresholds revealed normal function of inner hair cells. The identified abnormalities in the central auditory system therefore reveal a critical role of Ca_\text{v}1.3 in the auditory system beyond the cochlea. Next to governing neurotransmission from the inner hair cells to the auditory nerve, the protein directly sculptures development and function of central auditory neurons. A dual role in the auditory system was previously established for the transcription factor Gata3, as its loss results in degeneration of hair cells in the cochlea (45).
abnormal projection of olivocochlear neurons in the SOC (9).
In the course of our study, such a dual role in the auditory system has been revealed for two more genes. The proneural basic helix-loop-helix transcription factor Atoh1 is essential for hair cell development (46) and proper maturation and function of the auditory brainstem (29). Finally, Slc17a8, encoding Vglut3, is required for glutamate release from hair cells (47,48) and circuit refinement of auditory brainstem projections (8). These data bear important clinical implications, as mutations in Cacna1d (12), Gata3 (49,50) or Slc17a8 (48) have been associated with human hearing loss. In addition to peripheral deafness, affected patients will suffer from gene-specific deficits in the central auditory system. It will therefore be important to decipher the retrocochlear deficits after functional loss of deafness genes in more detail in order to better tailor hearing devices. In certain cases, it might turn out to be more appropriate to use auditory brainstem implants, as already used in patients with neurofibromatosis type 2 (51). The retrocochlear function of at least three peripheral deafness genes also makes a good case for routine functional analyses of deafness genes in the central auditory system.

Figure 5. Malformed SOC in Cacna1d<sup>−−</sup> mice. (A and B) Vglut1 immunoreactivity in coronal brainstem sections of P25 control (A) and Cacna1d<sup>−−</sup> mice (B), demonstrating a malformed SOC. (C and D) The malformed LSO was observed already at P4 in Cacna1d<sup>−−</sup> mice. (E and F) The volumes and cell numbers of the LSO and MNTB were determined at P25 from Nissl-stained serial sections (six SOCs and three animals per genotype). Significant decrease in the volume and cell number was observed in the LSO (E and F) and MNTB (G and H) of Cacna1c<sup>-</sup> mice. LSO, lateral superior olive; MNTB, medial nucleus of the trapezoid body. Dorsal is up, lateral is to the right. ***<i>P</i> < 0.001; Student’s t-test.
Another benefit from a detailed characterization of retrocochlear functions of deafness genes such as Cav1.3 will be a better insight into the underlying mechanisms and functional consequences of CAPD. These disorders are characterized by impaired sound processing in the central auditory system, which results in the absence of considerable peripheral hearing loss in perceptual dysfunction (52,53). The etiology of CAPD is poorly understood. About 2–3% of children and 10–20% of the elderly are affected. The high prevalence and a 2-to-1 ratio between boys and girls point to a genetic component, and genes important for auditory brainstem function represent attractive candidates. So far, the lack of appropriate clinically exploitable methods for differential diagnosis of the causes have impeded progress in dissecting the underlying disease mechanisms by human genetic approaches. Mouse models, such as Cacna1dcko mice, therefore represent important tools to explore the pathophysiological processes and functional consequences in CAPD patients.

Figure 6. Altered ABR in Cacna1dcko mice. (A) ABR thresholds for pure tone stimuli were invariant between Cacna1dfl/fl (control; closed circles) and Cacna1dcko mice (open squares; P > 0.05, two-way ANOVA). Mean values ± SE are shown. No difference was observed for ABR thresholds in response to click stimuli (lower left corner in the panel) or noise burst stimuli (lower right corner; P > 0.05, one-sided t-test). (B) Thresholds of the 2f1–f2 DPOAE revealed a slight increase in the threshold for 8 kHz and 11.3 kHz in Cacna1dcko mice. Statistical significance is indicated for two-way ANOVA analysis comparing the genotype (**) and post hoc one-sided t-test comparing pairs of thresholds at single frequencies with (*) indicating P < 0.05. (C) Amplitude of the DPOAE at 50 dB SPL f2 stimulation level (mean values ± SD) was slightly but significantly reduced for Cacna1dcko mice (open bars; P = 0.012, one-sided t-test) when compared with Cacna1dfl/fl (closed bar). Individual ear emission signals are shown as circles within each bar. (D) Click-evoked ABR waves (mean values ± SE) from Cacna1dfl/fl (black) and Cacna1dcko (gray) mice for an 86 dB SPL stimulus were overlaid for comparison. ABR wave amplitudes differed between genotypes, and differences were most pronounced at latencies between waves II and IV. The peaks corresponding to ABR wave-I to wave-IV are indicated by the arrows (negative peaks) and arrow heads (positive peaks). Waves are defined by the latency of their leading negative (n) and following positive (p) peak, as illustrated for wave-I. (E) Altogether, no difference in peak latencies between Cacna1dfl/fl (black) and Cacna1dcko (gray), but changes of average peak amplitude for waves I, II and III (arrows, arithmetic mean of ABR peak amplitude for stimuli between 20 and 80 dB). (F) Ablation of Cacna1d resulted in reduced peak amplitudes in wave I between stimulation levels of 50–70 dB, whereas peak amplitudes increased for waves II and III >60 dB. Average I/O functions from control mice are shown as black lines and from Cacna1dcko mice as gray lines (± SE). Data were obtained from 10 to 14 ears per genotype.
differs from that in sensory hair cells, as the ABR recordings demonstrate neurotransmission in the central auditory pathway of Cacna1d^{−/−} mice. In neurons, L-type Ca$^{2+}$ channels most often represent an important postsynaptic entry point for signaling cascades involved in excitation–transcription coupling (54–56). In the visual system, for instance, targeted ablation of the calcium channel β3-subunit disrupts axonal refinement (57). Our data demonstrate disturbed SOC architecture already at P4, only few days after the establishment of afferent input. Major SOC nuclei, such as the MNTB, complete their

---

**Figure 7.** Immunohistochemistry of Kv1 channels in the SOC of WT and Cacna1d^{−/−} mice. (A–D) Strong Kv1.1 immunoreactivity was observed in the LSO (A and B) and MNTB (C and D) of WT (A and C) and Cacna1d^{−/−} (B and D) neurons. (E–H) Compared with WT mice (E and G), Kv1.2 immunoreactivity was reduced in the LSO (F) and the MNTB (H) of Cacna1d^{−/−} mice. Inserts show high magnifications. Images show representative results from ≥3 independent experiments. LSO, lateral superior olive; MNTB, medial nucleus of the trapezoid body. Dorsal is up, lateral is to the right.
migration approximately 2 days prior to birth (P0 ≈ E19) (58,59). At the same day, SOC neurons become functionally innervated by neurons of the anteroventral cochlear nucleus and show elevated Ca\textsuperscript{2+} levels upon presynaptic stimulation (58–60). A crucial role of Cav1.3 in perinatal excitation–transcription coupling is in agreement with our eGFP expression data in the Cacna1d\textsuperscript{cko} mouse and RT–PCR analyses, which demonstrated abundant expression of Cacna1d in the SOC from P4 onwards. Furthermore, electrophysiological analyses in transgenic animals revealed functional Ca\textsubscript{v}1.3 in the SOC of P4- and P12-old animals (17). Support for the importance of signaling cascades between P0 and P4 comes from a previous analysis in mice with ablation of Atoh1. In these animals, ~50% of MNTB neurons underwent apoptotic cell death between P0 and P3. It was suggested that this loss reflects a lack of neurotrophic support due to the almost complete absence of their afferent projection neurons (29). Indeed, several analyses demonstrated a tight link between LTCCs and neurotrophic factors. LTCC-mediated Ca\textsuperscript{2+} influx promotes release of brain-derived neurotrophic factor (BDNF) (61) and transcription of the BDNF gene in cortical neurons (62) or retinal Müller cells (63). It is therefore interesting to analyze whether Cav1.3 regulates BDNF levels in the auditory brainstem or whether both act as independent survival promoting factors, as suggested for chicken nodose ganglion neurons (64).

In Cacna1d\textsuperscript{cko} mice, neurotransmission from the inner ear to the auditory nerve is preserved. This enabled us to interrogate function of the central auditory brainstem by ABR measurements, which reflect the activity of the auditory brainstem. We observed increased amplitudes of waves II and III in

![Figure 8. Immunohistochemistry against KCC2 and ChAT in the SOC of WT and Cacna1d\textsuperscript{cko} mice. (A–D) Strong KCC2 immunoreactivity was observed in the LSO (A and B), and MNTB (C and D) of WT (A and C) and Cacna1d\textsuperscript{cko} (B and D) neurons. (E and F) Strong ChAT immunoreactivity was observed in the LSO and VNTB of WT (E) and Cacna1d\textsuperscript{cko} (F) neurons. These data indicate that KCC2 expression and the olivocochlear bundle are not affected in Cacna1d\textsuperscript{cko} animals. Images show representative results from ≥2 independent experiments. LSO, lateral superior olive; VNTB, ventral nucleus of the trapezoid body. Dorsal is up, lateral is to the right.](https://academic.oup.com/hmg/article-abstract/21/17/3896/562597/3905)
Cacna1d<sup>ko</sup> animals, which suggest a higher excitation in auditory brainstem nuclei. One explanation is reduced expression of K<sub>1.1</sub> channels, which are important for rapid repolarization. In constitutive Cacna1d<sup>-/-</sup> mice, pharmacological blocking of K<sub>1.1</sub> channels by α-dendrotoxin converted LSO neurons with a single firing pattern into neurons with a multiple firing pattern upon current injections (16). Furthermore, loss of this channel reduces inhibitory drive from MNTB neurons into the LSO (65). The reduced expression of K<sub>1.2</sub> in Cacna1d<sup>ko</sup> animals might therefore partially account for the increased ABR amplitudes in waves II and III. However, other proteins might also be affected. For instance, LTCCs mediate activity-dependent up-regulation of K<sub>s</sub>1.1 in the inferior colliculus, another auditory brainstem structure (66). Detailed expression analyses have therefore to be performed to fully understand the molecular changes giving raise to altered ABRs.

In contrast to waves II and III, we observed between 50 and 70 dB a decrease in the amplitude of wave I, which reflects activity of the auditory nerve. In addition, the DPOAE amplitude was slightly reduced in Cacna1d<sup>ko</sup> mice. Since auditory thresholds are not affected in Cacna1d<sup>ko</sup> mice, we investigated olivocochlear neurons. This efferent feedback system adjusts the sensitivity of the cochlea and influences both the activity of the auditory nerve and DPOAE amplitudes (43,67,68). However, ChAT staining did not reveal any difference on the cellular level between controls and Cacna1d<sup>ko</sup> mice. This finding contrasts observations in mice with targeted ablation of Atoh1, where the loss of SOC neurons resulted in more densely packed and more ventrally located somata of the olivocochlear neurons (29). This difference might be in part due to the apparently stronger reduction in SOC nuclei after targeted ablation of Atoh1 compared with Cacna1d<sup>ko</sup>. In summary, our analyses identified a crucial role of Cacna1d<sub>1.3</sub> for the development of central auditory centers, and Cacna1d<sup>ko</sup> mice will provide a valuable mouse model to study CAPD. Furthermore, the newly generated Cacna1d-eGFP<sup>flex</sup> paves the way for spatial and temporal control of Cacna1d<sub>1.3</sub> loss, which will result in better tailored mouse models to study the physiological and pathophysiological roles of the channel.

**MATERIALS AND METHODS**

**Animals**

The Cre lines Egr2::Cre (28) and EIIa::Cre (19), the Cre-reporter line ROSA26R (69) and the Flp-deleter mouse (70) were described previously. All protocols were in accordance with the German Animal Protection law and approved by the local animal care and use committee (LAVES, Oldenburg, Karlsruhe). Protocols also followed the NIH guide for the care and use of laboratory animals.

**Generation of the Cacna1d-eGFP<sup>flex</sup> mouse line**

To generate mice with a conditional Cacna1d-eGFP<sup>flex</sup> allele, exon 2 of Cacna1d was targeted, as its ubiquitous deletion resulted in a complete silencing of the gene (11). The exon trapping cassette consisted of the promoterless reporter gene eGFP with an upstream general adenovirus splice acceptor and a downstream transcriptional termination sequence (polyadenylation sequence) (71). As a template for the Cacna1d locus, the cosmID J14279Q4 from a Lawrist7 genomic library of the 129 mouse strain was obtained from the RZPD (Berlin, Germany). The cassette was flanked by one pair of wild-type loxp sites and one pair of mutant L3 sites, with alternate organization and head-to-head orientation within each pair (Fig. 1A). This organization results in an inversion of the floxed cassette upon Cre expression (18). For positive selection in embryonic stem (ES) cells, the construct contained an FRT-flanked neomycin resistance gene (neo). The cassette was flanked on the 5′ end by a 2.6 kb homology arm encompassing exon 1a and at the 3′ end by a 5.6 kb homology arm containing exon 3. A thymidine kinase from Herpes simplex virus was introduced downstream to the 3′ homology arm as a negative selection marker (Fig. 2A). Recombineering techniques based on phage recombination were used to assemble the construct (72). To analyze functionality of the resulting construct in HeLa cells, the Caggs promoter [chicken beta-actin promoter with CMV enhancer (73)] was cloned into the 5′ homology arm 2 kb upstream of eGFP. The final targeting construct (without the Caggs promoter) was electroporated into R1 ES cells (74), which are derived from agouti-colored 129/SvJ mice. Positive ES cells were injected into blastocysts derived from the mouse strain C57Bl/6n. To remove neo, heterozygous Cacna1d-eGFP<sup>flex/+</sup> mice were crossed with the Flp-deleter mouse strain, which expresses the Flpe recombinase under the promoter of the human ACTB gene (Tg(ACTFLPe)9205Dym/J). This promoter is active in all tissues, including germ cells (70). The resulting germline knock-in of eGFP into the Cacna1d gene was maintained and analyzed as a heterozygote in the absence of Cre allele. The Egr2::Cre allele was genotyped with primers 5′-CACTACACCAGCAACTCTGCTCC-3′ and 5′-ATGTCGATAAACGGCCTGGCATCC-3′. The Cacna1d-GFP<sup>flex/+</sup> allele was identified with primers 5′-GGAGTTGGTG ATATCTGGTAAAGCCATG-3′ and 5′-CTCTGTCCATATCTCAACTCCCATA-3′ (product sizes: 2448 bp from the recombinant Cacna1d-GFP<sup>flex/+</sup> locus and 1000 bp from the wild-type Cacna1d locus).

**RT-PCR analysis**

For RT–PCR analyses of the mouse SOC, the brainstem was dissected from animals aged P4 or P25 and 200-μm-thick coronal sections were cut with a vibratome (Leica VT 100 S, Leica, Nussloch, Germany). Subsequently, the SOC was dissected manually. At P4, Socs were bilaterally collected from one slice and at P25 from three slices. For RNA isolation, Socs from five P4 animal or two P25 animals were pooled for each biological replica. Total RNA was isolated by the guanidine thiocyanate method (75). After reverse transcription, PCR was performed with primer pairs Cacna1d<sub>-/-</sub>-for 5′-TGCAACATCACAAGGAGCAC-3′ and Cacna1d<sub>-/-</sub>-rev 5′-CTTAATGCAGGCTTCTCGGTG-3′ (334 bp product) to amplify the 5′ of the open reading frame of Cacna1d, Cacna1d<sub>-/-</sub>-for 5′-ACATTCTGAACATGTCACGAC-3′ and Cacna1d<sub>-/-</sub>-rev 5′-AGGACTTGAAGGTCCACAG-3′ (283 bp product) to amplify a part towards the 3′ of

Downloaded from https://academic.oup.com/hmg/article-abstract/21/17/3896/562597 by guest on 30 January 2019
Cacna1d (76), and γ-actin-for 5'-ACAATGGCTCCGGCA TGTGC-3' and γ-actin-rev 5'-CCACATCTGCTGGAA GGTG-3' (1029 bp product) at an annealing temperature of 56°C for 30 cycles. PCR products were analyzed in a 2% agarose gel.

DAB staining, immunofluorescence and X-gal staining

For DAB staining, tissue sections were permeabilized in 1% H2O2 in phosphate-buffered saline (PBS) (150 mM NaCl, 10 mM Na-phosphate, pH 7.4) for 10 min at room temperature and washed with PBS three times for 10 min. To block unspecific binding sites, sections were incubated in 2% normal goat serum in 1% BSA/0.3% Triton X-100/PBS for 1 h at room temperature. The primary antibody anti-GFP (Molecular Probes Invitrogen, Karlsruhe, Germany) was diluted 1:5000 in blocking solution and slices were incubated at 4°C overnight. After two washes in 0.3% BSA/0.1% Triton X-100/PBS, slices were incubated for 1 h at room temperature with the biotinylated secondary anti-rabbit IgG antibody (Vector Laboratories, Lörach, Germany), diluted 1:600 in 0.3% BSA/0.1% Triton X-100/PBS. Thereafter, slices were washed twice in the same buffer without antibody. To enhance staining, sections were incubated with an avidin-biotin complex for 1 h followed by one washing step in 0.3% BSA/0.1% Triton X-100/PBS and two washing steps with PBS. Sections were then incubated in DAB solution (0.4 mg/ml) in 20 mM Tris–HCl with 0.012% H2O2 until staining was observed. The reaction was stopped by washing three times with PBS. Stained slices were mounted on glass slides and cover slipped with Eukitt (Sigma Aldrich, Taufkirchen, Germany). Slices were imaged with a stereoscope (Stemi 2000-C, Zeiss, Oberkochen, Germany).

For immunohistochemistry, a rabbit anti-GFP antibody was obtained from Invitrogen, a goat anti-ChAT from Millipore (Schwalbach, Germany), the mouse anti-Kv1.1 and anti-Kv1.2 from Neuromabs (Davis, USA), anti-KCC2 was reported previously (77) and rabbit anti-Vglut1 antibody was a generous gift from Dr S. El Mestikawy (Cetreil, Cedex, France). During immunohistochemistry against eGFP, the experimenter was blind to the genotype for two experiments for each stage and assigned eGFP expression correctly to the genotype. All comparisons between wild-type and knockout animals were done on slices processed strictly in parallel on the same day. Antibodies were diluted 1:2000 (anti-GFP), 1:200 (anti-ChAT), 1:50 (anti-Kv1.1 or anti-Kv1.2), 1:500 (anti-KCC2) and 1:1500 (anti-Vglut1) with carrier solution containing 1% bovine serum albumin, 1% goat serum and 0.3% Triton X-100 in PBS. Sections were incubated overnight with agitation at 7°C. They were then rinsed three times for 10 min in PBS, transferred again to carrier solution and incubated with secondary antibodies coupled to Alexa Fluor Dyes (diluted 1:1000, Invitrogen) for 1–2 h. After several washes in PBS, slices were mounted and images were taken with a BZ 8100 E fluorescence microscope (Keyence, Neu-Isernbgen, Germany). When comparing immunoreactivity in wild-type and knockout mice, images were taken under identical conditions for a given antibody. Image data were processed using Adobe Photoshop 7.0 software (Adobe Systems).

Nissl staining was performed on 30-µm thick sections. The volume of auditory nuclei was calculated by multiplying the outlined area with the thickness of each section (16). Three animals were analyzed for each genotype. For X-gal staining of brain sections, mice were perfused transcardially with a buffer containing 1× PBS, 1% formaldehyde, 0.2% glutaraldehyde, 0.2% NP-40 and 0.1% cholic acid. X-gal staining was performed in the following staining solution: 1× PBS, 2 mM MgCl2, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 1 mg/ml X-gal, 0.2% NP-40 and 0.1% cholic acid. Cochleae were decalcified in Rapid Bone Decalifier (Apex Engineering Producers Corporation, IL, USA) and cryosectioned at 10 µm. Slices were postfixed in 4% PFA and 2.5% glutaraldehyde and stained in X-gal solution for 24–48 h at 37°C prior to analyses using a Keyence BZ 8100 E microscope (brain slices) or an Olympus AX70 microscope (cochlea). Statistical analysis was performed using Student’s t-test.

Auditory evoked brainstem responses and otoacoustic emissions

ABR and DPOAEs were recorded in 6–9-week-old mice, anesthetized with a mixture of ketamine hydrochloride (75 mg/kg body weight, Pharmacia, Erlangen, Germany) and xylazine hydrochloride (5 mg/kg body weight, Bayer, Leverkusen, Germany). Electrical brainstem responses to free field click (100 µs), noise burst (1 ms) and pure tone (3 ms, 1 ms ramp) stimuli were recorded with subdermal silver wire electrodes at the ear, the vertex and the back of the animals. After amplification and bandpass filtering (200 Hz–5 kHz), signals were averaged for 64–256 repetitions at each sound pressure presented (usually 0–100 dB SPL in steps of 5 dB). Thresholds were determined by the lowest sound pressure that produced visually distinct evoked potentials from above threshold to near threshold. The cubic 2f1−f2 DPOAE was measured for f2 = 1.24 × f1 and L2 = L1−10 dB. Emission signals were recorded during sound presentation of 260 ms and averaged four times for each sound pressure and frequency presented. First, the 2f1−f2 distortion product amplitude was measured with L1 = 50 dB SPL and f2 between 4 and 45.2 kHz. Subsequently, the 2f1−f2 distortion product amplitude was measured for L1 ranging from −10 to 65 dB SPL at frequencies of f2 between 4 and 32 kHz.

Average ABR-wave curves are presented as mean ± SE. Peak amplitudes and latencies were collected, grouped in clusters of similar peak amplitudes and latencies and averaged for ABR-wave input–output analysis. Clusters of peaks were found at average latencies n0.9-p1.2 (wave I), n1.5-p2.2 (wave II), n2.9-p3.5 (wave III) and n3.9-p4.9 (wave-IV) (n = negative peak, p = positive peak, number = peak latency in ms). Differences of the mean were compared for statistical significance by Student’s t-test, alpha-levels corrected for multiple testing by Bonferroni–Holms, and two-way ANOVA (GraphPad Prism 2.01). Statistical significance was tested at alpha = 0.05, and resulting P-values are reported in the text and figures.
ACKNOWLEDGEMENTS

We wish to thank Anja Feistner, Jasmin Schröder, Martina Reents and Ariana Frömig for excellent technical assistance.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the EU grant CAVNET (MRTN-CT-2006-035367 to H.G.N. and D.B.), the DFG grants No. 428/5-1 and No. 428/10-1 to H.G.N., the SPP1608, and the BMBF Bernstein Center for Computational Neuroscience Heidelberg/Mannheim 01GQ1003A (TPA3) to D.B.

REFERENCES


