Deletion of the Ca\(^{2+}\) Channel Subunit \(\alpha_2\delta3\) Differentially Affects Ca\(_{\text{v}}\)2.1 and Ca\(_{\text{v}}\)2.2 Currents in Cultured Spiral Ganglion Neurons Before and After the Onset of Hearing

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Voltage-gated Ca\(^{2+}\) channels are composed of a pore-forming \(\alpha_1\) subunit and auxiliary \(\beta\) and \(\alpha_2\delta\) subunits, which modulate Ca\(^{2+}\) current properties and channel trafficking. So far, the partial redundancy and specificity of \(\alpha_1\) for \(\alpha_2\delta\) subunits in the CNS have remained largely elusive. Mature spiral ganglion (SG) neurons express \(\alpha_2\delta\) subunit isoforms 1, 2, and 3 and multiple Ca\(^{2+}\) channel subtypes. Differentiation and in vivo functions of their endbulb of Held synapses, which rely on presynaptic P/Q channels (Lin et al., 2011), require the \(\alpha_2\delta3\) subunit (Pirone et al., 2014). This led us to hypothesize that P/Q channels may preferentially co-assemble with \(\alpha_2\delta3\). Using a dissociated primary culture, we analyzed the effects of \(\alpha_2\delta3\) deletion on somatic Ca\(^{2+}\) currents (\(I_{\text{Ca}}\)) of SG neurons isolated at postnatal day 20 (P20), when the cochlea is regarded to be mature. P/Q currents were the dominating steady-state Ca\(^{2+}\) currents (54% of total) followed by T-type, L-type, N-type, and R-type currents. Deletion of \(\alpha_2\delta3\) reduced P/Q- and R-type currents by 60 and 38%, respectively, whereas L-type, N-type, and T-type currents were not altered. A subset of \(I_{\text{Ca}}\) types was also analyzed in SG neurons isolated at P5, i.e., before the onset of hearing (P12). Both L-type and N-type current amplitudes of wildtype SG neurons were larger at P5 compared with P20. Deletion of \(\alpha_2\delta3\) reduced L-type and N-type currents by 23 and 44%, respectively. In contrast, small P/Q currents, which were just being up-regulated at P5, were unaffected by the lack of \(\alpha_2\delta3\). In summary, \(\alpha_2\delta3\) regulates amplitudes of immature cultured SG neurons, whereas it regulates P/Q- and R-type currents at P20. Our data indicate a developmental switch from dominating somatic N- to P/Q-type currents in cultured SG neurons. A switch from N- to P/Q-type channels, which has been observed at several central synapses, may also occur at developing endbulbs of Held. In this case, reduction of both neonatal N- (P5) and more mature P/Q-type currents (around/after hearing onset) may contribute to the impaired morphology and function of endbulb synapses in \(\alpha_2\delta3\)-deficient mice.

Keywords: Ca\(^{2+}\) channel, Ca\(^{2+}\) current, P/Q-type, N-type, postnatal development, auditory, auxiliary subunit, primary culture
INTRODUCTION

Voltage-gated calcium channels (VGCCs) consist of a pore-forming α1 subunit and auxiliary β and α2δ subunits (Catterall et al., 2005; Dolphin, 2012; Zamponi et al., 2015). The extracellular α2δ proteins assist in trafficking and proper surface expression of the channel complex (Catterall, 2000; Dolphin, 2012, 2013, 2018). In heterologous expression systems, any α1 subunit can co-assemble with any of the four α2δ subunits, but in native systems there are partially specific functions for particular α2δ proteins (Dolphin, 2012, 2013, 2018; Geisler et al., 2015, 2019). The reasons for this partial specificity are so far unclear. Isoforms α2δ1–3 are widely expressed in the brain and often co-expressed in the same type of neuron (Cole et al., 2005; Schlick et al., 2010; Geisler et al., 2015). Recently, additional functions have been found for particular α2δ subunits including channel trafficking along axons, synapse development, and trans-synaptic alignment (Eroglu et al., 2009; Kurshan et al., 2009; Fell et al., 2016; Kadurin et al., 2016; Dolphin, 2018; Ferron et al., 2018; Geisler et al., 2019).

Cochlear spiral ganglion (SG) neurons transmit sound-evoked information transduced by hair cells to the brain. They consist of 95% myelinated type I neurons innervating inner hair cells (IHC) and 5% unmyelinated type II neurons integrating information from multiple outer hair cells. Each of the 10–20 ribbon synapses of one IHC is innervated by a type I SG neuron in a 1:1 manner. The axon of each SG neuron, which represents one fiber of the auditory nerve, branches and forms synapses at multiple targets in the cochlear nuclear complex in the brainstem (Malmierca and Merchán, 2004; Rusznak and Szucs, 2009). Bushy cells (BC) in the anteroventral cochlear nucleus receive large axosomatic synapses from auditory nerve fibers called endbulbs of Held (Ryugo, 1992; Limb and Ryugo, 2000), and each BC receives input from 5–7 SG neurons as shown for rat (Nicol and Walmsley, 2002). These glutamatergic excitatory synapses operate at high rates and with utmost temporal precision to preserve temporal information of sound signals (Joris et al., 1994). Glutamate release at the juvenile endbulb synapse is triggered by CaV2.1 influx, which is largely (85% of total) flowing through P/Q (CaV2.1) channels (Oleskevich and Walmsley, 2002; Lim et al., 2011).

Current knowledge about ion currents in SG neurons of hearing mice is sparse because these neurons are housed in the bony middle axis of the ossified cochlea, preventing slice recordings. Moreover, their soma is completely covered by the soma of satellite cells and their myelin from P0 onward in mice (Wang et al., 2013), which prevents recordings from acutely dissected SG tissue and requires dissociated primary cultures (Nicol and Walmsley, 2002). Primary cultured SG neurons from 3-month-old mice express a variety of voltage-gated CaV2+ channels since L-, P/Q-, N-, R-, and T-type currents have been identified by whole cell and single channel recordings (Lv et al., 2012, 2014). Single cell RNA sequencing confirmed transcripts for these CaV channels and revealed α2δ1, α2δ2, and α2δ3 but not α2δ4 transcripts in SG neurons of 4-week-old mice (Shrestha et al., 2018). Because of strong glycosylation of α2δ proteins, labeling them with antibodies is challenging and to date no specific antibodies exist to label α2δ3 protein in tissue at the cellular and subcellular level.

Previously, we have shown that lack of α2δ3 in mice causes impaired in vivo function of the endbulb of Held synapse resulting in an auditory processing disorder (Pirone et al., 2014). Endbulb synapses are smaller and malformed in 5-week-old α2δ3−/− mice. The observed reduction in auditory evoked input-output functions of the endbulb synapse suggests that impaired synaptic transmission may be caused by reduced presynaptic CaV2+ currents, malformed endbulbs of Held or both. A reduced number of CaV2.1-immunolabeled puncta at SG neuron somata of 5-week-old α2δ3−/− mice (Pirone et al., 2014) is in line with a preference of P/Q channels for α2δ3 and may suggest that presynaptic endbulb CaV2+ currents are reduced, too. Unfortunately, CaV2+ current recordings from tiny α2δ3−/− endbulbs of Held are not feasible. Because presynaptic CaV2+ channels need to be synthesized and trafficked to synapses, we aimed at defining whether genetic ablation of α2δ3 differentially alters the composition of somatic voltage-activated CaV2+ currents in primary cultured SG neurons of 3-week-old wildtype and α2δ3−/− mice, 8 days after the onset of hearing at P12 (Ehret, 1985). We also studied the presence of Cacna2d3 (α2δ3) transcripts and characterized a subset of voltage-activated CaV2+ currents in SG neurons of pre-hearing (P5) mice to ascertain whether neonatal L-type (CaV1.2/CaV1.3) channels as well as CaV2.1 and CaV2.2 channels require α2δ3 subunits at this young age.

MATERIALS AND METHODS

Animals

All experimental procedures were conducted in agreement with the European Communities Council Directive (2010/63/EU) in accordance with the German law and the regional board for scientific animal experiments of the Saarland. Additional ethics approval was not required according to the local and national guidelines. Prehearing and hearing mice of either sex were studied. Cacna2d3-deficient mice generated by Deltagen (B6.129P2-Cacna2d3tm1Dgen, San Mateo, CA, United States) (Neely et al., 2010) were obtained from the Jackson Laboratories, back-crossed on C57Bl6/N background for ≥10 generations and used for electrophysiological and immunohistological analysis. Knockout was obtained by targeted insertion of a bacterial LacZ cassette into exon 15 (of 39) of the Cacna2d3 gene such that the endogenous promoter drives the expression of β-galactosidase and of a truncated Cacna2d3 mRNA (exons 1–14) (Neely et al., 2010). Cochleae were dissected after mice had been sacrificed with isoflurane anesthesia and cervical dislocation (P19–P21, denoted as P20) or by decapitation (P4–P6, denoted as P5). Animals were housed with free access to food and water at 22°C and a 12 h light-dark cycle. Every experimental observation was confirmed in at least three experiments using different animals.

Dissociated Primary Culture of SG Neurons

SG neurons were isolated from neonatal (P5) and juvenile mice (P20) (Lee et al., 2016). To allow proper attachment of the
isolated neurons, coverslips with a diameter of 13 mm were coated with poly-D-lysine (0.5 mg/ml) for 7 h at 37°C and overnight with laminin (1 mg/ml; both Sigma-Aldrich, St. Louis, MO, United States). The cell culture medium was prepared as follows: Neurobasal A medium (Invitrogen, Carlsbad, CA, United States) was supplemented with B27 (2% v/v), L-glutamine (200 mM, Invitrogen, Carlsbad, CA, United States), and penicillin G (100 U/µl, Sigma-Aldrich, St. Louis, MO, United States). The cell culture medium was prepared overnight with laminin (1 mg/ml; both Sigma-Aldrich, St. Louis, MO, United States). The dissection solution contained 50 ml minimal essential medium (MEM, Invitrogen, Carlsbad, CA, United States), 400 µl MgCl₂ (stock solution 1 M), 110 µl D-glucose (450 mg/ml), and 50 µl penicillin G (100 U/µl). The solution was mixed and 2 ml were taken away to prepare the digestion solution, supplemented with B27 (2% v/v). 2 ml FBS (Invitrogen, Carlsbad, CA, United States) were added to the dissection solution and mixed. To prepare the centrifugation solution, 3 ml of the dissection solution was mixed with the same volume of solution 2, consisting of 25 ml 10× HBSS (Invitrogen, Carlsbad, CA, United States) and 154 g sucrose, final volume 500 ml, pH 7.5. For a primary culture of P20 SG neurons, tissue of four mice (eight cochleae) of the same genotype was pooled. For neonatal mice, tissue from three mice (six cochleae) was sufficient. After decapitation, the head was sagitally cut into halves and stored in ice-cold MEM. The cartilage-like (P5) or bony shell (P20) of the cochlea was carefully removed and the cochlear spiral was separated into an apical and a basal half in dissection solution under sterile conditions (Lee et al., 2016). The halves were transferred into petri dishes filled with 1 ml digestion solution. Tissue pieces were disintegrated and affiliated in 400 µl digestion solution in a 2 ml Eppendorf tube each. The tissue was incubated with 50 µl DNase I (1000 U/ml, Sigma-Aldrich, St. Louis, MO, United States) and 50 µl collagenase type I (10 mg/ml, Sigma-Aldrich, St. Louis, MO, United States) in a 37°C water bath for 15 min, followed by adding 1 µl 0.25 M NaOH and 50 µl 2.5% trypsin (Invitrogen, Carlsbad, CA, United States) and shaking for 10 min at 37°C at 180/min. To stop the enzymatic digestion, 400 µl FBS (Invitrogen, Carlsbad, CA, United States) was added; remainders of the tissue were carefully triturated and 800 µl centrifugation solution was added. Cells were centrifuged at room temperature for 5 min at 845 g. The supernatant was aspirated and the cell pellet was dissolved in 400 µl pre-warmed cell culture medium. After carefully re-suspending the pellet and filtering through a 40 µm cell strainer (Fisher Scientific, Hampton, NH, United States), 400 µl of cell suspension was plated onto two wells (400 µl/well) supplemented with 1 µl NT3 and 1 µl BDNF (both Sigma-Aldrich, St. Louis, MO, United States) at 10 ng/µl, respectively. When SG neurons were isolated from mature mice, 40 µl FBS was added. SG neurons were placed into an incubator at 37°C and 5% CO₂. The medium was replaced by medium supplemented with 10 ng/µl BDNF and NT3 each by 100% after 1 day and by 50% on the following days. SG neurons isolated at P5 were cultured for 2 days, SG neurons isolated at P20 were cultured for 3 days.

Electrophysiology
Currents from SG neurons of αδδ⁺/+ and αδδ⁻/− mice were recorded using an Axopatch 200B patch clamp amplifier (Molecular Devices, Sunnyvale, CA, United States) with PatchMaster V2x69 (Heka Electronics, Lambrecht, Germany) at room temperature (21 ± 1°C). The bath solution consisted of (in mM): 1.3 CaCl₂, 10 HEPES, 5.6 glucose, 5.8 KCl, 0.9 MgCl₂, 143 NaCl and 0.9 Na₂HPO₄ × H₂O, pH 7.35, 305 mosmol/kg. To isolate Ca²⁺ currents, cells were locally superfused with application solution (in mM): 15 4-AP, 1.3 CaCl₂, 2 CsCl, 5.6 glucose, 10 HEPES, 1 MgCl₂, 0.7 NaH₂PO₄ × H₂O, 30 TEA-Cl, 113 NMDG, pH 7.35, 305 mosmol/kg, via a custom-made gravity-fed application system. The pipette solution consisted of (in mM): 0.1 CaCl₂, 20 CsCl, 5 EGTA, 0.3 GTP, 5 HEPES acid, 4 MgCl₂, 4 Na₂ATP, 10 Na-phosphocreatine, 110 Cs-methanesulfonate, 295 mosmol/kg, pH was adjusted to 7.35 using 1 M CsOH.

For each recording involving application of a Ca²⁺ channel blocker, a new piece of the glass coverslip with cultured SG neurons was used that had been carefully cracked into pieces with the blunt end of a forceps. Patch pipettes were pulled from quartz glass with a laser micropipette puller P-2000 (Sutter Instruments, Novato, CA, United States) with a resistance of 6–8 MΩ. Cells with a large, round soma and one or two neurites were selected. After establishing the whole cell mode, neuron identity was confirmed by large and fast inactivating inward (Na⁺) currents, which were blocked after perfusion with application solution for 1 min, giving rise to smaller slowly inactivating inward Ca²⁺ currents. Recordings were accepted when the membrane resistance was > 1 GΩ. SG neurons were held at nominally −80 mV. Series resistance usually was between 5 and 15 MΩ, recordings with a larger series resistance were rejected. No compensation for the series resistance was employed because of relatively small Ca²⁺ currents. Data acquisition rate was 10 kHz, currents were filtered at 2 kHz.

L-, P/Q-, N-, and T-type Ca²⁺ currents were isolated with 10 µM nimodipine (Sigma-Aldrich, St. Louis, MO, United States), 1 µM α-agatoxin IVA, 1 µM ω-conotoxin, 1 µM SNX482 (from Alomone Labs, Jerusalem, Israel) and 5 µM mibebradil (Tocris Bioscience, Bristol, United Kingdom), respectively. Toxins were dissolved at 1 mM in distilled H₂O and stored in aliquots at −20°C until use. Mibebradil and nimodipine were dissolved at 5 and 10 mM in DMSO, respectively, and stored at −20°C. During application of the blocker we observed run-up of ICa in 8% of the recordings, resulting in negative difference currents. All data from these neurons were omitted from the analysis. Measurements of a particular Ca²⁺ current subtype of a given genotype and age included recordings of SG neurons from 3 to 5 (average: 3.6) culture preparations.

Analysis of Patch Clamp Data
Patch clamp data were analyzed using the software Igor Pro Version 6.12 (Wavemetrics, Lake Oswego, OR, United States) and custom-written routines. Linear leak subtraction was done off-line and voltages were corrected by subtracting a liquid junction potential (LJP) of 27.8 mV for the three-solution setting (Neher, 1992). Steady-state current-voltage (I/V) relations were calculated by averaging ICa during the last ms of the 100 ms depolarizing pulse. Voltage-gated Ca²⁺ currents (ICa) of a given genotype were variable within SG neurons from a particular
cochlear location but after statistical tests did not show systematic changes between apical or basal halves. Therefore, \(I_{Ca}\) data were pooled for the whole cochlear length for a given genotype and age.

**Quantitative Real-Time PCR for Cacna2d1, Cacna2d2, and Cacna2d3 in SG Tissue**

For qPCR analysis, spiral ganglia from \(\alpha_2\delta_3^{-/-}\) and \(\alpha_2\delta_3^{+/+}\) mice aged P5 were microdissected and separated into apical and basal halves. Because heterozygous \(\alpha_2\delta_3^{+/+}\) mice do not show a phenotype they served as controls for qPCR experiments (Neely et al., 2010). Pooled apical and basal SG from two animals each were transferred into cryotubes, frozen in liquid nitrogen and stored at \(-70^\circ\)C. Total RNA was isolated using the peqGOLD MicroSpin Total RNA Kit (PeqLab Biotechnologie GmbH, Erlangen, Germany) according to the protocol of the manufacturer. For reverse transcription (RT), isolated RNA (10 \(\mu\)l each) was incubated with 0.5 \(\mu\)l oligo dT20 primers (50 mM) and random primers pd(N)\(_6\) (50 \(\mu\)M, Applied Biosystems, Carlsbad, CA, United States) and 1 \(\mu\)l dNTP mix (10 mM; New England Biolabs, Ipswich, MA, United States) for 5 min at 65\(^\circ\)C and stored on ice for 1 min.

An RT mix (8 \(\mu\)l; Life Technologies, Carlsbad, CA, United States) consisting of 4 \(\mu\)l 5× RT buffer, 2 \(\mu\)l dithiothreitol (100 mM), 1 \(\mu\)l RNaseOUT\TM and 1 \(\mu\)l SuperScript\® III was added and each tube was incubated at 50\(^\circ\)C for 150 min followed by 70\(^\circ\)C for 30 min. cDNA was stored at \(-20^\circ\)C. The abundance of *Cacna2d1*–3 transcripts in SG cDNA was assessed by TaqMan quantitative PCR (qPCR) using a standard curve method (Schlick et al., 2010). TaqMan gene expression assays specific for *Cacna2d1*–3 isoforms were designed to span exon–exon boundaries and purchased from Applied Biosystems.

The following assays were used [name (gene symbol), assay ID (Applied Biosystems)]; \(\alpha_2\delta_1\) (*Cacna2d1*), Mm00486607_m1; \(\alpha_2\delta_2\) (*Cacna2d2*), Mm00457825_m1; \(\alpha_2\delta_3\) (*Cacna2d3*), Mm00486613_m1; \(\alpha_2\delta_4\) (*Cacna2d4*), Mm01190105_m1. Expression of hypoxanthine phosphoribosyl-transferase 1 (Hprt1; Mm00446968_m1) and succinate dehydrogenase, subunit A (*Sdha*; Mm01352363_m1) were used as endogenous controls. The qPCR (50 cycles) was performed in duplicates using total DNA (see above) and the specific TaqMan gene expression assay for each 20 \(\mu\)l reaction in TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, United States). Samples without cDNA were used as controls. Analyses were performed using the 7500 Fast System (Applied Biosystems, Foster City, CA, United States). The Ct values for each gene expression assay were recorded for each individual preparation and molecule numbers were calculated for each \(\alpha_2\delta\) subunit from their respective standard curve. Expression of *Hprt1* and *Sdha* were used for normalization of total mRNA abundance to allow a direct comparison between the expression levels in different preparations.

**Immunohistochemistry**

SG tissue from \(\alpha_2\delta_3^{+/+}\) and \(\alpha_2\delta_3^{-/-}\) mice was dissected after perfusion of the cochlea with Zamboni’s fixative (Stefanini et al., 1967) for 15 min, mounted on coverslips and double-labeled with rabbit polyclonal anti-Ca\(_{v}2.1\) (Synaptic Systems, Göttingen, Germany, #152203/7, 1:500) and mouse monoclonal anti-Golgi matrix protein 130 kDa (GM 130, BD Transduction Laboratories, San Jose, CA, United States, #610823, 1:50) as described (Fell et al., 2016). Donkey anti-mouse Alexa Fluor® 488 (Invitrogen, Carlsbad, CA, United States, #A-21202, 1:500) and donkey anti-rabbit Cy3 (Jackson Immuno, West Grove, PA, United States, #711-166-152, 1:1500) were used as secondary antibodies. Images were acquired with a confocal microscope LSM 710 using a 63\(\times\)/1.4 NA oil objective (both Zeiss Microscopy GmbH, Göttingen, Germany). Single optical slices with a thickness of 0.31 \(\mu\)m are shown.

**Statistical Analysis**

Igor Pro Version 6.12 (Wavemetrics, Lake Oswego, OR, United States) was used for statistical tests of \(I_{Ca}\). Because often data were not normally distributed, box plots and individual data points are shown, with boxes representing the 25th–75th percentiles, the median (horizontal bar) and the 10th–90th percentiles (whiskers). Comparisons between \(I_{Ca}\) without/with blocker (paired samples) were performed using the Wilcoxon signed test, comparisons of unpaired samples using the Wilcoxon rank test. Transcript data were analyzed on log10-transformed transcript numbers using two-way ANOVA with Holm–Sidak post hoc correction. Electrophysiological data are given as mean ± SD, transcript data as mean ± SEM.

**RESULTS**

L-Type \(Ca^{2+}\) Currents Were Unaltered in Cultured SG Neurons of 3-Week-Old \(\alpha_2\delta_3^{-/-}\) Mice

To assess the consequences of \(\alpha_2\delta_3\) deletion for the expression of different types of voltage-gated \(Ca^{2+}\) channels, we recorded \(Ca^{2+}\) currents (\(I_{Ca}\)) from primary cultured SG neurons that had been dissociated at P20 and cultured for 3 days. Cells with a large, round soma and one or two neurites were selected for patch-clamping, and there was no indication of an altered morphology of \(\alpha_2\delta_3^{-/-}\) SG neurons. L-type currents flowing through Ca\(_{v}1.2\) and Ca\(_{v}1.3\) channels, which both are expressed in mature SG neurons (Lv et al., 2014; Shrestha et al., 2018), were pharmacologically isolated using 10 \(\mu\)M nimodipine (Figures 1A, B). Control \(I_{Ca}\) values before application of the blocker were quite variable in both genotypes shown by box plots and single data points (Figure 1C). Superfusion of 10 \(\mu\)M nimodipine significantly reduced the steady state \(I_{Ca}\) in SG neurons in both \(\alpha_2\delta_3^{+/+}\) and \(\alpha_2\delta_3^{-/-}\) (Figure 1C and Table 1). The amplitude of L-type currents calculated as the differences between the respective control currents and the currents under superfusion with nimodipine was not different between genotypes (\(\alpha_2\delta_3^{+/+}\): 52.5 ± 30.4 pA, \(n = 21\); \(\alpha_2\delta_3^{-/-}\): 59.0 ± 28.5 pA, \(n = 19\); \(p = 0.17\); Figure 1D).

To summarize, lack of \(\alpha_2\delta_3\) did not affect the average L-type...
current amplitude of 3-week-old primary cultured SG neurons compared with wildtype.

**P/Q-Type Ca\(^{2+}\) Currents Were Strongly Reduced in Cultured SG Neurons of 3-Week-Old \(\alpha_2\delta3^{-/-}\) Mice**

Next we analyzed whether lack of \(\alpha_2\delta3\) affected Ca\(_{v}\)2.1 (P/Q) currents in cultured neurons at P20 (+ 3 DIV) using the P/Q-type channel blocker \(\omega\)-agatoxin IVA (Figure 2). Superfusion of 1 \(\mu\)M \(\omega\)-agatoxin IVA significantly reduced the steady state \(I_{Ca}\) in SG neurons of \(\alpha_2\delta3^{+/+}\) mice (Figures 2A,C and Table 1). A weaker but still significant block of \(I_{Ca}\) was exerted by \(\omega\)-agatoxin IVA in SG neurons of \(\alpha_2\delta3^{-/-}\) mice (Figures 2B,C and Table 1). This indicates that deletion of \(\alpha_2\delta3\) strongly reduced the amplitude of P/Q-type currents from 115.3 ± 58.8 pA (n = 23) in wildtype to 45.9 ± 32.0 pA (n = 27; \(p < 10^{-6}\)) in SG neurons from \(\alpha_2\delta3^{-/-}\) mice (Figure 2D). In other words, P/Q-type currents comprise 54% of total \(I_{Ca}\) in SG neurons of \(\alpha_2\delta3^{+/+}\) mice. Lack of \(\alpha_2\delta3\) reduced P/Q-type currents by as much as 60.2% compared with \(\alpha_2\delta3^{+/+}\) animals (Figure 2D).

Because \(\alpha_2\delta3\) proteins have been shown to participate in forward trafficking of Ca\(_{v}\)2 channels to the plasma membrane and their final presynaptic destination (Kadurin et al., 2016; Dolphin, 2018), we tested for a potential aberrant intracellular localization of Ca\(_{v}\)2.1 channels in \(\alpha_2\delta3^{-/-}\) SG neurons by double immunolabeling acutely dissected SG tissue from 3-week-old mice for Ca\(_{v}\)2.1 protein and the Golgi marker GM130 (Figure 2E). SG neurons showed both punctate and diffuse intracellular labeling and distinct punctate labeling for Ca\(_{v}\)2.1 at the plasma membrane, which was weaker in \(\alpha_2\delta3^{-/-}\) SG neurons compared with \(\alpha_2\delta3^{+/+}\) and correlates with their smaller P/Q-type currents in cultured SG neurons. There was minor overlap of Ca\(_{v}\)2.1 labeling with GM130 in both genotypes (Figure 2E), suggesting that the strongly reduced currents of \(\alpha_2\delta3^{-/-}\) SG neurons were not caused by retention of Ca\(_{v}\)2.1 channels within the cell, specifically in the Golgi apparatus.

**N-Type Ca\(^{2+}\) Currents Were Unaltered in Cultured SG Neurons of 3-Week-Old \(\alpha_2\delta3^{-/-}\) Mice**

Because N-type and R-type currents have been recorded in SG neurons cultured from 3-month-old mice (Lv et al., 2012) we tested for the presence of these Ca\(_{v}\)2 currents in \(\alpha_2\delta3^{-/-}\) SG neurons. N-type currents were pharmacologically isolated using 1 \(\mu\)M \(\omega\)-conotoxin (Figures 3A,B). For unknown reasons, the endogenous \(I_{Ca}\) recorded from wildtype SG neurons was unusually large here (384.0 ± 127.8 pA, Table 1) and in recordings for isolation of R-type currents (see below) compared with values of SG neurons used to isolate L-type and P/Q-type currents (Figures 2C, 3C; 200.0 and 198.4 pA, respectively). We assume that this reflects the rather high variability of SG neurons in our culture.

Application of \(\omega\)-conotoxin (1 \(\mu\)M) caused reductions of \(I_{Ca}\) in \(\alpha_2\delta3^{+/+}\) SG and \(\alpha_2\delta3^{-/-}\) SG neurons (Figure 3C and Table 1). The amplitudes of the N-type currents were small (\(\alpha_2\delta3^{+/-}\): 30.8 ± 25.8 pA, n = 18; \(\alpha_2\delta3^{-/-}\): 30.2 ± 24.8 pA, n = 15) and were not different between \(\alpha_2\delta3^{+/+}\) and \(\alpha_2\delta3^{-/-}\) mice (\(p = 0.47\); Figure 3D). These data indicate that lack of \(\alpha_2\delta3\) did not affect the small N-type currents in SG neurons of our preparation. Further, N-type currents did not compensate for the loss of P/Q-type channels in \(\alpha_2\delta3^{-/-}\) mice.

**Small R-Type Ca\(^{2+}\) Currents Were Reduced in Cultured SG Neurons of 3-Week-Old \(\alpha_2\delta3^{-/-}\) Mice**

To assess the contribution of R-type (Ca\(_{v}\)2.3) currents to total \(I_{Ca}\) of SG neurons and to test whether lack of \(\alpha_2\delta3\) affected their expression, we employed the Ca\(_{v}\)2.3 channel blocker SNX 482 (Figures 4A,B). SNX 482 (1 \(\mu\)M) significantly reduced \(I_{Ca}\) in \(\alpha_2\delta3^{+/+}\) and \(\alpha_2\delta3^{-/-}\) SG neurons (Figure 4C and Table 1).
α of P/Q-type currents in 38%, and R-type currents did not compensate for the reduction reduced the small amplitude of R-type currents in SG neurons by 

\[ n = 18; \]

isolation of different Ca_\(2+\) currents subtypes of SG neurons isolated at P20 from \(\alpha_2\delta3^{+/+}\) and \(\alpha_2\delta3^{-/-}\) mice.

### TABLE 1

| Isolation of/(drug) | control \(I_{Ca}\) (pA) | \(I_{Ca} + \) drug (pA) | n | \(p\) | control \(I_{Ca}\) (pA) | \(I_{Ca} + \) drug (pA) | n | \(p\) |
|---------------------|-------------------------|-------------------------|---|------|-------------------------|-------------------------|---|------|
| L-type (nimodipine) | 200.0 ± 80.6            | 147.5 ± 71.0            | 21| <10^{-6} | 271.4 ± 169.9          | 212.5 ± 160.0           | 19| <10^{-5} |
| P/Q-type (ω-agatoxin) | 212.6 ± 74.8          | 97.3 ± 35.3             | 23| <10^{-6} | 221.8 ± 88.3           | 175.9 ± 74.3            | 27| <10^{-7} |
| N-type (ω-conotoxin) | 384.0 ± 127.8          | 353.1 ± 112.6           | 18| <10^{-6} | 272.6 ± 99.4           | 242.3 ± 94.1            | 15| <10^{-4} |
| R-type (SNX 482)    | 373.9 ± 73.8           | 343.1 ± 68.0            | 13| <0.001  | 288.3 ± 82.8           | 269.2 ± 85.2            | 18| 0.018 |
| T-type (mibefradil) | 387.7 ± 197.2          | 316.7 ± 160.0           | 19| <10^{-5} | 247.5 ± 111.0          | 153.6 ± 66.5            | 20| <10^{-5} |

Number of SG neurons (n); \(p\) was determined using the Wilcoxon signed test.

The small R-type currents of \(\alpha_2\delta3^{+/+}\) (30.8 ± 17.6 pA, \(n = 13\)) were further reduced in \(\alpha_2\delta3^{-/-}\) SG neurons (19.1 ± 17.7 pA, \(n = 18\); \(p = 0.018\); Figure 4D). To summarize, deletion of \(\alpha_2\delta3\) reduced the small amplitude of R-type currents in SG neurons by 38%, and R-type currents did not compensate for the reduction of P/Q-type currents in \(\alpha_2\delta3^{-/-}\) mice.

**T-Type Ca\(^{2+}\) Currents Were Unaltered in Cultured SG Neurons of 3-Week-Old \(\alpha_2\delta3^{-/-}\) Mice**

Finally, we tested for T-type (Ca\(_\delta\)) currents, which activate at more negative potentials than Ca\(_\alpha1\) and Ca\(_\alpha2\) channels and do...
neither require αδ nor β subunits for their membrane expression (Dolphin, 2013). They were pharmacologically isolated using 5 μM mibebradil (Lv et al., 2012; Figure 5). The example I-V curves before/under mibebradil indicate a larger difference (T-type) component in the α2δ3−/− neuron of ~100 pA (Figure 5B) compared with the α2δ3+/+ neuron (Figure 5A). Under superfusion with mibebradil, ICa of the α2δ3−/− SG neuron indeed activated 5–10 mV more positive than before indicating that a substantial, negatively activating ICa (T-type) component was blocked (Figure 5B). On average, application of mibebradil reduced the steady state ICa in α2δ3+/+ and in α2δ3−/− SG neurons (Figure 5C and Table 1). Isolated T-type currents were highly variable between neurons of either genotype (Figure 5D). There was a tendency of increased T-type currents in α2δ3−/− SG neurons, which, however, was not significant (α2δ3+/+: 71.0 ± 52.1 pA, n = 19; α2δ3−/−: 94.2 ± 56.0 pA, n = 20; p = 0.081), most likely because of the large scatter in T-type currents (Figure 5D).

**Total Ca2+ Currents and Amplitudes of Ca2+ Current Subtypes in Cultured SG Neurons of 3-Week-Old α2δ3+/+ and α2δ3−/− Mice**

After determining the amplitudes of ICa in SG neurons mediated by L-, P/Q-, N-, R-, and T-type currents, we summarized their amplitudes and the total current in SG neurons of α2δ3+/+ and α2δ3−/− mice (Figure 6). The average total ICa of SG neurons dissociated at P20 (+ 3 DIV) was significantly smaller in α2δ3−/− mice (256.3 ± 113.9 pA, n = 99) corresponding to 85.3% of α2δ3+/+ (300.3 ± 147.9 pA, n = 94; p = 0.019; Figure 6A). In wildtype SG neurons, P/Q-type currents were the dominant Ca2+ current component (115.3 ± 58.8 pA), which was reduced to 45.9 ± 32.0 pA in α2δ3−/− (or 39.8% of the wildtype value, Figure 6B). Small R-type currents of α2δ3+/+ SG neurons (30.8 ± 17.6 pA) were significantly reduced to
Neither L- nor N-type currents compensated for the loss of P/Q-type currents in those neurons may suggest a reduced number of presynaptic P/Q channels at the endbulb synapse in 3-week-old mice \( \alpha_2\delta^3/-/\) mice. The question arises as to the role of \( \alpha_2\delta^3 \) on the composition of \( I_{Ca} \) well before the onset of hearing. We therefore analyzed a subset of \( I_{Ca} \) types in neonatal SG neurons dissociated at P5 and cultured for 2 DIV.

**L-Type Ca\(^{2+}\) Currents Were Reduced in Cultured SG Neurons of Neonatal \( \alpha_2\delta^3/-/ \) Mice**

Because L-type currents mediated by Ca\(_{v}1.2\) and Ca\(_{v}1.3\) channels play a role in Ca\(^{2+}\) signals controlling gene transcription, neurite growth, and neuronal differentiation (Dolmetsch et al., 2001; Roehm et al., 2008; Satheesh et al., 2012), we recorded those currents in SG neurons cultured at P5 (+2 DIV). SG neurons isolated from \( \alpha_2\delta^3/-/ \) mice did not show an immature or altered morphology compared with those of wildtype. L-type currents were present in SG neurons of both \( \alpha_2\delta^3/+/ \) and \( \alpha_2\delta^3/-/ \) mice (Figures 7A,B). Nimodipine (10 \( \mu \)M) blocked part of the total steady-state current in SG neurons of \( \alpha_2\delta^3/+/ \) and \( \alpha_2\delta^3/-/ \) mice (Figure 7C and Table 2). Lack of \( \alpha_2\delta^3 \) reduced the L-type current amplitude to 66.7 ± 43.4 pA (\( n = 23 \)) or 76.7% compared with \( \alpha_2\delta^3/+/ \) (87.0 ± 37.2 pA, \( n = 25 \); \( p = 0.017 \); Figure 7C). Notably, the average size of L-type currents in wildtype SG neurons was larger at P5 (87.0 ± 37.2 pA; Figure 7D) compared with P20 (52.5 ± 30.4 pA; \( p < 0.001 \); Figure 1D).

At the age of P5, the average unblocked \( I_{Ca} \) of all wildtype SG neurons amounted to 288.7 ± 78.5 pA (\( n = 60 \)), which was reduced to 249.8 ± 119.3 pA (86.5%) in \( \alpha_2\delta^3/-/ \) SG neurons (\( n = 54 \); \( p = 0.006 \)). These values are very similar to average total \( I_{Ca} \) values of SG neurons isolated at P20 of 300.3 pA for \( \alpha_2\delta^3/+/ \) and 256.3 pA for \( \alpha_2\delta^3/-/ \) mice (cf. Figure 6A).

**Small P/Q-Type Ca\(^{2+}\) Currents Were Not Altered in Cultured SG Neurons of Neonatal \( \alpha_2\delta^3/-/ \) Mice**

Next we analyzed P/Q-type currents in P5 (+2 DIV) SG neurons by using 1 \( \mu \)M ω-agatoxin IV A (Figures 8A,B). The decrease of \( I_{Ca} \) by the blocker was significant for SG neurons of \( \alpha_2\delta^3/+/ \) and \( \alpha_2\delta^3/-/ \) mice (Figure 8C and Table 2). The difference currents indicated a heterogenous but on average small contribution of P/Q-type currents to total \( I_{Ca} \) in both \( \alpha_2\delta^3/+/ \) (43.9 ± 29.9 pA, \( n = 14 \)) and \( \alpha_2\delta^3/-/ \) mice (32.4 ± 25.0 pA, \( n = 14 \); Figure 8D).

In contrast to our findings in P20 neurons, the P/Q-current amplitude was not different between the two genotypes (\( p = 0.17 \)). Double immunolabeling of acutely dissected SG tissue at P5 revealed sparse dot-like labeling of P/Q-type channels at the plasma membrane of SG neurons (Figure 8E). Most of
the Ca2.1 immunofluorescence was, however, co-localized with GM130 in the Golgi apparatus of both α2δ3+/+ and α2δ3−/− neurons (Figure 8E), much in contrast to 3-week-old SG neurons (Figure 2E). To summarize, Ca2.1 channels are just being up-regulated, synthesized, and inserted into the plasma membrane of SG neurons at P5 leading to similar small P/Q currents at this age, which was independent of the presence of the α2δ3 subunit. This raises the question whether α2δ3 is expressed in WT neurons at this age at all.

**Transcript Analysis of Cacna2d Isoforms in Spiral Ganglia of Neonatal Mice**

Transcripts for Cacna2d1, Cacna2d2, and Cacna2d3 encoding the three neuronal subunits α2δ1, α2δ2 and α2δ3 have been identified in SG neurons of 3-week-old mice (Shrestha et al., 2018) but it was unclear whether they are present in neonatal SG neurons. We performed transcript analysis for Cacna2d1, Cacna2d2, and Cacna2d3 with quantitative RT-PCR using SG tissue of P5 α2δ3−/− mice with α2δ3−/+ littermates serving as control. All three neuronal subunits Cacna2d1, Cacna2d2, and Cacna2d3 were expressed in α2δ3+/− SG tissue of the apical and basal cochlear halves (Figure 9). Note that due to the design of the α2δ3 knockout construct (introduction of a frameshift in exon 15) and the position of α2δ3-specific primers for qPCR (junction between exon 5 and exon 6), truncated, non-functional α2δ3-specific transcripts were still detected in α2δ3−/− tissue (hatched bars in Figure 9; see section “Materials and Methods”). There was no difference in the total amount of Cacna2d1–3 subunit mRNA between α2δ3+/+ and α2δ3−/− cells (two-way ANOVA; apex, $F = 4.11$, $p = 0.058$; base, $F = 0.03; p = 0.876$). Transcript numbers of individual subunits were not different between the two genotypes in the apical half ($F = 2.12; p = 0.149$). However, in α2δ3+/− neurons of the basal half, Cacna2d3 expression was significantly higher than expression of Cacna2d1 ($p = 0.03$) but not significantly higher than Cacna2d2 ($p = 0.085$). Expression of Cacna2d3 was significantly lower in α2δ3−/− compared with α2δ3+/− cells ($p < 0.001$). There was a tendency toward increased levels of both Cacna2d1 and Cacna2d2 in α2δ3−/− cells but these differences were not significant (Figure 9). To summarize, Cacna2d1 and Cacna2d2 transcripts partially compensate for non-functional Cacna2d3 transcripts in the knockout. Importantly, Cacna2d3 mRNA is present in α2δ3−/− SG neurons in the entire cochlea at P5, suggesting that the small amplitude of P/Q currents at this age was not caused by the developmental absence of α2δ3. Rather, the similar small amplitudes of P/Q currents in both α2δ3+/+ and α2δ3−/− SG neurons (Figure 8D) infer that they do not depend on the presence of the α2δ3 subunit at this age.

**N-Type Ca2+ Currents Were Severely Reduced in Cultured SG Neurons of Neonatal α2δ3−/− Mice**

A switch of N-type to P/Q-type currents has been reported in early postnatal development of the calyx of Held synapse, inhibitory thalamic and cerebellar synapses and the neuromuscular junction (Siri and Uchitel, 1999; Iwasaki et al., 2000).
Therefore we tested whether neonatal SG neurons express N-type currents and if they were affected by deletion of α2δ3 (Figures 10A,B). Application of ω-conotoxin (1 μM) caused significant reductions in ICa in α2δ3+/+ SG neurons (Figure 10C and Table 2). In SG neurons of α2δ3−/− mice, there was a weaker response to ω-conotoxin (Figure 10C and Table 2). Deletion of α2δ3 significantly reduced the amplitude of N-type currents in α2δ3+/+ SG neurons from 94.4 ± 55.2 pA (n = 21) to 53.3 ± 36.5 pA (n = 17; p = 0.009) in α2δ3−/− SG neurons (Figure 10D). Taken together, cultured SG neurons of wildtype mice at P5 + 2 DIV displayed N-type currents that were 3.1 times larger as at P20 + 3 DIV (30.8 pA, cf. Figure 3D). N-type currents of neonatal SG neurons deficient for α2δ3 were reduced to 56% of the wildtype value, suggesting that α2δ3 is indispensable for normal expression of CaV2.2 channels at this age.

### DISCUSSION

In this study, we have analyzed the role of the auxiliary Ca2+ channel subunit α2δ3 for the size and composition of Ca2+ currents of dissociated primary cultured SG neurons in the mature (P20) and immature cochlea (P5). The impaired in vivo function of the endbulb of Held synapse in α2δ3−/− mice (Pitro et al., 2014) led us to analyze whether lack of α2δ3 specifically affected voltage-activated Ca2+ currents. Due to the small size of endbulb synapses in α2δ3−/− mice, recordings of presynaptic currents are not feasible. Though the composition of Ca2+ currents differs between the presynapse and the soma (Doughty et al., 1998) our approach can nevertheless give insights into the specific dependence of somatic Ca2+ currents on the expression of α2δ3, which ultimately may also affect presynaptic (CaV2.1) currents (Lübbert et al., 2019).

### Composition of Ca2+ Currents in SG Neurons of 3-Week-Old Mice

SG neurons isolated from wildtype mice at P20 expressed different voltage-activated Ca2+ channels that conducted L-type, P/Q-type, N-type, R-type, and T-type currents. P/Q currents carried by CaV2.1 channels formed the largest ICa component comprising 54% of the total ICa, which contrasts findings from Lv et al. (2012) who found 11.5% P/Q current for basal and 17.2% for apical SG neurons in 3-month-old mice. However, the authors used a different subpopulation of neurons by separately culturing the most apical and the most basal part of the SG while discarding the middle part, which represents the range of best hearing. In contrast, our SG neurons were cultured from the apical and the basal half of the cochlea, respectively, such that SG neurons from the middle region were included in both cultures. The different age (3 weeks versus 3 months) may also be a factor.

### Table 2

| Isolation of (drug) | control ICa (pA) | ICa + drug (pA) | n  | p    | control ICa (pA) | ICa + drug (pA) | n  | p    |
|-------------------|-----------------|----------------|----|------|-----------------|-----------------|----|------|
| L-type (nimodipine) | 287.5 ± 73.7    | 200.5 ± 71.2   | 25 | <10−7 | 208.8 ± 95.9    | 142.1 ± 84.1    | 23 | <10−6 |
| P/Q-type (ω-agatoxin) | 279.3 ± 85.4    | 235.5 ± 63.3   | 14 | <10−5 | 282.3 ± 61.4    | 249.9 ± 77.4    | 14 | <10−3 |
| N-type (ω-conotoxin) | 296.9 ± 82.3    | 202.0 ± 52.5   | 21 | <10−6 | 278.3 ± 165.0   | 225.0 ± 166.8   | 17 | <10−4 |

Number of SG neurons (n); p was determined using the Wilcoxon signed test.
FIGURE 8 | Small P/Q-type Ca\(^{2+}\) currents are not altered in neonatal SG neurons of α\(_{2}\)δ\(_{3}\)−/− mice at P5 + 2 DIV. (A,B) Maximum I\(_{\text{Ca}}\) traces of an α\(_{2}\)δ\(_{3}\)+/+ (A, top) and an α\(_{2}\)δ\(_{3}\)−/− SG neuron (B, top) in response to 100 ms depolarizing voltage steps before (α\(_{2}\)δ\(_{3}\)+/+, black; α\(_{2}\)δ\(_{3}\)−/−, magenta) and during application of 1 µM ω-agatoxin IVA (blue). Corresponding steady-state I–V curves are shown below the traces. (C) Box-and-whisker plots of I\(_{\text{Ca}}\) before (ctrl) and under superfusion of 1 µM ω-agatoxin IVA (aga) of SG neurons isolated from α\(_{2}\)δ\(_{3}\)+/+ (+/+) and α\(_{2}\)δ\(_{3}\)−/− (−/−) mice. Numbers under the box plots denote the numbers of SG neurons. Wilcoxon signed test, **∗∗∗\(p<0.001\). (D) P/Q-type current amplitudes were unaltered between SG neurons from both genotypes; Wilcoxon rank test. (E) Double immunolabeling for Ca\(_{\text{v}}\)2.1 (cyan) and the Golgi marker GM 130 (magenta) in acutely dissected SG tissue of α\(_{2}\)δ\(_{3}\)+/+ (top) and α\(_{2}\)δ\(_{3}\)−/− (bottom) at P5. In both genotypes, most of the Ca\(_{\text{v}}\)2.1 protein was localized in the Golgi apparatus (overlap of magenta and cyan, resulting in white color) rather than in the membrane of the three SG neurons each (weak dotted outline in cyan). Confocal images (single optical slices); 63×/1.4 NA oil objective; scale bar, 10 µm.

potentially leading to reduced Ca\(_{\text{v}}\)2.1 currents in the fully mature subpopulation of SG neurons used by Lv et al. (2012). In line with our I\(_{\text{Ca}}\) data, recent single cell RNA sequencing demonstrated strong Cacna1a expression (encoding for Ca\(_{\text{v}}\)2.1) in 4-week-old mice, which dominated all transcripts for VGCCs in type I SG neurons (Shrestha et al., 2018).

An alternative explanation for the discrepancy in P/Q currents in SG neurons of wildtype mice, cf. (Lv et al., 2012), may lie in the dissociated primary culture of SG itself, which results in rather low neuronal survival (Vieira et al., 2007). Moreover, because type I SG neurons forming subclasses Ia, Ib, and Ic are molecularly and physiologically heterogeneous (Petitpré et al., 2018; Shrestha et al., 2018; Sun et al., 2018), subtle differences in dissociation protocols or chemicals may have resulted in a differential survival of these subclasses (Liu et al., 2016; Browne et al., 2017; Cai et al., 2017). Differential survival of subclasses may also explain the heterogeneity in our I\(_{\text{Ca}}\) values for a given genotype and age.

Lack of α\(_{2}\)δ\(_{3}\) Severely Affects Ca\(_{\text{v}}\)2.1 Currents in Cultured SG Neurons of 3-Week-Old Mice

The reduction of P/Q currents of α\(_{2}\)δ\(_{3}\)−/− SG neurons by 58% compared with the wildtype (Figures 2, 6) is in line with a significant reduction of immunopositive Ca\(_{\text{v}}\)2.1 puncta at the plasma membrane and within the somata of α\(_{2}\)δ\(_{3}\)−/− SG neurons (cf. Figure 2E). Reduction of somatic P/Q channels may lead to decreased axonal trafficking and channel density at the presynaptic terminal in vivo (Lin et al., 2011; Kadurin et al., 2016) and might contribute to the impaired function of the endbulb of Held synapse in α\(_{2}\)δ\(_{3}\)−/− mice (Pirone et al., 2014). In this context, a recent study showed that specific overexpression of Ca\(_{\text{v}}\)2.1 channels increased presynaptic currents and synaptic strength of the calyx of Held synapse, indicating that the somatic expression of Ca\(_{\text{v}}\)2.1 regulated the presynaptic abundance of Ca\(_{\text{v}}\)2.1 channels at this synapse (Lübbert et al., 2019).
Our results of reduced somatic P/Q currents in α2δ3−/− SG neurons contrast recent findings of Landmann et al. (2018) who reported unaltered expression of Ca2+ neurons in α2δ3−/− and α2δ3+/− mice (P5) using qRT-PCR. Transcript numbers (mean ± SEM) normalized to Hprt1 and Sdha of cDNA synthesized from SG tissue from the apical (left) or the basal cochlea cochlear half (right). Note that due to the design of the α2δ3 knockout construct (introduction of a frameshift in exon 15, see section “Materials and Methods”) and the position of α2δ3-specific primers for qPCR (junction between exon 5 and exon 6), α2δ3-specific transcripts were still detected in α2δ3−/− tissue (hatched bars). However, functional protein will not be produced. Each column is from 3 to 4 independent samples. Two-way ANOVA was performed on log10-transformed transcript numbers with Holm–Sidak post hoc test; *p < 0.05, **p < 0.01, ***p < 0.001.

In cerebellar Purkinje cells, Ca2.1 channels require the α2δ2 subunit for proper cellular function and normal morphology of the dendritic tree as shown for an α2δ2 null mutant, the ducky mouse (Brodebeck et al., 2002). Such a phenotype has not been found in α2δ3−/− mice (Neely et al., 2010) further corroborating the view that a specific pairing of a particular CaV channel type with a defined α2δ isoform does not exist in general. Co-assembly between the two partners rather depends on the cellular and extracellular context (Fell et al., 2016; Dolphin, 2018).

Not only P/Q-type currents, but also R-type currents were significantly affected by deletion of α2δ3. Mean R-type currents, which amounted to only 31 pA in wildtype SG neurons, were further reduced to 19.1 pA (or 62%) in α2δ3−/− SG neurons. The total ICa of SG neurons in our preparation was reduced in α2δ3−/− SG neurons to 85% of the wildtype yet to a lesser extent than the reduction in P/Q- and R-type currents should have inferred. A similar result has been found for total ICa in cultured DRG neurons from α2δ3−/− mice (Neely et al., 2010). The question arises as to the nature of the compensatory ICa component. T-type currents, which are present in SG neurons (Lv et al., 2012), are likely candidates to compensate for the reduction in P/Q currents because they can form functional channels without co-assembling with any α2δ subunit, for review see Dolphin (2012, 2018). Indeed, there was a tendency of increased T-type currents in α2δ3−/− SG neurons (Figures 5, 6). Notably, T-type currents were increased in thalamocortical relay neurons of a mouse line with loss-of-function of CaV2.1 (Zhang et al., 2002), which is partially recapitulated by the severe reduction of P/Q (CaV2.1) currents in P20 SG neurons of α2δ3−/− mice in the present study.
Opposing Effects of Deletion of α2δ3 on L-Type, N-Type, and P/Q-Type Currents in Cultured Neonatal SG Neurons

So far, voltage-activated Ca\(^{2+}\) currents have not been analyzed in neonatal SG neurons. Our recordings of L-, N-, and P/Q-type currents from P5 wildtype SG neurons revealed amplitudes of the \(I_{Ca}\) components that clearly differed from those at P20. At P5, L-type currents were 1.67-fold larger and N-type currents even 3.1-fold larger compared with P20. Moreover, deletion of \(\alpha_2\delta_3\) significantly reduced L-type and N-type currents in P5 SG neurons whereas it had no effect on L-type and N-type currents at P20.

Neonatal SG neurons (P5 + 2 DIV) of \(\alpha_2\delta_3^{+/+}\) and \(\alpha_2\delta_3^{-/-}\) mice exhibited similar small P/Q currents. This finding was supported by sparse \(Ca_{2.1}\) immunoreactivity in the somatic membrane whereas it was strongly enriched in the Golgi apparatus of SG neurons at P5 suggesting that \(Ca_{2.1}\) channels were just being up-regulated in both \(\alpha_2\delta_3\) wildtype and knockout (Figure 8). At P20, P/Q currents were 2.6-fold larger than at P5 and comprised the largest \(I_{Ca}\) component of cultured \(\alpha_2\delta_3^{+/+}\) SG neurons. In contrast, P/Q currents of \(\alpha_2\delta_3^{-/-}\) SG neurons at P20 had risen only 1.4-fold.

Immature SG neurons relay information from spontaneously active IHCs in a burst-firing mode with maximum firing rates of 100–300 Hz until the end of the first postnatal week (Trisch and Bergles, 2010; Trisch et al., 2010). With the onset of hearing, IHCs produce sound-evoked receptor potentials leading to much higher firing rates in type I SG neurons (Taberner and Liberman, 2005; Wu et al., 2016), which are accompanied by differentiation into SG neuron subtypes Ia, Ib, and Ic and respective changes in their ion channel expression (Petitpré et al., 2018; Shrestha et al., 2018; Sun et al., 2018).

At many synapses such as inhibitory synapses of thalamic and cerebellar neurons, the excitatory calyx of Held synapse, and at neuromuscular junctions, the composition of VGCCs changes during development with a prominent switch from \(Ca_{2.2}\) to \(Ca_{2.1}\) (Siri and Uchitel, 1999; Iwasaki et al., 2000). Fast excitatory synaptic transmission in the CNS usually is accomplished by \(Ca_{2.1}\) (Jun et al., 1999; Pagani et al., 2004; Lin et al., 2011). An increasing role for presynaptic \(Ca_{2.1}\) in postnatal development is underlined by the fact that \(Ca_{2.1}^{-/-}\) mice die at around 3 weeks of age, when P/Q currents are essential for motoneuron function, e.g., in the respiratory system (Jun et al., 1999).

Although our data represent somatic rather than presynaptic \(Ca_{2.1}\) currents, the prominent differences between N-type and P/Q-type currents of cultured \(\alpha_2\delta_3^{+/+}\) SG neurons at the two ages examined may suggest a similar developmental switch from the presynaptic \(Ca_{2.1}\) channels \(Ca_{2.2}\) at P5 to predominantly \(Ca_{2.1}\) at P20 for the endbulb of Held synapse. If this was the case, deletion of \(\alpha_2\delta_3\) would not only impact \(Ca_{2.1}\)-driven synaptic transmission in 3-week-old animals, but also \(Ca_{2.2}\)-driven synaptic transmission in the first postnatal week. A reduction of presynaptic N-type \(Ca_{2.1}\) currents in neonatal endbulb synapses may add to the impaired morphology and diminished sizes of endbulbs observed in \(\alpha_2\delta_3^{-/-}\) mice (Pirone et al., 2014) although \(Ca_{2.2}\)-independent functions of \(\alpha_2\delta_3\) in synapse formation and differentiation cannot be excluded.

Using a number of \textit{Drosophila} mutants, Kurshan et al. (2009) showed an indispensable role of the \(\alpha_2\delta_3\) ortholog \textit{straightjacket} for the development of the neuromuscular junction. This novel function was observed before the \(Ca_{2.1}\) channel ortholog \textit{cacophony} was expressed and thus was independent of the \(Ca_{2.2}\) channel function (Dickman et al., 2008; Ly et al., 2008; Kurshan et al., 2009). Notably, the \(\alpha_2\delta_3\) ortholog \textit{straightjacket} stabilizes the \(Ca_{2.1}\) channel ortholog \textit{cacophony} in \textit{Drosophila} (Ly et al., 2008). Because \(\alpha_2\delta_3\) proteins reside in the extracellular space and have protein–protein interaction domains, they may functionally interact with proteins of the extracellular matrix within the synaptic cleft or with proteins at the postsynapse. Thereby they might act as receptors for different factors involved in synaptogenesis such as thrombospondins (Resovi et al., 2014).

Of note, the extracellular matrix molecules thrombospondin I and II are involved in the innervation of cochlear inner hair cells by the peripheral dendrites of SG neurons in early cochlear development (Mendus et al., 2014). While \(\alpha_2\delta_1\) has been identified as thrombospondin receptor (Eroglu et al., 2009; Risher and Eroglu, 2012; Risher et al., 2018), interaction partners of \(\alpha_2\delta_3\) that mediate the developmental functions of thrombospondins remain to be established.

ETHICS STATEMENT

This study was carried out in agreement with the European Communities Council Directive (2010/63/EU) in accordance with the German law and the regional board for scientific animal experiments of the Saarland.

AUTHOR CONTRIBUTIONS

JE and FS conceived and designed the study. FS, VS, TE, KB, GO, and WW collected the data. FS, VS, GO, TE, KB, and JE analyzed and interpreted the data, and drafted the manuscript. FS, VS, GO, TE, KB, and JE carried out the experiments of the Saarland. We thank Jennifer Ihl, Kerstin Fischer, and Angela Di Turi for excellent technical assistance, Ebenezer Yamoah (University of Nevada, Reno, NV, United States) for instructing us to remain to be established.

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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.

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