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Different Ca_v1.3 Channel Isoforms Control Distinct Components of the Synaptic Vesicle Cycle in Auditory Inner Hair Cells

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The mechanisms orchestrating transient and sustained exocytosis in auditory inner hair cells (IHCs) remain largely unknown. These exocytotic responses are believed to mobilize sequentially a readily releasable pool of vesicles (RRP) underneath the synaptic ribbons and a slowly releasable pool of vesicles (SRP) at farther distance from them. They are both governed by Ca_v1.3 channels and require otoferlin as Ca²⁺ sensor, but whether they use the same Ca_v1.3 isoforms is still unknown. Using whole-cell patch-clamp recordings in posthearing mice, we show that only a proportion (~25%) of the total Ca²⁺ current in IHCs displaying fast inactivation and resistance to 20 μM nifedipine, a L-type Ca²⁺ channel blocker, is sufficient to trigger RRP but not SRP exocytosis. This Ca²⁺ current is likely conducted by short C-terminal isoforms of Ca_v1.3 channels, notably Ca_v1.3_{42A} and Ca_v1.3_{43S}, because their mRNA is highly expressed in wild-type IHCs but poorly expressed in *Otof*^{-/-} IHCs, the latter having Ca²⁺ currents with considerably reduced inactivation. Nifedipine-resistant RRP exocytosis was poorly affected by 5 mM intracellular EGTA, suggesting that the Ca_v1.3 short isoforms are closely associated with the release site at the synaptic ribbons. Conversely, our results suggest that Ca_v1.3 long isoforms, which carry ~75% of the total IHC Ca²⁺ current with slow inactivation and confer high sensitivity to nifedipine and to internal EGTA, are essentially involved in recruiting SRP vesicles. Intracellular Ca²⁺ imaging showed that Ca_v1.3 long isoforms support a deep intracellular diffusion of Ca²⁺.

Key words: auditory; calcium channels; hair cells; synaptic exocytosis

Significance Statement

Auditory inner hair cells (IHCs) encode sounds into nerve impulses through fast and indefatigable Ca²⁺-dependent exocytosis at their ribbon synapses. We show that this synaptic process involves long and short C-terminal isoforms of the Ca_v1.3 Ca²⁺ channel that differ in the kinetics of their Ca²⁺-dependent inactivation and their relative sensitivity to the L-type Ca²⁺ channel blocker nifedipine. The short C-terminal isoforms, having fast inactivation and low sensitivity to nifedipine, mainly control the fast fusion of the readily releasable pool (RRP); that is, they encode the phasic exocytotic component. The long isoforms, with slow inactivation and great sensitivity to nifedipine, mainly regulate the vesicular replenishment of the RRP; that is, the sustained or tonic exocytosis.

Introduction

The ribbon synapses of auditory inner hair cells (IHCs) encode critical information on sound timing and intensity into a specific

afferent discharge rate that shows fast onset, rapid adaptation, and a sustained pattern as long as the sound signal is maintained (Kiang, 1965). To perform this neural encoding faithfully, IHCs use two key elements at their ribbon active zones: Ca_v1.3 Ca²⁺ channels, which provide a fast and a focalized voltage-gated Ca²⁺ entry (Brandt et al., 2005; Frank et al., 2009; Vincent et al., 2014),

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and otoferlin, a large, six-C2 domain protein that acts as a high-affinity Ca²⁺ sensor that has been proposed to control vesicle fusion (Roux et al., 2006; Beurg et al., 2010). Exocytosis at this synapse displays a fast component reflecting the fusion of a readily releasable pool of vesicles (RRP) tethered to the ribbon and a slowly releasable pool (SRP) corresponding to the recruitment of distant vesicles (Moser and Beutner, 2000; Spassova et al., 2004; Johnson et al., 2005). Both exocytotic components are Ca²⁺ dependent and require the putative Ca²⁺ sensor otoferlin (Roux et al., 2006; Pangrsic et al., 2010; Levic et al., 2011; Vincent et al., 2014), but whether they involve similar Ca_v1.3 Ca²⁺-channels remains to be investigated.

In numerous tissues, the transcripts of Ca_v1.3 Ca²⁺ channels can undergo extensive alternative splicing resulting in protein isoforms, in particular in the C-terminal region underlying different gating properties (Bock et al., 2011; Huang et al., 2013). Notably, two Ca_v1.3 splice variants with early termination of the C terminus have been shown to yield to Ca_v1.3_{42A} and Ca_v1.3_{43S} isoforms. These short Ca_v1.3 isoforms Ca²⁺ channels are endowed with extremely fast inactivation and low sensitivity to dihydropyridine drugs (Huang et al., 2013). Interestingly, Ca_v1.3_{42A} mRNA transcripts have been detected in the mouse organ of Corti (OC) (Singh et al., 2008). Moreover, Ca_v1.3_{43S} mRNA transcripts have been found in the mouse IHCs (Scharinger et al., 2015), but their putative role in IHC exocytosis has not been investigated. In the present study, we used the different dihydropyridine sensitivity between Ca_v1.3 long and short isoforms to probe their specific role in IHC exocytosis. We also used the property of nifedipine of being switched instantaneously into an ineffective nitroso compound upon UV-flash illumination (Morad et al., 1983; Sanguinetti and Kass, 1984; Feldmeyer et al., 1995) to investigate the sequential role of long and short C-terminal isoforms of the Ca_v1.3 channels in RRP and SRP exocytosis. Furthermore, because Ca_v1.3 channels and otoferlin are thought to interact physically at the synapse (Ramakrishnan et al., 2009), the expression of these Ca_v1.3 isoforms was also investigated in mouse IHCs lacking otoferlin.

Materials and Methods

Tissue preparation

Experiments were performed in accordance with the guidelines of the Animal Care Committee of the European Communities Council Directive (86/609/EEC) and the ethics committee of the University of Bordeaux. All mice (C57BL/6 of either sex) were anesthetized by intraperitoneal injection of a xylazine (6 mg/ml) and ketamine (80 mg/ml) mixture (Sigma-Aldrich) diluted in physiological saline. Cochlear organs were dissected from littermate controls (*Otof*^{+/+}, *Otof*^{+/-}), here termed wild-type (WT), or from knock-out otoferlin C57BL/6 mice (*Otof*^{-/-}) as described previously (Vincent et al., 2014).

Electrophysiological recordings from IHCs were obtained in whole-mount OCs from mice at postnatal days 14–18 (P14–P18); that is, after the onset of hearing. The OC was freshly dissected under binocular magnification in an extracellular solution maintained at 4°C containing the following (in mM): NaCl 135, KCl 5.8, CaCl₂ 1.3, MgCl₂ 0.9, NaH₂PO₄ 0.7, glucose 5.6, Na pyruvate 2, and HEPES 10, pH 7.4, 305 mOsm. The tectorial membrane was carefully removed. The OC was placed in a recording chamber and the IHCs were observed with a 60× water-immersion objective [CFI Fluor 60× W near infrared (NIR), working distance (WD) = 2.0 mm, numerical aperture (NA) = 1; Nikon] attached to an upright Nikon FN1 microscope. The extracellular solution was complemented with 0.25 μM Apamin (Latoxan) and 0.2 μM XE-991 (Tocris Bioscience) to block SK channels and KCNQ4 channels, respectively. The external Ca²⁺ concentration was increased from 1.3 to 5 mM to enhance the amplitude of Ca²⁺ currents. All experiments were performed at room temperature (22–24°C).

Whole-cell recording and capacitance measurement

All IHC recordings were performed in the 20–40% normalized distance from the apex, an area coding for frequencies ranging from 8 to 16 kHz, using an EPC10 amplifier controlled by Patchmaster pulse software (HEKA Elektronik). Patch pipettes were pulled with a micropipette Puller P-97 Flaming/Brown (Sutter Instruments) and fire-polished with a Microforge MF-830 (Narishige) to obtain a resistance range from 3–5 MΩ. Patch pipettes were filled with a cesium-based intracellular solution containing the following (in mM): CsCl 145, MgCl₂ 1, HEPES 5, EGTA 1, TEA 20, ATP 2, and GTP 0.3, pH 7.2, 300 mOsm. In a few experiments, the concentration of the slow Ca²⁺ chelator EGTA was increased to 5 mM.

Ramp stimulation. Cells were held at –80 mV and depolarized from –90 mV to +30 mV in 120 ms giving a slope of voltage change of 1 mV/ms. IV curves were fitted with a Boltzmann function from –70 to –10 mV to obtain the half-maximal voltage activation potential ($V_{1/2}$) and the slope k using the following equation:

$$I_{(V)} = I_{\max} / (1 + e^{(V_{1/2} - V)/k})$$

Where I_{\max} is the maximal current.

Real-time capacitance measurement. Membrane capacitance (C_m) measurements were performed using Patchmaster Lock-in amplifier software (HEKA) as described previously (Vincent et al., 2014). A recruitment voltage depolarizing protocol was used to probe both the vesicular RRP fusion and the recruitment of SRP vesicles at the active zones. This protocol consisted in a train of 100 ms repetitive voltage steps from –80 mV to –10 mV, each stimulation being separated by a 100 ms interval.

Nifedipine experiments

Flash photoinactivation of nifedipine. We used the property of nifedipine to be inactivated by UV light (Morad et al., 1983; Feldmeyer et al., 1995) to restore the Ca²⁺ current instantaneously in IHCs. Ca²⁺ currents of IHCs were blocked by adding 20 μM the dihydropyridine blocker nifedipine to the external solution (Sigma-Aldrich, catalog #N7634). To allow efficient diffusion and blocking of Ca²⁺ currents by nifedipine, the OCs were always incubated for 5–10 min with the drug before starting to patch. Photoinactivation of nifedipine was achieved by applying brief UV flashes from a UV LED light source (Mic-LED 365, 128 mW; Prizmatix). The UV LED device was connected directly to the epi-illumination port at the rear of the upright Nikon FN1 microscope and illumination was focalized through the 60× objective (CFI Fluor 60X W NIR, WD = 2.0 mm, NA = 1; Nikon) and TTL triggered by the Patchmaster software.

Dose–response curve experiment. We incubated the OCs with different concentrations of extracellular nifedipine from 1 nM to 100 μM. All recordings started after 10–15 min of incubation with nifedipine. Ca²⁺ current amplitudes obtained at various nifedipine concentration were expressed as a percentage of the Ca²⁺ current recorded in the control bath (i.e., without nifedipine). Nifedipine was diluted in dimethyl sulfoxide (DMSO; Sigma-Aldrich, catalog #D8418). The final concentration of DMSO in the recording bath was always <1%. To be sure that DMSO did not affect the Ca²⁺ current, we performed recordings in the presence of 1% DMSO in the extracellular solution. Ca²⁺ current was similar with and without DMSO (data not shown). Data points were fitted with a sigmoidal Hill function as the following:

$$y = A_1 + (A_2 - A_1) x^n / (K^n + x^n)$$

where A_1 and A_2 are the minimum and the maximum Ca²⁺ current, respectively; x is the Ca²⁺ current at a given concentration of nifedipine; K is the Michaelis constant (i.e., IC₅₀), and n is the cooperative site.

Ca²⁺ imaging

Calcium imaging was performed from P14–P17 IHCs expressing the genetically encoded fast Ca²⁺ indicator GCaMP6f (Chen et al., 2013). These IHCs were obtained by injecting the adeno-associated virus AAV2/8 containing the encoding sequence of GCaMP6f through the round window of the cochlea of mice at age P3 *in vivo*. Whole mounts of the apical region of the OCs were dissected and mounted in the recording chamber as described above.

Image acquisition. As described by Vincent et al. (2014), changes in [Ca²⁺]_i were measured continuously with a C2 confocal system and

NIS-element imaging software (Nikon) coupled to the FN1 microscope. The dye was excited with a 488 nm solid-state laser (85-BCD-010-706; Melles Griot) and 500–530 nm emission recorded at 18 images/s.

Image analysis. Fluorescence emission was measured with ImageJ software by drawing a region of interest of 3.7 μm^2 (8 × 8 pixels) at the synaptic zones below the nucleus, at the region above the nucleus, and below the cuticular plate region (apical region). Emission fluorescent signals were analyzed and normalized by the ratio F/F_{min} ratio, where F_{min} was the minimum fluorescence found before starting stimulation.

Immunohistochemistry

Preparation of tissues. The ramps of the cochlear apparatus of P14–P16 *Otof*^{+/-} and *Otof*^{-/-} mice were dissected and prepared as described previously by Vincent et al. (2014). Briefly, they were rapidly perfused with 100% methanol at -20°C for 30 min and washed with cold PBS. The inner ear was then incubated for 2 h in PBS solution containing 10% EDTA. The OC was then dissected and the tectorial membrane removed. Tissue was first incubated with PBS containing 30% normal horse serum for 1 h at room temperature. Synaptic ribbons, Ca_v1.3 channels, or vesicular transporter 3 (VGLUT3) and otoferlin were simultaneously labeled with anti-CtBP2 (goat polyclonal; Santa Cruz Biotechnology, catalog #SC-5966), anti Ca_v1.3 (rabbit polyclonal, Alomone Labs, catalog #ACC-005), or anti-VGLUT3 (rabbit polyclonal; Synaptic Systems, catalog #135 203) and anti-otoferlin (mouse monoclonal; Abcam, catalog #ab53233) antibodies, respectively. Actin-F was also used to visualize hair cells (1:100, Phalloidin Fluoprobe 405; Interchim, catalog #FP-CA9870). Secondary antibodies were used at 1:500: first donkey anti-goat Fluoprobe 547H (Interchim, catalog #FP-SB2110) and donkey anti-mouse Fluoprobe 647 (Interchim, catalog #FP-SC4110) and then goat anti-rabbit Alexa Fluor 488 after a PBS rinse (Invitrogen, catalog #A-11008). The surface preparation of OCs (apical turns) was then mounted on Superfrost-Plus glass slides (Kindler) in a Prolong-Antifade medium (Invitrogen) and kept in the dark at -20°C until observation.

Image acquisition. Samples were analyzed using a confocal laser scanning microscope Leica SP8 with a 63× oil-immersion objective (NA = 1.4) and white light laser (470 to 670 nm) (Bordeaux Imaging Center). Phalloidin was imaged by using a diode laser at 405 nm also mounted on the microscope. Stack images were acquired with the same parameters as in our previous study (Vincent et al., 2014).

Western blot

The OCs from the apical cochlear turn of 11 (*Otof*^{-/-}) and 18 (*Otof*^{+/+}) mice (P12–P14) were microdissected and homogenized in a RIPA lysis and extraction buffer containing the following (in mM): Na-HEPES 25, EDTA 5, and DTT 1, along with 1% Triton X-100, 0.5% Na-deoxycholate, and 20% SDS, pH 7.2, containing a protease inhibitor mixture (Roche). The protein concentration was measured with a Bradford protein assay. For each genotype, equals amount of protein (~15 μg) were resolved on 8% SDS-PAGE and transferred onto a nitrocellulose membrane (Whatman Protran BA85). Membranes were incubated overnight at 4°C in the primary antibody solution against the target protein Ca_v1.3 (rabbit polyclonal antibody directed against the N-terminal region of the protein, amino acid residues 215–227, corresponding to the second extracellular loop, Alomone Labs, catalog #ACC-005; dilution 1/200) and anti-VGLUT3 (Synaptic Systems, catalog #135 203; dilution 1/500) as an internal reference. The Western blot membranes were then incubated in the HRP-conjugated secondary antibody solution (Bethyl Laboratories, catalog #A120–2018P) for 1 h at room temperature. For imaging and analysis, the blots were developed using ECL reagents (LI-COR, WesternSure Premium Chemiluminescent Substrate, catalog #926-95010) and read on a LI-COR C-Digit blot scanner. The Western blot images were analyzed using LI-COR Image Studio version 5.0 software. Western blots were repeated two times. To test for nonspecific staining, pre-absorption controls were performed. Primary Ca_v1.3 antibodies, pre-incubated with the antigenic peptide [Peptide (C)EQLTKETEGGNHS corresponding to amino acid residues 215–227 of rat Ca_v1.3] did not lead to any significant labeling of the blot membrane (data not shown).

Table 1. Sense and antisense primers used

cDNA	Name of primer	Primer sequence (5'–3')	Product size (bp)	
Myosin VIIa	6375s	CACCTGATGACTGGAAC	340	
	6715as	ATCATCCATTTGTATCCC		
Ca _v 1.3 (α_{1D})	Exons 42–46	5010s	AGATGACGACCCAGAAGACTCC	618
		5637as	GCTGCTGGGTACCTGTTGTA	
Exon 42	4664s	CAACCTGTTTCTTTGGTC	331	
	4995as	TATAGCCCGTCGGATTCTG		
Exon 42A	4946s	ATGCTTGAACGGATGCTTAG	200	
	5146as	CTTCTCCGGAGGAGTGC		
Exon 44	5010s	AGATGACGACCCAGAAGACTCC	440	
	5450as	CTAATGTAAGTCTCGTAATAGCGGGT		
$\Delta 44$	5010s	AGATGACGACCCAGAAGACTCC	452	
	5462as	CCTGACTCAGACCTTTAATACTG		

s, Sense; as, antisense.

RT-PCR

Isolation and precipitation of total RNA. Apical turns of 4 OCs from P35 *Otof*^{+/-} or P35 *Otof*^{-/-} mice were freshly dissected and stored in separate low-DNA-binding tubes containing 15 μl of RNase inhibitor (Amresco, catalog #E633) maintained in dry ice. Total RNA was extracted from tissues using the RNAzol protocol (Molecular Research Center, catalog #RN 190).

RT-mRNA (0.5 μg) was reverse transcribed to cDNA by using oligodT primers and AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene, catalog #600107). RT was performed at 37°C overnight.

PCR. Specific PCR amplification was performed by using 0.45 μg of cDNA and Herculase II Fusion DNA Polymerase (Stratagene, catalog #600677). Sense and antisense primers (Eurofins Genomics) are summarized in Table 1.

RT-PCR (pooled IHCs). Apical turns from P18 mice were placed in the same recording chamber as that used for patch-clamp recording. Using a large patch pipette ($R \leq 1.5 \text{ M}\Omega$), we collected 6–10 IHCs and broke the tip of the patch-pipette in a low-DNA-binding tube resting on dry ice and containing 15 μl of RNase inhibitor. RT-PCR from IHCs was performed as aforementioned for the whole OC.

Real-time semiquantitative PCR (Q-PCR)

We performed Q-PCR from 0.45 μg of cDNAs obtained from apical turns of OC as described above. PCR amplification was stopped at cycles 30, 31, 32, 33, 34, and 36 and analyzed by plotting fluorescence intensity. Data points were fitted using a sigmoidal function. Intensity was normalized to noise level. We used the cycle threshold parameter (Ct) to compare the amplification of each isoform. Ct was determined by tracing a line at the lowest intensity corresponding to the exponential phase of the sigmoidal curve (i.e., between the start of the signal increase and the start of the linear phase of the sigmoidal curve). The Ct parameter was the intersection point between this line and the sigmoidal curve.

All PCR products obtained from OCs and pooled IHCs were cloned and sequenced.

Statistical analysis

Electrophysiological results were analyzed with Patchmaster (HEKA Elektronik), OriginPRO 9.1 (OriginLab), and IgorPro 6.3 (Wavemetrics) software. Results are expressed as mean \pm SEM. Statistical analyses were performed with Student's *t* test. The limit of significance was set at $p < 0.05$.

Results

Nifedipine-resistant, fast-inactivating, Ca²⁺ current

The addition of 20 μM nifedipine to the extracellular medium, a concentration well above the submicromolar range IC₅₀ of full-length Ca_v1.3 channels (Huang et al., 2013) and the IC₅₀ of 69 nM that we have determined in a dose–response curve (see Fig. 2D, E) reduced the Ca²⁺ current amplitude in IHCs from posthearing mice (P14–P18) by nearly 75% from a mean peak value of

–184 ± 19 pA in the absence of nifedipine (control condition, black, $n = 8$) to a mean value of –46 ± 5 pA at –10 mV (nifedipine, green, $n = 17$; $p < 0.05$; Fig. 1A). Note that, even at 100 μM nifedipine, nearly 25% of the Ca²⁺ current remained unblocked (Fig. 2E). A similar effect of nifedipine on Ca²⁺ current has been found in rat cardiomyocytes, in which the IC₅₀ is ~100 nM and 20% of the Ca²⁺ current is resistant to nifedipine (Pignier and Potreau, 2000). This nifedipine-resistant current (Fig. 1A, inset) showed a similar voltage dependence compared with the total current in the control condition. Indeed, when fitting the IV curves from –70 mV to –10 mV with a sigmoidal Boltzmann function, there was no significant difference in the $V_{1/2}$ (–27.9 ± 0.9 mV and –26.9 ± 0.8 mV, nifedipine and control condition, respectively; $p = 0.45$) or k (5.9 ± 0.4 pA/mV and 5.7 ± 0.2 pA/mV, nifedipine and control condition, respectively; $p = 0.69$).

To determine the kinetics of the Ca²⁺ current reactivation when UV-inactivating nifedipine, IHCs were UV flashed 20 ms after the Ca²⁺ current onset evoked by a 100 ms step depolarization from –80 mV to –10 mV (Fig. 2A,B). UV-flash photolysis instantaneously inactivated nifedipine and unblocked Ca²⁺ channels, leading to an exponential increase of Ca²⁺ current with a mean time constant of 8.4 ± 0.7 ms (Figs. 1A,D, 2A–C). The reactivated Ca²⁺ current showed a nearly 2.5-fold increase in amplitude from its blocked state, rising from a peak value of –46 ± 5 pA in the presence of nifedipine to –108 ± 10 pA after the UV flash ($n = 10$; $p < 0.05$; Fig. 1A, purple trace). In our experimental conditions, IHCs were flashed in a nifedipine-containing extracellular solution. We believe that the UV flash, which is condensed through the 60× objective of the microscope, only inactivated nifedipine within few tens of micrometers around the IHCs. The uncomplete recovery of the Ca²⁺ current after the flash can be explained by a rapid diffusion and block by residual active nifedipine.

Comparison of the voltage-dependent parameters of the Ca²⁺ current before and after UV flash photolysis of nifedipine showed no significant changes either in $V_{1/2}$ (–27.9 ± 0.9 mV and –28.5 ± 1.5 mV, respectively, $p = 0.73$) or in the Boltzmann slope (k : 5.9 ± 0.4 pA/mV and 6.3 ± 0.2 pA/mV, respectively; $p = 0.42$; Fig. 1A, inset). The maximum reactivated Ca²⁺ currents reached nearly 60% of control values in the absence of nifedipine.

Control condition Ca²⁺ currents evoked during a 100 ms voltage step from –80 mV to –10 mV displayed a partial fast inactivation, corresponding to a reduction of 17.6 ± 2.2% ($n = 7$) of initial current at the end of the pulse (Fig. 1C, first pulse). A similar fast-inactivating component of Ca²⁺ currents was described previously in rat IHCs (Grant and Fuchs, 2008), turtle hair cells (Schnee and Ricci, 2003), and frog hair cells (Cho et al., 2014). This fast inactivation of Ca²⁺ channels was essentially due to Ca²⁺-dependent inactivation because it was largely reduced when Ba²⁺ ions replace Ca²⁺ ions as the charge carrier (Fig. 3), as described previously (Platzer et al., 2000; Michna et al., 2003). The inactivated current amplitude (obtained by subtracting the current at the first pulse from the second pulse) in the presence of Ca²⁺ was 39.8 ± 8.1 pA versus 6.7 ± 2.4 pA in the presence of Ba²⁺ (Fig. 3C; $p < 0.05$).

In the presence of nifedipine, Ca²⁺ currents showed a similar fast inactivation with an amplitude corresponding to 54.6 ± 3.7% ($n = 8$) of the initial current (Fig. 1D, first pulse). The kinetics and amplitude of the fast-inactivating component of the Ca²⁺ current in both control and nifedipine conditions were obtained by subtracting current values between two consecutive 100 ms pulses (pulse 1 – pulse 2) separated by 100 ms (Fig. 1F).

The fast-inactivating components in control and nifedipine conditions had a maximum amplitude of 40 ± 8 pA ($n = 7$) and 20 ± 3 pA ($n = 8$), respectively ($p < 0.05$). They showed similar fast-inactivating kinetics with a time constant of 3.8 ± 1.3 ms and 4.6 ± 0.9 ms in control and nifedipine conditions, respectively ($p = 0.57$). The inactivation kinetics of the Ca²⁺ current that we measured here could even be faster at physiological temperature, as shown previously by Grant and Fuchs (2008). Indeed, the biophysical properties of the ion channels, as well as exocytosis and vesicular recruitment, are known to be facilitated at body temperature (Kushmerick et al., 2006; Nouvian, 2007). Interestingly, our results showed that the fast-inactivating component of Ca²⁺ currents in IHCs is only poorly affected by nifedipine.

The time course of recovery of the Ca²⁺ current from inactivation was determined by using a paired-pulse protocol (each pulse of 50 ms duration from –80 mV to –10 mV) with different interpulse durations (Fig. 3D,E). The relationship between the amplitude of the Ca²⁺ current at the second pulse (normalized to the first pulse) and the interpulse interval could be best fitted with a single exponential function with $\tau = 33.7 ± 2.3$ ms ($n = 8$; Fig. 3E). The recovery of the fast-inactivating component of the Ca²⁺ current, obtained by normalizing the inactivation time constant of I_{Ca} at the second pulse to the one obtained at the first pulse, indicated a similar time constant with $\tau = 41.9 ± 9.3$ ms ($n = 8$; Fig. 3E). It is interesting that the time course of recovery of the Ca²⁺ current determined in the present study is in the range of the recovery time constant of sound-evoked short-term adaptation of the auditory nerve discharge rate (40–60 ms, Westerman and Smith, 1984; Spassova et al., 2004; Cho et al., 2011) but somewhat faster than the time constant of RRP recovery from depletion measured in IHCs (~140 ms; Moser and Beutner, 2000). These results suggest that the fast inactivation of the Ca²⁺ current could contribute to the short-term adaptation of transmitter release at the auditory ribbon synapses.

Reduced fast inactivation of Ca²⁺ current in IHCs lacking otoferlin

Inactivation of Ca²⁺ current was investigated in IHCs from P14–P18 otoferlin-deficient mice (*Otof*^{–/–}). We recall that otoferlin is thought to be the major Ca²⁺ sensor for IHC exocytosis (Roux et al., 2006; Beurg et al., 2010). This multi-C2 protein is believed to interact with the Ca_v1.3 II–III loop (Ramakrishnan et al., 2009). As described previously (Roux et al., 2006), we found that *Otof*^{–/–} IHCs displayed Ca²⁺ current peak amplitudes similar to WT (–164 ± 7 pA, $n = 9$ and –184 ± 19 pA, $n = 8$, respectively; $p = 0.33$; Fig. 1B). The sigmoidal Boltzmann fit of the IV curve did not display any significant differences either in $V_{1/2}$ (–27.0 ± 0.7 mV and –26.9 ± 0.8 mV, *Otof*^{–/–} and WT, respectively; $p = 0.93$) or in the slope factor k (6.3 ± 0.3 pA/mV and 5.7 ± 0.2 pA/mV, *Otof*^{–/–} and WT, respectively; $p = 0.1$; Fig. 1B, inset). However, Ca²⁺ currents of *Otof*^{–/–} IHCs displayed a largely reduced fast-inactivating component (Fig. 1E,G). Indeed, a 100 ms paired-pulse voltage-step protocol (pulse 1 to pulse 2) indicated a large reduction of the fast-inactivating component in *Otof*^{–/–} IHCs compared with WT (15.0 ± 2.2 pA and 40 ± 8 pA, respectively, $p < 0.05$; Fig. 1E,G). The residual inactivating component in *Otof*^{–/–} IHCs had similar kinetics to WT (4.1 ± 0.9 ms and 3.8 ± 1.3 ms, respectively, $p = 0.85$; Fig. 1E,G). These results suggest a reduced expression of fast-inactivating Ca²⁺ channels in IHCs lacking otoferlin.

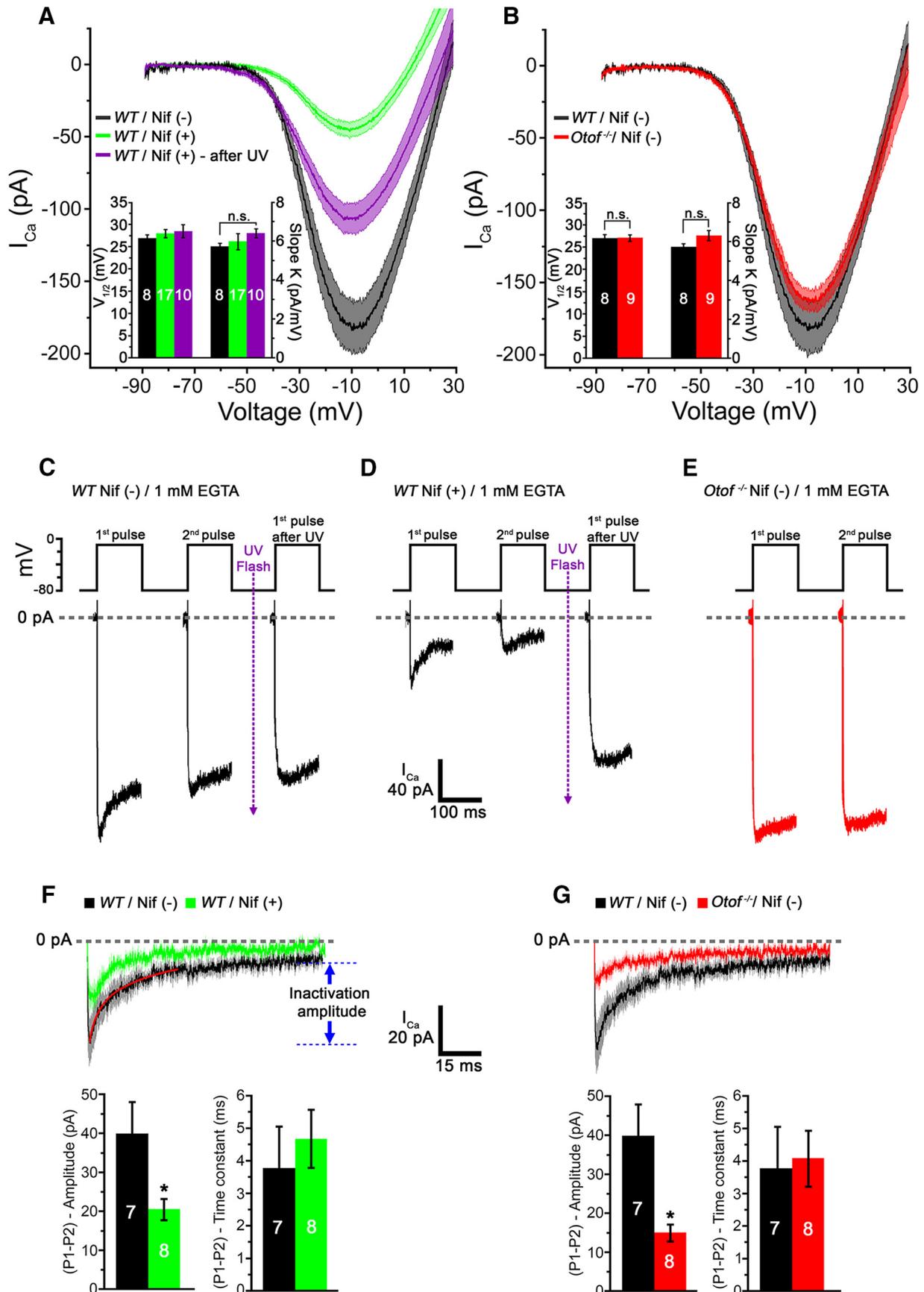


Figure 1. Nifedipine-resistant Ca²⁺ current in mature IHCs. **A**, IV-ramp activation curve of Ca²⁺ currents from -90 mV to +30 mV in WT mice without nifedipine (control condition, black), with 20 μ M nifedipine (green), and after the UV flash inactivation of nifedipine (purple). Inset shows the Boltzmann parameters ($V_{1/2}$ and k) obtained by fitting the IV curve from -70 mV to -10 mV with a Boltzmann sigmoidal function in the three different conditions. **B**, Same protocol as in **A** for comparison between WT (black, same trace as in **A**) and (*Figure legend continues*.)

Nifedipine-resistant Ca²⁺ currents trigger efficient fast RRP exocytosis

Surprisingly, although showing a large reduction in Ca²⁺ current in the presence of 20 μM nifedipine, WT P14–P18 IHCs displayed exocytotic amplitudes similar to control condition (20.15 ± 4.64 fF and 23.16 ± 2.53 fF, respectively, *p* = 0.64; Fig. 4A, B, D) when tested by 100 ms voltage steps from –80 to –10 mV. This indicated an apparent higher Ca²⁺ efficiency for RRP exocytosis in the presence of nifedipine (0.47 ± 0.08 fF/pA) compared with control IHCs in the absence of nifedipine (0.1 ± 0.02 fF/pA; *p* < 0.001). However, exocytosis in nifedipine-treated IHCs displayed larger paired-pulse depression (pulse 2/pulse 1 ratio) compared with controls (75 ± 5% and 32 ± 16%, respectively; *p* < 0.05; Fig. 4A, D, E). Increasing the concentration of intracellular EGTA from 1 to 5 mM in the presence of nifedipine only partially reduced the first exocytotic response to 13.5 ± 1.9 fF compared with 20.15 ± 4.64 fF, whereas the paired-pulse protocol showed similar depression to the nifedipine condition (59 ± 7.6% and 75 ± 5%, respectively; Fig. 4C, D; data in blue, *p* = 0.08).

Otof^{–/–} IHCs showed a first exocytotic response reduced to 10.58 ± 2.34 fF (*p* < 0.05) and a larger 77% paired-pulse depression compared with WT IHCs (Fig. 4D, E, data in red).

We also compared the Ca²⁺ efficiency of exocytosis obtained with a 100 ms step depolarization from –80 mV to +5 mV, in 10 mV increments, in the presence of nifedipine (green) and after UV inactivation of nifedipine in the same WT IHCs (Fig. 4F, G, purple). Again, we found that exocytosis showed a higher Ca²⁺ efficiency in the presence of nifedipine compared with responses obtained after UV-unblocking Ca²⁺ channels (0.25 ± 0.01 fF/pA and 0.16 ± 0.01 fF/pA before and after UV flash, respectively, *p* < 0.05). These results can be explained by the fact that the Ca²⁺ current amplitude increased after the flash but not exocytosis. Because the efficiency is the ratio of ΔC_m/I_{Ca}, the efficiency of exocytosis appears decreased after the UV flash. These results suggest that the IHC RRP release essentially depends on nifedipine-resistant Ca²⁺ currents.

Nifedipine severely impairs the sustained exocytotic component of the IHCs

A protocol of vesicle recruitment was tested by applying a train of 100 ms step depolarization from –80 mV to –10 mV with a 100 ms time interval in control and nifedipine-treated P14–P18 WT IHCs (Fig. 5A–C). To unblock Ca²⁺ channels, a UV flash was applied during the train of stimulation. In control WT IHCs, exocytosis first showed a slight depression after the second pulse and then a sustained linear increase that was not affected by the UV flash (Fig. 5A, C, black lines). In nifedipine-treated WT IHCs,

exocytosis displayed a strong depression after the second pulse and the exocytotic response was restored only after a long latency ranging from 700 to 800 ms after the UV flash, although the Ca²⁺ current was instantaneously unblocked (Fig. 5B, C, green lines). In WT IHCs loaded with 5 mM intracellular EGTA in the presence of nifedipine, exocytosis showed a constant profound depression with no recovery after UV-unblocking Ca²⁺ current (Fig. 5C, blue lines). Provided that endocytosis is not enhanced in presence of nifedipine, a reasonable assumption because it is considered a slow, calcium-dependent process (Moser and Beutner, 2000; Cho et al., 2011), the present results suggest that the noninactivating nifedipine-sensitive Ca²⁺ currents regulate vesicular recruitment, possibly by allowing a deep intracellular diffusion of Ca²⁺ ions. We also observed similar sustained depression in *Otof*^{–/–} IHCs (Fig. 5D, red lines), confirming the important role of otoferlin in the recruitment process (Pangrsic et al., 2010).

Imaging Ca²⁺ influx from nifedipine-resistant Ca²⁺ channels

Simultaneous Ca²⁺ imaging and whole-cell patch-clamp recordings were performed in P14–P18 WT IHCs expressing the genetically encoded fast Ca²⁺ indicator GCaMP6f (Chen et al., 2013). These experiments were performed in the presence or in the absence of 20 μM nifedipine during a train of 100 ms step depolarization (Fig. 6). In control condition, the recruitment protocol led to a parallel increase in intracellular Ca²⁺ (Fig. 6C) and membrane capacitance (Fig. 6A). Intracellular Ca²⁺ instantaneously increased at the synaptic zones in two phases (Fig. 6C, black trace): a first phase (Ph1) that mirrored the fast-inactivating Ca²⁺ currents and a second slow component (Ph2) that corresponded to the sustained Ca²⁺ currents (Fig. 6E, G). A rise in intracellular Ca²⁺ was also recorded at extrasynaptic sites around and above the nucleus, but with a long delay of several hundred milliseconds and a much smaller amplitude compared with that of the synaptic zones (Fig. 6C, G, H'; orange trace). These results suggested a diffusion of Ca²⁺ from the synaptic zones, where Ca²⁺ channels are concentrated. Remarkably, the Ca²⁺ rise in this supranuclear region (Fig. 6C, orange trace) perfectly mirrored the sigmoidal increase in cumulative exocytosis during the train protocol (Fig. 6A). These results indicated that a deep intracellular diffusion of Ca²⁺ is associated with vesicular recruitment.

In nifedipine condition, at the synaptic zones, Ca²⁺ responses of Ph1 showed similar kinetics compared with the control condition (4.57 ± 0.9 ΔF/s (*n* = 5) and 4.75 ± 1.2 ΔF/s (*n* = 5), respectively; *p* = 0.9), but Ph2 was largely diminished (Fig. 6D, F, H). At the supranuclear zone of the cells, the Ca²⁺ responses were completely suppressed by nifedipine (Fig. 6D, F, H'). These results suggest that most of the fast-inactivating nifedipine-resistant Ca²⁺ channels are located at the basal synaptic active zones. UV photolysis of nifedipine instantaneously increased the Ca²⁺ current amplitude (Fig. 6B) and Ca²⁺ signals (Fig. 6D, H, Ph3). As also shown in Figure 5, B and C, the recovery of the exocytotic response displayed a long latency of several hundred milliseconds after the UV flash. Because nifedipine does not affect the intracellular exocytotic machinery directly, as indicated by the conserved first exocytotic jump (Fig. 4B, D), our results suggest that the delayed exocytotic recovery after unblocking Ca²⁺ channels is due to the time of intracellular Ca²⁺ diffusion and/or recruitment of vesicular stores located far away from the synaptic sites of release.

Remarkably, fast Ca²⁺ responses were also recorded at the subcuticular zone of WT IHCs in control condition during voltage step depolarization (Fig. 7A–C, light blue trace; *n* = 7). These Ca²⁺ responses peaked with no apparent delay with the Ca²⁺

←

(Figure legend continued.) otoferlin-deficient mice (*Otof*^{–/–}, red). Inset shows the Boltzmann parameters (*V*_{1/2} and *k*) for WT and *Otof*^{–/–}. C–E, Example of Ca²⁺ current traces in response to a paired pulse of 100 ms depolarization from –80 mV (resting potential) to –10 mV. For control condition (C) and in the presence of 20 μM nifedipine (D), we applied a third 100 ms stimulation after the UV flash to test the UV unblocking of Ca²⁺ channels. F, G, Top traces show the inactivating Ca²⁺ currents (mean ± SEM) obtained after subtracting the second pulse response from the first. Histograms show the amplitudes and inactivation-time constants of the subtracted Ca²⁺ currents obtained in different conditions: control condition (black), with nifedipine (green), and in *Otof*^{–/–} without nifedipine (red). Amplitude of inactivation was measured between the Ca²⁺ peak and the steady state of inactivation (blue dashed lines in F). The fast-inactivating time constant was determined by fitting the current decrease with an exponential function (red line in F). Error bars indicate SEM. Nif (–), condition without nifedipine; Nif (+), condition with 20 μM nifedipine. **p* < 0.05 in A, B, F, and G. The number of cells is indicated for each condition in the histograms.

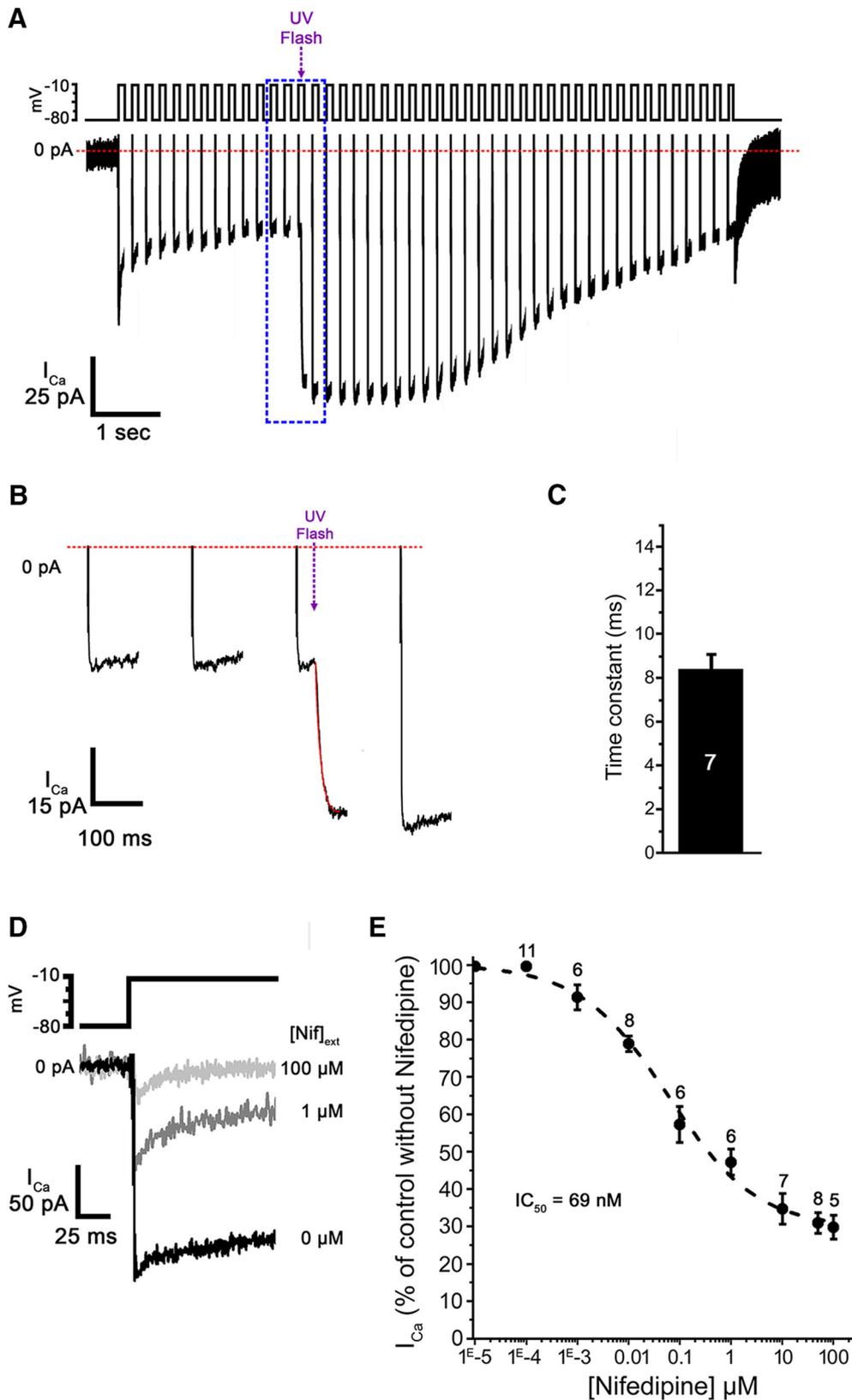


Figure 2. Fast UV reactivation of Ca²⁺ currents in the presence of extracellular nifedipine. **A**, Example of Ca²⁺ current trace recorded in response to a train of 100 ms stimulation from -80 to -10 mV. The purple arrow indicates the UV flash reactivation of the Ca²⁺ current. **B**, Magnification from blue dashed box in **A**. We used a single exponential function (red line) to fit the kinetics of unblocking Ca²⁺ current. Mean kinetics time constants are plotted in **C**. **D**, Examples of Ca²⁺ current recorded in response to a 100 ms depolarization from -80 mV to -10 mV in the presence of 0 μ M (black trace), 1 μ M (dark gray trace), and 100 μ M (light gray trace) extracellular nifedipine. **E**, Dose-inhibitory response curve of nifedipine on the amplitude of IHC Ca²⁺ currents. Data points were fitted with a sigmoidal Hill function with an IC₅₀ of 69 nM and $n_{Hill} = 1$. The cell number is indicated above each data point.

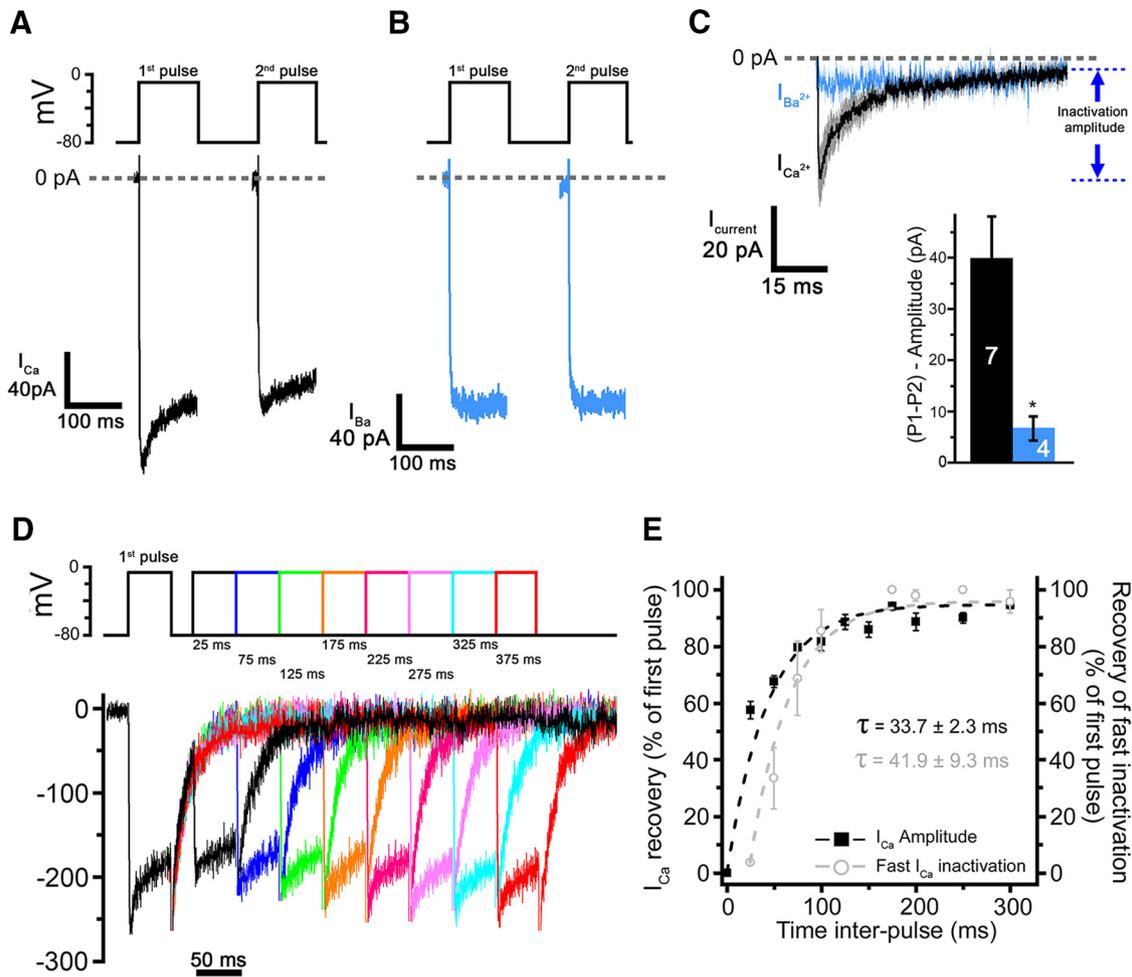


Figure 3. Absence of paired-pulse inactivation of Ba²⁺ currents. **A**, Paired-pulse protocol of Ca²⁺ currents as in Figure 1. **B**, Paired-pulse protocol of Ba²⁺ currents when extracellular Ca²⁺ ions were replaced by 5 mM Ba²⁺. **C**, Comparative averaged subtracted traces (pulse 1 – pulse 2). **D**, Ca²⁺ current recordings in response to a paired-pulse protocol (two pulses of 50 ms from –80 to –10 mV) with increasing interpulse durations. The protocol is represented at the top. Each color represents one given interpulse duration. **E**, Time recovery of the I_{Ca} amplitude (black squares) and the fast-inactivating component (light gray circles) of the Ca²⁺ current (n = 8 cells). The I_{Ca} amplitude was measured between the baseline and the maximum peak. The fast-inactivating component was determined with a single exponential from the maximum peak to the steady state of the inactivation. Data point were fitted with a single exponential (dotted lines).

synaptic responses despite being positioned >20 μm away from the synaptic zones, indicating that they were not due to Ca²⁺ diffusion or intracellular release, but rather from Ca²⁺ entry through Ca²⁺ channels. Reinforcing this hypothesis, our confocal immunohistochemistry experiments showed that small Ca_v1.3 clusters can indeed be detected below the cuticular area, whereas most Ca²⁺ channels are clustered at the basal synaptic ribbons (Fig. 7D). Conversely, in the subcuticular zones, the Ca²⁺ responses measured near the middle of the cell above the nucleus (orange trace) at ~9 μm away from the synaptic base showed a large delay (Δt) in their peak responses: 191 ± 19 ms (n = 7) compared with the synaptic responses (Fig. 7B,C). These Ca²⁺ responses above the nucleus were likely due to Ca²⁺ diffusion from the synaptic zones and the subcuticular area and/or to intracellular store release.

Expression of short and long Ca_v1.3 channel isoforms in IHCs
 Transcripts of the Ca_v1.3 long isoform (Ca_v1.3_{42L}) were detected in mature OCs of P20–P35 mice (Fig. 8A,B). Two short alternative splicing isoforms, 43S (Ca_v1.3_{43S}) and 42A (Ca_v1.3_{42A}), were also identified. RT-PCR of transcripts collected directly from P18 WT IHCs with a patch pipette also showed the expression of these long and short isoforms of Ca_v1.3 at the cellular level (Fig. 8E). Q-PCR showed that OCs from P20–P35 *Otof*^{-/-} mice had a

reduced transcript expression of Ca_v1.3_{43S} and Ca_v1.3_{42A} short isoforms compared with P20–P35 WT mice (Fig. 8A,C,D), whereas the long isoform Ca_v1.3_{42L} did not show any significant change (data not shown). The reduced expression of Ca_v1.3_{43S} and Ca_v1.3_{42A} short isoforms in the OC of *Otof*^{-/-} mice was confirmed at the protein level by Western blot analysis (Fig. 8F,G). The protein expression of Ca_v1.3_{43S} (187 kDa) and Ca_v1.3_{42A} (176 kDa), normalized to the vesicular glutamate transporter VGLUT3, was reduced by 21% and 24%, respectively, in the OC of *Otof*^{-/-} mice compared with *Otof*^{+/+}. *Otof*^{-/-} IHCs did not show any apparent lack or accumulation of synaptic vesicles (Roux et al., 2006) and VGLUT3 expression remained similar to *Otof*^{+/+} IHCs (Fig. 9A). Along with the reduced inactivation of Ca²⁺ current in *Otof*^{-/-} IHCs (Fig. 1E,G), these results suggest that Ca_v1.3_{43S} and Ca_v1.3_{42A} likely underlie the fast-inactivating and nifedipine-resistant currents described in WT IHCs (Fig. 1F). Moreover, we also found transcript coding the long Ca_v1.3 isoform deleted for the exon 44 (Ca_v1.3_{Δ44}; data not shown) in P18 WT IHCs. This long Ca_v1.3 isoform was characterized by a fast inactivating rate, but slower than the two short isoforms (Tan et al., 2011).

Mature *Otof*^{-/-} IHCs displayed a nearly 2-fold decrease in the number of ribbons per IHC (19 ± 1 ribbons per cell and 9 ±

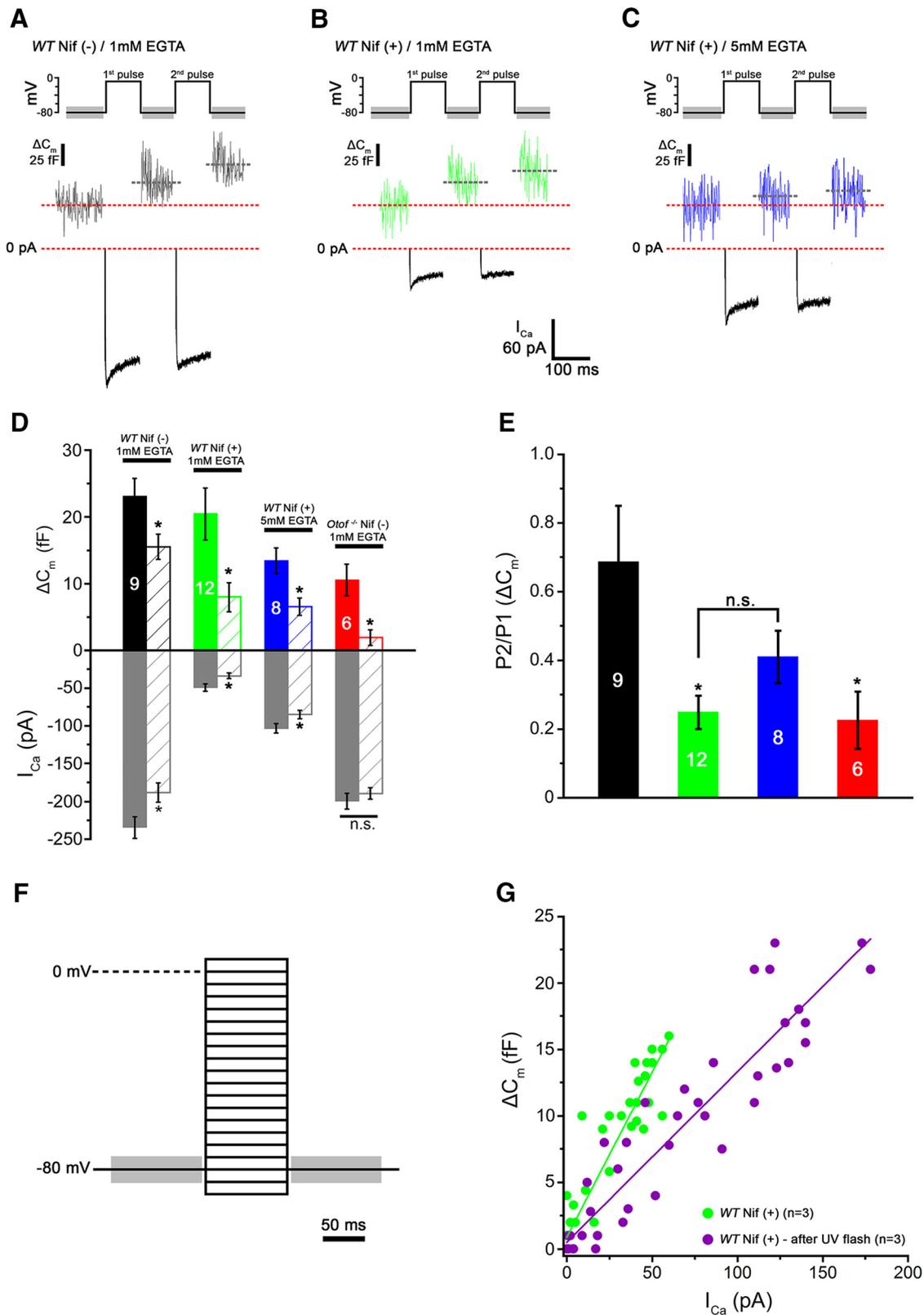


Figure 4. RRP exocytosis is weakly affected by nifedipine. **A–C**, Examples of capacitance response (ΔC_m , top) and Ca²⁺ currents (bottom) recorded in response to a 100 ms paired-pulse stimulation: control condition (**A**), condition with 20 μ M extracellular nifedipine (**B**), and condition with 5 mM intracellular EGTA and 20 μ M extracellular nifedipine (**C**). **D**, Comparative histograms of ΔC_m and Ca²⁺ currents recorded in control condition (black), with nifedipine (green), with nifedipine and 5 mM EGTA (blue), and in *Otof*^{-/-} without nifedipine (red). Filled histograms show data from the first pulse (P1, ΔC_{m1}) and hatched histograms from the second pulse (P2, ΔC_{m2}). ΔC_{m1} was calculated as the difference between responses at P1 and baseline (red dotted line). ΔC_{m2} was calculated as the difference between responses at P2 and P1. **E**, Comparative histograms of ΔC_m P2/P1 ratio. **F**, **G**, Ca²⁺ efficiency of RRP exocytosis in response to various voltage steps (100 ms duration, from -80 mV to +5 mV in 5 mV increments; **F**) in the presence of 20 μ M nifedipine before (green) and after UV-unblocking Ca²⁺ currents (purple) in WT IHCs. Error bars indicate SEM. Nif (-) indicates condition without nifedipine and Nif (+) condition with 20 μ M nifedipine. **p* < 0.05.

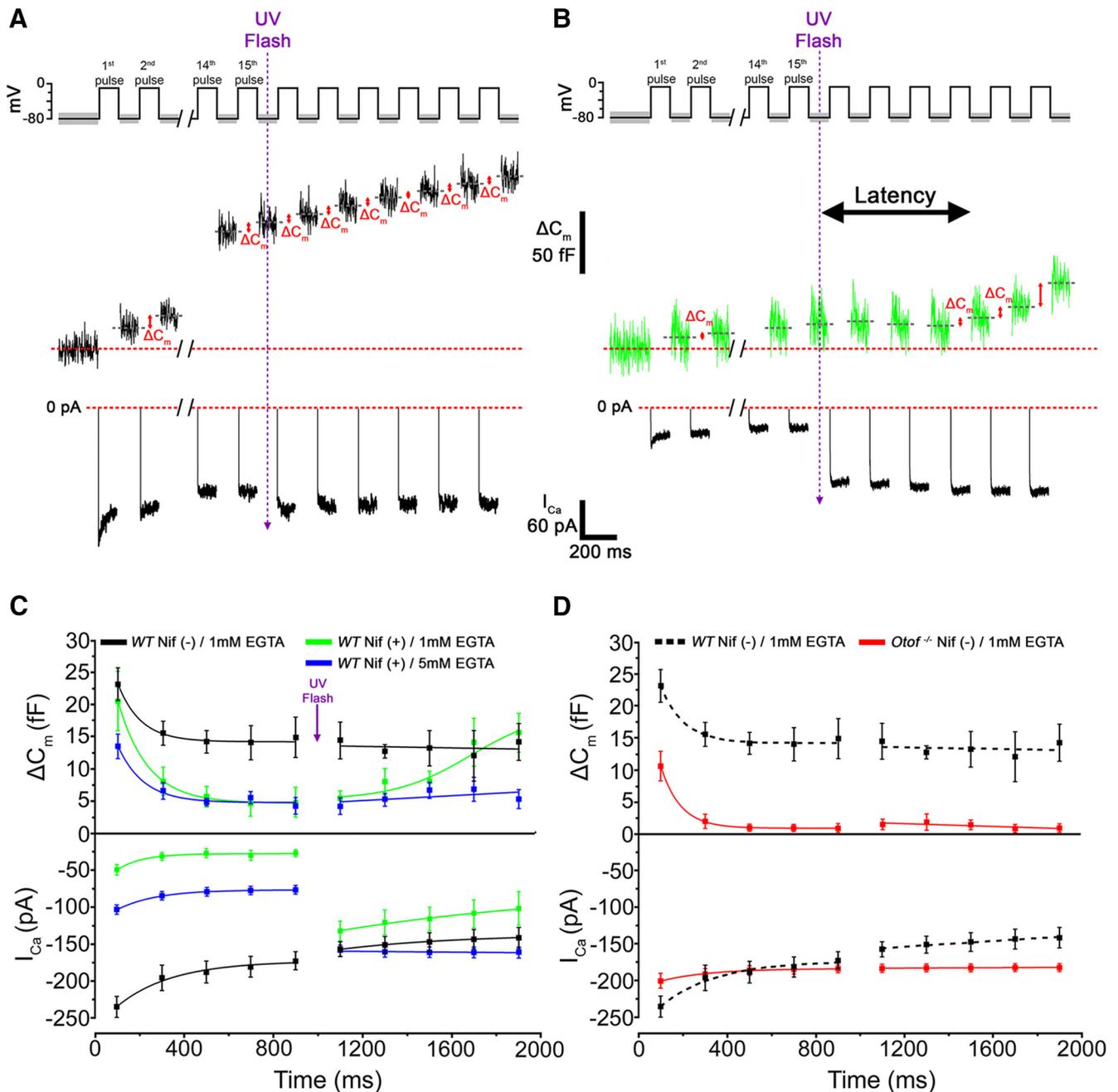


Figure 5. Nifedipine greatly reduces sustained exocytosis. Examples of comparative changes in ΔC_m and Ca^{2+} currents in control condition (**A**) and in the presence of 20 μM extracellular nifedipine (**B**) when applying a train of 100 ms stimulations. Dashed purple arrows indicate the illumination with UV. **C**, Plot of the sequential, 5 first, mean ΔC_m and Ca^{2+} current responses before and after the UV flash: control condition (black), condition with 20 μM nifedipine (green), condition with EGTA (5 mM) and nifedipine (blue). **D**, Similar comparative plot in control condition for WT IHCs (black dashed line) and *Otof*^{-/-} IHCs (red). Note the latency of the ΔC_m response after UV-unblocking Ca^{2+} channels in the presence of nifedipine (**B**, **C**). Error bars indicate SEM.

2 ribbons in P16-*Otof*^{+/-} and P16-*Otof*^{-/-} IHC, respectively; $p < 0.05$; Fig. 8*F, G*). The mean surface area of the ribbons was also decreased in *Otof*^{-/-} IHCs (Fig. 8*H*; $4.6 \pm 0.1 \mu m^2$ and $3.3 \pm 0.1 \mu m^2$, *Otof*^{+/-} and *Otof*^{-/-}; $p < 0.05$). These results suggest that otoferlin is required for both the expression of the short Ca_v1.3 isoforms and the organization of normal ribbons in IHCs.

Discussion

IHCs use Ca_v1.3 long and short isoforms for fine control of synaptic transmission

Here, we show that posthearing IHCs express a fast-inactivating nifedipine-resistant Ca^{2+} current that represents 25% of the total

Ca^{2+} current. The $V_{1/2}$ of this current was established at ~ -28 mV, thus excluding the implication of T-type Ca^{2+} channels that are characterized by a higher negative activation range (Carbone et al., 2014). T-type channels are believed to be expressed only in prehearing immature hair cells (Levic et al., 2007; Levic and Dulon, 2012). Moreover, the absence of fast inactivation in the presence of Ba²⁺ also argues against the implication of this Ca^{2+} channel family. Indeed, T-type Ca^{2+} channels are known to display a strong and fast inactivation even after Ba²⁺ replaces Ca^{2+} (Lacinova, 2005). A transient proton-mediated block of Ca^{2+} channels has been described during exocytosis in auditory

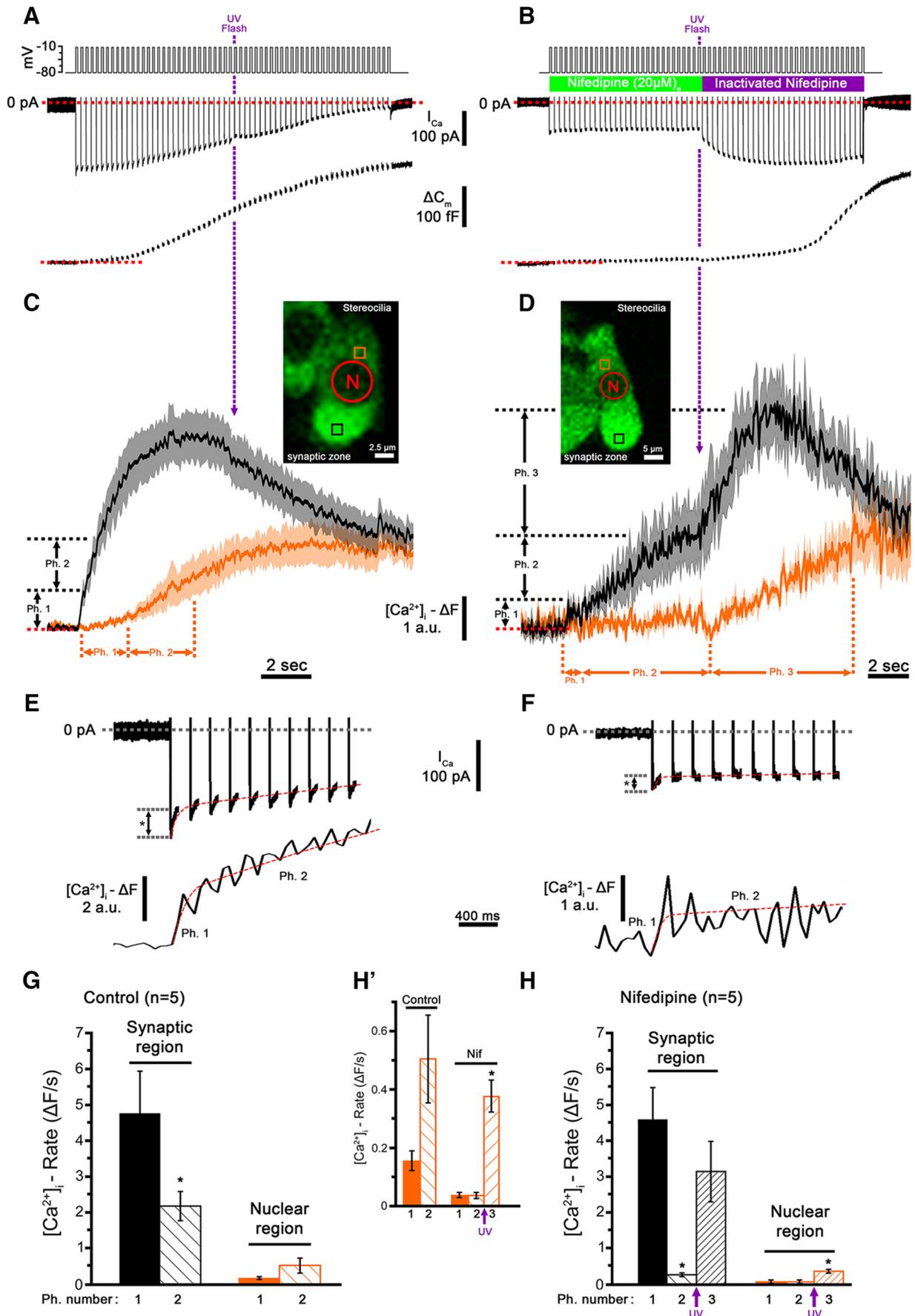


Figure 6. Ca²⁺ imaging during a train of stimuli reveals a fast synaptic response and a delayed, slowly developing extrasynaptic response in the region right above the nucleus. **A, B**, Examples of simultaneous recording of Ca²⁺ current (top) and capacitance response (bottom) in control condition (**A**) and in the presence of 20 μ M nifedipine (**B**) for WT IHCs during a (Figure legend continues.)

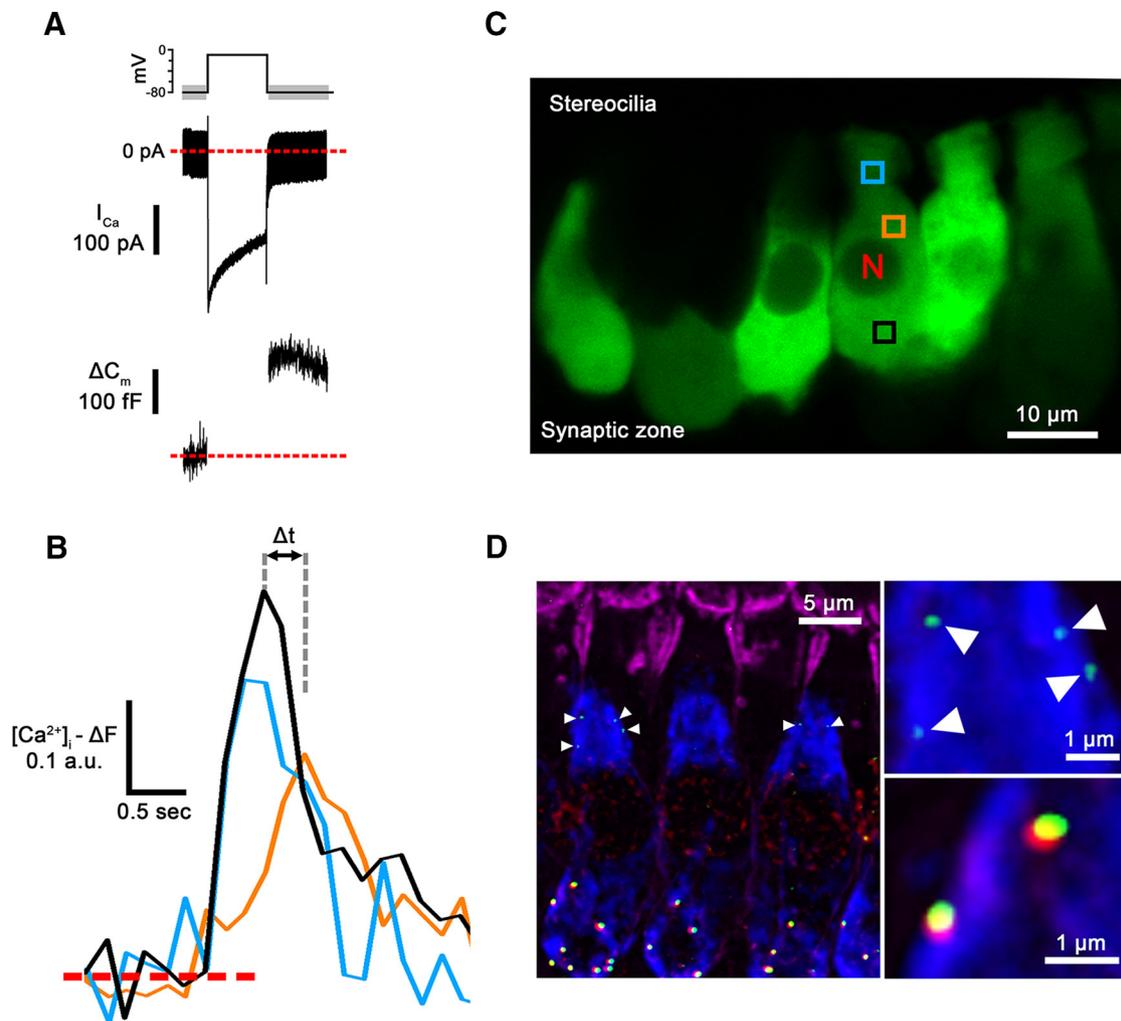


Figure 7. Ca^{2+} imaging and immunohistochemistry mostly localize $Ca_v1.3$ Ca^{2+} channels at the synaptic ribbons of WT IHCs but small $Ca_v1.3$ clusters are also seen extrasynaptically right below the cuticular plate region. **A, B**, Example of simultaneous recording of Ca^{2+} current (top) and capacitance response (bottom) in control condition (**A**) in a WT IHC evoked during a single 500 ms step depolarization from -80 mV to -10 mV. A simultaneous intracellular Ca^{2+} increase was recorded through the fast Ca^{2+} indicator, GCaMP6f (**B, C**). Ca^{2+} signals were measured from the synaptic zone (black line), above the nucleus (orange line), and under the cuticular plate region (blue line) as indicated in **C**, showing a row of IHCs genetically expressing the Ca^{2+} indicator GCaMP6f. Boxes represent the ROI used for the Ca^{2+} measurement shown in **B**. Δt represents the time difference between the maximum peak of intracellular Ca^{2+} measured at the synaptic area and the region above the nucleus. **D**, Typical example of $Ca_v1.3$ immunolabeling at the apical turn of a whole-mount OC from a P14 mouse. F-actin is labeled in pink, otoferlin in blue, CtBP2 (ribbon) in red, and Cav1.3 in green. Right top panel shows the cuticular plate region at higher magnification and the small clusters of $Ca_v1.3$ (arrowheads). Right bottom panel shows two synaptic active zones at higher magnification.

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(Figure legend continued.) train of 100 ms stimulations from -80 mV to -10 mV. Simultaneous intracellular Ca^{2+} increase (mean \pm SEM) was recorded in IHCs expressing the fast Ca^{2+} indicator GCaMP6f in control condition (**C**) and in the presence of nifedipine (**D**). Insets in **C** and **D** show examples of fluorescent IHCs expressing GCaMP6f. Black and orange boxes are the selected ROI for fluorescence measurements at the synaptic and subcuticular plate regions, respectively. Purple arrows and dotted lines represent the timing of UV-unblocking Ca^{2+} channels. The red circle with "N" indicates the position of the nucleus. Superimposed Ca^{2+} currents (top) and synaptic intracellular Ca^{2+} measurement (bottom) are shown during the first 10 stimuli for control condition (**E**) and nifedipine condition (**F**). Note that the fast-transient-inactivating component of Ca^{2+} current (asterisk) coincided with the first phase (Ph1) of Ca^{2+} increase, whereas the second phase (Ph2) mirrored the sustained Ca^{2+} current. Comparative histograms of the rates of Ca^{2+} increase in the basal synaptic region (SR) and above the nuclear region (NR) in control and nifedipine conditions are shown in **G** and **H**, respectively. Ph1, Ph2, and Ph3, Phase numbers 1, 2, and 3 (post-UV) in **C** and **D**, respectively. Rates were obtained by fitting Ph1, Ph2, and Ph3 with a linear function. **H'**, Magnification of the Ph1, Ph2, and Ph3 (post-UV) responses recorded at the subcuticular plate region shown in **G** and **H** in control and nifedipine conditions. * $p < 0.05$.

bullfrog hair cells (Cho and von Gersdorff, 2014). However, the Ca^{2+} current inactivation described here is unlikely to be due to a proton effect because we were working with 10 mM extracellular HEPES buffer, a concentration that prevents pH variations at the synaptic cleft of bullfrog auditory hair cells (Cho and von Gersdorff, 2014). The fast-inactivating nifedipine-resistant Ca^{2+} current uncovered in our study is essentially conducted by $Ca_v1.3$ channels. Indeed, $Ca_v1.3^{-/-}$ IHCs display $<10\%$ of WT Ca^{2+} current and this residual current does not show inactivation (Platzer et al., 2000; Brandt et al., 2003 see their Figs. 3F and 1A, respectively). Our results also suggest that the fast-inactivating nifedipine-resistant Ca^{2+} currents are driven by the $Ca_v1.3$ short isoforms 42A and 43S, the transcripts of which were found to be strongly expressed in IHCs. In agreement with our study, the $Ca_v1.3$ short isoform 43S transcript was shown recently to be expressed in IHCs (Scharinger et al., 2015). Expression in a heterologous system of these short $Ca_v1.3$ isoforms displays Ca^{2+} currents with similar fast inactivation and reduced sensitivity to nifedipine (Singh et al.,

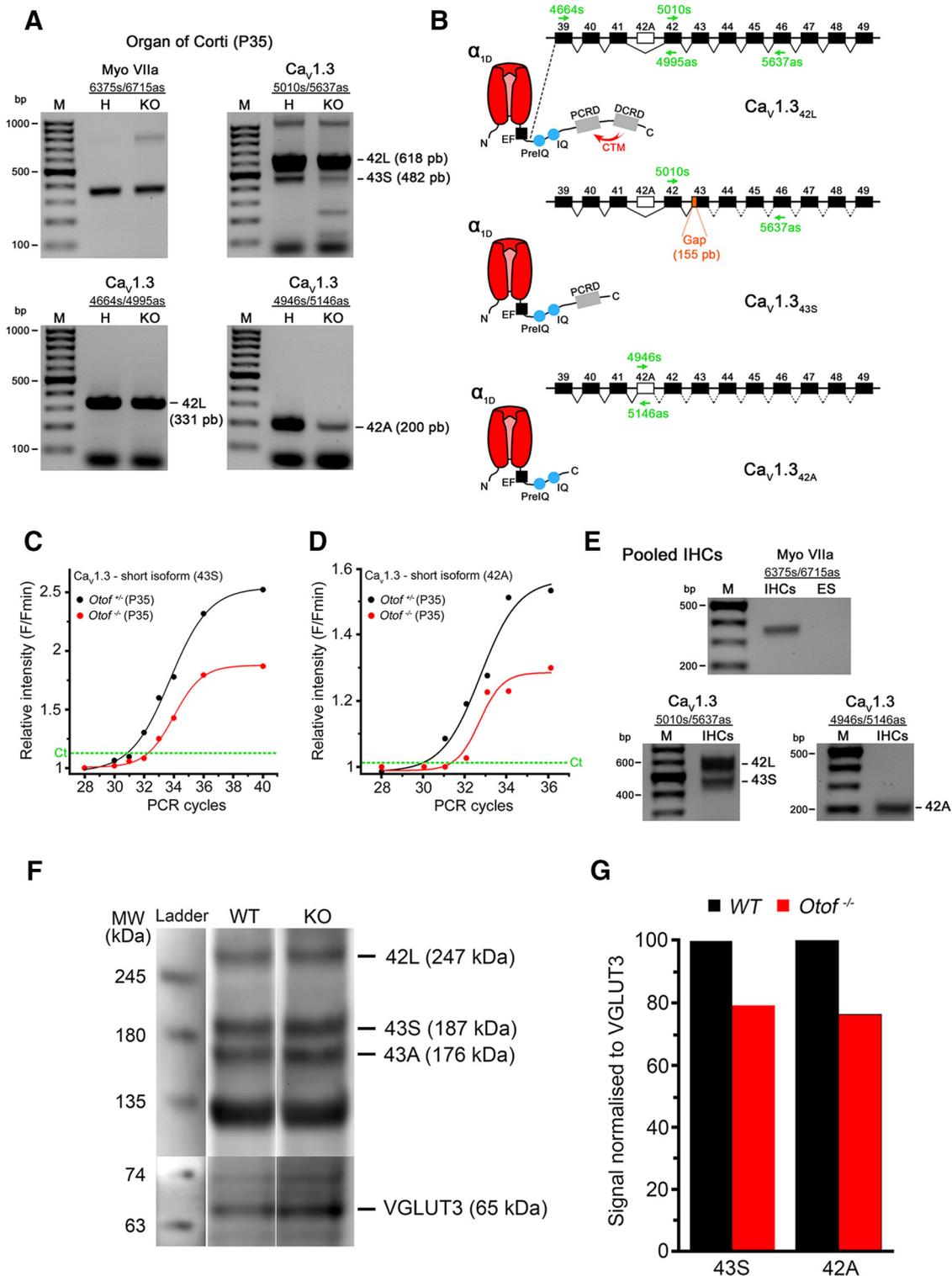


Figure 8. Transcript mRNA expression of Ca_v1.3 short and long isoforms. **A**, RT-PCR expression of myosin VIIa (Myo VIIa, top left), 42L isoform (long Cav1.3 isoform, bottom left) using 4664s/4995as, both 43S and 42L isoforms using 5010s/5637as (top right) and 42A (bottom right) using 4946s/5146as in P35 *Otof*^{+/-} (heterozygous: H) and *Otof*^{-/-} (KO) OCs. **B**, Schematic presentation of alternative splicing of Cav1.3 C-terminus (c-term) domain for long isoform 42L (top), short isoform 43S (middle), and short isoform 42A (bottom). Black and blank rectangles represent constitutive and alternative splicing, respectively. Incorporation of exon 42A instead of exon 42 induces a premature nonsense codon leading to a truncated c-term domain. Orange box represents the lack of 155 nt in the 43S sequence inducing a premature nonsense codon and a truncated c-term domain. Under each sequence, a schematic drawing of the translated α_{1D} c-term domain controlling Ca²⁺-dependent inactivation is shown. The red arrow (top schematic drawing) indicates the formation of the CTM by the interaction between the PCRD and DCRD for the long 42L isoform. Such a regulatory domain cannot be formed in the case of the two short 43S and 42A isoforms. Note that the green arrows above and under each sequence represent the hybridization site for each used primer. **C, D**, Q-PCR from P35 *Otof*^{+/-} (black) and *Otof*^{-/-} (red) OCs for short isoforms 43S (**C**) and 42A (**D**). Green dotted line represents the Ct. **E**, RT-PCR from pooled WT IHCs shows the transcripts of short (43S and 42A) and long (42L) Cav1.3 isoforms. ES: Extracellular solution was used as negative control. **F, G**, Western blot analysis indicating a reduction of 43S and 42A Cav1.3 isoforms in *Otof*^{-/-} OC.

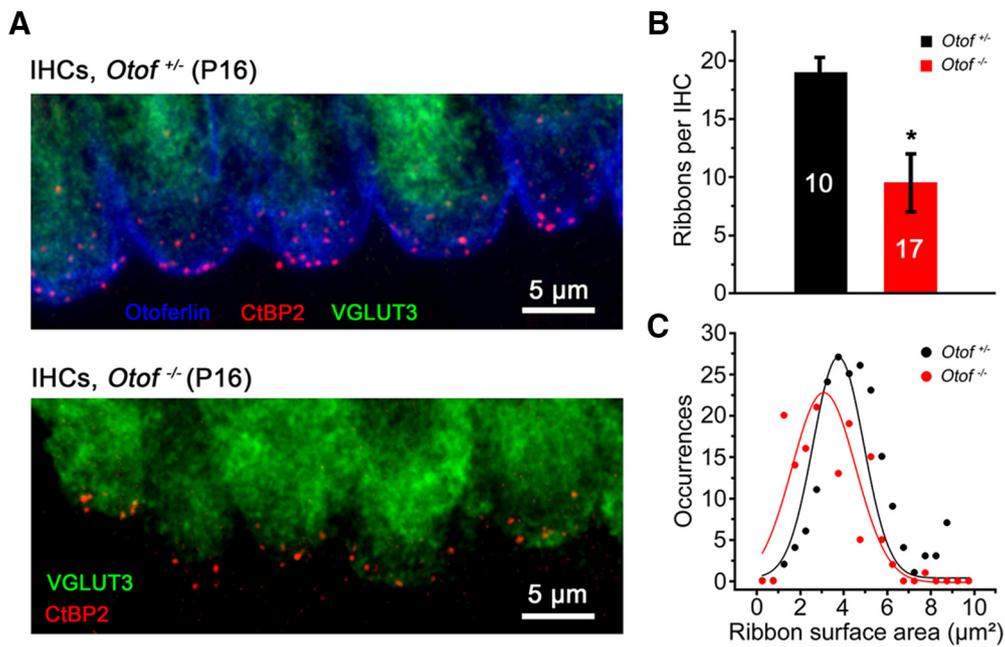


Figure 9. Comparison of ribbon immunolabeling between *Otof*^{+/+} and *Otof*^{-/-} mice. **A**, Stack-reconstructed confocal images from P16 *Otof*^{+/+} and *Otof*^{-/-} IHCs at the apical turn of OC. Tissues were immunolabeled for CtBP2 (ribbons; red), otoferlin (blue), and VGLUT-3 (green). **B**, **C**, Comparative number of ribbons per cell (**B**) and distribution of ribbon surface area (**C**) in *Otof*^{+/+} (black) and *Otof*^{-/-} (red) IHCs. **p* < 0.05.

2008; Bock et al., 2011; Huang et al., 2013). The implication of these short Ca_v1.3 isoforms in IHCs was further reinforced in our study by the concomitant decrease of the fast-inactivating component of the Ca²⁺ current and the reduced expression of the Ca_v1.3_{42A} and Ca_v1.3_{43S} in IHCs lacking otoferlin.

Remarkably, we found that the nifedipine-resistant Ca²⁺ current could still trigger a very efficient RRP exocytotic response when a short depolarizing pulse was applied. However, this exocytotic response showed a large depression during a paired-pulse or a train protocol. These results suggest that the fast-inactivating nifedipine-resistant Ca²⁺ channels are mainly involved in the release of RRP composed of ribbon associated vesicles, whereas the nifedipine-sensitive Ca²⁺ channels allowing deep intracellular diffusion of Ca²⁺ mainly control vesicular recruitment (SRP). Our results are in good agreement with previous studies showing the Ca²⁺ dependence of vesicular recruitment in hair cells (Cho et al., 2011; Levic et al., 2011; Schnee et al., 2011).

C-terminal domain of Ca_v1.3 channels shapes the inactivation kinetics

The C-terminal region of the Ca_v1.3 subunit contains regulatory domains that control calcium-dependent inactivation (CDI). This process occurs when the four EF-hand Ca²⁺ sensing protein calmodulin (CaM) interacts with the IQ and pre-IQ domains (Peterson et al., 1999; Qin et al., 1999; Erickson et al., 2003; Liu et al., 2010; Catterall, 2011; Striessnig et al., 2014). Calmodulin-like calcium-binding proteins (CaBPs), which have been found expressed to be in chick (Lee et al., 2007), mouse (Cui et al., 2007) and rat (Yang et al., 2006) auditory hair cells, can also regulate the CDI kinetics by competing with CaM interaction at the IQ domain (Lee et al., 2002; Yang et al., 2006; Cui et al., 2007; Striessnig et al., 2014). Multiple CaBPs isoforms are expressed in IHCs, notably CaBP1 and CaBP2, which are well colocalized at the IHC ribbon synapse (Cui et al., 2007). In addition, at the extreme C terminus of Ca_v1.3 long isoforms, two α helix proximal (PCRD) and distal C-terminal regulatory domains (DCRD; Fig. 8B) inter-

act with each other to form the C-terminal regulatory domain (CTM; Fig. 8B, red arrow). This CTM domain is thought to slow down the inactivation kinetics of long Ca_v1.3 isoforms by competing with the interaction of the Ca²⁺-CaM and IQ domains (Singh et al., 2008; Liu et al., 2010; Striessnig et al., 2014). The exon 44 encodes ~10 aa between PCRD and DCRD (Tan et al., 2011). The absence of these 10 aa shortened the length between these two domains and led to faster inactivation kinetics than the long Ca_v1.3_{42L} isoform but slower than the two Ca_v1.3_{42A} and Ca_v1.3_{43S} short isoforms (Bock et al., 2011; Tan et al., 2011). The speed up of the Cav1.3_{Δ44} inactivation compared with the long Cav1.3_{42L} isoform could be the result of a misfolding between the PCRD and the DCRD reinforcing the strength of the CDI. Although the short isoforms, Ca_v1.3_{42A} and Ca_v1.3_{43S}, lack the two last domains PCRD and DCRD or only the DCRD domain, respectively, they still possess the pre-IQ and IQ domains for Ca²⁺-CaM regulation (Huang et al., 2013). These truncated C-terminal regulatory domains in Ca_v1.3_{42A} and Ca_v1.3_{43S} channels is likely to explain their peculiar fast-inactivating kinetics in the millisecond range (Singh et al., 2008; Bock et al., 2011; Tan et al., 2011; Huang et al., 2013). The nifedipine-resistant Ca²⁺ current of IHCs displayed similar fast inactivation kinetics, suggesting that it could be driven by the Ca_v1.3_{42A} and Ca_v1.3_{43S} isoforms. Notably, the total Ca²⁺ current in IHCs displayed a small fast-inactivating component (with $t \sim 4$ –5 ms) and a second, large, slowly inactivating component (with $\tau \sim 65$ ms; data not shown), in agreement with results obtained in turtle auditory hair cells (Schnee and Ricci, 2003). This second slow-inactivating component, which is sensitive to nifedipine, likely corresponds to the slow inactivation of the Ca_v1.3_{42L} long isoform (bearing 75% of total Ca²⁺ current) that conserves intact PCRD and DCRD domains (Fig. 8B).

Reduced expression of Ca_v1.3_{42A} and Ca_v1.3_{43S} isoforms in *Otof*^{-/-} IHCs

In *Otof*^{-/-} IHCs, we found that expression of Ca_v1.3_{42A} and Ca_v1.3_{43S} transcripts is decreased and associated with a large-

amplitude reduction in the fast-inactivating Ca²⁺ currents. As shown previously by Roux et al. (2006), we found a 50% decrease in the number of synaptic ribbons in *Otof*^{-/-} IHCs. Otoferlin thus appears essential, not only for sensing Ca²⁺ and preserving ribbons at the presynaptic active zones, but also for maintaining a strong expression of the Ca_v1.3_{42A} and Ca_v1.3_{43S} channels. Disruption of the ribbon-associated cytomatrix protein bassoon in IHCs has also been shown to generate a loss of synaptic ribbons and a concomitant reduction in Ca_v1.3 channel expression (Frank et al., 2010). The reduction in the number of ribbons and the concomitant decrease in Ca²⁺ current inactivation observed in our study suggest that the Ca_v1.3_{42A} and Ca_v1.3_{43S} channels are functionally associated with the synaptic ribbons in IHCs.

Gain of using various Ca_v1.3 isoforms during IHC exocytosis

Auditory IHCs are remarkable in that they encode sound timing information precisely up to several kilohertz into phase-locked specific afferent discharge rates (Palmer and Russell, 1986). Presynaptic Ca_v1.3 short isoforms, inactivating in the millisecond range, would certainly be crucial for ensuring high-frequency phase locking and rapid adaptation of the afferent discharge rate. In central neurons (Tan et al., 2011) and in heart pacemaker cells (Bock et al., 2011), short Ca_v1.3 isoforms are believed to limit Ca²⁺ accumulation during fast-bursting activity. In IHCs, these short isoforms might help to reduce the size of Ca²⁺ domains below the synaptic ribbons. Notably, the ribbons themselves with their docked vesicles placed near the Ca²⁺ channels could further limit intracellular Ca²⁺ diffusion by acting as a diffusion barrier (Graydon et al., 2011). Conversely, slowly inactivating long Ca_v1.3 isoforms might allow a sustained and deeper Ca²⁺ diffusion in IHCs (Liu et al., 2010), a property essential for recruiting vesicles at large distances from the release sites. We propose that Ca²⁺ microdomains of IHCs may be composed of various proportions of long and short Ca_v1.3 isoforms that could lead to the observed heterogeneity of the Ca²⁺ signal between active zones (Frank et al., 2009).

Extrasynaptic Ca²⁺ channels at the subcuticular zone of IHCs

We also show here for the first time strong evidence for extrasynaptic Ca_v1.3 channel clusters below the cuticular plate, where BK channels (Pyott et al., 2004; Hafidi et al., 2005) and ryanodine receptors (Beurg et al., 2005) have been shown previously to be strongly expressed in IHCs. Their blockage by nifedipine suggested that they mainly involve Ca_v1.3 long isoforms. What is the physiological role for these apical Ca_v1.3 Ca²⁺ channel clusters? They could possibly trigger calcium-induced calcium release from intracellular Ca²⁺ stores (Beurg et al., 2005; Castellano-Muñoz and Ricci, 2014) and regulate the fast-repolarizing Ca²⁺-dependent BK currents in IHCs (Skinner et al., 2003; Beurg et al., 2005). Remarkably, IHCs have been proposed to contain two specialized areas of membrane delivery, the basal synaptic ribbons and the subcuticular area, where SNARE proteins are concentrated (Safieddine and Wenthold, 1999; Safieddine et al., 2002). It is therefore possible that the subcuticular Ca_v1.3 Ca²⁺ channels could regulate Ca²⁺-dependent apical extrasynaptic exo–endocytotic processes, as was suggested previously in the zebrafish's lateral line (Seiler and Nicolson, 1999) and in vestibular hair cells of the bull frog (Kachar et al., 1997). This process has been proposed to underlie communication between the endolymphatic compartment and IHC cytoplasm (Kachar et al., 1997; Seiler and Nicolson, 1999), but also to regulate the Ca²⁺ and calmodulin-dependent adaptation mechanism of the hair

bundles by sequestering the Ca²⁺ ATPase (Seiler and Nicolson, 1999).

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