CLC Anion/Proton Exchangers Regulate Secretory Vesicle Filling and Granule Exocytosis in Chromaffin Cells

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CIC-3, CIC-4, and CIC-5 are electrogenic chloride/proton exchangers that can be found in endosomal compartments of mammalian cells. Although the association with genetic diseases and the severe phenotype of knock-out animals illustrate their physiological importance, the cellular functions of these proteins have remained insufficiently understood. We here study the role of two Clcn3 splice variants, CIC-3b and CIC-3c, in granular exocytosis and catecholamine accumulation of adrenal chromaffin cells using a combination of high-resolution capacitance measurements, amperometry, protein expression/gene knock out/down, rescue experiments, and confocal microscopy. We demonstrate that CIC-3c resides in immature as well as in mature secretory granules, where it regulates catecholamine accumulation and contributes to the establishment of the readily releasable pool of secretory vesicles. The lysosomal splice variant CIC-3b contributes to vesicle priming only with low efficiency and leaves the vesicular catecholamine content unaltered. The related Cl−/H+ antiporter CIC-5 undergoes age-dependent downregulation in wild-type conditions. Its upregulation in Clcn3−/− cells partially rescues the exocytotic mutant defect. Our study demonstrates how different CLC transporters with similar transport functions, but distinct localizations can contribute to vesicle functions in the regulated secretory pathway of granule secretion in chromaffin cells.

Key words: anion/proton exchangers; chromaffin cells; CIC-3; exocytosis; neurosecretion

Significance Statement

Cl−/H+ exchangers are expressed along the endosomal/lysosomal system of mammalian cells; however, their exact subcellular functions have remained insufficiently understood. We used chromaffin cells, a system extensively used to understand presynaptic mechanisms of synaptic transmission, to define the role of CLC exchangers in neurosecretion. Disruption of CIC-3 impairs catecholamine accumulation and secretory vesicle priming. There are multiple CIC-3 splice variants, and only expression of one, CIC-3c, in double Cl−/H+ exchanger-deficient cells fully rescues the WT phenotype. Another splice variant, CIC-3b, is present in lysosomes and is not necessary for catecholamine secretion. The distinct functions of CIC-3c and CIC-3b illustrate the impact of expressing multiple CLC transporters with similar transport functions and separate localizations in different endosomal compartments.

Introduction

Adrenal chromaffin cells are prototypic neuroendocrine cells and represent a well-established model for studying presynaptic nerve terminals at high temporal resolution. They release catecholamine into systemic circulation by exocytosis of large dense-core vesicles (LDCVs), which accumulate catecholamines via proton-coupled vesicular monoamine transporters (Yaffe et al., 2018). In chromaffin cells, LDCVs are organized in four different vesicle pools, the depot pool, the unprimed pool (UPP), the slowly releasable pool (SRP), and the readily releasable pool (RRP; Becherer and Rettig, 2006). Vesicles move from the depot pool to the membrane and form the UPP in a process known as docking. Subsequently, in the

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Ca\(^{2+}\) -dependent step, the transition of vesicles from UPP to the slowly releasable pool (from which vesicles transit to-ward the RRP) is defined as priming (Becherer and Rettig, 2006). Physiologic stimuli cause an elevation of intracellu-lar calcium concentrations and mainly trigger fusion of release-competent, or primed, vesicles from the RRP (Voets et al., 1999), making this vesicle pool the principal determin-ant of the high spatiotemporal accuracy of catecholamine secretion (Schoch et al., 2001; Sørensen et al., 2003; Dhara et al., 2014; Mishima et al., 2014).

CLC chloride transporters have been proposed to contribute to vesicular acidification, neurotransmitter filling, and trafficking of intracellular organelles in various cells (Stauber and Jentsch, 2010, 2013). Adrenal chromaffin cells express all CLC antipor ters (Maritzen et al., 2008), but their functions and subcellular localizations have remained largely controversial (Barg et al., 2001; Maritzen et al., 2008; Deriy et al., 2009; Li et al., 2009; Jentsch et al., 2010). In experiments using CIC-3 antibodies with KO staining-controlled specificity, Maritzen et al. (2008) did not find CIC-3 in secretory granules of adrenal chromaffin cells and pancreatic islet cells. The authors observed re-duced exocytotic responses and lower catecholamine content in Cln\(^{−/−}\) chromaffin cells and postulated an indirect role of this transporter in exocytosis. Other groups reported localization in secretory vesicles and direct regulation of intragranular pH and secretion of insulin-containing gran-ules by CIC-3 in pancreatic β-cells (Barg et al., 2001; Deriy et al., 2009); however, antibodies were not tested for speci-ficity in Cln\(^{−/−}\) animals.

There are multiple splice variants of Cln3, and, thus far, the contributions of these distinct CIC-3 splice variants to chromaffin function have not been addressed. We combined high-resolution membrane capacitance measurements, am-perometric recordings, and confocal images to identify cellu-lar roles of CIC-3 splice variants. We aimed to determine the subcellular localization of CIC-3b and CIC-3c and elucidated at which vesicular maturation step Cl/\(H^+\) exchanger regu-lates exocytosis in chromaffin granules. We found that the exocytosis of secretory granules and their catecholamine content are reduced in the absence of CIC-3. Rescue experiments from double Cl/\(H^+\) exchanger-deficient cells revealed that expression of CIC-3c fully restore transmitter content and granule exocytosis. Expression of CIC-3b did not alter catecholamine accumulation of single secretory granules but partially rescued exocytosis in cells lacking CIC-3 and CIC-5. Our results demonstrate that CIC-3c is present in a subpopulation of large dense-core vesicles and directly regulates catecholamine accumulation and exocytosis in chromaffin cells, whereas CIC-3b contributes to the prim-ing of secretory granules but is not involved in the process of neurotransmitter accumulation.

Materials and Methods

**Mutant mice and cell culture. Cln3\(^{−/−}\) mice, provided by Thomas Jentsch (Leibniz-Forschungsinstitut für Molekulare Pharmakologie; Stobrawa et al., 2001), were maintained as heterozygotes by continuous cross-breeding with C57BL/6. Knock-out and WT littermates of either sex were obtained by crossing heterozygotes and were identified by PCR genotyping. Preparation and cultivation of adrenal chromaffin cells were performed as described previously (Sørensen et al., 2003; Borisovska et al., 2005). In brief, adrenal glands were removed and enzymatically treated (12 U/ml papain; Worthington Biochemical) for 20 min. Subsequently, cells were seeded on glass coverslips after trituration and cultured at 37°C and 10% CO\(_2\) in enriched DMEM [100 ml DMEM supplemented with 0.4 ml penicillin/streptomycin, 1 ml sodium pyruvate (100 mS), and 1 ml insulin-transferrin-selenium-X; Invitrogen]. For lenti-viral transduction, the viral suspension was added 2 h after plating. Patch-clamp and amperometric recordings were performed at room temperature either on the second or third day in vitro or 4–5 d after infection of the cells with viral particles. For confocal microscopy, cells were seeded on poly-D-lysin (catalog #P6407, Sigma-Aldrich) coated coverslips.

**Electrophysiology.** Whole-cell voltage-clamp recordings were performed using an EPC10 amplifier controlled by PatchMaster (HEKA Elektronik). Photolysis of caged Ca\(^{2+}\) and ratiometric measurements of intracellular calcium concentration were conducted as previously described (Borisovska et al., 2005). The standard extracellular solution consisted of the following (in mM): 130 NaCl, 4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 48 n-glucose, pH 7.4, with NaOH. For flash photolysis experiments, pipettes with resistances between 3 and 4 M\(\Omega\) were filled with the following (in mM): 110 Cs-Glumate, 8 NaCl, 3.5 CaCl\(_2\), 5 NP- EGTA (provided by Dieter Bruns, University of Homburg, Germany), 0.2 Fura-2, 0.3 Furaptra (Thermo Fisher Scientific), 2 MgATP, 0.3 Na\(_2\)GTP, 40 HEPES, pH 7.3, 320 mOsm. NP-EGTA was photolyzed by a flash of ultraviolet light (Xenon flash lamp, Rapp OptoElectronics) focused through an Olympus objective (60×, UPlanApo, 1.35 oil) of an inverted microscope (IX71, Olympus). Fura-2 and Furaptra were excited at 350/380 nm using a monochromator light. To maintain high [Ca\(^{2+}\)]\(_{int}\) after the flash small amounts of NP-EGTA were photolyzed. For quantifying depolarization-induced secretion we used patch pipettes with resistances between 3 and 4 M\(\Omega\) and containing the following (in mM): 135 Cs-glutamate, 48 CsCl, 1.8 CaCl\(_2\), 0.8 MgCl\(_2\), 0.5 MgATP, 0.5 Na\(_2\)GTP, 10 HEPES-CaOH, pH 7.3, 350 mmol calculated free [Ca\(^{2+}\)]\(_{int}\) 290 mOsm, pH 7.4. Capacitances were measured using the Lucia-Nehér technique (sine wave stimulus, 1000 Hz, 35 mV peak-to-peak amplitude, DC-holding potential –70 mV) using the lock-in extension of Patchmaster (HEKA Elektronik). Currents were low-pass filtered at 2.9 kHz and digitalized with a sampling rate of 20 kHz. Membrane ca-pacitance was analyzed using SigmaPlot 12.3 software with custom-ized routines (Systat Software). The flash-evoked capacitance response was fit with \(f(t) = A_0 + A_1(1-\exp^{t/\tau_1}) + A_2(1-\exp^{t/\tau_2}) + kt\). \(A_0\) denotes the cell capacitance before the flash, and \(A_1, \tau_1, \text{ and } A_2, \tau_2\) represent amplitudes and time constants of RRP and SRP, respec-tively (Rettig and Neher, 2002). Ca\(^{2+}\) currents from cultured chro-maffin cells were measured as described by Toft-Bertelsen et al. (2016). The standard extracellular solution contained the following (in mM): 135 NaCl, 10 HEPES, 2.8 KCl, 10 CaCl\(_2\), 1 MgCl\(_2\), 11 n-glucose, and 1 μM tetrodotoxin. The patch-pipette solution contained the following (in mM): 112.5 CsGlut, 36 HEPES, 9 NaCl, 3 MgATP, 0.45 Na\(_2\)GTP, and 10 EGTA (300 mOsm, pH 7.2). Ca\(^{2+}\) currents were measured during 100 ms test pulses ranging from –70 to +120 mV in 10 mV increments from a holding potential of –70 mV. We determined the voltage dependence of channel activation by plotting tail current amplitudes measured at fixed-test step to –50 mV following the variable test pulses applied.

Carbon fiber electrodes (7 μm diameter, Goodfellow) were prepared as previously described (Guzmán et al., 2007). Amperometric recordings were performed under voltage-clamp conditions with a polarization potential of +800 mV, filtered at 2.9 kHz, and sampled at 20 kHz, and analyzed using Origin (OriginLab) and an Igor-based macro (IgorPro 7.01, WaveMetrics) developed in Sulzer Lab (https://github.com/Dsulzerlab/Quanta_analysis). The analysis was restricted to events with peak amplitude >7 pA, total charge >10 fC, and 50–90% rise time faster than 0.9 ms. Foot signal events >2 ms in duration were analyzed. For K\(^{−}\)-induced secretion, the external solution [containing the following (in mM): 50 NaCl, 80 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 48 glucose, 10 HEPES, pH 7.3, adjusted with NaOH] was applied from a perfusion pipette. For KO\(^{−}\)-induced secretion, the external solution [containing the following (in mM): 50 NaCl, 80 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 48 glucose, 10 HEPES, pH 7.3, adjusted with NaOH] was applied from a perfusion pipette. Only one recording was made per coverslip to avoid the fatigue of the secre-tory response. For calcium infusion experiments, the pipettes solution contained the following (in mM): 110 Cs-glutamate, 8 NaCl, 1 CaCl\(_2\), 20 DPTA, 1 MgCl\(_2\), 2 MgATP, 0.5 Na\(_2\)GTP, 40 HEPES-CaOH, pH 7.3, 3 mOsm calculated free [Ca\(^{2+}\)]\(_{int}\).

**Expression constructs and lentiviral production.** We constructed the RNA-polymerase III promoter based on the retinal backbone vector
FsY 1.1-eGFP G.W. (provided by Mikhail Filippov, Nizhny Novgorod, Russia). The human H1 promoter was cloned upstream of the synapsin promoter (H1-FsY1.1-eGFP), and the short-hairpin targeting sequence found in the mouse ClC-5 nucleotide sequence NM_016691.2 (CCTATGATGATTTCAACCA) was cloned downstream of the H1 promoter (H1-shRNA-FsY 1.1-eGFP, Sigma-Aldrich; Clone ID, NM_016691.2-240s1c1; TRC number, TRCN0000069494, mean knock-down level of 94% and mean knockdown level tested in chromaffin cell cultures was of 92.6%, n = 8 cultures). In addition, a scrambled shRNA sequence (ACTACCGTGTATAGGTG) was inserted into the same vector and used as a control for all shRNA experiments (H1-shRNAscr-FsY 1.1-eGFP). To rescue CIC-3 function, the CIC-3 promoter was cloned downstream of the H1-shRNA sequence, replacing the synapsin promoter (H1-shRNA-FpromCIC-3 1.1-eGFP). We cloned the *Mus musculus* CIC-3b and CIC-3c sequence downstream to the

Figure 1. Exocytosis triggered by trains of depolarizing steps is impaired in Clcn3<sup>−/−</sup> cells from adult mice. A, Schematic representation of the depolarization protocol used to trigger exocytosis of secretory vesicles (top, 18 pulses, 100 ms to +10 mV delivered with 300 ms rest intervals) and the averaged ΔCm responses from WT and for Clcn3<sup>−/−</sup> cells obtained from P0 (postnatal day zero) mice (bottom). B, Mean value for the total ΔCm in newborn WT or Clcn3<sup>−/−</sup> chromaffin cells. C, Mean sizes of the RRP from newborn WT or Clcn3<sup>−/−</sup> chromaffin cells. D, E, The amplitude of the Ca<sup>2+</sup> currents measured during the first four depolarization episodes but not the Na<sup>+</sup> currents measured at the first depolarization step, was significantly higher in the Clcn3<sup>−/−</sup> chromaffin cells from young mice. F, Schematic representation of the depolarization protocol used to trigger secretion (top, 18 pulses, 100 ms to +10 mV delivered with 300 ms rest intervals) and the averaged ΔCm responses in adult WT or Clcn3<sup>−/−</sup> cells (bottom). G, Mean value for the total ΔCm from adult WT or Clcn3<sup>−/−</sup> cells. H, Mean sizes of the RRP from adult WT or Clcn3<sup>−/−</sup> cells. I, J, The amplitude of the Ca<sup>2+</sup> currents measured during the first four depolarization episodes and Na<sup>+</sup> currents measured at the first depolarization step were similar between WT and Clcn3<sup>−/−</sup> chromaffin cells isolated for adult mice. Young mice: WT, n = 25, black circles; Clcn3<sup>−/−</sup>, n = 26, red circles; adult mice: WT, n = 29, black circles; Clcn3<sup>−/−</sup>, n = 35, red circles, ***p < 0.001, **p < 0.01, *p < 0.05, ns, Not significant. n, denotes the number of analyzed cells. Student’s t test. Data were collected from five independent experiments per condition and are represented as mean ± SEM.
CIC-3 promoter, followed by 2A self-cleaving sequence and eGFP (FpromCIC-3c 1.1-eGFP or H1-shRNA- FpromCIC-3c 1.1-eGFP). The genomic localization of the promoter sequence used in this study is chromosome 8, NC_000074.6 (from 60955251–60956463). This position is located right in front of the starting codon of CIC-3c and is different from the promoter sequence of the other CIC-3 splice variants. CIC-3c (GenBank accession no. NM_173876.3) or CIC-3b (GenBank accession no. NM_173873.1; O’Leary et al., 2016). An empty vector carrying CIC-3c promoter and eGFP was used as a control (FpromCIC-3c 1.1-eGFP). Lentiviral particles were produced as described previously (Guzman et al., 2010) by coexpressing lentiviral expression vectors, the helper plasmids pRSVREV and pMDLg/pRRE, and vesicular stomatitis virus G-protein-expressing plasmid (provided by Thomas Südhof, Howard Hughes Medical Institute, Stanford University) in HEK293FT cells. The culture medium containing lentiviral particles was collected 72 h post-transfection and ultracentrifuged for 2 h. Lentiviral particles were immediately resuspended in culture medium, frozen in liquid nitrogen, and stored at −80°C.

Confocal microscopy. Lentiviral transduction was used to express CIC-3b and CIC-3c (Guzman et al., 2015) in combination with fluorescent markers such as Lamp1 (which was a gift from Walther Mothes, plasmid #1817, Addgene; http://www.addgene.org/1817; Sherr et al., 2003), Rab7, Rab11 (a gift from Richard Pagano, plasmid #12605 and #12674, Addgene; Choudhury et al., 2002), TIR (a gift from Gary Banker, plasmid #45060 Addgene; Burack et al., 2000); and VAMP3 and VAMP4 (a gift from Thierry Galli, plasmid #423310 and #423313, Addgene; Galli et al., 1998). Chromogranin A and neuropeptide Y were cloned into the pL56rl lentivector (Guzman et al., 2010) using genomic cDNA isolated from mouse adrenal gland and using the following set of primers: Chromogranin A (GenBank accession no. NM_007693.2; O’Leary et al., 2016), forward 5′- ATGGCGTCCACGGGTTCTTG-3′, reverse 5′-TCCCGCGGCAAGGCTC-3′, and neuropeptide Y (GenBank accession no. NM_023456.3; O’Leary et al., 2016), forward 5′- ATGCTAGGTAACAAGCGAATGGGGC-3′ and reverse 5′-ATGCTAGGTAACAAGCGAATGGGGC-3′. Chromaffin cells were imaged 4–5 d after transduction with a Leica TCS SP5 II inverted microscope (Leica Microsystems) using a 63 × oil immersion objective in PBS containing Ca2+ and Mg2+ (Invitrogen) at room temperature (22–24°C). eGFP (enhanced green fluorescence protein) fluorescence was excited with a 488 nm Argon laser, mRFP (monomeric red fluorescence protein) with a 594 nm He-Ne laser, and emission signals were detected after filtering at 500–550 nm or 600–650 nm bandpass filters. WT or Clcn3−/− chromaffin cells from postnatal day (P9–P1) were used for immunostaining after 4 d of culture. Cells were fixed for 10 min with 4% paraformaldehyde and permeabilized for 1 h with a solution containing the following: 0.5% Triton 100, 5% fetal bovine serum, and 0.5% bovine serum albumin in phosphate-buffer, pH 7.4. Thereafter the samples were immunostained using the primary CIC-3 antibodies, rabbit anti-CIC-3 ALC-001 (Alomone Labs) or Table 1. mRNA Transcripts levels of CIC-3 and CIC-5 in mouse adrenal gland

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<td>Clcn3−/−</td>
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<td>P60</td>
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Adrenal glands were collected from C57BL/6 mouse strain at two different developmental stages, P0 (postnatal day 0) (n = 5) and P60 (postnatal day 60) (n = 5) or Clcn3−/− mice at P0 (n = 6). **p < 0.01. Student’s t test. Data were collected from four independent experiments per condition and are represented as mean ± SEM; n denotes the number of analyzed cells.
rabit anti-CIC-3 (Sigma-Aldrich), at the concentration suggested by the manufacturer or using 1:100 dilution (both concentrations gave similar results) and mouse anti-chromogranin A (catalog #AB2604, Abcam) or with mouse anti-LAMP1 (catalog #HA3 SC-2011, Santa Cruz Biotechnology) using the concentrations recom-

mended by the manufacturer. Samples were washed twice and incubated with the secondary antibody (rabbit Alexa Fluor 488 and mouse Alexa Fluor 647, 1:1000, in phosphate buffer, catalog #A-11008 and #A-31571, Invitrogen). To determine the degree of colocalization between CIC-3b or CIC-3c or the anti-CIC-3 ACL-001 antibody and the different intracellular markers, the subcellular localization was quantified in confocal images as mRFP fluorescence intensity overlapping with eGFP fluorescence or Alexa 488 fluorescence intensity overlapping with Alexa 647 signal using Mander’s overlap coefficient. Images were analyzed and assembled for publication in ImageJ 1.44p software (National Institutes of Health; Schneider et al., 2012).

Electron microscopy. Adrenal glands from WT and Clcn3−/− littermates (P0–P1) were fixed for 24 h with 2.5% glutaraldehyde in 0.1 M phosphate buffer at room temperature. After fixation, glands were washed with phosphate buffer for a further 24 h, treated with 1% OsO4 (in 0.2 M phosphate buffer) for 3 h, washed twice with distilled water, and dehydrated by ascending alcohol concentrations (25–100%). Dehydrated glands were incubated with propylene oxide followed by an additional 20 min incubation with a 1:1 mixture of Epon containing propylene oxide [Epon; 47.5% glycidether, 26.5% dodecylsuccinic acid anhydride, 24.5% methylnadic anhydride, and 1.5% Tris (dimethylaminomethyl)pheno] Thereafter, samples were immersed in pure epoxy resin for 1 h at room temperature followed by polymerization (28°C for 8 h, 80°C for 2.5 h, and finally at room temperature for 4 h). Ultrathin sections (60–80 nm thickness) were mounted on copper grids. To enhance contrast, sections were treated with uranyl acetate and lead citrate. Samples were examined using an EM900 electron microscope (Zeiss) equipped with a slow-scan CCD Camera (TRS). We only used sections containing cells with visible nucleus and identified secretory vesicles by their round electron-dense core. For each condition, different fields of view were analyzed, the number of vesicles was counted in this area, and the diameter of secretory vesicles was measured. Data are presented as mean ± SEM, n indicating the number of vesicle. Statistical comparisons were made using one-way ANOVA (Tukey’s test).

RNA isolation and qRT-PCR. Adrenal glands were collected from C57/B6J mice in two different developmental stages P0 (n = 5 mice) and P60 (n = 3) or Clcn3−/− mice at P0 (n = 5). RNA was isolated using the TRIzol Reagent method following instructions from the manufacturer (catalog #15996026, Thermo Fisher Scientific). In brief, 20 mg of tissue sample were homogenized in 1 ml of TRIzol, mixed with chloroform, and vigorously shaken for 15 s. After 2–3 min incubation at room temperature, the sample was centrifugated at 12 000 × g for 20 min at 4°C. The upper aqueous phase containing the RNA was separated, mixed with isopropanol by vortexing, and incubated for 10 min at room temperature. After centrifugation at 12 000 × g for 10 min at 4°C, the supernatant was discarded, and the pellet was washed with 75% ethanol, vortexed, and centrifuged at 7 500 × g for 5 min at 4°C. The pellet was dried for 15–20 min and resuspended in 20 μl of RNasea-free water. Genomic DNA was eliminated by treating the isolated RNA with DNase using the DNase I kit (catalog #18068015, Thermo Fisher Scientific). To evaluate the expression of the different CLC exchangers, we performed RT-PCR using 1 μl of total RNA, SuperScript III One-step RT-PCR system (catalog #12574, Invitrogen), and the following set of primers: CIC-3a (GenBank accession no. NM_007713.3; O’Leary et al., 2016); 5′-GCGCCCATCTCTGATGCTTAAGG-3′, reverse 5′-AGCTAGTGCCCCTGTAGACGGC-3′; CIC-3b (GenBank accession no. NM_173873.1; O’Leary et al., 2016); 5′-GCCGCTACATGCGCGGTACAG-3′, reverse 5′-TGGTCTCTCAGGAGGAGG-3′; CIC-3c (GenBank accession no. NM_173876.3; O’Leary et al., 2016); 5′-ATGGAATGCTTTCTGATC-3′, reverse 5′-AGCTGTTGGCCCTC-3′; Clcn3 (GenBank accession no. NM_138741.1; O’Leary et al., 2016); 5′-TGCCCTCAAGAGACGTTGATTTG-3′, reverse 5′-AACGAAATCTGTTCTGCTGCTC-3′; Clcn3 isoform 2 (GenBank accession no. NM_001243762.1; O’Leary et al., 2016); 5′-CAGAGGCTTTCATCAGGGGAGTTTTAG-3′, reverse 5′-TGCCTGACATTCCAGACACCGTACG-3′; and the reference gene 18S rRNA 5′-CCCGGCTAGAGGTGAAATTCTTG-3′, reverse 5′-GCCCGTAGTTGAAATTCTTG-3′. Images subcellular localization was quantified in confocal images as mRFP fluorescence intensity overlapping with eGFP fluorescence or Alexa 488 fluorescence intensity overlapping with Alexa 647 signal using Mander’s overlap coefficient. Images were analyzed and assembled for publication in ImageJ 1.44p software (National Institutes of Health; Schneider et al., 2012).
Figure 4. Subcellular localization of ClC-3 splice variants. A, Diagram of the protein topology model of ClC-3 illustrating the transmembrane and cytoplasmic domains. The 18 α helices are labeled A–R, the two cytoplasmic cystathionine β-synthase (CBS) domains are shown in ovals (CBS1 and CBS2). The amino acid sequence of the cytoplasmic N- and C-amino termini are shown within the rectangles. Highlighted in red, we illustrated the clathrin binding dileucine motif and in blue, the recycling endosomes targeting signal. Note that ClC-3b and ClC-3c differ

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Results

CIC-3 regulates granule exocytosis in chromaffin cells

We studied granule exocytosis in chromaffin cells from newborn and adult mice on trains of depolarizing pulses using time-resolved capacitance measurements. Such pulses increase [Ca\textsuperscript{2+}], at the plasma membrane via activation of voltage-gated calcium channels and trigger the fusion of vesicles that are in close proximity to the calcium channels. We used a series of depolarizations (18 pulses, 100 ms to +10 mV delivered with 300 ms resting intervals at −70 mV) that is known to effectively deplete the RRP within the first four pulses of the stimulus trains but to be ineffective in releasing vesicles from the SRP (Voets et al., 1999). In chromaffin cells prepared from newborn mice, the total capacitance changes elicited by this stimulation protocol were larger in Clcn3−/− chromaffin cells than in WT. However, the fourth step of depolarization, which defines the size of the RRP, was not different between WT and Clcn3−/− (Fig. 1A–C). The amplitude of the Ca\textsuperscript{2+} current component during the first four depolarization episodes was on average 1.4 ± 0.08-fold higher in chromaffin cells isolated from young Clcn3−/− mice than for WT (Fig. 1D). Na\textsuperscript{+} currents were not altered in young Clcn3−/− cells (Fig. 1F). In cells from adult mice, we found the first four capacitance responses within the stimulus train to be reduced in Clcn3−/− (Fig. 1F–H), with unchanged calcium and sodium current amplitudes (Fig. 1I,J). Because exocytosis is triggered by Ca\textsuperscript{2+} influx in these experiments, the age-dependent differences might be because of regulatory adjustments in the number or function of voltage-gated Ca\textsuperscript{2+} channels. We therefore isolated Ca\textsuperscript{2+} currents with Cs\textsuperscript{−}-based intracellular solution and 2 μM of tetrodotoxin in the bath solution to block K\textsuperscript{+} and Na\textsuperscript{+} currents; these experiments revealed a 1.5 ± 0.25-fold increase in peak calcium current amplitudes in juvenile Clcn3−/− cells, with no changes in the voltage dependence of channel activation (Fig. 2A–D). Adult Clcn3−/− cells did not show differences in the peak calcium current amplitudes when compared with the WT.

Constitutive genetic ablation often causes compensatory changes in expression levels of other proteins. Therefore, we hypothesized that other proteins with similar functions as ClC-3, for example, ClC-4 or ClC-5, might compensate exocytosis in the Clcn3−/− condition at an early developmental stage and increase the number of voltage-gated calcium channels at the plasma membrane. ClC-4 and ClC-5 are also present in adrenal glands (Maritzen et al., 2008) and exhibit functional properties comparable to those of ClC-3 (Guzman et al., 2013). Because ClC-4 lacks an endosomal trafficking signal and requires association with ClC-3 to be exported from the endoplasmic reticulum to endosomal compartments (Guzman et al., 2017), it is unlikely that ClC-4 exerts direct effects on exocytosis in Clcn3−/− condition. Therefore, we focused on ClC-5 and quantified mRNA transcripts levels in WT mice at two different ages, P0 and P60. We found CLC5 transcripts to be reduced by ~70% in adult mouse adrenal tissue as compared with WT newborn condition (Log [cDNA] = 2.7 ± 0.2 for ClC-5 in WT P0 vs for ClC-5 in WT P60 = 1.8 ± 0.2, p = 0.02; Table 1). Moreover, CLC-5 expression
is upregulated in Clcn3−/− adrenal glands, \( \log [\text{cDNA}] = 2.7 \pm 0.2 \) for CIC-5 in WT P0 vs in 3.1 ± 0.15 for CIC-5 in Clcn3−/− P0, \( p = 0.02; \) Table 1). Altogether, these data show an age-dependent reduction in the CIC-5 mRNA expression levels that are associated with changes in the density of voltage-gated calcium channels.

CIC-3c localizes to a subpopulation of LDCVs that is positive for VAMP3/cellubrevin

There are five splice variants of Clcn3—CIC-3a, CIC-3b, CIC-3c, CIC-3d, and CIC-3e—that reside in distinct intracellular compartments (Gentzsch et al., 2003; Okada et al., 2014; Guzman et al., 2015). We tested the expression of CIC-3 in mouse adrenal glands using RT-PCR and found not only all Clcn3 splice variants but also CIC-3 in this tissue (Fig. 3A). In cultured mammalian cells, CIC-3a and CIC-3b localize to the lysosome (Guzman et al., 2015) and CIC-3e to the Golgi apparatus (Gentzsch et al., 2003). The exact subcellular localization of CIC-3d is currently not known; however, carboxy-terminal sequences are identical in CIC-3d and CIC-3e, suggesting that CIC-3d also localizes to Golgi (Okada et al., 2014; Fig. 4A). CIC-3c is sorted to the recycling endosome (RE) of HEK293T cells (Guzman et al., 2015) and thus represents a candidate Cl−/H+ exchanger in chromaffin cell LDCVs.

As there are no CIC-3-specific antibodies available that permit distinction between endosomal CIC-3 splice variants (Fig. 5), we expressed CIC-3b and CIC-3c as eGFP-fusion proteins in chromaffin cells and studied their subcellular localization using confocal microscopy and Mander’s correlation coefficient (Fig. 4B–E). To avoid overexpression artifact, we used the mouse CIC-3c promoter to drive the expression of CIC-3. Cells transduced with the exogenous CIC-3b or CIC-3c reached nearly the same mRNA transcripts levels as in the WT condition (endogenous CIC-3c 6.3e-3 ± 0.4e-3 vs exogenous CIC-3c 7.0e-3 ± 0.3e-3 \( p = 0.1 \) and endogenous CIC-3b 2.1e-2 ± 0.4e-2 vs exogenous CIC-3b 2.2e-2 ± 1.0e-2 \( p = 0.1; \) \( n = 4-5 \) cultures, Mann–Whitney rank sum test). Chromaffin cells were cotransfected with fluorescently tagged markers, such as VAMP3/cellubrevin, VAMP4, Chromogranin A (ChrA), or neuropeptide Y (NPY), and were coexpressed together with CIC-3c or CIC-3b. VAMP3 is a vSNARE protein present in recycling endosomes (Teter et al., 1998) and implicated in the exocytosis of chromaffin granules (Borisovska et al., 2005), NPY and ChrA are markers for LDCVs (Huttn et al., 1995), and VAMP4 has been used to identify immature LDCVs (Eaton et al., 2000; Bonnemaison et al., 2013). We found CIC-3c, but not CIC-3b, preferentially in a subpopulation of LDCV that are positive for VAMP3 (Fig. 4B,E(a),F(a),D). CIC-3c localizes to lysosomes (Fig. 4E(c),D), in agreement with Maritzen et al. (2008), but not to the recycling endosome (Fig. 4E(b),D). In contrast, >70% of CIC-3c positive puncta colocalize with VAMP3 and >50% with NPY or ChrA and with VAMP4 (Fig. 4B(b–d),D). There are two functionally distinct populations of recycling endosomes that either express Rab11 (Kobayashi and Fukuda, 2013) or the transferrin receptor TIR (Maxfield and McGraw, 2004). In agreement with our previous work (Guzman et al., 2015), we found colocalization of CIC-3c with both recycling endosomal marker Rab11 and TIR (Ullrich et al., 1996; Kobayashi and Fukuda, 2013; Fig. 4C(a,b),D), whereas colocalization with the lysosomal/late endosomal marker Lamp1/Rab7 was negligible (Figs. 4E(d),D, 6). These results identify CIC-3c as the CIC-3 splice variant present at a subpopulation of VAMP3-positive LDCVs.

Expression of CIC-3c but not of CIC-3b fully rescues exocytosis in double mutant Clcn3−/−/kdCIC-5 cells

Photolytic uncaging of Ca2+ causes a homogeneous step-like increase in the intracellular calcium concentration (\([Ca^{2+}]_{\text{i}}\)) from \( \sim 300 \text{ nM} \) to \( \sim 30 \text{ \mu M} \) that synchronizes fusion of catecholamine-containing vesicles and permits quantifying the time course of vesicle fusion (Fig. 7A). In such experiments, three kinetically distinct release components can be separated by fitting the capacitance time course with the sum of two exponentials and a linear function; the exocytotic burst, in which the RRP and the SRP of vesicles fuse with the membrane (Voets et al., 1999), and the sustained component, which reflects the continuous priming and fusion of granules at high \([Ca^{2+}]_{\text{i}}\) (Rettig and...
When calcium channels are bypassed with flash photolysis, the absence of CIC-3 indeed attenuates the exocytotic response in young cells (Fig. 7). The exocytotic burst as well as its two components of release, the RRP and SRP, are reduced in Clcn3−/−+Scr cells (Fig. 7B–D), without changes in time constants, and the sustained component of release remains unaltered. The reduced changes in membrane cell capacitance during exocytotic bursts might be because of smaller vesicles in the Clcn3−/− condition. We quantified the size of LDCV by electron microscopy and did not find differences in size distributions of LDCV between Clcn3−/− and WT condition from P0–P1 mice (Fig. 8A,B). On average, we obtained LDCVs diameters of 141.3 ± 2.6 nm in WT and 147.7 ± 5.0 nm in Clcn3−/− (p = 0.3, Mann–Whitney rank sum test, Fig. 8C), similar to values reported for mice at this age (Pinheiro et al., 2014). We conclude that the absence of CIC-3 does not alter the size of the LDCVs in chromaffin cells but rather the availability of vesicles for exocytosis.

We next corrected for the upregulation of CIC-5 in Clcn3−/− by using lentivirus-mediated shRNA transfer [Clcn3−/−/kdCIC-5, hereafter denoted as double mutant (DMut) cells]. As controls, WT or Clcn3−/− cells were transduced with a virus carrying the scrambled shRNA sequence (hereafter denoted as +Scr). DMut cells showed a significant reduction of the CIC-5 mRNA expression levels 4–5d after lentiviral transduction (mRNA CIC-5 levels relative to 18S for WT or +Scr = 0.014 ± 1.5e-3 and for kdCIC-5 = 9.62e-4 ± 1.99e-5, p < 0.003, n = 8 cultures, Student’s t test). Deletion of CIC-5 in Clcn3−/− cells leads to a further reduction of the exocytotic burst in the flash-evoked vesicle exocytosis by ~40% (Clcn3−/−+Scr 264.2 ± 20.8 fF vs DMut 158.9 ± 15.3 fF, p < 0.001) so that responses of WT+Scr or DMut using one-way ANOVA (Tukey’s HSD post hoc test). Data were collected from four to five independent cultures per condition and are represented as mean ± SEM. n denotes the number of analyzed cells.
Reduced ClC-5 expression in Clcn3<sup>−/−</sup> cells mainly impaired the size of the fast phase (RRP), whereas the slow phase (SRP) of the exocytotic burst remained almost unaffected (Fig. 7C,D). The subsequent sustained component of the release, as well as the release time constant of the RRP, was not altered by the absence of both transporters (DMut). Knock down of ClC-5 in WT cells did not change the exocytotic response (Fig. 7, orange trace and circles), arguing against an independent upstream function of ClC-5 in granule exocytosis.

Expression of ClC-3c in D Mut cells completely restores the flash-evoked response (Fig. 7B–D). The exocytotic burst and its two components of release were significantly different from the D Mut, but similar to control (Fig. 7B–D). ClC-3b expression was less effective in rescuing the exocytotic response (Fig. 7A). The magnitude of the exocytotic burst was similar in D Mut+ClC-3b cells to that observed in Clcn3<sup>−/−</sup>+Scr cells (Fig. 7B). The size of the RRP was almost identical to the Clcn3<sup>−/−</sup>+Scr condition but significantly higher than that of the D Mut cells, and the SRP was not statistically different from the D Mut (Fig. 7C,D). Expression of the exogenous ClC-3c or 3b in D Mut cells resulted in similar mRNA transcript levels of the splice variants as in the WT condition (endogenous ClC-3c 6.3e-3 ± 0.4e-3 vs exogenous ClC-3c 7.0e-3 ± 0.3e-3 p = 0.1 and endogenous ClC-3b 2.1e-2 ± 0.4e-2 vs exogenous ClC-3b 2.2e-2 ± 0.1e-2 p = 0.1; n = 4–5 cultures, Mann–Whitney rank sum test). We can therefore exclude the possibility that the rescue phenotypes are a consequence of lentiviral ClC-3c or ClC-3b overexpression. The rescue experiments indicate that ClC-3c is sufficient to support granule exocytosis and can fully substitute for the loss of Cl<sup>−</sup>/H<sup>+</sup> exchangers in secretory vesicles. They suggest that ClC-3c is present in LDCVs, which is in good agreement with our confocal results.

**CIC-3c but not ClC-3b contributes to catecholamine accumulation in secretory granules**

The absence of ClC-3 Cl<sup>−</sup>/H<sup>+</sup> exchangers is predicted to alter the luminal ionic composition of chromaffin granules and thus their neurotransmitter content. To assess the catecholamine content of individual secretory vesicles and define the contribution of ClC-3c and ClC-3b to catecholamine accumulation, we used carbon fiber amperometry and stimulated secretion by extracellular application of 80 mM of potassium solution or by infusion of 3 μM of free Ca<sup>2+</sup> transporters. On stimulation with high K<sup>+</sup>, which mimics electrical stimulation with >15 Hz (Fulop and Smith, 2007), spike amplitudes and amperometric signal (which reports on the vesicular catecholamine content) as well as frequencies of the amperometric signal were significantly smaller in D Mut than in WT+Scr (Fig. 9A–E). Half-width and 50–90% rise times were unchanged (Fig. 9F,G). No differences in any amperometric parameter were observed between Clcn3<sup>−/−</sup>+Scr and D Mut (Fig. 9B–G). Expression of ClC-3c in D Mut cells restored WT event frequency, amplitude, and charge (Fig. 9B–D). In contrast, expression of ClC-3b in D Mut left quantal sizes as small as in D Mut cells (Fig. 9B–D), with no changes in the half-width and in the rise time (Fig. 9F,G).

Infusion of 3 μM free Ca<sup>2+</sup> permits resolving individual secretory responses independently of voltage-gated calcium channel opening. However, Ca<sup>2+</sup> infusion does not only trigger the fusion of highly primed vesicles but also the unprimed pool of vesicles. In D Mut, amperometry experiments confirmed the reduced catecholamine content of secretory granules in the absence of both transporters (Fig. 10, D Mut). The amplitude, frequency, and quantal charge were significantly reduced in D Mut cells when compared with the WT+Scr condition (Fig. 10A–E). The half-width of D Mut fusion events triggered by intracellular Ca<sup>2+</sup> infusion was larger than that of WT+Scr-mediated events (Fig. 10B,F) but unaltered on stimulation by K<sup>+</sup> perfusion. Expression of ClC-3c rescued the frequency and the quantal size in D Mut cells also for this stimulation paradigm to WT levels (Fig. 10C–E). The rescue was only evident during the first 40 s of amperometric recording, suggesting that only newly formed vesicles were equipped with the transduced protein (Duncan et al., 2003; Estévez-Herrera et al., 2016; Fig. 11). Again, expression of ClC-3b in D Mut cells (D Mut+ClC-3b) failed to restore the
Figure 9. ClC-3c but not ClC-3b regulates catecholamine accumulation in chromaffin granules. A, Representative amperometric traces for WT+Scr, DMut, or DMut+ClC-3c condition. Secretion of catecholamine was triggered by the application of a high K⁺ extracellular solution (80 mM KCl, 60 s duration). B, Representative single exocytotic events from amperometric recordings on WT+Scr (black), Clcn3⁻/⁻ + Scr, (red), DMut (blue), DMut+ClC-3b (brown), or DMut+ClC-3c (gray). C–G, Properties of secretory amperometric events for WT+Scr (n = 24, black circles), Clcn3⁻/⁻ + Scr, (n = 20, red circles), DMut (n = 23, blue circles), DMut+ClC-3b (n = 21, brown circles), or DMut+ClC-3c (n = 25, gray circles), represented as data distribution. ***p < 0.001, **p < 0.01, *p < 0.05. ns, Not significant. The different conditions were compared with WT+Scr or DMut using one-way ANOVA (Tukey’s HSD post hoc test). Data were collected from seven independent experiments per condition and are represented as mean ± SEM. n denotes the number of analyzed cells.

Table 2. Properties of individual fusion events stimulated by extracellular application of high [K⁺] solution in WT or Clcn3⁻/⁻ chromaffin cells expressing scrambled/eGFP (WT+Scr or Clcn3⁻/⁻+Scr) or Clcn3⁻/⁻ chromaffin cells expressing kDClc-5 (DMut) or kDClc-5+ClC-3b (DMut+ClC-3b) or kDClc-5+ClC-3c (DMut+ClC-3c)

<table>
<thead>
<tr>
<th>Genotype (no. of cells)</th>
<th>No. events/stimulus</th>
<th>Charge (pC)</th>
<th>50–90% Rise time (ms)</th>
<th>Half-width (ms)</th>
<th>% of events with foot</th>
<th>Foot charge (pC)</th>
<th>Foot duration (ms)</th>
<th>Foot charge (pC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT+Scr (n = 24)</td>
<td>64.6 ± 7.0</td>
<td>0.206 ± 0.02</td>
<td>86.9 ± 6.2</td>
<td>0.34 ± 0.01</td>
<td>1.6 ± 0.1</td>
<td>41 ± 2.5</td>
<td>5.3 ± 0.2</td>
<td>8.1 ± 0.6</td>
</tr>
<tr>
<td>Clcn3⁻/⁻ + Scr (n = 20)</td>
<td>39.0 ± 5.6*</td>
<td>0.158 ± 0.01*</td>
<td>59.9 ± 6.3*</td>
<td>0.37 ± 0.01*</td>
<td>1.7 ± 0.1*</td>
<td>43.7 ± 3.4*</td>
<td>4.7 ± 0.3*</td>
<td>8.6 ± 0.8*</td>
</tr>
<tr>
<td>DMut (n = 23)</td>
<td>40.0 ± 4.0*</td>
<td>0.129 ± 0.009**</td>
<td>52.7 ± 4.5**</td>
<td>0.35 ± 0.01*</td>
<td>1.6 ± 0.1*</td>
<td>43 ± 3.4*</td>
<td>4.7 ± 0.3*</td>
<td>7.4 ± 0.5*</td>
</tr>
<tr>
<td>DMut+ClC-3b (n = 21)</td>
<td>46.7 ± 6.1</td>
<td>0.147 ± 0.011**</td>
<td>61.0 ± 7.4*</td>
<td>0.34 ± 0.01</td>
<td>1.6 ± 0.1</td>
<td>43.5 ± 3.3**</td>
<td>4.8 ± 0.4**</td>
<td>7.8 ± 0.6**</td>
</tr>
<tr>
<td>DMut+ClC-3c (n = 25)</td>
<td>68.3 ± 6.4</td>
<td>0.208 ± 0.016**</td>
<td>92.3 ± 7.2**</td>
<td>0.34 ± 0.01</td>
<td>1.6 ± 0.1</td>
<td>47.7 ± 2.5**</td>
<td>5.3 ± 0.3**</td>
<td>9.2 ± 0.5**</td>
</tr>
</tbody>
</table>

No. of events/stimulus refers to the mean number of amperometric events obtained during the application of 80 mM potassium solution (60 s application). ***p < 0.001, **p < 0.01, *p < 0.05. ns; Not significant, versus WT+Scr and DMut. One-way ANOVA (Tukey’s HSD post hoc test). Data were collected from five to seven independent experiments per condition and are represented as mean ± SEM. Bold-italic ns represent the statistical comparison of the different rescues versus the DMut.
Table 3. Properties of individual fusion events stimulated by intracellular perfusion of 3 μM free Ca\(^{2+}\) solution in WT chromaffin cells expressing scrambled/eGFP (WT + Scr) or Clcn3\(^{-/-}\) chromaffin cells expressing kClC-3 (DMut) or kClC-3c + Clcn3b (DMut + CIC-3b) or kClC-5 + CIC-3c (DMut + CIC-3c)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Event frequency (Hz)</th>
<th>Charge (pC)</th>
<th>50–90% Rise time (ms)</th>
<th>Half-width (ms)</th>
<th>% of events with foot</th>
<th>Foot amplitude (pA)</th>
<th>Foot duration (ms)</th>
<th>Foot charge (FC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT + Scr (n = 23/1462)</td>
<td>1.6 ± 0.24</td>
<td>0.241 ± 0.01</td>
<td>97.7 ± 5.0</td>
<td>0.33 ± 0.01</td>
<td>1.6 ± 0.1</td>
<td>25.9 ± 1.8</td>
<td>6.0 ± 0.4</td>
<td>6.3 ± 0.5</td>
</tr>
<tr>
<td>DMut (n = 20/801)</td>
<td>0.84 ± 0.11*</td>
<td>0.163 ± 0.009***</td>
<td>52.2 ± 4.2***</td>
<td>0.35 ± 0.01*</td>
<td>1.9 ± 0.1*</td>
<td>23.8 ± 2.2**</td>
<td>4.7 ± 0.3**</td>
<td>5.5 ± 0.3**</td>
</tr>
<tr>
<td>DMut + CIC-3b (n = 18/717)</td>
<td>0.8 ± 0.1***</td>
<td>0.139 ± 0.001***</td>
<td>64.8 ± 7.9***</td>
<td>0.32 ± 0.01***</td>
<td>1.4 ± 0.1***</td>
<td>23.9 ± 2.3**</td>
<td>4.7 ± 0.5**</td>
<td>5.6 ± 0.4**</td>
</tr>
<tr>
<td>DMut + CIC-3c (n = 17/1375)</td>
<td>1.9 ± 0.19**</td>
<td>0.224 ± 0.015***</td>
<td>95.8 ± 6.1**</td>
<td>0.32 ± 0.01**</td>
<td>1.5 ± 0.1**</td>
<td>29.5 ± 3.0**</td>
<td>5.6 ± 0.2**</td>
<td>4.8 ± 0.3**</td>
</tr>
</tbody>
</table>

No. of cells/total no. of events denotes the number of cell and the total number of events analyzed per condition. Event frequency was calculated over the first 40 s of amperometric recording. *p < 0.01, **p < 0.001, ***p < 0.0001. ns, Not significant, versus WT. One-way ANOVA (Tukey’s HSD post hoc test). Data were collected from four to five independent experiments per condition and are represented as mean ± SEM. Bold-italic ns represent the statistical comparison of the different rescues versus the DMut.

Figure 10. Ca\(^{2+}\) infusion experiments confirm the Cl\(^{-}/\)H\(^{+}\) exchanger dependency of the catecholamine accumulation process. A, Representative amperometric recording for WT, DMut, or DMut + CIC-3c (area highlighted in gray within the recording represents the first 40 s of analyses showed in C–G) stimulated by the pipette infusion of 3 μM free Ca\(^{2+}\). B, Representative single exocytotic events from amperometric recordings on WT + Scr (black), DMut (blue), DMut + CIC-3b (brown), or DMut + CIC-3c (gray). Spikes with values closer to the mean average amplitude, charge, and half for each condition were selected within the amperometric recordings. C–G, Properties of secretory amperometric events for WT + Scr (n = 23 cells, black circles, with 63.5 ± 9.6 events per cell), DMut (n = 20 cells, blue circles; with 33.9 ± 4.5 events per cell), DMut + CIC-3b (n = 18 cells, brown circles; with 31.2 ± 5.46 events per cell), or DMut + CIC-3c (n = 17 cells, gray circles; with 76.4 ± 7.7 events per cell), represented as data distribution. ***p < 0.001, **p < 0.01, *p < 0.05. ns, Not significant. Different conditions were compared with the WT + Scr or DMut using one-way ANOVA (Tukey’s HSD post hoc test). Data were collected from four independent experiments per condition and are represented as mean ± SEM.
catecholamine content of the individual secretory granules; quantal amplitude and charge were significantly smaller than in the WT + Scr but not statistically different from DMut (Fig. 10C, E). Event frequency was not different between DMut+ClC-3b and DMut. ClC-3b and ClC-3c did not cause major changes in foot signal or in the 50–90% rise time of the amperometry currents (Fig. 10G, Table 3), reinforcing the notion that Cl\(^{-}\)/H\(^{+}\) exchangers are not involved in the formation and expansion of the fusion pore of secretory granules. Expression of ClC-3b or ClC-3c both accelerated the half-width to values closer to WT + Scr condition (Fig. 10F). The presence of ClC-3c but not of ClC-3b in secretory vesicles is in line with the notion that Cl\(^{-}\)/H\(^{+}\) exchangers are required to regulate the vesicular catecholamine content of chromaffin granules.

**Discussion**

We here studied the role of CLC Cl\(^{-}\)/H\(^{+}\) exchangers in chromaffin cells. We found that one ClC-3 splice variant, ClC-3c, localizes to the LDCVs, regulates the catecholamine accumulation process, and efficiently contributes to the establishment of secretory granules for exocytosis. ClC-3b is not present in LDCVs and does not regulate the granular neurotransmitter content. It contributes to vesicle priming, but only with reduced efficiency. ClC-5 is developmentally downregulated in WT chromaffin cells, and ClC-3 deficiency causes compensatory changes in the ClC-5 mRNA expression levels.

The contribution of Cl\(^{-}\)/H\(^{+}\) exchangers to granule exocytosis in chromaffin cells can be demonstrated in rescue experiments, in which exocytosis was triggered by flash photolysis of caged calcium (Fig. 7). The expression of ClC-3c in double Cl\(^{-}\)/H\(^{+}\) exchanger-deficient cells fully rescues the exocytotic response of secretory granules to WT levels (Fig. 7, DMut + ClC-3c), showing that ClC-3c is essential and functionally sufficient for neurosecretion. In contrast, the expression of ClC-3b in DMut cells supports exocytosis with low efficiency (Fig. 7, DMut + ClC-3b). In the absence of ClC-3, upregulation of ClC-5 (Table 1) was not able to compensate for the flash-evoked response (Fig. 7, Clcn3\(^{-/-}\) + Scr). Its removal in WT cells does not alter granule exocytosis (Fig. 7, WT + kdClC-5), indicating that ClC-5 plays a minor, if any, role in exocytosis in WT condition.

To study the fusion of individual vesicles we used two different experimental approaches, high K\(^{+}\) or Ca\(^{2+}\)-infusion stimulation (Figs. 9, 10). We used amperometry to analyze the quantal neurotransmitter content of the chromaffin granules and found reduced quantal amperometric charges in the absence of ClC-3 alone (Fig. 9, Clcn3\(^{-/-}\)) or absence of ClC-3 and ClC-5 (DMut, Figs. 9, 10). Because the size of LDCVs is similar in WT and Clcn3\(^{-/-}\) (Fig. 8), such reduction must be caused by differences in LDCV catecholamine concentrations. In rescue experiments from DMut cells, the expression of ClC-3c was the only Cl\(^{-}\)/H\(^{+}\) exchanger that fully recovers all amperometric parameters to control levels (Figs. 9, 10). Lentiviral expression of ClC-3b left quantal sizes in DMut cells unaffected (Figs. 9, 10), indicating that only ClC-3c, but not ClC-3b, contributes to catecholamine accumulation. None of our genetic maneuvers changed the prespike signal or the rise time of amperometric events (Figs. 9G, 10G, Tables 2, 3), arguing against an important role of ClC-3 in the initial formation and expansion of the fusion pores in chromaffin granules. Application of 80 mM of extracellular K\(^{+}\) as well as Ca\(^{2+}\) infusion lead to a fast increase in the cytosolic Ca\(^{2+}\) at the release site and promotes full-collapse vesicle exocytosis (Elhamdani et al., 2001; Fulop and Smith, 2007). The observed smaller unitary events in DMut under such full-collapse fusion
exclude the possibility of partial catecholamine discharge from mutant granules.

This functional evidence is in good agreement with our colocalization analyses. It supports the notion that CIC-3c, but not CIC-3b, is present in LDCVs, with CIC-3c in a subpopulation of LDCV positive for VAMP3 and CIC-3b in lysosomes (Fig. 4). Our conclusion that CIC-3c is present in LDCVs disagrees with earlier reports on the CIC-3 localization in chromaffin cells. Maritzen et al. (2008) used immunolabeling and viral infection to conclude that CIC-3 neither colocalizes with chromogranin in chromaffin cells, a marker for secretory granules, nor with insulin in β-pancreatic cells. The discrepancy between these and our results is likely because of the existence of multiple splice variants with distinct subcellular localization. The antibodies used in the earlier study cannot differentiate between splice variants, and Maritzen et al. (2008) virally expressed only CIC-3b, so no conclusion can be drawn about CIC-3c from these experiments. Weinert et al. (2020) used localization experiments with fluorescently tagged CIC-3 and functional studies to conclude that CIC-3 is not present in synaptic vesicles. They took advantage of a novel knock-in mouse model (Clcn3<sup>ven/ven</sup>) with a Venus tag sequence fused to the start of exon 1. This genetic modification results in the expression of only certain fluorescently tagged Clcn3 splice variants, that is, of CIC-3a (M_007711.3), but not of others, most importantly not of CIC-3c. Moreover, Weinert et al. (2020) assessed synaptic transmission in hippocampal slices and found that the frequency and amplitude of quantal events were similar between WT and Clcn3<sup>+/-</sup>; however, the authors did not correct for the upregulation of CIC-5 at the age in which the experiments were performed (P14–16). The novel experiments of Weinert et al. (2020) thus do not contradict the functional role of CIC-3c in exocytosis.

CLC-3 knockout mice might affect the recycling of other proteins that regulate catecholamine secretion. Genetic ablation of SNAP-25, a SNARE protein essential for Ca<sup>2+</sup>-dependent exocytosis in neuroendocrine cells, causes defective priming of LDCVs (Sørensen et al., 2003; Washbourne et al., 2002) and alters Ca<sup>2+</sup> current densities in chromaffin cells (Toft-Bertelsen et al., 2016), quite similar to the Clcn3<sup>-/-</sup> phenotype. However, deletion of SNAP-25 leaves the neurotransmitter content of individual granules unaffected (Sørensen et al., 2003), thus excluding the possibility that a trafficking defect of SNAP-25 is the sole basis of the observed functional alterations. Secretory vesicles contain a granule matrix that binds catecholamines (Helle et al., 1985). Two major constituents, chromogranin A and B, have been identified and shown to be involved in granulogenesis as well as in catecholamine sequestration (Videen et al., 1992; Yoo, 1996; Kim et al., 2006; Díaz-Vera et al., 2012). The absence of chromogranin A/B in chromaffin cells reduces the quantal charge and the frequency of amperometric events (Díaz-Vera et al., 2012), similar to the DMut phenotype. However, whereas deletion of chromogranin A/B affects the rising phase of quantal events, the DMut-mediated events do not show changes in the kinetic of fusion neither by high K<sup>+</sup> stimulation nor by Ca<sup>2+</sup>-infusion; the rise time was affected by the double CIC deletion (Figs. 9, 10). This indicates that a reduced granular content of granins is not responsible for the DMut phenotype.

Figure 12 depicts our current concepts about the functions of CIC-3b and CIC-3c in chromaffin cells. After exocytosis, chromaffin granule components are retrieved through the early endosome to the trans-Golgi network (Ceridono et al., 2011), and nascent granules undergo additional maturation steps that involve reduction of the luminal pH (Kim et al., 2006). VMATs exchange one catecholamine for two luminal protons so that pH gradients across the LDCV membrane are main determinants of the vesicular catecholamine content. CIC-3 is a chloride/proton exchanger, and its pronounced voltage dependence makes CIC-3 perfectly suited for chloride-driven vesicle acidification (Guzman et al., 2013; Rohrbough et al., 2018). Our results are compatible with the idea that CIC-3c regulates the catecholamine accumulation process by fine-tuning the luminal ion concentration of secretory vesicles. CIC-3c traffics from the trans-Golgi network to REs and consequently to immature secretory granule, where it might contribute to reducing the luminal pH as a maturation step. Lack of CIC-3 impairs vesicle acidification and makes catecholamine accumulation less efficient, resulting in lower levels of neurotransmitters. CIC-3b contributes to vesicle priming (Fig. 7C, DMut+ CIC-3b) and is unable to restore the quantal content (Figs. 9, 10, DMut+CIC-3b), suggesting that CIC-3b is present at an early stage of vesicle maturation and is subsequently sorted to
the lysosomes (Fig. 12). In addition to modifying the vesicular ion homeostasis, CIC-3 might also recruit novel binding partners that modify secretory vesicle pools (Weinert et al., 2014).

We observed increased densities of voltage-gated calcium channels (Fig. 2) and CIC-5 mRNA transcript levels in chromaffin cells from young Clcn3−/− mice (Table 1). Moreover, exocytosis elicited by a train of depolarizing pulses was significantly impaired in adults but not in young Clcn3−/− cells (Fig. 1). These results suggest that CIC-5 regulates the trafficking of calcium channels in an early developmental stage. Clcn5 is the disease gene for Dent’s disease (Fisher et al., 1995), and its gene product, CIC-5, was shown to be involved in endocytosis of various epithelial cells (Piwon et al., 2000). Age-dependent downregulation in neuronal tissues, as well as the lack of neurologic symptoms in Dent’s disease patients (Wrong et al., 1994), suggested that CIC-5 does not play an important role in the mature CNS. Our work on chromaffin cells—a model system for presynaptic function—demonstrates how regulating the expression of CIC-5 minimizes the impact of CIC-3 ablation on exocytosis by increasing the number of calcium channels on the plasma membrane. This mechanism predicts a neuroprotective function of CIC-5 in neuronal cells, which likely prevents neurodegeneration in Clcn3−/− before P20 (Stobrawa et al., 2001).

Our results identify CIC-3 as a key element of the regulated exocytotic pathway of neuroendocrine cells. They indicate that CIC-3c plays an active role in the neurotransmitter accumulation process, most likely adjusting luminal ionic concentrations of secretory vesicles and thereby facilitating catecholamine uptake. This work pinpoints the subcellular role of CIC-3 in neuroexocytosis and sets down the bases for a better understanding of the functions of Cl−/H+ exchangers in cellular physiology.

References


