Vti1a functions in dense-core vesicle biogenesis

Julia Kurps$^{2,*}$, Alexander M. Walter$^{1,2,*}$, Heidi de Wit$^2$, Susanne Schöning$^3$, Trine L. Toft-Bertelsen$^1$, Juliane Lauks$^2$, Iwona Ziomkiewicz$^4$, Annita Ngatchou Weiss$^5$, Alexander Schulz$^4$, Gabriele Fischer von Mollard$^3$, Matthijs Verhage$^2$, and Jakob B. Sørensen$^{1,6}$

$^1$Neurosecretion group, Department of Neuroscience and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, DK-2200 Copenhagen N, Denmark
$^2$Department of Functional Genomics and Clinical Genetics, Center for Neurogenomics and Cognitive Research, VU University Amsterdam and VU Medical Center, 1081 HV Amsterdam, The Netherlands
$^3$Biochemie III, Fakultät für Chemie, Universität Bielefeld, 33615 Bielefeld, Germany
$^4$Department of Plant and Environmental Sciences, University of Copenhagen, DK1871 Frederiksberg C, Denmark
$^5$Department of Pharmacology, University of Michigan, Ann Arbor, MI 48109-5632, USA
$^6$Lundbeck Foundation Center for Biomembranes in Nanomedicine, University of Copenhagen, DK-200 Copenhagen N, Denmark

* These authors contributed equally to this paper. Published in EMBO Journal (2014): 33(15), 1681-97
Abstract

The SNARE-protein Vti1a is proposed to drive fusion of intracellular organelles, but recent data also implicated Vti1a in exocytosis. Here we show that vti1a is absent from mature secretory vesicles in adrenal CCs, but localizes to a compartment near the Trans-Golgi-Network, partially overlapping with Syntaxin6. Exocytosis is impaired in vti1a null cells, partly due to fewer Ca$$^{2+}$$-channels at the PM, partly due to fewer vesicles of reduced size and Synaptobrevin-2 content. In contrast, release kinetics and Ca$$^{2+}$$-sensitivity remains unchanged, indicating that the final fusion reaction leading to transmitter release is unperturbed. Additional deletion of the closest related SNARE, Vti1b, does not exacerbate the Vti1a phenotype and vti1b null cells show no secretion defects, indicating that vti1b does not participate in exocytosis. Long-term re-expression of vti1a (days) was necessary for restoration of secretory capacity, whereas strong short-term expression (hours) was ineffective, consistent with Vti1a involvement in an upstream step related to vesicle generation, rather than in fusion. We conclude that Vti1a functions in vesicle generation and Ca$$^{2+}$$-channel trafficking, but is dispensable for transmitter release.
Communication between intracellular compartments in eukaryotic cells, but also communication between cells, relies mostly on the fusion of cargo-containing vesicles with target membranes driven by evolutionarily conserved SNARE-proteins (Jahn and Scheller, 2006; Südhof and Rothman, 2009). SNARE complexes consist of four α-helices in a coiled coil structure, with 16 layers of amino acids facing the center that add to complex stability through mostly hydrophobic interactions (Sutton et al., 1998). The exception is the central zero-layer of the complex, which contains highly conserved polar side-chains of either glutamine (Q) or arginine (R) residues, leading to the classification into Q- and R-SNAREs (Fasshauer et al., 1998). All known SNARE complexes are made up of three Q-SNARE motifs, one from each separate subfamily (denoted Qa, Qb and Qc), and one R-SNARE (Antonin et al., 2002; Sutton et al., 1998; Zwilling et al., 2007).

A large body of work has focused on the SNARE complex involved in neuronal exocytosis, which consists of Syntaxin1 (a Qa-SNARE), SNAP-25 (contributes both the Qb and the Qc helices), and VAMP2/Synaptobrevin-2 (an R-SNARE). Genetic ablation of neuronal SNAREs leads to a loss of triggered neurotransmitter release, demonstrating the essential role of the SNAREs in exocytosis (Borisovska et al., 2005; Sørensen et al., 2003a).

Vps-ten-interacting-1a (vti1a) is classified as a Qb-SNARE, although it is unique by harboring an aspartate residue within the zero-layer (Antonin et al., 000a). This aspartate is involved in similar interactions as the glutamine in other complexes (Zwilling et al., 2007). Vti1a was suggested to play a role in (1) homotypic early endosome fusion with Syntaxin13 (or Syntaxin16), Syntaxin6 and VAMP4 (Brandhorst et al., 2006; Kreykenbohm et al., 2002), (2) early endosome to golgi fusion with Syntaxin16, Syntaxin6 and VAMP3 (or VAMP4) (Mallard et al., 2002) and (3) late endosome to golgi fusion with Syntaxin16, Syntaxin10 and VAMP3 (Ganley et al., 2008; Medigeshi and Schu, 2003). Notably, Syntaxin6 has been implicated in the maturation of secretory vesicles, by stimulating homotypic fusion of immature secretory granules (ISGs) (Tooze et al., 2001; Wendler et al., 2001), and VAMP4 was also found in ISGs (Wendler et al., 2001). Based on the fact that Vti1a participates in two other pathways together with Syntaxin6 and VAMP4, the question arises whether there is a role for Vti1a in vesicle biogenesis or maturation.

Vti1a is one of the two yeast-orthologs of Vti1p in mammals, the other is Vti1b (Advani et al., 1998; Fischer von Mollard and Stevens, 1998). Loss of vti1p in yeast is lethal (Lupashin et al., 1997; Fischer von Mollard et al., 1997), but individual loss of vti1a or vti1b is tolerated in mouse, while the simultaneous deletion of both results in widespread neurodegeneration and peri-natal lethality (Atlashkin et al., 2003; Kunwar et al., 2011). Vti1b is mainly found on late endosomes (Kreykenbohm et al., 2002) and has been associated with the fusion of late endosomes together with Syntaxin7, Syntaxin8 and the R-SNARE VAMP8 (Antonin et al., 000a), whereas Vti1b interaction with VAMP7 is required for transport from late endosomes to lysosomes (Pryor et al., 2004).

Recent investigations of Vti1a and VAMP4 in neurons have implicated these “endosomal” SNAREs in regulated exocytosis reactions: VAMP4 is involved in asynchronous neurotransmitter release (Raingo et al., 2012), whereas Vti1a was suggested to play a role in the action-potential-independent spontaneous fusion of synaptic vesicles (Ramirez et al., 2012).
This is controversial, since Vti1a-carrying vesicles also fused during prolonged stimulation trains (Hoopmann et al., 2010; Ramirez et al., 2012), and other studies have shown that spontaneous release is modulated by mutation of the neuronal SNAREs (Deak et al., 2006; Weber et al., 2010). The question remains whether endosomal SNAREs compete with neuronal ones to directly drive exocytosis, or whether their apparent involvement in exocytosis is due to a function in upstream processes.

Here, we used adrenal CCs to answer this question as it applies to large dense-core vesicles (LDCVs) in neurosecretory cells. Adrenal CCs secrete catecholamines and various peptides into the blood as part of the stress response, and isolated cells constitute a powerful assay system, where exocytosis can be monitored with sub-millisecond time resolution (Rettig and Neher, 2002). The basal fusion mechanism appears to be largely conserved between LDCVs in CCs and synaptic vesicles, but notable differences include the biogenesis pathway of vesicles. In neurosecretory cells (and in neurons), LDCVs are formed by budding from the TGN, whereas small synaptic vesicles are formed via fast (within few minutes) recycling of endocytosed components (Burgoyne and Morgan, 2003; Südhof, 2004).

Results

To investigate the role of vti1a and vti1b in the regulated exocytosis pathway in neurosecretory cells, we here studied adrenal CCs.

Vti1a localizes to a peri-nuclear compartment positive for Syntaxin6

In NRK epithelial cells, vti1a is found localized to the Golgi/(TGN) (Kreykenbohm et al., 2002), but in addition Vti1a is localized to a subset of synaptic vesicles in neurons (Antonin et al., 000a; Kreykenbohm et al., 2002; Ramirez et al., 2012). The localization in neurosecretory cells is not clear, as these cells contain a different kind of secretory vesicle with an electron-dense core, hence denoted large dense-core vesicles (LDCVs). We stained cultured mouse adrenal CCs using two Vti1a-specific antibodies and imaged cells using 3D-structured illumination microscopy (3D-SIM)(Schermelleh et al., 2010). This resulted in Vti1a-specific staining of a peri-nuclear compartment (fig2.1A, compare wild type and vti1a null). Co-staining against Syb-2 (VAMP2/Synaptobrevin-2), the R-SNARE responsible for LDCV fusion (Borisovska et al., 2005), resulted in a clearly vesicular staining, with many vesicles in the periphery of the cell (fig2.1A), consistent with the localization of mature vesicles in CCs (Toonen et al., 2006). Staining for Syntaxin6 which has been described from the TGN, immature vesicles, and endosomes (Bock et al., 1997; Brandhorst et al., 2006; Klumperman et al., 1998; Kreykenbohm et al., 2002; Mallard et al., 2002; Wendler et al., 2001) showed partial colocalization with Vti1a (fig2.1B). The compartment positive for Vti1a was distinct from the cis-Golgi, as revealed by GM130-staining, (fig2.1C), and might therefore be TGN. However, staining against a classical TGN-marker, TGN38, also did not reveal overlap with Vti1a (fig2.1D). Instead, Vti1a appears to be surrounded by the TGN38 positive compartment. Consistent with the partial co-localization, Syntaxin6 was also found surrounded by TGN38 staining (fig2.1E).
Thus, the compartment positive for Vti1a appears very similar to the Syntaxin6 positive, but TGN38 negative, subdomain of the TGN shown to be involved in the recycling of GLUT4 (Shewan et al, 2003) and vesicle formation in pancreatic beta-cells (Kuliawat et al., 2004). This compartment has recently been shown to contain PICK1, which is involved in vesicle generation in growth hormone secreting cells, and it was suggested that it might constitute immature vesicles (Holst et al., 2013).

Recent analysis of vti1a/b double knockouts revealed that more lysosomal hydrolases are secreted, probably due to defects in transport between TGN and endosomes (Kunwar et al., 2011). We found no apparent changes in the morphology of lysosomes stained with Lamp1 in the vti1a null; their number, size and area were not different from wild type littermate controls (fig2.2Ai-Aii, fig2.13Di-Dii).

Since 3D-SIM is not a strictly quantitative method, we quantified staining intensities using images obtained in the confocal microscope. Interestingly, this showed that the expression of the presumed vti1a-partner Syntaxin6 was depressed in vti1a null cells (fig2.2Ai-Aii). Since the Syntaxin6 positive compartment is involved in vesicle formation, its reduction might lead to fewer mature vesicles.
Quantification of Syb-2 staining indeed revealed that the mean cellular Syb-2 level was significantly reduced in \textit{vti1a} null CCs (fig2.2Ai-Aii). In contrast, the levels of GM-130 were unchanged by elimination of \textit{vti1a} (fig2.2Ai-Aii).

To understand whether elimination of \textit{vti1a} causes up regulation and compensation by other SNAREs, we performed immunoblotting from whole adrenal glands from newborn \textit{vti1a} null and \textit{wild type} mice. Protein levels of Syntaxin16, SNAP-23, -25, -29, -47, and VAMP-4 were unchanged in the \textit{vti1a} null (fig2.2Bi-Bii). However, the level of syb-2 was reduced (fig2.2Bii), consistent with the results from immunostaining. The level of Syntaxin6 was unchanged in this analysis, which appears inconsistent with the results from immunostaining. However, Syntaxin6 is a ubiquitous SNARE, which is also present in the adrenal cortex, and therefore a selective reduction in the CCs of the adrenal medulla might go undetected. Alternatively, the apparent reduction in immunostaining might have been caused by a partial collapse of the Syntaxin6 positive compartment in the absence of Vti1a, leading to impaired immuno-availability.

To investigate whether Vti1a might be present on Syb2-positive mature LDCVs as a prerequisite for driving secretion, we scrutinized 3D-SIM image planes obtained close to the footprint of the cells, where peri-nuclear staining was absent (fig2.3). The background staining for Vti1a in the \textit{vti1a} null appears as speckles (fig2.1A-C and 2.3B), which is an artifact of the 3D-SIM reconstruction algorithm when applied to weak homogeneous staining (compare to fig2.13A-D outside of the Golgi area). A few vesicular structures positive for Vti1a were found in the periphery, which were negative for Syb-2 (fig2.3A, line profiles), but such structures were also found occasionally in \textit{vti1a} null cells (fig2.3B) and thus they were not further investigated. Vti1a-staining on Syb-2 positive vesicles was generally not detected (fig2.3A), but the speckled nature of the Vti1a-staining made the assessment difficult. To circumvent this problem we averaged subimages, selected such that the vesicle was centered in the middle. Averaging sub-images of 76 vesicles from the \textit{vti1a} \textit{wild type} cells, we obtained an averaged vesicular spot of Gaussian shape, as expected (fig2.3C). Strikingly, averaging the same sub-images in the Vti1a-channel resulted in a homogeneous signal, with no sign of Vti1a accumulation on the vesicle (fig2.3C). This shows that the "speckles" do not constitute a vesicle-associated signal and is strong evidence against localization of Vti1a on Syb2-positive LDCVs in CCs. Similar averaging of 63 vesicles in the \textit{vti1a} null CCs revealed homogeneous staining almost indistinguishable from \textit{wild type} cells (fig2.3D), indicating that background staining dominates the Vti1a-channel outside the TGN. Strikingly, the Syb2-signal of the averaged vesicle in \textit{vti1a} null cells was significantly weaker than in \textit{wild type} cells (fig2.3C and 2.3D, right panels), indicating that the Syb-2 level on the vesicle is depressed (see also below). Co-staining against Vti1a and chromograninB, another vesicular marker, also did not reveal co-localization (fig2.13E).

Overall, immunofluorescence combined with confocal and 3D-SIM established that Vti1a is present in a compartment near the TGN, where it partly co-localizes with and stabilizes Syntaxin6, a known participant in vesicle biogenesis. During biogenesis Vti1a is sorted away from the nascent vesicle, since it is absent from mature vesicles. The important role of Vti1a in vesicle biogenesis is demonstrated by the fact that its absence results in fewer mature vesicles (see also below) containing less Syb-2.
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Figure 2.2: MCCs of vti1a null mice show decreased levels of Syntaxin6 and Synaptobrevin-2. Ai. Confocal images of wild type (top) and vti1a null (bottom) CCs co-stained against Vti1a (green) together with Syb-2, Syntaxin6, GM130 or Lamp1 (magenta). Aii. Quantification of syb-2 (wild type: n=40 cells, vti1a null: n=40 cells), Syntaxin6 (wild type: n=43, vti1a null: n=41) and GM130 (wild type: n=29, vti1a null: n=23) fluorescence intensities and Lamp1 positive lysosomes (wild type: n=26, vti1a null: n=26). Bi. Western blots of wild type and vti1a null whole adrenal homogenates probed against Syntaxin6 (syx6), Synaptobrevin-2 (syb-2), SNAP-47, SNAP-29, SNAP-23, Syntaxin16 (syx16), SNAP-25 and VAMP4. Bii. Quantification of protein levels in the vti1a null relative to wild type. Note that a similar reduction in Synaptobrevin-2 levels was detected by both analyses, while a reduction in Syntaxin6 was only detected in immunostainings. Data are mean ± SEM, ♯: p=0.06, paired t-test, two-tailed. Scale bar: 2 μm.
Figure 2.3: Mature secretory vesicles are devoid of Vti1a staining A-B. 3D-SIM optical section obtained near the footprint of a wild type cell (A) and a vti1a null cell (B) stained for Vti1a (green) and Syb-2/VAMP2 (magenta). Syb-2 positive granules appear to be largely devoid of Vti1a, as shown by the line profile (A, bottom panels). In 3D-SIM, the homogeneous background staining in the vti1a null appears as “speckles” (B, bottom panel). C. To investigate whether Vti1a is enriched on LDCVs, individual vesicles were aligned by their Syb-2 fluorescence and averaged. The Syb-2 staining (middle panel) was highly enriched on the vesicle, while the averaged Vti1a staining (left panel) was homogenous, showing that there is no enrichment of Vti1a on the vesicle. The line is a fit of a Gaussian to the Syb-2 signal. D. Single vesicle fluorescence analysis in the vti1a null. The peak fluorescence values for Syb-2 are highly significantly reduced compared to the wild type (panel C). The line shows the best fit of a Gaussian. Data are means, error bars SEM. Number of cells (n) and vesicles (N) that were analyzed in C and D: wild type: n=3, N=76; vti1a null: n=3, N=63. Scale bar: 2 µm.
Exocytosis and Ca$^{2+}$-channel abundance is reduced by deletion of vti1a

In order to understand how exocytosis depends on Vti1a, we performed cellular capacitance measurements. During exocytosis, the addition of vesicular membrane to the PM leads to an increase in the surface area of the cell, which is proportional to the increase in cellular capacitance. We used a well characterized stimulation protocol to elicit exocytosis: six 10 ms depolarization followed by four depolarizations of 100 ms duration (Voets et al., 1999). We found that the total amount of exocytosis triggered by this type of stimulation was reduced in vti1a null cells compared to littermate controls (fig2.4Ai). The difference between the groups was augmented during a second stimulation 60 s after the first one, leading to a highly significant decrease in release from Vti1a deficient cells (fig2.4Aii, 2.4C). In these experiments we simultaneously performed amperometric measurements which showed decreased transmitter release from the Vti1a deficient cells (fig2.4A bottom panels). Intracellular Ca$^{2+}$-concentrations were measured simultaneously using microfluorimetry and showed significantly lower Ca$^{2+}$-levels in response to the depolarizations in knockout cells (fig2.4A, top panels). We therefore quantified the sizes of Na$^{+}$ and Ca$^{2+}$-currents measured during the first depolarization and indeed found that Ca$^{2+}$ currents were significantly reduced in Vti1a-deficient cells (fig2.16B).

To further investigate this point, we argued that the Ca$^{2+}$-current reduction could either be due to (I) a change in Ca$^{2+}$-channel activation, (II) a decrease in the single channel conductance or (III) a reduction in Ca$^{2+}$-channel number. We investigated hypothesis 1 by performing tail-current analysis and plotted the activation curve (fig2.14A). Wild type and vti1a null CCs had overlapping activation curves and indistinguishable activation thresholds (fig2.14A), ruling out hypothesis (I). Next, we performed non-stationary fluctuation analysis on the activation of the Ca$^{2+}$-currents (Fenwick et al., 1982). Fitting the variance-mean relationship with parabolas allowed us to estimate the single channel conductance and the number of channels. We found that single channel currents were not significantly different between vti1a null and wild type, although a trend towards even larger currents in the knockout was found. In contrast, the number of channels was significantly lower in vti1a null CCs (fig2.14B). To verify this finding by independent means, we performed live staining (on ice to prevent endocytosis) with an antibody recognizing the extracellular part of the α2δ4 subunit of all Ca$^{2+}$-channel subtypes expressed in CCs. Quantitative confocal analysis revealed a highly significant reduction in the Ca$^{2+}$-channel levels (fig2.4D), in line with our electrophysiological analysis. Thus, we conclude that Vti1a is involved in maintaining normal Ca$^{2+}$-channel numbers at the PM, consistent with a role for Vti1a in Ca$^{2+}$-channel trafficking.

Exocytosis is decreased by vti1a-deletion even after stimulation by-passing Ca$^{2+}$-channels

In order to test whether the observed exocytosis defect in knockout cells was secondary to the reduction in Ca$^{2+}$-currents we progressed with a stimulation protocol that is independent of Ca$^{2+}$-channel-activation.

We used Ca$^{2+}$-uncaging to trigger exocytosis, which evoked a robust multi-phasic increase of cellular capacitance in wild type cells, due to the fusion of catecholamine-containing vesicles, illustrated by a concomitant oxidative current through the amperometric fiber (fig2.5A).
Figure 2.4: Vti1a-deficient cells show reduced secretion and depressed surface levels of voltage gated Ca\textsuperscript{2+}-channels Ai and Aii. Top panel: depolarization protocol used to elicit secretion. The resulting increase in intracellular Ca\textsuperscript{2+}-levels (second panel) was paralleled by an increase of cellular capacitance (third panel) and cumulative amperometric charge (bottom panel), indicating release of secretory vesicles. The second stimulation (Aii) was applied 60 s after the first one. B. Average Na\textsuperscript{+} and Ca\textsuperscript{2+} currents induced by the first voltage depolarization. The bar diagram shows the quantification. C. Quantification of capacitance increase during and between the two depolarization protocols. D. Confocal midsections (left panels) of wild type and vti1a null CCs stained live with an antibody against an extracellular stretch of the α2δ loop of voltage-gated Ca\textsuperscript{2+}-channels. The bar diagram shows the quantification. All panels: wild type control is shown in black (or white bars in B and C histograms), vti1a null in blue. Traces and bar diagrams show means ± SEM. Number of cells (n) in A-C: wild type: n=61; vti1a null n=66. Number of cells (n) in D wild type: n=59; vti1a null: n=60. Scale bar: 2 µm.
Ca\textsuperscript{2+}-triggered release from Vti1a deficient CCs was significantly impaired, resulting in approximately 50% less capacitance increase (fig2.5A and 2.5B). The size of the exocytotic burst (exocytosis elicited within 1 s of the calcium stimulus) reports on the number of pre-primed vesicles at the time of the stimulus, whereas the sustained component (exocytosis between 1 and 5 s after the stimulus) reports on the rate of ongoing vesicle priming at high [Ca\textsuperscript{2+}]. Both components were reduced in similar proportions (fig2.5B). However, the temporal behavior of the capacitance traces was very similar between the wild type and knockout cells when the responses were normalized (fig2.5C). This shows that although the magnitude of the response is affected by the loss of vti1a the kinetics of release is unaltered.

Thus, vti1a null cells display a clear defect in the extent, but not the kinetics, of secretion, when using a stimulus independent of Ca\textsuperscript{2+}-channels. The defect appears overall stronger when cells are tested with Ca\textsuperscript{2+}-uncaging (fig2.5) than when probed by depolarization (fig2.4). This is most likely due to the higher basal Ca\textsuperscript{2+} concentration used during uncaging experiments (784 ± 23 nM in wild type cells; 831 ± 31 nM in vti1a null cells) than during depolarization experiments (68.8 ± 5.8 nM in wild type cells; 69.0 ± 6.3 nM in vti1a null cells). This increase in basal Ca\textsuperscript{2+} concentration causes an increase in the releasable vesicle pools by stimulating priming (Voets, 2000) and therefore increases the demand for secretory vesicles, which the vti1a null cells cannot meet (see also below). This is consistent with the finding that the decrease in secretion was stronger during the second round of depolarizations (fig2.3). We conclude that vti1a null cells suffer from a secretion defect, which is more severe during stronger stimulation.

Loss of Vti1a does not affect the Ca\textsuperscript{2+}-sensitivity of exocytosis

We directly tested the Ca\textsuperscript{2+}-sensitivity of exocytosis in so-called ”ramp-experiments”, where the intracellular Ca\textsuperscript{2+}-concentration is increased slowly (ramp-like) while the cellular capacitance and Ca\textsuperscript{2+} concentrations are measured simultaneously (Sørensen et al., 2002). The Ca\textsuperscript{2+} level at the point of fastest secretion acceleration the release threshold was determined as a sensitive read-out of the Ca\textsuperscript{2+}-sensitivity for release (vertical lines in fig2.15A and B). This parameter is changed when mutating components of the release machinery including synaptotagmin-1 (syt-1) (Sørensen et al., 2003b) and the syt-1:SNARE interaction site (Mohrmann et al., 2013). The average release threshold (vertical dashed lines and error bars in fig2.15C) was similar in both conditions (wild type: 1.29 ± 0.11 µM, n=50; vti1a null: 1.22 ± 0.08 µM, n=48), demonstrating normal Ca\textsuperscript{2+} sensitivity of the release machinery in Vti1a deficient CCs.

Since neither the kinetics, nor the Ca\textsuperscript{2+}-sensitivity of release were changed in the vti1a null CC, we conclude that exocytosis triggering is independent of Vti1a, and the secretion defect in knockout cells must be due to reactions upstream of exocytosis itself.
Figure 2.5: Loss of Vti1a results in a decreased number of releasable (primed) vesicles. A. Exocytosis induced by Ca$^{2+}$-uncaging is reduced in vti1a nulls. Ca$^{2+}$-uncaging at 0.5 s (at red arrow) led to a rapid increase in the global Ca$^{2+}$-concentration (top) which resulted in vesicle fusion leading to an increase in cellular capacitance (middle), amperometric current (bottom, left ordinate) and cumulative charge (bottom, right ordinate). B. Quantification of the cellular capacitance prior to uncaging (cell size) and changes in capacitance at 1 s (burst) and 5 s (total) after uncaging. The sustained release (sust.) is the difference between total and burst secretion. C. Release kinetics is unaltered in vti1a nulls: capacitance curves (from the middle panel of A) scaled to their respective values at 1 s have similar shapes.
Vti1a-loss decreases the number of secretory granules

In order to investigate which upstream reactions were affected by the loss of Vti1a we performed ultra-structural analyses of CCs from vti1a null mice (fig2.6 and 2.7). We found that the overall cell morphology was normal in the vti1a null, but loss of vti1a profoundly decreased the total number of LDCVs by approx. 40% (fig2.6Ai, 2.6Bi, 2.7Ci and 2.7Cii). The number of vesicles docked to the PM was reduced to a similar extent (fig2.6Aii, 2.6Bii and 2.7Ciii), indicating that the defect in docking is secondary to the reduction in the number of granules. The reduction in exocytotic burst size (fig2.5A) indicative for vesicle priming also roughly agrees with the reduction in vesicle numbers. Overall, these findings are consistent with the reduction in exocytosis being caused by the paucity of vesicles in the cell, not by a defect in the docking, priming or triggering mechanisms.

We analyzed the morphology of individual vesicles and found that Vti1a deficient mice had slightly, but significantly, smaller vesicles (fig2.6Aiii, 2.6Biii, 2.7Civ and 2.7Cv). Docked vesicles showed the same trend as non-docked (fig2.6Civ). This finding correlates with the lower Syb-2 staining per vesicle identified previously (fig2.3C-D), and, indeed, when we calculated the average Syb-2 staining per vesicle (fig2.3C-D) and normalized to the membrane area of the vesicles, calculated from the diameters (fig2.6Civ), the specific Syb-2 abundance was indistinguishable between wild type and vti1a null (normalized data: wild type=100 ± 14%; vti1a null: 93 ± 16%). Thus, vesicles are fewer and smaller in the vti1a null CCs, but maintain similar specific Syb-2 density in the membrane. These findings again points to a function of Vti1a in the formation and maturation of LDCVs.

Lack of re-release of vesicular components in chromaffin cells

The reduction in exocytosis and vesicle numbers found in the vti1a null CCs would be consistent with a role for Vti1a in vesicle biogenesis, in agreement with its localization to immature vesicles and the TGN-compartments leading to formation of mature LDCVs. However, another possibility is that the reduction in vesicle number is caused indirectly, by a defect in recycling of vesicular components back to the TGN, consistent with the proposed role of Vti1a in endosome-to-golgi fusion (Mallard et al., 2002). However, the extent to which recycling of vesicular components plays a role in LDCV biogenesis in CCs is unknown.

In an attempt to distinguish between those two possibilities, we examined whether the vesicular component Syt-1 would recycle to functional vesicles and thus be available for re-release at later times. We incubated CCs undergoing exocytosis with a Syt-1 antibody covalently liked to cypHer, a pH-sensitive cyanine dye, which is quenched at neutral, but fluorescent at low pH (Hua et al., 2011) (fig2.8A). Vesicle cycling (exocytosis followed by endocytosis) was stimulated by short incubation of the cells in a solution with a high K⁺-concentration in the presence of the antibody. This was followed by 1 hour incubation in culture medium at 37o in a CO2-incubator (fig2.8B). The cells were washed, and imaged. Overall, staining was weak, but specific, as it was quenched by the alkalization of intracellular compartments with NH4Cl (pH 8.2, fig2.8D), unaffected by acidic wash (acetic acid, pH 5.5, fig2.8D), reduced by preimubcation of the antibody with the antigenic peptide (fig2.16B) and identical treatment of cells in the absence of the antibody resulted in no fluorescence (fig2.16A). Staining in wild type cells was two times stronger than in vti1a null CCs (fig2.8D), consistent with the difference in secretory capacity.
Figure 2.6: *VTI1A* null cells have fewer and smaller large dense core vesicles A-B. Electron micrographs of cultured wild type (A) and *vti1a* null (B) cell, showing overall normal morphology. Scale bars = 1000 nm. Aii-Bii. Close-up of wild type (Aii) and *vti1a* null cells (Bii) showing docked vesicles. Aiii-Biii. Examples of docked and undocked vesicles in the wild type (Aiii) and the *vti1a* null (Biii).
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Figure 2.7: Vti1a null cells have fewer and smaller large dense core vesicles Ci. Cumulative distribution of the number of vesicles from the surface of the cell. Vti1a null cells have overall fewer vesicles. Insert: Close-up of the first 100 nm, showing differences in docked vesicles (corresponds to first bin). Cii-Ciii. Bar diagrams showing a reduction in total number of vesicles and docked vesicles in the vti1a null. Number of cells (n): wild type n=28, vti1a null n=23. Civ. Average diameter (mean of cell means) of undocked vesicles, docked vesicles and all vesicles. Cv. Size distribution of all vesicles in wild type and vti1a null cells. Number of cells (n): wild type: n=21, vti1a null: n=21. *: p=0.0677.
A second stimulation with high-K⁺-solution did not lead to any detectable exocytosis of cypHer-loaded compartments, as shown by the lack of a significant decrease in fluorescence (fig2.8D).

The lack of significant re-release indicates that Syt-1 does not recycle to releasable vesicles in either wild type or vti1a null CCs. These data suggest that a normal secretion capacity of CCs can be maintained without recycling constituents of the release machinery, at least on a timescale of hours. Longer incubations led to weaker signals probably indicative of Syt1-breakdown, rather than targeting to the TGN (fig2.16A). The fluorescence decay in vti1a null and wild type cells occurred at similar speeds, suggesting that protein degradation occurs with the same capacity independent of Vti1a. However, we cannot rule out that a minor fraction of Syt-1 recycles to new vesicles after longer times. Our experiment overall suggests that a possible role of vti1a in retrograde trafficking is not likely to explain the observed secretion phenotype and a role in the anterograde pathway is the most likely explanation for the observed impairment of the secretion response.

Vti1b does not compensate for the loss of Vti1a

The closest related protein to Vti1a in mouse is Vti1b and previous analysis of Vti1b-deficient mice showed only subtle defects (Atlashkin et al, 2003), whereas double vti1a/vti1b null mice are not viable (Kunwar et al., 2011). This led to the suggestion that these proteins might compensate for each other. We studied the role of Vti1b in secretion and secretory vesicle biogenesis and possible compensation by vti1b in the vti1a null by analyzing vti1b null and vti1a/b double null mice.

Loss of Vti1b neither had any effect on exocytosis evoked by Ca²⁺-uncaging (fig2.17), nor did it change the number or distribution of LDCVs (fig2.10Bvi and -Bvii).
Figure 2.8: Antibody uptake experiment fails to demonstrate re-release of Syt-1. A. The antibody against Syt-1 was labeled with CypHer5E, a pH-dependent dye, which is fluorescent in acidic compartments. This antibody would therefore label Syt-1 in endosomes or recycling vesicles. B. Cells were incubated with high-K$^+$ solution (KCl Sol.) and washed with normal extracellular solution in the continuous presence of the antibody and then transferred to a CO2-incubator for 1 h at 37°C. Images were captured twice in extracellular solution (to assess bleaching), then once in the presence of high-K$^+$ solution, then in acidic buffer (AcOH: acetic acid, 50 mM), and finally in ammonium-buffer (NH4Cl: 50 mM). C. Transmission (left) and fluorescent images in the presence of acetic acid (AcOH, middle) and NH4Cl. Scale bar = 2 µm. Notice that dimmer staining in the presence of ammonium overlaps with staining in the presence of AcOH, indicating incomplete quenching of fluorescence, rather than unspecific staining. D. Left: Total fluorescence in wild type and vti1a null cells in extracellular solution. Middle: Fluorescence in extracellular solution (2nd image, see panel B, "Rest"), high-K$^+$ ("KCl"), AcOH and NH4Cl-solution; all are normalized to the intensity during the 1st image. The difference between the "Rest" and the value 1.0 reports on the bleaching from one image to the next. Right: same as middle, in vti1a null cells. Data are means, error bars SEM. ♯: p=0.06. Number of cells: wild type: n=40; vti1a null: n=29.
Figure 2.9: The phenotype of \textit{vti1a/vti1b} double null CCs is similar to that of the \textit{vti1a} null Ai. Ca\(^{2+}\)-uncaging experiment. Panels arranged like in Figure 5A. Aii. Quantification of cell size, total-, burst- and sustained secretion reveal a reduction of all release phases in \textit{vti1a/b} double nulls (DKO) compared to littermate double heterozygous (DHet) controls. Aiii. Capacitance curves scaled to their respective values at 1 s have similar shapes in \textit{vti1a} DKO and DHet cells. Number of cells: \textit{vti1a/b} DHet: n=38; \textit{vti1a/b} DKO: n=52.

In the double \textit{vti1a/vti1b} null, secretion, total- and docked vesicle numbers were reduced to a similar extent as in the \textit{vti1a} null (fig2.9Ai-2.10Bvii). This demonstrates that the additional loss of Vti1as closest relative Vti1b did not increase the severity of the \textit{vti1a} null phenotype and establishes that \textit{vti1b} does not compensate for \textit{vti1a} loss in its absence. Therefore, we conclude that Vti1a and Vti1b subserve non-overlapping roles in adrenal CCs.

\textbf{Long-, but not short-term expression of \textit{vti1a} restores secretion}

In conventional knockout animals the deletion of the gene affects the whole organism. Therefore, our observations from CCs taken shortly after birth could theoretically be due to developmental defects that affected the creation of these cells at earlier stages, without \textit{vti1a} playing a direct role in the biogenesis of vesicles at the time of the experiment. Therefore, we tested whether the acute re-expression of the protein rescued the knockout phenotype.
Figure 2.10: The phenotype of vti1a/vti1b double null CCs is similar to that of the vti1a null Bi-Biv. Electron micrographs of CCs in the intact gland of vti1a/vti1b DHet animals (Bi, Bii) and a vti1a/b DKO animal (Biii, Biv). Bi, Bii: scale bars = 1000 nm; Bii, Biv: scale bars = 200 nm. Bv. Size distribution of secretory vesicles in DHet (grey bars), and the DKO (red bars), respectively. The double null has smaller vesicles, consistent with findings in vti1a null cells. Bvi, the number of docked vesicles is reduced in the vti1a null and the vti1a/b DKO s. Animals prepared in parallel (as littermates) are shown together. For the vti1b null, we did not obtain wild type littermates. Bvii, the total number of vesicles is reduced in the vti1a null and the vti1a/b DKO s. Data are mean, error bars SEM. Number of cells: vti1a/b DHet: n=20; vti1a/b DKO: n=20; wild type: n=20; vti1a null: n=20; vti1b null: n=18.
We first used the Semliki forest virus (SFV), which is widely used for rescue experiments in CCs (e.g., Borisovska et al., 2005; Liu et al., 2008; Sørensen et al., 2003b; Tian et al., 2005; Walter et al., 2010)). Infection with the virus resulted in robust expression of \textit{vti1a} as early as six hours after infection with protein levels in 20-fold excess of the endogenous \textit{wild type}-levels. However, exocytosis from cells expressing the protein for six to eight hours showed no rescue (fig2.11C); they were as impaired in their release as Vti1a-deficient cells, while \textit{wild type} littermates displayed robust secretion in parallel experiments. At longer times, the health of the CCs suffered due to the transient nature of Semliki Forest infection, which eventually leads to the death of the cells.

Our previous (successful) use of the SFV-system to induce rescue within 6-8 hours was focused on proteins directly involved in exocytosis, including the SNAREs and Syt-1. We suspected that rescue with Vti1a might require longer times, because of its role in vesicle generation, which has a longer turnover time (Duncan et al., 2003). Therefore, we used the lentiviral (LV) expression system, which allows stable and long-term expression. Infection of \textit{vti1a}-knockout-cells with LVs resulted in 2-fold expression of \textit{vti1a} 48 h after infection, with similar localization of the protein as in the \textit{wild type} (fig2.11A). Re-expression of \textit{vti1a} with LVs rescued the observed defect in secretion. Only the sustained component seemed to be still slightly depressed after rescue. However, statistical analysis showed significant rescue during all phases of exocytosis (fig2.11B). This experiment demonstrates that the \textit{vti1a} null phenotype is cell autonomous and reversible upon re-expression of the protein. The lack of rescue using a strong, transient expression system (like the SFV) appears inconsistent with a direct involvement of Vti1a in exocytosis, but in line with the action on an upstream step involved in vesicle generation.
Figure 2.11: Rescue of secretion in nulls requires long-term expression of \( vti1a \)

A. Staining of \( vti1a \) nulls rescued with Lentiviruses expressing \( vti1a \) (magenta) and eGFP (green). The right panel shows two cells, one is infected with Lentivirus, one is not. Scale bars: 2 µm. Bi. \( \text{Ca}^{2+} \)-uncaging experiment, panels arranged like in Figure 5A. Shown are three groups: wild type littermates (black traces), \( vti1a \) nulls (blue traces), and \( vti1a \) nulls rescued with Lentiviruses expressing \( vti1a \) (lilac trace). Bii. Quantification of cell size, total-, burst- and sustained secretion reveals significant rescue of all components (total, burst and sustained release) by lentiviral expression. Biii. Release kinetics is unaltered in all groups: capacitance curves (from panel Bi) scaled to their respective values at 1 s have similar shapes. C. Rescue of burst component as a function of expression time, using Semlikivirus, or Lentivirus. Data are means ± SEM. Number of cells: wild type: \( n=34 \); \( vti1a \) null: \( n=30 \); \( vti1a \) null + \( vti1a \) (Lenti): \( n=30 \); \( vti1a \) null + \( vti1a \) (Semliki, 6h): \( n=9 \); \( vti1a \) null + \( vti1a \) (Semliki, 7h): \( n=15 \); \( vti1a \) null + \( vti1a \) (Semliki, 8h): \( n=9 \). The wild type and knockout data in B are the same as shown in Figure 5. All three groups of cells were measured in parallel, from the same animals in the case of knockout and rescue, and from littermates in the case of wild types.
This tentative model for secretory vesicle biogenesis postulates that sequential vesicle fusion of immature vesicles utilizing a full complement of SNAREs, at least including Vti1a and Syntaxin6, and probably VAMP4, is a prerequisite for formation of secretory granules. Immature vesicles form by budding from the TGN using PICK1 and ICA69 (Cao et al., 2013; Holst et al., 2013). To account for the observation that Synaptobrevin-2 can be re-supplied to Synaptobrevin-2 knockout cells within several hours to create fully fusogenic vesicles, whereas Vti1a requires long-term (days) re-expression, we suggest that Synaptobrevin-2 is supplied to almost mature (“pre-mature”) vesicles at a very late stage. The model does not show the return of Vti1a/Syntaxin6/VAMP4 to the TGN.

Figure 2.12: Rescue of secretion in nulls requires long-term expression of vti1a

Chromogranins, other LDCV cargo
PICK1/ICA-69
Discussion

We found that vti1a-deletion results in a reduction of catecholamine exocytosis without changes in fusion kinetics or Ca$^{2+}$-dependence of fusion. This indicates that not the exocytosis mechanism, but the number of fusing vesicles is changed by vti1a deletion. Indeed, electron microscopy demonstrated a proportional decrease in docked and total vesicle numbers in vti1a null cells, indicating Vti1a involvement in upstream processes leading to the formation of vesicles. Furthermore, functional rescue required long-term (2 days) expression of vti1a, whereas short-term overexpression was inefficient. This points again to an upstream role of vti1a (see below). Finally, using 3D-structured illumination microscopy we showed that Vti1a is not present on mature chromaffin vesicles. The conclusion that Vti1a acts on an upstream step aligns with previous findings that docking, priming and fusion in these cells depend on other SNAREs, namely Syntaxin1a, Synaptobrevin-2 and SNAP-25 together with the Ca$^{2+}$-sensor Synaptotagmin-1 (Borisovska et al., 2005; de Wit et al., 2006, 2009; Sørensen et al., 2003a).

Vti1a is found on a subset of synaptic vesicles (Antonin et al., 2000a; Hoopmann et al., 2010; Ramirez et al., 2012), and recent data implicated Vti1a in spontaneous fusion (Ramirez et al., 2012), which is controversial (Hoopmann et al., 2010). Adrenal CCs show little spontaneous release and we here show that Vti1a does not localize to LDCVs, and that release rates and Ca$^{2+}$-sensitivities are unchanged in the vti1a null, ruling out a direct role for Vti1a in fusion of these vesicles. However, our findings indicate three different ways in which Vti1a affects release indirectly. First, by participating in vesicle biogenesis at or near the TGN, Vti1a is a prerequisite for generating LDCVs in normal numbers. Second, Vti1a also plays a role for the properties of generated vesicles, with LDCVs in the vti1a null being smaller, although interestingly their specific Syb-2/VAMP2 density was maintained. Third, in vti1a null cells, fewer Ca$^{2+}$-channels were present at the membrane, consistent with a trafficking defect of these channels. This finding is reminiscent of a previous report that Vti1a participates in trafficking of Kv4 K$^+$ channels and KChIPs (K$^+$ channel-interacting proteins) to the PM (Flowerdew and Burgoyne, 2009).

Given our findings it is possible that Vti1a participates in the generation of neuropeptide-containing dense-core vesicles in neurons, and/or in trafficking of Ca$^{2+}$-channels. Thus, elimination of vti1a might influence neurotransmission indirectly, by changing secretion of neuropeptides, as is the case for Synaptotagmin-4 (Dean et al., 2009), or by reducing Ca$^{2+}$-currents. Synaptotagmin-4 has been implicated in dense-core vesicle maturation via interaction with Syntaxin6 (Ahras et al., 2006), and it appears likely that Vti1a is part of the same complex. Further studies will be required to understand the function of Vti1a in neurons.

Our data clearly demonstrate that Vti1a is involved in generation of LDCVs. In its absence, the total number of vesicles was reduced, resulting in fewer vesicles that were docked to the PM and in a reduction of the RRP. The role of Vti1a in vesicle generation is in line with previous data showing impaired delivery of GLUT4 to the membrane following vti1a knockdown (Bose et al., 2005). Notably, the Vti1a partner VAMP4 has been implicated in biosynthetic entry of GLUT4 into insulin-responsive vesicles (Williams and Pessin, 2008), whereas fusion of GLUT4 vesicles with the PM requires a different SNARE complex consisting of Syntaxin4, SNAP-23 and Syb-2 (Kawanishi et al., 2000), which aligns well with our findings of an upstream function for Vti1a in vesicle generation.
Immunofluorescence localized Vti1a to a compartment near the TGN, which contains the Vti1a-partner Syntaxin6, but not TGN38. A similar Syntaxin6 positive and TGN38-negative compartment was previously found to be involved in GLUT4 recycling (Shewan et al., 2003). Recently, the BAR-domain proteins PICK1 and ICA69 were found to be involved in dense-core vesicle generation in growth hormone secreting cells (Holst et al., 2013) and in pancreatic beta-cells (Cao et al., 2013). Interestingly, PICK1 co-localized better with Syntaxin6 than with TGN38 (Holst et al., 2013), pointing to a similar localization as Vti1a. Thus, a picture is emerging that a Syntaxin6 positive compartment close to the TGN is specifically involved in vesicle generation. This compartment has been referred to as "a subdomain of the TGN" (Shewan et al., 2003), or based on the colocalization with Syntaxin6 and experiments with brefeldin A simply "immature vesicles" (Cao et al., 2013; Holst et al., 2013). Experiments using immuno-EM will be required to characterize this compartment in more detail. Together with Vti1a this compartment then contains Syntaxin6 and possibly VAMP-4 (Wendler et al., 2001). The final partner needed for a functional SNARE-complex is likely Syntaxin13 or Syntaxin16, which are known partners of Vti1a (Brandhorst et al., 2006; Kreykenbohm et al., 2002; Mallard et al., 2002; Zwilling et al., 2007). A full complement of SNAREs in this compartment prompts the question why a membrane fusion step is required during vesicle biogenesis?

An interesting finding was that long-term (2 days) re-expression of vti1a was necessary for rescue of the vti1a null phenotype, whereas short-term expression was ineffective. This contrasts with functional rescue within 8 hours, which has been demonstrated for the integral vesicular membrane proteins Syb-2 and Syt-1 that are directly involved in exocytosis triggering (Borisovska et al., 2005; Nagy et al., 2006; Walter et al., 2010). One idea, which would integrate this finding with the need for membrane fusion in vesicle biogenesis, is that mature vesicles are generated by several sequential fusion reactions between immature vesicles (Wendler et al., 2001), until the full vesicle complement is achieved (fig2.12). In this cascade, incorporation of Syb-2 and Syt-1 might be the last events to occur, because they will only be needed for fusion with the PM, and thus they can be supplied to almost-mature vesicles within a few hours. However, the formation of vesicles through fusion of immature precursors might start further upstream, and involve fusion reactions taking place over the course of days (fig2.12). Therefore, eliminating the basal fusion machinery between these precursor vesicles will also take days to remedy. This idea aligns well with the demonstrated need for Syntaxin6 in immature vesicle biogenesis, is that mature vesicles are generated by several sequential fusion reactions between immature vesicles (Wendler et al., 2001), until the full vesicle complement is achieved (fig2.12). In this cascade, incorporation of Syb-2 and Syt-1 might be the last events to occur, because they will only be needed for fusion with the PM, and thus they can be supplied to almost-mature vesicles within a few hours. However, the formation of vesicles through fusion of immature precursors might start further upstream, and involve fusion reactions taking place over the course of days (fig2.12). Therefore, eliminating the basal fusion machinery between these precursor vesicles will also take days to remedy. This idea aligns well with the demonstrated need for Syntaxin6 in immature vesicle biogenesis (Wendler et al., 2001). It further accounts for the lack of co-localization between Syb-2 and Vti1a within the TGN-area, which is hard to explain if Syb-2 is incorporated into immature vesicles at the beginning of the biosynthetic cascade.

Alternatively to an anterograde function in homotypic fusion of immature vesicles, vesicle formation could be affected indirectly by deletion of Vti1a, by impairing recycling of components of the fused vesicles back to the TGN (retrograde pathway). However, studying Syt-1 cycling we demonstrated that re-release is not a predominant mechanism. This suggests that a normal secretion capacity in CCs can be maintained without recycling constituents of the release machinery. This appears more consistent with a function of Vti1a in an anterograde pathway, although recycling of components over longer times than resolved in our experiment cannot be ruled out.

Even in the absence of Vti1a homotypic fusion of immature vesicles must be able to proceed, but with decreased efficiency. This is reflected by the observation of mature albeit fewer and slightly smaller LDCVs and substantial exocytosis even in the vti1a null.
Thus, other SNAREs must be able to compensate for Vti1a-loss, or alternatively this fusion reaction can be bypassed altogether during the formation of mature vesicles. Compensation of one SNARE by another is expected, because SNAREs display a certain degree of promiscuity, as long as the QabcR-rule of formation is obeyed (Jahn and Scheller, 2006). Thus, candidates for SNAREs compensating for Vti1a are other Qb-SNAREs.

The closest related Qb SNARE is Vti1b, even though the two proteins only share 30% homology (Fischer von Mollard and Stevens, 1998). We found that vti1b knockout cells showed no defect in LDCV generation or release. The simultaneous loss of both vti1a and vti1b did not result in additional defects, with vesicle priming, vesicle numbers and vesicle sizes similar to those in the vti1a single knockout. This shows that Vti1b cannot compensate for Vti1a in the secretory pathway and that the two proteins which have a single homologue in yeast have evolved to serve separate functions in mammals. Consequently, the neurodegenerative phenotype of the vti1a/vti1b double knockout mouse (Kunwar et al., 2011) in the absence of the same phenotype in the single knockouts (Atlashkin et al., 2003; Kunwar et al., 2011) might be a synergistic consequence of simultaneous defects in secretion (due to Vti1a loss) and late endosomal or lysosomal function (Vti1b loss, (Atlashkin et al., 2003)).

In conclusion, we have demonstrated that Vti1a, but not Vti1b, supports exocytosis in adrenal CCs by maintaining correct vesicle numbers, vesicle sizes, and number of Ca\(^{2+}\) channels in the PM. We suggest that Vti1a acts together with Syntaxin6 in an intermediate fusion step near the TGN, which eventually leads to the formation of mature dense-core vesicles.

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Materials and Methods

Mouse strains
We used mice of the vt1a and vt1b strains (Atlashkin et al., 2003; Kunwar et al., 2011). The mouse strains were kept heterozygous for the null allele and heterozygote crosses were used to generate null animals (knockouts). wild type littermates were used for control unless noted otherwise. The double vt1a/vt1b strain was kept in the double heterozygous condition and double knockouts were compared to double heterozygotes.

Expression viruses
The vt1a cDNA was ligated using BamHI and EcoRI-sites into a plasmid containing an IRES2 (EMCV-IRES)-EGFP element. The resulting vt1a-IRES2-EGFP cassette was ligated into the pSFV1 plasmid using SmaI and AvrII sites. For the generation of the lenti viral plasmid, the vt1a-IRES2-EGFP cassette was ligated into the multiple-cloning site of pLenti. All constructs were verified by sequencing. Generation of Semliki Forest Virus (SFV) and Lentiviral particles followed standard protocols.

Adrenal chromaffin cell cultures
Adrenal chromaffin cell cultures were prepared from P0-P1 pups, or occasionally from E18 embryos, as described previously (Sørensen et al., 2003a). The cells were incubated at 37°C and 8% CO2 and used within 4 d.

Immunoblotting
Western blot analysis was carried out using standard methods.

Immunofluorescence staining and confocal imaging
For staining of fixed cells, E18 or P1 chromaffin cells and paraformaldehyde fixation was used.

Confocal images were acquired with a 63X Plan-Neofluar lens (Numerical aperture 1.4, Carl Zeiss b.v. Weesp) and a zoomfactor 5 on a Zeiss 510 Meta Confocal microscope (Carl Zeiss). 3D-Structured illumination microscopy (3D-SIM) was performed using the Elyra PS.1 platform (Carl Zeiss) equipped with a 63X Plan-Apochromat lens (Numerical aperture 1.46, Carl Zeiss).

For stainings of Ca^{2+}-channels, live CCs were blocked (4% BSA in PBS cooled to 4 °C) for 5 min and incubated with an antibody against voltage-dependent Ca^{2+} channel subunits (rabbit anti-Cavα2δ4; dilution 1:200, Alomone labs) at 4 °C for 30 min, followed by washing and incubation with a secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit antibody; dilution 1:1000, Invitrogen) at 4 °C for 1 hr. The cells were fixed at room temperature with 4% paraformaldehyde for 15 min. Samples were mounted with Prolong Gold. Images were acquired using a Zeiss LSM710 point laser (Argon Lasos RMC781272) scanning confocal microscope with a Zeiss Plan-Apochromat 63X/NA 1.4 oil objective (Carl Zeiss, Germany). Analysis of immunofluorescence imaging data was done using ImageJ.
2. Vti1a in biogenesis

**Live cell imaging of Syt-cycling**
For antibody feeding experiments, cells were densely plated on 12 mm coverslips. Cells were first washed for a few seconds in extracellular solution (solution A). They were then placed into a high potassium containing depolarizing solution (solution B) containing a rabbit antibody (Synaptic Systems, cat. no. 105103; 1:50 dilution) against the luminal domain of mouse-Syt-1 coupled to the pH sensitive fluorescent probe CypHer5E by Synaptic Systems (custom made). Experiments were performed at 1:50 dilution. Cells were depolarized for 2.5 min at room temperature and then transferred for 5 min to solution A also containing the syt-1-cypHer antibody. Cells were washed for a few seconds in solution A without antibody and incubated in culture medium in the incubator for 1 h prior to image acquisition.

During imaging two images were acquired in ordinary extracellular solution (solution A), to evaluate photo-bleaching. This was followed by an image in depolarizing solution, one in low pH-solution (pH 5, buffered with MES), and one after treatment with NH4Cl, which quenches cypHer. All solutions were applied for 3 min prior to image acquisition.

**Electron microscopy**
Electron microscopy of intact glands (fig2.9 and 2.10): adrenal glands were removed on embryonic day 18 and emersion fixed for 2.5 h at room temperature with 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and further processed as previously described (Voets et al, 2001).

Electron microscopy of cultured cells (fig2.6): chromaffin cells were cultured on rat tail type I collagen-coated coverslips (Cellocate, Eppendorf, Germany). Cells (DIV2) were fixed for 45 min at room temperature with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), washed, embedded and analyzed as explained before (Toonen et al., 2006).

Analysis of secretory vesicle distribution was done blinded for the genotype of the animal. For each condition the distribution of secretory vesicles was analyzed in ultrathin sections (90 nm) of randomly selected chromaffin cells from different animals (and three different grids per animal). Only cells with a visible nucleus and clear-cut PM were taken into account. Secretory vesicles were recognized by their round, dense core. Docked vesicles were without any measurable distance between granule and PM. Distances from the granule membrane to the PM were measured on digital images acquired at 20,000x magnification by a Kodak MegaPlus 1.4i camera controlled by analySIS (Soft Imaging Systems / Olympus, Germany).

**Electrophysiology**
Calcium uncaging, measurement of cellular capacitance, amperometry, and cell calcium concentrations were carried out essentially as previously described (Pinheiro et al., 2013).

**Statistical analysis**
Data are represented as mean ± SEM, with n denoting the number of cells and statistical analysis was performed using two-tailed t tests if not noted otherwise in the text; *: p < 0.05, **: p < 0.01, ***: p < 0.001.
Figure 2.13: Confocal images of wild type and vti1a null CCs stained for Vti1a and Syb-2, GM130, Syntaxin6, Lamp1 and ChromograninB. A. Confocal images of wild type (top) and vti1a null (bottom) CCs co-stained against Vti1a (green) and Syb-2 (magenta). B. Confocal images of wildtype (top) and vti1a null (bottom) CCs co-stained against Vti1a (green) and GM130 (magenta). C. Confocal images of wild type (top) and vti1a null (bottom) CCs co-stained against Vti1a (green) and Syntaxin6 (magenta). D. Confocal images of CCs co-stained against Vti1a (green) and Lamp1 (magenta). Dii. Quantification of lysosome number, area and size. E. Confocal images of wild type CCs co-stained against Vti1a (green) and ChromograninB (magenta). The left panel in Dii shows the same data as in Figure 2Aii. Numbers on bars in (D) give the number of cells analyzed. Scale bars: 2 μm.
Figure 2.14: Vti1a deletion leads to a reduction of voltage gated Ca\(^{2+}\)-channels the plasma membrane

A. Examples of whole-cell voltage clamp experiments to test the current-to-voltage (I-V) relationship in wild type (black) and vti1a null (blue) CCs are shown on the left. Cells were clamped at -70 mV at rest and depolarized for 5 ms from -80 mV to +80 mV in 10 mV intervals. Channel activation was determined by analysis of tail currents and the average plotted against the membrane potential (error bars too small to be visible). This relationship was fit with a Boltzmann function to determine the membrane potential at which 50 % of the channels activated (V50). These values are shown in the right hand histogram. B. Non-stationary fluctuation analysis on repetitive depolarizations to 20 mV was performed. The two graphs on the left show two examples of the variance (var) in wild type (black) and vti1a null (blue) cells plotted against the average current (I) obtained during the activation-phase at the beginning of depolarizations. The inserts show 50 sweeps of the measured current (note that the variation in the current increases as the currents increase). The red lines show the best fit of a parabola to the data. The offset of the fit was fixed to match the variance measured in the currents prior to depolarization. The right hand histograms summarize the quantification of the fluctuation analysis. "i" signifies the single-channel current. The number of channels was divided by the cellular capacitance to compare the density of channels on the cell surface. Scale bars in B: 50 pA (vertical) and 1 ms (horizontal). Histograms show means, error bars are SEM, except for the histogram in panel A where the error of the fit is displayed. Number of cells (n): wild type: n=11; vti1a null: n=11; *: p=0.04.
Figure 2.15: Experiments with slowly increasing Ca\textsuperscript{2+}-levels (ramp experiments) reveal similar release thresholds in \textit{vti1a} nulls and littermate wildtypes (A and B) Representative examples of ramp experiments in \textit{wild types} (A) and \textit{vti1a} nulls (B). The top panel shows the increase in intracellular Ca\textsuperscript{2+}-concentration. The middle panels show the resulting capacitance increase and the bottom panel the amperometric current. The vertical lines represent the time point of maximal acceleration of the capacitance response that provides a sensitive readout of the Ca\textsuperscript{2+}-sensitivity of exocytosis. C. Many capacitance responses were replotted from experiments like the ones shown in A and B to reveal the Ca\textsuperscript{2+}-dependency of the capacitance increase. The dashed vertical lines represent the average Ca\textsuperscript{2+}-concentrations of maximum acceleration. All panels: \textit{wild type} control is shown in black, \textit{vti1a} nulls in blue. Traces and histograms show means, error bars are SEM. Number of cells (n): \textit{wild type}: n=48; \textit{vti1a} nulls: n=50.
Figure 2.16: Loss of Vti1a leads to less vesicle cycling, but protein degradation occurs at similar rates as in control cells. A. The total fluorescence of the cypHer-conjugated antibody in wild type and vti1a null cells is shown at two time points (1 and 5 h, the 1h data are the same as in Fig2.4). Both conditions show a decrease in fluorescence values at longer intervals, suggesting protein degradation with similar speed (similar slope). In all conditions the total fluorescence was subtracted by the fluorescence upon NH4Cl-treatment. The grey symbol and dashed line illustrate the fluorescence of wildtype cells stimulated and imaged in the same manner, but without exposure to the antibody (no AB, negative control). B. Antibody uptake occurs by recognition of to the Syt-1 epitope. As a specificity test, we investigated whether cypHer uptake into cycling vesicles occurred via the fluid phase (without antibody binding) or required the binding to the Syt-1 epitope. Fluorescence was assessed using the same antibody as in panel A (and Figure 8), but labelled with Oyster 550 instead of cypHer. Stimulation of wild type CCs in the presence of the antibody led to a robust increase in cellular fluorescence. In contrast, pre-incubation of the antibody with a peptide containing the antibody epitope lead to a partial block of dye uptake, signified by lower fluorescence intensity values. The (unspecific) fluorescence of cells measured in parallel which were stimulated in the absence of the antibody was subtracted from both datasets (baseline fluorescence) indicated by the grey dashed line (the SEM is shown as shaded region in the lower section of the graph). Data are means, error bars are SEM. Number of cells (n) in A at 1 h in the wild type (control): n=40; vti1a null: n=29; no AB: n=19. Number of cells (n) at 5 h in the wildtype: n=10; vti1a null: n=10. Number of cells (n) in B in the wild type: n=110; vti1a null: n=64; no AB: n=33.
Figure 2.17: Vti1b is dispensable for neurosecretory release from CCs. A. Ca$^{2+}$-uncaging at 0.5 s led to a rapid increase in the global Ca$^{2+}$-concentration (top), an increase in cellular capacitance (middle), amperometric current (bottom, left ordinate) and -charge (bottom, right ordinate). B. Quantification of cell size, total-, burst- and sustained secretion reveals no difference between *vti1b* null and littermate wild type controls. C. Release kinetics is unaltered in *vti1b* nulls: capacitance curves (from the middle panel of A) scaled to their respective values at 1 s have similar shapes. Data are means, error bars SEM. Wildtype control is shown in black (white bars in B histogram), *vti1b* nulls in green. Number of cells: wild type: n=42; *vti1b* null: n=48.