Vti1A and Neurobeachin: two trafficking proteins with different fates?

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In preparation
Abstract

The trans-Golgi/early endosomal SNARE protein Vti1a (Vacuolar protein sorting 1er p tail interacting 1a) and the brain-enriched scaffolding protein Neurobeachin (Nbea) have both been implicated in protein transport. We have shown in our earlier study that these two proteins reside in the same cellular compartment. In this study we set out to examine the impact of Nbea loss on the subcellular distribution of Vti1a and vice versa. In addition, we studied Vti1a’s function in more detail given the possibility that these two proteins act in the same cellular pathway. We show that depletion of Nbea does not affect the somatic localization of Vti1a, while loss of Vti1a leads to a small reduction in the number of Nbea puncta per unit dendrite in cultured hippocampal neurons, without perturbing their general connectivity and dendritic complexity. Adrenal chromaffin cells deficient in Vti1a exhibit a reduction in catecholamine release, while the overall architecture of the organelles constituting the secretory- and endo-lysosomal pathway is not significantly affected. Taken together, although in the soma Vti1a, just like Nbea, aligns closely with the Golgi apparatus, and its loss does have a minor impact on Nbea’s subcellular distribution, its lack of colocalization in dendrites and results from chromaffin cells do not support the premise that Vti1a and Nbea act in the same cellular pathway.
Introduction

When studying potential interaction partners of a specific protein, researchers often scrutinize the subcellular localization looking for colocalization of the proteins of interest. Colocalization only implies that the proteins are located within close proximity, without any indication of actual interaction. However, sometimes proteins that reside within close proximity do interact with each other or/and are involved in the same cellular function. Since, as observed in Chapter 2, Nbea resides in the same compartment as Vti1a (also referred to as Vti-2rp), it is only reasonable to examine Vti1a’s function in more detail, given the possibility that these two proteins act in the same functional pathway.

Protein transport and membrane flow in eukaryotic cells is mediated by transport vesicles that bud from the donor compartment and then target to and fuse with the appropriate acceptor compartment (Lupashin et al., 1997). A key role in such intracellular membrane fusion events is played by SNAREs (N-ethylmaleimide-sensitive factor attachment protein receptors), which have been conceptually divided into those associated primarily with vesicles (v-SNAREs) and those on target membranes (t-SNAREs; Holthuis et al., 1998; Rothman, 1994). All known SNARE-dependent fusion steps involve a member of the syntaxin family of t-SNARES (Holthuis et al., 1998).

Vti1p, the yeast counterpart of Vti1a, is a v-SNARE that binds to five of the eight yeast syntaxins: Sed5 (the yeast counterpart of syntaxin 5), Tlg1p (syntaxin 6), Tlg2p (syntaxin 16), Pep12p and Vam3p (Fischer von Mollard and Stevens, 1999; Fisher von Mollard et al., 1997; Holthuis et al., 1998; Lupashin et al., 1997; Ungermann et al., 1999). Vti1p seems to be involved in numerous transport pathways, ranging from several pathways to the vacuole, homotypic vacuolar fusion, intra-Golgi retrograde transport, and membrane retrieval from early and/or late endosomes (pre-vacuolar compartment - PVC) to the early and/or late Golgi (Fischer von Mollard and Stevens, 1999; Fisher von Mollard et al., 1997; Holthuis et al., 1998; Lupashin et al., 1997; Ungermann et al., 1999). It is tempting to speculate that the two mammalian counterparts, i.e. Vti1a and Vti1b (also referred to as Vti-rp1) evolved to mediate distinct transport events separately (Xu et al., 1998).

Indeed, in vitro experiments suggest distinct functions for the two proteins, with Fab fragments specific for Vti1a blocking fusion of early endosomes and the ones specific for Vti1b blocking fusion of late endosomes, but not vice versa (Antonin et al., 2000a). Vti1a exists in at least two distinct Golgi SNARE complexes - one containing syntaxin 5 (enriched in the cis-Golgi) and the other containing syntaxin 6 (enriched in the TGN; Xu et al., 1998). The latter, together with syntaxin16 and the endosomal VAMP4 has been implicated in an early/recycling endosomes-to-TGN transport pathway (Mallard et al., 2002). In addition, microinjection of antibodies against Vti1a into Vero cells specifically arrests the transport of the envelope protein (G-protein) of vesicular stomatitis virus from the ER to the plasma membrane at the Golgi apparatus (Xu et al., 1998). Thus, Vti1a, like Vti1p seems to be involved in the secretory as well as endosomal pathway. Vti1b together with syntaxin 7 and syntaxin 8, both of which have been previously associated with late endosomes and their fusion with
lysosomes (Mullock et al., 2000), forms a SNARE complex with endobrevin/VAMP8 (Antonin et al., 2000a).

The notion of a functional segregation of Vti1a and Vti1b is further strengthened by the fact that the Arabidopsis homologs AtVTI1a and AtVTI1b substitute for yeast Vti1p in two different trafficking steps. AtVTI1a can function in the traffic from the TGN to the PVC, but cannot replace Vti1p in the two alternative traffic pathways to the vacuole that do not pass through the PVC, i.e. along either the alkaline phosphatase (ALP) or the aminopeptidase I (API) (Zheng et al., 1999). The latter does not even pass through either the ER or the Golgi apparatus, but instead oligomerizes in the cytosol and is enclosed in a double membrane to form cytosol to vacuole (CTV) transport vesicles (Fischer von Mollard and Stevens, 1999). Alternatively, AtVTI1B can partially fulfill the function of Vti1p in API traffic along the CVT pathway (Zheng et al., 1999).

Also the subcellular localization of Vti1a and Vti1b differ. Vti1a, as shown by fractionation and immunofluorescence microscopy of rat kidney cells, as well as by immuno-electron microscopy of human fibroblasts, associates preferentially with the Golgi apparatus, especially the TGN (Kreykenbohm et al., 2002; Xu et al., 1998). Also, in cultured hippocampal neurons and hippocampal tissue sections Vti1a shows the familiar, perinuclear pattern in the cell body (Antonin et al., 2000b). However, in addition, it also colocalizes with synaptobrevin in the nerve terminals (Antonin et al., 2000b). The portion of Vti1a that is enriched on small synaptic vesicles is a brain-specific Vti1a splice variant, i.e. Vti1a-β (Antonin et al., 2000b). Vti1b on the other hand, is mainly localized to early endosomes and multivesicular late endosomes, with only low levels being present on the TGN, lysosomes or synaptic vesicles (Antonin et al., 2000b; Kreykenbohm et al., 2002).

Given the colocalization of Nbea with Vti1a, in this study we investigated whether and how the loss of Vti1a influences Nbea’s subcellular distribution and vice versa. We show that loss of Nbea does not disrupt the subcellular localization of Vti1a, while depletion of Vti1a results in a reduction of the number of Nbea puncta per unit dendrite. Furthermore, by performing exocytosis measurements in chromaffin cells, an established model for neurosecretion (Toonen et al., 2006) we demonstrate that Vti1a knock-out chromaffin cells possess fewer primed vesicles, while their organelle morphology is not perturbed.

Materials and Methods

Laboratory animals

Nbea and Vti1a KO mice have been described before (Kunwar et al., 2011; Su et al., 2004). For cultures of Nbea and Vti1a KO and WT hippocampal neurons mouse embryos were obtained at embryonic day 18 by caesarian section of pregnant females from timed mating of heterozygous animals (C57/B16 background). Vti1a KO and WT adrenal chromaffin cells, were obtained from postnatal day 1 (P1) mice. All animals were housed and bred according to the institutional and Dutch governmental
guidelines for animal welfare.

**Dissociated hippocampal cultures**

Dissected hippocampi from embryonic day 18 (E18) Nbea and Vti1a mice were collected in ice-cold Hanks Buffered Salt Solution (HBSS; Sigma), buffered with 7 mM HEPES (Invitrogen). They were incubated in Hanks-HEPES with 0.25% trypsin (Invitrogen) for 20 min at 37°C. After washing, neurons were triturated using a fire-polished Pasteur pipette and counted in a Fuchs-Rosenthal chamber. The cells were plated in pre-warmed Neurobasal medium (Invitrogen) supplemented with 2% B-27 (Invitrogen), 1.8% HEPES, 0.25% glutamax (Invitrogen) and 0.1% Pen/Strep (Invitrogen) at a density of 25k/well on 18 mm glass coverslips coated with a mixture of 0.1 mg/ml poly-D-lysine (Sigma), 0.2 mg/ml rat tail collagen (BD Biosciences) solution and 10.2 mM acetic acid solution (Sigma) containing a glial feeder layer.

**Adrenal chromaffin cell cultures**

Adrenal chromaffin cell cultures were prepared as described previously (Walter et al., 2010). In short, embryonic adrenal glands were dissected out, placed in filtered Locke’s solution (154 mM NaCl, 5.6 mM KCl, 0.85 mM NaH2PO4, 2.15 mM Na2HPO4, and 10 mM glucose, pH 7.0), and cleaned free of connective tissue. The glands were incubated with 0.2 ml of papain solution (250 ml DMEM (Invitrogen) supplemented with 50 mg l-cysteine, 2.5 ml of 0.1 M CaCl2, 2.5 ml of 50 mM EDTA, and 20-25 U/ml papain (Worthington Biochemical) and equilibrated with 5% CO2 at 37°C for 40 min, followed by the addition of 150 µl of inactivating solution (225 ml DMEM supplemented with 25 ml of heat-inactivated fetal calf serum (Gibco), 625 µg albumin, and 625 µg trypsin inhibitor (Sigma-Aldrich)) for 5 min. The solution was then carefully replaced by 0.2 ml enriched DMEM, and the glands were triturated gently through a 200 µl pipette tip. 50 µl of the cell suspension was plated on each sterile coverslip in 6-well plates, and the cells were allowed to settle before supplementing with enriched medium (500 ml DMEM supplemented with 2 ml penicillin/streptomycin (Invitrogen) and 5 ml insulin-transferrin-selenium-X (Invitrogen). The cells were incubated at 37°C and 10% CO2 and used within 3 d.

**Exocytosis measurements**

Exocytosis measurements were performed as described previously (Walter et al., 2010). Conventional whole cell patch-clamp recordings were performed at room temperature with Sylgard-coated 3-5 MΩ pipettes (Kimax-51; Kimble Chase) and an EPC-9 patch-clamp amplifier was used together with the Pulse software package (HEKA). The pipette solution contained 100 mM Cs-glutamate, 8 mM NaCl, 4 mM CaCl2, 32 mM Hepes, 2 mM Mg-ATP, 0.3 mM GTP, 1 mM ascorbic acid, 5 mM nitrophenyl-EGTA (Invitrogen), 0.4 mM fura-4F (Invitrogen), and 0.4 mM furaptra.
Isolated chromaffin cells were bathed in a solution containing 145 mM NaCl, 2.8 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, and 10 mM Hepes plus 2 mg/ml D-glucose, pH 7.2 (osmolarity was adjusted to 310 mOsM). Capacitance measurements were performed by using the Lindau-Neher technique implemented as the “sine + dc” mode of the software lock-in extension of Pulse, which allows long-duration capacitance measurements in a single sweep. A 1,000-Hz, 70-mV peak to peak sinusoid voltage stimulus was superimposed onto a DC holding potential of -70 mV. Currents were filtered at 3 kHz and sampled at 12 kHz. Flashes of UV light were generated by a flash lamp (Rapp Optoelektronik), and fluorescence excitation light was generated by a monochromator (TILL Photonics); these were coupled into the epifluorescence port of an inverted microscope (Axiovert 100; Carl Zeiss, Inc.) with a 40x Fluor objective (Carl Zeiss, Inc.). The fluorescent dyes were excited at 350/380 nm, and the illumination area was reduced to cover only the diameter of the cell. Emitted light was detected with a photomultiplier, filtered at 3 kHz, and sampled at 12 kHz by Pulse software. The fluorescence ratio signal was calibrated in separate experiments by infusing the cell with solutions with known $[\text{Ca}^{2+}]_i$ buffered by 20 mM BAPTA (Invitrogen) or 1,3-Diamino-2-hydroxy-propane-N,N,N',N'-tetraacetic acid (DPTA; Sigma-Aldrich). The $[\text{Ca}^{2+}]_i$ was calculated using a Kd of 0.222 µM for BAPTA and 80 µM for DPTA while taking into account Ca$^{2+}$ binding to the dyes, nitrophenyl-EGTA and ATP, using a custom-written macro running in IgorPro (WaveMetrics). Fluorescent excitation light was used not only to measure $[\text{Ca}^{2+}]_i$ but also to adjust $[\text{Ca}^{2+}]_i$ by inducing gradual photolysis of the Ca$^{2+}$ cage before the flash, such that cells could be studied under conditions of uniform pre-flash $[\text{Ca}^{2+}]_i$ (Voets, 2000). For details on data analysis see Walter et al., 2010.

Immunofluorescence staining and imaging

E18 hippocampal neurons were fixed at DIV15 by incubation in 3.7% formaldehyde (Electron Microscope Sciences) in Dulbeccos Phosphate Buffered Saline (D-PBS, Gibco) for 20 min. Subsequently, they were permeabilized for 5 min in D-PBS containing 0.5% Triton X-100 (Sigma), followed by a 30 min incubation in D-PBS containing 0.1% Triton X-100 and 2% normal goat serum to block aspecific binding. The same solution was used for diluting antibodies. Neurons were incubated for 2 h in primary antibodies at room temperature (RT), washed 3 times with D-PBS and incubated in secondary antibodies for 1 h. After additional 3 washes they were mounted on microscopic slides with ProLong$^{[6]}$Gold (Invitrogen) and imaged with a 63X Plan-Neofluar lens (Numerical aperture 1.4, Carl Zeiss b.v. Weesp) on a Zeiss 510 Meta Confocal microscope (Carl Zeiss). The same protocol was followed for staining P1 adrenal chromaffin cells that were fixed at DIV2.

Antibodies

Specific primary antibodies were used against EEA1 (mouse monoclonal 14, BD Transduction Laboratories, 1:100), GM130 (mouse monoclonal 35, BD Transduction Laboratories, 1:100), and GM130$'$ (mouse monoclonal 35, BD Transduction Laboratories, 1:100).
Vti1A and Neurobeachin Laboratories 1:400), Lamp1 (rat monoclonal 1D4B, Abcam 1:100), MAP2 (chicken polyclonal, Abcam, 1:20000), Nbea (rabbit polyclonal, Synaptic Systems, 1:1000), human TFR (mouse monoclonal H68.4, Zymed, 1:500), VAMP2 (mouse monoclonal 69.1, Synaptic Systems, 1:1000) and Vti1a (for hippocampal neurons: mouse monoclonal 45, Transduction Laboratories, 1:200; for adrenal chromaffin cells: rabbit polyclonal, 1:200, a kind gift from Gabriele Fischer von Mollard). As secondary antibodies goat α-mouse, goat α-rabbit and goat α-chicken Alexa Fluor 488, 546 and 647 were used (all Molecular Probes, 1:1000).

Data analysis For the quantification of synapses and Nbea clusters, as well as for the Sholl analysis, in Vti1a WT and KO hippocampal neurons (Figure 5.2) single plane images of neurons (1024x1024) were analyzed using SynD software (Schmitz et al., 2011). Imaging conditions were held constant for the VAMP2 and Nbea channel across the different genotypes. Images were processed using ImageJ software (Version 1.44I, National Institutes of Health, Bethesda MD). For the analysis of lysosomes in adrenal chromaffin cells the position of the top and bottom of the chromaffin cell along the z-axis were determined and the middle section of the z-stack images was taken. The images were preprocessed by using the MaxEntropy thresholding method (Kapur et al., 1985) in ImageJ and then analyzed using a custom-written macro running in ImageJ. The somas in Supplementary material Figure 5.6 were blown up using the bicubic resampling tool in Adobe Photoshop CS5 software (Version 12, Adobe Systems Inc, San Jose, California). We used MATLAB R2007a (The MathWorks Inc, Natick, Massachusetts) to test our datasets for normality with Lilliefors goodness-of-fit and heterogeneity of variance with Bartlett’s test for equality of variance. If data allowed a two-tailed unpaired Student’s t-test was performed. Alternatively, the Wilcoxon-Mann-Whitney test was performed.

Results Loss of Nbea does not disrupt the subcellular localization of Vti1a To examine whether Nbea loss influences the subcellular localization of Vti1a, we co-stained DIV15 cultured Nbea WT and KO hippocampal neurons with a α-Vti1a (for Vti1A specificity see Supplementary material Figure 5.6) and α-Nbea antibody. Vti1a immunofluorescence was observed exclusively in the soma (Figure 5.1 A). Although we did not co-stain Vti1a with any specific Golgi marker, its co-localization with Nbea (Figure 5.1; see also Chapter 2) indirectly confirmed Vti1a’s reported localization at the Golgi (Antonin et al., 2000b; Kreykenbohm et al., 2002). However, we did not observe additional punctate staining typical for Vti1a-β (Antonin et al., 2000b).
Figure 5.1 Loss of Nbea does not affect subcellular localization of Vti1a. DIV15 Nbea WT and KO hippocampal neurons (E18) fixed in 3.7% formaldehyde and co-stained with α-MAP2 (not shown in the merge), α-Vti1a (in red) and α-Nbea antibody (in green). Scale bar = 5 µm.

The commercially available α-Vti1a antibody that we used for colocalization with Nbea shows only one band on the immunoblot of rat brain (Transduction Laboratories, Cat. No. 611220). While the immunogen that was used to raise this antibody encompassed mouse Vti1a amino acids (AA) 114-217 (Transduction labs, Cat. No. 611220), the antibody that identified Vti1a-β contained mouse Vti1a AA 1-187, and showed two bands on the immunoblot (Antonin et al., 2000b). Vti1a-β contains an insertion of seven AA after residue 114 (Antonin et al., 2000b). Hence, the commercially available antibody does not seem to recognize the brain-specific Vti1a variant. Unfortunately, we could not use the antibody that recognizes also Vti1a-β for our
colocalization study, since it was raised in rabbit, just like the only available α-Nbea antibody. However, we can conclude that at least Vti1a’s somatic localization was not visibly affected by the depletion of Nbea.

Vti1a KO neurons show a small reduction in the number of Nbea puncta per unit dendrite

Since we were interested whether the depletion of Vti1a influences the subcellular localization of Nbea, as well as the general connectivity (in terms of number of synapses formed), we co-stained DIV15 Vti1a WT and KO hippocampal neurons for the dendritic marker MAP2, the presynaptic marker VAMP2 and Nbea (Figure 5.2 A). Quantification of the Nbea and VAMP2 immunofluorescence detected in dendrites revealed that the number of synapses per unit dendrite (Figure 5.2 B), the synapse area (Figure 5.2 D), as well as the total number of synapses (Supplementary material Figure 5.7 A) were not affected by loss of Vti1a. Furthermore, Vti1a does not seem to influence synapse formation per se. However, synapses of Vti1a KO neurons were brighter than the synapses of their WT counterparts (Figure 5.2 D), indicating that more VAMP2 was present in synapses when Vti1a was depleted.

Interestingly, Vti1a KO neurons showed a reduction in the number of Nbea clusters per unit dendrite (Figure 5.2 C), while the total number of dendritic Nbea clusters, their area and intensity remained unchanged (Supplementary material Figure 5.7 C, Figure 5.2 E and G, respectively). The total dendritic length was also not altered in Vti1a KO neurons (Supplementary material Figure 5.7 B and D). Sholl analysis (Shol, 1953) was used for the analysis of the dendrite complexity. Vti1a WT and KO neurons exhibited similar dendritic complexity. They only diverged at a distance of 30 µm from the cell bodies, where the dendrites of KO neurons were more complex (Figure 5.2 H). These results indicate that loss of Vti1a does influence Nbea’s subcellular distribution, however the effect is not very prominent.

Loss of Vti1a affects catecholamine release in chromaffin cells

To test whether Vti1A loss affects exocytic control mechanisms, we used chromaffin cells, which are widely used as a model system to study fast Ca\(^{2+}\) exocytosis (Neher, 2006). By means of caged-[Ca\(^{2+}\)] flash photolysis exocytosis was elicited, which was monitored by membrane capacitance measurements and carbon fiber amperometry (Walter et al., 2010). In chromaffin cells catecholamine release occurs as a fast initial increase in capacitance, i.e. the secretory burst (with two kinetically distinct components), which is followed by a sustained phase of release (Stevens et al., 2011; Voets et al., 1999). The two components of the burst (fast and slow burst) represent two releasable pools, i.e. the readily releasable pool (RRP) and the slowly releasable pool (SRP) of vesicles, which are thought to be primed (Voets et al., 1999; Walter et al., 2010). The later, much slower part of the capacitance increase, i.e. the sustained component, is triggered by release of vesicles that are being primed during the stimulation period (Stevens et al., 2011; Voets et al., 1999).
Figure 5.2 Hippocampal neurons deficient in Vti1a show a reduction in the number of Nbea puncta per unit dendrite. (A) DIV15 Vti1a WT and KO hippocampal neurons (E18) fixed in 3.7% formaldehyde and co-stained with the dendritic marker α-MAP2, the synaptic marker α-VAMP and the α-Nbea antibody. Scale bar = 5µm. (B) Comparison of the number of synapses per unit dendrite between Vti1a WT and KO hippocampal neurons as calculated from the total number of synapses and total dendritic length (as shown in A and B of Supplementary material Figure 5.7). (C) Comparison of the number of Nbea puncta per unit dendrite between Vti1a WT and KO hippocampal neurons, again calculated from the total number of Nbea clusters and the according total dendritic length (see C and D Supplementary material Figure 5.7). (D) Quantification of the average synapse area in Vti1a WT and KO hippocampal neurons. (E) Quantification of the average area of Nbea clusters in Vti1a WT and KO hippocampal neurons. (F) Quantification of the average synapse intensity Vti1a WT and KO hippocampal neurons. (G) Quantification of dendritic Nbea puncta intensity in Vti1a WT and KO hippocampal neurons. (H) Sholl analysis conducted on Vti1a WT and KO neurons. Error bars indicate SEM. (*P <0.05 in t-test)
As can be seen in Figure 5.3, in Vti1a KO cells the burst size was reduced, indicating that there are fewer primed vesicles. No apparent changes were observed in the sustained phase of release rate, suggesting that the forward priming rate is the same. There was no difference in kinetics of release between WT and KO cells (see normalized average traces in Figure 5.3 B), which means that triggering of release is not affected by Vti1a loss. Taken together, chromaffin cells deficient in Vti1a show a defect in catecholamine release that might be due to a reduction in the number of primed vesicles.

Figure 5.3 Chromaffin cells deficient in Vti1a show a defect in catecholamine release. (A) Averaged recordings from Vti1a KO (in grey) and wildtype littermate (control, black) cells. Top panel: mean SEM of the intracellular Ca2+ concentration after UV-induced calcium uncaging at T=0.5s. Middle panel: mean capacitance increase. Bottom panel: mean amperometric current (thick traces, left ordinate) and amperometric charge (thin traces, right ordinate). (B) A detailed view of normalized capacitance traces in (A) allows comparison of release kinetics. (C) Quantification of capacitance values prior to the stimulation (cell size) and of burst (one second after stimulation), total (five second after stimulation) and sustained (difference of total and burst) secretion. Data are represented as mean SEM. Control: n=32 cells; Vti1a KO: n=41 cells.

**Chromaffin cells of Vti1a KO mice show intact organelle morphology**

To study organelle morphology in chromaffin cells of Vti1a KO and WT mice, we fixed these cells at DIV2 and stained them with the α-Vti1a antibody in combination with different markers including GM130 for Golgi, Lamp1 for lysosomes, EEA1 for early endosomes and TfR for recycling endosomes (Figure 5.4 A and Figure 5.5 A and B). As was indirectly the case in neurons (see Chapter 2), also in chromaffin cells...
Vti1a was closely aligned with the Golgi apparatus (Figure 5.4 A). No significant differences were observed in the staining patterns between Vti1a WT and KO cells (except for Vti1a, of course). Additionally, we did not find any differences in the number of lysosomes, their total area and their average size between both genotypes (Figure 5.4 B - D).

![Image of Figure 5.4](image)

**Figure 5.4 Loss of Vti1a does not affect the number nor size of lysosomes in adrenal chromaffin cells.** (A) DIV2 Vti1a WT and KO adrenal chromaffin cells (P1) fixed at DIV2 in 3.7% formaldehyde and co-stained for the lysosomal marker Lamp1 (not shown in the merge), the cis-Golgi marker GM130 (in green) and Vti1a (in red). Scale bar = 2.5 µm. (B) Comparison of the number of lysosomes between Vti1a WT and KO chromaffin cells. (C) Quantification of the total lysosomal area in Vti1A WT and KO chromaffin cells. (D) Comparison of the average size of lysosomes between Vti1a WT and KO neurons. Error bars indicate SEM.

Interestingly, the colocalization experiments revealed large overlap between Lamp1 and EEA1, as well as Lamp1 and TfR. As we did not image control slides in which only one dye is present using our multiple-channel settings, we can not completely exclude the possibility of bleed-through, i.e. the appearance of fluorescence from one
fluorophore in the detection channel set up to detect another fluorophore. However, the images of Figure 5.4, which were taken with similar settings as the images of Figure 5.5, did not show any indication of bleed-through. The large overlap between Lamp1 and EEA1/TfR might therefore indicate that the separate steps of the endocytic pathway in chromaffin cells is not as sharply delineated as for instance in neurons.

![Figure 5.5 The early endosomal marker EEA1 and the recycling endosomal marker TfR show substantial overlap with the lysosomal marker Lamp1. Vti1a WT and KO adrenal chromaffin cells (P1) fixed at DIV2 in 3.7% formaldehyde and co-stained using α-Lamp1 (in green), α-Vti1a (not shown in the merge) and α-EEA1 (A) or α-TfR (B; both in red). Scale bar = 2.5 µm.](image)

Taken together, these data imply that the overall architecture of the organelles, con-
Substituting the secretory and endo-lysosomal pathway, was not significantly affected by the lack of Vti1a.

Discussion

Vti1a (Vacuolar protein sorting ten p tail interacting 1a), a SNARE protein implicated in protein trafficking in the secretory, as well as in the endosomal pathway (Xu et al., 1998) showed high colocalization with somatic Nbea (see Chapter 2). The depletion of Vti1a led to a small reduction in the number of Nbea puncta per unit dendrite and although loss of Nbea did not affect the somatic localization of Vti1a, it is impossible to rule out that the brain specific Vti1a variant - Vti1a-β was unaltered as well. Chromaffin cells deficient in Vti1a showed reduced catecholamine release, while their general organelle morphology was not perturbed.

Vti1a’s colocalization with Nbea in neurons and its juxtaposition to GM130 in chromaffin cells (Figure 5.1 and Figure 5.4 A), matches Vti1a’s previously described association with the Golgi apparatus (Kreykenbohm et al., 2002; Xu et al., 1998). Vti1a, just like Nbea (see Chapter 2), also disperses upon BFA treatment, i.e. loses its perinuclear localization (Kreykenbohm et al., 2002; Xu et al., 1998), which further strengthens the point that both proteins associate with the Golgi. Although, we could not detect Vti1aβ in our preparation (possibly because of the lack of immunoreactivity of the antibody due to the additional amino acids in the β variant), a potential colocalization with Nbea seems rather unlikely, given the fact that Nbea is located exclusively in dendrites (Chapter 2), while Vti1a-β resides on synaptic vesicles, which are pertinent to axons (Antonin et al., 2000a; Takamori et al., 2006). These results indicate that Vti1a and somatic Nbea both play a role in the early secretory pathway, while the brain-specific splice variant Vti1a-β and Nbea exhibit a clearly different subcellular localization and therefore probably engage in different cellular functions.

The exocytosis measurements in chromaffin cells clearly revealed a defect in the burst phase of catecholamine release (Figure 5.3). This phase represents the releasable pool of vesicles (RRP and SRP), which is thought to be primed (Voets et al., 1999; Walter et al., 2010). Hence, the observed defect might be a consequence of a reduction in the number of primed vesicles. However, alternative explanations are possible, e.g. a decrease in the size of the granules. Future EM studies could help reveal the underlying cause for these results, e.g. by analyzing the number of total and docked vesicles.

In hippocampal pyramidal neurons knockdown (KD) of Vti1a selectively impairs spontaneous neurotransmission. Both, miniature inhibitory and excitatory postsynaptic currents (mIPSCs/mEPSCs) are reduced in frequency, while their amplitudes are not affected (Ramirez et al., 2012). Also, no differences are seen in the peak amplitudes of paired pulse ratios among Vti1a KD and wild-type neurons, indicating that Vti1a does not affect evoked inhibitory release (Ramirez et al., 2012). However, Vti1a has been shown to reside on vesicles of the readily releasable pool, i.e. initial vesicles to be released upon stimulation, participating in the recycling of synaptic vesicles by
mediating their fusion with endosomes (Hoopmann et al., 2010; Rizzoli et al., 2006). Although the question whether spontaneous and activity-dependent release originate from the same vesicle pool is far from resolved (for review see Denker and Rizzoli, 2010), it seems that Vti1a is involved in both forms of release. The increase in VAMP2 signal parallel to the reduction in the number of Nbea clusters per unit dendrite in Vti1a deficient neurons (Figure 5.2 C and F) might therefore be a compensatory effect for the reduced neurotransmission. However, further experiments are necessary to thoroughly explain this observed phenotype.

Vti1a KO hippocampal neurons exhibited similar total dendritic length (Supplementary material Figure 5.7 B and D) and general dendrite complexity (Figure 5.2 H) as Vti1a WT neurons. Since Vti1a/Vti1b double knock-out (DKO) hippocampal neurons show reduced neurite outgrowth (in terms of the number of cultured neurons and the length of their neurites) compared to double heterozygous neurons (Kunwar et al., 2011), the lack of a profound effect on total dendritic length and complexity might be due to a partial functional redundancy between Vti1a and Vti1b. Since DKO mice die at birth, while Vti1a deficient mice (lacking also Vti1a-β), as well as Vti1b deficient mice are viable and fertile (Atlashkin et al., 2003; Kunwar et al., 2011), Vti1a and Vti1b can at least partially substitute for each other.

The fact that chromaffin cells of Vti1a KO mice show intact organelle morphology is in line with studies in immortalized fibroblasts isolated from DKO and double heterozygous (DHET) embryos, as well as hippocampal neurons cultured from these embryos. These displayed a similar overall architecture of the organelles constituting the secretory and endo-lysosomal pathway, indicating that lack of Vti proteins does not have a significant impact on general organelle morphology (Kunwar et al., 2011).

Taken together, despite Nbea’s colocalization with Vti1a in the soma, Vti1a loss does not result in a phenocopy of Nbea depletion. Although there is some minor effect on Nbea puncta, based on the arguments presented above, it does not seem likely that these two proteins interact or engage in the same cellular pathway. Nevertheless, further experiments, like overexpressing Vti1a in Nbea KO neurons and trying to rescue the Nbea null phenotype, could give additional insights to this notion.


Supplementary Material

Figure 5.6 The localization of Vti1a pursued by specific α-Vti1a antibody in Vti1A WT and KO hippocampal neurons. (A) DIV15 Vti1a WT and KO hippocampal neurons (E18) fixed in 3.7% formaldehyde and co-stained with α-MAP2 (in green) and α-Vti1a (in red). (B) Blown up somas in A. The Vti1a signal observed in Vti1a KO images belongs to the WT glia cells, which were used as the neurons’ feeder cell layer. Scale bar = 5µm.
Figure 5.7 Quantification of the number of synapses, Nbea clusters and the according total dendritic length in Vti1a WT and KO hippocampal neurons. Error bars indicate SEM.
Chapter 5