Vti1a/b regulate synaptic vesicle and dense core vesicle secretion via protein sorting at the Golgi

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The SNAREs Vti1a/1b are implicated in regulated secretion, but their role relative to canonical exocytic SNAREs remains elusive. Here, we show that synaptic vesicle and dense-core vesicle (DCV) secretion is indeed severely impaired in Vti1a/b-deficient neurons. The synaptic levels of proteins that mediate secretion were reduced, down to 50% for the exocytic SNARE SNAP25. The delivery of SNAP25 and DCV-cargo into axons was decreased and these molecules accumulated in the Golgi. These defects were rescued by either Vti1a or Vti1b expression. Distended Golgi cisternae and clear vacuoles were observed in Vti1a/b-deficient neurons. The normal non-homogeneous distribution of DCV-cargo inside the Golgi was lost. Cargo trafficking out of, but not into the Golgi, was impaired. Finally, retrograde Cholera Toxin trafficking, but not Sortilin/Sorc1 distribution, was compromised. We conclude that Vti1a/b support regulated secretion by sorting secretory cargo and synaptic secretion machinery components at the Golgi.
Regulated secretion of signaling molecules from synaptic vesicles (SVs) and dense-core vesicles (DCVs) is the primary means of neuronal communication. SVs store neurotransmitters and are locally recycled after exocytosis\(^1\)–\(^3\), whereas DCVs, which contain neuropeptides and neurotrophins\(^4\)–\(^5\), are continuously generated at the trans-Golgi\(^6\)–\(^7\). Immature DCVs are proposed to undergo initial fusion reactions, homotypic fusion\(^8\)–\(^9\) or heterotypic fusion to endosomes\(^10\), before they are fusion-competent. Fusion requires formation of SNARE complexes consisting of four SNARE protein domains (R, Qa, Qb, and Qc)\(^11\). In addition to the canonical exocytic SNARE proteins (VAMP2, Syntaxin-1, and SNAP25)\(^12\)–\(^13\), other R- and Q-SNAREs are implicated in regulated secretion. However, how these additional SNAREs regulate secretion in relation to the canonical exocytic SNAREs is still poorly understood.

In this context, the Qb-SNARE Vti (VPS10 interacting) proteins are particularly enigmatic. Two Vti proteins are ubiquitously expressed in mammals, Vti1a and Vti1b\(^12\)–\(^14\). These proteins function in endo-biosomal trafficking\(^15\)–\(^19\) and combined gene inactivation in mice triggers neurodegeneration and perinatal death\(^12\). Vti1a knockdown results in reduced spontaneous SV fusion\(^20\)–\(^39\). However, it is unclear which domain Vti1a would contribute to exocytic SNARE complexes because SNAP25 supplies the Qb-SNARE domain for spontaneous SV fusion\(^21\)–\(^23\). Vti1a knock-out chromaffin show reduced exocytosis by unknown pathways that result in decreased numbers of secretory granules\(^24\). Hence, it is possible that, also in neurons, dysregulation of Vti1a/b-dependent upstream pathways contributes to downstream secretion defects.

Here, Vti1a/b-null neurons were used to test this hypothesis. We found that the number of DCVs and synapses, and the synaptic levels of proteins that mediate secretion were all reduced. The delivery of DCV-cargo and SNAP25 into axons was reduced. DCV-cargo and SNAP25 accumulated in the Golgi. These phenotypes were rescued by expression of either Vti1a or Vti1b. Furthermore, Golgi cisternae were distended and the export of cargo from the Golgi was impaired. We conclude that Vti1a/b regulate secretion by sorting secretory cargo and proteins of the secretion machinery out of the Golgi.

**Results**

**Fewer synapses and DCVs in Vti1a/b-deficient neurons.** To study the role of Vti1a and Vti1b in regulated secretion, we used Vti1a/Vti1b double knock-out (DKO) mice\(^12\), with double heterozygotes (DHZ) as controls (Supplementary Figure 1A). Expression of Vti1a or Vti1b in DKO neurons at 1 day in vitro (DIV1) using lentiviral vectors produced perinuclear expression, similar to endogenous proteins (Supplementary Figure 1B). Cell death was observed in DKO neurons between DIV3-10 (Supplementary Figure 1C), affecting glutamatergic and GABAergic subpopulations equally (Supplementary Figure 1D–E). The surviving DKO neurons had 30% shorter dendrites at DIV-14 (Fig. 1a; Supplementary Figure 1F-G, 2A), without altered arborization (Supplementary Figure 2B). This reduction was rescued by either Vti1a or Vti1b expression (Fig. 1a; Supplementary Figure 1F-G, 2A). The axonal length was reduced to a similar extent and was only partially rescued (Supplementary Figure 1F, H, I). The length reduction was not a property of the surviving neurons, as DIV-4 neurons had similar alterations (Supplementary Figure 1J–M).

Synaptic vesicles (SVs) were quantified by staining for endogenous Synaptophysin-1 (Fig. 1a), and DCVs by expressing an established DCV cargo reporter, Neureptide-Y (NPY) fused to pHluorin (Fig. 1f), which co-localizes with endogenous DCV markers\(^23\)–\(^26\). At DIV-14, expression of both markers was punctate (Fig. 1a, f). In DKO neurons, the number of Synaptophysin-1 and NPY-pHluorin puncta was reduced by 65% and 50%, respectively (Supplementary Figure 2C, D), which resulted in 40% (Fig. 1b) and 15% (Fig. 1g) reductions after compensating for differences in neurite length. Puncta distribution relative to the soma was unaltered (Fig. 1e, j). Furthermore, puncta intensity in DKO neurons was 30% lower for Synaptophysin-1 (Fig. 1c, d) and 50% lower for NPY-pHluorin (Fig. 1h, i). The endogenous DCV cargo BDNF was analyzed to corroborate the differences observed using the DCV reporter. The proportion of BDNF-positive neurons was similar between groups (83.2 ± 7.1, 79.7 ± 9.3, 71.7 ± 2.5, 81.1 ± 12.8% for DHZ, DKO and rescue with Vti1a or Vti1b, respectively). The density and intensity of BDNF puncta were reduced in DKO neurons (Supplementary Figure 2E–I), without alterations in distribution (Supplementary Figure 2K).

Expression of Vti1a or Vti1b rescued all these parameters (Fig. 1; Supplementary Figure 2A–J), except for DCV puncta intensity, which was only partially rescued by Vti1b (Fig. 1a–h; Supplementary Figure S2 H, I).

At the ultrastructure level, random cross sections of synapses contained similar numbers of SVs, docked SVs per synapse, active zone length and postsynaptic density length between DHZ and DKO (Fig. 1k–m, Supplementary Figure 2K, L). However, synaptic profiles contained DCVs twofold less often in DKO neurons (DHZ, 30.27 ± 2.10%; DKO, 16.05 ± 5.81%) and, among DCV-containing micrographs, DKO synapses contained 40% fewer DCVs (Fig. 1n). The DCV diameter was not altered in DKO neurons (Fig. 1o, p).

These results show that Vti1a/b deficiency results in in vitro cell death, and that the surviving neurons are smaller and have fewer synapses and DCVs. Furthermore, expression of either Vti1a or Vti1b rescues virtually all these phenotypes equally well.

**Reduced SV and DCV secretion in Vti1a/b-deficient neurons.** We investigated how regulated secretion is affected by loss of Vti1a/b expression by quantifying SV and DCV fusion. To study SV fusion, we applied live dual-color imaging of Synaptophysin-pHluorin\(^27\) (SypHy) and FM4-64-labeled SVs (Supplementary Figure 3A), and whole-cell patch clamp. SypHy-fluorescence was quenched under resting conditions (Fig. 2a; Supplementary Movie 1). High frequency stimulation (HFS, 100 action potentials (APs) at 40 Hz) triggered SV fusion from most synapses (Fig. 2a). A higher percentage of non-responsive synapses was observed in DKO neurons (Fig. 2b). In responsive synapses, the fraction of SVs that fused was 50% lower (Fig. 2c, d). Neither kinetics of fluorescence increase nor fluorescence decay after HFS, a measure for SV endocytosis and re-acidification\(^27\), were affected in DKO neurons (Fig. 2e; Supplementary Figure 3E). Consistent with SypHy, DKO neurons also showed reduced FM4-64 uptake upon 60 mM K\(^+\) depolarization, and reduced release upon HFS (Supplementary Figure 3F-I). These parameters were rescued by expression of either Vti1a or Vti1b (Fig. 2a–d; Supplementary Figure 3B–I).

In whole-cell patch clamp recordings, evoked transmitter release upon single AP or HFS (100 APs, 40 Hz) stimulation was decreased by 80–90% (Fig. 2f, g; Supplementary Figure 3J, L, M). The amplitude and charge transfer of spontaneous transmitter release was 20–25% lower (Fig. 2f, h; Supplementary Figure 3O). After correcting for the decreased SV charge, evoked release remained reduced to a similar extent, 80–90% (Supplementary Figure 3K, N). The frequency of spontaneous fusion events was
reduced by 65% (Fig. 2f, i). The readily releasable pool (RRP), quantified as charge transferred during hypertonic sucrose superfusion, was almost seven fold smaller (Fig. 2j; Supplementary Figure 3P). The release probability, calculated as charge transfer evoked by one AP over the RRP, was 45% lower (Fig. 2k).

Finally, the paired pulse ratio was larger in DKO neurons (Supplementary Figure 3L, Q).

To investigate DCV secretion, we performed live imaging of NPY-pHluorin-labeled DCVs. NPY-pHluorin fluorescence was initially quenched (Fig. 3a; Supplementary movie 2). HFS
stimulation (16 trains of 50APs at 50 Hz), reported to elicit efficient DCV fusion21,25,26, triggered similar calcium transients in all groups (Supplementary Figure 4A-F). Fusion of individual DCVs, characterized by dequenching of NPY-pHluorin puncta (Supplementary Figure 4G), was 85% lower in DKO neurons (Fig. 3a, b). The DCV fusion probability, calculated as DCV fusion events (Fig. 3b) over total DCV pool (Fig. 3c), was 3.5-fold lower (Fig. 3d; Supplementary movie 2). The release kinetics were similar between groups, with ~30% events occurring during the first 3 stimulation trains (Fig. 3e, f). Individual fusion events were more transient in DKO neurons (Supplementary Figure 4H). However, the event amplitude was unaffected (Supplementary Figure 4, I, J). The fraction of fusion events that occurred at synapses (within the point spread function of the synapse marker Synapsin-ECFP, Supplementary Figure 4K), was 30% in DKO neurons and 50% in DHZ (Supplementary Figure 5L). All these phenotypes were rescued by expression of either Vti1a or Vti1b (Fig. 3a–f; Supplementary Figure 6H–L), except for the total DCV pool, which was only partially rescued by Vti1b. A reduction in DCV fusion was not observed in Vti1a or Vti1b single KO neurons (Supplementary Figure 4M–R). These results indicate that DKO neurons have impaired secretion, characterized by smaller readily releasable SV pools, decreased SV release probability, reduced spontaneous SV fusion frequency and amplitude, and decreased DCV fusion probability. Vti1a/b are dispensable for fusion kinetics during stimulation, SV endocytosis and acidification or DCV cargo loading and acidification.

Lower levels of exocytic proteins in Vti1a/b DKO neurons. To understand why regulated secretion is impaired in DKO neurons, we measured the levels of eight synaptic proteins involved in exocytosis by immunocytochemistry, using Synaptophysin-1 as synaptic marker. DKO neurons had a significant reduction in staining intensity for synaptic SNAP25 (61.3%), Munc13-1 (46.1%), Bassoon (35.5%), Synaptogamin-1 (29.8%), and RIM1/2 (19.2%) (Fig. 4; Supplementary Figure A–H & Table 1). These reductions were rescued by either Vti1a or Vti1b expression. The staining intensity of extrasynaptic SNAP25 was decreased to a similar extent (Supplementary Figure 5I). Staining for Synaptobrevin-2, Syntaxin-1, and Munc18-1 showed a trend towards reduced intensity (19.3, 16.4, and 10.4%, respectively, not significant). The reduction in synaptic protein levels correlated with known protein half-lifes28 ($r^2 = 0.719$, $p < 0.0001$; Supplementary Figure 5J).

To test whether the reduced secretion and synaptic protein levels were features specific of the surviving DKO neurons, Vti1a and Vti1b were expressed in DKO neurons after most cell death had occurred (DIV10; Supplementary Figure 6A). This late expression rescued the fraction of responsive synapses, SV fusion in responsive synapses (Supplementary Figure 6B–D) and levels of SNAP25 and Munc13-1 (Supplementary Figure 6E–H).

These results indicate that Vti1a/b are required to maintain normal levels of secretion machinery components, and that the reduced number of responsive synapses, synaptic secretion and synaptic protein levels are not features specific for surviving DKO neurons.

Reduced protein influx into axons in Vti1a/b DKO neurons. Increased protein degradation or reduced delivery to synapses are the two main explanations for the decreased synaptic levels of proteins that mediate secretion. To address the first possibility, we blocked protein translation for 24 h with cycloheximide. Cycloheximide treatment reduced synaptic SNAP25 and Munc13-1 levels to a similar extent in DHZ and DKO neurons (Supplementary Figure 5K–O). To address protein delivery, SNAP25-EGFP transport was monitored in DIV-5 neurons after photo-bleaching the proximal axon, identified with the axon initial segment marker NaVII/III-mCherry (Fig. 5a). SNAP25 was selected as reporter because its levels in DKO neurons were decreased the most. SNAP25-EGFP puncta were detected entering the axon in all groups, at an average speed of around 1 µm/s (Fig. 5b). In DHZ neurons, 3.9 puncta/min entered the axon, as opposed to 2.2 in DKO neurons (Fig. 5c).

The reduced number of DCVs detected in DKO neurons (Fig. 1) may be the consequence of a similar inefficient delivery to axons. To address this, we expressed the DCV-reporter NPY-mCherry and NaVII/III-YFP (Fig. 5d). NPY puncta entered the axon in all groups, at a velocity of 1.0–1.3 µm/s (Fig. 5e). However, similar to SNAP25-EGFP, 40% fewer NPY puncta entered the axon in DKO neurons (Fig. 5f). NPY delivery to the axon was rescued by expression of either Vti1a or Vti1b (Fig. 5d, f). These results indicate that Vti1a/b control the efficient delivery of SNAP25 and DCV cargo to axons.

Abnormal Golgi and cargo accumulation in Vti1a/b DKO neurons. To understand the reduced cargo delivery to axons in DKO neurons, we analyzed the location of Vti proteins. 90% of the Golgi-marker MannosidaseII-ECFP (ManII-ECFP) co-localized with Vti1a and Vti1b (Fig. 6a, b; Supplementary
Figure 7A). Vti1a and Vti1b strongly co-localized at ManII-ECFP positive regions (93%), while in the rest of the neuron co-localization was low (22%, Fig. 6c). In ManII-ECFP negative regions, Vti1a and Vti1b puncta partially co-localized with endolysosomal markers (Supplementary Figure 7), as expected15–19.

Because of the preferential localization of Vti1a/b to the Golgi, we examined the structure of this organelle in DKO neurons. At DIV-5, the areas stained by GM130, ManII-EGFP, and TGN46 were reduced in DKO (Supplementary Figure 8D). Mitochondria and the trans-Golgi marker TGN46 were reduced in DKO neurons (Supplementary Table 1). Abnormal Golgi defects and the reduced cargo delivery to axons at DIV-5. Mitochondria were already smaller in DKO neurons (Supplementary Figure 8D). Mitochondria defects and the reduced cargo delivery to axons at DIV-5. Mitochondria were already smaller in DKO neurons (Supplementary Figure 8D).

We subsequently investigated a potential relationship between Golgi defects and the reduced cargo delivery to axons at DIV-5. At DIV-5, the areas stained by GM130, ManII-EGFP, and TGN46 were already smaller in DKO neurons (Supplementary Table 1). Endogenous SNAP25 was found in ManII- EGFP- and TGN46-positive areas (Fig. 6k). SNAP25 staining in these areas was greater than in the rest of the soma (Fig. 6k). In DKO neurons, the ratio of SNAP25 expression in ManII- EGFP- or TGN46-positive areas were already smaller in DKO neurons (Supplementary Table 1). Abnormal Golgi defects and the reduced cargo delivery to axons at DIV-5. Mitochondria were already smaller in DKO neurons (Supplementary Figure 8D). Mitochondria defects and the reduced cargo delivery to axons at DIV-5. Mitochondria were already smaller in DKO neurons (Supplementary Figure 8D).
over the rest of the soma was 16% and 23% higher, respectively (Fig. 6l–m; Supplementary Figure 8E–G). NPY-pHluorin was also enriched in GM130- and TGN46-positive areas and the ratio of expression was greater in DKO than controls at GM130- (57%) and TGN46-positive areas (63%) (Fig. 6n–p, Supplementary Figure 8H–J). The expression ratios for SNAP25 and NPY were rescued by expression of either Vti1a or Vti1b (Fig. 6k–p). The expression of the postsynaptic protein PSD95 and TrKB receptors were also decreased and, like NPY-pHluorin and SNAP25, the ratio of expression inside/outside of the Golgi was greater in DKO neurons (Supplementary Figure 9). Hence, in the absence of Vti1a/b, Golgi export appears to be compromised for both presynaptic and postsynaptic proteins, as well as DCV-cargo.

### Fig. 3 Reduced dense-core vesicle secretion in Vti1a/b-deficient neurons.

**a** Representative examples of single DIV-14 neurons expressing NPY-pHluorin, before, during HFS (16 trans, 50APs at 50 Hz) and after NH4⁺ superfusion. Scale bar = 5 µm. Images show maximum projections of time-lapse recordings.

**b** DCV fusion is reduced in DKO neurons (DHZ: 123.6 ± 26.4; DKO: 18.9 ± 4.5; DKO + Vti1a: 108.5 ± 20.7; DKO + Vti1b: 119.8 ± 22.1 fusion events/neuron; Kruskal–Wallis).

**c** Reduced DCV pool, calculated upon NH4⁺ superfusion, in DKO neurons (DHZ: 3259.9 ± 395.8; DKO: 1865.0 ± 182.3; DKO + Vti1a: 2997.6 ± 314.7; DKO + Vti1b: 2697.0 ± 294.4 DCVs/neuron; Kruskal–Wallis).

**d** Decreased DCV fusion probability in DKO neurons (DHZ: 3.29 ± 0.51; DKO: 0.96 ± 0.17; DKO + Vti1a: 3.60 ± 0.43; DKO + Vti1b: 4.59 ± 0.67%; Kruskal–Wallis).

**e**, **f** Histogram and cumulative plots of the total (top) and fraction (bottom) of DCV fusion events per stimulation train (gray bars). Bars show mean ± SEM. Bullets and columns represent individual observations and independent litters, respectively. Gray bars represent 16 × 50 AP at 50 Hz stimulation. N = 25, 29, 28, 27 for DHZ, DKO, DKO + Vti1a and DKO + Vti1b, respectively. *p < 0.05, **p < 0.001.
Figure 4: Reduced synaptic levels of proteins that mediate secretion in Vti1a/b-deficient neurons. 

- **a**–**d** Representative examples of single DIV-14 neurons immunostained for Synaptophysin-1 as synaptic marker and Syntaxin-1 and SNAP25 (**a**), Munc18-1 and Munc13-1 (**b**), RIM1/2 and Bassoon (**c**), and Synaptobrevin-2 and Synaptophysin-1 (**d**). 
- **e**–**l** Intensity distribution of the levels of Syntaxin-1 (**e**), SNAP25 (**f**), Munc13-1 (**g**), Munc18-1 (**h**), Bassoon (**i**), RIM1/2 (**j**), Synaptotagmin-1 (**k**), Synaptobrevin-2 (**l**) in all synapses (n = Over 15,000 synapses per protein and group). Statistical significance tested in Supplementary Figure 5. Scale bar = 2 µm.
To further analyze this cargo accumulation, we acquired higher resolution images of the somata. The Golgi was labeled with GM130 and TGN46. Discrete NPY-pHluorin puncta were observed in somata, surrounding the Golgi area (Supplementary Figure 8K). The location of these somatic puncta relative to the Golgi was not altered in DKO neurons (Supplementary Figure 8L). Within the Golgi area, NPY-pHluorin fluorescence was non-homogeneous, with sub-areas of NPY-pHluorin enrichment (Supplementary Figure 8M). In DKO neurons NPY-pHluorin distribution was more homogeneous (Supplementary Figure 8M, N). NPY-pHluorin puncta co-localized to a similar (low) extent with the lysosomal marker Lamp1 (Supplementary Figure 8O). The accumulation and abnormal distribution of cargo at the Golgi in DKO neurons prompted us to investigate the trafficking to/from this organelle using the RUSH system29. NPY-EGFP fused to Streptavidin-binding protein (SBP) was co-expressed in DIV-5 neurons with the ER-resident protein KDEL fused to Streptavidin. The interaction between Streptavidin and SBP traps cargo at the ER29 (Fig. 7a). Trafficking of the constitutive cargo, GPI, was studied in the same manner. Reversion of the SBP-Streptavidin binding by Biotin application triggered NPY or GPI transport to neurites (Fig. 7b, c). Prior to biotin application, the distribution of GPI and NPY was dispersed at the somata (Fig. 7d, i), consistent with ER retention29. Biotin application triggered GPI and NPY translocation to ManII-ECFP-labeled areas (Golgi) within minutes (Fig. 7d, i), with similar kinetics between groups (Fig. 7d–f, i–k). However, the NPY export kinetics were slower

Reduced cargo export from the Golgi in Vti1a/b DKO neurons.

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Fig. 5 Reduced influx of SNAP25 and DCV cargo into axons in Vti1a/b-deficient neurons. a Representative examples of DIV-5 neurons, in mass cultures, expressing SNAP25-EGFP and the axonal-initial-segment (AIS) marker NaVII/III-mCherry before, after photobleaching and three minutes after photobleaching. Kymographs show NPY-mCherry puncta in the AIS photobleached segment. b No differences in SNAP25 puncta velocity in the AIS (DHZ: 1.08 ± 0.10, n = 24; DKO: 0.99 ± 0.04 μm/s, n = 24; Mann–Whitney). c Less SNAP25 puncta enter the AIS in DKO neurons (DHZ: 3.93 ± 0.37, n = 24; DKO: 2.17 ± 0.30 puncta/min, n = 24; t-test). d Representative examples of DIV-5 neurons, in mass cultures, expressing the AIS marker NaVII/III-YFP and NPY-mCherry before, after photobleaching and two minutes after photobleaching. Kymographs show NPY-mCherry puncta in the AIS photobleached segment. e No differences in NPY puncta velocity in the AIS (DHZ: 1.00 ± 0.09, n = 23; DKO: 1.30 ± 0.11, n = 27; DKO + Vti1a: 1.20 ± 0.15, n = 14; DKO + Vti1b: 1.28 ± 0.10 μm/s, n = 15; Wilcoxon). f Less NPY puncta enter the axon in DKO neurons (DHZ: 3.39 ± 0.23, n = 24; DKO: 2.13 ± 0.21, n = 27; DKO + Vti1a: 3.71 ± 0.28, n = 14; DKO + Vti1b: 4.47 ± 0.43 puncta/min, n = 15; Wilcoxon). Bars show mean ± SEM. Bullets and columns represent individual observations and independent litters, respectively. In c and f, some individual observations had the same value and overlapped in the graph. **p < 0.01, ***p < 0.001. Scale bar = 10 s and 5 μm
in DKO neurons (Fig. 7d, e) as indicated by the lower fraction that exited the Golgi 64 or 104 minutes after biotin application (Fig. 7g, h). In astrocytes, similar results were obtained (Supplementary Figure 10). For GPI, the export kinetics were initially slower in DKO neurons (32% less cargo exited the Golgi 64 min after biotin application; Fig. 7l), but later converged (Fig. 7m). These data indicate that Vti1a/b are necessary for efficient Golgi export of regulated cargo and, to a lesser extent, constitutive cargo, but not for ER-to-Golgi transport.
Impaired retrograde transport in Vti1a/b DKO neurons.

Because SNARE proteins mediate membrane fusion, the abnormal Golgi structure and cargo export may be the consequence of defective fusion reactions at the Golgi in DKO neurons. Since anterograde transport from the ER was unaltered (Fig. 7), we investigated retrograde transport to the Golgi using Cholera Toxin subunit-B fused to Alexa-488 (CTB-A488) as reporter30. In DIV-14 neurons, CTB-A488 expression was predominantly observed at the Golgi 2 h after application (Fig. 8a). This fluorescence was 37% lower in DKO neurons (Fig. 8b) and 27% lower after normalizing to the fluorescence outside this organelle (Fig. 8c, d). Vti1a expression rescued the relative fluorescence at the Golgi, but Vti1b expression only showed partial rescue (Fig. 8a–d). Because CTB transport relies on synaptic activity30, we repeated the experiment at DIV-5, but no alterations were observed 2 h after incubation (Supplementary Figure 11). Because we previously observed that defects in GPI export from the Golgi were only detected after short, but not long, incubation times (Fig. 7l–m), we reasoned that a similar time-dependency may affect CTB-A488 and explain the absence of a measurable phenotype 2 h after CTB-A488 incubation in DKO neurons. To explore this possibility, we measured CTB-A488 expression at the Golgi 30 min after incubation and observed that, at this shorter time point, the relative fluorescence at the Golgi was decreased in DKO neuron (Supplementary Figure 11A-D).

The mammalian VPS10 orthologs Sortilin and Sorcs1 sort regulated cargo31–33. Therefore, we studied these proteins as candidates to explain altered Golgi export in DKO neurons. However, cellular levels and distribution of these proteins were normal (Supplementary Figure 12A-D), and Vti1a/b and Sortilin or Sorcs1 did not co-immunoprecipitate (Supplementary Figure 12, E, F), as opposed to Vti1a/b and a Vti interactor, Syntaxin-1616, or Vti1p and VPS10 in yeast34 (Supplementary Figure 12G).

These results indicate that DKO neurons have reduced retrograde transport of CTB-A488, which is fully rescued by Vti1a, and partially by Vti1b. Furthermore, expression or distribution of the VPS10 proteins Sortilin and Sorcs1 provide no explanation for altered Golgi sorting in DKO neurons.

Discussion

We investigated the function of Vti1a/b in regulated secretion. We showed that in the absence of Vti1a/b neurons form less synapses, contain fewer DCVs and have impaired secretion capacity. Furthermore, the synaptic levels of proteins that regulate secretion were lower, the influx of SNAP25 and DCV cargo into the axon was decreased and these molecules accumulated in the Golgi. The expression of either Vti1a or Vti1b rescued all these phenotypes. Finally, Golgi morphology was abnormal, with distended cisternae and clear vacuoles, cargo export from the Golgi was compromised, and retrograde, but not anterograde, trafficking to the Golgi was impaired.

Vti1a and Vti1b are the two mammalian orthologs of the single-yeast Vti1p gene34. Vti1a and Vti1b share only 31% homology35. In vitro and ex vivo studies have reported separate functions for these proteins in distinct steps of intracellular trafficking15–19. In line with this, we observed (partial) colocalization of Vti proteins with endo-lysosomal markers. We also found that Vti proteins co-localize at the Golgi and that many Vti1a/b-null phenotypes in neurons were fully rescued by the expression of either Vti1a or Vti1b, indicative of redundant functions. Furthermore, the fact that DKO mice show neurodegeneration and die at birth, while single null mutants are viable and fertile12,36 supports shared functions for these proteins. Hence, Vti1a and Vti1b support many cellular functions in neurons in an equally efficient and largely redundant manner. Such redundancy may have remained unnoticed because most of the previous studies selectively addressed single Vti proteins and/or used systems where only a single-Vti protein may be expressed. Alternatively, redundancy may be specific for neurons, which is supported by the fact that neurons are the most affected cell type in the DKO mice12. The reduced DCV pool and impaired CTB delivery to the Golgi were the only two phenotypes more efficiently rescued by Vti1a than Vti1b. This aligns with the decreased number of secretory granules in Vti1a KO chromaffin cells24 and the Vti1a-dependent transport of Shiga toxin to the Golgi in cell lines16. Hence, although few aspects of intracellular trafficking are more efficiently supported by Vti1a, Vti1a and Vti1b generally support cellular functions in a largely redundant manner, at least in neurons.

Vti1a is detected in SV fractions35,37 and local roles in endosomal sorting of SVs38 and spontaneous SV fusion30,39 have been proposed. This latter proposal was supported by the spontaneous fusion of Vti1a-labeled acidic organelles (which were distinct from Syntaxin6– or transferrin receptor-labeled organelles) and by a reduced spontaneous SV fusion upon single Vti1a or combined Vti1a/VAMP7 knockdown30,39. We observed that SV and
DCV secretion were severely impaired in Vti1a/b-deficient neurons. Because SNAP25 supplies the Qb domain in the SNARE complex that drives SV exocytosis, also spontaneous22,23, it seems improbable that the Qb-SNAREs Vti1a/b have a direct role in SV fusion with the plasma membrane. Our data demonstrates that Vti1a/b maintain normal levels of, at least, SNAP25, Munc13-1, RIM, Bassoon, and Synaptotagmin-1 and, to a lesser extent, Syntaxin-1, Synaptobrevin-2, and Munc18-1, and that at least SNAP25 accumulates at the Golgi. Deiciency for most of these proteins inhibits SV fusion3,40,41 and DCV fusion21,25,42, and haploinsufficiency also affects synaptic transmission for at least, SNAP25 or Munc18-143,44. Hence, the combined reduction of the
levels of these proteins provides a plausible explanation for the impaired secretion in the absence of Vti1a/b. Although our data cannot exclude local synaptic functions for Vti1a/b, we conclude that Vti1a/b support synaptic secretion by sorting and targeting components of the secretion machinery to synapses.

Fewer DCVs were observed in synaptic micrographs of DKO neurons and staining for DCV-cargo was reduced, whereas DCV cargo loading per vesicle was unaltered. This indicates that DKO neurons express fewer DCVs with probably unaltered cargo composition. In contrast, the number of SVs per synapse was unaffected, while staining intensity for synaptic markers was decreased in DKO synapses. This suggests that, unlike DCV biogenesis, SV biogenesis is unaffected by the loss of Vti1a/b, despite the reduced levels of synaptic proteins. We conclude that SV biogenesis is coupled to the rate of exocytosis, as suggested by recent studies, and does not depend on Vti1a/b. Furthermore, given the reduced staining of synaptic proteins, SVs most likely contain a reduced number of native proteins per vesicle in DKO neurons. This provides a plausible explanation for the impaired synaptic transmission in Vti1a/b neurons.

The influx of DCV-cargo and proteins of the secretion machinery into the axon was decreased in DKO neurons, but colocalization of these proteins with lysosomes or their cellular stability were unaltered. Therefore, the reduced levels of DCVs and proteins that drive secretion are most likely explained by decreased axonal influx, not by aberrant protein turnover. The two main cellular pathways that can explain the decreased influx into axons are impaired maturation of transport vesicles (by homotypic or heterotypic fusion) or impaired protein export from the Golgi. The Vti partner Syntaxin-6 mediates homotypic fusion of secretory organelles ex vivo, arguing for the first scenario. However, the somatic distribution of DCVs and the cargo content of DCVs were normal in Vti1a/b-deficient neurons, suggesting unaffected maturation. A different Qb-SNARE may support vesicle maturation or, alternatively, this process may not be essential in neurons. In contrast, we observed independent lines of evidence for the second scenario, impaired Golgi export. The Golgi was smaller and cisternae were abnormal, the normal non-homogeneous distribution of DCV-cargo inside the Golgi was lost, cargo accumulated at the Golgi and the transit time of labeled cargo through this organelle was abnormally long. In addition, the export of labeled cargo was also delayed in astrocytes, indicating that Vti1a/b do not only support this function in neurons. Therefore, we conclude that Vti1a/b are required for normal protein export from the Golgi.

The accumulation of tumor necrosis factor-α in a Golgi-like compartment in macrophages after Vti1b inhibition. Our study shows that targeting of cargo presumably not sorted to the regulated secretory pathway, like SNAP25, GPI, PSD95, and Trkβ receptors, was also impaired in DKO neurons. This is in line with the impaired delivery to the plasma membrane of Ca2+-channels in Vti1a-null chromaffin cells and Kv4 K+ channels in HeLa cells deficient for Vti1a or the Vti SNARE partner VAMP7, and indicates that the exit of many proteins from the Golgi relies on Vti1a/b.

Neurons degenerate in the absence of Vti1a/b. Because neuronal death was already abundant at DIV-4, when regulated secretion is minimal, and because abolishing regulated secretion per se does not trigger neuronal death, the degeneration observed in DKO neurons is probably not a direct consequence of impaired exocytosis. However, we cannot exclude that the combined loss of (many) membrane proteins contributes to the degeneration observed. Despite the accelerated cell death of DKO neurons, many cellular functions were normal. The distribution of synapses and DCVs was unaffected, DCV trafficking speed (around 1 μm/s) was in the range of reported values, and SV endocytosis, calcium influx and efflux and vesicular acidification were similar to DHZ neurons, confirming that Vti1a/b DKO neurons can cope with energy-demanding tasks. Furthermore, protein stability, mitochondrial morphology and ER-to-Golgi cargo trafficking were unaffected. This is in line with previous observations that many intracellular trafficking pathways, including recycling of transferrin receptors, delivery of endocytosed cargo to lysosomes and autophagy, were fully functional in Vti1a/b DKO fibroblasts. Golgi abnormalities in the absence of Vti1a/b were also observed in acute brain slices, indicating that this is not a feature unique for cultured neurons. Furthermore, the levels of synaptic proteins and SV fusion were rescued by expression of Vti1a or Vti1b after most DKO neurons died, which confirms that these features are not intrinsic of the surviving neurons. Hence, we conclude that loss of Vti1a/b function does not trigger generalized cellular dysfunctions, but selectively affects regulated secretion by impaired Golgi export of relevant proteins, which is not a unique feature for surviving neurons or culture conditions.

Molecules are delivered to the Golgi anterogradely from the ER, or retrogradely from the plasma membrane or intracellular organelles. ER-Golgi trafficking was normal in Vti1a/b-deficient neurons and astrocytes, in line with the fact that other Qb-SNAREs mediate this anterograde pathway. In contrast, retrograde transport from the plasma membrane to the Golgi was impaired in Vti1a/b-deficient neurons, consistent with the decrease in Shiga toxin transport in cell lines upon interference.
with Vti1a-function using antibodies. The preferential localization of Vti1a/b to the Golgi aligns with a role in retrograde transport to this organelle. Vti1b localized to the Golgi in neurons to a larger extent than what was reported in fibroblast cell lines, which may indicate cell type differences. Vti1a/b deficiency had a larger impact on retrograde transport at DIV-14 than at DIV-5. This can generally be explained by a progressive nature of this defect: components that regulate this trafficking probably become progressively missorted over time. Alternatively, different Qb-SNARE may be involved in similar pathways during different developmental stages, or synaptic activity influences CTB retrograde trafficking, as suggested. Expression of either Vti1a or Vti1b in DKO neurons increased CTB delivery to the Golgi, but only Vti1a fully rescued it. Retrograde transport of Shiga toxin to
the Golgi depends on EpsinR33, a Vti1b interactor34, which confirms the relevance of Vti1b in plasma membrane–to–Golgi transport. In addition to plasma membrane–Golgi transport, additional retrograde transport routes may be affected in DKO neurons. Indeed, Vti1a mediates late endosome–to–Golgi transport17, also in yeast55. Defective transport of sorting molecules between endo-lysosomal organelles and the Golgi may also help to explain Golgi export defects in Vti1a/b DKO neurons, and would fit with the role of endo-lysosomal proteins in sorting of regulated secretory cargo10,56,57.

Several cellular phenotypes show striking similarities to those observed for Vti1a/b deficiency. First, blocking expression of the Golgi-Associated Retrograde Protein (GARP)-complex subunit Vps54 in cell lines resulted in inefficient transit of GPI from the Golgi to the cell surface58. Second, disrupting either the Conserved Oligomeric Golgi (COG) or the GARP tethering complexes, which function in recycling to or within the Golgi, resulted in distended Golgi cisternae and vacuolization53,59–61. These similarities suggest that Vti1a/b may work together with one or both of these complexes to regulate transport to or within the Golgi and that impaired delivery of molecules that control protein sorting may explain the cellular phenotypes described in our study (Fig. 8e). Interestingly, Vti1a indeed interacts with COG and GARP subunits62,63 but the functional implications of these interactions remains unknown.

In yeast, Vti1p binds to the sorting receptor Vps10 and a Vti1p mutation causes cargo misorting64. In mammals, the VPS10 receptors Sortilin and Sorcs1 bind and sort regulated cargo31–33,37–39, and would provide a plausible link between retrograde trafficking defects and other cellular phenotypes observed in Vti1a/b DKO neurons. However, the levels and distribution of these receptors were unaltered in DKO neurons, and binding to mammalian Vti1a or Vti1b was not detected. Additional molecules have been implicated in protein sorting at the Golgi, including calcium transporters64, proton pumps65, HHD166, CAB456, and lipid66,67. Cytosolic proteins, such as actin regulators64, adapter complexes65, enzymes65, and Arf members7,12, also contribute to Golgi sorting, but the correct localization of these soluble proteins probably does not rely directly on Vti1a/b-dependent membrane trafficking. An overarching model for protein sorting at the Golgi is lacking and the link between molecules that function in this process and Vti1a/b remains unclear. However, the current study clearly indicates that Vti1a/b function is central to coordinate Golgi import/export, with major downstream consequences if this aspect is not functional.

**Methods**

**Laboratory animals and primary cultures.** Vti1a/b null mice, generated by replacing a 6.5 Kb fragment in Vti1a exon 6 and a 7 Kb fragment in Vti1b exon 4 with neomycin resistance cassettes, have been described before30,36. Embryonic day (E) 18.5 embryos were obtained by cesarean section of pregnant females from timed mating of Vti1a± and Vti1b± mice, or vice versa. Mouse hippocampi were dissected from these embryos in HBSS (Sigma), digested in 0.25% trypsin (Sigma) and 1 mg/ml poly-D-lysine (Sigma) and 0.7 mg/ml rat tail collagen (BD Biosciences). Animals were housed and bred according to institutional and Dutch guidelines.

**Plasmids.** Vti1a, Vti1b, NPY-pHluorin, NPY-mCherry, Synapsin-ECFP, and Synaptophysin-pHluorin plasmids have been described24. A plasmid for α2δ1-pHluorin was engineered by substituting YFP by mCherry in NaVa122-YFP (Addgene, 26056). ManII-ECFP was made by substituting GFP by ECFP in ManII-GFP (gift from V. Malhotra, Centre for Genomic Regulation, Barcelona, Spain). The above plasmids were cloned into pLenti vectors under the human Synapsin promoter and constituting GPI from Streptavidin-KDEL/SBP-EGFP-GPI (Addgene, 65294). Standard calcium phosphate precipitation was used to deliver these plasmids, in combination with ManII-ECFP. Cells were analyzed 20–24 h after transfection.

For immunoprecipitation, Vti1a and 1b were fused to Myc tags, and Sorcs1 (gift from J. de Wit, VIB Center for the Biology of Disease, Leuven, Belgium) and Sortilin (gift from C. M. Petersen, Aarhus Universiteit, Denmark) were fused to Flag tags. These plasmids were expressed under CMV promoters and transfected into HEK293T cells.

**Immunostaining.** Neurons, fixed in 3.7% paraformaldehyde (EM) for 20 min, were permeabilized with 0.5% Triton-X (Fisher Scientific) for 5 min and blocked with 2% normal goat serum (Life Technologies) and 0.1% Triton-X for 20 min. Incubation with primary and secondary antibodies was done at room temperature (RT) for 2 or 1 h, respectively. All solutions were in PBS (composition in mM: 137 NaCl, 2.7 KCl, 10 Na2HPO4, 1.8 KH2PO4, pH = 7.4). Coverslips were mounted in Mowiol (Sigma) and Z-stacks were obtained on a confocal microscope (Nikon Eclipse Ti) equipped with ×10 (NA = 0.45) and ×63 oil (NA = 1.40) objectives controlled by NisElements 4.30 software. For retrograde labeling experiments, neurons were incubated at 37 °C with CTB-A488 (Thermo Fisher; 100 ng/ml) for 15 min and fixed 30 min or 2 h after incubation. For protein stability, neurons were incubated with cycloheximide (Sigma, 100 µg/ml) in 0.1% DMSO, or 0.1% DMSO alone for 24 h before fixation.

Primary antibodies used for immunocytochemistry: Vti1a (1:1000, BD, 611220), Vti1b (1:1000)35,36, MAP2 (1:1000, Abcam, ab5392), Munc18-1 (1:100, BD, 610336), SNAP25 (1:1000, Abcam, ab8481), Syntaxin-1 (1:1000, SIGMA), Syntaxin-1 (1:1000, Santa Cruz), Syntaxin-1 (1:1000, SySy, mouse), Syntaxin-1 (1:1000, SySy, mouse), Sortilin (gift from C. M. Petersen, Aarhus Universiteit, Denmark) were fused to Myc tags, and Sorcs1 (gift from J. de Wit, VIB Center for the Biology of Disease, Leuven, Belgium) and Sortilin (gift from C. M. Petersen, Aarhus Universiteit, Denmark) were fused to Flag tags. These plasmids were expressed under CMV promoters and transfected into HEK293T cells.
citrate buffer (pH 6) and incubated with 1% normal goat serum in PBS for 1 h. Renatured sections were incubated with primary mouse anti-GM130 antibody (1:400 in PBS, 1% goat serum) overnight at 4 °C, followed by Cy3-conjugated anti-mouse antibodies (Jackson Immunoresearch, 1:400 in PBS with 1% goat serum) for 1 h at RT. Nuclei were stained in Hoechst dye (1:1000; Thermo Fisher, 33342).

Live imaging. Coverslips were placed in imaging chambers perfused with Tyrode’s buffer (composition in mM: 2 CaCl2, 2.5 KCl, 119 NaCl, 25 HEPES, pH 7.4) and recorded at RT using a Zeiss AxioImager.Z1 (SV and DCV fusion) or at 37 °C (FRAP and RUSH) using a Nikon Eclipse Ti microscope. Microscopes were equipped with laser-based illumination (444, 488, and 561 nm), appropriate filter sets, ×40 oil (NA = 1.3) or ×63 oil (NA = 1.4) objectives and an EM charge-coupled device camera (C9100-02, Hamamatsu). Time-lapse recordings were acquired using Axiovision 4.8 or NisElements 4.30. The acquisition frequency was 2 Hz for DCV fusion, FRAP and calcium imaging; 0.6 Hz for dual color (SypHy/FM4-64) SV fusion and 1 Hz for single (SypHy) SV fusion at DIV10. HFS was applied using parallel platinum electrodes delivering 50 mA, 1 ms pulses controlled by a Master-8 (AMPI) and a stimulus generator (A-365, WPI). Intracellular pH was neutralized by gravity flow application of modified Tyrode’s solution (50 mM NaCl replaced by 50 mM NHE4·Cl). For FRAP experiments, laser intensity and pulse duration for bleaching were optimized to reach >90% fluorescence decrease of NPY-mCherry or SNAP25-EGFP at the axon initial segment (labeled using NaVII/III-mCherry or -EYFP). For calcium imaging, neurons were incubated with 2 µM Fluo5F AM (Molecular Probes) in supplemented Neurobasal solution (50 mM NaCl replaced by 50 mM NH4Cl). For FRAP experiments, the number and speed of NPY-mCherry or SNAP25-EGFP fusion events with >50% pixels within the binary mask were considered synaptic.

Statistics. Shapiro and Levene’s test was used to test distribution normality and homogeneity of variances, respectively. When assumptions of normality or homogeneity of variances were met, parametric tests were used: t-test or ANOVA (Tukey as post hoc). Otherwise, non-parametric tests were used: Mann–Whitney U or Kruskal–Wallis test (Holm as post hoc). R was used as software. No predetermined sample size or randomization were used. No data was excluded. Data was analyzed blindly.

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Author contributions
J.E.M., R.F.T., and M.V. designed the experiments. J.E.M. collected and analyzed experimental data, except for electrophysiology (V.H), electron microscopy (J.v.W.) and brain slice stainings (C.B.). G.F.v.M. facilitated the mouse line. J.E.M, R.F.T., and M.V designed figures and wrote the manuscript with input from all authors.

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