

Rabphilin-3A negatively regulates neuropeptide release, through its SNAP25 interaction

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Abstract

Neuropeptides and neurotrophins are stored in and released from dense-core vesicles (DCVs). While DCVs and synaptic vesicles (SVs) share fundamental SNARE/SM proteins for exocytosis, a detailed understanding of DCV exocytosis remains elusive. We recently identified the RAB3-RIM1 pathway to be essential for DCV-, but not SV exocytosis, highlighting a significant distinction between the SV- and DCV secretory pathways. Whether RIM1 is the only RAB3 effector that is essential for DCV exocytosis is currently unknown. In this study, we show that rabphilin-3A (RPH3A), a known downstream effector of RAB3A, is a negative regulator of DCV exocytosis. Using live-cell imaging at single vesicle resolution with RPH3A-deficient hippocampal neurons, we show that DCV exocytosis increased 3-fold in the absence of RPH3A. RAB3A-binding deficient RPH3A lost its punctate distribution, but still restored DCV exocytosis to WT levels when re-expressed. SNAP25-binding deficient RPH3A did not rescue DCV exocytosis. In addition, we show that RPH3A did not travel with DCVs, but remained stationary at pre-synapses. RPH3A null neurons also had longer neurites, which was partly restored when ablating all regulated secretion with tetanus neurotoxin. Taken together, these results show that RPH3A negatively regulates DCV exocytosis, potentially also affecting neuron size. Furthermore, RAB3A interaction is required for the synaptic enrichment of RPH3A, but not for limiting DCV exocytosis. Instead, the interaction of RPH3A with SNAP25 is relevant for inhibiting DCV exocytosis.

eLife assessment

This **important** study advances our understanding of the mechanisms of neuronal large dense-core vesicle (LDCV) secretion, which mediates neuropeptide and neurotrophin release. It describes a negative regulatory process involving the interaction of the Rab3-effector Rabphilin-3A with the SNARE fusion protein SNAP25, which limits LDCV secretion and neurite growth. The evidence in support of the authors' claims is generally **convincing**, but some conclusions, e.g. regarding the role of Rabphilin-3A-controlled neurotrophin signaling in neurite growth, are **incompletely** supported. This study will be of interest to the fields of cell biology, cellular neuroscience, and neuroendocrinology.

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Introduction

Neuropeptides and neurotrophins are crucial neuronal signaling molecules that play diverse roles in brain development, neurogenesis and synaptic plasticity (Malva et al., 2012 [↗](#); Pang et al., 2004 [↗](#); Park & Poo, 2013 [↗](#); van den Pol, 2012 [↗](#); Zaben & Gray, 2013 [↗](#)). These neuromodulators are sorted at the trans-Golgi network (TGN) and packaged into dense core vesicles (DCVs). Similar to neurotransmitter release from synaptic vesicles (SVs), neuropeptide release is calcium and activity-dependent. However, unlike SVs, DCVs can fuse at various locations in the neuron, requiring higher and more sustained stimulation frequencies (Balkowiec & Katz, 2002 [↗](#); Hartmann et al., 2001 [↗](#); Persoon et al., 2018 [↗](#)). Interestingly, all SNARE/SM proteins essential for exocytosis are shared between these two secretory pathways (Arora et al., 2017 [↗](#); Farina et al., 2015 [↗](#); Hoogstraaten et al., 2020 [↗](#); Puntman et al., 2021 [↗](#); Südhof, 2013 [↗](#); van de Bospoort et al., 2012 [↗](#)). However, while SV secretory pathways are well characterized, a detailed understanding of DCV exocytosis is still emerging. We recently discovered that RAB3, a protein largely dispensable for SV exocytosis (Schlüter et al., 2004 [↗](#), 2006 [↗](#)), and its effector RIM1 to be essential for DCV exocytosis (Persoon et al., 2019 [↗](#)). This indispensable role of the RAB3-RIM pathway signifies a main difference between SV and DCV exocytosis. Whether RIM is the only RAB3 effector that is essential for DCV exocytosis is currently unknown.

Rabphilin-3A (RPH3A) is a downstream effector of RAB3A that is highly expressed in the brain (Schlüter et al., 1999 [↗](#)). RPH3A expression increases throughout development, similar to established synaptic proteins (Baldarelli et al., 2021 [↗](#); Blake et al., 2021 [↗](#); Krupke et al., 2017 [↗](#)). RPH3A binds RAB3A and RAB27 and contains two lipid- and calcium-binding C2 domains (Guillén et al., 2013 [↗](#); Tsuboi & Fukuda, 2005 [↗](#)). The second C2 domain (C2B) binds SNAP25 in a calcium independent manner (Deák et al., 2006 [↗](#)). Similar to RAB3 null mutants, depletion of RPH3A shows no detectable synaptic phenotype in mice under normal physiological conditions (Schlüter et al., 1999 [↗](#)), and only a mild phenotype in *C. elegans* (Staunton et al., 2001 [↗](#)). However, mutant mice do show increased synaptic recovery after intense stimulation, which is rescued by reintroducing full length (FL) RPH3A but not a mutant that lacks the C2B domain. This suggests that RPH3A negatively regulates SV recycling after depletion of the releasable vesicle pool, and that this function depends on its interaction with SNAP25 (Deák et al., 2006 [↗](#)). In *C. elegans*, the absence of RBF-1, the homolog of RPH3A, exacerbates the phenotypes of other SNARE mutants, as observed in the *rbf-1/ric-4* (RPH3A/SNAP25) double mutant, suggesting that RPH3A contributes to SNARE protein function (Staunton et al., 2001 [↗](#)).

The role of RPH3A in the context of DCV exocytosis within mammalian neurons remains unclear. Notably, a recent genetic screen in *C. elegans* revealed that *rbf-1* depletion increases the release of fluorescently tagged neuropeptides without affecting the frequency of spontaneous mini-EPSCs. This suggests a potential negative regulatory role for RPH3A in neuropeptide release (Laurent et al., 2018 [DOI](#)), opposite to RIM1 (Persoon et al., 2019 [DOI](#)). However, it remains uncertain whether RPH3A functions as a negative regulator in mammalian neurons, and whether, similar to synaptic transmission, the interaction with SNAP25 is important.

We set out to determine the role of RPH3A in DCV exocytosis in hippocampal mouse neurons, and to investigate the relevance of RAB3A and SNAP25 binding. We found that RPH3A did not travel with DCVs, but remained stationary at synapses. Using RPH3A null mutant mice (Schlüter et al., 1999 [DOI](#)) we show that the absence of RPH3A indeed increased DCV exocytosis. Furthermore, RPH3A null neurons exhibit longer neurites and an increased number of DCVs. This effect was partly reduced when all regulated secretion was eliminated by tetanus neurotoxin (TeNT, Hoogstraaten et al., 2020 [DOI](#); Shimojo et al., 2015 [DOI](#)), indicating that the increased neurite length partially depends on regulated secretion. Finally, expressing a mutant RPH3A unable to bind RAB3A/RAB27A restored DCV exocytosis, but not when expressing a mutant RPH3A unable to bind SNAP25. This suggests that limiting DCV exocytosis does not depend on the interaction with RAB3A, but at least in part on the interaction with SNAP25.

Results

RPH3A is enriched in presynaptic structures

RPH3A is reported to localize to both pre- and post-synaptic sites (Stanic et al., 2015 [DOI](#)), and on DCVs through its RAB-binding domain (RBD) in PC12 cells (Fukuda et al., 2004 [DOI](#)). We first determined whether RPH3A is expressed in excitatory or inhibitory mouse neurons. To test this, we immunostained for RPH3A in hippocampal VGLUT+ wildtype (WT) neurons and striatal VGAT+ WT neurons at 14 days *in vitro* (DIV14, **Figure S1A** [DOI](#)). We found no difference in RPH3A expression between hippocampal and striatal neurons (**Figure S1B** [DOI](#)). To localize RPH3A with higher spatial precision and confirm localization on DCVs, we performed stimulated emission depletion (STED) microscopy in hippocampal mouse neurons at DIV14. RPH3A staining was predominantly punctate and overlapped with the presynaptic marker Synapsin1 (Syn1, **Figures 1A, B** [DOI](#)) and, to a lesser extent, with the post-synaptic marker Homer (**Figures 1A, C** [DOI](#)). RPH3A partly colocalized with DCV marker chromogranin B (ChgB, **Figures 1A, D** [DOI](#)). Pearson's correlation coefficients were similar for RPH3A with either Syn1, Homer and ChgB (**Figure 1E** [DOI](#)), however, Mander's overlap coefficient analysis (Manders et al., 1992 [DOI](#)) revealed that RPH3A colocalized significantly more with Syn1 than Homer or ChgB (**Figure 1F** [DOI](#)). In addition, most RPH3A puncta contained Syn1, but not all Syn1 puncta contained RPH3A (**Figure 1G** [DOI](#)). These data suggest that RPH3A is a synaptic protein with predominantly presynaptic accumulation.

We next determined which known interactions are important for RPH3A's synaptic localization by expressing mutant RPH3A constructs fused to mCherry, lacking specific interactions or a phosphorylation site, in hippocampal WT neurons at DIV1-2 (**Figure S1C** [DOI](#)). FL RPH3A-mCherry and all mutant constructs were expressed to a similar level in neurites (**Figures S1G, H** [DOI](#)). We performed STED microscopy at DIV14 and quantified the colocalization of mCherry with Syn1 and Homer (**Figures S1D, E** [DOI](#)). Similar to endogenous RPH3A, FL WT RPH3A-mCherry showed a punctate distribution (**Figure S1D** [DOI](#)) that colocalized strongly with Syn1, and to a lesser extent with Homer (**Figure S1F** [DOI](#)). Four mutant versions of RPH3A: a C-terminal truncation, a Ca²⁺-binding deficient mutant (Δ Ca²⁺-binding) and a mutant that does not bind SNAP25 (Δ SNAP25), all retained a similar punctate presynaptic distribution (**Figures S1D, E** [DOI](#)). However, mutant RPH3A unable to bind RAB3A/RAB27A (Δ RAB3A/RAB27A, Fukuda et al., 2004 [DOI](#)) and a mutant that lacked a CaMKII dependent phosphorylation site (Δ CaMKII-phos site) showed no punctate distribution

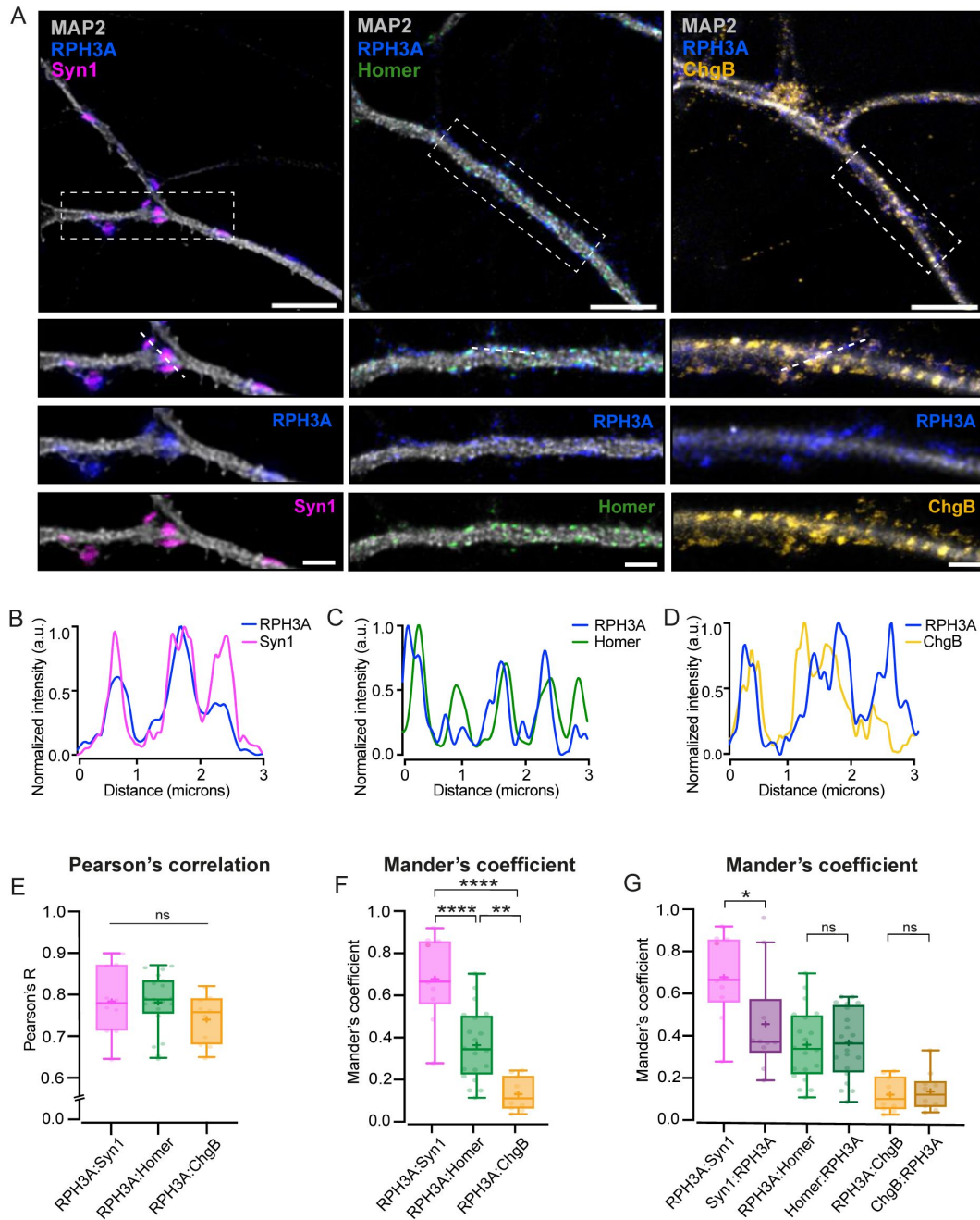


Figure 1.

RPH3A localizes to the pre-synapses.

(A) Representative images of WT hippocampal neurons (top) with zooms (bottom) co-stained for RPH3A (blue) and Syn1 (magenta), Homer (green) or ChgB (yellow). Scale bar, 5 μ m (top) and 2 μ m (bottom). (B, C, D) Line plots show normalized fluorescent intensity across dotted line in example zooms. (E) Pearson's correlation coefficient and (F) Mander's overlap coefficient comparing the colocalization of RPH3A with Syn1, Homer and ChgB. (G) Mander's overlap coefficient comparing the colocalization of RPH3A in either Syn1, Homer or ChgB puncta, and vice versa. RPH3A:Syn1, RPH3A:Homer and RPH3A:ChgB show the same dataset as in F. Dots represent a field of view. Boxplots represent the median (line), mean (+) and Tukey range (whiskers). Each dot represents a field of view: Syn1: 2(10); Homer 2(20); ChgB: 2(10). Kruskal-Wallis H test with Dunn's correction: * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. ns = non-significant, $P > 0.05$.

(**Figures S1D-F**). These data suggest that the interaction with RAB3A is required for RPH3A's synaptic localization. CaMKII dependent phosphorylation may also be involved in the synaptic localization of RPH3A.

RPH3A does not travel with DCVs

Recent evidence has demonstrated that RAB3A is transported together with DCVs (Persoon et al., 2019). To test if RPH3A travels with DCVs on both axons and dendrites, we co-expressed neuropeptide Y (NPY)-mCherry and FL WT EGFP-RPH3A (Tsuboi & Fukuda, 2005) in DIV14 RPH3A knockout (KO) neurons. In addition, we tested co-transport of NPY fused to pH-sensitive EGFP (NPY-pHluorin), with two RPH3A mutants: a truncated RPH3A lacking both C2 domains but retaining the RAB3A and RAB27A binding sites, and Δ RAB3A/RAB27A mutant (**Figure 2A**, Fukuda et al., 2004). Live-cell imaging was performed before and after photobleaching a fixed-size area in neurites, without distinguishing between axons and dendrites, to enhance visualization of moving vesicles entering the bleached area (**Figures 2B-D**). The number of moving versus immobile puncta was determined for each construct prior to photobleaching. Both FL- and truncated RPH3A exhibited low mobility (7-14% moving), although they occasionally showed small movements over short distances (less than 10 μ m within 4 minutes). In contrast, 47% of NPY puncta were mobile (**Figure 2E**), as shown before (Hoogstraaten et al., 2020; Persoon et al., 2019). Only a small fraction of RPH3A (14%) and truncated RPH3A (7%) puncta travelled at velocities characteristic for DCV transport (Bittins et al., 2010; de Wit et al., 2006; Kwinter et al., 2009). These results indicate that the majority of RPH3A organizes in immobile puncta that colocalize with synaptic markers (see above), but do not travel through the axons or dendrites like DCVs do.

We next determined the fraction of RPH3A that travelled together with NPY-labelled vesicles. Few moving NPY vesicles contained RPH3A or truncated RPH3A (NPY:RPH3A = 6%, NPY:Truncated RPH3A = 1%). Conversely, of the already small fraction of moving FL- and truncated RPH3A, only a few travelled together with NPY (RPH3A:NPY = 18%, truncated RPH3A:NPY = 8%; **Figure 2F**). Truncated RPH3A showed almost no fluorescence recovery after photobleaching compared to NPY (**Figure 2G**), however Δ RAB3A/RAB27A RPH3A showed a faster recovery compared to FL- and truncated RPH3A (**Figure 2H**). Overall, these findings suggest that RPH3A does not travel with DCVs and that the stationary organization of RPH3A relies on RAB3A/RAB27A interactions.

RPH3A deficiency increases DCV exocytosis

Since RPH3A appeared to remain mostly stationary at the pre-synapse (**Figures 1**, 2), we examined the role of RPH3A in neuropeptide release in hippocampal neurons. We recorded DCV fusion events in single hippocampal mouse neurons from RPH3A KO and WT littermates at DIV14-16. We confirmed the loss of RPH3A expression in RPH3A KO neurons with immunocytochemistry (**Figures 3A, B**). NPY-pHluorin, a validated DCV fusion reporter (Arora et al., 2017; Farina et al., 2015; Persoon et al., 2018; van de Bospoort et al., 2012), was used to quantify single fusion events (**Figure 3C**). To elicit DCV fusion, neurons were stimulated twice with 8 bursts of 50 action potentials (AP) at 50 Hz, separated by 30 s (**Figure 3D**) or once with 16 bursts of 50 APs at 50 Hz. The acidity of DCVs quenches NPY-pHluorin, but upon fusion with the plasma membrane, the DCV deacidifies resulting in increased fluorescent NPY-pHluorin intensity (**Figure 3C**). After stimulation, neurons were briefly perfused with NH_4^+ to de-quench all NPY-pHluorin labelled DCVs (**Figures 3C, D**) to determine the number of remaining DCVs per cell. We have previously demonstrated that the fluorescent intensity of NPY-pHluorin in confocal imaging directly correlates with the number of fluorescent puncta for endogenous DCV markers using super-resolution imaging (Persoon et al., 2018).

RPH3A KO neurons showed a 3-fold increase in the total number of fusion events compared to WT (**Figures 3F, G**, S2A, B). To test if re-expressing RPH3A in KO neurons could restore DCV fusion to WT levels, we infected KO neurons with a FL RPH3A construct at DIV0 (**Figure 3E**). This

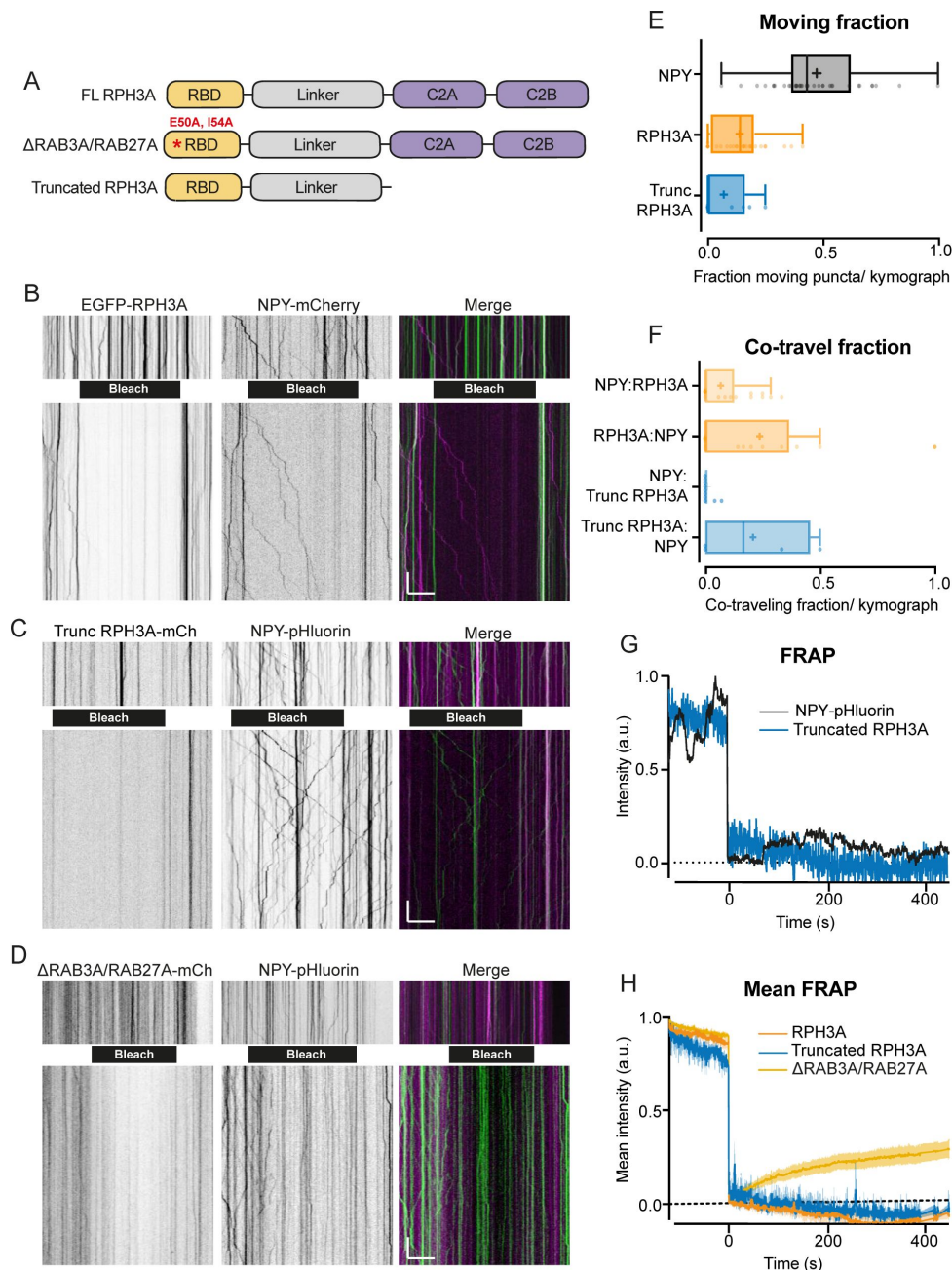


Figure 2.

RPH3A does not travel with DCVs.

(A) Domain structures of FL RPH3A and mutant RPH3A constructs lacking specific interactions: Δ RAB3A/RAB27A mutant RPH3A and truncated RPH3A that lacked its calcium and SNAP25 binding C2A and C2B domain. (B) Kymographs of EGFP-RPH3A and NPY-mCherry, (C) mCherry-truncated RPH3A and NPY-pHluorin, and (D) mCherry- Δ RAB3A/RAB27A RPH3A and NPY-pHluorin before (upper) and after (lower) photobleaching (black bar) to remove most stationary puncta and facilitate analysis. NPY-pHluorin showed more resistance for bleaching. Merged images show mCherry (pseudo-coloured magenta) and EGFP/pHluorin (green). Scale bar, 20 μ m (x-axis) and 20 s (y-axis). (E) Moving fraction of NPY, FL RPH3A and truncated RPH3A puncta per kymograph. (F) Fraction of co-travel of NPY puncta with either FL- or truncated RPH3A puncta, and co-travel of FL- or truncated RPH3A with NPY puncta. Dots represent a kymograph. (G) Fluorescent recovery of the traces shown in Figure 2C after photobleaching truncated RPH3A or NPY-pHluorin. (H) Mean fluorescent recovery traces from multiple kymographs after photobleaching FL-, truncated- or Δ RAB3A/RAB27A RPH3A. Lines $\pm$ shading represents mean $\pm$ SEM. Boxplots represent median (line), mean (+) and Tukey range (whiskers).

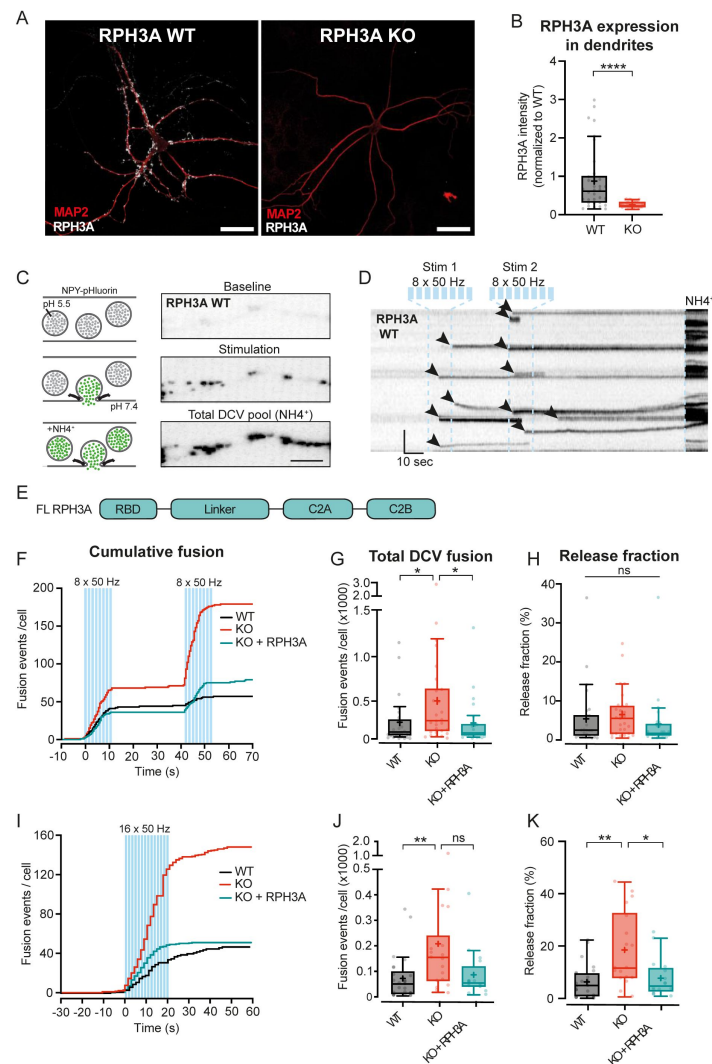


Figure 3.

RPH3A deficiency increases DCV exocytosis.

(A) Typical example of RPH3A WT and KO neurons immunostained for MAP2 (red) and RPH3A (white). Scale bar, 50 μ m. (B) RPH3A expression in dendrites of WT and KO neurons normalized to WT per independent experiment. N numbers of individual experiments and single neuron observations in brackets: WT: 4(34); KO: 4(33). (C) Schematic representation (left) and imaging example of a WT neurite stretch (right) infected with NPY-pHluorin as optical DCV fusion reporter. NPY-pHluorin is quenched in the acidic DCV lumen before fusion (baseline) but dequenches upon fusion (stimulation). NH_4^+ perfusion dequenches all NPY-pHluorin labelled DCVs (remaining DCV pool). Scale bar, 5 μ m. (D) Kymograph of a WT neurite stretch with the stimulation paradigm used to elicit DCV fusion (two bursts of 8 x 50 AP trains at 50 Hz interspaced by 0.5 s between each train and 30 s between each burst, blue bars) and NH_4^+ perfusion (NH_4^+) used to dequenches all NPY-pHluorin labelled vesicles. Arrowheads indicate fusion events. (E) Domain structure of FL RPH3A construct. (F) Cumulative median histogram of fusion events over time in WT (black), RPH3A KO (red) and KO neurons infected with FL RPH3A (cyan). Blue bars indicate the stimulation paradigm (two bursts of 8 x 50 AP bursts at 50 Hz). (G) Total number of DCV fusion events per condition (two bursts of 8 x 50 AP bursts at 50 Hz). (H) Released fraction defined as the number of fusion events normalized to the remaining pool of DCVs. N numbers of individual experiments and single neuron observations in brackets: WT: 5(51); KO: 5(43); KO + RPH3A: 5(39). (I) Cumulative median histogram of events over time in WT (black), RPH3A KO (red) and KO neurons infected with FL RPH3A (cyan). Blue bars indicate the stimulation paradigm (16 x 50 AP bursts at 50 Hz). (J) Total number of DCV fusion events per condition (16 x 50 AP bursts at 50 Hz). (K) Release fraction per cell. N numbers of individual experiments and single neuron observations in brackets: WT: 4(25); KO: 4(18); KO + RPH3A: 4(16). Boxplots represent the median (line), mean (+) and Tukey range (whiskers). Each dot represents an individual neuron. Kruskal-Wallis H test with Dunn's correction: * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. ns = non-significant, $P > 0.05$.

construct localized to synapses (**Figure S1D** [↗](#)), similar to endogenous RPH3A expression (**Figures 1A-C** [↗](#)). Re-expression of RPH3A in KO neurons restored the number of fusion events to WT levels (**Figures 3F, G** [↗](#)). The released fraction, i.e. the number of fusion events divided by the remaining DCV pool, did not differ between WT and KO neurons (**Figure 3H** [↗](#)), and a trend towards a larger remaining DCV pool in these KO neurons was observed (**Figure S2C** [↗](#)).

We have recently shown that different stimulation protocols influence certain fusion dynamics like event duration, but not the total number of fusion events (Baginska et al., 2023 [↗](#)). To test this and the robustness of our findings, we used a prolonged stimulation protocol (16 bursts of 50 APs at 50 Hz). The event duration was similar in WT and KO neurons, for both stimulation paradigms (**Figures S2D-F** [↗](#)). During prolonged stimulation, we observed an increase in DCV fusion events in KO neurons compared to WT (**Figures 3I, J** [↗](#)), similar to the 2 x 8 stimulation protocol. In addition, the released fraction was significantly increased in KO neurons compared to WT (**Figure 3K** [↗](#)). We did not observe a difference in remaining pool size between KO and WT in these neurons (**Figure S2G** [↗](#)). Overexpression of FL RPH3A in KO neurons again restored the number and released fraction of fusion events to WT levels (**Figures 3I-K** [↗](#)). We did not observe an effect on spontaneous DCV fusion in RPH3A KO neurons (**Figure S2H** [↗](#)). Together, these findings indicate that RPH3A is an inhibitor of DCV exocytosis.

The SNAP25-, but not the RAB3A-interaction domain of RPH3A contributes to limiting DCV exocytosis

To investigate whether the interactions with RAB3A or SNAP25 are relevant to limit DCV exocytosis, we overexpressed FL WT RPH3A, Δ RAB3A/RAB27A mutant RPH3A (Fukuda et al., 2004 [↗](#)), and Δ SNAP25 mutant RPH3A (Ferrer-Orta et al., 2017 [↗](#)) in RPH3A KO neurons (**Figure 4A** [↗](#)). Expression of Δ RAB3A/RAB27A restored DCV fusion and the released fraction to WT levels, similar to FL RPH3A (**Figures 4B, C** [↗](#)). However, expression of Δ SNAP25 did not fully rescue the number of fusion events or the released fraction (**Figures 4E, F** [↗](#)). We did not observe any difference in the number of remaining DCVs upon Δ RAB3A/RAB27A or Δ SNAP25 expression (**Figures 4D, G** [↗](#)). Taken together, these results suggest that RAB3A/RAB27A binding is not essential for the limiting effect of RPH3A on DCV exocytosis, but that the interaction with SNAP25 appears to contribute to this effect.

RPH3A deficiency leads to increased neurite length and DCV numbers

Since we observed a trend towards a bigger DCV pool in KO neurons (**Figure S2C** [↗](#)), and the total number of DCVs per neuron correlates with dendrite length (Persoon et al., 2018 [↗](#)), we examined the neuronal morphology of RPH3A KO neurons. Single hippocampal neurons (DIV14) were immunostained for MAP2 to quantify the total dendritic length, and for endogenous DCV cargo ChgB to determine the number of DCVs (**Figure 5A** [↗](#)). We have previously shown that this correlates well with the number of dSTORM ChgB puncta (Persoon et al., 2018 [↗](#)). Indeed, neurons lacking RPH3A had longer dendrites that harbored more DCVs than WT neurons (**Figures 5B, C** [↗](#)) and KO neurons contained more DCVs per μ m (**Figure 5D** [↗](#)). The number of DCVs correlated with the total dendrite length in both genotypes (**Figure 5F** [↗](#)), as shown previously for WT neurons (Persoon et al., 2018 [↗](#)). Moreover, the intensity of endogenous ChgB was decreased in RPH3A KO neurons (**Figure 5E** [↗](#)), suggesting affected vesicle loading or reduced clustering. However, the peak intensity of fusion events during live recording was unchanged (**Figure S2I** [↗](#)), indicating that the decrease in DCV cargo intensity is potentially due to reduced clustering or accumulation. Taken together, these results suggest that lack of RPH3A leads to longer dendrites with a concomitant increase in the number of DCVs. RPH3A depletion does not seem to affect the neuropeptide content in DCVs, instead it might reduce the clustering of DCVs.

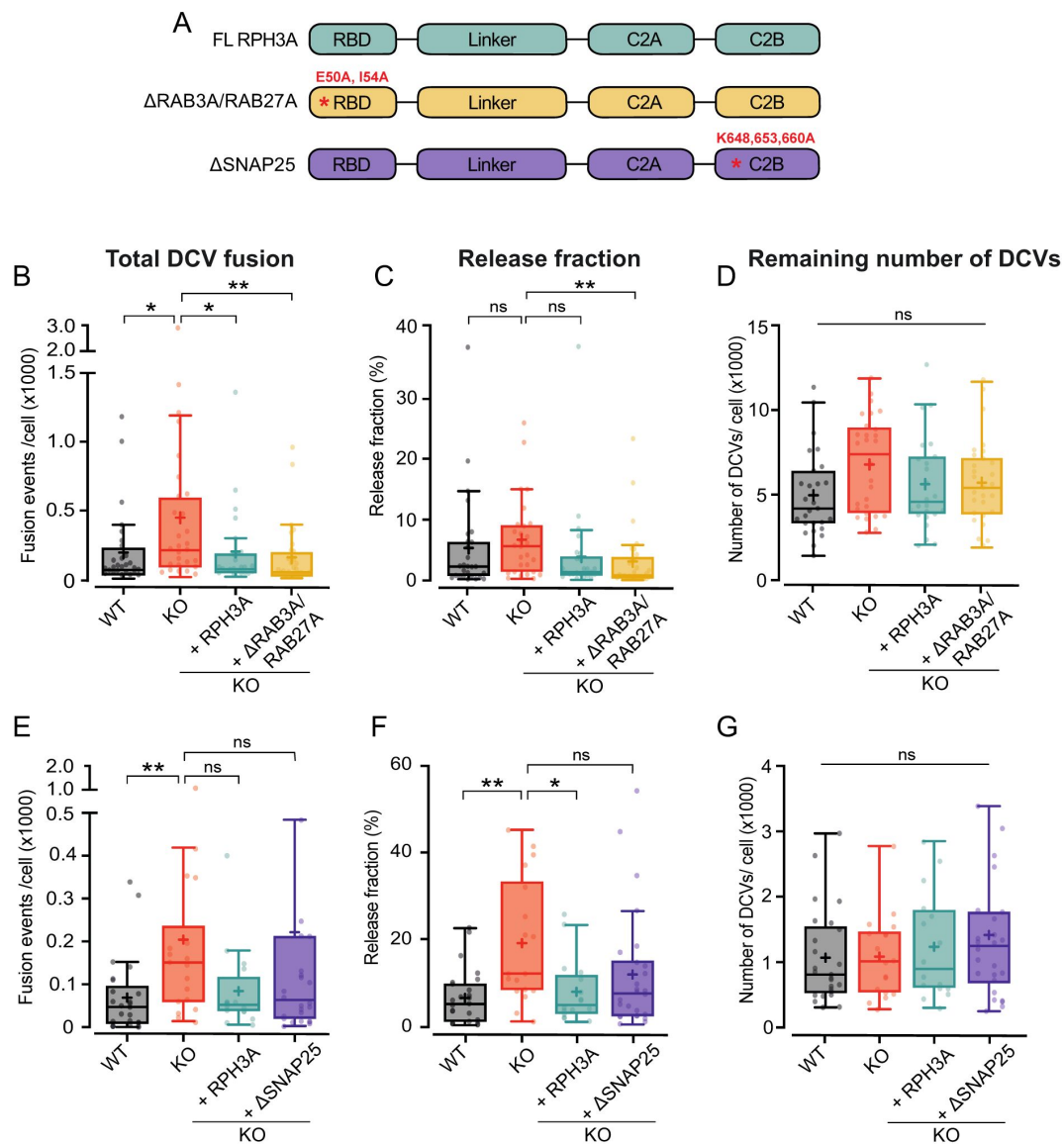


Figure 4.

RPH3A interaction with SNAP25, but not RAB3A, partly contributes to limiting DCV exocytosis.

This Figure shows the same dataset for WT, KO and FL RPH3A as in [Figure 3](#). **(A)** Domain structures of FL RPH3A (cyan), Δ RAB3A/RAB27A- (yellow) and Δ SNAP25 mutant RPH3A (purple) with the corresponding mutant sites in red. **(B)** Total DCV fusion events in WT (black), KO (red) and KO neurons expressing RPH3A (cyan) or Δ RAB3A/RAB27A mutant RPH3A (yellow). **(C)** Released fraction of the number of fusion events normalized to the remaining DCV pool per cell. **(D)** Remaining NPY-pHluorin labelled DCV pool estimates derived from NH_4^+ perfusion after stimulation. N numbers of individual experiments and single neuron observations in brackets: WT: 4(27); KO: 4(28); KO + RPH3A: 4(24); KO + Δ RAB3A/RAB27A: 4(28). **(E)** Total number of DCV fusion events in WT (black), KO (red) and KO neurons expressing RPH3A (cyan) or Δ SNAP25 mutant RPH3A (purple). **(F)** Release fraction per cell. **(G)** Remaining NPY-pHluorin labelled DCV pool per cell. N numbers of individual experiments and single neuron observations in brackets: WT: 4(25); KO: 4(18); KO + RPH3A: 4(16); KO + Δ SNAP25: 4(26). Boxplots show the mean (+), median (line) and Tukey range (whiskers). Each dot represents a single neuron. Kruskal-Wallis H test with Dunn's correction: * $P < 0.05$, ** $P < 0.01$. ns = non-significant, $P > 0.05$.

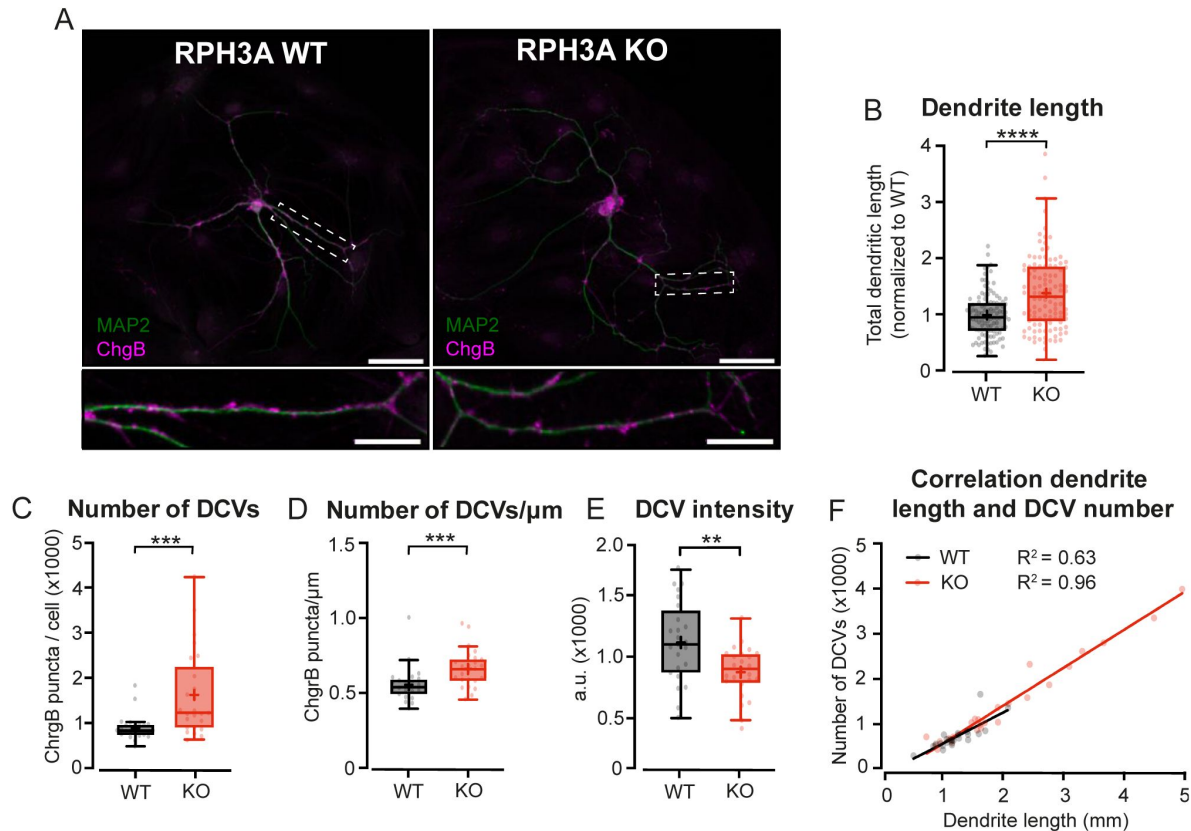


Figure 5.

Increased neurite length and DCV number upon RPH3A deficiency.

(A) Typical example of a single WT and RPH3A KO hippocampal neuron (top) with zooms (bottom) stained for MAP2 (green) and the DCV marker ChgB (magenta). Scale bars, 50 μ m (top) and 20 μ m (bottom). (B) Total dendritic length of single hippocampal RPH3A WT or KO neurons normalized to WT per independent experiment. N numbers per condition: WT: 14(112); KO: 14(113). (C) Total number of ChgB labelled DCVs per neuron for each group. N numbers per condition: WT: 3(24); KO: 3(25). (D) Total ChgB labelled DCVs per μ m for each neuron per group. (E) Mean intensity of ChgB labelled DCVs per neuron for each group. (F) Correlation between ChgB labelled DCVs and dendritic length (mm). Linear regression goodness of fit (r^2) is given for each group. Boxplots represent median (line), mean (+) and Tukey range (whiskers). Each dot represents an individual neuron. Mann-Whitney U or unpaired t test: ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Increased neurite length upon RPH3A deficiency partly depends on regulated secretion

RPH3A deletion resulted in increased DCV exocytosis (Figure 3) and dendrite length (Figure 5B). We reasoned that increased release of neuropeptide- and neurotrophic factors throughout development could contribute to the longer neurites observed. To test this, we inhibited both SV- and DCV exocytosis by cleaving VAMP1, VAMP2, and VAMP3 using TeNT (Hoogstraaten et al., 2020; Humeau et al., 2000), followed by immunostainings for MAP2 and Tau to assess dendritic and axonal length, respectively. Neurons infected with TeNT at DIV1 lacked VAMP2 staining at DIV14, confirming successful cleavage (Figure 6A). TeNT expression in WT neurons had no effect on dendritic (Figure 6B) or axonal length (Figure 6C), as shown before (Harms & Craig, 2005). TeNT expression in KO neurons restored neurite length to WT levels (Figures 6B, C). When comparing KO neurons with and without TeNT, KO neurons with TeNT show a trend towards decreased neurite length, similar to WT. (Figures 6C, B). Re-expression of RPH3A in KO neurons without TeNT restored neurite length to WT levels (Figures 6B, C). To identify the downstream pathway, we overexpressed mutant RPH3A constructs, lacking specific interactions (Figure S1A). No significant differences in dendrite or axon length were observed for any of the mutants compared to WT (Figures S3A-C). These results indicate that regulated secretion is not required for neurite outgrowth, but that the increased neurite length upon RPH3A depletion depends, at least in part, on regulated secretion.

Discussion

In this study, we addressed the role of RPH3A in DCV exocytosis in hippocampal neurons. RPH3A predominantly localized to the pre-synapse (Figure 1) and did not travel with DCVs (Figure 2). RPH3A null mutant neurons showed an increase in DCV exocytosis (Figure 3). Expression of a RAB3A/RAB27A-binding deficient, but not a SNAP25-binding deficient RPH3A, in RPH3A KO neurons, restored DCV exocytosis to WT levels. (Figure 4). Finally, RPH3A null neurons had longer neurites that contained more DCVs (Figure 5). The increase in neurite length may partially depend on regulated secretion, as TeNT expression showed a strong trend towards reduced neurite length to WT levels (Figure 6). Taken together, we conclude that RPH3A limits DCV exocytosis, partly through its interaction with SNAP25.

Presynaptic enrichment and accumulation of RPH3A

We found that RPH3A is enriched at the pre-synapse. This is in line with previous studies showing synaptic enrichment of RPH3A (Li et al., 1994; Mizoguchi et al., 1994; Stanic et al., 2015, 2017). RPH3A showed mostly a punctate distribution, except in the pre-synapse, where it tends to accumulate (Figure 1A). Previous studies showed that inactivation of *synapsin1* and 2 genes decreased RPH3A levels but increased phosphorylation of the remaining RPH3A (Lonart & Simsek-Duran, 2006). Hence, synapsins may, directly or indirectly, contribute to presynaptic RPH3A accumulation, for instance as part of the phase separation of the vesicle cluster. In addition, phosphorylation reduces RPH3A's affinity for membranes (Foletti et al., 1994) and therefore preferential presynaptic dephosphorylation may also help to explain the presynaptic RPH3A accumulation.

RPH3A does not travel with DCVs in hippocampal neurons

We demonstrated that FL and truncated RPH3A are highly stationary at synapses. However, a RPH3A mutant unable to bind RAB3A/RAB27A was more mobile (faster recovery after photobleaching, Figure 2), and lost its punctate distribution. This indicates that the synaptic enrichment of RPH3A depends, at least in part, on RAB3A/RAB27A interactions. This is in line with

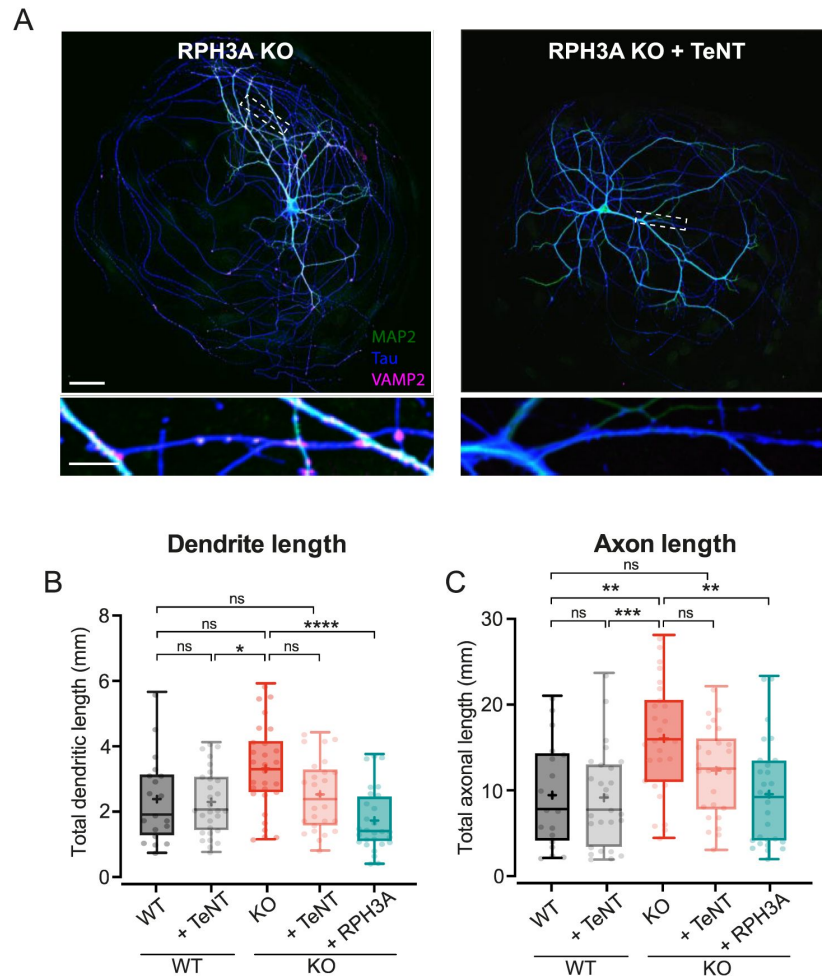


Figure 6.

Increased neurite length upon RPH3A deficiency partly depends on regulated secretion.

(A) Typical example of a single RPH3A KO neurons either infected with TeNT or not, showing successful VAMP2 cleavage with zooms (bottom) stained for MAP2 (green), Tau (blue) and VAMP2 (magenta). Scale bars, 50 μ m (top) and 20 μ m (bottom). (B) Total dendritic and (C) axonal length (mm) of WT and KO neurons - / + TeNT, and KO neurons expressing RPH3A. N numbers per condition: WT: 3(19); WT + TeNT: 3(27); KO: 3(28). KO + TeNT: 3(26), KO + RPH3A: 3(28). Boxplots represent median (line), mean (+) and Tukey range (whiskers). Each dot represents an individual neuron. Kruskal-Wallis H test with Dunn's correction: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns = non-significant, $P > 0.05$.

previous findings showing the expression levels and localization of RPH3A depend on RAB3 in mammalian neurons (Schlüter et al., 1999 [↗](#)). These data do not exclude that RPH3A interacts with immobile DCVs at synapses, potentially in a docked or primed state at the release sites, consistent with our conclusion that RPH3A serves as a negative regulator of DCV exocytosis. Based on these findings, we propose that RAB3A recruits RPH3A to DCV release sites, where it interacts with (immobile) DCVs and competes with essential DCV proteins, such as synaptobrevin/VAMP2 and synaptotagmin-1/synaptotagmin-7 (Hoogstraaten et al., 2020 [↗](#); van Westen et al., 2021 [↗](#)), for SNAP25 binding (**Figure 7** [↗](#)).

RPH3A limits DCV exocytosis in hippocampal neurons

DCV exocytosis was increased by 3-fold upon RPH3A depletion and this effect was rescued by overexpressing FL RPH3A (**Figures 3F, I** [↗](#)). The increase in released fraction upon RPH3A depletion was most significant during intense, prolonged stimulation (16 bursts of 50 action potentials, **Figure 3K** [↗](#)). During more distributed stimulation, a similar trend was observed (**Figure 3F** [↗](#)). Together, these results suggest that RPH3A is a negative regulator of DCV exocytosis (**Figures 7A, B** [↗](#)). This is in line with *C. elegans* data, showing that the lack of the nematode homolog *rbf-1* increased DCV exocytosis, but had no effect on spontaneous release of SVs. This suggests that *rbf-1* limits DCV exocytosis only (Laurent et al., 2018 [↗](#)). In addition, expression of FL RPH3A in PC12 cells reduced high KCl-dependent neuropeptide release (Fukuda et al., 2004 [↗](#)). In this study, we demonstrated that RPH3A also negatively regulated neuropeptide release in mammalian hippocampal neurons. Our prior estimations in mouse hippocampal neurons indicated that merely 1-6% of the total DCV pool undergo exocytosis upon strong stimulation (Persoon et al., 2018 [↗](#)), implying the existence of an inhibitory release mechanism. To our knowledge, RPH3A is the only negative regulator of DCV exocytosis in mammalian neurons identified so far.

The limiting effect of RPH3A on DCV exocytosis partially depends on SNAP25 binding

We recently discovered that RAB3 and its effector RIM1 are positive regulators of DCV exocytosis in mammalian hippocampal neurons (Persoon et al., 2019 [↗](#)). In contrast, we demonstrated that RPH3A serves as a negative regulator of DCV exocytosis. Given that RPH3A is a downstream effector of RAB3, and that RAB3 is necessary for synaptic RPH3A enrichment, we expected that the interaction with RAB3 plays a role in limiting DCV exocytosis. However, enhanced DCV exocytosis was rescued upon expression of a RPH3A mutant that was unable to bind RAB3A/RAB27A, suggesting that the limiting effect of RPH3A on DCV exocytosis is independent of an interaction with RAB3A (**Figure 7C** [↗](#)). This indicates that even though RAB3A is important for the localization of RPH3A, RPH3A can still limit exocytosis when its punctate organization is lost, suggesting that the interaction with RAB3A is not rate limiting. One other potential mechanism by which RPH3A could directly limit exocytosis is by limiting SNAP25 availability. Previous research has demonstrated that RPH3A binding to SNAP25 negatively regulates SV recycling (Deák et al., 2006 [↗](#)). We found that expressing mutant RPH3A that lacks SNAP25 binding in KO neurons does not fully restore DCV exocytosis to WT levels. This suggests that RPH3A limits DCV exocytosis by interacting with SNAP25 (**Figure 7D** [↗](#)). The partial selectivity for the DCV secretory pathway may be attributed to RPH3A functioning as a downstream effector of RAB3A. RAB3 is essential for DCV exocytosis (Persoon et al., 2019 [↗](#)) but largely redundant for synaptic transmission (Schlüter et al., 2004 [↗](#), 2006 [↗](#)). Based on our findings, we propose that RAB3A plays a role in recruiting RPH3A to synaptic exocytosis sites, where RPH3A might bind available SNAP25, potentially restricting the assembly of SNARE complexes and thereby inhibiting DCV exocytosis (**Figure 7** [↗](#)).

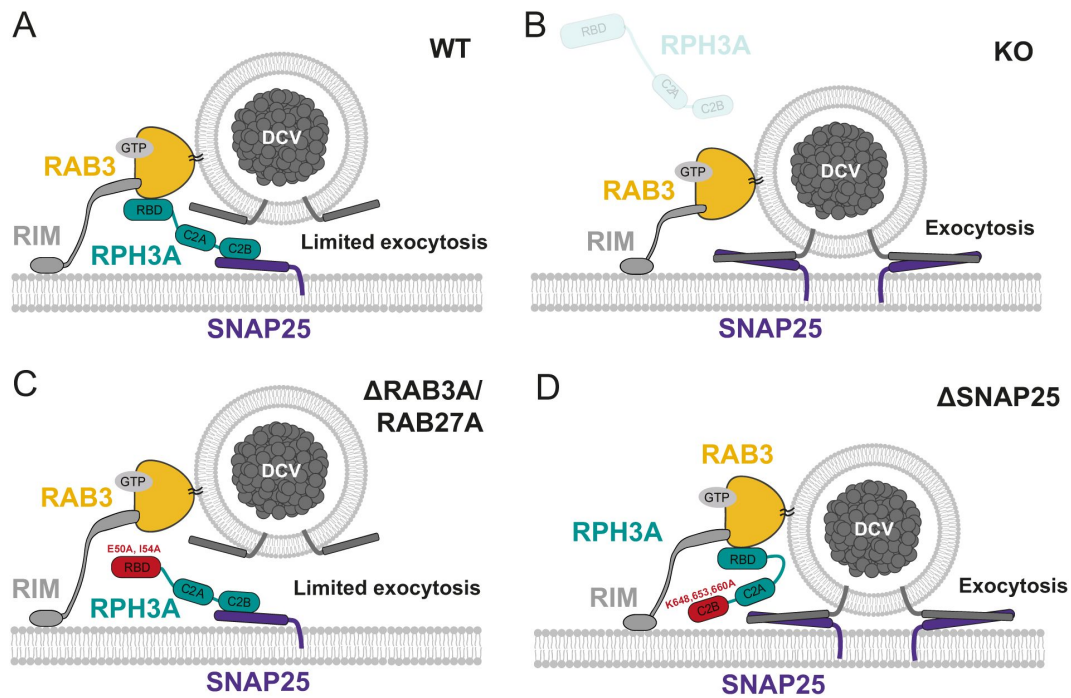


Figure 7.

Role of RPH3A in DCV exocytosis.

(A) RPH3A binding to RAB3 through its RAB-binding domain (RBD) ensures confined presynaptic localization of RPH3A. RPH3A binding to SNAP25 through its C2B domain inhibits DCV exocytosis, potentially by inhibiting SNAP25-binding of essential DCV proteins synaptobrevin/VAMP2 and/or synaptotagmin (not depicted). (B) In the absence of RPH3A, DCV exocytosis is not limited. (C) Upon expression of a RPH3A mutant that is unable to bind RAB3A/RAB27A, DCV exocytosis is limited. (D) When RPH3A is unable to bind SNAP25, the SNARE assembly is not restricted and therefore DCV exocytosis is not limited, while RPH3A is still recruited to synapses/release sites via RAB3.

Increased regulated secretion in RPH3A KO neurons might lead to longer neurites

RPH3A KO neurons have longer neurites that correlated with the number of DCVs as shown before (Persoon et al., 2018 [↗](#)). In agreement with previous findings (Ahnert-Hilger et al., 1996 [↗](#); Grosse et al., 1999 [↗](#); Osen-Sand et al., 1996 [↗](#)), we find that TeNT expression did not affect neurite length of WT neurons, but showed a trend towards shorter neurites in RPH3A KO neurons. Based on this, we speculate that the increased neurite length in RPH3A KO neurons might, at least partially, be driven by TeNT-dependent regulated secretion, in particular VAMP1, 2 or 3 mediated exocytosis.

Given that neuropeptides and neurotrophic factors are key modulators of neuronal maturation and outgrowth (Mu et al., 2010 [↗](#)), and that RPH3A depletion leads to increased DCV exocytosis, it stands to reason that we observed longer neurites in RPH3A KO neurons.

The partial rescue by TeNT suggests RPH3A-dependent mechanisms that could explain the increase in neuron size besides regulated secretion. RPH3A could control neurite length by regulating the actin cytoskeleton. RPH3A interacts with actin via binding to α -actinin and β -adducin (Baldini et al., 2005 [↗](#); Coppola et al., 2001 [↗](#); Kato, Sasaki, et al., 1996 [↗](#)). *In vitro* experiments showed that RPH3A together with α -actinin can bundle actin (Kato, Sasaki, et al., 1996 [↗](#)). Regulation of the actin cytoskeleton has extensively been linked to neurite outgrowth (Meberg & Bamberg, 2000 [↗](#)) making it plausible that lack of RPH3A alters actin regulation, resulting in longer neurites.

Material and methods

Animals

Animals were housed and bred in accordance with Dutch and institutional guidelines. All animal experiments were approved by the animal ethical committee of the VU University / VU University Medical Centre (license number: FGA 11-03 and AVD112002017824). All animals were kept on a C57Bl/6 background. For RPH3A KO (Schlüter et al., 1999 [↗](#)) and WT primary hippocampal neuron cultures, RPH3A heterozygous mice mating was timed and P1 pups were used to dissect hippocampi. Pups were genotyped prior to dissection to select RPH3A KO and WT littermates.

Neuron culture

Primary hippocampal neurons were cultured as described before (De Wit et al., 2009 [↗](#); Farina et al., 2015 [↗](#)). In short, dissected hippocampi were digested with 0.25% trypsin (Gibco) in Hanks' balanced salt solution (Sigma) with 10 mM HEPES (Life Technologies) for 20 minutes at 37 °C. Hippocampi were washed, triturated, and counted prior to plating. For single hippocampal neurons, 1000-2000 neurons per well were plated on pre-grown micro-islands generated by plating 6000 rat glia on 18 mm glass coverslips coated with agarose and stamped with a solution of 0.1 mg/ml poly-D-lysine (Sigma) and 0.7 mg/ml rat tail collagen (BD Biosciences, Mennerick et al., 1995 [↗](#); Wierda et al., 2007 [↗](#)). For continental hippocampal cultures, 20,000 neurons per well were plated on pre-grown glial plates containing 18 mm glass coverslips. All neurons were kept in neurobasal supplemented with 2% B-27, 18 mM HEPES, 0.25% Glutamax and 0.1% Pen-Strep (all Gibco) at 37 °C and 5% CO₂.

Lentiviral vectors and infections

All constructs were cloned into lentiviral vectors containing a synapsin promoter to restrict expression to neurons. Lentiviral particles were produced as described before (Naldini et al., 1996 [↗](#)). NPY-mCherry, NPY-pHluorin and TeNT-IRES-mCherry were described before (Emperador

Melero et al., 2017 [↗](#); Nagai et al., 2002 [↗](#)). For re-expression of RPH3A in single RPH3A KO neurons, FL RPH3A was cloned into a lentiviral vector containing an IRES-NLS-mCherry to verify infection during live cell experiments. For DCV fusion experiments at DIV14-DIV16, neurons were infected with RPH3A-IRES-NLS-mCherry, RAB3A and RAB27A binding deficient RPH3A with two-point mutations (E50A and I54A, Fukuda et al., 2004 [↗](#)) and SNAP25 binding deficient RPH3A (K648A, K653A and K660A, Ferrer-Orta et al., 2017 [↗](#)) at DIV1-2. The E51A/I54A double mutant of RPH3A was previously validated to lose RAB3A and RAB27A binding activity (Fukuda et al., 2004 [↗](#)), and the K651A/K656A/K663A mutant was shown to not bind rat WT SNAP25 (Ferrer-Orta et al., 2017 [↗](#)). We determined the corresponding residues to be mutated in a mouse SNAP25-binding deficient RPH3A construct (K648,653,660A). Neurons were infected with NPY-pHluorin at DIV8-9. For neurite length and co-travel experiments, FL RPH3A, truncated RPH3A (1-375), RAB3A/RAB27A binding deficient RPH3A (Fukuda et al., 2004 [↗](#)), RPH3A with two point-mutations in the C2B domain (D568N, D574N) and RPH3A phosphorylation deficient (S217A, Foletti et al., 2001 [↗](#); Fykse et al., 1995 [↗](#)) were N-terminally tagged with EGFP or mCherry interspaced by a short glycine linker sequence (Tsuboi & Fukuda, 2005 [↗](#)). For neurite length experiments at DIV14, all constructs were infected at DIV1-2. For co-travel experiments at DIV10 constructs were infected at DIV4.

Immunocytochemistry

Cells were fixed with 3.7% paraformaldehyde (PFA, Merck) in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) for 12 minutes at room temperature (RT). Cells were immediately immunostained or kept in PBS at 4 °C. For ChgB immunostainings, cells were permeabilized in 0.5% Triton X-100 (Fisher Chemical) for 10 minutes and blocked with 5% BSA (Acro Organic) in PBS for 30 minutes at RT. For all other immunostainings, cells were permeabilized with 0.5% Triton X-100 for 5 minutes and blocked with 2% normal goat serum (Fisher Emergo) in 0.1% Triton X-100 at RT. Cells were incubated with primary antibodies overnight at 4°C. Primary antibodies used were: polyclonal ChgB (1:500, SySy), monoclonal RPH3A (1:1000, Transduction Labs), polyclonal MAP2 (1:500, Abcam), polyclonal Syn1 (1:1000, #P610; 1:500, SySy), VGLUT1 (1:500, Millipore), polyclonal Homer 1 (1:500, SySy), polyclonal Tau (1:1000, SySy), monoclonal VAMP2 (1:1000, SySy) and monoclonal mCherry (1:1000, Signalway Antibody). Alexa Fluor conjugated secondary antibodies (1:1000, Invitrogen) were incubated for 1 hour at RT. Abberior secondary antibodies (1:50, Abberior) for STED imaging were incubated for 2 hours at RT. Coverslips were mounted on Mowiol (Sigma-Aldrich).

STED imaging

STED images were acquired with a Leica SP8 STED 3x microscope with an oil immersion 100x 1.44 numerical aperture objective. Alexa Fluor 488, Abberior STAR 580 and Abberior STAR 635p were excited with 592 nm and 775 nm using a white light laser. The Abberior STAR 635p was acquired first in STED mode using 4x line accumulation, followed by Abberior STAR 580, both were depleted with 775 nm depletion laser (50% of max power). Alexa Fluor 488 was acquired in STED mode and depleted with 592 nm depletion laser (50% of max power). The Alexa Fluor 405 channel was acquired in confocal mode. The signals were detected using a gated hybrid detector (HyD) in photon-counting mode (Leica Microsystems). Z-stacks were made with a step size of 150 nm and pixel size of 22.73 × 22.73 nm. Finally, deconvolution was performed with Huygens Professional software (SVI).

Live and fixed imaging

For live cell experiments, neurons were continuously perfused in Tyrode's solution (2 mM CaCl₂, 2.5 mM KCl, 119 mM NaCl, 2 mM MgCl₂, 30 mM glucose, 25 mM HEPES; pH 7.4) at RT. For DCV fusion experiments, imaging was performed at DIV14-16 with a Zeiss AxioObserver.Z1 equipped with 561 nm and 488 nm lasers, a polychrome V, appropriate filter sets, a 40x oil objective (NA 1.3) and an EMCCD camera (C9100-02; Hamamatsu, pixel size 200 nm). Images were acquired at 2 Hz

with AxioVision software (version 4.8, Zeiss). Electrical stimulation was performed with two parallel platinum electrodes placed around the neuron. After 30 seconds of baseline, 2 x 8 (separated by 30 seconds) or 1 x 16 train(s) of 50 APs at 50 Hz (interspaced by 0.5 s) were initiated by a Master-8 (AMPI) and a stimulus generator (A-385, World Precision Instruments) delivering 1 ms pulses of 30 mA. NPY-pHluorin was dequenched 50 or 80 seconds after the last stimulation train by superfusing Tyrode's with 50 mM NH₄Cl (replacing 50 mM NaCl). For a detailed protocol of DCV fusion analyses see (Moro et al., 2021 [link](#)).

For co-travel experiments, neurons were infected with NPY-mCherry or NPY-pHluorin and FL- or mutant RPH3A fused to EGFP or mCherry. Imaging was performed at DIV9-10 on a Nikon Ti-E eclipse microscope with a LU4A laser system, appropriate filter sets, 60x oil objective (NA = 1.4) and EMCCD camera (Andor DU-897). Sequential images for both 488 and 561 color channels were acquired for 2 minutes at 2 Hz. After 2 minutes, both axonal and dendritic stretches were photobleached to facilitate analysis. A galvano laser scanner was used to scan a selected area with both lasers at 100% (26.9 μ s pixel dwell time). Bleaching was followed by an 8-minute acquisition at 2 Hz in both channels. To visualize NPY-pHluorin, neurons were continuously perfused in Tyrode's containing 25 mM NH₄Cl (replacing 25 mM NaCl). NPY-pHluorin showed more resistance for bleaching.

For fixed experiments, confocal images were acquired using a Zeiss LSM 510 confocal laser-scanning microscope (40x objective; NA 1.3) and LSM510 software (version 3.2 Zeiss) or a A1R Nikon confocal microscope with LU4A laser unit (40x objective; NA 1.3) and NIS elements software (version 4.60, Nikon). To determine both dendrite (MAP2) and axon (Tau) length, the whole glial island was visualized by stitching 4 images (604.7 x 604.7 microns).

Analyses

For DCV fusion experiments, ImageJ was used to manually place 3x3 pixel ROIs around each NPY-pHluorin fusion event on both axons and dendrites, excluding the soma. An NPY-pHluorin event was considered a DCV fusion event if it suddenly appeared and if the maximal fluorescence was at least twice the SD above noise. Custom written MATLAB (MathWorks, Inc.) scripts were used to calculate the number and timing of fusion events. The remaining DCV number per neuron was determined as the number of fluorescent puncta during NH₄⁺ perfusion, corrected for overlapping puncta. The released fraction was calculated by dividing the total number of fusion events by the remaining pool size. For a detailed protocol of DCV fusion analyses see (Moro et al., 2021 [link](#)).

For DCV co-travel experiments, segmented lines were drawn along the neurites and kymographs were created using the KymoResliceWide plugin in ImageJ. Puncta were considered moving if the minimal displacement during the whole 2- or 8-minute acquisition was at least $\frac{3}{4}$ microns within a 10 second period. For colocalization experiments, the Pearson's and Manders' correlation coefficients were determined using the JACoP plugin (Bolte & Cordelières, 2006 [link](#)).

For fixed experiments, the number of endogenous ChgB+ puncta were counted using SynD (Schmitz et al., 2011 [link](#); van de Bospoort et al., 2012 [link](#)) software (version 491). All puncta were divided by the mode of the first quartile of puncta intensity values (an estimate for a single DCV) to estimate the total number of DCVs per neuron. Based on this, the total number of DCVs per neuron was quantified. To determine dendritic and axonal length, a mask was created using MAP2 and Tau immunostaining, respectively. Single-pixel images were obtained from the masks. The distances between the neighboring pixels within the dendrite mask are summed together to obtain the dendrite or axon length (Schmitz et al., 2011 [link](#)).

Statistical analysis

Statistical tests were performed using R or GraphPad Prism. Normal distributions of all data were assessed with Shapiro-Wilk normality tests and Levene's test of homogeneity of variances. Multi-level models were used to account for variance within the animals when variance between animals significantly improved the model (Aarts et al., 2014 [DOI](#)). To compare two groups, unpaired Student's t-test in the case of normal distributed data or Mann-Whitney U tests for non-parametric data were used. For multiple comparisons the Kruskal-Wallis test was used for non-parametric data followed by Dunn's multiple comparisons test to compare conditions. Data is represented as boxplots (25- 75% interquartile range) with the median (line), mean (+) and Tukey whiskers or bar graphs with SEM. N numbers represent number of independent experiments, with the number of individual neurons in brackets. Dots in all graphs indicate single neuron observations, unless stated otherwise.

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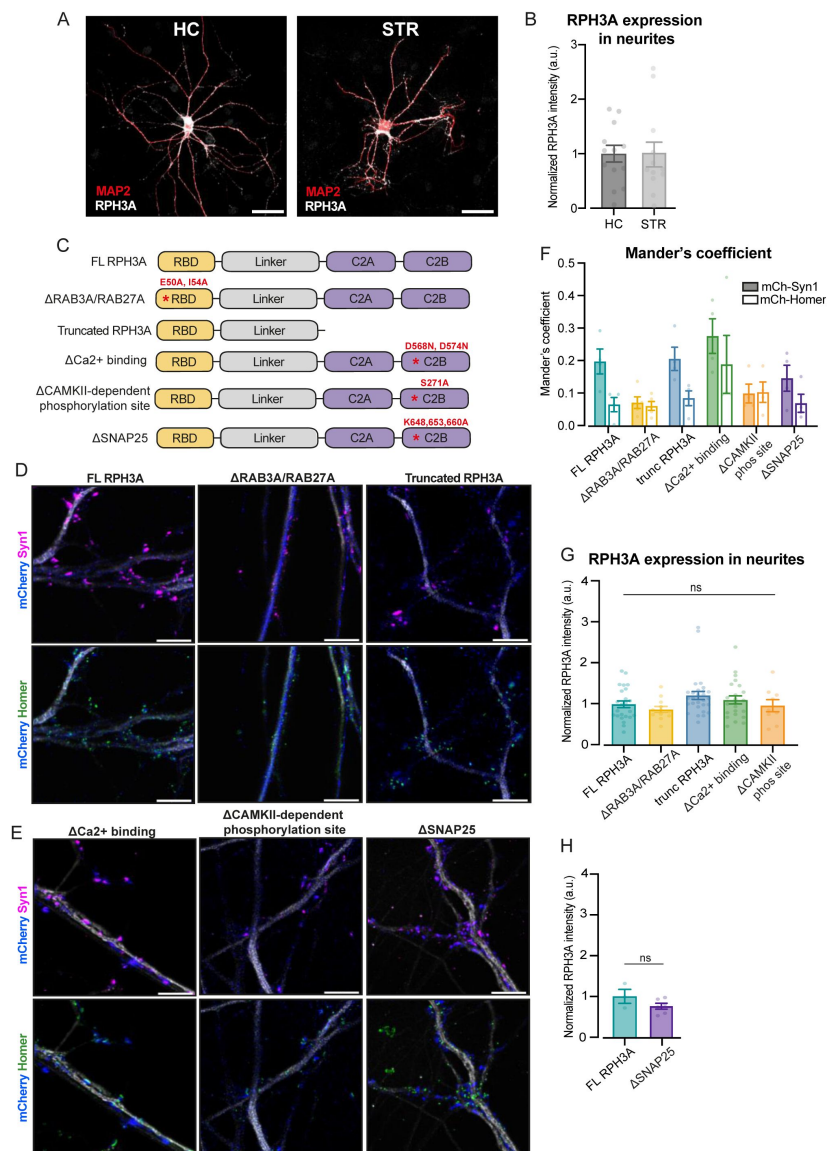
Author contributions

Adlin Abramian*: Validation; formal analysis; investigation; data curation; visualization; writing original draft; writing review and editing. **Rein Hoogstraaten***: Conceptualization; validation; formal analysis; investigation; data curation; visualization; writing original draft; writing review. **Fiona H. Murphy**: Formal analysis; investigation; writing review. **Kathryn F. McDaniel**: Investigation; formal analysis. **Ruud F. Toonen**: Supervision; conceptualization; writing review. **Matthijs Verhage**: Supervision; conceptualization; writing review; funding acquisition.

Data availability statement

All data and constructs used will be made available upon reasonable request.

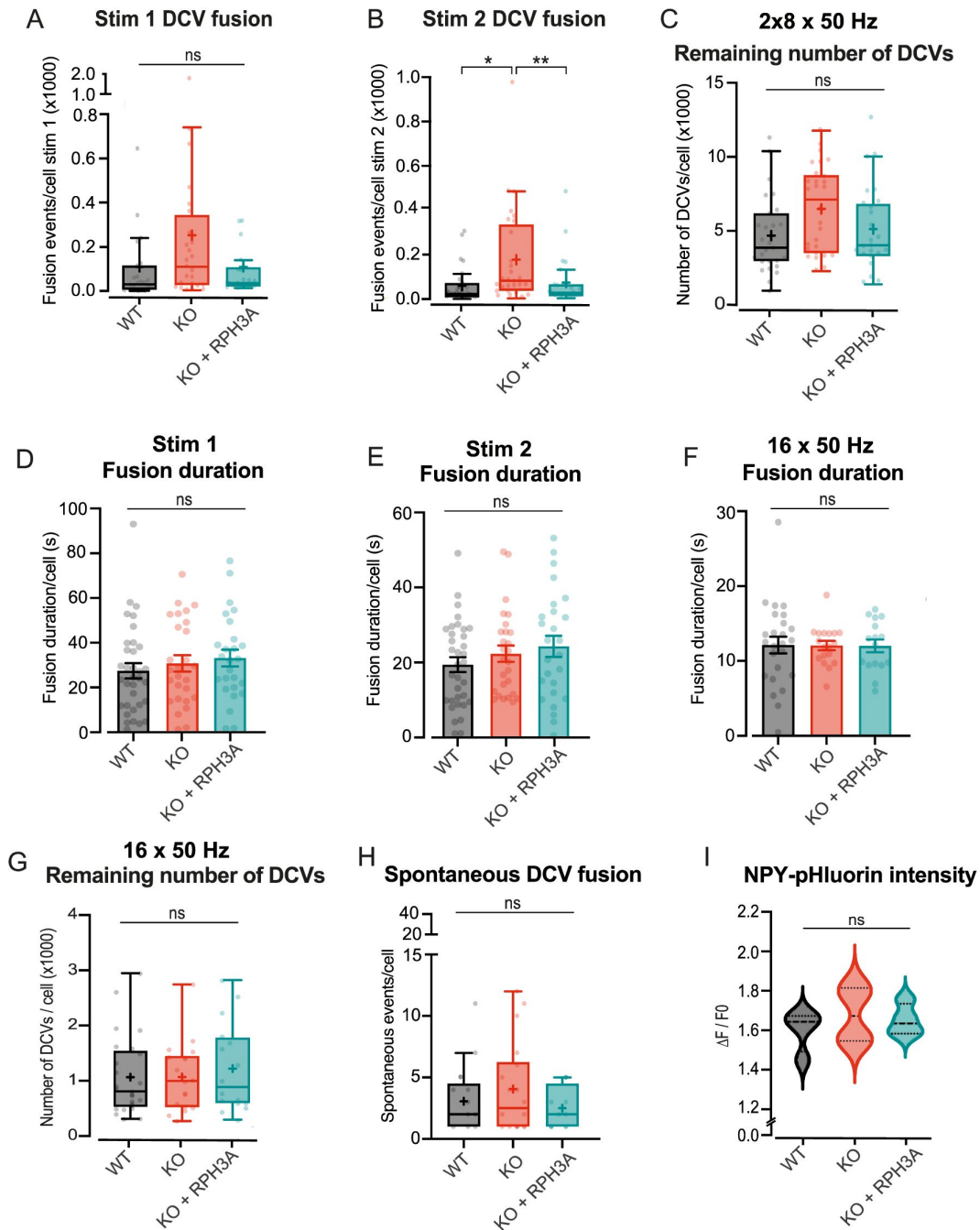
Supplementary figures



Supplementary figure 1.

RPH3A localization depends on RAB3A/RAB27A-binding.

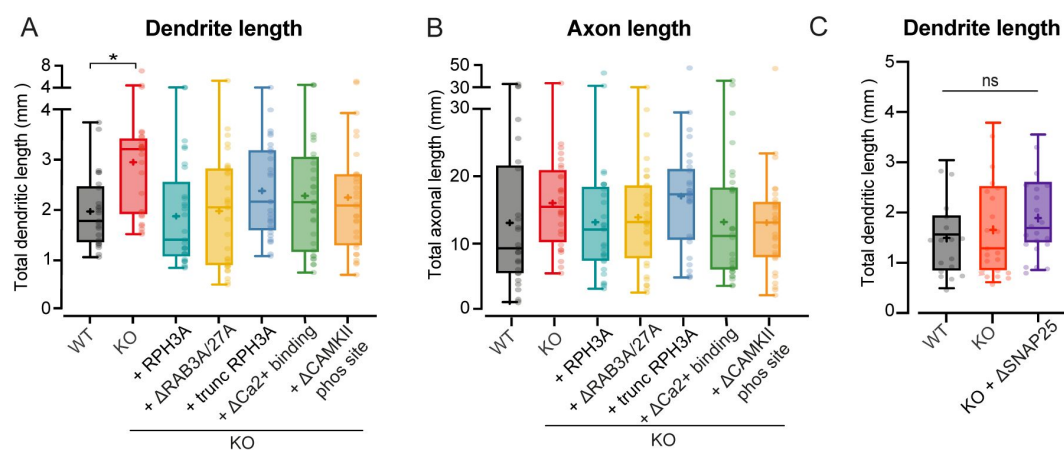
(A) Representative images of WT hippocampal and striatal neurons stained for RPH3A (white), VGLUT/VGAT (not shown) and MAP2 (red). N numbers of individual experiments and single neuron observations in brackets: HC: 2(13); STR: 2(12). Scale bar, 50 μ m. (B) RPH3A intensity in MAP2+ neurite mask in hippocampal and striatal neurons, normalized to batch. (C) Domain structures of FL RPH3A and mutant RPH3A constructs lacking specific interactions: Δ RAB3A/RAB27A; truncated RPH3A that lacked its calcium and SNAP25 binding C2A and C2B domains; RPH3A with two C2B domain mutations to reduce calcium binding affinity (Δ Ca²⁺ binding); RPH3A that lacked a CaMKII dependent phosphorylation site (Δ CaMKII-dependent phosphorylation site) and Δ SNAP25. The corresponding mutation sites are indicated in red. (D, E) Representative STED images of WT neurons expressing either FL RPH3A, Δ RAB3A/RAB27A, truncated RPH3A, Δ Ca²⁺-binding, Δ CaMKII-dependent phosphorylation site or Δ SNAP25, immunostained for mCherry (pseudo-colored blue), MAP2 (grey), Syn1 (magenta) or Homer (green). Scale bar, 5 μ m. (F) Mander's overlap coefficient analyses of mCherry with either Syn1 or Homer, in WT neurons expressing FL or mutant RPH3A constructs. (G) RPH3A expression in KO neurons expressing FL RPH3A or mutant RPH3A constructs normalized to WT per independent experiment. N numbers per condition: KO + RPH3A: 6(24); KO + Δ RAB3A/RAB27A: 3(12); truncated RPH3A: 6(28); KO + Δ Ca²⁺ binding: 5(23); and KO + Δ CaMKII-dependent phosphorylation: 2(9). (H) RPH3A expression in KO neurons expressing FL RPH3A or mutant RPH3A unable to bind SNAP25. N numbers per condition: KO + RPH3A: 1(3); KO + Δ SNAP25: 1(6). Each dot represents a single neuron. Kruskal-Wallis H test with Dunn's correction: ns = non-significant, $P > 0.05$.



Supplementary figure 2.

RPH3A depletion increases DCV exocytosis, but does not affect remaining DCV pool size or content.

(A, B) DCV fusion events per condition for the first and second 8 x 50 APs bursts at 50 Hz. (C) Estimate of the remaining NPY-pHluorin labelled DCV pool during 2 x 8 x 50 APs at 50 Hz. (D, E) Fusion duration during first and second 2 x 8 burst stimulation protocol. (F) Fusion duration during 16 x 50 Hz stimulation protocol. (G) Estimate of the remaining NPY-pHluorin labelled DCV pool during 16 x 50 APs at 50 Hz. (H) The number of spontaneous DCV fusion events per cell during baseline. (I) Mean peak intensity of NPY-pHluorin during live imaging per independent experiment. The black dashed line represents the median. Boxplots show the mean (+), median (line) and Tukey range (whiskers). Each dot represents a single neuron. Kruskal-Wallis H test with Dunn's correction: * $P < 0.05$, ** $P < 0.01$. ns = non-significant, $P > 0.05$.



Supplementary figure 3.

Increased neurite length upon RPH3A depletion does not depend on RAB3A/RAB27A-binding, calcium binding or phosphorylation of RPH3A.

(**A**) Total dendritic and (**B**) axonal length of WT, KO and KO neurons expressing FL or mutant RPH3A (Δ RAB3A/RAB27A, truncated, Δ Ca2+ binding or Δ CAMKII-dependent phosphorylation). N numbers per condition: WT 3(25); KO 3(28); KO + RPH3A: 3(29); KO + Δ RAB3A/RAB27A: 3(32); truncated RPH3A: 3(29); KO + Δ Ca2+ binding: 3(30); and KO + Δ CAMKII-dependent phosphorylation: 3(30). (**C**) Total dendritic length of WT, KO and KO neurons expressing Δ SNAP25 mutant. N numbers per condition: WT 3(17); KO 3(21); KO + Δ SNAP25: 3(20). Each dot represents a single neuron. Kruskal-Wallis H test with Dunn's correction: * $P < 0.05$. ns = non-significant, $P > 0.05$.

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Joint Public Review

The molecular mechanisms that mediate the regulated exocytosis of neuropeptides and neurotrophins from neurons via large dense-core vesicles (LDCVs) are still incompletely understood. Motivated by their earlier discovery that the Rab3-RIM1 pathway is essential for neuronal LDCV exocytosis, the authors now examined the role of the Rab3 effector Rabphilin-3A in neuronal LDCV secretion. Based on live, confocal, and super-resolution imaging approaches, the authors provide evidence for a synaptic enrichment of Rabphilin-3A and for independent trafficking of Rabphilin-3A and LDCVs. Using an elegant NPY-pHluorin imaging approach, they show that genetic deletion of Rabphilin-3A causes an increase in electrically triggered LDCV fusion events and increased neurite length. Finally, knock-out-replacement studies, involving Rabphilin-3A mutants deficient in either Rab3- or SNAP25-binding, indicate that the synaptic enrichment of Rabphilin-3A depends on its Rab3 binding ability, while its ability to bind to SNAP25 is required for its effects on LDCV secretion and neurite development. The authors conclude that Rabphilin-3A negatively regulates LDCV exocytosis and propose that this mechanism also affects neurite growth, e.g. by limiting neurotrophin secretion. These are important findings that advance our mechanistic understanding of neuronal large dense-core vesicle (LDCV) secretion.

The major strengths of the present paper:

- (i) The use of a powerful Rabphilin-3A KO mouse model.
- (ii) Stringent lentiviral expression and rescue approaches as a strong genetic foundation of the study.
- (iii) An elegant FRAP imaging approach.
- (iv) A cutting-edge NPY-pHluorin-based imaging approach to detect LDCV fusion events.

Weaknesses of the present paper:

- (i) It remains unclear why a process that affects a general synaptic SNARE fusion protein - SNAP25 - would specifically affect LDCV but not synaptic vesicle fusion.
- (iii) The mechanistic links between Rabphilin-3A function, LDCV density in neurites, neurite outgrowth, and the proposed underlying mechanisms involving trophic factor release remain unresolved.

<https://doi.org/10.7554/eLife.95371.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Joint Public Review:

The molecular mechanisms that mediate the regulated exocytosis of neuropeptides and neurotrophins from neurons via large dense-core vesicles (LDCVs) are still incompletely understood. Motivated by their earlier discovery that the Rab3-RIM1 pathway is essential for neuronal LDCV exocytosis, the authors now examined the role of the Rab3 effector Rabphilin-3A in neuronal LDCV secretion. Based on multiple live and confocal imaging approaches, the authors provide evidence for a synaptic enrichment of Rabphilin-3A and for independent trafficking of Rabphilin-3A and LDCVs. Using an elegant NPY-pHluorin imaging approach, they show that genetic deletion of Rabphilin-3A causes an increase in electrically triggered LDCV fusion events and increased neurite length. Finally, knock-out-replacement studies, involving Rabphilin-3A mutants deficient in either Rab3- or SNAP25-binding, indicate that the synaptic enrichment of Rabphilin-3A depends on its Rab3 binding ability, while its ability to bind to SNAP25 is required for its effects on LDCV secretion and neurite development. The authors conclude that Rabphilin-3A negatively regulates LDCV exocytosis and propose that this mechanism also affects neurite growth, e.g. by limiting neurotrophin secretion. These are important findings that advance our mechanistic understanding of neuronal large dense-core vesicle (LDCV) secretion.

The major strengths of the present paper are:

- (i) The use of a powerful Rabphilin-3A KO mouse model.*
- (ii) Stringent lentiviral expression and rescue approaches as a strong genetic foundation of the study.*
- (iii) An elegant FRAP imaging approach.*
- (iv) A cutting-edge NPY-pHluorin-based imaging approach to detect LDCV fusion events.*

We thank the reviewers for their positive evaluation of our manuscript.

Weaknesses that somewhat limit the convincingness of the evidence provided and the corresponding conclusions include the following:

- (i) The limited resolution of the various imaging approaches introduces ambiguity to several parameters (e.g. LDCV counts, definition of synaptic localization, Rabphilin-3A-LDCV colocalization, subcellular and subsynaptic localization of expressed proteins, AZ proximity of Rabphilin-3A and LDCVs) and thereby limits the reliability of corresponding conclusions. Super-resolution approaches may be required here.*

We thank the reviewer for their constructive suggestion. We fully agree that super-resolution imaging would produce a more precise localization of RPH3A and co-localization with DCVs. We have now repeated our (co)-localization experiments with STED microscopy. We find that RPH3A colocalized with the pre-synaptic marker Synapsin1 and, to a lesser extent, with the post synaptic marker Homer and DCV marker chromogranin B (new Figure 1). This indicates that RPH3A is highly enriched in synapses, mostly the pre-synapse, and that RPH3A partly co-localizes with DCVs.

(ii) The description of the experimental approaches lacks detail in several places, thus complicating a stringent assessment.

We apologize for the lack of detail in explaining the experimental approaches. We have included a more detailed description in the revised manuscript.

(iii) Further analyses of the LDCV secretion data (e.g. latency, release time course) would be important in order to help pinpoint the secretory step affected by Rabphilin-3A.

We agree. To address this comment, we have now included the duration of the fusion events (new Figure S2D-F). The start time of the fusion events are shown in the cumulative plots in now Figure 3F and I. The kinetics are normal in the RPH3A KO neurons.

(iv) It remains unclear why a process that affects a general synaptic SNARE fusion protein - SNAP25 - would specifically affect LDCV but not synaptic vesicle fusion.

We agree that we have not addressed this issue systematically enough in the original manuscript. We have now added a short discussion on this topic in the Discussion of the revised manuscript (p 15, line 380-386). In brief, we do not claim full selectivity for the DCV pathway. Some effects of RPH3A deficiency on the synaptic vesicle cycle have been observed. Furthermore, because DCVs typically do not mix in the synaptic vesicle cluster and fuse outside the active zone (and outside the synapse), DCVs might be more accessible to RPH3A regulation.

(v) The mechanistic links between Rabphilin-3A function, LDCV density in neurites, neurite outgrowth, and the proposed underlying mechanisms involving trophic factor release remain unclear.

We agree that we have not addressed all these links systematically enough in the original manuscript, although we feel that we have at least postulated the best possible working model to link RPH3A function to DCV exocytosis/neurotrophic factor release and neurite outgrowth (p 15-16, line 396-400). Of course, a single study cannot support all these links with sufficient experimental evidence. We have now added a short text on what we can conclude exactly based on our experiments and how we see the links between RPH3A function, DCV exocytosis/neurotrophic factor release, neurite outgrowth and DCV density in neurites (p 13-14, line 317-325).

Reviewer #1 (Public Review):

Summary:

The manuscript by Hoogstraaten et al. investigates the effect of constitutive Rabphilin 3A (RPH3A) ko on the exocytosis of dense core vesicles (DCV) in cultured mouse hippocampal neurons. Using mCherry- or pHluorin-tagged NPY expression and EGFP- or mCherry tagged RPHA3, the authors first analyse the colocalization of DCVs and RPH3A. Using FRAP, the authors next analyse the mobility of DCVs and RAB3A in neurites. The authors go on to determine the number of exocytotic events of DCVs in response to high-frequency electrical stimulation and find that RPH3A ko increases the number of exocytotic events by a factor 2-3, but not the fraction of released DCVs in a given cell (8x 50Hz stim). In contrast, the release fraction is also increased in RBP3A KOs when doubling the stimulation number (16x 50Hz). They further observe that RPH3A ko increases dendrite and axon length and the overall number of ChgrB-positive DCVs. However, the overall number of DCVs and dendritic length in ko cells directly correlate, indicating that the number of vesicles per dendritic length remains unaffected in the

RPH3A KOs. Lentiviral co-expression of tetanus toxin (TeNT) showed a non-significant trend to reduce axon and dendrite length in RPH3a KOs. Finally, the authors use co-expression of RAB3A and SNAP25 constructs to show that RAB3A but not SNAP25 interaction is required to allow the exocytosis-enhancing effect in RPH3A KOs.

While the authors' methodology is sound, the microscopy results are performed well and analyzed appropriately, but their results in larger parts do not sufficiently support their conclusions. Moreover, the experiments are not always described in sufficient detail (e.g. FRAP; DCV counts vs. neurite length) to fully understand their claims.

Overall, I thus feel that the manuscript does not provide a sufficient advance in knowledge.

Strengths:

- The authors' methodology is sound, and the microscopy results are performed well and analyzed appropriately.

- Figure 2: The exocytosis imaging is elegant and potentially very insightful. The effect in the RPH3A KOs is convincing.

- Figure 4: the logic of this experiment is elegant. It shows that the increased number of DCV fusion events in RPH3A KOs is related to the interaction of RPH3A with RAB3A but not with SNAP25.

We thank the reviewer for their positive evaluation of our manuscript.

Weaknesses:

- The results in larger parts do not sufficiently support the conclusions.

- The experiments are not always described in sufficient detail (e.g. FRAP; DCV counts vs. neurite length) to fully understand their claims.

- Not of sufficient advance in knowledge for this journal

- The significance of differences in control experiments WT vs. KO) varies between experiments shown in different figures.

- Axons and dendrites were not analyzed separately in Figures 1 and 2.

- The colocalization study in Figure 1 would require super-resolution microscopy.

To address the reviewers' comments, we have provided a more detailed explanation of our analysis (p 19-20, line 521-542). In addition, we have repeated our colocalization experiments using STED microscopy, see Joint Public Review item (i).

Reviewer #2 (Public Review):

Summary:

Hoogstraaten et al investigated the involvement of rabphilin-3A RPH3A in DCV fusion in neurons during calcium-triggered exocytosis at the synapse and during neurite elongation. They suggest that RPH3A acts as an inhibitory factor for LDV fusion and this is mediated partially via its interaction with SNAP25 and not Rab3A/Rab27. It is a very elegant study although several questions remain to be clarified.

Strengths:

The authors use state-of-the-art techniques like tracking NPY-PHluorin exocytosis and FRAP experiments to quantify these processes providing novel insight into LDCs exocytosis and the involvement of RPH3A.

We thank the reviewer for their positive evaluation of our manuscript.

Weaknesses:

At the current state of the manuscript, further supportive experiments are necessary to fully support the authors' conclusions.

We thank the reviewer for their comments and suggestions. We have performed additional experiments to support our conclusions, see Joint Public Review items (i) – (iv)

Reviewer #3 (Public Review):

Summary:

The molecular mechanism of regulated exocytosis has been extensively studied in the context of synaptic transmission. However, in addition to neurotransmitters, neurons also secrete neuropeptides and neurotrophins, which are stored in dense core vesicles (DCVs). These factors play a crucial role in cell survival, growth, and shaping the excitability of neurons. The mechanism of release for DCVs is similar, but not identical, to that used for SV exocytosis. This results in slow kinetic and low release probabilities for DCV compared to SV exocytosis. There is a limited understanding of the molecular mechanisms that underlie these differences. By investigating the role of rabphilin-3A (RPH3A), Hoogstraaten et al. uncovered for the first time a protein that inhibits DCV exocytosis in neurons.

Strengths:

In the current work, Hoogstraaten et al. investigate the function of rabphilin-3A (RPH3A) in DVC exocytosis. This RAB3 effector protein has been shown to possess a Ca²⁺ binding site and an independent SNAP25 binding site. Using colocalization analysis of confocal imaging the authors show that in hippocampal neurons RPH3A is enriched at pre- and post-synaptic sites and associates specifically with immobile DCVs. Using site-specific RPH3A mutants they found that the synaptic location was due to its RAB3 interaction site. They further could show that RPH3A inhibits DCV exocytosis due to its interaction with SNAP25. They came to that conclusion by comparing NPY-pHluorin release in WT and RPH3A KO cells and by performing rescue experiments with RPH3A mutants. Finally, the authors showed that by inhibiting stimulated DCV release, RPH3A controlled the axon and dendrite length possibly through the reduced release of neurotrophins. Thereby, they pinpoint how the proper regulation of DCV exocytosis affects neuron physiology.

We thank the reviewer for their positive evaluation of our manuscript.

Weaknesses:

Data context

One of the findings is that RPH3A accumulates at synapses and is mainly associated with immobile DCVs.

However, Farina et al. (2015) showed that 66% of all DCVs are secreted at synapses and that these DCVs are immobile prior to secretion. To provide additional context to the data, it would be valuable to determine if RPH3A KO specifically enhances secretion at

synapses. Additionally, the authors propose that RPH3A decreases DCV exocytosis by sequestering SNAP25 availability. At first glance, this hypothesis appears suitable. However, due to RPH3A synaptic localization, it should also limit SV exocytosis, which it does not. In this context, the only explanation for RPH3A's specific inhibition of DCV exocytosis is that RPH3A is located at a synapse site remote from the active zone, thus protecting the pool of SNAP25 involved in SV exocytosis from binding to RPH3A. This hypothesis could be tested using super-resolution microscopy.

We thank the reviewer for their suggestion. We have now performed super resolution microscopy, see Joint Public Review item (i). However, these new data do not necessarily explain the stronger effect of RPH3A deficiency on DCV exocytosis, relative to SV exocytosis. We have added a short discussion on this topic to the revised manuscript, see Joint Public Review item (iv).

Technical weakness

One technical weakness of this work consists in the proper counting of labeled DCVs. This is significant since most findings in this manuscript rely on this analysis. Since the data was acquired with epi-fluorescence or confocal microscopy, it doesn't provide the resolution to visualize individual DCVs when they are clumped. The authors use a proxy to count the number of DCVs by measuring the total fluorescence of individual large spots and dividing it by the fluorescence intensity of discrete spots assuming that these correspond to individual DCVs. This is an appropriate method but it heavily depends on the assumption that all DCVs are loaded with the same amount of NPY-pHluorin or chromogranin B (ChgB). Due to the importance of this analysis for this manuscript, I suggest that the authors show that the number of DCVs per μm^2 is indeed affected by RPH3A KO using super-resolution techniques such as dSTORM, STED, SIM, or SRRE.

The reviewer is correct that this is a crucial issue, that we have not addressed optimally until now. We have previously devoted a large part of a previous manuscript to this issue, but have not referred to this previous work clearly enough. We have now clarified this (p 7, line 187-190). In brief, we have previously quantified the ratio between fluorescent intensity of ChgB and NPY-pHluorin in confocal microscopy over the number of dSTORM puncta in sparse areas of WT mouse hippocampal neurons (Persoon et al., 2018). This quantification yielded a unitary fluorescence intensity per vesicle that was very stable of different neurons. Although there might be some underestimation of the total number of DCVs when using confocal microscopy, the study of Persoon et al. (2018) has demonstrated that these parameters correlate well and that the estimations are accurate. Considering that the rF/F0 is similar in RPH3A WT and KO neurons (now Figure S2I), meaning that the intensity of NPY-pHluorin of one fusion event is comparable, we can presume that this correlation also applies for the RPH3A KO neurons.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Major points:

(1) The authors perform an extensive analysis regarding the colocalization of RPH3A and DCVs (Figure 1 upper part). This analysis is hampered by the fact that the recorded data has in relation to vesicle size limited resolution ($> 1 \mu\text{m}$) to allow making strong claims here. In my view, super-resolution microscopy would be required for the co-localization studies shown in Figure 1.

We fully agree and have now performed super-resolution microscopy, see Joint Public Review item (i)

(2) The FRAP experiments (Figure 1 lower part) cannot be sufficiently understood from what is presented. The methods say that both laser channels were activated during bleaching but NPY-pHluorin is not bleached in Fig. 1E. Explanation of the bleaching is not very circumspect. In 1D, it is rather EGFP-RPH3A that is entering the bleached area than the NPY vesicles. These experiments require a more careful explanation of methodology, observed results, and their interpretation. Overall, the observed effects in the original kymograph traces require a better explanation.

We acknowledge that NPY-pHluorin in Figure 1E (now Figure 2C) is not completely bleached. NPY-pHluorin appeared to be more difficult to bleach than NPY-mCherry. However, it is important to clarify that we merely bleached the neurites to remove the stationary puncta and facilitate our analysis of DCV/RPH3A dynamics. This bleaching step does not affect the interpretation of our results. We apologize that this was not clearly stated in the text and have made the necessary adjustments in legend, results- and methods section, (p 6-7, line 162-163; p 5, line 140-142 and p 19, line 508-513). Additionally, we apologize for the accidental switch of the kymographs for NPY-mCherry and EGFP-RPH3A in Figure 1D (now Figure 2B, C). We greatly appreciate identifying this error.

(3) Figure 1: The authors need to mention whether axons, dendrites, or both were analyzed throughout the different panels and how they were identified. Is it possible that axons were wrapping around dendrites in their cultures (compare e.g. Shimojo et al., 2015)? Given the limited spatial resolution and because of this wrapping, interpretation of results could be affected.

We completely agree with the reviewer's assessment and conclusion. We are unable to distinguish axons from dendrites using this experimental design. We have made sure to specify in the text that our observation that RPH3A does not co-travel with DCVs is true for both dendrites and axons, (p 5, line 150).

(4) Figure 2: The exocytosis imaging is elegant and potentially very insightful. The effect in the RPH3A KOs is convincing. However, the authors determine the efficacy of exocytosis from NPY-pHluorin unquenching of DCVs only. This is only one of several possible parameters to read out the efficiency of exocytosis. Kinetics like e.g. delay between stimulation and start of exocytosis events or release time course of NPY after DCV fusion were not determined. Such analysis could give a better insight into what process before or after the fusion of DCVs is affected by RPH3A ko.

We fully agree with the reviewer. We have now included the duration of the fusion events (new Figure S2D-F). The start time of the fusion events are shown in the cumulative plots in now Figure 3F and I. The kinetics are normal in the RPH3A KO neurons.

Moreover, it needs to be mentioned whether 2C and D are from WT or ko cultures. It would be best to show representative examples from both genotypes.

We have now adjusted this in the new figure (now Figure 3C, D).

The number of fusion events is much increased but the release fraction is not significantly changed. While this is consistent with results in Figure 4C it is at variance with 4F. This raises questions about the reliability of the effects in RPH3A KOs.

The release fraction indicates the number of fusion events normalized to the total DCV pool. In Figure 4D, we observed a slightly bigger pool size, which explains the lack of significance when analyzing the released fraction. In Figure 4G, however, DCV pool sizes are similar between KO and WT, leading to a statistically significant effect on release fraction in KO neurons. Furthermore, Figures 4B and E distinctly show a substantial increase in fusion events in RPH3A KO neurons. This variability in pool size observed could potentially be attributed to variation in culture or inherent biological variability.

Given the increased number of ChgrB-positive DCVs in RPH3A KOs (shown in Figure 2) and that only the cumulative number of exocytosis events were analysed, how can the authors exclude that the RPH3A ko only affects vesicle number but not release, if the % change in released vesicles is not different to WT? Kinetics of release don't seem to be affected. Importantly, what was the density of NPY-pHluorin vesicles in WT vs. ko?

In Figure 2 (now Figure 5) we show that RPH3A KO neurons are larger and contain more endogenous ChgB+ puncta than WT neurons. This increased number of ChgB+ puncta scales with their size as puncta density is not increased. A previous study (Persoon et al., 2018) has demonstrated a strong correlation between DCV number and neuron size. Our data show that RPH3A deficiency increased DCV exocytosis, but the released fraction of vesicles depends on the total number of DCVs, which we determined during live recording by dequenching NPY-pHluorin using NH₄⁺. Considering that this is an overexpression of a heterologous DCV-fusion reporter, and not endogenous staining of DCVs, as in the case of ChgB+ puncta, some variability is not unexpected.

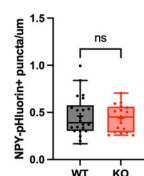
Also in these experiments, the question arises of whether the authors analyse axons, dendrites, or both throughout the different panels and how they were identified.

In our experimental design we record all fusion events per cell, including both axons and dendrites but excluding the cell soma. We have clarified this in the method section, (p 19, line 508 and p 19, line 521-522).

(5) Figure 3: in D the authors show that ChgrB-pos. DCV density is slightly increased in KOs. How does this relate to the density of NPY-pHluorin DCVs in Figure 2?

We do not observe a difference in NPY-pHluorin density (see Author response image 1). However, it is important to note that we relied on tracing neurites in live recording images to determine the neuronal size. In contrast, the ChgB density was based on dendritic length using MAP2 (post-hoc) staining was limited. In addition, Chgr+ puncta represent an endogenous DCV staining, NPY-pHluorin quantification is based on overexpression of a heterologous DCV-fusion reporter. These two factors likely contribute some variability.

Author response image 1.



The authors show a non-significant trend of TeNT coexpression to reduce axon and dendrite lengths in RPH3A KOs. While this trend is visible, I think one cannot draw conclusions from that when not reaching significance. The argument of the authors that

the increased axon and dendrite lengths are created by growth factor peptide release from DCV during culture time is interesting. However, the fact that TeNT expression shows a trend toward reducing this effect on axons/dendrites is not sufficient to prove the release of such growth factors.

We agree. We have toned down this speculation in the revised manuscript, (p 15-16, line 395-400).

Lastly, the authors don't provide insight into the mechanisms, of how RPH3A ko increases the number of DCVs per μm dendritic length in the neurons. In my view, there are too many loose ends in this story of how RPH3A ko first increases spontaneous release of DCVs and then enhances neurite growth and DCV density. Did the authors e.g. measure the spontaneous release of DCVs in their cultures?

We measured spontaneous release of DCVs during the 30s baseline recording prior to stimulation. We observed no difference in spontaneous release between WT and KO neurons (now Figure S2H). However, baseline recording lasted only 30 seconds. It is possible that this was too short to detect subtle effects.

Other points:

(1) Figure 4: the logic of this experiment is elegant. It shows that the increased number of DCV fusion events in RPH3A KOs is related to the interaction of RPH3A with RAB3A but not with SNAP25. As mentioned above, it is irritating that the reduction of fusion events in KOs and on the release fraction is sometimes reaching significance, but sometimes it does not. Likewise, the absence of significant effects on DCV numbers is not consistent with the results shown in Figures 3C and D.

DCV numbers in Figure 3 (now Figure 5) are determined by staining for endogenous ChgB, whereas in Figure 4D and G DCV numbers are determined by overexpressing NPY-pHluorin and counting the dequenched puncta following a NH_4^+ puff.

(2) Figure 1B: truncation of the y-axis needs to be clearly indicated.

We have replaced this figure with new Figure 1 and have indicated truncations of the y-axis when needed (new Figure 1E).

(3) Page 10: "Given that neuropeptides are key modulators of adult neurogenesis (Mu et al., 2010), and that RPH3A depletion leads to increased DCV exocytosis, it is coherent that we observed longer neurites in RPH3A KO neurons." I cannot follow the argument of the authors here: what has neurogenesis to do with neurite length?

We apologize for the confusion. We have clarified this in the revised text, (p 16, line 398-400).

Minor point:

There are some typos in the manuscript. e.g., page 8: "... may partially dependent on regulated secretion..."); page 6: "...to dequence all..."

Thank you for noticing, we have corrected the typos.

Reviewer #2 (Recommendations For The Authors):

(1) Supplementary Figure S1A, in my opinion, should be in Figure 1A as it illustrates all the constructs used in this study and helps the reader to follow it up.

We thank the reviewer for their suggestion. However, we feel that with the adjustments we have made in Figure 1, the illustrations of the constructs fit better in Figure S1, since new Figure 1 shows the localization of endogenous RPH3A and not that of the constructs.

(2) One of the conclusions of the manuscript is the synaptic localization of the different RPH3A mutants. The threshold for defining synaptic localization is not clear either from the images nor from the analysis: for example, the Manders coefficient for VGut1-Syn1 which is used as a positive control, ranges from 0.65-0.95 and that of RPH3A and Syn1 ranges from 0.5-0.95. These values should be compared to all mutants and the conclusions should be based on such comparison.

We agree. We have now repeated our initial co-localization experiment with all the RPH3A mutants (now Figure S1D-F).

(3) Strengthening this figure with STED/SIM/dSTORM microscopy can verify and add a new understanding of the subtle changes of RPH3A localization.

We fully agree and have now added super-resolution microscopy data, see Joint Public Review item (i).

(4) As RAB3A/RAB27A (Δ RAB3A/RAB27A) loses the punctate distribution, please clarify how can it function at the synapse and not act as a KO. Is it sorted to the synapse and how does it is sorted to the synapse?

We used lentiviral delivery to introduce our constructs, resulting in the overexpression of Δ RAB3A/RAB27A mutant RPH3A. This overexpression likely compensates for the loss of the punctate distribution of RPH3A, thereby maintaining its limiting effect on DCV exocytosis. It is plausible that under physiological conditions, the mislocalization of RPH3A would lead to increased exocytosis, similar to what we observed in the KO.

(5) Is RPH3A expressed in both excitatory and inhibitory neurons?

We agree this is an important question. Single cell RNA-seq already suggests the protein is expressed in both, but we nevertheless decided to test expression of RPH3A protein in excitatory and inhibitory neurons, using immunocytochemistry with VGAT and VGLUT as markers in hippocampal and striatal WT neurons. We found that RPH3A is expressed in both VGLUT+ hippocampal neurons and VGAT+ striatal neurons (new Figure S1A, B).

(6) The differential use of ChgB and NPY as markers for DCVs should be clarified and compared as these are used at different stages of the manuscript.

We have previously addressed the comparison between ChgB and NPY-pHluorin (Persoon et al., 2018). We made sure to indicate this more clearly throughout the manuscript to clarify the use of the two markers.

(7) FRAP experiments- A graph describing NPY recovery should be added as a reference to 2H and discussed.

We agree. We have made the necessary adjustments (new Figure 2G).

(8) Figure 2E shows some degree of "facilitation" between the 2 8x50 pulses RPH3A KO neurons. Can the author comment on that? What was the reason for using this dual stimulation protocol?

There is indeed some facilitation between the two 8 x 50 pulses in KO neurons and to a lesser extent also in the WT neurons, which we have observed before in WT neurons (Baginska et al., 2023). Baginska et al. (2023) showed recently that different stimulation protocols can influence certain fusion dynamics, like the ratio of persistent and transient events and event duration. We used two different stimulation protocols to thoroughly investigate the effect of RPH3A on exocytosis, and assess the robustness of our findings regarding the number of fusion events. Fusion kinetics was similar in WT and KO neurons for both stimulation protocols (new Figure 2D-F).

(9) Figure 3 quantifies dendrites length and then moves to quantify both axon and dendrites for the Tetanus toxin experiment. What are the effects of KO on axon length? In the main figures, it is not mentioned but in S3 it seems not to be affected. How does it reconcile with the main conclusion on neurite length?

Figure 3H (now Figure 6C) shows the effect of the KO on axon length: the axon length is increased in RPH3A KO neurons compared to WT, similar to dendrite length. Re-expressing RPH3A in KO neurons rescues axonal length to WT levels. In Figure S3, we observe a similar trend as in main Figure 3 (new Figure 6), yet this effect did not reach significance. Based on this, we concluded that neurite length is increased upon RPH3A depletion.

(10) For lay readers, please explain the total pool and how you measured it. However, see the next comment.

We agree. We have now defined this better in the revised manuscript, (p 19, line 524-527 and p 20, line 535-539).

(11) It is a bit hard to understand if the total number of DCV was increased in the KO and if the pool size was increased and in which figure it is quantified. Some sentences like: "A trend towards a larger intracellular DCV pool in KO compared to WT neurons was observed" do not fit with "No difference in DCV pool size was observed between WT and KO neurons (Figure S2D)" or with "During stronger stimulation (16 bursts of 50 APs at 50 Hz), the total fusion and released fraction of DCVs were increased in KO neurons compared to WT". They are not directly supported, or not related to specific figures. Please indicate if the total DCVs pool, as measured by NH4, was increased and based on that, the fraction of the releasable DCVs following the long stimulation. From Figure 2H, the conclusion is an increase in fusion events. In general, NH4 is not quantified clearly- is it quantified in Figure S2C? And if it is a trend, how can it become significant in Figure 3?

We agree there has been some inconsistency in the way we describe the data on the total number of DCVs. We have addressed this in the revised text to ensure better clarity. The total DCV pool measured by NPY-pHluorin was not significantly increased in KO neurons, we see a trend towards a bigger DCV pool in the 2x8 50 Hz stimulation paradigm (now Figure S2C), therefore the released fraction of vesicles is not increased in Figure 1G (now Figure 3G). The number of DCV in Figure 3 (now Figure 5) is based on endogenous ChgB staining and not overexpression like the DCV pool measured by NPY-pHluorin. In Figure 3 (now Figure 5) we show that RPH3A KO neurons have slightly more ChgB⁺ puncta compared to WT.

(12) In Figure 3, the quantification is not clear, discrete puncta are not visible but rather a smear of chromogranin staining. How was it quantified? An independent method to count DCV number, size, and distribution like EM is necessary to support and add further understanding.

We acknowledge that discrete ChgB puncta are not completely visible in Figure 3 (now Figure 5). Besides the inherent limitation in resolution with confocal imaging, we believe that this is due to ChgB accumulation in the KO neurons, as shown in now Figure 5D. Nonetheless, to address this concern of the reviewer, we have selected other images that represent our dataset (now Figure 5A). Furthermore, the number of ChgB+ DCVs was calculated using SynD software (Schmitz et al., 2011; van de Bospoort et al., 2012) (see previous reply). EM would offer valuable independent confirmation on the total DCV number, size and distribution. However, with the current method we already know that vesicle numbers are at least similar. Does that justify the (major) investment in a quantitative EM study? Moreover, this issue does not affect the central message of the current study.

(13) Can the author discuss if the source of DCVs that are released at the synapse is similar or different from the source of DCVs fused while neurites elongate?

With our current experimental design, we are unable to draw conclusions regarding this aspect. We are not sure how experiments to identify this source (probably the Golgi?) would be crucial to sustain the central message of our study.

(14) An interesting and related question: what are the expression levels of RPH3A during development and neuronal growth during the nervous system development?

While we have not specifically examined the expression levels of RPH3A over development, public databases show that RPH3A expression increases over time in mice, consistent with other synaptic proteins (Blake et al., 2021; Baldarelli et al., 2021; Krupke et al., 2017). We have now added this to the revised manuscript (p 2, line 55-56).

(15) The conclusion from Figure 4 about the contribution of SNAP25 interaction to RPH3A inhibitory effect is not convincing. The data are scattered and in many neurons, high levels of fusion events were detected. Further or independent experiments are needed to support this conclusion. For example, is the interaction with SNAP25 important for its inhibitory activity in other DCV-releasing systems like adrenal medulla chromaffin cells?

We agree that further studies in other DCV-releasing systems like chromaffin cells would provide valuable insight into the role of SNAP25 interaction in RPH3A's inhibitory effect on exocytosis. However, we believe that starting new series of experiments in another model system is outside of the scope of our current study.

(16) Furthermore, the number of DCVs in the KO is similar in this experiment, raising some more questions about the quantification of the number of vesicles, that differ, in different sections of the manuscript (points # 10, 11).

The total DCV pool in the fusion experiments is measured by overexpression NPY-pHluorin, this cannot be directly compared to the number of endogenous ChgB+ DCV in Figure 3 (now Figure 5), see also item (11)

(17) The statement - "RPH3A is the only negative regulator of DCV" is not completely accurate as other DCV inhibitors like tomosyn were described before.

We agree. By this statement, we intend to convey that RPH3A is the only negative regulator of DCVs without substantial impact on synaptic vesicle exocytosis, unlike Tomosyns. We have clarified this in the revised text, (p 15, line 366-367).

(18) The support for the effect of KO on the "clustering of DCVs" is not convincing.

The intensity of endogenous ChgB puncta was decreased in RPH3A KO neurons (now Figure 5E). However, the peak intensity induced by single NPY-pHluorin labeled DCV fusion events (quanta) was unchanged (now Figure S2I). This indicates that the decrease in ChgB puncta intensity must be due to a reduced number of DCVs (quanta) in this specific location. We have interpreted that as ‘clustering’, or maybe ‘accumulation’. However, we only put forward this possibility. We are now more careful in our speculations within the text, (p 11 line 271-277).

(19) Final sentence: “where RPH3A binds available SNAP25, consequently restricting the assembly of SNARE complexes” should be either demonstrated or rephrased as no effect of trans or general SNARE complex formation is shown.

We agree. We have made the necessary adjustments in the text, (p 15, line 387-389).

(20) A scheme summarizing RPH3A's interaction with synaptic proteins and its effects on DCVs release, maybe even versus its effects on SVs release, should be considered as a figure or graphic abstract.

We have included a working model in Figure 7.

(21) Figure 4 logically should come after Figure 2 to summarize the fusion-related chapter before moving to neurite elongation.

We have placed Figure 4 after Figure 2 (now Figure 3).

Reviewer #3 (Recommendations For The Authors):

One important finding of this study is that RPH3A downregulates neuron size, possibly by inhibiting DCV release. Additionally, the authors demonstrated that the number of DCVs is directly proportional to the number of DCVs per μm^2 , and that RPH3A KO reduces DCV clustering. This conclusion was drawn by comparing ChgB with NPY-pHluorin loading of the DCVs. However, this comparison is not valid as ChgB is expressed at an endogenous level and NPY-pHluorin is over-expressed. In the KO situation where DCV exocytosis is enhanced, the available endogenous ChgB may be depleted faster than the overexpressed NPY-pHluorin. Hoogstraaten et al. should either perform a study in which ChgB is overexpressed to test whether the difference in DCV remains or at least provides an alternative interpretation of their data.

We thank the reviewer for this comment. The reviewer challenges one or two conclusions in our original manuscript (It is not entirely clear to what exactly “This conclusion” refers): (a) “the number of DCVs is directly proportional to the number of DCVs per μm^2 ”, and (b) “that RPH3A KO reduces DCV clustering”. The reviewer probably means that the number of DCVs per neuron is directly proportional to size of the neuron (a) and states this (these) conclusion(s) are “not valid as ChgB is expressed at an endogenous level and NPY-pHluorin is over-expressed” because “endogenous ChgB may be depleted faster than the overexpressed NPY-pHluorin”. We have three arguments to conclude that faster depletion of ChgB cannot affect these two conclusions: (1) DCVs bud off from the Golgi with newly synthesized (fresh) ChgB. Whether or not a larger fraction of DCVs is released does not influence this initial ChgB loading into DCVs (together with over-expressed NPY-pHluorin); (2) in hippocampal neurons merely 1-6% of the total DCV pool undergoes exocytosis (the current study and also extensively demonstrated in Persoon et al., 2018). RPH3A KO neurons release few percent more of the total DCV pool. Hence, “depletion of ChgB” is only marginally different between experimental groups; and (c) the proposed experiment overexpressing ChgB will not help scrutinize our current conclusions as ChgB overexpression is known to affect DCV biogenesis

and the total DCV pool, most likely much more than a few percent more release by RPH3A deficiency.

Hoogstraaten et al. conducted a thorough analysis of the impact of RPH3A KO and its rescue using various mutants on dendrite and axon length (see Supplementary Figure 3). However, they did not test the effect of the Δ SNAP25 mutant. The authors demonstrated that this mutant is the least efficient in rescuing DCV exocytosis (Figure 4E). Hence the neurons expressing this mutant should have a similar size to the KO neurons. This finding would strongly support the argument that DCV exocytosis regulates neuron size. Otherwise, it would suggest that RPH3A may have a function in regulating exocytosis at the growth cones that is independent of SNAP25. Since the authors most probably have the data that allows them to measure the neuron size (acquired for Supplementary Figure 2), I suggest that they perform the required analysis.

We agree this is important and performed new experiments to determine the dendrite length of RPH3A WT, KO and KO neurons expressing the Δ SNAP25 mutant. We observed that the dendrite length of RPH3A KO neurons expressing Δ SNAP25 mutant is indeed similar to KO neurons (new Figure S3C). Although not significant we observe a clear trend towards bigger neurons compared to WT. This strengthens our conclusion that increased DCV exocytosis contributes to the observed increased neuronal size.

The authors displayed the result of DCV exocytosis in two ways. One is by showing the number of exocytosis events the other is to display the proportion of DCVs that were secreted. They do the latter by dividing the secreted DCV by the total number of DCVs. These are visualized at the end of the experiment through NH4⁺ application. While this method works well for synaptic secretion as the marker of SV is localized to the SV membrane and remains at the synapse upon SV exocytosis, it cannot be applied in the same manner when it is the DCV content that is labeled as it is released upon secretion. Hence, the total pool of vesicles should be the number of DCV counted upon NH4⁺ application in addition to those that are secreted. This way of analyzing the total pool of DCV might also explain the difference in this pool size between KO neurons stimulated two times with 8 stimuli instead of one time with 16 stimuli (Sup Fig 2 C and D). This is an important point as it affects the conclusions drawn from Figure 2.

We thank the reviewer for this comment. We agree, and we have made the necessary adjustments throughout the manuscript.

The kymogram of DCV exocytic events displayed in Figure 2D shows a majority of persistent (>20s long) events. This is strange as NPY-pHluorin corresponds to the released cargo. Previous work using the same labeling and stimulation technique showed that content release occurs in less than 10s (Baginska et al. 2023). The authors should comment on that difference.

In Baginska et al. (2023), the authors distinguished between persistent and transient events. The transient events are shorter than 10s for the 2x8 and 16x stimulation paradigms, whereas persistent events can last for more than 10s. In our study we did not make this distinction. However, in response to this reviewer, we have now quantified the fusion duration per cell. These new data show that the mean duration is similar between genotypes for both stimulation paradigms. We have added these new data (new Figure S2D-F).

In Figures 1D and E, some puncta in the kymogram appeared to persist after bleaching. This raises questions about the effectiveness of the bleaching procedure for the FRAP experiment.

The reviewer is correct that NPY-pHluorin in Figure 1E (now Figure 2C) is not fully bleached. NPY-pHluorin was more resistant to bleaching than NPY-mCherry. However, we merely bleached the neurites to facilitate our analysis by reducing fluorescence of the stationary puncta without causing phototoxicity. Some remaining fluorescence after bleaching does not affect our conclusions in any way.

In the discussion, the paragraph titled "RPH3A does not travel with DCVs in hippocampal neurons" is quite confusing and would benefit from a streamlined explanation.

We thank the reviewer for this comment. We made the necessary adjustments to make this paragraph clearer, (p 14, line 339-351).

First paragraph of page 8 "TeNT expression in KO neurons restored neurite length to WT levels. When compared to KO neurons without TeNT, neurite length was not significantly decreased but displayed a trend towards WT levels (Figure 3G, H)." These two sentences are confusing as they seem contradictory.

We agree that this conclusion has been too strong. However, we do not see a contradiction. The significant effect between KO and control neurons on both axon and dendrite length is lost upon TeNT expression (which forms the basis for our conclusions cited by the reviewer, now Figure 6B, C). While the difference between KO neurons +/- TeNT did not reach statistical significance. The (strong) trend is clearly in the same direction. We have refined our original conclusion in the revised manuscript, (p 12, line 304-306).

The data availability statement is missing.

We have added the data availability statement, (p 21, line 571-572).

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