Supplementary Information

Materials and Methods

cDNAs.

Full length human Varp coding sequence, Interaction Domain (ID, aa 641 to 709) were subcloned from pEntr221-Varp (RZPD, Berlin, Germany) in pcDNA3 and pEGFP-N1. Mutant lacking the Vps9 domain (ΔVps9-Varp-GFP, aa 307 to 1050) was obtained by enzymatic digestion of Varp-EGFP. Rat brain TI-VAMP was cloned in pcDNA3 and in mRFP. GST-tagged TI-VAMP construct was previously described (Martinez-Arca et al, 2003b). PeGFP-Rab21, myc-Rab21 and peGFP-Rab5, plasmids were previously characterized (Lanzetti et al, 2000; Simpson et al, 2004), peGFP-Rab11 was a kind gift of Dr Bruno Goud (Institut Curie, Paris).

Cell culture and cDNA transfection.

HeLa and L-929 cells were grown in DMEM supplemented with 10% fetal calf serum (PAA, Lesmoreaux, France), PC12 cells in RPMI with 10% horse serum and 5% fetal calf serum on collagen-coated dishes. HeLa and PC12 cells were transfected with LipofectAMINE 2000 as described by the supplier. Cells were fixed after 24-36 h with 4% paraformaldehyde and processed for immunofluorescence microscopy as described previously (Martinez-Arca et al, 2000). Hippocampal neurons from newborn [postnatal day P0] mice were prepared as described previously (Danglot et al, 2003) and grown on poly-Lysine-coated (Sigma-Aldrich, Saint Quentin Fallavier, France) 12-mm coverslips at a density of 75000-100000 per coverslip in Neurobasal media supplemented with 2% B27 and 2 mM glutamine. Neurons were transfected at DIV1 by using LipofectAMINE 2000 according to the manufacturer's instructions. After 48h (72 h from the beginning of the culture), neurons were fixed with 4% paraformaldehyde and processed for immunofluorescence microscopy as described previously. Experiments in figure 3G-I were repeated four times. Mean of axon length corresponding to percentile graph (fig. 3H) was: GFP 314.2±14.9 µm, Varp-GFP 325.3±19.7 µm and GFP-ID 182.2±13.9 µm.

All other reagents were from Invitrogen (Cergy-Pontoise, France) unless specified.
Antibodies.

Rabbit polyclonal Varp antibodies (pAb TG40) were raised by injecting rabbits with recombinant GST-ID (amino acids 641 to 709) (Eurogentec, Leuven, Belgium). Sera were immunopurified against the antigen on Sulfolink Columns (Pierce, Illinois, USA). Rabbit serum against TI-VAMP (TG18) and mouse monoclonal antibodies (mAb) against TI-VAMP (Cl 158.2) and Syb2 (Cl 69.1) were described previously (Alberts et al, 2006; Muzerelle et al, 2003). Mouse anti β-tubulin was from Hybridoma bank (University of Iowa, IA, USA). Rat mAb anti Tyr-Tubulin was from Chemicon. Mouse mAb anti-myc (clone 9E10) and anti-GFP (clone 7.1 and 13.1) were from Roche Diagnostic (Indianapolis, IND, USA). Chicken anti-MAP2 and rabbit anti-GFP were from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Sheep anti human TGN46 was from AbDserotec (Oxford, UK). All other reagents were from Sigma-Aldrich (Saint Quentin Fallavier, France) unless specified.

Immunoprecipitation assay.

Cells were lysed in TSE (50 mM TrisHCl pH8.0, 150 mM NaCl, 1 mM EDTA) plus 1% Triton x100 (Chaineau et al, 2008) and immunoprecipitation experiments were carried out as described in (Martinez-Arca et al, 2003a). Briefly, 1.2 mg of protein extract was submitted to immunoprecipitation overnight at 4°C, with 25 µl of protein G or protein A sepharose beads (GE Healthcare, USA), washed with TSE-1% Triton and loaded on 4-12% NuPAGE gels. Gels were ran in MOPS buffer (Invitrogen), processed for Western blotting using fluorescent secondary antibodies and scanned in a Odyssey infrared imaging system (LI-COR, Lincoln, Nebraska, USA).

Time lapse imaging.

In dual-color imaging for colocalization time-lapse experiments, channels were collected sequentially every 5-10 sec with an inverted microscope Leica DMI6000B (Leica Microsystem, Mannheim, Germany) equipped with a high-pressure mercury arc lamp (HBO 100W, Osram), 63x/1.4 Plan-Apochromat oil-immersion objectives and a digital camera (Cascade:512B; Roper Scientific, Evry, France). Imaging was conducted in RPMI without phenol red + 10% horse serum + 5% foetal bovine serum or in modified Krebs-Ringer-HEPES buffer (140 mM NaCl, 2.8 mM KCl, 2 mM MgCl2,
1 mM CaCl$_2$, 10 mM HEPES, 5.5 mM glucose pH 7.4 with NaOH). Temperature and pH were controlled by an air-CO$_2$ warmed (37°C) mixture. Lamp power and exposure time were the lowest possible (10-30% Hg lamp, 50-100 ms) to avoid phototoxicity and reduce the bleed-through between the two channels.

**Image acquisition, deconvolution and colocalization analysis.**

Image scans for each probe were acquired in series at a step-size of 0.3 μm with an inverted microscope described above. At least 15 sections were scanned per sample for each probe. Specimen magnification was 630x or 1006x. Deconvolution and image analysis were performed by using MetaMorph software (Roper Scientific, Evry, France) based on an algorithm produced on the system from the convolution of a point spread function (PSF) to differentiate and reduce extraneous light or scattered light captured by the camera. A PSF describes the imaging and resolution characteristics of light collected by the optics of the microscope and was derived by scanning a 0.3 μm fluorescent bead (PS-Speck™ Microscope Point source Kit; Invitrogen, Cergy-Pontoise, France). The resulting PSF was Fourier transformed into an optical transfer function that manipulated the data to produce images with a higher signal-to-noise resolution of the probe emission patterns. All data sets were used for image analysis and image reconstructions. Subtraction of background fluorescence and change of intensity gain were optimally set for each emission. Colocalization analysis was performed in at least 20 selected cells for each experimental condition. Cells were processed for deconvolution for each emission channel as described above and the best plane was selected for colocalization analysis. The two channels were then manually thresholded and the Mander’s coefficient was calculated by using Mander’s coefficient plug in bundled with WCIF ImageJ (http://www.uhnres.utoronto.ca/facilities/wcif/).

Alternatively, thresholded images were processed to measure the total number of labelled pixels for every channel. Afterwards the number of pixels that overlap between the two channels was calculated by using the image calculator option of ImageJ. Data were represented as percentage of number of pixels that overlap and the total number of pixels for one staining.
siRNA knockdown and neurite/axon length assay.

Varp, Syb2 and Tl-VAMP RNA interference were achieved by using specific pre-designed siRNA duplexes (Qiagen, Courtaboeuf, France, see Table S2 below for details). For every experiment performed non-targeting siRNA (scramble, luciferase) and mock transfection were used as controls.

Human HeLa cells and mouse L-929 cells were transfected once with oligonucleotides at concentration of 100 nM by using oligofectAMINE (Invitrogen, Cergy-Pontoise Pointoise, France) according to the manufacturer’s instructions and cultured for additional 72-96 h. Cells were treated for Western blot analysis or coverslips fixed and processed for immunofluorescence as described previously (Martinez-Arca et al, 2000). HeLa and L-929 siRNA experiments were repeated respectively at least ten and three times.

PC12 cells were transfected by using AMAXA NucleofectorTM technology (Kit V, Amaxa, Koln, Germany) twice on two consecutive days with 1.5-3 μg of oligonucleotides per sample. After the second transfection, cells were plated on collagen-coated glass coverslips and cultured for additional 48 h (72 h from the first transfection). Cells were then differentiated with 100 nM staurosporine for 2 to 12 h at 37°C. Cells were treated for Western blot analysis or coverslips fixed and processed for immunofluorescence as described previously (Martinez-Arca et al, 2000). Between three and nine hundred randomly chosen cells were analysed for each treatment condition in at least five independent experiments. Neurites were defined as thin process longer than 5 μm, from the cell body limit to the end of the process and measured, based on tubulin staining, by using the MetaMorph software (Roper Scientific, Evry, France) or the NeuronJ plugin of ImageJ (Abramoff et al, 2004).

Mouse hippocampal neurons were cotransfected at DIV1 with oligonucleotides at concentration of 100 nM and 0.25 μg of EGFP by using lipofectAMINE 2000 according to the manufacturer’s instructions and cultured for additional 72 h (96 h total from beginning of the culture). Neurons were then fixed with 4% paraformaldehyde and processed for immunofluorescence. GFP expressing neurons were selected and their axons (identified by the lack of MAP-2 staining) were measured by using the MetaMorph software. Data were collected by four independent experiments and at least from 50 neurons for each treatment condition. Mean of axon length corresponding to percentile graph (fig. 3D) was: siRNA
scramble 407.9±22.4 µm, siRNA Varp_m1 308.7±23.8 µm and siRNA Varp_m2 303.6±24.4 µm.

Table S2. siRNA duplexes classification.

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<th>Oligo name</th>
<th>Target sequence</th>
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**Total RNA purification and Real Time RT-PCR.**

HeLa, L-929 cells, PC12 cells or mouse hippocampal neurons were cultured and treated as described in the Methods section and total cellular RNA was isolated and purified using Nucleospin®RNA II kit (Macherey-Nagel) according to the manufacturer’s instructions. The integrity of the RNA was confirmed by agarose gel electrophoresis (data not shown) and the concentration was quantified in a Nanodrop spectrophotometer (Wilmington, DE, USA).

Real Time RT-PCR was performed on LightCycler™480 (Roche Diagnostics, Mannheim, Germany) by using SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, Cergy-Pontoise, France) according to the manufacturer’s instructions. Briefly, reverse transcription and DNA amplification was carried out in a total volume of 10 µl containing 10 µM of each primer, 5 µl of 2X SYBR Green Reaction Mix, 0.4 µl Superscript II RT/Platinum Taq mix, 0.5 µl ultrapure BSA (1 mg/ml) and 100 ng of template RNA. Each RNA sample was processed in triplicate. Standard curve was created by using serial dilutions of RNA template of not treated cells (wild type). For detection of Varp, the sense primer 5’-AATGCTCTCTACACCAATGCGCTCC-3’ and antisense primer 5’-GATCTTGAAGGCTTCTTGATTTTGTTAAAG-3’ were used. The same primers
were employed for the different cellular species amplifying one region of 211 bp in the Varp sequence (from 534 to 745). The PCR reaction was cycled 50 times after initial step of reverse transcription (50°C, 5 min) and denaturation (92°C, 1 min) with the following parameters: denaturation at 92°C, 5 s; annealing-extension at 50°C, 30 s. Fluorescence was acquired after every cycle. Melting curve analysis of amplification products was performed at the end of each PCR reaction by increasing the temperature to 95°C at 0.2°C/s. Fusion curve allowed us to ensure that a single amplicon is obtained for each reaction. Five to ten μl of each RT-PCR product were visualized after agarose gel electrophoresis and ethidium bromide staining (data not shown). Quantifications of mRNA coding for Varp expression in siRNA treated cells (see Table S1 for details) were obtained by using LightCycler®480 software (Roche Diagnostics, Mannheim, Germany) and expressed as percentage of mRNA expression of the control siRNA (scramble) treated cells.
Supplementary Figure Legends

Figure S1. Varp antibody characterization and Varp-Rab21 interaction.
A, Characterization of the anti-Varp TG40 antibody directed against ID in control HeLa cells or HeLa cells expressing GFP-ID, Varp or Varp-GFP. The antibody recognized a band in HeLa cell extracts of the expected molecular weight (117kDa, arrowhead). This signal was reinforced upon overexpression of Varp. The antibody also recognized GFP-tagged ID (double circle) and GFP-tagged full length Varp (dash) as seen by Western blotting (WB; SM, 10% of starting material) and immunoprecipitated endogenous as well as overexpressed forms of Varp (IP Varp). The same amounts of extracts and immunoglobulin were used as seen by ponceau staining (PS). Control, untransfected HeLa cells; IP, immunoprecipitation; HC, IgG Heavy Chain; LC, IgG Light Chain. B, Endogenous Varp coprecipitates with Rab21-T33N more intensively than with Rab21-WT and Rab21-Q78L in HeLa cells. Control, untransfected HeLa cells; Arrowhead, Varp; Dash, Varp-GFP; Double circle, GFP-ID; Single asterisk, Rab21; SM, 10% of starting material; IP, immunoprecipitation; WB, Western blot; HC, IgG Heavy Chain; LC, IgG Light Chain.

Figure S2. Colocalization of Rab21, Rab5 or Rab11 with Varp and TI-VAMP in PC12 cells.
Cells were cotransfected with the wild-type (WT) form of GFP-Rab21, GFP-Rab5 or GFP-Rab11 and Varp and then differentiated with 100 nM staurosporine for 24 h. Cells were fixed and triple labelled for GFP (revealing overexpressed Rabs), Varp and TI-VAMP. Images were acquired with a piezo-driven 63x objective and processed for deconvolution with Metamorph software. Overlays on the right panels show colocalization between Varp and TI-VAMP (arrows) or between Rab21, Rab5 or Rab11, Varp and TI-VAMP (arrowheads). Scale bar, 10 μm.

Figure S3. ΔVps9-Varp-GFP overexpression impairs axonal growth in mouse hippocampal neurons.
A, DIV1 mouse hippocampal neurons were transfected with Varp-GFP, ΔVps9-Varp-GFP or GFP, and stained for GFP (green) and MAP2 (red). The longest process is the axon (as confirmed by the lack of MAP2 staining) is emphasized by a set of
arrowheads in each condition (merge panel), Scale bars, 20 μm. B, Quantification of the effect of ΔVps9-Varp-GFP on axonal length in GFP positive cells represented as average. Significance determined by two-tailed unpaired t-test **P<0.01. Data are shown as mean ± s.e.m. n, number of measured axons. C, TI-VAMP coprecipitates ΔVps9-Varp-GFP and GFP-tagged Varp from HeLa cell extracts. IP, immunoprecipitation.

Figure S4. Partial colocalization between TI-VAMP, Rab21 and the trans-Golgi network marker TGN46 in HeLa cells.
HeLa cells were transfected with GFP-Rab21-WT, and labelled for GFP (green), TI-VAMP (red) and TGN46 (blue). Images were acquired with a piezo-driven 63x objective and processed for deconvolution with Metamorph software. Magnifications of boxed regions are shown in the right panels. Some colocalization spots between Rab21, TI-VAMP and TGN46 (arrows) are pointed out in the merge panel. Scale bar, 10 μm.
Supplementary Movie Legends

**Movie S1. Varp and TI-VAMP traffic in mouse hippocampal neurons.**
DIV1 mouse hippocampal neurons were co-transfected with Varp-GFP (green) and RFP-TI-VAMP (red). After 48 h neurons were treated with Brefeldin A (BFA, 1 μg/ml) for 16 h at 37°C to block the exit from the Golgi complex (Jareb & Banker, 1997). After BFA washout, neurons were recovered for 1 h in fresh culture medium and then imaged as described in Materials and Methods. Scale bar, 10 μm. Time is indicated in min:sec. Acceleration 30x.

**Movie S2. Varp and TI-VAMP are cotransported in mouse hippocampal neurons.**
2x magnification of the boxed regions of Movie S1. Vesicular cotransport of Varp-GFP and RFP-TI-VAMP (arrowheads) in anterograde and retrograde (not shown) directions is shown merged. Scale bar, 10 μm. Time is indicated in min:sec. Acceleration 33x.

**Movie S3. Dynamics of TI-VAMP vesicles in the peripheral region of axonal growth cone.**
RFP-TI-VAMP vesicles were tracked in a growth cone expressing GFP-Rab21-WT. TI-VAMP positive vesicles (arrowheads) moved into the peripheral region of the axon whereas Rab21 seems to be retained in the central region. Time is indicated in min:sec. Acceleration 13x.
References


Jareb M, Banker G (1997) Inhibition of axonal growth by brefeldin A in hippocampal neurons in culture. J Neurosci 17: 8955-8963


Figure S1
Figure S2
Figure S3

A

GFP  MAP2  Merge

GFP
Varp-GFP
ΔVps9-Varp-GFP

B

Axon length (% GFP)

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<th></th>
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C

IP Ti-VAMP

GFP
ΔVps9 Varp-GFP
Ti-VAMP
Figure S4
Table S1. Quantification of the effects of siRNA duplexes in Varp mRNA expression by Real-Time RT-PCR. Data are shown as mean ± s.e.m. N.D., not determined.

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