**Science Supporting Online Material**

**Nanotubular Highways for Intercellular Organelle Transport**
Amin Rustom, Rainer Saffrich, Ivanka Markovic, Paul Walther, Hans-Hermann Gerdes
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**Materials and methods**

*Cell culture and transfection*
PC12 cells (rat pheochromocytoma cells, clone 251) \( (S1) \) were cultured in DMEM supplemented with 10% fetal calf serum and 5% horse serum as described \( (S2) \). For light microscopic analysis, PC12 cells were plated on LabTek™ chambered 4-well coverglasses (Nalge Nunc Int., Wiesbaden, Germany) or 9-mm coverslips. Transfection was performed as described \( (S2) \). The following cDNA constructs were used: pEGFP-f (Clontech Laboratories, Palo Alto, CA, USA), pEYFP-N1 (Clontech), pβactin-eGFPgamma(149), referred to as EGFP-actin \( (S3) \), syn-EGFP, pcDNA3-hCgB-EGFP \( (S2) \), marker for secretory granules, pEGFP-Mito (Clontech), marker for mitochondria.

*Dye stainings and immunofluorescence labeling*
For wheat germ agglutinin (WGA) staining, WGA Alexa Fluor® 594 conjugate (Molecular Probes, Inc., Eugene, OR, USA, 1 mg/ml concentration) was added directly to the culture medium (1:300). For LysoTracker™ staining LysoTracker Green DND-26 (Molecular Probes) was added directly to the culture medium (100 nM final concentration). Vybrant™ DiI, DiO cell labeling solutions (Molecular Probes) were used for labeling of cells in suspension according to supplier's instructions. CellTracker™ solution (Molecular Probes, 20 µM final concentration) was used according to supplier's instructions. F-actin was fluorescently labeled with phalloidin-TRITC/FITC conjugate (Sigma Chemical Co., Saint-Louis, MO, USA, 250 nM final concentration) as described \( (S2) \). For nuclear staining 4,6-diamino-2-phenylindol-di-
hydrochlorid (DAPI, Molecular Probes, 1:500) was used. Indirect immunofluorescence labeling was performed as described (S2). Primary antibodies: monoclonal anti-synaptophysin antibody Sy-38 (Chemicon, Temecula, CA, 1:100), polyclonal anti-myosin Va antiserum Dil2 (S4) (1:300), monoclonal anti-α-tubulin antibody (clone DM1A, Sigma Chemical Co., 1:500), polyclonal anti-GFP antibody (Molecular Probes, 1:500). Secondary antibodies (Jackson Immuno Research Labs, Inc., West Grove, PA, USA): Goat anti-mouse lissamine (1:500), goat anti-rabbit TRITC (1:200), goat anti-rabbit Cy5 (1:300).

**Light Microscopy**

High resolution, bright field and fluorescence videomicroscopy was performed with a monochromator-based imaging system (T.I.L.L. Photonics GmbH, Martinsried, Germany) as described (S5), except that a tripleband filterset DAPI/FITC/TRITC F61-020 (AHFanalysentechnik AG, Tübingen, Germany) was used as was a piezo z-stepper (Physik Instrumente GmbH & Co., Karlsruhe, Germany) for 3D analysis. Confocal analyses were performed with a Leica SP2 confocal microscope equipped with a 100x HCX PL APO 100x/1.40 NA oil objective (Leica, Microsystems Vertrieb GmbH, Bensheim, Germany). Both imaging systems were equipped with a 37°C heating control device and a 5% CO₂ supply (Live Imaging Services, Olten, Switzerland). For 3D analysis, 40 z-sections were taken, optionally processed by using the deconvolution extension of TILLvisION (T.I.L.L. Photonics GmbH) and analyzed by the 3D extension of IPLab software v3.2.2 (Scanalytics, Inc., Fairfax, VA, USA).

**Transmission Electron Microscopy**

For transmission electron microscopy (TEM) cells were prepared following standard protocols. PC12 cells were fixed with 2.5% glutaraldehyde and postfixed with 1% OsO₄/1,5% K₄Fe(CN)₆, dehydrated in graded series of 1-propanol and block stained in 1% of uranyl acetate and embedded in „Epon“. Consecutive ultra thin sections (80 nm) were contrasted with lead citrate and imaged in a Zeiss EM 10 at an accelerating voltage of 80 kV.

**High Resolution Scanning Electron Microscopy**

For scanning electron microscopy (SEM) PC12 cells were fixed with 2.5% glutaraldehyde, dehydrated in graded series of ethanol and critical point dried using CO₂. Afterwards cells were coated using a BAF 300 freeze etching device (Bal-Tec, Balzers, Principality of Liechtenstein). Cells were rotary coated as described (S6) using platinum-carbon (coating thickness: 3 nm). The samples were imaged in an Hitachi S-5200 in-lens field emission scanning electron microscope at an accelerating voltage of 4 kV using the secondary electron signal.
Microinjection
For microinjection PC12 cells were plated on MetTek™ glass bottom culture dishes (MatTek Corporation, Ashland, MA, USA). WGA and cycloheximide (10 µg/ml) were added directly to the culture medium. Cells were analyzed using a Leica Leitz DM IRB fluorescence microscope (Leica Mikrosysteme Vertriebs GmbH) equipped with an Leica PL APO 100x/1.4-0.7 objective, a Quantix CCD camera (Photometrics, Roper Scientific Germany, Ottobrunn) and microinjected with 5-20 fl 10x PBS solution according to standard procedures using an Eppendorf Transjector 5246 and an Eppendorf Micromanipulator 5171 (Eppendorf AG, Hamburg, Germany). Capillaries were made with a P-87 capillary puller (Sutter Instruments Inc., Novarto, USA).
Supporting figures

Fig. S1 3D fluorescence microscopy reveals TNTs between non-neuroendocrine cells. Cultured human embryonic kidney cells HEK 293 (ATCC CRL 1573) (A) or (normal) rat kidney cells (NRK) (B) were stained with WGA and analyzed by 3D fluorescence microscopy (see Materials and methods). One (x-y)-section through the middle of the connected cells is shown. The inlay in (B) shows the corresponding (x-z)-section through the marked TNT (arrow). Note the stretched membrane-tubes with a diameter...
of approximately 150 nm, attached at the nearest distance between cells without contacting the substrate (arrows). Bar, 20 µm.
Fig. S2 Rupture of TNTs after prolonged light exposure. PC12 cells stained with WGA were analyzed by high resolution live cell fluorescence videomicroscopy (see Materials and methods). Shown here are selected frames of a videosequence acquired at 1 frame/s. Notably excitation with light of 565 nm leads to visible vibrations of the stretched TNT (A, B) and its subsequent rupture (C, D). The timepoints of image acquisition are indicated. Bar, 20 µm.
Fig. S3 TNT connections are resistant to trypsin/EDTA treatment. WGA stained PC12 cells were analyzed by fluorescence (A1, A2) or bright field (B1, B2) videomicroscopy (see Materials and methods) at indicated timepoints after replacing the culture medium by 1.25% or 2.5% trypsin (Boehringer Mannheim GmbH, Germany) supplemented with 2 mM EDTA (Sigma Chemical Co.), respectively. After prolonged trypsin/EDTA treatment cells start to round up, lose contact to each other (A1, A2, arrowhead) and eventually detach from the substrate (B2). Note that the TNT connections between the cells (arrows) are maintained. Bar, 20 µm.
Fig. S4 Analysis of \textit{de novo} formation of TNTs between PC12 cells. WGA stained cells (see Materials and methods) were analyzed in the presence and absence of 5 µM latrunculin-B (lat-B) (Calbiochem, La Jolla, CA, USA) as indicated. Analysis was performed by 3D fluorescence microscopy (see Materials and methods) at indicated timepoints after plating of singularized cells. For a quantitative analysis at each
timepoint, 10 areas depicting approximately 150 cells were chosen randomly and the number of TNTs was assessed. Note the strong increase (8.75 fold) in the number of TNTs from 0.5 to 2 hours after plating (A) and the absence of TNTs after 1 hour of incubation in the presence of lat-B (B). Each graph shows mean values of two independent experiments.
Fig. S5 TNTs are not a relic of incomplete cytokinesis. PC12 cells were cultured for 14 hours in the presence or absence of 2.5 mM thymidine (Sigma Chemical Co.) and then
passaged. Thymidine treatment arrests cells in the G1/ S phase of cell division. One hour after passaging, cells were stained with WGA (see Materials and methods), fixed for 20 minutes in 4% paraformaldehyde containing 4% sucrose, treated with 0.2% Triton X-100 for 3 minutes and finally stained with DAPI (see Materials and methods). Analysis by fluorescence microscopy (see Materials and methods) revealed that in both, the absence and presence of thymidine, TNTs are formed (A, top panel, arrowheads), although in the presence of thymidine the number of mitotic cells (A, arrows) was strongly reduced (A, compare bottom panel left and right). A quantitative analysis revealed that although the number of mitotic cells dropped to 20.09% as compared to control cells, almost the same number of TNTs (86.5%) was formed (B). Notably, this slight reduction may have its reason in the reduced cell number (69.1%) due to the block of cell division by the thymidine treatment. Bar, 20 µm.
Fig. S6 Ectopically expressed EGFP-actin displays discrete punctuated signals in PC12 cells. PC12 cells were transfected with a cDNA encoding for EGFP-actin, plated on LabTek™ chambers and cultured for 12 hours (see Materials and methods). EGFP-actin expressing cells were then analyzed by high resolution 3D fluorescence microscopy (see Materials and methods). Shown are two selected single (x-y)-planes of a 3D reconstruction cutting a cell at the bottom (A) and through the middle (B). Magnifications of the boxed areas in (A) and (B) are shown in (A1), (B1) and (B2), respectively. Note the punctuated signals of EGFP-actin (arrows) in the cytoplasm (A1, B2) as well as in the cell cortex (B1). Bar, 10 µm.
Fig. S7 Calcein enters efficiently filopodia but not TNTs. PC12 cells were stained 24 hours after plating with WGA (red) (see Materials and methods) and calcein AM (green) (Molecular Probes, 1:3000) and were analyzed by confocal microscopy. (A-C1) Analysis of filopodia: optical section at the bottom of the cell where numerous filopodia are located. Images of WGA (A) and calcein staining (B) together with the corresponding overlay (C) are shown. Note that calcein enters efficiently filopodia even those displaying a small diameter. This is highlighted by magnifications of the boxed...
areas in (A-C) shown in (A1-C1), respectively (arrowheads). (D-F1) Analysis of TNTs: optical section at the middle of the cells where a TNT is located. Images of WGA (D) and calcein staining (E) together with the corresponding overlay (F) are shown. Note that calcein is not entering the WGA stained TNT structure highlighted by the magnifications of the boxed areas in (D-F) displayed in (D1-F1), respectively (arrowheads). Bar, 15 µm.
Fig. S8 Biochemical and morphological characterization of ectopically expressed EGFP-f in PC12-cells. (A) EGFP-f is tightly membrane-associated. PC12-cells were
transfected with a cDNA encoding for EGFP-f (see Material and methods) or, as a control, were left untreated (wt). After 12 hours of incubation, post nuclear supernatants (PNS) were prepared from transfected and control cell lysates as described (S7). The PNS of control cells was kept on ice (lane 1) and that from transfected cells was divided into three aliquots. One aliquot was kept on ice (lane 2). The second aliquot was centrifuged at 100 000 x g for 1 hour resulting in a pellet (lane 3) and a supernatant fraction (lane 4). The third aliquot was centrifuged at 100 000 x g for 1 hour. The supernatant was discarded and the resulting pellet was resuspended in 250 µl H₂O and mixed with an equal volume of 2x carbonate buffer consisting of 0.2 M Na₂CO₃-NaHCO₃, pH 11.0, 2 M KCl and 2x protease inhibitors (leupeptin, aprotinin, PMSF). This solution was incubated for 30 minutes on a head over tail rotor at 4°C and then centrifuged at 100 000 x g for 1 hour resulting in a pellet (lane 5) and supernatant fraction (lane 6). Equal aliquots of the obtained pellets, supernatants and the PNS were subjected to 12% SDS-PAGE either directly (pellets) or after TCA-precipitation (supernatants, PNS). The amount of EGFP-f in each fraction was determined by western blotting using anti-GFP primary (1:5000, see Materials and methods) and HRP-conjugated secondary antibody (1:5000). As a control, rat secretogranin II (SgII), a matrix protein of secretory granules, was analyzed by the use of anti-SgII serum (S8).

The total amount of protein in each fraction was analyzed by Coomassie staining. Note that EGFP-f was detected as a specific band of 30 kDa in samples prepared from transfected (lane 2) but not non-transfected cells (lane 1). After centrifugation 88% of EGFP-f was found in the membrane pellet (lane 3) and of that 95.1% remained membrane-associated after alkaline carbonate treatment (lane 5). After centrifugation of the PNS, a specific band for SgII at 90 kDa was detected to 85.9% in the membrane-pellet (lane 3). In contrast to EGFP-f, only 14.5% of SgII remained membrane-associated after alkaline carbonate treatment (lane 5). (B, C) Ectopically expressed EGFP-f is exclusively localized at the plasmamembrane and displays punctuated and raft-like signals. PC12 cells were transfected with a cDNA encoding for EGFP-f, plated on LabTek™ chambers and cultured for 2 hours (see Materials and methods). EGFP-f expressing cells were then analyzed by high resolution 3D fluorescence microscopy (B, C) (see Materials and methods). Shown are two EGFP-f expressing cells (B, C) displaying unstained areas (open arrowheads) as well as punctuated and raft-like fluorescence signals of EGFP-f exclusively at their plasmamembrane (B, C, arrows). (D) EGFP-f is specifically targeted to the plasmamembrane (3D analysis). PC12 cells stained with CellTracker™, were mixed with PC12 cells transfected with EGFP-f, co-cultured for 48 hours, processed for immunocytochemistry using an antibody directed against GFP and FITC-phalloidine, and analyzed by 3D confocal microscopy (see Material and methods). A single (x-y)-section as well as single (x-z)- and (y-z)-sections (the respective planes are indicated by lines in the (x-y)-image) through the middle of a
cell expressing the GFP fusion protein is shown. Note, that the exclusive surface staining of EGFP-f labeled cells contrasts the intracellular, vesicular staining-pattern of DiO-labeled cells (E, arrowheads). Bar, 10 µm.
Fig. S9 3D analysis of PC12 cells displaying transferred EGFP-f signals. One PC12 cell population was transfected with a cDNA encoding for EGFP-f (green) and mixed with a second population stained with CellTracker™ (blue) as described for Fig. 4A-C of our
TNT based transfer was analyzed after 24 (A, B) or 48 hours (C, D) after co-plating using high resolution 3D fluorescence microscopy (see Materials and methods). Shown are selected (x-y)-sections of 3D reconstructions of pairs of TNT-connected cells (A, C, top images) as well as (x-z)-sections (A, C, bottom images) corresponding to the gray lines in the top images depicting only the EGFP-f channel recordings. In the (x-z)-sections the membranes of the EGFP-f-expressing (cell 1, green) and receiving cells (cell 2, blue) are highlighted by broken lines and the TNT connection is marked by an arrowhead. Please note that the outlines of the TNT connections were extrapolated from the corresponding (x-y)-planes because the insufficient z-resolution does not resolve the very thin TNT connection. The indicated boxes in (A) and (C) are magnified in the top images of (B) and (D), respectively. Note that the transferred, cap-like EGFP-f signals cover large surface areas of the receiving cells (dotted white lines) and are in continuity with the respective TNT. For two representative planes indicated by red lines (plane 1 and 2) in the top images of (B) and (D), the corresponding (x-y)-sections are presented as indicated (B, D, middle and bottom images). Note that the punctuated (arrows) and raft-like (dotted white lines) signals of EGFP-f are located at the plasmamembrane of the receiving cells. The haze in the cell periphery derives from out of focus information. The icons at the left indicate the orientation of the presented sections (black frames) with respect to the 3D reconstructions (grey cubes). Bars, 10 µm.
Fig. S10 Induced transfer of plasmamembrane-associated EGFP-f between TNT-connected cells. PC12 cells were transfected with a cDNA encoding for EGFP-f, plated
and incubated for 12 hours (see Materials and methods). Cells were stained with WGA and analyzed by fluorescence microscopy in the presence of cycloheximide (10 µg/ml). A selected EGFP-f-positive cell (cell 1), connected via a TNT (arrow) with a non-expressing cell (cell 2) was microinjected with hyperosmotic solution and analyzed after indicated time periods (see Materials and methods). Note the continuous increase in EGFP-f surface fluorescence selectively for the TNT-connected cell over the entire observation period of 20 min (B1-B3), whereas for neighboring cells (asterisks) no increase in EGFP-f fluorescence was detected. For quantitation, line profiles displaying grey value intensities (B1-3, right) were performed as indicated (red arrows). Arrowheads indicate the position of the cell border. Bar, 20 µm.
Fig. S11 TNT-based transfer of fluorescent organelles between cells. PC12 cells were stained with DiI and analyzed by fluorescence videomicroscopy (see Material and methods). Three video sequences corresponding to Fig. 4F, G were acquired consecutively at 4 frames/s: movie S4 covers 25 s, movie S5 and movie S6 cover 101 s.
each. (A, B) Overview pictures of a TNT (arrowhead) connecting two cells and facilitating uni-directional, intercellular transfer of DiI-labeled organelles. The broken lines in (A) outline the TNT together with the cell borders of the connected cells (asterisks) and the arrow indicates the direction of organelle transfer. In (B) four color-coded overlapping tracks (indicated by numbers 1-4) corresponding to the transport of four distinct fluorescent organelles are shown. The arrows indicate the direction of transport for the respective organelles. For each track displayed in (B) four selected frames depicting the analyzed organelle (arrowheads in galleries 1-4) are shown. The time points of image acquisition are indicated. Bar, 20 µm. Corresponding video sequences are available as supporting online video sequences movie S7 and movie S8. The vesicles corresponding to tracks 1-4 are highlighted in the video sequences by the track number.
Fig. S12 Uni-directional transfer of DiI-labeled organelles between TNT-connected cells. A mixed population of DiI and DiO stained PC12 cells (red and green cells, respectively) was analyzed at indicated timepoints after plating of singularized cells by confocal 3D fluorescence microscopy and subsequent maximum projection (see Materials and methods). After 1 hour, cells accommodate either DiI or DiO positive vesicular structures (A). After two hours, distinct cells contain DiI and DiO positive structures (B1, arrows). A 3D analysis of corresponding single (x-y)- (B2) and (x-z)-sections (B3) of the cell in B1 reveals that DiI-positive structures are located inside the DiO-labeled cell. At later timepoints, distinct cells harbor mostly vesicular structures positive for both dyes (yellow structures in C, arrowheads). Bar, 20 µm.
Fig. S13 Uni-directional transfer of DiI-labeled organelles between TNT-connected NRK cells. A mixed population of DiI- and DiO-labeled NRK cells (red and green cells, respectively) was analyzed 12 hours after plating of singularized cells by 3D fluorescence microscopy following deconvolution (see Materials and methods). Shown is a single (x-y)-section through a pair of cells connected by a TNT (arrow). The inlay shows the corresponding (x-z)-section through the marked TNT (arrow). Note that only the right cell of the TNT-connected pair of cells harbors DiI- and DiO-labeled organelles. Bar, 20 µm.
Fig. S14 TNTs facilitate the intercellular transfer of DiI/DiO labeled organelles by a mechanism different from endo-/exo- and phagocytosis. (E) Two differentially labeled PC12 cell populations were co-plated on LabTek™ chambers for 1 hour at 37°C and then further cultivated at different temperatures as indicated in the absence or presence of 5 µM latrunculin-B (lat-B). Note that further incubation at 0.7°C for 4 hours led to a significant increase of double-labeled cells (second column from right) whereas after further incubation at 37°C for 4 hours in the presence of lat-B no increase of double-labeled cells was detected (right column). (A) Two differentially labeled PC12 cell populations were co-plated on LabTek™ chambers for 1 hour at 37°C and then further cultivated for 4 hours at 0.7°C. A solitary, double-labeled cell (upper right cell) is connected via a TNT (arrow) to a neighboring cell (asterisk). (B) Magnification of the
boxed area in (A). Note the red and green organelles (arrowheads) in the cytoplasm of the double-labeled cell. (C, D) To exclude that at 0.7°C endocytosis or phagocytosis takes place, PC12 cells were stained 1 hour after plating with WGA and kept on 0.7 (C) or 37°C (D) for additional 4 hours. Note that cells kept at 37°C show a prominent perinuclear staining (asterisk) as well as numerous endocytotic structures in the cytoplasm (arrowheads). In contrast, cells kept at 0.7°C show a prominent plasmamembrane staining and no detectable endocytotic structures in the cytoplasm. Bar, 10 µm.

**Online movies**

Videosequences according to Figures 2A-D; 2G,H; 3A,B; 4F,G1; 4F,G2; 4F,G3 and to Fig. S11 are provided as supporting online material (Movie S1-8).

**Supporting references**