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Molecular Brain Research xx (2004) xxx–xxx

MOLECULAR BRAIN RESEARCH

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Research report

Rapid dendritic transport of TGN38, a putative cargo receptor

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Accepted 4 May 2004

Abstract

Protein transport to and from the postsynaptic plasma membrane is thought to be of central importance for synaptic plasticity. However, the molecular details of such processes are poorly understood. One mechanism by which membrane and secretory proteins may be transported to and from postsynaptic membranes is via cargo receptors. We studied the dendritic transport of TGN38, a putative cargo receptor thought to mediate protein transport between the *trans*-Golgi network (TGN), endosomes, and the plasma membrane. With fluorescence time-lapse imaging of neurons expressing a TGN38-green fluorescent protein fusion protein (GFP-TGN38), we observed rapid bidirectional dynamics of the protein in dendritic shafts. In addition, the protein was present on the surface and on intracellular membranes of dendrites and dendritic spines. Finally, GFP-TGN38 was found to cycle rapidly between the plasma membrane and intracellular membranes within dendrites, including those of spines. Together, our results suggest a role for TGN38 in facilitating rapid changes in the protein composition of postsynaptic membranes.

Theme: Excitable membranes and synaptic transmission
Topic: Postsynaptic mechanisms

Keywords: *Trans*-golgi network; Protein transport; Synaptic plasticity; Dendrite; Cargo receptor

1. Introduction

Dendritic protein transport is thought to be important for translating altered patterns of synaptic activity into changes in synaptic function. In particular, the transport of ion channels to and from the dendritic plasma membrane has been implicated in synaptic plasticity [22,38]. While there is currently intensive study of the cellular and molecular aspects of ion channel transport in dendrites, many additional dendritic transport processes might contribute to synaptic plasticity. A greater understanding of the various protein transport processes occurring in dendrites would also provide a context for understanding dendritic ion channel transport. It is for these reasons that we have studied the dendritic transport of TGN38, a protein that has been used as a model trafficking receptor in nonneuronal cells. It is thought that the function of TGN38 is to mediate the transport of secretory or membrane proteins within cells [4,39].

There are various mechanisms by which membrane and secretory proteins are selectively transported to or from particular cellular membranes. For instance, some proteins are known to directly interact with the clathrin adapter complex which mediates their endocytosis. Others, including many secretory and membrane proteins, associate with membrane transport machinery indirectly, through an interaction with another protein. A class of proteins, thought to function primarily as intermediaries in the latter case, are known as cargo receptors. These proteins are typically single-pass integral membrane proteins that exhibit rapid dynamics between distinct cellular membranes.

The best characterized cargo receptors are the mannose-6-phosphate receptors (MPRs). These proteins bind phos-

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doi:10.1016/j.molbrainres.2004.05.013

phomannosyl residues of lysosomal hydrolases (the cargo) in the TGN and mediate their transport to late endosomes where the hydrolases dissociate in response to the lower pH in the lumen of the late endosomes [12]. The MPRs then either return to the TGN to repeat the process or move to the plasma membrane to retrieve hydrolases from the extracellular space [12]. Whereas the lumenal domains of the MPRs are responsible for binding the cargo proteins, the cytoplasmic domains mediate the transport of the protein complexes by interactions with cellular trafficking machinery, such as proteins involved in membrane budding [12].

While MPRs mediate protein transport from the TGN to late endosomes, other cargo receptors are thought to mediate TGN to plasma membrane transport of secretory and membrane proteins. TGN38 is thought to have such a role [4,39]. Like MPRs, TGN38 is a single-pass integral membrane protein that exhibits rapid dynamics between the TGN, endosomes, and the plasma membrane [23,26,34]. The cytoplasmic domain of TGN38 is also known to mediate its dynamic localization by interacting with the AP-2 clathrin adapter complex [6,19,31,41,45].

β1-integrin and plasminogen activator inhibitor-1 have been implicated as candidate cargo proteins of TGN38 [24,43]. β1-integrin is an integral membrane protein that mediates cellular adhesion to the extracellular matrix (ECM) [3]. Its cytoplasmic domain indirectly binds the actin cytoskeleton and activates intracellular signaling pathways. Plasminogen activator inhibitor-1 is a secreted protein that functions as an inhibitor of the extracellular proteases urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) [2].

Because both β1-integrin and plasminogen activator inhibitor-1 have roles in modulating cell–ECM interactions, it is possible that TGN38, in its capacity as a cargo receptor, could play an important role in such processes [24]. This idea is supported by the observation that high levels of overexpressed TGN38 in cultured cells result in detachment of the cells from the substratum [26]. Dynamic modulation of cell–ECM interactions is thought to be important for both synapse formation and plasticity. In fact, both β1-integrin and tPA have been implicated in hippocampal long-term potentiation [5,27].

The localization of TGN38 in the dendritic plasma membrane and intracellular membranes of dendritic spines is consistent with its potential involvement in modulating cell–ECM interactions of synapses [32]. However, if TGN38 has such a role, it would also need to exhibit a dynamic localization on dendritic membranes. The dynamics of TGN38 in dendrites has not been addressed. In order to shed light on possible roles for TGN38 in neurons, we studied its dynamics in dendrites of hippocampal neurons. In particular, we addressed the hypothesis that TGN38 cycles rapidly between the plasma membrane and intracellular membranes of dendrites. Our results are consistent with the idea that TGN38 facilitates rapid changes in the protein composition of the postsynaptic plasma membrane.

2. Materials and methods

All experimental data sets include a minimum of 10 cells taken from at least two separate cultures. Images shown are representative examples of the respective data sets.

2.1. Cell culture

Hippocampal brain tissue from E18 rats was obtained from Brainbits in Hibernate/B27 media [7]. Cells were dissociated and plated at 20,000 cells/cm^2 on poly-D-lysine-coated glass-bottomed culture dishes (Mattek). Cells were cultured as described [8], except that they were fed only weekly.

2.2. DNA constructs

Plasmids encoding cyan fluorescent protein (CFP), green fluorescent protein (GFP), and yellow fluorescent protein (YFP) were obtained from Clontech. cDNA for rat TGN38 was generated via PCR amplification with Clontech's Marathon rat brain cDNA as template. CFP was inserted between amino acids 40 and 41 of TGN38 [17] using established techniques of molecular biology. Glycine residues were inserted at both the amino and carboxy termini of CFP to facilitate the proper folding of both CFP and TGN38. GFP-TGN38 was made exactly the same way, except that the GFP cDNA was used instead of that for CFP. The construct encoding the transferrin receptor-YFP fusion was made by excising the GFP coding sequence of the transferrin receptor-GFP plasmid [9] with Age I and BsrG I and replacing this sequence with the YFP coding sequence. Transferrin receptor-GFP cDNA was a kind gift of Dr. Gary Banker (Oregon Health Sciences University).

2.3. Transfections

Cultures were transfected with plasmid DNA using Lipofectamine 2000 (Life Technologies). For each transfection, 3 μl of Lipofectamine 2000 was added to 50 μl Neurobasal, and 2 μg plasmid DNA was added to a separate 50 μl Neurobasal. Both mixtures were incubated at room temperature for 5 min. The mixtures were then combined and incubated at room temperature for 20 min. Media on culture (~ 2 ml) was then replaced with Neurobasal/Lipofectamine 2000/DNA mixture, and cells were incubated at 35 °C for 10 min. Original culture media was then returned to the culture, and the culture was returned to the incubator. For cotransfections shown in Figs. 1 and 5, 1 μg of each plasmid was used; for the cotransfection shown in Fig. 4, 1 μg of EYFP and 2 μg of CFP-TGN38 were used. Neurons were transfected after 18–23 days in vitro. Cells were fixed or imaged live 14–16 h after transfection.

Cells cotransfected with CFP-TGN38 and YFP for Fig. 1 were fixed with 4% paraformaldehyde (PFA), 4% sucrose, in PBS at 4 °C for 20 min, and then permeabilized with

−20 °C methanol at −20 °C for 20 min before being imaged in Dulbecco's phosphate-buffered saline. Cells cotransfected with CFP-TGN38 and transferrin receptor-YFP (shown in Fig. 5) were fixed with −20 °C methanol at −20 °C for 20 min before being imaged in Dulbecco's phosphate-buffered saline. Cells that were imaged live (shown in Figs. 4 and 6) were rinsed once with 35 °C Dulbecco's phosphate-buffered saline, and then imaged in Dulbecco's phosphate-buffered saline (initially 35 °C) in a room warmed to 26–27 °C.

2.4. Immunofluorescence

For syntaxin 6/CFP-TGN38 double labeling, neurons expressing CFP-TGN38 were rinsed twice in Dulbecco's phosphate-buffered saline and then fixed with −20 °C methanol at −20 °C for 20 min. Cells were blocked with 10% goat serum, 2% BSA, in phosphate-buffered saline at room temperature for 1 h. Then they were incubated in anti-syntaxin 6 monoclonal antibody (StressGen) diluted 1:1000 in block at room temperature for 1 h. Cells were washed three times with block at room temperature and then incubated with AF488-conjugated goat anti-mouse IgG (Molecular Probes) diluted 1:1000 in block at room temperature for 1 h. Cells were again washed three times in block at room temperature, and then imaged in Dulbecco's phosphate-buffered saline.

For immunofluorescence of endogenous TGN38, untransfected hippocampal neurons (18–24 days in vitro) were fixed and processed the same way as described above for the anti-syntaxin 6 experiments, except that an anti-TGN38 monoclonal antibody (Transduction Labs) was used (diluted 1:1000) as the primary antibody. For antibody uptake/surface-labeling experiments, mouse monoclonal anti-GFP antibody (Boehringer Mannheim) was diluted 1:1000 in Dulbecco's phosphate-buffered saline and applied to cells at the temperature and for the duration indicated in the figure legends. Immediately following primary antibody incubations, cells were rinsed in ice-cold Dulbecco's phosphate-buffered saline and fixed with 4% paraformaldehyde, 4% sucrose, 1× PBS at 4 °C for 20 min. Then cells were again rinsed with Dulbecco's phosphate-buffered saline and permeabilized with −20 °C methanol at −20 °C for 20 min. After permeabilization, cells were blocked with 10% goat serum, 2% BSA, 1× PBS for 1 h at room temperature. Secondary antibody, AF488-conjugated goat anti-mouse IgG was then added to cells, diluted 1:1000 in block. Cells were incubated in secondary antibody at room temperature for 1 h. Finally, cells were washed in block three times for 10 min each. Prior to imaging cells, block was replaced with Dulbecco's phosphate-buffered saline.

For acid washes, cells were first rinsed three times with ice-cold Dulbecco's phosphate-buffered saline. Then the Dulbecco's phosphate-buffered saline was removed, and cells were incubated at 4 °C for 5 min after addition of ice-cold 0.2 M acetic acid, 0.5 M NaCl. Cells were then rinsed again, three times with ice-cold Dulbecco's phosphate-buffered saline prior to fixation.

2.5. Imaging

Both living and fixed cells were imaged in Dulbecco's phosphate-buffered saline. Cells were imaged with a Nikon Diaphot inverted fluorescence microscope. A Princeton Instruments cooled CCD digital camera (MicroMax) was used to record images. ImagePro version 4.0 imaging software was used to control image acquisition and for image analysis. Filters for imaging ECFP, EGFP, EYFP, and AF488 were purchased from Chroma. The "bleed-through" of CFP into the YFP-filter set and of YFP and AF488 into the CFP filter set was found to be negligible.

3. Results

In order to study the dynamics of TGN38 in dendrites of hippocampal neurons, we expressed a recombinant fusion protein of TGN38 with CFP (a mutant of GFP), CFP-TGN38. However, before studying the dynamics of CFP-TGN38, it was important to determine whether it was correctly localized when expressed in hippocampal neurons. We therefore began by examining the subcellular localiza-

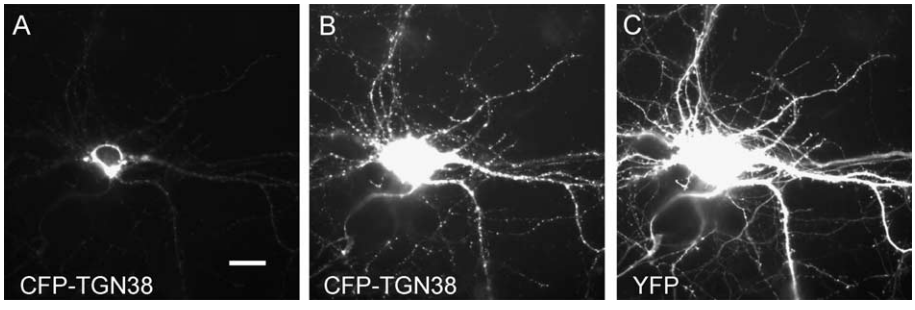

Fig. 1. Expression of CFP-TGN38 in hippocampal neuron. Cultured hippocampal neurons were cotransfected with CFP-TGN38 and YFP, fixed 14–16 h posttransfection and imaged in Dulbecco's phosphate-buffered saline. Hippocampal neuron coexpressing CFP-TGN38 and YFP is shown in panel A (CFP-TGN38 channel; contrast and brightness were adjusted to show detail in the cell body), panel B (CFP-TGN38 channel; contrast and brightness were adjusted to show detail in the dendrites), and panel C (YFP channel). Scale bar in panel A = 20 μm.

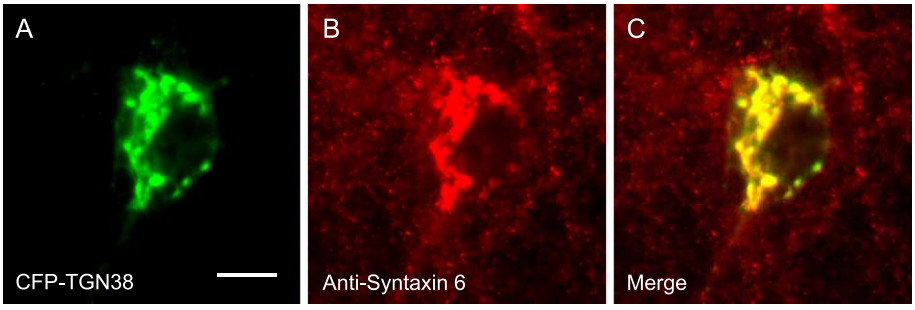

Fig. 2. CFP-TGN38 colocalizes with syntaxin 6 in hippocamal neurons. Cultured hippocampal neurons were transfected with CFP-TGN38, fixed 14–16 h posttransfection, and processed for immunofluorescence with an anti-syntaxin 6 antibody. Syntaxin 6 is a TGN resident protein. Immunofluorescence labeling for syntaxin 6 in a hippocampal neuron expressing CFP-TGN38 is shown in panel A (CFP-TGN38 channel), panel B (antisyntaxin 6 channel), and panel C (panels A and B, merged; colocalization indicated with yellow). Scale bar in panel A = 10 μm.

tion of CFP-TGN38. Because TGN38 is predominantly localized to the TGN, CFP-TGN38 should be most concentrated in the cell body in a perinuclear localization where the Golgi apparatus is concentrated in neurons.

Indeed, cultured rat hippocampal neurons expressing CFP-TGN38 (Fig. 1A,B) exhibited the strongest fluorescence in the cell bodies adjacent to the nucleus (Fig. 1A). A weaker, punctate fluorescence can be seen throughout the dendrites (Fig. 1B, which is the same image shown in Fig. 1A, but with the brightness/contrast adjusted to show the structures of weaker fluorescence). The anatomic extent of the transfected cells was visualized by coexpression of the spectrally distinct YFP, which effectively "filled" the cells (Fig. 1C). The large, perinuclear structures found in the cell bodies resemble the immunofluorescence patterns of endogenous TGN38 in neurons [42,25] (JOM, unpublished observations). A further indication that CFP-TGN38 is correctly localized to the Golgi apparatus is its colocalization with syntaxin 6, a distinct TGN resident protein, in neuronal cell bodies (Fig. 2).

Immunofluorescence of endogenous TGN38 in hippocampal neurons (Fig. 3) revealed a punctate pattern of the protein in dendrites of a subset of the neurons [42]. This pattern is comparable to that of CFP-TGN38 (Fig. 1), suggesting that the recombinant protein is correctly localized in dendrites. In addition to the punctate fluorescence seen in dendritic shafts, CFP-TGN38 was also found in a subset of dendritic spines (Fig. 4). That is, some (filled arrows, Fig. 4), but not other (hollow arrows, Fig. 4), spines exhibit both YFP and CFP-TGN38. Why CFP-TGN38 is present in some but not all dendritic spines remains uncertain.

Next, we sought to identify the dendritic organelles containing CFPTGN38. Because the Golgi apparatus has not been observed in dendrites to the same degree as we found CFP-TGN38, it seemed unlikely that many of the dendritic organelles expressing CFP-TGN38 corresponded to the Golgi apparatus. However, recycling endosomes have been observed in dendrites of hippocampal neurons with fluorescence microscopy [44], and chimeric forms of TGN38 have been found on recycling endosomes in nonneuronal cells [16]. We therefore visualized the early/recycling endosomes of cells expressing CFP-TGN38 by coexpressing the transferrin receptor fused to YFP [9]. In neurons expressing both proteins, the large dendritic CFP-TGN38 puncta also contained transferrin receptor-YFP (Fig. 5, solid arrows), consistent with the conclusion that these organelles are early/recycling endosomes. However, only a subset of the organelles expressing CFP-TGN38 can thus be identified as early/recycling endosomes as many of the smaller (< 1 μm in length) organelles containing CFP-TGN38 did not contain

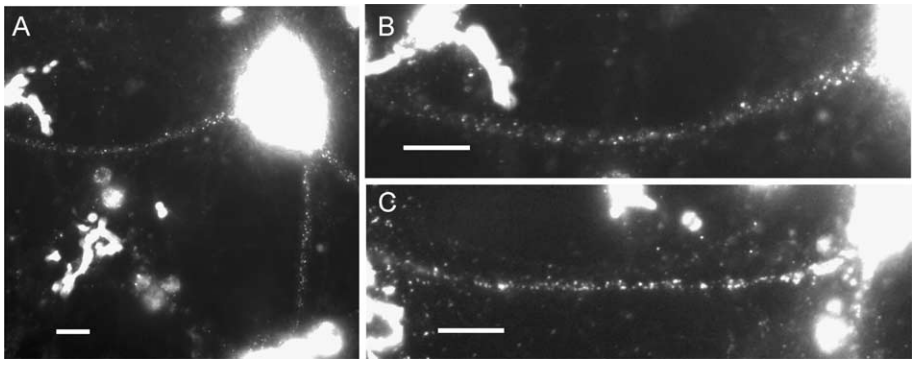

Fig. 3. Immunofluorescence of endogenous TGN38 in dendrites. Cultured hippocampal neurons were processed for immunofluorescence with an anti-TGN38 antibody. Note the punctate pattern in the dendrites of the neuron in the upper right hand corner of (A). One of the dendrites from this neuron is shown at a higher magnification in (B). A dendrite from another neuron is shown in (C). Scale bars = 10 μm.

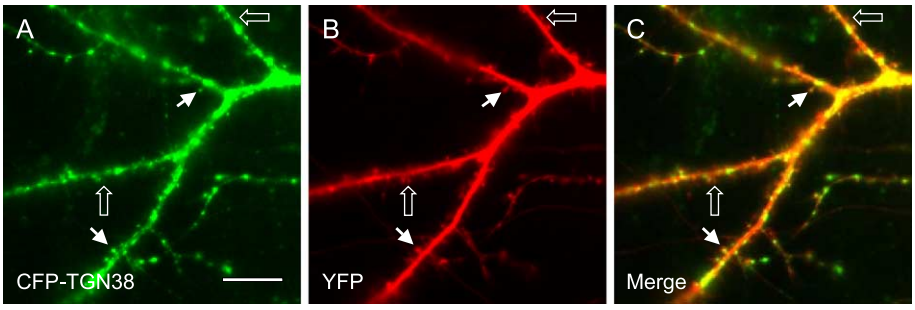

Fig. 4. Presence of CFP-TGN38 in a subset of dendritic spines. Cultured hippocampal neurons were cotransfected with CFP-TGN38 and YFP, fixed 14–16 h posttransfection and imaged in Dulbecco's phosphate-buffered saline. Hippocampal neuron coexpressing CFP-TGN38 and YFP is shown in panel A (CFP-TGN38 channel), panel B (YFP channel), and panel C (panels A and B merged; colocalization indicated with yellow). Note the presence (solid arrows) or absence (hollow arrows) of CFP-TGN38 in dendritic spines revealed by YFP expression. Scale bar in panel A = 10 μm.

transferrin receptor-YFP (Fig. 5, hollow arrows). These smaller organelles likely represent transport carriers.

Time-lapse imaging of living, transfected neurons revealed rapid movements of GFP-TGN38-containing transport carriers in dendrites (Fig. 6). These organelles correspond in size and relative fluorescence intensity to the smaller organelles shown in Fig. 5. In Fig. 6B, a "difference image" of two consecutive frames in a time-lapse series of a neuron expressing GFP-TGN38 provides a static representation of the dynamics. A difference image is generated by subtracting one image from another. It therefore represents the difference between the two original images, and in this case, it highlights the movements of fluorescent structures in the specimen that occurred in the time interval between the acquisition of the

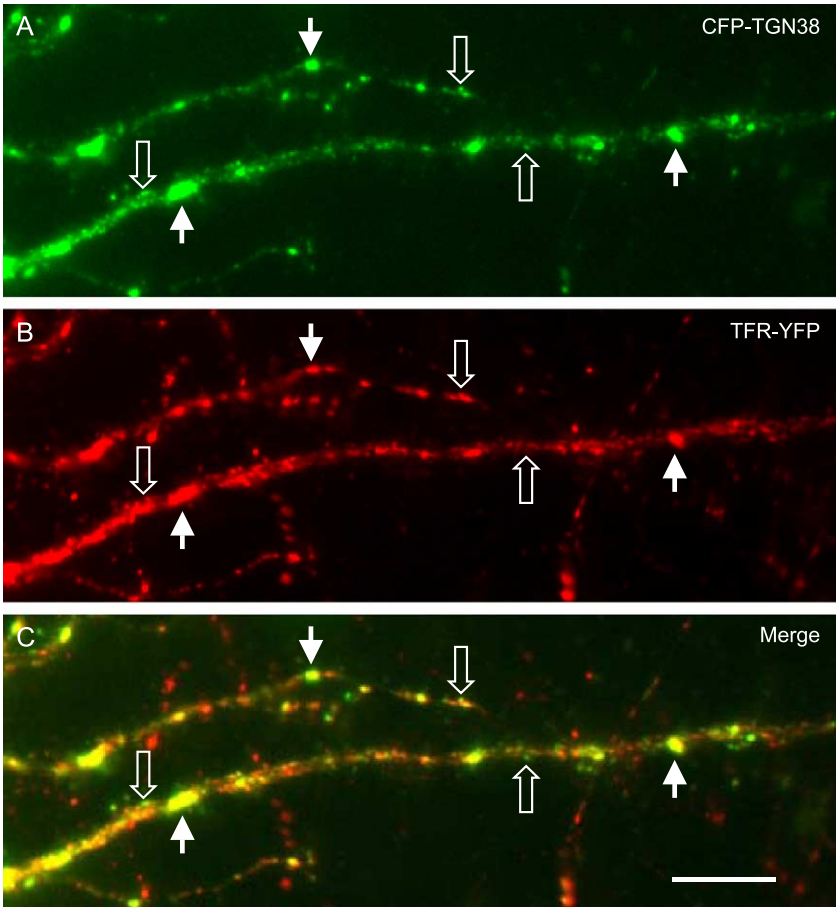

Fig. 5. Dendritic structures containing CFP-TGN38 include early/recycling endosomes. Cultured hippocampal neurons were cotransfected with CFP-TGN38 and transferrin receptor-YFP, fixed 14–16 h posttransfection and imaged in Dulbecco's phosphate-buffered saline. Hippocampal neuron coexpressing CFP-TGN38 and transferrin receptor-YFP is shown in panel A (CFP-TGN38 channel), panel B (transferrin receptor-YFP channel), and panel C (panels A and B merged; colocalization indicated with yellow). Note colocalization of the larger puncta indicated by solid arrows and the absence of transferrin receptor-YFP in smaller CFP-TGN38 containing structures, indicated with hollow arrows. Scale bar in panel C = 10 μm.

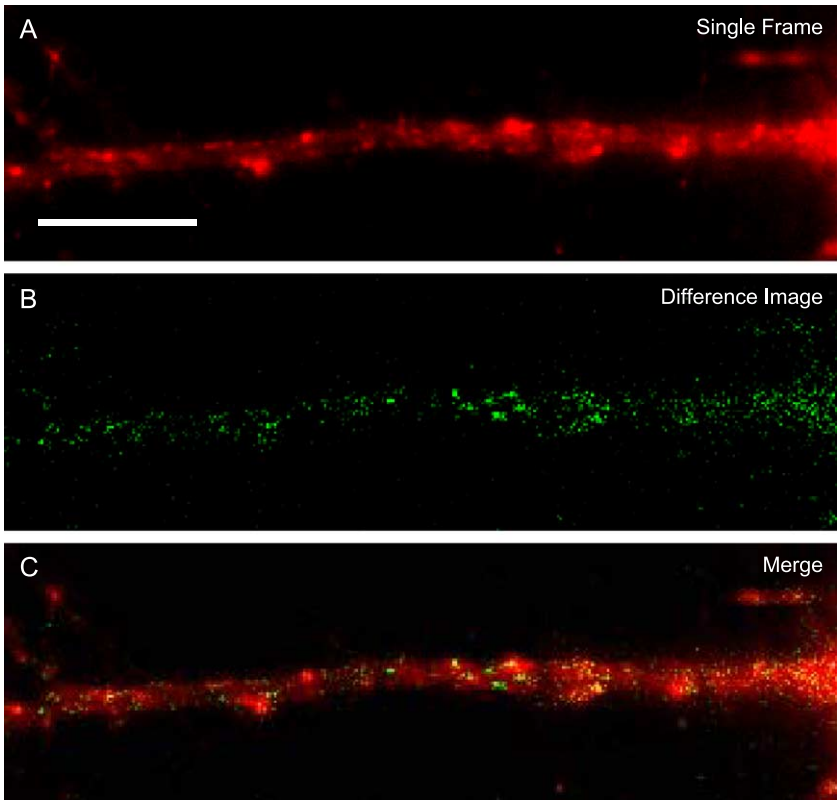

Fig. 6. Dynamics of GFP-TGN38 in dendrites. Cultured hippocampal neurons were transfected with GFP-TGN38 and imaged 14–16 h posttransfection in Dulbecco's phosphate-buffered saline. 50 frames were acquired of living neurons expressing GFP-TGN38 in 72-s time-lapse series. One of these frames is shown in (A). In (B), the frame in (A) was subtracted from that immediately following it to produce this difference image. These frames were acquired approximately 1.4 s apart. In (C), this difference image (in green) was merged with the image in (A) to illustrate the localization of the movements. The entire 72-s time-lapse series is played 10 times faster than real time in (D). Note the relative stability of the larger organelles (likely the early/recycling endosomes as shown in Fig. 3), some of which are indicated with arrows. Also note the rapid dynamics of the small, faint organelles. A 72-s time-lapse series of a different neuron is shown in (E). This movie also plays 10 times faster than real time. Scale bar in panel A = 10 μm.

two images. This difference image (green) is merged with the first of the two frames (red, Fig. 6A) in Fig. 6C. As the frames were acquired only 1.4 s apart, the difference image demonstrates substantial dynamics within a short time interval.

While these static images show robust dendritic transport of the protein on a fast time-scale, an online movie of this time-lapse series (Fig. 6D, http://www.duke.edu/~av8/vandongen_lab/Figure_6D.mov) provides information regarding the directionality and the sizes of the moving organelles. A movie of a different neuron expressing GFP-TGN38 is included in Fig. 6E (http://www.duke.edu/~av8/vandongen_lab/Figure_6E.mov) as an additional example. Fluorescent transport carriers moved in both directions in dendrites (Fig. 6D,E). The speeds of the fastest organelles were approximately 1 μm/s. Some of the transport carriers can be seen traveling many microns in the course of a 72-s time-lapse series, suggesting that some of these organelles move between the cell body and various points in the dendrites, perhaps carrying signals between the nucleus and synapses. In contrast to the fast dynamics of the transport carriers, the larger, brighter organelles (i.e., the early/recycling endosomes) tended to remain stationary (Fig. 6D, arrows).

Next, we investigated the possibility that CFP-TGN38 might cycle between the plasma membrane and intracellular membranes of dendrites in a manner similar to that described for TGN38 in nonneuronal cells. If this occurs, we would expect to find a subset of the CFP-TGN38 on the surface of the dendrites. We thought this would be the case because TGN38 has been observed on the surface of various nonneuronal cell types [6,23,35] and on the dendritic plasma membrane of hippocampal neurons [32].

To test for the surface expression of CFP-TGN38 on dendrites, living neurons expressing CFP-TGN38 were incubated at 4 °C for 30 min in the presence of an anti-GFP antibody (which also recognizes CFP). At 4 °C, membrane transport is inhibited so the antibody will not be internalized under these conditions. Because CFP is attached to the lumenal/extracellular side of TGN38, it should be accessible to the antibody if the protein resides in the plasma membrane. However, the antibody did not have access to intracellular pools of CFP-TGN38, because the cells were not permeabilized prior to incubation with the antibody.

Neurons "surface-labeled" with the anti-GFP antibody were then fixed, permeabilized, and incubated with an AF488-labeled secondary antibody. A strong immunofluo-

rescence signal was observed on the dendrites of transfected cells (Fig. 7A′ ,A″). Dendritic spines were also labeled (Fig. 7A″ , arrows). Nontransfected cells did not exhibit fluorescence in the AF488 channel, indicating that the antibody was specific for CFP. Furthermore, antibody incubation followed by an acid wash prior to fixation (to remove surface-bound antibodies) reduced the antibody signal almost to background levels (Fig. 7B,B′), indicating that the antibody was bound to the exterior of the cells.

To test whether TGN38 cycles between the cell surface and intracellular membranes of dendrites, we used an antibody uptake assay. In such an assay, living cells are incubated in the presence of a primary antibody, followed by an acid wash to remove any antibodies remaining on the cell surface. The cells are then fixed, permeabilized, and incubated with a secondary antibody. If the primary antibody binds a protein on the cell surface that is subsequently endocytosed, the antibody will also be endocytosed and will not be removed with the acid wash.

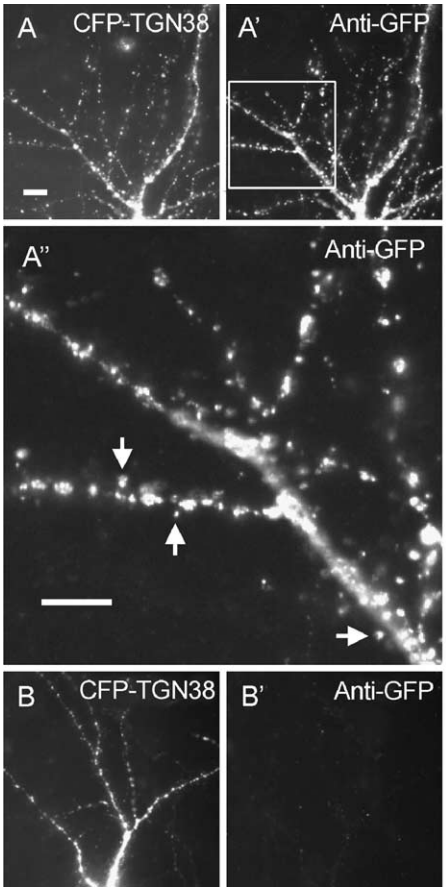

Fig. 7. CFP-TGN38 is expressed on the dendritic surface. Cultured hippocampal neurons were transfected with CFP-TGN38. Posttransfection (14–16 h), cells were incubated at 4 °C for 30 min in Dulbecco's phosphate-buffered saline plus anti-GFP antibody, and then fixed and permeabilized (A–A′) or subjected to an acid wash (to remove surface-bound antibodies) and then fixed and permeabilized (B and B′). Cells were then processed for immunofluorescence with an AF488-conjugated secondary antibody. (A) and (B) show the CFP-TGN38 channel while (A′), [A″ (inset of A′)], and (B′) show the anti-GFP (AF488) channel. Scale bars are 10 μm. Exposure times, contrast and brightness of (A′) and (B′) are equal. Note antibody-labeled dendritic spines indicated with arrows in (A″).

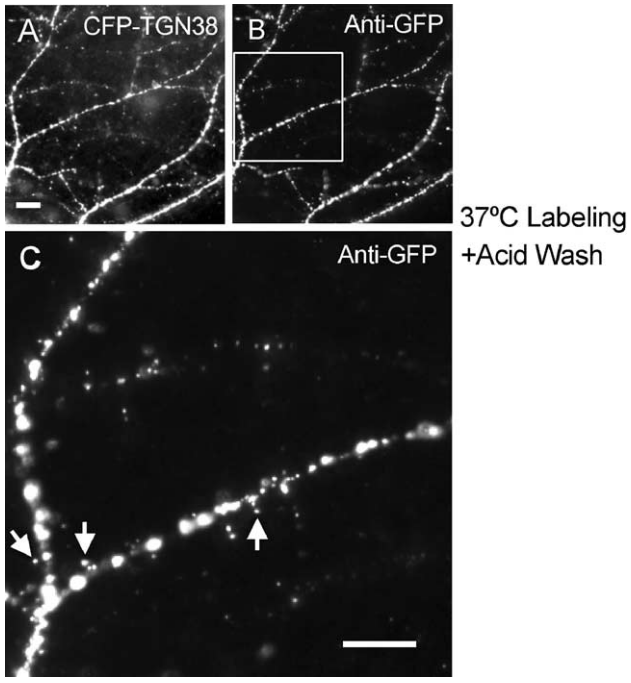

Fig. 8. CFP-TGN38 cycles rapidly from the dendritic plasma membrane to intracellular membranes in the dendritic shaft and spines. Cultured hippocampal neurons were transfected with CFP-TGN38. Posttransfection (14–16 h), the cells were incubated at 35 °C for 6 min in Dulbecco's phosphate-buffered saline plus anti-GFP antibody. The cells were then subjected to an acid wash, fixed, and permeabilized. Finally, the cells were processed for immunofluorescence with an AF488-conjugated secondary antibody prior to imaging. In (A), the CFP-TGN38 channel of a representative neuron is shown. In (B), the anti-GFP (AF488) channel is shown, and in (C), the inset of (B) is shown enlarged. Note in (C), the signal in dendritic spines (arrows). Scale bars = 10 μm.

When transfected cells were incubated with the anti-GFP antibody at 35 °C for 6 min, followed by processing as described above (see Materials and methods for a detailed description), a strong, punctate fluorescence was observed throughout the dendrites in the AF488 (antibody) channel (Fig. 8). The punctate immunofluorescence signal was due to internalization of the primary antibody as it was not eliminated by the acid wash. Acid washes of surface-labeled neurons (shown in Fig. 7B,B′) were performed side-by-side with these procedures to confirm the effectiveness of the acid wash. The punctate pattern was not produced in neurons when the antibody was applied in the presence of 0.45 M sucrose (Fig. 9), a condition known to inhibit clathrin-mediated, but not fluid-phase, endocytosis [18]. Antibody uptake was dependent upon interaction with CFP because nontransfected cells were not labeled. Strong labeling of dendritic spines could also be seen (Fig. 8C, arrows). These puncta remained despite the acid wash, indicating that recently endocytosed CFP-TGN38 had accumulated on intracellular membranes within spines. Comparing the anti-GFP pattern (Fig. 8B) with the CFP-TGN38

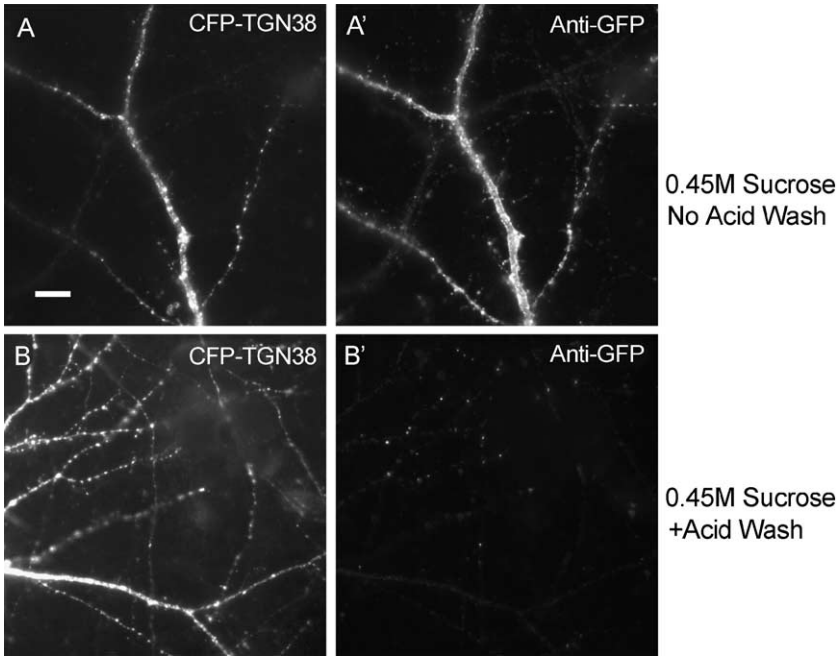

Fig. 9. Hypertonic media inhibits the endocytosis of CFP-TGN38. Cultured hippocampal neurons were transfected with CFP-TGN38. Posttransfection (14–16 h), the cells were incubated at 35 °C for 10 min in Dulbecco's phosphate-buffered saline supplemented with 0.45 M sucrose. Anti-GFP antibody was then added, and the cells were incubated at 35 °C for an additional 6 min. Then the cells were either fixed and permeabilized [(A) CFP-TGN38 channel; and (A′) anti-GFP (AF488) channel] or subjected to an acid wash and then fixed and permeabilized [(B) CFP-TGN38 channel; and (B′) anti-GFP (AF488) channel]. Exposure times, contrast and brightness of (A′) and (B′) are equal. Scale bar = 10 μm.

pattern (Fig. 8A), it is clear that most of the CFP-TGN38-containing organelles were labeled with the antibody after only 6 min. Together, these data demonstrate that CFP-TGN38 is endocytosed rapidly from the cell surface of neurons. Furthermore, the fact that the protein is found on the dendritic surface and that even short antibody incubation times produce a strong intracellular immunofluorescence signal in the dendrites suggests that endocytosis is occurring within the dendritic compartment, possibly from synaptic membranes.

4. Discussion

We have studied the dendritic transport of TGN38, a putative cargo receptor implicated in modulating cell–ECM interactions. Time-lapse imaging of dendrites expressing GFP-TGN38 revealed fast, bidirectional trafficking of transport carriers containing the protein. In contrast, the early/recycling endosomes, on which GFP-TGN38 was predominantly localized in dendrites, were relatively immobile. GFP-TGN38 was found on the surface of dendrites as well as dendritic spines. Rapid endocytosis of GFP-TGN38 was observed within dendrites, and the endocytosed protein was found in a punctate pattern in dendritic shafts as well as dendritic spines. The localization and dynamics of GFP-TGN38 in dendrites imply a role for TGN38 in rapidly shuttling cargo proteins into or out of postsynaptic membranes. Because such processes are known to be important for synaptic plasticity, future study of the role of TGN38 in synaptic plasticity models such as long-term potentiation (LTP) and long-term depression (LTD) would be logical extensions of this work. In addition, by revealing dendritic transport processes of a protein used as a model trafficking receptor in nonneuronal cells, this study provides important contextual information for understanding such transport events as neurotransmitter receptor shuttling, which may be of primary importance for synaptic plasticity.

Because we have employed a recombinant protein, GFP-TGN38, to infer information about the transport of its endogenous counterpart, TGN38, it is important that the localization and behavior of the recombinant protein reflect that of the endogenous protein. There are multiple indications that this is the case. Like TGN38, GFP-TGN38 was most concentrated in the cell bodies of the neurons where it colocalized with syntaxin 6, indicating that it was correctly localized to the Golgi apparatus. The punctate pattern of GFP-TGN38 in dendrites also resembles the immunofluorescence pattern of its endogenous counterpart. In dendrites, GFP-TGN38 was found on the plasma membrane, large (≥ 1 um, i.e., early/recycling endosomes) and small (< 1 um, i.e., transport carriers) organelles, and on intracellular membranes within dendritic spines. Likewise, with electron microscopy and immunogold labeling of TGN38 in distal dendrites of rat hippocampus, Pierce et al. [32] found TGN38 immunoreactivity on the plasma membrane, large and small membrane-bound compartments as well as intracellular membranes within dendritic spines. The localization

of GFPTGN38 on only a subset of dendritic spines and on a set of transport carriers distinct from that of transferrin receptor-YFP are indications that the protein was not simply flooding the secretory pathway as a result of overexpression, but rather that it was specifically localized to particular membranes by virtue of its structure. In contrast, when GFP-TGN38 was expressed in hippocampal neurons at substantially higher levels than those used in this study, the protein became concentrated on the plasma membrane, perhaps reflecting a saturation of certain transport machinery (JOM, unpublished observations). In addition to the localization of the recombinant protein reflecting that of the endogenous protein, GFP-TGN38 undergoes clathrin-mediated endocytosis and thus behaves like TGN38. Altogether, these data indicate that GFP-TGN38 is localizing and behaving like endogenous TGN38 in the conditions of the experiments described here.

TGN38 is thought to be a cargo receptor, binding secretory, or membrane proteins in the TGN with its lumenal domain and mediating their transport to the plasma membrane with its cytoplasmic domain [4,39]. This model is based on findings that TGN38 interacts with membrane budding machinery on both the plasma membrane and the TGN, as well as the observation that it cycles between the TGN and the plasma membrane [20,21,23,31,34]. More recently, β1-integrin and plasminogen activator inhibitor-1 have been identified as candidate cargo proteins of TGN38 [24,43].

β1-integrin was found to interact with the lumenal domain of TGN38 [43]. Expression of a TGN38 mutant, which mislocalizes but retains its ability to bind β1-integrin, caused a parallel mislocalization of β1 integrin [43]. Integrins are integral membrane proteins known to function as heterodimeric receptors for cell surface and extracellular matrix proteins. Each integrin is made up of one α subunit paired with a β subunit (see Ref. [3] for review). Of the 24 known integrin subunits, three, α8, β1, and β3 have been examined at the ultrastructural level in the hippocampus, and immunoreactivity for each was found on postsynaptic densities of spine synapses [14,30,37]. Interestingly, in all three cases, only a subset of the spines (and a subset of the postsynaptic densities) was labeled. Whether the population of spines expressing β1-integrin corresponds to those we have found to express TGN38 remains to be seen. In any case, the possibility that TGN38 is responsible for shuttling β1-integrin into and out of synaptic membranes is apparent. Because integrins have been implicated in hippocampal LTP, [27] this is a scenario that warrants further study.

Overexpression of TGN38 in normal rat kidney (NRK) cells was found to increase the secretion of plasminogen activator inhibitor-1 [24]. Because plasminogen activator inhibitor-1 can mediate cell detachment from the ECM, the idea that it is a cargo protein of TGN38 could explain the observation that overexpression of TGN38 results in cell detachment from the substratum [11,13,26,24]. Plasminogen activator inhibitor-1 is the physiological inhibitor of the extracellular serine protease, tissue plasminogen activator (tPA). The mRNA for tPA is induced as an immediate-early gene in the hippocampus in response to LTP-evoking stimuli [33]. tPA has been implicated in the late phase of hippocampal LTP [5]. Perhaps of relevance to its role in LTP are the observations that tPA is released from cortical neurons in response to membrane depolarization and that it cleaves the NR1 subunit of the *N*-methyl-D-aspartate (NMDA) receptor, apparently resulting in an increase in NMDA-triggered calcium signals [29]. If TGN38 is involved in the transport of plasminogen activator inhibitor-1 to the cell surface, the rapid cycling of TGN38 within dendritic spines would suggest that TGN38 might have an important role in regulating the activity of tPA at synapses. With this in mind, it would be interesting to know whether overexpression of TGN38 in neurons might inhibit the cleavage of NR1, and thereby attenuate changes in synaptic strength.

In addition to binding β1-integrin and proteins involved in membrane budding, TGN38 also interacts with two closely related proteins, neurabin I and II [40]. Found in dendritic spines, the neurabins are actin-binding proteins with PDZ and coiled-coil domains [1,28,36]. The significance of their interaction with TGN38 is not yet clear. However, the fact that GFP-TGN38 was found on intracellular membranes within dendritic spines supports a hypothesis of Stephens and Banting [40] regarding the role of the TGN38–neurabin interaction. The authors suggested that the F-actin-binding protein neurabin may interact with TGN38 in order to link TGN38-containing membranes to the actin cytoskeleton; but they found no colocalization of neurabin with TGN38 in PC12 cells. Because neurabin is known to be concentrated in dendritic spines, the proteins likely do colocalize in these specializations of neurons. As spines are rich in F-actin [15] and are not thought to contain microtubules in their cytoplasm [10], the interaction of TGN38 with neurabin could provide a means of anchoring intracellular membranes within spines, a step that may be essential for protein transport to and from spine synapses.

Time-lapse imaging of GFP-TGN38 in dendrites revealed long-distance transport of this protein. Because the transport organelles moved bidirectionally (either towards or away from the cell body), it is plausible that some of these organelles move between the cell body and various points in the dendrites. If this is the case, then these organelles could mediate communication between synapses and the nucleus. They also may be involved in the delivery of proteins synthesized in the cell body to particular synapses. Despite the substantial internalization of GFP-TGN38 observed with the antibody uptake assay, dynamic events corresponding to endocytosis and subsequent fusion of fluorescent organelles were not observed with the time-lapse experiments. This is likely a result of our inability to detect the organelles mediating these processes due to the small number of TGN38 proteins residing on them.

It is important to note that the transport of GFP-TGN38 in neurons reported here was observed without manipu-

lating synaptic activity. There is thus a robust basal level of TGN38 dynamics in dendrites. If TGN38 is involved in synaptic plasticity, one might expect to see a change in its dynamics in response to altered synaptic activity. However, we have not found gross changes in the dynamics of GFP-TGN38 in response to removal of calcium from the media, application of the AMPA receptor antagonist CNQX, or manipulations designed to increase synaptic activity such as removal of magnesium or application of 30 mM potassium chloride (JOM and JCG, unpublished observations). Of course, there may be subtler, activity-dependent changes in TGN38 dynamics that we were not able to detect. Another possibility is that synaptic activity could regulate the interactions of TGN38 with its cargo proteins.

The rapid dynamics of GFP-TGN38 in dendrites raises a number of questions regarding the role of TGN38 in synaptic structure and plasticity. For instance, would TGN38 overexpression or depletion (in the postsynaptic cell) have an effect on LTP or LTD? Also, what is the precise role that TGN38 plays in neurons regarding its cargo proteins? Does TGN38 transport cargo to or from synaptic membranes as the dynamics would suggest? It is also not yet clear exactly which proteins are truly cargo for TGN38. As for plasminogen activator inhibitor-1, additional study is needed to determine if it interacts with TGN38, as one would expect if it were a cargo for TGN38. In addition, while the data implicating β1-integrin and plasminogen activator inhibitor-1 as cargo of TGN38 have been obtained from nonneuronal cells, it is important to know whether the same scenarios exist in neurons. Finally, it is possible that there are additional, as yet, unidentified cargo proteins for TGN38. Identifying any additional cargo proteins might be critical to understanding the cellular role of TGN38.

Acknowledgements

This work was supported by grant NS31557 from the National Institutes of Neurological Disorders and Stroke to A.M.J.V.D.

The authors thank Dr. James O. McNamara Sr. for critical feedback on this manuscript, and Dr. Gary Banker for kindly providing the plasmid encoding Transferrin Receptor-GFP.

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