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Dominant-Negative Myosin Va Impairs Retrograde but Not Anterograde Axonal Transport of Large Dense Core Vesicles

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Abstract

Axonal transport of peptide and hormone-containing large dense core vesicles (LDCVs) is known to be a microtubule-dependent process. Here, we suggest a role for the actin-based motor protein myosin Va specifically in retrograde axonal transport of LDCVs. Using live-cell imaging of transfected hippocampal neurons grown in culture, we measured the speed, transport direction, and the number of LDCVs that were labeled with ectopically expressed neuropeptide Y fused to EGFP. Upon expression of a dominant-negative tail construct of myosin Va, a general reduction of movement in both dendrites and axons was observed. In axons, it was particularly interesting that the retrograde speed of LDCVs was significantly impaired, although anterograde transport remained unchanged. Moreover, particles labeled with the dominant-negative construct often moved in the retrograde direction but rarely in the anterograde direction. We suggest a model where myosin Va acts as an actin-dependent vesicle motor that facilitates retrograde axonal transport.

Keywords

Axonal transport; Hippocampal neurons; Large dense core vesicles; Myosin Va; Neuropeptide Y

Introduction

Large dense core vesicles (LDCVs) are intracellular organelles that store, transport, and release neuropeptides and hormones. They are found in nerve cells and neuroendocrine cells (here termed secretory granules, SGs) and release their content through regulated secretion. They contain compounds such as brain-derived growth factor, enkephalin or neuropeptide Y (Burgess and Kelly 1987). The SGs of neuroendocrine cells have been studied intensively

due to their abundance in these cells (Huttner et al. 1995; Glombik and Gerdes 2000; Kim et al. 2006). Key features include the biogenesis at the trans-Golgi network (Tooze and Huttner 1990), a complex maturation process (Tooze 1998), microtubule-dependent transport to the cell periphery, immobilization in the actin cortex (Rudolf et al. 2001), and stimulus-induced fusion with the plasma membrane.

Secretory granules of neuroendocrine cells, like melanosomes in melanocytes, are associated with the actin-based motor protein myosin Va (Wu et al. 1997; Rose et al. 2003; Rudolf et al. 2003). Interaction of myosin Va with this type of organelle is involved in active actin-based transport (Wu et al. 1998), the capturing and anchoring in the actin-rich cell cortex (Wu et al. 1998; Rudolf et al. 2003), or the docking at the plasma membrane prior to exocytosis (Varadi et al. 2005; Eichler et al. 2006; Desnos et al. 2007). Myosin Va is highly expressed in the brain (Mercer et al. 1991), and the lack of myosin Va causes the human disease *Griscelli syndrome*, which is characterized by hypopigmentation and seizures leading to premature death (Pastural et al. 1997), thus demonstrating the necessity for myosin Va in the nervous system. It is involved in the transport of various organelles including ER-derived vesicles from squid axoplasm (Tabb et al. 1998; Brown et al. 2004), synaptic vesicles in superior cervical ganglion neurons (Bridgman 1999), tetanus-toxin-containing endosomes in motor neurons (Lalli et al. 2003), neurofilaments in dorsal root ganglion cells (Alami et al. 2009), mRNA-containing particles in mouse hippocampal neurons (Yoshimura et al. 2006), and AMPA receptor-containing vesicles in rat hippocampal neurons (Correia et al. 2008).

We have recently shown that myosin Va regulates the exocytosis of LDCVs in cultured nerve cells (Bittins et al. 2009), but its influence on the neuronal transport of these vesicles has not been addressed. It is known that long-range LDCV transport is dependent on microtubules in neurons (de Wit et al. 2006), neuroendocrine cells (Rudolf et al. 2001; Varadi et al. 2005), and melanocytes (Wu et al. 1998). The vesicles are localized to synapses where they undergo regulated secretion (Wu et al. 2004; Brigadski et al. 2005; Lochner et al. 2006). Here, we demonstrate that the motor protein myosin Va participates in the transport of LDCVs in axons and dendrites. In axons, the expression of a dominant-negative myosin Va-tail construct caused a significant reduction in the speed of retrogradely transported LDCVs. Interestingly, anterograde vesicle speed was unaffected. We propose a mechanism where myosin Va selectively binds to retrogradely moving vesicles and contributes to regulate their transport.

Methods

Cells and Transfection

Primary hippocampal neurons from fetal E18 Wistar rat were prepared as described (Goslin and Banker 1998) and plated on 4-chamber LabTek chambers (Nunc) or 1 cm glass coverslips coated with poly-L-ornithin and laminin at 12,000 cells/cm². Culture medium: N-MEM (Gibco) with B27 neurobasal supplement (Gibco) and PSN antibiotic mix (Gibco) conditioned on astrocyte cultures for 3 days. At 9–15 days in vitro, neurons were transfected using magnet-assisted transfection (MATra; IBA, Göttingen, Germany) or lipofectamine 2000 transfection (Invitrogen). MATra generally yielded lower transfection efficiency, which was sometimes preferred for live-cell imaging when the traced neurites should not intermingle with neurites from transfected neighboring cells.

DNA

Neuropeptide Y (NPY) cDNA fused to EGFP or mRFP cDNA in a standard Clontech vector was provided by Wolfhard Almers, Vollum Institute, Portland, OR, USA. Myosin Va-tail-

mGFP and myosin Va-tail-mCherry were constructed as described elsewhere for MCLT-FLAG (Wu et al. 1998) except that the brain myosin Va cDNA was used as the starting material instead of the melanocyte myosin Va cDNA, and the resulting *Eco*R1 fragment was cloned into *Eco*R1-cut mGFP-C1 vector (Clontech) or mCherry-C1 vector (Clontech), respectively. Empty cloning vectors encoding EGFP, mRFP, or mCherry were used as transfection controls. Plasmids were amplified in *E. coli* strain DH5alpha and purified with Marligen Biosciences Maxi DNA purification kits.

Immunocytochemistry

Cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, treated with 50 mM NH_4Cl /PBS for 20 min, permeabilized with 0.2% Triton X-100/PBS for 5 min, and blocked with 0.2% gelatine/PBS for 10–30 min. Primary and secondary antibodies were diluted with 0.2% gelatine/PBS and successively applied for 30–60 min at room temperature. Cells were washed with PBS and mounted in Moviol. Primary antibody: rabbit anti-chicken myosin Va C-terminus, Sigma LF12, 1:200. Secondary antibody: goat anti-rabbit IgG coupled to Alexa Fluor 647, Invitrogen, Molecular Probes A21245, 1:1,000.

Imaging

Transfected cells were imaged 20–30 h after transfection on a Zeiss Axiovert M200 microscope equipped with a Till Photonics Polychrome V monochromator, a sensicam imago QE CCD camera (PCO), Till Vision 4.0 software, a heating chamber, and a humidified CO_2 -perfusion chamber. For live-cell imaging, the culture medium was replaced with imaging medium (NMEM without phenol red, containing Gibco B27 supplement and PSN antibiotic mix). For analysis of vesicle dynamics, time-lapse recordings were made using a 40 $\times$ objective (Zeiss EL Plan Neofluar), a triple band filter set (FITC/Cy3/Cy5, Chroma F-61005) at 488 and 555 nm excitation wavelength, 200–500 ms exposure for each wavelength, and 1 frame per second for 100 s. All dual- or triple-color experiments were accompanied by singlecolor control conditions and thorough observation to eliminate cross talk. Confocal imaging of fixed cells was performed with a Leica TCS SP5 microscope.

Image Analysis

Image processing and analysis was performed using Image J version 1.38t (Rasband WS, Image J, US National Institutes of Health), Imaris versions 4.1.3 and 5.7.1 (Bitplane AG, Zurich, Switzerland), and Adobe Photoshop CS. Colocalization of NPY-labeled organelles with antibody-labeled endogenous myosin Va in neurites was determined using Image J. The dual-color wide field images were subjected to a median filter to enhance the punctate signals. A line was laid over the neurites, and an intensity profile of the red and green signals was generated (see Fig. 3b2). A line profile along an empty region adjacent to the neurite was used to subtract the background fluorescence. Peaks above background in the profile of each color were marked by hand, and peaks that were double marked were considered colocalizing organelles.

Time-lapse recordings were analyzed using the kymograph plug-in for Image J written by Jens Rietdorf and Arne Seitz. Axons were identified morphologically by their length and uniformly by thin diameter. Axonal vesicle movement was measured starting at 40 μm distance from the soma and until the end of the image, including all clearly traceable branches. The most proximal 30 μm of the axon was considered axon initial segment (AIS) and was evaluated separately. Dendritic vesicle movement was measured for the entire length of the dendrites, usually about 30 μm at this stage of development. Two dendrites per cell were counted on average. For the comparison of LDCV traffic between myosin Va-tail transfected cells and control cells, analysis was done blinded to the protocol. Definitions of vesicle movement: Anterograde/retrograde (also referred to as unidirectional): movement

that is clearly biased toward one direction, and the travelled distance is longer than 5 μm during the imaging period of 100 s. Occasional jumps to the opposite direction may occur. Bidirectional: any kind of movement that is neither unidirectional nor immobile. Stationary/immobile: no detectable movement during the imaging period.

Results

LDCV Traffic in Cultured Neurons

We expressed neuropeptide Y (NPY) fused to EGFP or mRFP in cultured hippocampal neurons to label LDCVs. Cells were analyzed by light microscopy 24 h after transfection. At this stage, brightly labeled vesicles were enriched in the cell soma, specifically in the perinuclear region where the Golgi apparatus is located (Fig. 1b, asterisk). Both dendrites and axons contained vesicles (Fig. 1a, d), which occasionally accumulated in tips of the neurites (Fig. 1c). This distribution is similar to that observed for the endogenous granule marker secretogranin II in cultured neurons (Bittins et al. 2009). We subsequently analyzed the movement of the NPY-labeled LDCVs and compared the two main compartments axon and dendrites. Live-cell imaging was performed with cultured neurons transfected with NPY-mRFP and pEGFP as a cytoplasmic marker for 24 h. A time resolution of 1 Hz allowed us to follow the fast movement of all labeled vesicles in dendrites and axons. With the use of kymographs (a projection of one-dimensional movement on a time axis, Fig. 2a1, a2), moving particles were identified and classified into four categories, namely retrograde (toward the cell body), anterograde (away from the cell body), bidirectional (moving back and forth), and stationary. Example traces of vesicles for each category are shown in Fig. 2b.

Dendritic LDCVs Move Rapidly in Both Directions

In dendrites, it was obvious that most vesicles move bidirectionally and change the direction frequently. This was readily observed by watching the time-lapse recordings, and it became even more apparent on the kymographs that are dominated by zigzag lines caused by particles that reverse their movement during the 100 s observation period (Fig. 2a1, c). In addition, some vesicles were found to move unperturbed in either the retrograde or the anterograde direction, and a substantial amount of immobile vesicles was present (Fig. 2c).

Vesicle movement in the axon initial segments (AIS) was extremely variable between individual cells. This may be a result of arbitrarily defining the AIS as the first 30 μm of the axon. The true length was not determined due to the lack of a live marker for this compartment and may vary strongly between cells. Thus, our measurements likely contain a mixture of axon proper and axon initial segment. The numbers obtained from this region are, however, reminiscent of dendritic vesicle movement, rather than axonal movement, as judged from the relatively high amount of bidirectional traffic (Fig. 2d).

Directed Axonal LDCV Movement: Anterograde Transport is Faster than Retrograde Transport

In the axons, NPY-EGFP-labeled particles were counted starting at 40 μm distance from the soma and until the end of the field of view. Vesicles moved rapidly in both directions, like they did in dendrites, but in axons the transport was much more directed than in dendrites: many vesicles maintained their transport direction—anterograde or retrograde, respectively—during the observation time, and there was no apparent preference for either direction (Fig. 2e). To characterize the dynamics of LDCV movement, the speed of the NPY-mRFP-labeled LDCVs in the axons was measured. We traced all labeled vesicles for the entire imaging time or as long as they remained within the field of view. Axonal vesicle speed often changed during the imaging period, and it is of note that we hardly observed what might be considered “pause times” or “stop-and-go” movement for other organelles (Cui et

al. 2007; Roy et al. 2007). Here, LDCVs rarely came to a complete stop, so that we chose to include the periods of slower movement in the measurements. We found that anterograde axonal transport was significantly faster than retrograde transport (1.14 and 0.75 $\mu\text{m/s}$, respectively, Fig. 2f). Frequency histograms of the mean velocities show a wide distribution, where the fastest anterograde vesicle moved at 3.75 $\mu\text{m/s}$, and the fastest retrograde vesicle moved at 2.75 $\mu\text{m/s}$ (Fig. 2g). These values are in agreement with the reported range for vesicle transport and indicative of fast microtubule-dependent transport (Hirokawa and Takemura 2005).

Myosin Va-Tail Expression Alters Vesicle Dynamics in Dendrites

It is well documented that the actin-based motor protein myosin Va is involved in the movement of dense core vesicles in melanocytes (Wu et al. 1997, 1998, 2002) and secretory cells (Rudolf et al. 2003). In cultured hippocampal neurons, myosin Va is distributed widely in a punctate fashion along the axons and dendrites (Fig. 3a). When neurons were transfected with the LDCV marker NPY-EGFP, endogenous myosin Va colocalized with $67.7 \pm 2.8\%$ of LDCVs (mean $\pm$ SEM, $n = 468$ LDCVs in five cells; Fig. 3b1, b2), with no apparent difference between axons and dendrites. We next tested whether myosin Va was involved in the transport of LDCVs in neurons by expressing a dominant-negative tail of myosin Va (myosin Va-tail). This tail contains the cargo-binding C-terminus and the coiled-coil region, but lacks the motor domain and the neck region. The construct used in this work represents the tail domain of the brain splice variant. It includes the three amino acid exon B that is necessary to bind to the regulatory light chain Dlc8 (Hodi et al. 2006; Wagner et al. 2006). This exon is absent in other isoforms of myosin Va and may account for some cell-type-specific differences. Brain myosin Va lacks exons D and F found in the melanocyte splice form (Seperack et al. 1995).

For live-cell imaging, myosin Va-tail fused to mGFP was expressed in cultured hippocampal neurons. The subcellular localization of myosin Va-tail-mGFP was reminiscent of the distribution of the endogenous protein, as seen by immunofluorescence (Fig. 4a, compare with Fig. 3a). The protein was enriched in the perinuclear region and produced punctate staining in the soma (not shown), axons, and dendrites (Fig. 4a). Coexpression of myosin Va-tail-EGFP with NPY-mRFP showed colocalization in all compartments, including small punctate structures in dendrites and axons (Fig. 4a).

The most noticeable effect of myosin Va-tail expression was the elevated number of LDCVs in axons (Fig. 4c). In the AIS and dendrites, the vesicle number was not significantly higher than before (Fig. 4b). Also, the dynamics of NPY-labeled LDCVs were altered in cells expressing myosin Va-tail. In dendrites, the LDCVs became more stationary, and both retrograde and anterograde transport were slightly reduced compared to mock-transfected cells (Fig. 4b). As the number of dendritic vesicles in total did not change significantly, this suggests that myosin Va-tail inhibits retrograde and anterograde traffic and renders dendritic LDCVs immobile. In the AIS, vesicle movement was again extremely variable between cells such that no significant changes could be measured (not shown).

Myosin Va-Tail Reduces Retrograde LDCV Speed in the Axon and is Cotransported with Retrograde LDCVs

Upon expression of myosin Va-tail, more vesicles were located in the axon than under control conditions. This increase was caused by a massive increase of stationary vesicles, while the numbers of retrograde, anterograde, and bidirectional vesicles were unchanged (Fig. 4c). A second effect on axonal LDCV traffic was the finding that unidirectional transport was impaired, not in numbers but in velocity, when myosin Va-tail was present. Strikingly, this impairment only affected the retrograde flux of LDCVs, but not anterograde

movement. Myosin Va-tail expression resulted in a reduced mean velocity (Fig. 4d) that corresponded to a downward shift in the frequency distribution of retrograde vesicle movement (Fig. 4e). The frequency histogram demonstrates that fast retrograde vesicle movement still occurred when myosin Va-tail was expressed, but that there were much more slow vesicles when compared to control conditions (Fig. 4e). It is important to point out that while retrograde transport was slower during expression of myosin Va-tail, the total number of retrograde vesicles was unchanged. Thus, the reduced flow could only marginally contribute to the strong increase in total and immobile vesicles. Rather, it seems that myosin Va-tail caused retrograde vesicles to move slower, and in addition it increased the total number of vesicles in the axon.

We next tested whether the population of slower moving retrograde vesicles was preferentially associated with the myosin Va-tail construct. Myosin Va binds to its cargo via the tail domain (Wu et al. 1998), and accordingly myosin Va-tail colocalized with NPY-labeled organelles (Fig. 4a) and dual-color time-lapse recordings clearly showed a cotransport of myosin Va-tail with NPY, demonstrating the presence of myosin Va-tail on NPY-containing LDCVs (Fig. 5a, arrowheads). There was, however, no correlation between the speed of retrograde vesicles and the presence of myosin Va-tail on the vesicles. About half (56%) of the retrograde LDCVs were cotransported with myosin Va-tail, and they had the same average speed as the LDCVs that were not cotransported with myosin Va-tail (mean $\pm$ SEM, 0.58 ± 0.08 and 0.54 ± 0.09 $\mu\text{m/s}$, respectively). Both populations moved significantly slower than the retrograde LDCVs in control cells (0.75 ± 0.06 $\mu\text{m/s}$, Fig. 2f). The frequency histogram also demonstrates that myosin Va-tail association was not correlated with vesicle speed (Fig. 5d).

Myosin Va-Tail is Not Associated with Anterogradely Moving Particles in the Axon

Despite its significant effect on retrograde axonal LDCV transport, myosin Va-tail expression caused no significant changes of anterograde traffic: The number of anterogradely moving LDCVs was not changed (Fig. 4c), the mean velocity was not significantly reduced (Fig. 4d), and the frequency histogram showed little difference between control cells and myosin Va-tail expressing cells (Fig. 4e). Moreover, most of the anterogradely moving LDCVs were not cotransported with myosin Va-tail. Only 16% of anterograde LDCVs colocalized with myosin Va-tail (Fig. 5b). This was in striking contrast to the comparatively high colocalization of myosin Va-tail with retrograde and stationary LDCVs (Fig. 5b). The fact that myosin Va-tail preferentially bound to retrogradely transported and stationary LDCVs indicates that the myosin Va-tail–cargo interaction was specific and that myosin Va-tail predominantly interfered with retrograde axonal transport.

Seeing that myosin Va-tail associated with only a small subset of the anterogradely moving LDCVs, we then investigated the movement of all myosin Va-tail-labeled particles. This population is composed of myosin Va-tail-labeled LDCVs (containing the marker protein NPY-EGFP) and myosin Va-tail-labeled non-LDCV particles (without NPY-EGFP). Again, we found that very few particles moved anterogradely, although retrograde movement was abundant (Fig. 5c).

In summary, we found that myosin Va-tail predominantly interacts with retrogradely moving axonal vesicles, and that its expression causes a significant impairment of retrograde axonal LDCV traffic without affecting anterograde traffic.

Discussion

Live-cell imaging and dynamic colocalization of LDCVs with myosin Va-tail revealed that myosin Va-tail preferentially associated with retrogradely moving axonal vesicles rather

than with anterogradely transported vesicles and that it exerted a dominant-negative function by reducing the velocity of retrograde LDCVs in axons.

LDCV Transport in the Axon Initial Segment and Dendrites

We observed that the vesicle density in the AIS was significantly higher than in the more distal axon, and that vesicle movement in the most proximal region of the axon was more reminiscent of dendritic movement than axonal movement. The steep gradient from high to low vesicle density was often observed by eye and on the kymographs indicating that the differences were not due to simple dilution of vesicles. Rather, it suggests the presence of a filter within the AIS that restricts the entry of vesicles into the axon. Our time-lapse recordings and kymographs show that vesicles in the AIS move bidirectionally and rarely enter the axon proper as if held back by some barrier. This behavior is reminiscent of vesicles that carry the dendritic membrane protein transferrin receptor (Burack et al. 2000). It was proposed that the AIS constitutes a “selectivity filter” to prevent the entry of dendritic proteins into the axon (Burack et al. 2000; Song et al. 2009). While LDCVs in neurons are not to be considered dendritic cargo, one could envision a scenario where the AIS selectively filters a subpopulation of LDCVs and prevents the entry of other LDCVs. Our observations support the idea of the AIS as a “selectivity filter” and concur with the notion of soma, dendrites, and AIS as one continuous compartment that differs from the axon.

Within the axon, vesicle movement was much more directed than in dendrites. This is consistent with previous observations of LDCVs and other vesicles in nerve cells (Overly et al. 1996; de Wit et al. 2006; Kwinter et al. 2009). The phenomenon is commonly explained by the fact that microtubule orientation is unipolar in axons, with the plus end of microtubules oriented away from the cell soma, but it is bipolar in dendrites (Baas et al. 1988). For a vesicle, this means that if movement is driven by a plusend- directed motor protein (kinesin), it can move anterogradely over a long distance in an axon. This mechanism provides the possibility of efficient long-range transport along axons as long as the activity of the motor protein is tightly coordinated (Welte 2004). In a dendrite, the same vesicle will change the direction every time it detaches from a microtubule and encounters one with opposite polarity, causing the observed frequent reversal of direction.

Dominant-Negative Myosin Va-Tail in Vesicle Transport

Myosin Va-tail is a frequently used tool to impair the function of endogenous myosin Va, and it often has the same effect as the absence or downregulation of endogenous myosin Va (Wu et al. 1998; Varadi et al. 2005; Desnos et al. 2007; Yoshizaki et al. 2007; Correia et al. 2008). The exact mechanism by which it exerts this effect is, however, still unclear. Among the possible mechanisms are (a) blocking binding sites on the cargo, so that full-length myosin Va cannot associate with the cargo, (b) interaction with full-length myosin Va on the cargo, so that the cargo cannot be moved by full-length myosin Va, or (c) interaction with full-length myosin Va in the cytoplasm, so that full-length myosin Va cannot interact with the cargo or actin. Our aim was not to study these mechanisms; however, our results provide some interesting clues as to which mechanism is predominant. Here, we saw that myosin Va-tail expression slowed down retrogradely moving vesicles, and that myosin Va-tail was associated with retrogradely moving vesicles. If myosin Va-tail acted as in scenarios (a) and (b), then the vesicles that contain myosin Va-tail should move slower on average than the vesicles devoid of myosin Va-tail. This was, however, not the case, suggesting that myosin Va-tail also acts via scenario (c), by removing full-length myosin Va from the vesicles which would then cause the reduced speed as discussed later. It was in fact previously shown that myosin Va-tail can release full-length myosin Va from vesicles (Brown et al. 2004).

The Role of Myosin Va in Fast Neuronal Vesicle Transport

Noticeable features of LDCV movement were the frequent changes of velocity when vesicles were transported along the axon. Few progressed with constant speed—rather, they slowed down or accelerated frequently. Considering that vesicle movement may be carried out by several identical motor complexes bound to one vesicle simultaneously (Hill et al. 2004; Kural et al. 2005; Levi et al. 2006), the changes in speed could reflect the engagement and disengagement of these motor complexes. There is, however, evidence indicating that increasing the number of active motor complexes does not affect the vesicle speed (Gross et al. 2007). Alternatively, it was suggested that vesicles may occasionally detach from the microtubule track and perform actin-based or diffusive movement until they are reloaded onto the microtubules (Gross et al. 2007). The slower periods of vesicle movement could be such actin-dependent movement, especially, the rare events when axonal LDCVs reverse their direction for a short period. A myosin motor could support loading of vesicles onto microtubule tracks, by transporting them along the actin cytoskeleton until they encounter a microtubule. In the absence of a functional myosin motor, these vesicles would then be less efficiently loaded back onto the microtubule tracks and get stranded on their journey through the axon. Myosin Va is the prime candidate to perform this function for LDCVs, since it is highly expressed in the brain and associated with LDCVs/SGs in various cell types (Wu et al. 1997; Rose et al. 2003; Rudolf et al. 2003). Recently, it was found that myosin Va performs diffusive motion along microtubules in addition to the known actin-dependent movement, which makes it an even better candidate for linking microtubule-dependent and actin-dependent vesicle transport (Ali et al. 2007). Accordingly, we found that inhibiting myosin Va function slows down retrograde axonal LDCVs, increases the number of immobile axonal vesicles, and inhibits the retrograde and anterograde dendritic traffic. These effects can be explained by a model where myosin Va moves LDCVs along actin filaments to facilitate (re-) attachment to microtubules. The great increase in immobile vesicles observed upon myosin Va-tail expression could represent vesicles that should be transferred from actin filaments to microtubules, but fail to do so in the absence of functional myosin Va. Consequently, they accumulate in the axons as immobile vesicles. The reduced speed of retrograde axonal traffic could be caused by a delay in reattaching to microtubules whenever the active microtubule-dependent motor protein detaches. This mechanism may also apply to transport of other types of vesicles along axons, e.g., the impaired retrograde transport of tetanus toxin carriers (Lalli et al. 2003) and longer pausing times of neurofilament transport (Alami et al. 2009) in neurons from *dilute* mice, the stalled vesicles from squid axoplasm upon myosin Va-tail expression (Brown et al. 2004), or upon injection of a myosin Va antibody (Tabb et al. 1998).

Myosin Va and its Preference for Retrograde Transport

Expression of myosin Va-tail reduced the speed of retrograde axonal vesicles, but not of anterograde vesicles. We also found a surprisingly low amount of anterograde myosin Va-tail movement in axons, of both LDCVs and other vesicles labeled with myosin Va-tail. A previous study showed that neuronal vesicles labeled with myosin Va-tail moved equally well in the anterograde and in the retrograde direction (Bridgman 1999). In that work, vesicle movement was measured in “neurites,” which implies that the distinction between dendritic and axonal transport was not made. Our results, on the other hand, clearly show that vesicle transport in these two compartments must be considered differentially. However, Bridgman (1999) also reported that there were less retrogradely transported particles when endogenous myosin Va was absent, i.e., in *dilute lethal* neurons. The author suggests that myosin Va is involved in retrograde transport (Bridgman 2004), which is in accordance with our conclusion.

Thus, it emerges that myosin Va as a motor protein is required for certain forms of vesicle movement that specifically affect retrograde axonal transport. How can this be explained? Retrograde transport of LDCVs at first instance seems paradoxical, since the vesicles are thought to be stored and released from exocytotic sites (Hartmann et al. 2001). Several recent publications, however, report that retrograde transport of LDCVs in axons is common (Adachi et al. 2005; de Wit et al. 2006; Park et al. 2008), and it was suggested that this pool of retrogradely moving LDCVs could be used to replenish the exocytotic stores when these are depleted after neuronal activity (Shakiryanova et al. 2006; Frischknecht et al. 2008). Myosin Va may primarily be located at exocytotic sites and bind to LDCVs only once they reach these sites, so that anterograde traffic will not be affected by inhibition of myosin Va. At exocytotic sites, myosin Va contributes to regulate LDCV exocytosis (Bittins et al. 2009), and vesicles that have visited these sites but have not undergone exocytosis, will now be tagged with myosin Va. This could then aid the transition from the cortical actin network to microtubules to allow retrograde axonal transport, and it could facilitate fast axonal transport as discussed earlier and elsewhere (Bridgman 2004). The inhibition of myosin Va would then cause vesicles to accumulate at exocytotic sites, and also impair retrograde transport, which is in accordance with our results. Future research will determine whether anterograde and retrograde axonal LDCVs indeed represent different vesicle populations with regard to age and will elucidate the contribution of actin-dependent movement on long-range axonal transport.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AIS	Axon initial segment
EGFP	Enhanced green fluorescent protein
LDCV	Large dense core vesicle
mGFP/mRFP	Monomeric green/red fluorescent protein
NPY	Neuropeptide Y
SEM	Standard error of the mean
SG	Secretory granule
DIV	Days in vitro

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Fig. 1. Subcellular localization of fluorescently labeled NPY expressed in cultured hippocampal neurons. Cells were transfected with a plasmid encoding NPY-mRFP or NPY-EGFP at 11 DIV and fixed and imaged with a confocal microscope (**a–c**) or a wide field microscope (**d**) 24 h later. **a** Confocal image with brightness and contrast adjusted to visualize punctate NPY-mRFP fluorescence in the axon (*arrow*). **b** Neuronal cell body with punctate vesicular fluorescence and abundant perinuclear signal (*asterisk*). **c** Dendrite tip with punctate fluorescence. **d** Wide field image showing NPY-EGFP distribution in dendrites and axon (*arrow*). Scale bars **a, b, d**, 30 μ m; **c**, 10 μ m

Fig. 2.

Dynamics of LDCVs in neurons. Cells were double-transfected with a plasmid encoding NPY-mRFP and one encoding EGFP as a cytoplasmic marker at 11 DIV. After 24 h, time-lapse recordings were taken at 1 s intervals for 100 s. **a1** Kymograph of dendritic LDCVs. The neuronal cell body lies to the *left*, the time axis is vertical. Vesicles moving in the anterograde direction appear as diagonal stripes from *top left* to *bottom right*; immobile vesicles are *vertical stripes*. **a2** Kymograph of axonal LDCVs. **b** Example traces of axonal vesicles to demonstrate the classification as retrograde, anterograde, or bidirectional (stationary not shown). **c, d, e** Distribution of LDCVs into different categories in (c)

dendrites, $n = 300$ vesicles, (*d*) proximal axon/AIS, $n = 174$ vesicles, and (*e*) axons, $n = 591$ vesicles. **f** Axonal vesicle speed. Mean velocity of all unidirectional axonal LDCVs, $n = 201$ vesicles. **g** Frequency histogram of retrograde and anterograde LDCV velocities. Dendrites $n = 32$, AIS $n = 17$, axons $n = 19$, four independent experiments. Re, retrograde, Ant, anterograde, Bi, bidirectional, Stat, stationary. *Error bars* = SEM. *Scale bars* = 10 μm . *** $P < 0.001$ (two-tailed unpaired *T*-test)

Fig. 3. Colocalization of NPY-EGFP with endogenous myosin Va. **a** Immunofluorescence with an antibody directed against myosin Va reveals extensive punctate staining in dendrites and axons of cultured hippocampal neurons at 12 DIV. **b1** Punctate structures of endogenous myosin Va colocalize with LDCVs labeled with NPY-EGFP in transfected neurons. **b2** Intensity line profile along the neurite shown in B1 after background subtraction; *red*, myosin Va; *green*, NPY-EGFP. *Arrow*, NPY-EGFP that colocalizes with endogenous myosin Va. *Arrowhead*, NPY-EGFP that does not colocalize with myosin Va. *Scale bars a* 30 μ m, *b1* 5 μ m

Fig. 4.

Effect of myosin Va-tail expression on LDCV traffic. **a** Coexpression of myosin Va-tail and NPY in neurons. Plasmids-encoding myosin Va-tail-mGFP and NPY-mRFP were cotransfected in cultured hippocampal neurons and livecell imaging was performed 24 h later. **A'** myosin Va-tail-mGFP, **A''** NPY-mRFP, **A'''** overlay, high resolution images of the boxed region of the axon in **a**. **Arrows**, colocalizing punctate of myosin Va-tail and NPY. **b** LDCV dynamics in dendrites. Vesicles $n = 300$ control, $n = 444$ myosin Va-tail. **c** LDCV dynamics in axons. Vesicles $n = 591$ control, $n = 1,673$ myosin Va-tail. **d** Mean velocity of axonal LDCVs. Vesicles $n = 201$ control, $n = 291$ myosin Va-tail. **e** Frequency histograms

of axonal retrograde and anterograde LDCV velocities. Control, cotransfection of NPY-mRFP, and pEGFP. myoVa-tail, cotransfection of NPY-mRFP, and myosin Va-tail-mGFP. Cells control $n = 19$, cells myosin Va-tail $n = 26$, four independent experiments. LDCV analysis was done blinded to the treatment of the cells. *Scale bars* **a** = 50 μm , $A''' = 10 \mu\text{m}$. *Error bars* = SEM. * $P < 0,05$; ** $P < 0,01$; *** $P < 0,001$

Fig. 5.

Cotransport of myosin Va-tail with LDCVs. **a** dual-color axon kymograph. Neurons expressing myosin Va-tail-mCherry and NPY-EGFP were imaged for 100 s at 1 frame per second. A kymograph was made from the axon-showing myosin Va-tail-mCherry-labeled vesicles in *red* and NPY-EGFP-containing vesicles in *green*. Arrowheads, a vesicle labeled with both colors appears *yellow*. **b** Differential binding of myosin Va-tail to retrograde and anterograde LDCVs. LDCVs in the axons of myosin Va-tail-transfected cells were counted, and cotransport with myosin Va-tail was analyzed. **c** Axonal movement of myosin Va-tail. Cells were transfected with myosin Va-tail-mCherry and NPY-EGFP and movement of all

myosin Va-tail-labeled particles was analyzed. LDCV, particles labeled with NPY-EGFP, and myosin Va-tail-mCherry (*yellow* in the kymograph), others, particles only labeled with myosin Va-tail-mCherry (*red* in the kymograph). **d** Frequency histograms showing the cotransport of myoVa-tail with retrograde and anterograde axonal LDCVs. +myoVa-tail, LDCVs that are cotransported with myosin Va-tail-mCherry; -myoVa-tail, LDCVs in the same cells that are not cotransported with myosin Va-tail. *Scale bar* 10 μ m. *Error bars* SEM. Cells, $n = 23$, vesicles $n = 1,234$, four independent experiments. * $P < 0.05$; ** $P < 0.01$