Anterograde Glycoprotein Dependent Transport of Newly Generated Rabies Virus
in Dorsal Root Ganglion Neurons

Anja Bauer¹, Tobias Nolden¹, Josephine Schröter¹, Angela Römer-Oberdörfer¹, Shani Gluska², Eran Perlson², Stefan Finke¹

¹Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Molecular Virology and Cell Biology, D-17493 Greifswald – Insel Riems, Germany
²Tel Aviv University, Sackler Faculty of Medicine, Department of Physiology and Pharmacology, Sagol School of Neuroscience, Ramat Aviv, Tel Aviv 69978, Israel

running title: rabies virus anterograde axonal transport

abstract: 250 words
importance: 149 words
text: 5.991 words
number of figures: 9
supplementary files: 5

*Corresponding author: Mailing address: Friedrich-Loeffler-Institut, Suedufer 10, D-17493 Greifswald – Insel Riems, Germany.

Phone: ++49 38351 71283. Fax: ++49 38351 71151. E-mail: stefan.finke@fli.bund.de
Abstract

Rabies virus (RABV) spread is widely accepted to occur only by retrograde axonal transport. However, examples of anterograde RABV spread in peripheral neurons such as dorsal root ganglion (DRG) neurons indicated a possible bidirectional transport by an uncharacterized mechanism. Here, we analyzed the axonal transport of fluorescence labeled RABV in DRG neurons by live-cell microscopy. Both, entry-related retrograde transport of RABV after infection at axon endings and post-replicative transport of newly formed virus were visualized in compartmentalized DRG neuron cultures. Whereas entry-related transport at 1.5 μm/sec occurred only retrogradually, after two days of infection multiple particles were observed in axons moving in both the anterograde and retrograde directions. The dynamics of post-replicative retrograde transport (1.6 μm/sec) were similar to entry-related retrograde transport. In contrast, anterograde particle transport at 3.4 μm/sec was faster, indicating active particle transport. Interestingly, RABV missing the glycoproteins did not move anterogradually within the axon. Thus, anterograde RABV particle transport depended on the RABV glycoprotein. Moreover, co-localization of GFP-labeled ribonucleoproteins (RNP) and glycoprotein in distal axonal regions as well as co-transport of labeled RNPs with membrane anchored mCherry reporter confirmed that either complete enveloped virus particles, or vesicle associated RNPs were transported. Our data show that anterograde RABV movement in peripheral DRG neurons occurs by active motor protein dependent transport. We propose two models for post-replicative long distance transport in peripheral neurons: either transport of complete virus particles or co-transport of RNPs and G-containing vesicles through axons to release virus at distal sites of infected DRG neurons.
Rabies virus retrograde axonal transport by dynein motors supports virus spread over long distances and lethal infection of the central nervous system. Though active rabies virus has been widely accepted to be unidirectional, evidence for anterograde spread in peripheral neurons supports the hypothesis that in some neurons RABV also enters the anterograde pathway by so far unknown mechanisms.

By live microscopy we visualized fast anterograde axonal transport of rabies virus. The velocities exceeded those of retrograde movements, suggesting that active, most likely kinesin-dependent transport machineries are involved. Dependency of anterograde transport on the expression of virus glycoprotein G and co-transport with vesicles further suggest, that complete enveloped virus particles or co-transport of virus ribonucleoprotein and G-containing vesicles occurred. These data provide first insight in the mechanism of anterograde rabies virus transport and substantially contributes to the understanding of RABV replication and spread of newly formed virus in peripheral neurons.
Introduction

Although many viruses are able to enter the nervous system, only a limited set of viruses has evolved specific mechanisms for directed axonal transport to ensure neuroinvasion and virus replication in the peripheral and central nervous system (CNS) (1). Among those viruses, rabies virus (RABV; rhabdoviridae family) is a classic example of a pathogen that enters the nervous system by retrograde axonal transport. Though retrograde and anterograde transport of any cargo within the axon of a neuron depend on microtubules and associated motor complexes (2), detailed knowledge about checkpoints regulating the directionality of virus transport processes in neurons remain limited.

RABV is a non-segmented negative-strand RNA virus that is transmitted from rabid animals by bites. The virus is transported through retrograde microtubule dependent axonal transport (3) from the site of inoculation to the CNS, where virus replication is accompanied with progressive neuronal dysfunctions and lethal outcome of the disease. The genome of rabies virus encodes five virus proteins: nucleoprotein N, phosphoprotein P, matrix protein M, glycoprotein G and large polymerase L. Rabies virus replication requires a ribonucleoprotein (RNP) complex consisting of the genomic virus RNA tightly packaged by nucleoprotein. Phosphoprotein P is associated with the RNP in chaperoning N to ensure specific RNA packaging and as polymerase co-factor within the P/L-polymerase complex. Identification of a dynein light chain 1 (DLC1) binding motif in the rabies virus phosphoprotein (4, 5) supported the hypothesis, that RABV RNPs directly bind to dynein motor complexes and thus enter the retrograde transport pathway after release from endosomes. Subsequent studies revealed that destruction of the DLC1 binding motif in P did not abrogate the ability of RABV to invade the CNS (6) but had a supportive effect on early virus transcription (7).

Fluorescence protein fusion to P has been used to label RNPs of RABV (8, 9) and other rhabdoviruses (10) to visualize intracellular RNP transport. By combination of GFP-tagged rabies virus RNPs and incorporation of a membrane anchored red fluorescent protein into virus particles, double labeled
rabies virus particles were further used to show retrograde axonal transport of complete, membrane
enveloped virions (11), suggesting that in neurons, receptor usage by the viral glycoprotein and
subsequent receptor-dependent endocytosis are crucial for entering the long distance transport to the
cell soma. In neurons from mouse dorsal root gangliaons (DRGs), most of the retrogradely transported
RABV was co-transported with low affinity Nerve Growth Factor receptor (p75NTR) and colocalized
with acidified transport vesicles (12), confirming RABV transport in vesicles. This provided direct
evidence that one of the three proposed neuronal RABV receptors (13) is indeed a receptor that directs
incoming virus particles in the retrograde transport pathway. Notably, RABV was not only co-
transported with p75NTR but also accelerated the p75NTR retrograde axonal transport machinery,
indicating that RABV modulates axonal transport in the course of retrograde infection (12).

In accordance to the model of receptor dependent axonal transport, closely related vesicular stomatitis
virus and unrelated retrovirus vectors have been directed to retrograde infection of neurons by
incorporation of rabies virus glycoprotein (14-16) and co-transport with the potential RABV receptors
with pseudotyped lentiviruses was shown (17).

RABV axonal transport has been considered unidirectional and this led to the use of RABV as a
retrograde transneuronal marker for tracing neuronal connections within the CNS (reviewed in (18,
19)). Monosynaptically restricted trans-synaptic tracing by GFP expressing G-gene deleted rabies
viruses (20, 21) represented a pioneering step in neurobiology as the system allowed reliable tracing of
retrograde trans-synaptic neuron connections. Similar to RABV, which was also modified to allow non-
retrograde infection by introduction of vesicular stomatitis virus (VSV) G sequences (22, 23), the
system was also adapted to VSV vectors for use as retro- and anterograde neuronal tracers in a G-
dependent manner (16, 24).

Recent evidence for anterograde trans-synaptic transfer of rabies virus in sensory neurons emphasized
that rabies virus G protein also supports anterograde trans-synaptic spread, at least in peripheral
neurons (25). Accordingly, although unidirectional trans-synaptic RABV spread in the CNS is commonly accepted from a large body of evidence, intraneuronal spread of rabies in peripheral neurons appears to be special and the proposed exclusive retrograde RABV transmission to the CNS through motor neurons (18) may represent only one part of involved transport processes. In agreement with anterograde RABV transport in sensory neurons (25), in vitro and in vivo virus tracking in DRG neurons showed that anterograde virus transport through axons of peripheral DRG and other peripheral neurons is possible and that anterograde axonal transport of infectious RABV occurred at remarkable velocities of 100 to 400 mm/day (26, 27). However, mechanisms that explain the transport of RNPs or complete intracellular virus particles from the cell soma into axons have not been described. In addition to the infection of the CNS by anterograde pathways (25), a role of anterograde RABV transport in centrifugal spread from infected DRGs to peripheral sites such as skin and hair follicles has also been proposed. However, inefficient transport or passive diffusion in the anterograde direction was concluded from the late appearance of virus in these organs (28). Thus, a central unsolved question is, whether anterograde transport of RABV in peripheral neurons indeed is mediated by passive diffusion or by directed anterograde transport processes of virus particles or subviral complexes. In the latter case, unknown mechanisms that allow anterograde RABV transport have to be unraveled. In particular, differences in these axonal transport processes in peripheral DRG and CNS neurons may be decisive for infections routes and pathogenesis of RABV and other neuroinvasive viruses.

In a compartmentalized cultivation system that allowed reliable directed outgrowth of more than 1.7 cm long axons, we characterized the axonal transport of GFP-labeled RABV directly after infection of peripheral DRG neurons at distal axon endings to follow entry-related retrograde axonal transport. Moreover, after virus replication in the neurons soma (post-replicative), axonal virus transport was further investigate to see, whether virus is (i) indeed transported in the anterograde direction, (ii) whether anterograde transport velocities differ from early retrograde transport processes and (iii) to
determine whether virus transport depends on the presence of viral glycoprotein. Finally, we propose two possible models of glycoprotein-dependent anterograde axonal transport of newly formed RABV in peripheral neurons: either RABV is transported as a complete particle after intracellular budding in axonal transport vesicles or as pre-budding complex with cytoplasmic RNPs connected to glycoprotein-containing transport vesicles.
Material and Methods

Cells and viruses. BSR T7/5 cells (29) were used for virus rescue from recombinant cDNA as described previously (30). Conditional expression of RABV matrix and glycoproteins in MGon cells (31) was used for amplification of G-gene deleted RABV. Virus stocks of autonomously replicating RABV were prepared on NA neuroblastoma cells provided by the Collection of Cell Lines in Veterinary Medicine (CCLV), FLI Riems.

All recombinant virus cDNAs were constructed by standard techniques on the basis of recombinant cDNA derived from attenuated RABV vaccine strain SAD B19 (32). The EGFP-P expressing recombinant rabies viruses (rRABV) encoded a fusion protein of N-terminal EGFP and downstream phosphoprotein P as previously described (8). In the mutant EGFP-P DLC1mut two amino acid exchanges within a dynein light chain 1 (DLC1) binding motif were introduced (142-EDKSTQTT-149 → 142-EDASTATT-149) to eliminate binding to cellular DLC1 (9). In rRABV EGFP-P Gcvs and rRABV EGFP-P DLC1mut Gcvs, the authentic G protein of the attenuated RABV was replaced by the G coding sequence (EU126641.1) of the neuroinvasive RABV strain CVS-11. In addition to the authentic RABV G, rRABV tmCherry encoded membrane anchored mCherry protein with RABV G signal-, transmembrane and cytoplasmic domain sequences from an additional cistron. Recombinant Newcastle disease virus (rNDVF1 EGFP-P) used as control is based on NDV Clone 30 modified by a polybasic amino acid sequence at the fusion protein cleavage site (33, 34) and the insertion of an extra gene coding for an EGFP-NDV P fusion protein introduced between the P and M genes.

DRG neuron preparation and cultivation. Dorsal root ganglia were prepared from E15.5 embryos of pregnant Sprague-Dawley rats that were obtained from the Department of Experimental Animal Facilities and Biorisk Management (Friedrich-Loeffler-Institut). After euthanization with CO₂, embryos were removed from the uterus and transferred to a tissue culture dish. The embryos were rinsed with Hanks balanced salt solution (HBSS; Fisher Scientific), decapitated, laminectomized and
the spinal cord was removed. The DRGs were collected in HBSS at 4 °C. After dissolution of the DRG neurons by trypsin treatment for 20 min at 37 °C, neurons were cultivated in Neurobasal medium with B27-supplement, GlutaMax (all Invitrogen), Penicillin/Streptomycin (Sigma-Aldrich) and nerve growth factor 2.5S (50 ng/ml; Invitrogen). The neuron cultures were incubated at 37 °C and neuronal medium was replaced every 3 days.

**Compartmentalized neuron culture.** After coating of µ-Slide 6 0.4 cultivation chambers (Ibidi) with poly-DL-ornithine (500 µg/ml; Sigma-Aldrich) and natural mouse laminin (10 µg/ml; Invitrogen), the channels of the µ-Slide connecting the two opposite chambers were filled with 30 µl of 0.5% agarose in Neurobasal medium. After agarose polymerization, the two chambers were filled with Neurobasal medium and 30µl of a DRG neurons suspension containing cells from 2.5 dorsal root ganglia were seeded into the “proximal” chamber. After cultivation over night at 37 °C, 40 µl medium were removed from the chambers and replaced by the same volume of Neurobasal medium with 10 µM Arabinofuranosyl Cytidine (Ara C). After 3 days of Ara C-treatment, the medium was replaced with Ara C-free medium and the neurons were further cultivated for 2 to 3 weeks.

**Indirect immunofluorescence and live virus imaging.** Indirect immunofluorescence with mouse monoclonal antibody E559 (35) recognizing RABV G protein and AlexaFluor 568 conjugated secondary antibodies (Molecular Probes) was performed by standard techniques after fixation with 3.7 % formaldehyde in PBS and permeabilization with 0.5 % Triton-X100 in PBS. Images were acquired with a Leica SP5 confocal laser scan microscope (63x objective; numerical aperture: 1.4) with sequential acquisition of the fluorophores in double fluorescent specimen. Images were processed with the ImageJ software version 1.48b (36).

Live imaging of neuronal cultures was performed at 37 °C on a Leica SP5 confocal lasscan microscope equipped with a resonant scanner for fast image acquisition. Images were acquired at frame rates indicated in the results section. To achieve better particle detection, in some experiments the...
pinhole was widened as indicated. Detection of mCherry and EGFP fluorescence occurred simultaneously without any time loss between acquisition of red and green channels. Quantitation of image stacks was performed by using ImageJ and the Manual Tracking plugin developed by F.P. Cordelières (http://imagej.nih.gov/ij/plugins/track/Manual\%20Tracking\%20plugin.pdf). Transport velocities [µm/sec] of virus particles were calculated for each time frame by the particle displacement divided by time required for image or z-stack acquisition (0.276 sec - 0.503 sec). Velocities were categorized by 0.5 µm/sec intervals.
Results

RABV infection and live imaging of DRG neurons. To study RABV axonal transport we established a compartmental platform as described in the Material and Methods. DRG neurons were cultured from 15.5 days old rat embryos and the cells were seeded into the proximal chamber of the compartmentalized channel slides (Figure 1A). To prevent movement of cell bodies towards the opposite culture chamber, the connecting channels were filled with 0.5% agarose as a diffusion barrier. Neurons were cultivated for two weeks and axon outgrowth through the 1.7 cm long channels towards the opposite distal chamber was regularly controlled by microscopy. In addition, the absence of cell bodies in channels and distal medium reservoirs was verified.

When axon growth cones reached the distal chamber after two weeks, neurons were infected at the axon endings with 10^4 focus forming units (ffu) of recombinant RABV expressing EGFP-tagged phosphoprotein P. In addition to the EGFP, the recombinant virus rRABV EGFP-P DLC1mut GcvS (Figure 1B) contained a mutation in the dynein light chain 1 (DLC1) binding motif of P. Moreover, the glycoprotein G of the attenuated RABV backbone was replaced by a G sequence of the pathogenic CVS-11 strain. Whereas the glycoprotein replacement was intended to increase neuroinvasive properties to the attenuated rRABV backbone (9), the DLC1 binding mutation was inserted to exclude any effect of dynein motor complex binding of incoming RNPs during entry and the intracellular transport of newly formed RNPs after virus replication in the infected neuron.

Infection of DRG neurons was monitored by GFP fluorescence. After one day of infection, green fluorescence in proximal cell bodies and in axons (not shown) was detectable. After 2 to 5 days of infection, the neurons were completely filled with EGFP-tagged P protein and axonal transport of EGFP-P particles in both directions was observed. Bundles of parallel fluorescent axons were detected in the channel, whereas infected cell bodies remained exclusively in the proximal chamber (Figure 1B). Detection of fluorescent neurons even after 5 days of infection revealed that the compartmentalized...
neuron cultures not only allowed retrograde infection of the neurons but also to follow non-lytic RABV infections over a long time period. Successful infection at the axon terminals with rRABV EGFP-P DLC1mut Gcvs confirmed that DLC-1 binding by P is not required for retrograde transport. Similar results were obtained with rRABV EGFP-P Gcvs in which the DLC-1 binding motif in P was not affected (not shown).

**Retrograde transport of virus particles.** To visualize entry-related retrograde transport of virus particles after infection at the axon endings, $10^4$ focus forming units (ffu) of rRABV EGFP-P DLC1mut Gcvs were added to the distal chambers, and axons were monitored by live confocal laser scan microscopy. After 170 min, individual fluorescent particles were observed in the middle of the channel. The fluorescent particles were tracked by image acquisition at 0.276 sec/frame. Trajectories of individual particles (Figure 2AB; Figure S1) were captured and transport velocities of virus particles were calculated (Figure 2C). A mean transport velocity of $1.49 \mu$m/sec was calculated from instantaneous velocities (n = 817) from 8 individual retrograde trajectories.

**Anterograde transport of EGFP-particles.** Strong GFP fluorescence in axons of rRABV EGFP-P DLC1mut Gcvs infected neurons (see Figure 1B) indicated anterograde transport of soluble EGFP-tagged phosphoprotein and of newly formed EGFP-P containing protein complexes in axons after virus replication in the cell soma. To clarify whether EGFP-P particles were indeed transported in axons, DRG neurons were infected with $10^4$ ffu of rRABV EGFP-P DLC1mut Gcvs at the cell soma, and two days later, transport was analyzed in the mid part of the channel. Multiple particulate EGFP-P structures were detected within the channels (Figure 3A; Figure S2), indicating that newly formed EGFP-P particles have entered the axon after virus replication in the cell soma. Moreover, fast and directed transport of EGFP-P particles in both, antero- and retrograde directions was observed, further indicating that the EGFP-P particles indeed hijacked active cellular transport machineries within the
axons. Trajectories of individual particles confirmed transport of particles either into the antero- or retrograde direction (Figure 3A, left and right panel; Figure S3).

To assess whether different transport mechanisms were involved in the observed post-replicative retro- and anterograde transport processes, the mean transport velocities of particles from anterograde and retrograde trajectories were determined (Figure 3B). Notably, anterograde particle transport at 3.37 µm/sec (STDV = 0.18) was about twice faster than retrograde transport processes. With 1.6 µm/sec (STDV = 0.39), the mean velocity of retrogradely transported particles was comparable to the velocity of entry-related retrograde transport after infection in the distal chamber, although the distribution of retrograde transport velocities slightly differed between entry-related and post-replicative retrograde transport processes (see Figure 2C). The different transport kinetics for antero- and retrograde transport processes strongly suggested that different cellular transport mechanisms were involved, most likely by the use of different cellular motor complexes. Similar results were obtained with rRABV EGFP-P Gcvs (not shown), indicating that both entry-related retrograde and, after virus replication, axonal transport in both directions was independent of DLC-1 binding by P.

Co-localization of EGFP-P particles with nucleoprotein N. Although EGFP-P has been previously used as a marker for extracellular virions (8) and retrograde axonal transport during virus entry (9, 11, 12), we here checked co-localization with nucleoprotein N to see, whether EGFP-P particles transported in the axons at later phases of DRG infection indeed could represent viral RNPs. Therefore DRG neurons were infected at the cell soma and 3 days later the cells were fixed and immunostained with an N specific antibody. Confocal laser scan analysis in distal areas of the axons revealed, that most of the EGFP-P particles also contained nucleoprotein N. This was evident from both, maximum z-projections demonstrating complete axon bundles (Figure 4A) and single confocal slices (Figure 4B). These data indicated that the transported particles indeed represent viral RNPs.
Exclusion of Newcastle Disease Virus (NDV) derived EGFP-P from anterograde transport in axons. To assess whether the cultured DRG neurons still had mechanisms regulate transport of specific protein complexes in axons, we investigated, whether EGFP labeled phosphoprotein of NDV is also transported into axons after NDV replication and EGFP-P-particle formation in the neurons cytoplasm.

In contrast to RABV, NDV only occasionally infects the CNS (37) and we considered the existence of specific motifs in NDV mediating axonal transport of NDV particles as unlikely. After 4 days of infection at the cell soma with RABV EGFP-P Gcvs or rNDVF1 EGFP-P, GFP fluorescence was monitored in the cell soma, in a proximal area within the channel close to the cell soma (~1 mm relative to proximal chamber) and in a distal part (~12 mm relative to proximal chamber) of the channel (Figure 5). Both viruses led to the accumulation of strong GFP-fluorescent cytoplasmic inclusions, indicating that both viruses successfully infected the DRG neurons at the cell soma. In contrast to RABV EGFP-P, where small EGFP-P particles were detected in outgrowths of the neurons, in rNDVF1 EGFP-P infected cultures, particulate EGFP-P fluorescence was restricted to the cell soma and only a faint, unequally distributed GFP-fluorescence was detectable in neuritic outgrowths (see Figure 5, proximal chamber, brightened area). In the channels, NDV-infected neurons only led to a faint axoplasmic GFP fluorescence that decreased from the more proximal to the more distal part of the channel (Figure 5, proximal and distal area). Particulate structures were absent in the axons, showing that rNDVF1 EGFP-P particles do not enter the axonal transport machinery. In contrast to rNDVF1 EGFP-P, infection with RABV EGFP-P resulted in the appearance of particulate structures in proximal and distal parts of the axons. These data support the idea, that in the used DRG neuron cultures specific axonal sorting occurs. Accordingly, we assume that GFP-labeled RABV particles are not randomly transported through axons, for instance as a result of abundant protein levels, but that specific mechanisms exist that allow intra-axonal transport of RABV particles into the anterograde direction.

Anterograde transport of EGFP-P particles depends on glycoprotein G. Axonal transport of newly
formed EGFP-P particles could either be due to the transport of enveloped virus particles after budding in cellular membrane compartments or to the transport of cytoplasmic subviral protein complexes. To assess whether the transported EGFP-P particles represented enveloped and glycoprotein G-containing virus particles, post-replicative EGFP-P transport in rRABV EGFP-P Gcvs infected DRG neurons was compared to DRG neurons that were infected with G-gene deleted RABV. Four days after infection of DRG neurons at the cell soma with $10^4$ ffu of rRABV EGFP-P Gcvs or G-gene deleted virus rRABV EGFP-P $\Delta G$, the GFP fluorescence was monitored in the cell soma (proximal chamber) and in the middle part of the channels. Both viruses efficiently infected the DRG neurons as multiple cytoplasmic inclusions became visible in the cell soma (Figure 6). Immunostaining against glycoprotein G confirmed the absence of G protein in the post-replicative phase of rRABV EGFP-P $\Delta G$ infection. Whereas in close proximity to the soma some EGFP-P particles were detected, images from mid parts of the channels revealed that in the absence of G only diffuse GFP fluorescence was detectable in the axons, indicating that no EGFP-P-particles entered the anterograde transport pathway (Figure 6). In contrast, RABV EGFP-P Gcvs led to intra-axonal transport of EGFP-P particles. These data strongly suggested the axonal transport of glycoprotein containing virus particles or at least co-transport of EGFP-P particles and vesicles of the cellular transport machinery containing RABV-G. Moreover, dependence of the anterograde axonal transport on newly synthesized G also excluded the possibility that the observed EGFP-P particles in the axons were due to an accumulation of input virus. 

**Colocalization of P particles with glycoprotein.** To assess whether G-dependent axonal transport of P particles correlated with colocalization of G and P, DRG neuron cultures were infected with rRABV EGFP-P Gcvs at the cell soma. After 2 days of infection the DRGs were fixed and immunostained with G-specific Mab E559. Detection of EGFP-P particles and G vesicles (Figure 7; green and red, respectively) in distal axon parts confirmed anterograde transport of both proteins. Colocalization of EGFP-P and G (arrowheads) further indicated co-transport of both virus proteins in particulate
structures. In addition, non-colocalized EGFP-P and G particles were also observed in the distal chamber (arrows). Due to the deletion of G, neither EGFP-P particles nor G signals were detected in distal axon parts of rRABV EGFP-P ΔG infected DRGs (Figure 7, g-i), whereas diffusely distributed axoplasmatic GFP-fluorescence still indicated presence of soluble EGFP-P protein even at distal parts of the axons. These data indicated that G and EGFP-P particles indeed accumulated in distal axon regions either as a result of co-transport through neurons or of particle assembly at distal membrane sites. As transport of EGFP-P particles depended on the presence of G, we hypothesized that co-transport of both viral proteins components was causative for the observed colocalization. Some non-colocalizing particles may be due to the additional transport of G-containing secretory vesicles in the case of red particles, and partial dissociation of G and EGFP-P from co-transported particles at distal sites.
Co-transport of newly formed EGFP-P particles and tmCherry-labeled vesicles. Since anterograde transport of EGFP-P particles depended on the expression of G in the infected cell, transport of newly formed virus particles or subviral structures through neurons was most likely. To show that EGFP-P particles were indeed co-transported with G-containing membranes, neurons were coinfectected at the cell soma with rRABV EGFP-P Gcvs and rRABV tmCherry at the proximal chamber. In addition to its authentic G, rRABV tmCherry expressed a membrane anchored mCherry-fusion protein in which the ectodomain of RABV G was replaced by the red-fluorescent mCherry protein (Figure 8B). As colocalization of EGFP-P and membrane anchored tmCherry depended on co-infection and subsequent formation of new virus particles, anterograde co-transport of both labels was expected in case of vesicles associated axonal RNP transport.

Indeed, at 3 days post infection, anterograde axonal transport of EGFP-P particles and mCherry fluorescent vesicles was detectable in the axons. Double-infected neurons exhibited double stained particles in axons and co-transport of EGFP-P and mCherry (Figure 8A, arrows), as demonstrated by time-projections and by image sequences of individual transported particles. These data clearly showed that newly formed EGFP-P particles were co-transported with membrane anchored mCherry protein. Analysis of individually tracked particles further revealed that the ratio of red and green fluorescence was not constant over time (Figure 8C; Figure S4), suggesting that the mCherry-labeled membrane was not as tightly bound to the EGFP-P particle as may be expected in the case of complete bullet shaped RABV particles. Nevertheless, these data strongly supported the hypothesis that anterograde axonal transport of EGFP-P particles either occurs in or at RABV glycoprotein containing membrane vesicles.
Discussion

By the use of EGFP-P labeled viruses, we here show that newly formed RABV particles are anterogradely transported into axons of peripheral rat DRG neurons. Though early and more recent work provided evidence for anterograde spread of RABV in peripheral neurons (25, 26), the involved mechanisms of anterograde RABV transport remained unclear. Here, by demonstrating the dependency of the anterograde transport on the expression of viral glycoprotein in the infected DRGs, we now provide a deeper insight in the mechanism of anterograde RABV spread. Glycoprotein dependent transport of RABV particles (Fig. 6) and co-transport of membrane anchored mCherry comprising the cytoplasmic and transmembrane domains of RABV G (Fig. 8; Fig. S4) strongly support a model in which either complete virus particles are transported within cellular transport vesicles or cytoplasmic RNPs are co-transported with glycoprotein-containing vesicles by sticking to the cytoplasmic side of the vesicles (Fig. 9). Moreover, direct observation of fast and directed anterograde RABV transport in peripheral sensory neurons strongly supports a model in which active anterograde transport machineries are involved. In view of current concepts of exclusive retrograde axonal transport of RABV in neurons (reviewed in (18)), our data and recent evidence for anterograde trans-synaptic RABV spread in sensory neurons (25) strongly support a more differential model of axonal RABV transport in which peripheral neurons could substantially differ in permitting RABV transport in the anterograde direction.

Earlier work already supported anterograde axonal RABV transport in DRG and other peripheral neurons (26, 27, 38). Tsiang and colleagues were able to demonstrate virus release after anterograde transport through DRG neurons, strongly supporting the hypothesis that anterograde axonal transport indeed led to the release of infectious virus at pre-synaptic membranes of DRG neurons. Although anterograde spread of RABV in peripheral neurons has also been recognized in vivo in the course of viral spread to peripheral sites, only inefficient transport or passive diffusion in the anterograde...
direction was concluded (28) and mechanistic insight into the involved processes remained poor. Fast
and directed anterograde transport at velocities clearly exceeding those determined for retrograde
RABV transport during entry (Fig. 2) and after virus replication in the cell (Fig. 3) strongly supports
the idea that active, energy dependent transport by kinesins is involved in anterograde RABV transport.
This is also consistent with current concepts in directed intraneuronal transport (reviewed in (39)).
Physical barriers that prevent uncontrolled transport of large complexes into axons (40) may be one
reason for the requirement of specific mechanisms to allow RABV particle transport. Since passive
diffusion of virions, subviral particles or even large viral protein complexes into axons is unlikely, the
dependency of axonal transport on the expression of glycoprotein G suggests, that the viral
glycoprotein represents a sorting signal that either directs budding of virions into transport vesicles or
that allows recruitment of virus RNPs to vesicles that are transported into axons.
Different transport velocities for retro- and anterograde transport of viruses have been reported. For
instance, bidirectional fast-axonal transport of pseudorabies virus in chicken DRG neurons was
characterized by average velocities of 1.97 and 1.2 µm/s for anterograde and retrograde transport
processes (41), confirming that anterograde axonal virus transport occurs about twice as fast as
retrograde virus transport. Higher velocities as determined for RABV transport in rat DRGs may be
due to the use of different cellular motor proteins or to species-specific differences in the velocity of
axonal transport in DRGs derived from rat or chicken embryos. The same reasons may also contribute
to minor differences in retrograde RABV transport velocities in murine DRG neurons. In the murine
DRG neurons, the average velocity of retrograde RABV transport 0.93 µm/sec (12) remained below
the velocities of 1.5 µm/sec and 1.6 µm/sec as observed here for retrograde axonal RABV transport in
the rat DRG neurons (Figs. 2 and 3). Notably, also in the murine system, subpopulations of fast RABV
transport were identified with velocities of 1.2 to 1.4 µm/sec (12), indicating that also in the murine
system RABV can achieve transport kinetics similar to those observed here in the rat DRG system.
Importantly, with average anterograde velocities of 3.4 µm/sec (Fig. 3) we were clearly able to distinguish fast anterograde RABV transport processes from retrograde transport. The most reasonable explanation for the differences in the kinetics of retro- and anterograde transport processes is the use of different cellular motor complexes. The possibility to differentiate between slower retrograde and faster anterograde transport processes also revealed that the observed particle transport in opposite directions (Figure 6) indeed depended on different transport mechanisms and were not just an effect of back-folding and growing of axons terminals towards the proximal chamber.

Although we do not yet have a possible role of kinesin motors in the anterograde axonal rabies virus transport and no kinesin binding motifs in any RABV protein have been described, we hypothesize that kinesin-dependent axonal transport was involved in post-replicative anterograde transport processes. In view of the suggested models, vesicle associated RABV transport indeed may not require specific kinesin binding sites in virus proteins since cellular components of the transport vesicles could be used.

Similar retrograde transport velocities in virus entry and after virus replication further indicated dynein mediated back-transport into the retrograde direction, either by virus release and re-infection at axonal termini or by exchange of motor proteins and subsequent switch in transport directionality. Indeed, back-transport of excess vesicles back to the soma has been described in Drosophila (42) and could easily explain the observed bi-directional transport of RABV particles after virus replication.

To show restricted transport of cytoplasmatically expressed RNP proteins, we included recombinant NDVEGFP-P expressing Newcastle Disease Virus. Exclusion of NDV EGFP-P particles from transport into axons of infected DRG neurons (Figure 5) strongly supports the hypothesis, that the particle transport for RABV occurs in an active and regulated way, Indeed, in hippocampal neurons a physical barrier for both, lateral diffusion of membrane proteins and lipids (43, 44) and transport through the cytoplasm (40) in the axon initial segment (AIS) serves as filter for cargoes that are excluded from axons. In an actin-dependent manner, large 70 kDa dextrans were excluded from diffusion into axons.
by the cytoplasmic filter, whereas smaller GFP molecules or 10 kDa dextrans were not excluded (40). Remarkably, the molecular weight of monomeric 74 kDa NDV EGFP-P fusion protein is similar to that of 70 kDa dextran, and exclusion of even monomeric rNDVF1 EGFP-P is conceivable. At least partial exclusion of soluble rNDVF1 EGFP-P is supported by the finding that diffuse rNDVF1 EGFP-P fluorescence was detectable mainly in proximal axon areas, whereas fluorescence was hardly detectable in more distal parts (Figure 5). More intense signals for the 62 kDa soluble RABV EGFP-P within axons may rely on a less restricted transport into axons because of the lower molecular weight or on the presence of sorting signals in P that may allow transport of soluble P through a cytoplasmic barrier within the proximal axon areas. In case of transported RNPs, which represent large complexes of RNA and N, L and P proteins (see colocalization of EGFP-P particles with N in Figure 4) a physical barrier in the axon initial segment is most likely to preclude passive diffusion in axons.

Glycoprotein dependent axonal transport of EGFP-P particles (Figure 6) revealed that transport of EGFP-P particles into axons requires envelope dependent sorting or transport signals. This conclusion was supported (i) by G-dependent appearance of P particles in axons, (ii) by co-localization of EGFP-P particles and G protein in distal axon areas (Figure 7) and (iii) by intraaxonal co-transport of EGFP-P and tmCherry (Figure 8). Co-transport of EGFP-P and membrane anchored tmCherry further revealed that post-replicative anterograde axonal transport of EGFP-P is connected to vesicle transport, leading to the hypothesis that either complete virions were transported in cellular vesicles (Figure 9A) or that cytoplasmic P-containing RNPs are associated with RABV G containing vesicles of the secretory pathway (Figure 9B). Whereas the first model may result in release of preformed infectious virus at pre-synaptic membranes by exocytosis, the latter would lead to local enrichment of G in the pre-synaptic membrane with the RNP already bound at the cytoplasmic side of the membrane. This would allow local concentration of virus components needed for budding at pre-synaptic membranes after long distance transport through axons.

21
Secretory transport of complete virions within cellular vesicles (Figure 9A) would imply intracellular budding of RABV particles. Indeed, intracellular virus particle formation is known within RABV infected cells (45, 46) and more recent data provided evidence for matrix protein dependent accumulation of virus-like particles within the rough endoplasmic reticulum (rER) (30, 47). Transport of virus particles within secretory Golgi-vesicles, however, has not been observed so far. Since most investigations have been performed in non-neuronal cell cultures, it is not yet clear whether ER virions indeed are not mobilized in infected neurons and whether the observed co-transport of EGFP-P and tmCherry could be due to mobilization of ER-associated RABV virions. However, the dynamics of the ER in neurons and motor-assisted vesicular ER transport within axons (48) could mobilize post-replicative virion accumulation within the ER of neurons without a requirement for Golgi-vesicles.

EGFP-P and G co-localization in distal axon areas was not complete, as indicated by particles with EGFP-fluorescence only (Figure 7), and the red and green fluorescence intensities of transported particles were not perfectly overlaying (Figure 8BC), suggesting that no complete virions with a rigid bullet shaped morphology were transported. Co-transport of G-containing vesicles and cytoplasmic RNP on the cytoplasmic side of the vesicles would explain imperfect co-localization of EGFP-P particles and tmCherry, and would support a model in which co-enrichment of virus envelope proteins and RNP allow coordinated budding at distal pre-synaptic membranes (Figure 9B).

Our data confirm early results on RABV infection of peripheral neurons that claimed anterograde axonal transport in cultivated DRG neurons (26) and in vivo after intranasal and intramuscular inoculation (27, 38), the latter indicating that anterograde virus transport observed in primary peripheral DRG neuron cultures may not be a result of cultivation artefacts. Infection of mice by the olfactory route is an excellent example for anterograde spread of RABV towards the CNS after a first round of replication in olfactory receptor neurons, where infection of 2nd order neurons mitral cells as well as trans-synaptic transmission to higher ordered neurons must have included anterograde axonal...
spread (27). Recent evidence for anterograde RABV spread in vivo through sensory neurons (25) also supported the conclusion of different axonal transport modes in RABV infected peripheral sensory and CNS neurons. Whereas previous work mainly relied on virus spread by the detection of infected neurons, we present here for the first time a dynamic insight into the post-replicative transport of newly formed RABV. We provide evidence that envelope-dependent transport of either complete anterograde virus particles or vesicle-RNP co-transport occurs in peripheral neurons, both providing the possibility of virus release and transmission to higher ordered neurons at distal pre-synaptic membranes (Figure 9).

As our data strongly indicate fast anterograde axonal transport after replication in DRG neurons, we conclude that anterograde axonal transport through sensory neurons, either towards the CNS or centrifugally to peripheral sites, occurs in an active manner, in contrast to previously suggested passive diffusion (28). Obviously, in addition to receptor-dependent retrograde entry into neurons (11), the G protein also determines long distance transport in DRG neurons after virus replication in the cell soma (16). Similar to RABV, intracellular transport of complete VSV within vesicles of the secretory pathway has not been demonstrated, and co-transport of cytoplasmic RNPs with G-containing vesicles, as discussed here for RABV, cannot be excluded.

We have shown that anterograde transport of newly formed RABV virions or subviral particles in axons of peripheral DRG neurons occurs in a glycoprotein dependent manner. In view of the large body of evidence for exclusive unidirectional spread of RABV in motor-neurons and within the CNS, our and others’ findings suggest that RABV transport depends on neuron type specific mechanisms and that peripheral DRG neurons, and maybe also other peripheral neurons, are exceptional in supporting active anterograde RABV transport. Future identification of distinct mechanisms in peripheral and central axonal virus transport and their impact on virus spread and pathogenesis may be crucial for a detailed understanding of nervous system infections by RABV and other neuotropic viruses.
Acknowledgements

We thank Angela Hillner and Dietlind Kretzschmar for technical assistance and are grateful to Thomas C. Mettenleiter for helpful discussions. We thank Karl-Klaus Conzelmann for critical comments on the manuscript. We further thank the Department of Experimental Animal Facilities and Biorisk Management of the Friedrich-Loeffler-Institut for providing embryonated rats for DRG preparations. This study was supported by the German Israeli Foundation for Scientific Research and Development (grant no. 1107-73.1/2010) and German Federal Ministry of Education and Research (grant no. 01KI1016A).
References


29. Buchholz UJ, Finke S, Conzelmann KK. 1999. Generation of bovine respiratory syncytial...
virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. Journal of virology 73:251-259.


Figure Legends

**Figure 1:** Chambered dorsal root ganglion (DRG) neuron cultivation, directed infection and EGFP-P detection in infected neurons. (A) DRG neurons were prepared from rat embryos and seeded in cell culture devices of which the two chambers were connected by an agarose filled channel. After two weeks, axonal growth cones reached the distal chamber. Directed infections at growth cones (distal chamber) or at the cell bodies (proximal chamber) allowed imaging of virus infections and intra-axonal transport within axons. Virus infections were monitored directly after infection (0 dpi; retrograde virus entry) or post-replicative after 1 – 5 days of infection. (B) DRG neuron cultures were infected at the growth cones (right side) with rRABV EGFP-P DLC1mut Gcvs (top: genome organization). After 5 days of infection, GFP autofluorescence was detected in the cell bodies (left side), in bundled axons (middle) and in distal chambers (right side). Upper two rows: Bright field and fluorescence images of cultivation devices. The images were assembled from 48 field of views. Lower two rows: magnifications from proximal and distal chambers (right and left side, respectively) and from the middle of the channel (middle).

**Figure 2:** RABV retrograde axonal transport. DRG neurons were infected with rRABV EGFP-P DLC1mut Gcvs at the distal growth cones. After 170 min virus particle transport was observed by image acquisition in the middle part of the channels (0.276 sec/frame; optical slice = 1.5 μm). (A) Transport of a single virus particle into the retrograde direction. From a total of 181 time frames, every 20th image is shown. Transported virus particle is marked by circles. (B) Time-projection of 842 time frames. (C) Particle transport velocities were determined for each time frame and were categorized as indicated in the diagram. The frequencies of different transport velocities are shown. Data have been generated from eight trajectories, comprising a total of 817 individual transport events.

32
Figure 3: Post-replicative axonal transport. DRG neurons were infected in the proximal chamber and EGFP-P particle transport in axons was monitored after 48 h of infection by z-stacks acquisition (5 optical slices per stack; acquisition rate: 0.503 sec/stack; optical slice = 1.5 µm; size depth = 6 µm).

(A) Directed transport into the anterograde (left) and retrograde (right) direction was visualized by trajectories for individual particles. Note: The left and right images represent identical time points and areas. (B) Particle transport velocities were determined for each time frame and were categorized as indicated in the diagram. The frequencies of different transport velocities are shown for retro- and anterograde transport processes (black and grey bars, respectively). Anterograde transport velocities were calculated from fifteen trajectories consisting of 636 individual transport events. Retrograde transport velocities were calculated from seven trajectories consisting of 512 transport events.

Figure 4: Colocalisation eGFP-P and nucleoprotein N in axons. Immunostaining against nucleoprotein N after 3 days of infection in distal parts of DRG axons. (A) Maximum z-projection of 34 optical slices (0.772 µm each; size depth = 11.1 µm). (B) Detail from A. Single optical slice (z = 0.772 µm). Scale bar: 3 µm.

Figure 5: Exclusion of NDV phosphoprotein from anterograde transport in axons. DRG neurons were infected at the cell soma with rRABV EGFP-P Gcs and recombinant NDV expressing EGFP-tagged NDV P protein (rNDVF1 EGFP-P). EGFP-P fluorescence was monitored at 4 dpi within the cell soma (proximal chamber) and in the channel at proximal areas close to the cell soma side (middle row) and in more distant distal areas (bottom row). Note: brightness/contrast was increased (B/C set to 100) for rNDVF1 EGFP-P fluorescence within the image section (cell soma) and in images from channel areas.
**Figure 6: Post-replicative axonal transport of EGFP-P particles is G protein dependent.** DRG neurons were infected with rRABV EGFP-P Gcvs and G-gene deleted rRABV EGFP-P ΔG at the cell soma. Upper row: cells were fixed after 2 days of infection and were immunostained with G-specific monoclonal antibody E559. Merged images of red G signals and green EGFP-P autofluorescence are shown. Small detail: G-specific fluorescence only. Lower row: After 4 days of infection, axonal EGFP-P fluorescence of living neurons was monitored within channels. The images represent maximum z-projections of seven optical slices (0.772 µm each; size-depth = 4.5 µm).

**Figure 7: Colocalization of RABV G and EGFP-P in distal chambers.** DRG neuron cultures were infected with rRABV EGFP-P Gcvs or rRABV EGFP-P ΔG at the cell soma. At 2 dpi neurons were fixed and immunostained with G-specific Mab E559. Optical slice = 0.772 µm. (a-c) Axons of rRABV EGFP-P Gcvs infected neurons in the distal chamber. (d-f) Magnification of distal axons with EGFP-P and G co-localization indicated by open arrowheads. Particles exhibiting only green or red fluorescence are indicated by arrows. Scale bar: 1 µm. (g-i) Axons of rRABV EGFP-P ΔG infected neurons in the distal chamber. Note: only diffusely distributed GFP-fluorescence in axons and bouton like axon swellings was observed, indicating the presence of soluble EGFP-P in the distal axon parts.
Figure 8: Co-transport of EGFP-P particles and tmCherry-vesicles. DRG neuron cultures were co-infected with rRABV EGFP-P Gcvs and rRABV tmCherry in the cell soma. After 3 days of infection, axonal transport of EGFP-P (green) and tmCherry (red) was monitored within the channels (0.336 sec / frame, optical slice = 0.772 µm). (A) Colocalization of EGFP and mCherry fluorescence in transported particles. Upper three rows: t-projections with red, green and merged images. Arrows: co-infected neurons. The dashed lines indicate the time period and area used for single particle tracking as shown below. The image sequence was derived from 25 time frames and every 3rd image shown. Scale bar: 1 µm. (B) Genome organization of rRABV EGFP-P Gcvs and rRABV tmCherry. The tmCherry protein consists of RABV G derived signal peptide, transmembrane and cytoplasmic sequences (SP, TM and Cyt). (C) Single particle analysis with time-projection of an anterograde particle transport process. Shown are merged images (1 µm² details of and individual transported particle) from 60 consecutive frames. The frame order is indicated at the left side. (D) T-projections of neurons exclusively infected with rRABV EGFP-P Gcvs or rRABV tmCherry. Scale bar: 1 µm.

Figure 9: Model of intra-neuronal post-replicative RABV transport. Either complete, enveloped virus particles are transported within exocytotic vesicles (A) or co-transport of cytoplasmic RNPs and G-containing transport vesicles (B) may occur. Whereas the former mode may allow release of complete virus particles at axon termini or pre-synaptic membranes, the co-transport model would allow local concentration of both, viral glycoprotein and RNPs at distal sites of virus assembly. After fusion of the transport vesicle with pre-synaptic membranes, the RNP is positioned directly beneath the G-enriched pre-synaptic membrane and virus particles may be assembled by a subsequent budding event.