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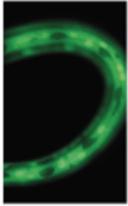
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An endocytic CARriage tale

Adenoviruses internalization and trafficking in neurons

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In immune-competent hosts, adenoviruses (Ads) are mild pathogens that cause mainly infections of the respiratory and ocular tracks. The advent of Ad-based gene transfer vectors made the understanding of their interaction with the host cellular machinery an intensive field of research over the last decade. As studies focused primarily on epithelial-like cells, the mechanism of neuronal uptake of Ads was still unknown. Using a combination of biochemical and cell biology approaches, we characterized the axonal trafficking pathway used by the canine adenovirus serotype 2 (CAV-2) to reach the neuronal soma. We showed that CAV-2 and CAR (coxsackievirus and adenovirus receptor) are entering a vesicular pathway coupled to the axonal transport machinery. The lumen of the multivalent Rab7⁺ vesicles that transport CAV-2 and CAR is, surprisingly, pH neutral. Among other issues, our study opens numerous questions concerning the neuronal function of CAR.

Adenoviridae is a rapidly expanding family of more than 150 serotypes. Adenoviruses (Ads) are non-enveloped, double-stranded DNA viruses associated mainly with mild infections.¹ Due to their advantageous features such as their modular organization and broad range of cellular tropisms, Ads have been intensively studied over the last 20 years in gene and cancer therapies. Numerous efforts have been undertaken to characterize the cellular interactions during viral binding, intracellular trafficking and replication. To enter host cells, Ads use a variety of

primary and auxiliary receptors, such as the coxsackievirus and adenovirus receptor (CAR) and integrins.² Most studies addressing the intracellular trafficking of Ads during the first steps of infection have concentrated on human serotypes 2 and 5 (HAd2 and HAd5) in epithelial cells.² Following receptor-mediated endocytosis and a drop in pH as the vesicle matures, Ads escape early endosomes, possibly due to conformational changes in the capsid that favor endosomal lysis via protein VI.³ Once in the cytoplasm, capsids recruit cytoplasmic dynein to be transported to the nuclear membrane and deliver their genome in the nucleus.^{4,5} However, several Ads use alternative mechanisms/pathways, making studies on Ad trafficking cell type- and serotype-dependent.⁶

Often, Ads infect the gut, respiratory and ocular tracts leading to self-limited morbidity. However, Ads also infect cells of the brain and have been associated with brain tumors.⁷ Early reports concerning the transport of Ads in neurons suggested that vectors derived from these viruses could be promising platforms for gene therapy of the central nervous system (CNS).^{8,9} In particular, canine adenovirus serotype 2 (CAV-2) is efficiently retrogradely transported and leads to stable gene expression in rodents, dog and primate CNS.^{10,11} Compared to HAd5, which transduces neurons and glial cells, CAV-2 infects almost exclusively neurons in vitro and in vivo. Although it appears that CAV-2 is dependent on CAR to enter and infect cells,¹² the mechanism used to be transported to and within the CNS was uncharacterized.

Key words: adenovirus, axonal transport, coxsackievirus and adenovirus receptor, endosomes

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To better understand CAV-2 axonal trafficking, we first took an *in vitro* approach by imaging fluorescently-labeled CAV-2 in primary rodent motor neurons (MNs). *In vivo*, CAV-2 can be retrogradely transported in MNs of the spinal cord after intra-muscular injection in hind-legs of mice. *In vitro*, CAV-2 transport was bi-directional, suggesting the involvement of several motors with opposite polarity. In axons, where the microtubule tracks are polarized with the minus end towards the cell body, dynein is responsible for the retrograde trafficking, whereas the kinesin family ensures mainly anterograde delivery of cargoes to nerve terminals.¹³ To understand the role of these two classes of motors in CAV-2 transport, we used a dominant-negative approach by overexpressing dynamitin p50, known to disrupt dynein-dependent transport, and the TPR domain of kinesin light chain, which inhibit kinesin-driven movement. Not surprisingly, inhibiting dynein and kinesin led to a similar impairment of CAV-2 transport, demonstrating the need of multiple molecular motors for its efficient axonal transport.¹⁴

To further dissect CAV-2 axonal traffic, we performed electron microscopy analyses of CAV-2 infected MNs. At early time point of entry, we found virions in clathrin-coated pits, similarly to that seen with HAd5 entry in epithelial cells.² However, at 45 minutes post-internalization, when the transport is robust, the majority of virions were still found inside endosomes, in contrast with Ad traffic in epithelial cells. Vesicular transport was confirmed using labeled-dextran, a fluid phase marker that labels endocytic organelles, and that was found with transported CAV-2. Furthermore, tetanus toxin (TeNT), which is only retrogradely transported in MNs,¹⁵ was also found in endosomes containing CAV-2 “en route” to the cell body. Endocytic transport of CAV-2 is rather surprising because capsids of Ads can recruit directly dynein to be transported. Because of the key role of intra-endosomal pH in Ads’ escape into the cytosol,¹⁶ and the fact that TeNT enters some pH neutral carriers in axons,¹⁷ we measured the pH environment of CAV-2-transported organelles by

using carboxyfluorescein, a pH-sensitive dye, directly coupled to virions. We found that axonal vesicles containing CAV-2 had a lumen with a pH close to neutral, whereas CAV-2-containing endosomes in cell bodies were acidic. These features of axonal vesicles could explain why CAV-2 remains inside endosomes during its long-range transport. Interestingly, these axonal endosomes matured from Rab5 (marker of early endosomes) to Rab7 (classical marker of late, acidic endosomes),¹⁸ on transported carriers. These data suggest that in neurons, some Rab7 carriers, which lumens are at neutral pH, provide a protective environment for long-range axonal transport that allows endogenous molecules, as well as pathogens, to be transported in the CNS.¹⁴

Finally, we asked what led to the efficient axonal retrograde transport of CAV-2. Because of its apparent dependence on CAR to enter cells in culture, we assayed the localization of this adhesion molecule during viral binding and transport. Interestingly, the role of CAR as a key component of tight junctions of endothelial and epithelial cells is well documented,¹⁹ but its function in neurons remains uncharacterized. In MNs, we found co-localization between CAR and CAV-2 as expected during binding, but more surprisingly also at late stage of internalization, when axonal transport occurs. Using an approach consisting of magnetic purification of endosomes using TeNT coupled to iron beads, we also found CAR in axonal carriers. Furthermore, the use of recombinant CAV-2 FK allowed us to visualize directly CAR dynamics. Surprisingly, the fiber knob had similar transport properties compared to CAV-2, suggesting that CAR is somehow linked to the endocytic and transport machineries in neurons. These unsuspected intracellular dynamics of CAR prompted us to monitor the transport of this molecule *in vivo* in the sciatic nerve. Our data, using sciatic nerve ligation to stop anterograde and retrograde axonal transports, confirmed the axonal trafficking of CAR after FK uptake at the neuromuscular junction. Finally, using competition experiments with unlabeled fiber knob, we found that the main binding site on FK, most

likely to CAR, is the main determinant in CAV-2 entry in MNs (Fig. 1).

This set of observations suggests a key role of CAR in uptake and transport of adenoviruses in the CNS and potentially in neuronal homeostasis. However, it is still unclear whether this molecule can directly target Ads to the axonal transport machinery by actively recruiting motors and adaptors necessary for sorting along the endocytic pathway and microtubule-dependent traffic, or would act more passively as a first step for viral binding that will be followed by general internalization mechanisms.

Numerous questions concerning the molecular mechanisms used by viruses to enter the CNS remain open. In particular, the signals activated during viral endocytosis that lead to the recruitment of the axonal transport machinery are still poorly understood. Similarly, the regulation of bi-directional transport by cytoplasmic dynein and kinesin is not yet fully characterized. Finally, little is known about the fate of viral capsids once they arrive in the cell body. With our data concerning the lumen environment in axonal vesicles, it is tempting to speculate that acidification takes place close to the cell body and would allow viral escape and targeting to the nucleus. This protective environment could also allow other Ad serotypes, such as HAd5, to be transported in vesicles in axons. Further experiments will be needed to address these questions by using compartmentalized culture systems to recreate neuronal polarization *in vitro*. Due to the essential role of axonal transport in neuronal homeostasis and its defect in many neurodegenerative disorders,²⁰ understanding the molecular mechanisms involved in long-range traffic is of crucial importance. Using viruses as tools will also lead to a better characterization of this cellular process.

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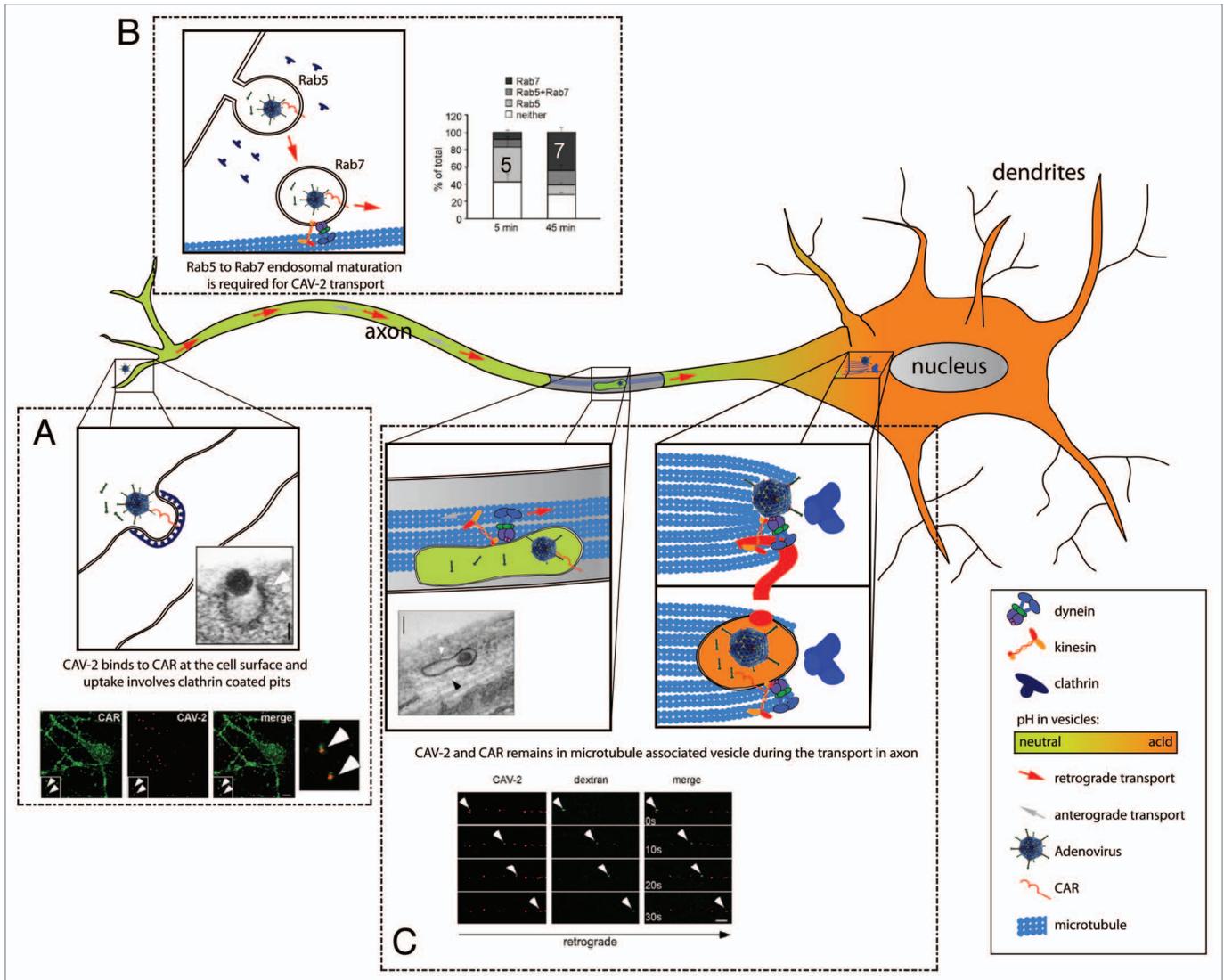


Figure 1. The different steps of CAV-2 transport. First, the virus binds to CAR at the cell surface followed by internalization in clathrin-coated pits. (A) upper part shows electron microscopy of CAV-2 in a coated pit; lower part shows colocalisation of CAV-2 and CAR at the surface of motor neurons. During entry, maturation of CAV-2 containing vesicles occurs, with a shift from Rab5 to Rab7 endosomes that will eventually lead to CAV-2 axonal transport. (B) shows quantification of colocalisation between CAV-2, Rab5 and Rab7 during internalization. Despite Rab7 maturation, the pH of axonal endosomes stays neutral, and CAV-2 remains inside vesicles during transport. (C) shows electron microscopy of CAV-2 at late stage of endocytosis, when the transport occurs, and live-cell imaging of co-transport of CAV-2 and the endocytic marker dextran. CAV-2 traffic appears to have a bias for the retrograde direction and both dynein and kinesin are involved. Surprisingly, CAR is also found in CAV-2 positive vesicles. The somato-dendritic fate and endosomal escape of CAV-2 in neurons are still unclear and remain to be described.

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