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Disruption of the Coxsackievirus and Adenovirus Receptor-Homodimeric Interaction Triggers Lipid Microdomain- and Dynamin-dependent Endocytosis and Lysosomal Targeting

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Background: The coxsackievirus and adenovirus receptor (CAR) acts as a docking factor during infection.

Results: Adenovirus fiber knob induces CAR internalization via a pathway involving lipid microdomain integrity, actin dynamics, and dynamin.

Conclusion: In neurons, CAR is linked to endocytic pathways that could modulate its function or regulate viral infection.

Significance: Learning how viruses use receptors to enter and spread in the nervous system.

The coxsackievirus and adenovirus receptor (CAR) serves as a docking factor for some adenovirus (AdV) types and group B coxsackieviruses. Its role in AdV internalization is unclear as studies suggest that its intracellular domain is dispensable for some AdV infection. We previously showed that in motor neurons, AdV induced CAR internalization and co-transport in axons, suggesting that CAR was linked to endocytic and long-range transport machineries. Here, we characterized the mechanisms of CAR endocytosis in neurons and neuronal cells. We found that CAR internalization was lipid microdomain-, actin-, and dynamin-dependent, and subsequently followed by CAR degradation in lysosomes. Moreover, ligands that disrupted the homodimeric CAR interactions in its D1 domains triggered an internalization cascade involving sequences in its intracellular tail.

Cell surface molecules are often responsible for the initial recognition of pathogens by the host, and thus much effort has been devoted to characterize how cells capture pathogens. In the nervous system, several cell adhesion molecules (CAMs)7

located at nerve terminals bind viruses, including members of Herpesviridae and Picornaviridae (1). Virus propagation and/or latency depend on their ability to be internalized and to recruit the axonal transport machinery to be transported to the cell body of neurons. Axonal transport is a key mechanism in neuronal homeostasis that maintains efficient communication between the somatodendritic compartment and nerve termini. The integrity of this process is crucial for neuronal survival as impairment of factors regulating this pathway can be linked to neurodegenerative disease (2, 3). In this light, viruses are ideal tools to understand cellular processes: for instance, α-herpesvirus and rabies virus have been used as neuronal tracers and to understand signals regulating intracellular trafficking in neurons (1, 4).

The coxsackievirus and adenovirus receptor (CAR, encoded by CXADR) is a member of the immunoglobulin (Ig) superfamily (5). It has a single membrane-spanning domain, an extracellular domain of 216 residues composed of two Ig-like domains (D1 and D2), and an intracellular tail composed of 107 residues. The D1 domain can be recognized at the cell surface by many adenovirus (AdV) types and the group B coxsackieviruses (CVB). Our current understanding of its role in AdV entry is that it serves as “docking platform,” but does not significantly participate in internalization. This is based primarily on the finding that when the entire intracellular tail of CAR is deleted AdV infection still progressed efficiently (6). In addition, during CVB entry at tight junctions in epithelial cells, CAR remained at the plasma membrane (7). However, during canine adenovirus type 2 (CADV-2, commonly referred as CAV-2) entry in motor neurons, CAR and CAV-2 were co-internalized within axons and transported bidirectionally in pH-neutral endosomes (8). Incubation of motor neurons with recombinant CAV-2 fiber knob...
Viral Ligands Induce CAR Endocytosis

Briefly, hippocampi were isolated, dissociated with 0.025% trypsin, and plated in Neurobasal medium (Invitrogen) containing B27 (Invitrogen), l-glutamine (Sigma), Glutamax (Invitrogen), 10% fetal bovine serum (FBS, Sigma), and antibiotics. Hippocampal neurons were then incubated at 37 °C and 5% CO2 under a humidified environment. At day in vitro (DIV) 4, 5% of the medium was replaced with medium without l-glutamine and FBS. Neurons were used at DIV 10–14. Neu2A cells were cultured in DMEM (Invitrogen) containing 10% FBS and antibiotics.

**siRNA Treatments**—siRNAs against the murine clathrin heavy chain (CHC) were previously described (chc-1, 5'-AACAUUGCCUCAGCUCCUGTT-3'; chc-2, 5'-AAGUG-GAUCCUCUUUGAAUACCGTT-3) (20). Control siRNA was designed using the siRNA CHC-1 sequence and replacing 2 central nucleotides (control, 5'-AACAUUGGCAACAGCU-CUUGTT-3'). siRNA duplexes were purchased from IDT Technologies and transfected in primary neurons according to a published method (21). Briefly, neurons at DIV 11 were co-transfected with a plasmid encoding GFP (pEGFP) and siRNA duplexes using Lipofectamine 2000. Three days later, ligands (FKCAV or Tf) were applied and internalization was monitored. Knock-down of CHC was monitored using anti-clathrin antibody (clone X22) from Abcam.

**Adenoviral Vectors and Recombinant Fiber Knob (FKCAV)**—CAV-GFP, CAV-CRE, and HAdV5-βGal were produced and purified using established methods (22, 23). To produce FKCAV (residues 358–542), the cDNA was cloned into pPROEX HTb (Invitrogen), expressed with a cleavable His6 tag, and purified as previously described (8). Labeled FKCAV was obtained as previously described (8).

**Flow Cytometry**—Neu2A cells transfected with a plasmid encoding for CAR-RFP were collected in PBS (Invitrogen) and incubated for 1 h at 4 °C with anti-CAR Rmcb. Cells were washed with PBS and incubated for 30 min with Alexa Fluor 488-labeled anti-mouse IgG antibody. As control, nontransfected cells incubated with anti-CAR and cells transfected and incubated with only the secondary antibody were used. Cells were washed with PBS, fixed with 2% paraformaldehyde, and analyzed with FACSCalibur (BD Biosciences). Compensation to avoid overlapping fluorescence between RFP and Alexa Fluor 488 was performed. Data analysis was performed using the FlowJo software.

**Lipid Microdomain Isolation**—Lipid microdomains were isolated with a modified protocol previously described (24). Briefly, cells were lysed using 250 mM sucrose, 1 mM MgCl2, 1 mM CaCl2, and 20 mM Tris-HCl, pH 7.5. The cellular homogenate was passed through a 25-gauge needle several times and centrifuged at 16,000 × g for 10 min at 4 °C. The supernatant was put aside, the procedure repeated on the pellet, and the two supernatants pooled. Lipid microdomain fractions were obtained by ultracentrifugation in OptiPrep™ step density gradients. Two milliliters (ml) of the supernatant was mixed with an equal volume of 50% OptiPrep diluted in lysis buffer and transferred to a SW40 ultracentrifuge tube. 1.7 ml of 20, 15, 10, and 5% OptiPrep and lysis buffer (v/v) were successively overlaid. The tube was centrifuged at 75,000 × g for 90 min at 4 °C. Fractions of 1 ml were removed from the top of the tube.
Viral Ligands Induce CAR Endocytosis

and precipitated in 10% trichloroacetic acid overnight at 4 °C. Precipitates were then washed with cold acetone and solubilized in SDS-PAGE loading buffer.

**Immunofluorescence and Confocal Microscopy**—Hippocampal neurons plated on poly-D-ornithine coverslips or in microfluidic chambers (see below) were imaged using Leica LSM780 and Zeiss SP5 confocal microscopes, with ×63 1.4 NA Plan Apochromat oil-immersion objectives.

For indirect immunofluorescence, neurons were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 5 min at room temperature (RT), followed by a blocking step with 2% bovine serum albumin (BSA) and 10% horse serum for 30 min to 1 h at RT. Primary and secondary antibodies were diluted in blocking solution and incubated sequentially for 1 h at RT. Samples were then mounted with fluorescent mounting medium (Dako) with DAPI (Sigma) and imaged by confocal microscopy.

Live cell imaging was performed as previously described (8). Briefly, 100–150 frames were acquired at 0.5 frames/s. Kymographs were generated using the Image J software.

**Microfluidic Chambers**—Microfluidic chambers (MCs) for neuronal compartmentalization were produced as previously described (25, 26). Briefly, polydimethylsiloxane inserts were covalently bound to glass bottom dishes (Wilco Dish) using a plasma cleaner (Deiner Electronic). MCs were then coated with 4% BSA for 2 h at 37 °C followed by poly-d-ornithine for 2 h at 37 °C. Devices were washed and further coated with laminin for 90 min at 37 °C. Dissociated neurons were plated in the proximal compartment and axonal growth through the microgrooves to the distal compartment was allowed for 5–8 days. To perform axonal transport experiments, distal compartments were fluidically isolated by using less volume than in proximal compartments.

**Ethic Statement**—The animals were treated in accordance with the European Community Council Directive 86/609, modified by the decrees 87/848 and 2001/464. The Animal Welfare Committee at the University of Montpellier 2 approved all protocols and all efforts were made to minimize the number of animals used and potential pain and distress. Pertinent license and permits (34–132) were obtained prior to institutional and regional animal welfare committee approval.

**Intraparenchymal Injection of FKCAV**—6–7-Week-old female OF1 mice (Charles River) weighing 26–30 g were housed in groups prior to injections, and allowed food and water ad libitum. They were maintained in a controlled environment (22 ± 1 °C, 55 ± 5% humidity) with a 12 h:12 h light/dark cycle. Intrastratial injections were performed in anesthetized animals with an intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). To cover the entire striatum, we targeted four coordinates of injection: anterior-posterior, +0.14 mm; lateral, +2 mm; dorsal-ventral, −3/−4 mm; third and fourth sites of injection: anterior-posterior, +1.10 mm; lateral, +1.5 mm; dorsal-ventral, −3/−3.75 mm). Animals were divided into several groups. One group received injections of PBS and a second group injections of FKCAV (0.2, 1, or 5 μg/striatum). Each mouse was injected in one hemisphere; the contralateral striatum was used as internal control. Mice were sacrificed by intracardiac fixation or decapitation 1, 7, 14, or 30 days post-injection. For the study of CAR expression by immunoblot, striata were dissected and proteins were extracted by sonication with 5% SDS. The location of the Cy5-tagged FKCAV was evaluated in the striatum and the substantia nigra 1 day post-injection.

**Statistical Analyses**—Data were analyzed using Student’s t test for unpaired data and one sample t test for data compared with control set as 100% (*, p value <0.05; **, p value <0.01; ***, p value <0.001 versus control). Results are expressed as mean ± S.E.

**RESULTS**

Multiple Ligands Induced CAR Internalization in Primary Neurons and Neu2A Cells—Receptor engagement by viruses or viral proteins is often linked with conformational changes that can ultimately lead to endocytosis of the receptor-pathogen complex. To better understand the mechanism leading to CAR internalization, the role of ligand valency and CAR-CAR interactions, we incubated hippocampal neurons for 20 min at 37 °C after a pre-binding step on ice with multiple CAR ligands: FKCAV, CAV-2, HAdV5, and anti-CAR antibodies (Fig. 1, A–E).

Cultured hippocampal neurons from E18 express CAR at the membrane and possibly in intracellular compartments (data not shown), as we previously showed in primary motor neurons (8, 27). CAV-2 and FKCAV led to CAR internalization in puncta that were also positive for Tf, which label endocytic structures as observed in motor neurons (8)) (Fig. 1, A, B, and E). We also detected internalized CAR after incubation with HAdV5, suggesting that other CAR-tropic AdVs also triggered CAR internalization (Fig. 1, C and E). One possible explanation for ligand-induced CAR internalization was that its clustering through multivalent interactors initiate subsequent endocytosis. To test whether clustering per se was responsible for CAR entry, we used anti-CAR antibodies recognizing epitopes on the extracellular domains of CAR (see “Experimental Procedures”). In contrast to trivalent (FKCAV) or multivalent (AdV) ligands, anti-CAR antibodies did not lead to CAR internalization in hippocampal neurons (Fig. 1, D and E), suggesting that clustering of CAR through multimeric ligands was not sufficient for its internalization. Notably, recombinant FKs and AdVs can disrupt CAR D1-D1 interactions (28).

This differential ligand-induced internalization was also seen in CAR-transfected Neu2A cells, a mouse line derived from a murine neuroblastoma with undetectable CAR expression (Fig. 2A). In Neu2A cells transfected with a plasmid expressing CAR-RFP and then incubated with FKCAV, CAV-2, or HAdV5, the ligands were found in vesicular structures together with CAR, similarly to hippocampal neurons. Notably, cells incubated with anti-CAR antibodies did not contain intracellular puncta of CAR (Fig. 2, B and C). To test these observations using another method, we monitored CAR plasma membrane levels using flow cytometry. Neu2A cells were transfected with a plasmid encoding CAR-RFP, and then incubated overnight with FKCAV, CAV-2, or HAdV5. In these conditions, we found a decrease in plasma membrane CAR (Fig. 2D), and therefore conclude that viral ligands triggered CAR internalization. Of note, at similar physical particle per cell ratio (pp/c), CAV-2
induced CAR internalization more efficiently than HAdV5 (Fig. 2D). Together, these data showed that CAR can be internalized in neuronal cells, but different efficacies in ligand-dependent internalization existed.

**CAR Is Located within Lipid Microdomains at the Plasma Membrane and during Internalization in Neurons—**The plasma membrane is organized in subdomains where proteins and lipids can elicit specific signals, regulate cell adhesion, and other processes. Among these domains, lipid microdomains have unique properties that diversify membrane trafficking and signaling (29). Because numerous viral and toxin receptors, including CAR (30, 31), are located within lipid microdomains, we assayed the membrane sublocalization of CAR in primary neuron cultures and in the mouse brain. When using a detergent-free method (24) to isolate lipid microdomains from primary cortical neurons, flotillin-1, a marker for lipid microdomains, was found in the top fractions of the density gradient (Fig. 3A). CAR was mainly found in flotillin-1 fractions, suggesting that CAR was a resident of lipid microdomains in neurons (Fig. 3A). To monitor the dynamic association of CAR with lipid microdomains, we used methyl-β-cyclodextrin to remove cholesterol from membranes and shift lipid microdomain-associated proteins toward the bottom of the density gradient.

Under these conditions, CAR and flotillin-1 were found in the bottom fractions of gradient of cortical neurons (Fig. 3A).

AdVs use multiple receptors to engage and enter cells. The paradigm is that CAR-bound AdVs are internalized via interaction with integrins through an RGD motif in the penton base (32). To circumvent the role of co-receptors, which could bias the interpretation of CAR membrane dynamics, we used FKCAV as a CAR-specific ligand. We previously showed that when motor neurons were incubated with a mutant form of FKCAV, FKCAV/CAR, where the CAR-binding site was ablated, we could not detect CAR interaction via surface plasma resonance, cell binding, or CAR endocytosis (8, 9). These data were consistent with the specificity of FKCAV to detect CAR.

Some proteins, such as the epidermal growth factor receptor (EGFR), can diffuse laterally out of lipid microdomains during clathrin-dependent internalization (33). To test whether CAR would also leave these microdomains during binding or the first steps of entry, we assayed its lipid microdomain location after ligand engagement. FKCAV was incubated with cells at 4 °C to allow attachment, and then 20 min at 37 °C to allow internalization. Using this assay, we found that CAR did not exit lipid microdomains after FKCAV binding or internalization (Fig. 3B). Similarly, neither CAV-2 nor HAdV5 displaced CAR from
these microdomains (Fig. 3B). We then asked whether lipid microdomain integrity was needed for CAR internalization. We incubated hippocampal neurons with filipin, a lipid microdomain-disruptor, prior to FKCAV binding. In this condition, the internalization of FKCAV/CAR and the binding domain of CTB, which recognizes the ganglioside GM1, a well-characterized lipid microdomain component, was prevented. By contrast, Tf internalization, which uses a non-lipid microdomain entry pathway, was not (Fig. 3C and D). This suggested that the main route of entry of CAR in neurons required the integrity of lipid microdomains.

Together, these data demonstrated that CAR was a permanent resident of lipid microdomains in neurons in native conditions, and during ligand-induced internalization. Furthermore, the association of CAR with lipid microdomains was crucial in its ability to undergo endocytosis as sequestration of cholesterol blocked its internalization.

Clathrin-independent, Dynamin- and Actin-dependent CAR Endocytosis in Neurons—Lipid microdomain-dependent and clathrin-dependent endocytosis were, until recently, thought to be largely independent. This is illustrated by the data concerning EGF/EGFR endocytosis, which can happen in lipid microdomains, or after lateral diffusion outside them, and then engaging the clathrin machinery (33). However, this view has been tempered by reports suggesting that clathrin-dependent endocytosis could take place into lipid microdomains (34). In epithelial cells, numerous studies showed that AdV entry involves the clathrin machinery (35). Notably, the intracellular domain of CAR has a functional tyrosine-based AP (adaptor protein) binding site YXXΦ (YNQV; Fig. 4A) that is present in numerous receptors that use clathrin machinery for endocytosis. This motif was reported to be involved in the basolateral sorting of CAR through AP1 in epithelial cells (36). However, the role of YNQV as an internalization sequence has not been characterized for CAR. We therefore used transfected Neu2A cells with plasmids encoding CAR-RFP deletion mutants lacking the PDZ (PSD-95/Discs-large/ZO-1) binding domain and the potential AP binding site (Fig. 4, A and B). When we incubated FKCAV with cells expressing the full-length (FL) CAR-RFP at 4 °C, we found FKCAV bound only on transfected cells, showing the specificity for plasma membrane CAR (data not shown). We then shifted cells to 37 °C to monitor CAR endocytosis. Under these conditions, FKCAV and CAR were co-endocytosed in endocytic vesicles (Fig. 4, C and D). Neu2A cells expressing CAR-RFP with a deletion in the AP binding site (CAR-315) also bound FKCAV at 4 °C. After inducing internalization at 37 °C, endocytosis was similar to full-length CAR (Fig.
suggesting that clathrin-dependent endocytosis was unlikely to be the major route of internalization of CAR.

To identify the sequence involved in CAR internalization, we carried out a more extensive deletion analyses. We found that the sequence necessary for FKCAV-mediated endocytosis of CAR was between amino acids 261 and 274, as CAR-274 was internalized but CAR-261 was not (Fig. 4, C and D). To test if this conclusion held true in primary neurons, CAR-RFP plasmids were transfected in hippocampal neurons that were subsequently incubated with FKCAV. Similarly to that found in Neu2A, CAR-261 failed to internalize FKCAV (Fig. 4, E and F).

To address the role of clathrin-mediated endocytosis using another approach, we transfected Neu2A cells and hippocampal neurons with a plasmid expressing AP2/H92622T156A, a dominant-negative construct that blocks clathrin-dependent endocytosis (37). AP2/H92622T156A inhibited Tf uptake, which is dependent on the clathrin machinery (Fig. 5A). However, in Neu2A (Fig. 5, A and B) and hippocampal neurons (Fig. 5, C and D) expressing AP2μ2T156A and CAR-GFP, FKCAV was efficiently internalized. Similarly, hippocampal neurons co-transfected with a plasmid harboring a GFP expression cassette to identify transfected cells and two different siRNAs against the CHC was not blocking FKCAV internalization, which supports our conclusion that CAR did not use the clathrin machinery for its internalization upon FKCAV engagement (Fig. 5, E and F).

As actin filaments are involved in the endocytosis of numerous receptors (40), we then assayed the role of actin in CAR internalization. Huang et al. (41) previously showed that CAR interacts with actin, suggesting that the actin cytoskeleton could play a role in its trafficking. To monitor the role of actin, we used LatA, which induces the disassembly of actin polymers. We transfected Neu2A cells with CAR-GFP, incubated them at
4 °C with FKCAV and then shifted them at 37 °C for 1 h. In these conditions, cells pre-treated with LatA failed to internalize FKCAV, whereas in DMSO-treated cells FKCAV was found in endosomes (Fig. 6, A and B). Similarly, in mock-treated cells FKCAV was present in endosomes 1 h post-internalization, whereas LatA treatment maintained a membrane-bound pattern of FKCAV in hippocampal neurons, consistent with an inhibition of internalization (Fig. 6, C and D).

Taken together, these data suggested that in neurons, CAR was linked to a lipid microdomain-, actin-, and dynamin-dependent route of internalization. In addition, the AP binding site in the intracellular tail of CAR was not necessary for its endocytosis, consistent with its uptake via a clathrin-independent route.

**FKCAV-induced CAR Internalization in Neurons Is Coupled to the Endolysosomal Pathway**—Receptor function can be modulated by the preferential targeting to subcellular compartments upon ligand-induced internalization. After endocytosis, ligand-receptor complexes can be recycled back to the plasma membrane, targeted to the trans-Golgi network and endoplasmic reticulum, or degraded by lysosomes. During their endocytosis, AdVs trigger endosomal lysis to reach the cytosol. This phenomenon precludes the identification of the trafficking of CAR after endocytosis. We therefore used FKCAV to induce CAR internalization and characterized its trafficking post-entry. When hippocampal neurons were incubated with FKCAV, we detected a relocalization of endogenous CAR that led to its disappearance from neurites and its accumulation in the cell body (data not shown). This could be interpreted in at least two ways: active transport (as previously shown) or distal degradation. To determine whether CAR entered the recycling pathway, we co-incubated hippocampal neurons with FKCAV and Tf on ice, then shifted the neurons to 37 °C for various times. Tf and its receptor TfR are markers of the recycling pathway and reach early endosomes prior to targeting recycling endosomes and the plasma membrane. FKCAV and Tf colocalized at the early time point but then separated, suggesting that CAR was not targeted to recycling endosomes (Fig. 7, A and B).

As CTB uses a lipid microdomain-dependent endocytic pathway (42), which transports the toxin from endosomes to the Golgi apparatus, we monitored the possible co-transport of CAR within this route. To this end, we co-incubated hippocampal neurons with FKCAV and CTB as above. Although FKCAV/CAR colocalized with CTB at early time points and was in peri-
nuclear organelles at late time points, they did not colocalize
with CTB at the trans-Golgi network (Fig. 7, C and D), as seen
by three-dimensional rendering (Fig. 7E).

We repeated the above assay and stained FK\textsuperscript{CAV}-incubated
neurons with LAMP1, a lysosomal marker. At 2 h post-inter-
nalization, numerous organelles were FK\textsuperscript{CAV}/LAMP1\textsuperscript{+}(Fig.
7F). To control for targeting of FK\textsuperscript{CAV} to lysosomes, we assayed
the colocalization of FK\textsuperscript{CAV} and endogenous CAR 2 h post-
internalization by immunofluorescence (data not shown). Sim-
ilarly, when we incubated hippocampal neurons with FK\textsuperscript{CAV}
oncertain, we detected an ~60% decrease in CAR levels by
immunoblot analyses, consistent with ligand-induced degrada-
tion (Fig. 7, G and H). CAR degradation was almost completely
blocked by pre-treating cells with pepstatin A/E-64d or chloro-
quione, drugs that perturb lysosomal function (Fig. 7, G and H).
Incubating cells with an anti-CAR antibody did not lead to its
degradation, consistent with the immunofluorescence data
regarding its internalization (Fig. 7, G and H). From these data,
we concluded that the primary fate of CAR after ligand-induced
internalization was toward lysosomal degradation, and not the
retrograde or recycling pathways.

**Lipid Microdomain- and Actin-dependent Axonal-specific CAR Endocytosis andSomatodendritic Lysosomal Targeting—**
The composition of neuronal subcompartments such as den-
drites and axons can differ greatly and possibly influence
ligand-receptor endocytosis. To better understand ligand
uptake at different neuronal sites, we used MCs (43). Hip-
pocampal neurons were cultured for 5–8 days to allow axons to
cross the microgrooves. Incubation in the axonal compartment
with FK\textsuperscript{CAV} led to efficient retrograde transport of FK\textsuperscript{CAV}
and accumulation of CAR in cell bodies (data not shown). As
observed in batch cultures, ~75% of the CAR\textsuperscript{+} retrograde car-
rriers were also CTB\textsuperscript{+} when the axonal compartment was co-
inubated with FK\textsuperscript{CAV} and CTB, suggesting that lipid microdo-

![Diagram](image-url)
main-rich structures mediated the axonal retrograde transport of CAR (Fig. 8, A and B). However, after axonal transport, FK<sub>CAV</sub> and CTB did not colocalize in the trans-Golgi network (Fig. 8C), whereas retrograde FK<sub>CAV</sub> was found in LAMP1<sup>+</sup> somatodendritic structures (Fig. 8D). These data suggest a sorting mechanism once axonal cargoes reached the cell body and that CAR was targeted to lysosomes after retrograde transport.

To monitor whether the endocytic pathway regulating axonal entry and transport was similar to that in batch cultures, we assayed the effect of lipid microdomain disruption on FK<sub>CAV</sub>/CAR axonal transport. When axon termini were preincubated with filipin, FK<sub>CAV</sub> and CTB failed to accumulate in the soma, whereas rhodamine/dextran, a fluid phase marker, did (Fig. 9, A and B). Similarly, when axons were treated with LatA prior to FK<sub>CAV</sub> incubation, we did not detect efficient FK<sub>CAV</sub> accumulation in cell bodies after retrograde transport (Fig. 9, C and D).

Together, these data showed that axonal endocytic trafficking of CAR was coupled to lipid microdomain- and actin-mediated endocytosis. In addition, retrograde co-transport with other lipid microdomain-dependent ligands was followed by a sorting to different somatodendritic destinations.

**Ligand-induced CAR Axonal Transport and Degradation Occur in Vivo**—Finally, we asked whether CAR trafficking and degradation occurred in the mammalian brain. To address this, we injected FK<sub>CAV</sub> in the striatum of 8-week-old mice. Using immunofluorescence, we found that FK<sub>CAV</sub> was efficiently retrogradely transported from the striatum to the cortex and substantia nigra (SN) (Fig. 10A). This retrograde transport was also observed in the sciatic nerve after intramuscular injections (8). Eighteen hours post-injection, ipsilateral and contralateral striata were removed and proteins extracted. An ~80% decrease in CAR levels in FK<sub>CAV</sub>-injected striata was detected compared with the controlateral structure, whereas mock (PBS) did not induce this phenomenon (Fig. 10B). Ligand-induced CAR degradation was dose-dependent (Fig. 10B) and CAR levels started to return to normal around 1 month post-injection (Fig. 10, C and D). Together, these data showed that after internalization, CAR was targeted to lysosomes for degradation in vivo.

**DISCUSSION**

In this study, we defined the mechanisms regulating CAR endocytic trafficking upon ligand binding. We showed that CAR-tropic AdVs and adenoviral proteins induced CAR internalization in neurons and neuronal cells. Endocytosis was lipid microdomain-, actin-, and dynamin-dependent, and did not require clathrin and its adaptors. Somatodendritic and axonal endocytosis targeted CAR to lysosomes leading to its degradation. Although CAR and CTB/GM1 shared common a lipid-microdomain entry/transport pathway, they differed in terms
of their final destination after axonal retrograde transport, suggesting a sorting mechanism at the soma-axon interface.

Numerous pathogens use plasma membrane receptors to access and spread in the CNS (1, 4). For instance, herpes simplex virus 1 (HSV-1) interacts with nectin-1, a CAM located at sensory terminals (44). Similarly, in motor neurons poliovirus binds CD155 to be endocytosed and retrogradely transported through direct interaction of the receptor with cytoplasmic dynein (45). As viral diseases can lead to severe pathologies such as paralysis or dementia, numerous efforts have been made to better characterize the host-pathogen interaction and the endogenous function of their cellular receptor(s).

Although AdVs are not quintessential human brain pathogens, they can be associated with encephalitis and brain tumors in humans and other species (46–50). Moreover, some AdV vectors are particularly promising for gene transfer to the CNS (51, 52). For instance, vectors derived from CAV-2 preferentially transduce neurons and their efficient axonal retrograde transport allows a widespread transduction in the CNS of rodents and dogs (27, 53). To better understand CAV-2 tropism and transport, we previously analyzed its axonal transport in motor neurons (8). We showed that CAV-2 transport from nerve terminals to the cell body was in pH-neutral vesicles, which underwent a maturation process requiring the sequential recruitment of the small GTPases Rab5 and Rab7. These axonal carriers are also used by clostridial neurotoxins and neurotrophins (26, 54), as well as poliovirus (55), and may represent a common organelle providing a protective environment responsible for long-range transport in neurons (1). We also showed that CAR, the docking molecule of CAV-2 (56, 57), was found in endosomes together with CAV-2, suggesting that it used CAR to access the CNS (8).

As a virus receptor, the role of CAR was mainly considered as a docking platform, with no primordial function described in internalization. Typically, it is thought that some AdVs use CAR to dock, followed by integrin-mediated internalization. This is supported by data showing that deletion of the intracellular tail of CAR still allowed AdV-mediated transduction (6).
Similarly, during CVB endocytosis in polarized epithelial cells CAR remained at the plasma membrane, whereas CVB was internalized (7). However, we previously showed that FKCAV triggered efficient CAR axonal transport, demonstrating that CAR could be directly linked to an endocytic pathway in motor neurons (8). We have now shown that CAV-2 and FKCAV triggered CAR internalization in different neuronal types and cell lines. Moreover, unlike that reported in other cell types, HAdV5 also triggered CAR internalization in neurons. As CAR engagement can regulate signaling pathways (10, 11), it is conceivable that CAR plays a yet undescribed role in endocytosis of AdVs and/or signaling cascades affecting AdV propagation in some cell types. Whether the neuronal signaling of CAR is pro- or antiviral is obviously unknown.

Notably, we show that not all CAR “ligands” behaved similarly. Antibodies directed against the extracellular D1 and D2 domains did not lead to CAR internalization, whereas FKCAV, CAV-2, and HAdV5 did. CAR can form homodimers in trans and in cis (18, 19, 28). The binding sites and affinity of viral and non-viral ligands have been measured and could explain why we observed differences in terms of ligand-induced internalization. Indeed, FKCAV, as a trimer, is theoretically able to engage three CAR molecules (9, 58), whereas CAV-2 and HAdV5 have 12 trimeric fibers and therefore are, in theory, able to engage 36 CAR monomers. Moreover, it is worth considering that the CAR-binding affinity of these ligands could also regulate its membrane trafficking: notably, FKCAV binds to CAR with a lower $K_d$ than FKHAdV5 (1.1 versus 7.9 nM) (9, 59). Similarly, FK-CAR affinity is higher than the CAR D1-D1 homotypic interaction ($K_d \sim 16$ nM) (60). Excess fiber production during HAdV5 propagation may perturb CAR-CAR homotypic interaction, as it binds similar residues in the D1 domain, and disrupts tight junctions during AdV epithelial infection (28).

The hypothesis we favor for ligand-induced CAR internalization is that CAR-CAR interaction needs to be displaced, as during FKCAV or AdV engagement: it would not be clustering, but disengagement that triggers CAR internalization. In support of this view, polyclonal anti-CAR antibodies, in conditions that mimic multimeric ligand engagement, did not induce CAR endocytosis. We postulate that disruption of the CAR-CAR interaction then favors, or unmasks, intra- or intermolecular interactions that will induce endocytosis. This, coupled to
amino acids between 261 and 274, could induce signals necessary for CAR entry (Fig. 11). Interestingly, CAR-binding CVB do not bind at the D1–D1 interface, but to residues situated on the side of the D1 domain (61). This observation is consistent with our hypothesis and why CAR would not be endocytosed during CVB entry in epithelial cells (7, 31). As viruses have been selected for their ability to structurally mimic endogenous host ligands (62), it is likely that a CAR ligand could trigger CAR-CAR disengagement and subsequent internalization. Interestingly, junctional adhesion molecule type L and AdVs bind similar domains on CAR with an affinity higher than CAR-CAR interaction ($K_d$ 5 versus $16\mu M$) to elicit signaling (10). However, the junctional adhesion molecule type L-CAR interaction occurs in trans and was not reported to trigger CAR internalization.

We found that CAR is recruited to cholesterol-rich microdomains in neurons. In contrast to EGFR, which exits lipid microdomains prior to endocytosis, CAR location was maintained during ligand-induced entry. Furthermore, the integrity of lipid microdomains was necessary for CAR endocytosis. Lipid microdomain-mediated entry is a general term to describe cholesterol-dependent mechanisms. However, lipid microdomains are heterogenous by nature and can differ in terms of composition and size (63, 64). Caveolin-mediated entry is probably the best described lipid microdomain-mediated endocytic pathway. However, although neurons express caveolin (65), there is no evidence that caveolae can form in neurons (66), which would exclude this endocytic route in CAR internalization. Flotillin-dependent entry is another cholesterol-dependent pathway. In neurons, flotillin-1 can regulate the endocytosis of several proteins including the amyloid precursor protein (67) and semaphorin 3A (68). Our data does not exclude the possibility that CAR could enter via this pathway.

Because the intracellular domain of CAR has a potential AP2 binding site, we also tested the involvement of clathrin and dynamin in the internalization of CAV-2. This site has been well characterized in the basolateral sorting of CAR through AP1 in epithelial cells, but its role in endocytosis was recently questioned (36, 69). Using deletion mutants lacking this site and overexpression of a dominant-negative AP2 mutant, we showed that CAR was still efficiently endocytosed following FK$^{CAV}$ engagement, suggesting that clathrin had a nonessential role in this process. Perturbation of dynamin function and actin depolymerization, however, inhibited CAR internalization.

Clathrin-independent, dynamin- and lipid microdomain-mediated endocytosis has been previously reported for cholera toxin (70), SV40 (71), and proteoglycan (72). Actin plays major roles in numerous internalization pathways (40), but its
FIGURE 10. CAR is transported and degraded in vivo. A, intraparenchymal injections of FK\textsuperscript{CAV} led to its axonal transport in connected regions. Scheme of brain regions showing Cy5-FK\textsuperscript{CAV} localization after injection in the striatum. Slices were stained for β3-tubulin (Tuj-1). B and C, injection of 5 μg of FK\textsuperscript{CAV} led to CAR degradation in the striatum. Representative immunoblots of at least three independent experiments show CAR expression in the mouse striatum. B, CAR levels 1 day post-injection of PBS or different doses of FK\textsuperscript{CAV}. A dose effect of FK\textsuperscript{CAV} injection on CAR expression was observed. C, time course effect of FK\textsuperscript{CAV} injection on CAR expression. CAR levels were restored one month post-injection (Ipsi, ipsilateral; Cont, contralateral; NI, non-injected). D, quantification of three independent experiments; results are expressed as mean ± S.E. compared with control set as 100%. *, p value <0.05; ***, p value <0.001 versus control). Scale bars, 20 μm.

FIGURE 11. Mechanisms of ligand-induced CAR endocytosis in neuronal cells. a, CAR (in green) is located in lipid rafts (in blue) and homodimeric interaction occurs through the D1 domain. b, the trimeric fiber knob (FK\textsuperscript{CAV}, in magenta), the CAR-interacting domain of some adenovirus types, binds with higher affinity to the D1-D1 interacting site and triggers D1-D1 disengagement. FK\textsuperscript{CAV} can engage one, two, or three CAR molecules leading to either clustering, or D1 disengagement in FK\textsuperscript{CAV} saturating conditions. Lipid raft integrity, dynamin, and actin (in purple) play crucial roles in subsequent internalization that will target CAR to endolysosomes for degradation (lysosomal enzymes represented by yellow Pacman). c, a region (amino acids 261–274) in the tail of CAR was essential for ligand-induced CAR endocytosis. d, clustering through antibody (in red) or coxsackievirus (7) binding, was insufficient to trigger CAR internalization, likely because they do not disrupt D1-D1 interactions and therefore do not favor or unmask, intra- or intermolecular interactions that regulate ligand-induced CAR endocytosis.
involvement in mammalian endocytosis remains controversial. Its role is best studied during clathrin-mediated endocytosis, but other routes also require actin dynamics, such as macropinocytosis and lipid-dependent entry (40). The analogy with SV40 and GM1-mediated internalization is pertinent, as SV40 also uses dynamin and lipid microdomains and also actin during entry (71). It was suggested that viral diffusion at the plasma membrane is blocked by actin assembly leading to its immobilization in lipid microdomains (73). Indeed, the formation of cholesterol-sensitive clusters may be regulated by cortical actin (74). CAR drifting at the plasma membrane has also been reported and was influenced by the actin cytoskeleton (75). Notably, actin interacts with the CAR cytoplasmic domain (41). Whether this interaction and its role in the regulation of CAR lateral diffusion at the plasma membrane are necessary for CAR endocytosis remain to be tested.

After FKCAV engagement ex vivo and in vivo, the majority of CAR was not recycled or transported to the Golgi network, but targeted to lysosomes for degradation. CTB and FKCAV shared CAR was not recycled or transported to the Golgi network, but endocytic machinery may also help to better understand how interactions with molecules regulating internalization and the function of CAR in the CNS as was reported for other CAMs. Whether this interaction and its role in the regulation of CAR lateral diffusion at the plasma membrane are necessary for CAR endocytosis remain to be tested.

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Viral Ligands Induce CAR Endocytosis

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VOLUME 289 • NUMBER 2 • JANUARY 10, 2014

JOURNAL OF BIOLOGICAL CHEMISTRY

694
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