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CAV-2 — why a canine virus is a neurobiologist's best friend

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Canine adenovirus type 2 (CAV-2) vectors are powerful tools for fundamental and applied neurobiology due to their negligible immunogenicity, preferential transduction of neurons, widespread distribution via axonal transport, and duration of expression in the mammalian brain. CAV-2 vectors are internalized in neurons by the selective use of coxsackievirus and adenovirus receptor (CAR), which is located at the presynapse in neurons. Neuronal internalization and axonal transport is mediated by CAR, which potentiates vector biodistribution. The above characteristics, together with the ~30 kb cloning capacity of helper-dependent (HD) CAV-2 vectors, optimized CAV-2 vector creation, production and purification, is expanding the therapeutic and fundamental options for CNS gene transfer.

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Introduction

Adenoviruses (AdVs) are 150-mDa nonenveloped pathogens. They have a linear double-stranded DNA genome of 28 — 42 kilobase pairs (kbp) packaged in a 90 nm icosahedral shell. An increasing number of the >200 AdVs isolated from human and nonhuman hosts are partially characterized and are being exploited for their potential as a gene transfer tools. Unfortunately, the characteristics of vectors derived from human type 5 (HAdV-C5) in mice have subjugated the majority of global research and medical communities' perception of AdV vectors for long-term gene transfer (in contrast to the use of AdV vectors for vaccines). We believed that it was unlikely that vectors derived from other AdV types would have a similar set of characteristics, and therefore we pushed forward with the development of nonhuman AdV vectors. One of these 'other' AdVs is canine adenovirus type 2 (CAdV-2, or commonly referred to as CAV-2). In most hosts, AdVs cause minor infection in the epithelium. In domestic dogs, CAV-2 typically causes an upper respiratory track disease. Back in the early 1990s the impetus to treat cystic fibrosis with gene therapy seemed feasible. Why create a vector from a virus that naturally infects members of the Canidae family [1]? CAV-2 infects the respiratory track, is mass-produced as a vaccine against CAV-1, and at the time was partially sequenced. CAV-2 seemed like an ideal choice to make the first nonhuman AdV vector to avoid the pre-existing immune response and treat cystic fibrosis. Although CAV-2 vectors efficiently transduce lung epithelial cells *in vivo* [2], gene transfer to lung for long-term therapy has encountered many obstacles.

Notably though, vector tropism is not restricted to tissues that manifest disease symptoms following wild type virus infections. During the development of CAV-2 vectors we found that intranasal, intramuscular and intracerebral injections preferentially transduce neurons [3]. In rat olfactory cavity, CAV-2 vectors preferentially transduce the olfactory neurons (in contrast to the neighboring columnar epithelial cells). When CAV-2 vectors are injected in muscles, they poorly transduce myofibres — while efficiently transducing the innervating motor and sensory neurons. To transduce motor and sensory neurons, whose somas are located near the spinal cord, CAV-2 traffics via retrograde axonal transport. In brain parenchyma CAV-2 vectors preferentially transduce neurons at the site of injection as well as the neurons that projected to the injected structure via retrograde axonal transport [3,4].

Because of these characteristics CAV-2 vectors are being used to understand higher-order brain functions and anatomical organization of neural circuits [5••,6,7,8•,9••,10,11••,12]. In addition to fundamental studies, the characteristics of CAV-2 vectors permit us to treat global brain diseases as well (see below).

Vectors

In the mid-1990s, creating replication-defective, E1-deleted (ΔE1) CAV-2 vectors was not as straightforward as that for HAdV vectors [13,14]. Transfection of overlapping fragments of the CAV-2 vector genomes in canine cells and expecting homologous recombination [13] never worked (even transfection of the intact 32 kb CAV-2 genome poorly produces viruses). Now, ΔE1 CAV-2 vector genomes are created by homologous recombination in

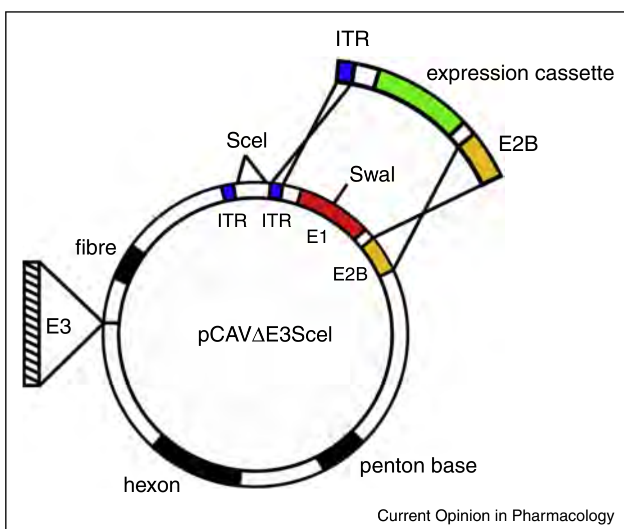
Escherichia coli [13,14] using the method described by Chartier *et al.* [15].

To generate ΔE1 CAV-2 vectors from plasmids that contained the ΔE1 vector genome, we introduced in canine cells (DK cells) a CAV-2 E1 region expression cassette to make DKE1 cells [16]. However, in contrast to 293 cells [17], DK cells are notoriously difficult to transfect with >30 kb linear fragments and initiation of replication of the CAV-2 vector genome liberated from a plasmid is also limited. Although we produced >100 ΔE1 CAV-2 vectors, we needed to remove this labor intense and expensive bottleneck. To circumvent the poor transfection efficacy of linear 30-kb fragments in DK cells, we created DKSce1 cells [18•]. DKSce1 cells are E1-transcomplementing cells expressing the estrogen receptor (ER) fused to I-SceI, a yeast meganuclease. Plasmids containing the I-SceI recognition sites flanking the CAV-2 genome were generated and, together with DKSce1 cells, these tools allow us to increase the efficiency of transfection with supercoiled DNA, and induce intracellular genome release due to the 4-OH-tamoxifen mediated nuclear translocation of I-SceI and excision of the vector genome [18•]. This technical improvement increased CAV-2 vector generation by ~1000 fold. We also deleted the E3 region from this plasmid to create pCAVΔE3Sce1, which increases the cloning capacity to ≥8 kb (Figure 1).

Helper-dependent vectors

The most efficient AdV vectors for gene transfer are called helper-dependent (HD) vectors [19]. They retain the ~200 bp inverted terminal repeats (ITRs) and the ~150 bp packaging signal (ψ), which are needed to replicate the genomes, but are void of regions coding for viral proteins. To generate a HD CAV-2 vector, the initial cloning of the HD vector genome is again in *E. coli* via homologous recombination using a combination of plasmids that maintains the size of the HD vector genome between 25 and 35 kbp [20]. The linearized HD genome is transfected into DKE1 cells and co-infected with a ΔE1 'helper' vector, which sequentially provides the viral proteins in *trans* during the 36 h propagation cycle. Both HD and helper genomes replicate, but to prevent helper vector packaging, the ψ in the helper vector is flanked with 34 bp loxP sequences that, in combination with constitutive or transient expression of Cre recombinase excises the ψ. This precludes packaging of the helper vector genome, while preferentially packaging the HD vector genome.

Figure 1



pCAVΔE3Sce1 plasmid, which is E3-deleted and contains two Sce1 sites between ITRs allowing intracellular genome release in DKSce1 cells. The expression cassette of interest is introduced by homologous recombination between ITR and E2B regions, replacing E1 region.

However, in DKE1 cells constitutively expressing Cre (DKCre cells), Cre recombinase activity negatively impacts cell viability, E1 region-encoded protein expression, and the production of HD CAV-2 vectors [21]. Because multiple amplification steps are needed to produce HD CAV-2 vectors, which hampers robust production and in turn the availability of high-quality HD vectors, we analyzed the progression of HD vector propagation cycle [22]. When compared with ΔE1 vectors, the helper genome replicates faster during HD CAV-2 vector production. This is mirrored by an upregulation of the CAV-2 polymerase, pre-terminal protein, and higher and earlier expression of structural proteins. Although genome packaging occurs similar to ΔE1 vectors, more immature capsids are generated during HD production. This leads to a ~4-fold increase in physical-to-infectious particles ratio and is concordant with increased autophagy and cell death, in which early cell death compromises volumetric productivity [22]. Therefore, there are still minor technical challenges that need to be overcome to optimize and democratize HD CAV-2 use.

CAV-2 tropism, neuron connectivity and nuclei function

Studies addressing AdVs receptor interactions and trafficking have helped pave the way toward the characterization of how CAV-2 engages the coxsackievirus and adenovirus receptor (CAR) and the endocytic machinery [23–30]. CAR is a member of the CTX subfamily of immunoglobulin (Ig) superfamily. In epithelial cells, CAR is a component of the tight junction complex at the basolateral membrane [31]. CAR was initially identified as a cellular protein involved in attachment and infection by group B coxsackieviruses (CVB) and later found to be an AdV receptor [32,33]. The knob region of the CAV-2 fiber (and fibers knobs of many human AdV types) interacts with the extracellular distal D1 domain region [32]. In epithelial-like cells, CAR is a docking factor for HAdV-C5, because CAR lacking its tail it is not significantly different from full length CAR during

HAdV-C5 capsid internalization [34]. These data led to the conclusion that HAdV-C5 internalization is mediated by integrins via engagement of the conserved RGD motif in the penton [23,35,36].

Interestingly, CAR is co-endocytosed upon engagement of CAV-2 and HAdV-C5 in neurons and in neuron-like cells, raising the possibility that CAR actively participates in endocytosis [26,28•]. During CAV-2 entry in motor neurons, CAV-2 and CAR are co-internalized at axon termini and transported in pH-neutral/Rab7^{+} endocytic vesicles [26]. Ligand-induced CAR endocytosis occurs via lipid rafts, and dynamin and actin play a crucial role to target CAR to lysosomes for degradation [28•]. Interestingly, the cytoplasmic tail of CAR appears to play a key role in CAV-2, but not HAdV-C5, internalization [37]. Several sequences in, and post-translational modifications of, the CAR tail such as the PDZ domain, clathrin adaptor protein binding site [33,38], palmitoylation and/or phosphorylation probably influence CAV-2 engagement/internalization. In fibroblast-like cells CAR is also co-endocytosed with CAV-2 and HAdV-C5, and the deletion of the tail does not impact the transduction of HAdV-C5 but impacts CAV-2 internalization showing that that the CAR tail differentially influences AdV internalization. Although HAdV5 is also a 'CAR-tropic' virus, and HAdV-C5 vectors can transduce neurons, HAdV-C5 preferentially transduces glia. We can only speculate as to why HAdV-C5 does not efficiently use neuronal CAR: it is conceivable that integrin-mediated internalization of HAdV-C5 is poorly functional CAR-rich lipid rafts of the neuron membrane, or that intracellular transport of HAdV-C5 is inefficient because HAdV-C5 is prematurely released from CAR-positive vesicles during axonal transport and precludes efficient delivery to the soma.

Most would agree that the CNS is a complex combination of partially characterized functional and neural circuits [5••,6,7,8•,9••,10,11••]. Identifying the involvement of a given neuronal pathway in a specific behavior is challenging due to the billion of neurons synapsing to 10–100 000 other neurons in the brain. Here, CAV-2 vectors are filling a void to help unravel the functional connectivity between neurons and nuclei [7,9••,10,11••]. Neurobiologists are taking advantage of CAV-2 transport from axon termini to distant somas to label and/or modify a group of neurons that projects to a defined region [12].

State-of-the-art techniques to activate specific neuronal pathways include using a combination of optogenetics [39] and 'designer receptor exclusively activated by designer drugs' (DREADD) technologies [40] via Flp-mediated or Cre-mediated recombination. By combining CAV-2 vectors and DREADDs one can activate or inactivate targeted neuronal pathways *in vivo* [11••,41•]. Thus, the use of CAVCre, a CAV-2 vector expressing Cre recombinase, and a second vector containing Cre-inducible DREADD expression cassette allows one to activate a group of neurons from one area of the nervous system that innervate a distal area (Figure 2). Using this approach Boender *et al.* probed the ventral tegmental area (VTA) to nucleus accumbens (Acb) pathway. A vector expressing hSyn-DIO-hM$_3$D(G$_q$)-mCherry was infused in VTA where the cell bodies of neurons that projects to Acb are located. Following this, CAVCre was infused in Acb, an area innervated by VTA neurons. CAVCre induced the expression of DREADD in neurons that project to Acb. Another study used the same tools to identify a subpopulation of neurons from parabrachial nucleus that project to the central nucleus of the amygdala, which are involved in appetite suppression [11••]. In some cases, this approach can circumvent the need to implement glass fibers for optogenetic stimulation and complements the use of transgenic Cre mice.

CAV-2 vector retrograde transport is also used to determine the molecular profiling of neurons on the basis of their connectivity [5••]. To this end, ribosomes were tagged with an anti-GFP camelid nanobodies, which captured translating mRNAs from neurons transduced with CAV-GFP vector (Figure 3). This technique identifies marker genes for neuronal populations on the basis of their connections, which are potentially relevant to a variety of behaviors. Selective optogenetic manipulation of a subset of neurons depending on their projections is also possible via the expression of channelrhodopsin2 (ChR2) or halorhodopsin, which allows one to determine the function of neurons of a given pathway [42,43].

CAV-2 vectors to understand and treat neurodegenerative diseases

What is the ideal therapeutic target for a vector with these capabilities? Therapies for diseases caused by deficiency in lysosomal enzymes, in particular the mucopolysaccharidoses (MPS) that affect the brain, are an unmet need. It is likely that the entire MPS brain needs to be treated and therefore a vector that is capable of widespread brain distribution is essential. In addition, the phenomena of cross correction — where lysosomal enzymes produced from a cell can be secreted and captured by neighboring cells [44] — will synergize with CAV-2 vectors to allow global brain therapy.

MPS VII is an extremely rare ($<$1/1 000 000 live births) autosomal recessive disorder caused by deficiency in the enzymatic activity of β-glucuronidase. MPS VII patients display a range of clinical variability, from the most severe with *hydrops fetalis* to an attenuated phenotype with late onset and almost normal intelligence. Impaired β-glucuronidase activity results in partial degradation of chondroitin sulfate, dermatan sulfate, heparan sulfate, and gangliosides, which progressively accumulate and are associated with hepatic, cardiovascular, respiratory, skeletal, corneal, and CNS lesions. β-glucuronidase deficiency has also been

Figure 2

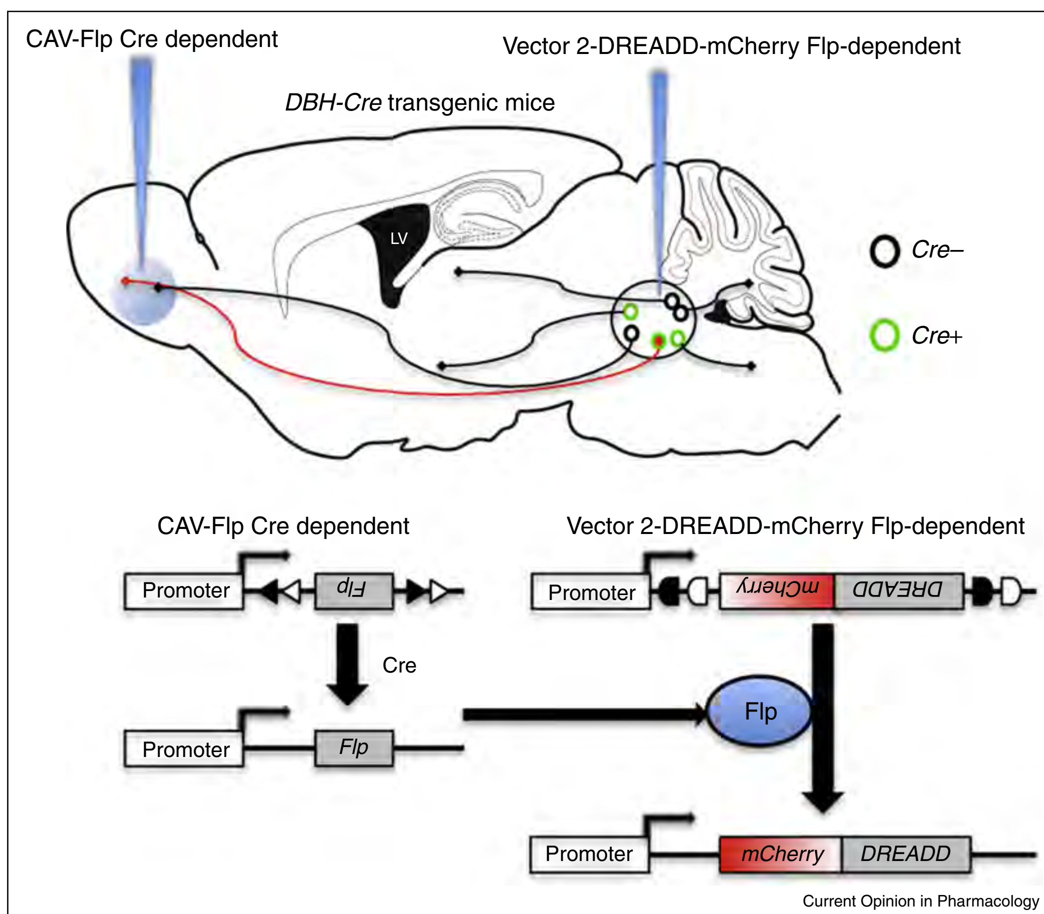
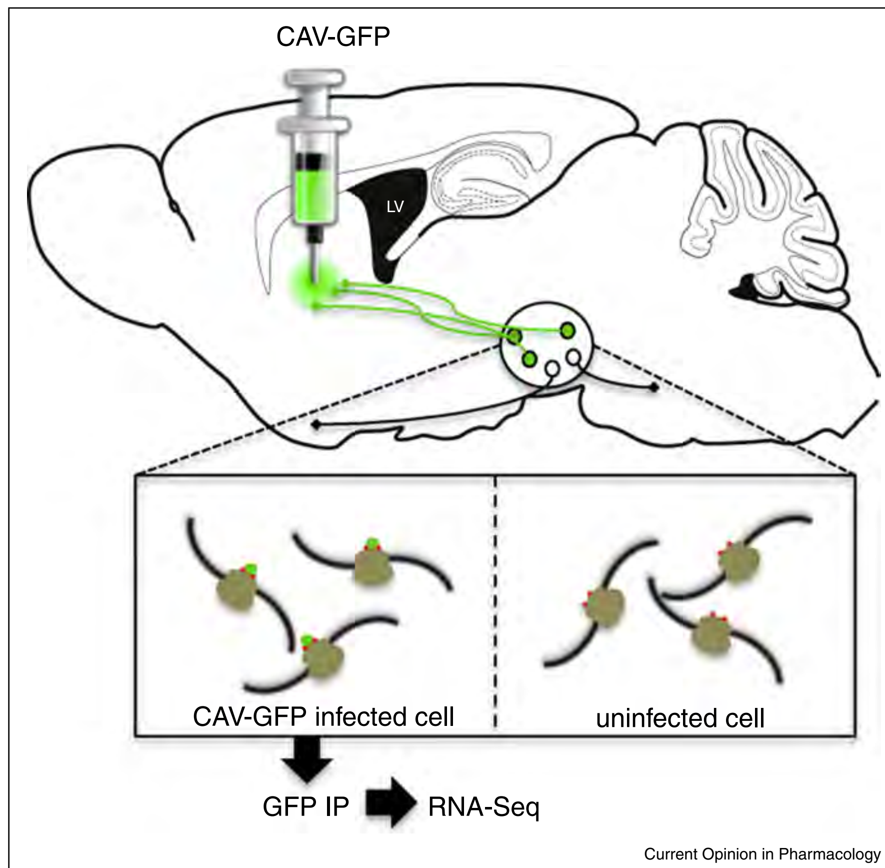


Diagram showing the use of Cre recombinase, Flpase and DREADDs to study the function of a specific subset of neurons. Vector harboring a Flp-dependent DREADD-mCherry cassette is injected to one specific area of the brain of mice expressing Cre under the control of dopamine beta-hydroxylase (DBH) promoter. CAV harboring a Cre-dependent Flp cassette is injected to another area of the brain and will be transported to the soma of neurons that project to this area. Flp will be expressed in infected dopaminergic neurons, and will turn-on the expression of DREADD in dopaminergic neurons in the area where vector 2 was injected and only in those neurons that project to the area where CAV was injected.

linked to neuroinflammation. We demonstrated that as little as 10^{10} physical particles of a HD CAV-2 vector expressing β-glucuronidase restores the global brain β-glucuronidase activity, reduces GAG accumulation, corrects the histological hallmarks (enlarge of GAG^{+} storage vesicles and irregular lysosome morphology) of MPS VII in the brains of MPS VII mice and dogs brains [8•,45,46]. More strikingly, MPS VII mice display improved cognitive functions following HD CAV-2 vector injections [46]. A notable result is the comparable efficacy of HD-CAV-2 vector expressing β-glucuronidase: the dose injected into the MPS VII dogs was 50 — 200-fold lower than that used for adeno-associated virus vectors in the brains of MPS I and MPS III dogs [47]. To put this dose into a more global preclinical gene transfer comparison, it would not be surprising to see similar doses of a vector used in the rodent brain during preclinical testing. The efficacy of HD CAV-2 vectors translates into using significantly less vector/patient, which reduces the costs and diminishes the risk of adverse effects, in particular a dose-dependent immune response to the vector in a diseased brain. Equally clinically relevant, and in contrast to the above study using adeno-associated virus vectors for MPS I/III therapy, only transient immunosuppression is necessary when using a HD CAV-2 vector and there is no obvious reduction in efficacy in the MPS VII dogs sacrificed at four versus one month postinjection.

MPS IIIA is also a rare autosomal recessive disorder leading to a severe neurodegenerative disorder caused by a deficiency of N-sulfoglucosamine sulfohydrolase (SGSH) activity. Deficiencies in SGSH activity cause the accumulation of partially degraded heparan sulfate glycosaminoglycan fragments, with patients exhibiting severe and progressive neurological deterioration usually resulting in death in the mid to late teenage years. Using CAV-2 vectors expressing SGSH Lau *et al.* corrected

Figure 3



Specific molecular profiling on the basis of neuronal connectivity after CAV-GFP injection. CAV-GFP is injected in a specific brain area of mice that express nanobody-L10 fusion protein in neurons. Only projective neurons to the site of injection will express GFP, which will bind to nanobody-tagged ribosomes. Immunoprecipitation for GFP will allow identifying marker genes for neurons on the basis of their projections.

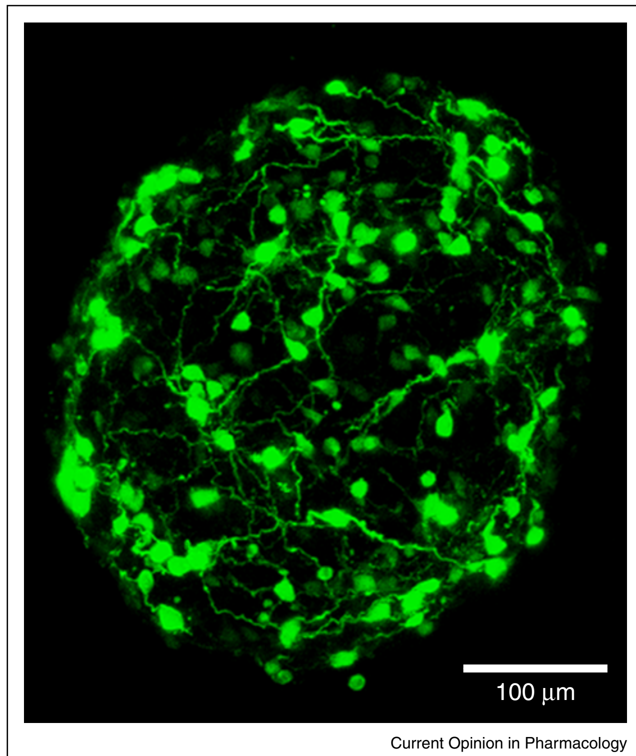
numerous areas of the MPS IIIA mouse brain [48,49]. In another line of therapy, a CAV-2 vector expressing neuroglobin, in combination with a *c-Jun N-terminal kinase* inhibitor, protects against oxidative stress and neuronal apoptosis induced by stroke in hypertensive rats [50]. In this study a direct comparison of CAV-2 vector biodistribution in the rat brain also showed that CAV-2 vectors significantly outperform HIV-1 vectors.

CAV-2 vectors can also be used to better understand disease etiology and progression. Because CAV-2 vector injection in the striatum can lead to transduction of ≥75% of the tyrosine hydroxylase-expressing neurons in the substantia nigra (SN) [3], CAV-2 vectors have the potential to mimic and/or treat Parkinson disease (PD). PD is characterized by the loss of dopaminergic neurons projecting from the SN to the striatum. One could either use a protective strategy to try to prevent dopaminergic neuron loss by expressing neurotrophic factors (e.g. glial cell-derived neurotrophic factor) that promote neuron survival, or create genetic models of PD by expressing disease-inducing proteins in dopaminergic neurons. Leucine rich repeat kinase 2 (LRRK2) mutations are the most common genetic cause of PD in northern Africa [51]. However, the size of the LRRK2 cDNA (~8 kb) precludes its efficient expression from most vectors — except HD CAV-2 vectors. Notably, HD CAV-2 LRRK 2 vector can induce Parkinsonism in nonhuman primates (unpublished data).

Preparing for clinical use

Although ΔE1 CAV-2 vectors allow expression >12 months in some species, HD vectors further improve the safety and durability of transgene expression. To prepare for a potential clinical use, scalable and robust production processes are required. The laboratory-grade DKE1/DKSce1 cell lines are unlikely to obtain regulatory approval for clinical grade vector production. Therefore, a GMP-compliant bioprocess was developed generating MDCK-E1 cells — propagated in scalable stirred bioreactors and using serum-free medium — to produce CAV-2 vectors that are then purified using column chromatography

Figure 4



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CAVGFP transduction of differentiated human neurospheres. Spinning disk confocal microscopy analysis of transduced 3D differentiated cultures: neurospheres were incubated with 100 infectious particles/cell for 4 h and imaged 5 days post-transduction; GFP expression in green (image provided by Catarina Brito).

steps [52]. Productivity, purity and quality of the CAV-2 vectors meet all the specifications set by the regulatory authorities for HAdV vectors. These results constitute key steps toward a scalable process for CAV-2 vector production compliant with clinical material specifications.

Finally, the efficacy to transduce human neurons (Figure 4) was compared to pseudotyped self-inactivating HIV-1 vectors and HD HAdV-C5 vectors. We found that CAV-2 vectors transduced significantly more neurons than HIV-1 vectors and HD HAdV-C5 vectors. In addition, with the goal of dissecting the toxicogenomic signatures of HIV-1, HD HAdV-C5, and HD CAV-2 vectors, we analyzed the transcriptional response of more than 47 000 transcripts in human neurons [53]. Both HD CAV-2 and HIV-1 vectors activate the innate arm of the immune response, including Toll-like receptors and hyaluronan circuits. HIV-1 vector also induce a type 1 interferon response, and affect the DNA damage pathways — but in opposite direction to HD-CAV-2 vectors — suggesting a differential response of the p53 and ATM pathways to the vector genomes. As a general response to the vectors, human neurons activate pro-survival genes and neuron morphogenesis, presumably with the goal of re-establishing homeostasis. However, in spite of this transcriptional signature induced by HD CAV-2 vectors, human neurons do not appear to develop a mechanistic response and therefore this sterile response does not perturb neuron homeostasis [54]. These data allow a better understanding of the impact of vectors on human neurons, and possible approaches to improve the therapeutic impact of brain-directed gene transfer.

Conclusion

As a colleague recently wrote '*Pardon the pun, but the use of CAV-2 vectors is going viral*'. Undaunted by (or ignorant of) the reputation of AdV vectors, the neurobiology community is exploiting the use of CAV-2 vectors by expressing DREADDs, inducible Cre and Flp recombinases, variants of channelrhodopsins, and other proteins in the mammalian brain. The use of CAV-2 vectors is impacting the understanding of brain biology and treatment of neurodegenerative diseases. Will CAV-2 vectors find a niche to treat human brain disease? In spite of their remarkable efficacy and encouraging results, the combination of the notorious reputation of 'adenovirus vectors', the technical challenges of producing HD vectors, and the emboldened results with some adeno-associated virus vectors, CAV-2 vector use may need to wait until the trend changes. But, they will be there, like a sleeping dog, when needed.

Conflict of interest

The authors declare that they have no relevant conflict of interest.

Note added in proof

The TRIO approach [12] used to map input–output connections in specific brain regions was also recently used by Beier *et al.* [55] to systematically map the relationships of ventral tegmental area dopaminergic neurons of the mouse brain.

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