Title: A next step in adeno-associated virus-mediated gene therapy for neurological diseases: regulation and targeting.
Authors: Chtarto A, Bockstael O, Tshibangu T, Dewitte O, Levivier M, Tenenbaum L
Journal: British journal of clinical pharmacology
Year: 2013 Aug
Volume: 76
Issue: 2
Pages: 217-32
DOI: 10.1111/bcp.12065

A NEXT STEP IN ADENO-ASSOCIATED VIRUS (AAV)-MEDIATED GENE THERAPY FOR NEUROLOGICAL DISEASES: REGULATION AND TARGETING.

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• **running head** : Pharmacology of AAV-mediated gene therapy in CNS.

• **keywords** : adeno-associated virus, gene therapy, Parkinson's disease, lysosomal storage diseases, leukodystrophies, gene regulation, tetracycline-inducible transcription, immune response.

• **word count** : 5,761 words.

• **number of figures** : 3.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bcp.12065

SUMMARY

rAAV vectors mediating long-term transgene expression are excellent gene therapy tools for chronic neurological diseases. While rAAV2 was the first serotype tested in the clinics, more efficient vectors deriving from the rh10 serotype are currently being evaluated and other serotypes are likely to be tested in the near future. In addition, aside from the currently used stereotaxy-guided intraparenchymal delivery, new techniques for global brain transduction (by intravenous or intra-cerebrospinal injections) are very promising.

Various strategies for therapeutic gene delivery to the CNS have been explored in human clinical trials in the past decade. Canavan disease, a genetic disease caused by an enzymatic deficiency, was the first to be approved. Three gene transfer paradigms for Parkinson's disease have been explored : converting L-dopa into dopamine through AADC gene delivery in the putamen ; synthesizing GABA through GAD gene delivery in the overactive subthalamic nucleus; and providing neurotrophic support through Neurturin gene delivery in the nigro-striatal pathway.

These pioneer clinical trials demonstrated the safety and tolerability of rAAV delivery in the human brain at moderate doses. Therapeutic effects however, were modest, emphasizing the need for higher doses of the therapeutic transgene product which could be achieved using more efficient vectors or expression cassettes. This will require re-addressing pharmacological aspects, with attention to which cases require either localized and cell-type specific expression or efficient brain-wide transgene expression, and when it is necessary to modulate or terminate the administration of transgene product. The ongoing development of targeted and regulated rAAV vectors is described.

INTRODUCTION

AAV vectors in the central nervous system

The isolation of a molecular clone of adeno-associated virus (AAV) serotype 2 into the pBR322 plasmid by Samulski in 1982 opened the door for the genetic analysis of this virus [1] [2]. The origin of replication, consisting of the two 145-nucleotide non-coding extremities (the inverted terminal repeats, or ITRs) of the viral genome was shown to be the only *cis*-acting element needed for replication and encapsidation of the viral DNA and for rescue from plasmids. This discovery enabled the generation of recombinant AAV (rAAV) vectors through the cloning of heterologous genes instead of the AAV genes between the ITRs. Recombinant viral particles are then produced by providing AAV genes and some helper adenoviral genes *in trans* [3] [4] [5] [6].

rAAV vectors are excellent tools in gene therapy for treatment of neurological diseases as they transduce post-mitotic cells [7] [8] [9] that mediate the sustained, long-term gene expression [10] [11] that is required to treat chronic diseases. rAAV serotype 2 was the first discovered serotype and is still the best-understood but vectors deriving from several other human or simian serotypes have demonstrated both a higher efficiency and a wider distribution of transgene expression in the CNS [12] [13] [14].

rAAV vectors have an excellent safety profile [15]. First, they elicit only low titer and transient neutralizing antibodies and no inflammation when administered in the brain [10] [16]. Given the high seropositivity for AAV in the human population, this immune response against rAAV in the presence of circulating anti-AAV antibodies [17, 18], is a particularly important issue [19]. It has been demonstrated recently that after intraparenchymal injections into the CNS, transgene expression is unaffected in immune-primed mice harbouring physiologically-relevant anti-AAV2 antibodies levels [20]. Notably, there are significant differences in the immune responses toward the different AAV serotypes in the human population. In particular, healthy

donors present lower antibody titers against AAV5, AAV8 and AAV9 as compared to AAV2 and AAV1 [21]. In one study however, probably due to the infection of antigen-presenting cells in the brain, high titer rAAV9 encoding non-self proteins elicited a strong cell-mediated and humoral immune response accompanied by a prominent inflammation [22]. Therefore, when using rAAV9 encoding non-human proteins (such as engineered transasctivators; see below), the immune and inflammatory responses should be carefully evaluated. In addition, caution should be taken when interpreting data of rAAV9-mediated transfer of human cDNAs in animal models.

Another risk, albeit a rare one, is vector DNA integration is a rare event resulting in a low risk of inadvertent oncogene activation [23]. Despite the increasing number of long-term studies in rodents and non human primates, none has reported deleterious DNA integration event in the brain [24]. In fact, increased tumor rates, clearly related to the vector have only been evidenced in a particular tumor-prone model-in the liver- after a partial hepatectomy, both of which conditions artificially increase cell proliferation and DNA rearrangements [25].

After intraparenchymal delivery, rAAV vectors target mainly neurons in addition to a low percentage of glial cells depending on the transcriptional regulatory elements [26] [27], the region [28] [29], the serotype [30] and the mode of delivery [31] [32] [33] [34] [35] [36] [37]. In a recent report, though, in striking contrast with previous studies [34] [35], rAAV9 intrastriatal delivery has been shown to mediate transgene expression equally efficiently in astrocytes and neurons [22]. It should be noted, however, that the volume of virus injected by Ciesielska and coll. (i.e. 10 µl) was 5-fold higher and the infusion speed 2.5-fold higher than in the other studies, conditions which might affect the transduction pattern [38].

The advent of new techniques for global brain transduction [39, 40] has opened new doors for the treatment of CNS diseases. These consists of injecting the virus intravenously exploiting the trans-blood-brain barrier delivery of rAAV9 [39] [32] [31] [41] or injecting it into the cerebrospinal fluid (CSF) via the cisterna magna [40]. With these new delivery modes, however, the immune and inflammatory responses to the vectors need to be revisited. In particular, it has been reported that

even a low neutralizing antibody titer in the blood of non-human primates abrogated transduction after intravenous delivery of rAAV9 [41]. Infusion of rAAV9 into the CSF via the cisterna magna led to dramatically stronger expression of the transgene and a wider distribution of gene transfer throughout the brain but, as with intravenous injection, a significant preexisting anti-AAV antibody titer abrogated brain transduction [33].

Gene therapy clinical trials in the CNS with AAV

Various strategies for therapeutic gene delivery to the CNS have been explored in pre-clinical studies and in the past decade in human clinical trials. We will only illustrate the main strategies which have reached the clinics. More extensive reviews on rAAV-mediated gene transfer in the CNS have been published recently [9] [6] [42].

Canavan disease (CD), is a leukodystrophy caused by genetic mutations of the aspartoacylase (ASPA) gene, a metabolic enzyme restricted in the CNS to oligodendrocytes and it was the first disorder to be approved for AAV gene therapy in the CNS [43] [44]. ASPA deficiency results in accumulation of N-acetyl-aspartate in oligodendrocytes and subsequent hypomyelination/dysmyelination. Although the first clinical trial testing rAAV therapy on CD demonstrated no therapeutic effect, it did demonstrate the safety and tolerability of rAAV2 vectors delivery in the brain. In a subset (3/10) of CD subjects, low to moderate levels of AAV neutralizing antibody with respect to baseline but, no increased inflammation or cellular immune response were reported [45]. These data suggest that, at the dose used (1×10^{12} vector genomes (vg) per patient) and with intraparenchymal administration, the approach is relatively safe. Potential explanations for the lack of therapeutical effect are the low efficiency and restricted distribution of rAAV2-mediated transgene expression and the absence of transgene expression in oligodendrocytes .

Correction of lysosomal enzyme deficiencies has also generated promising data. Late infantile neuronal ceroid lipofuscinoses (Batten Disease) is an autosomal recessive lysosomal storage disorder that results in progressive neurological degeneration. The disease is caused by

mutations in the CLN2 gene encoding a lysosomal tripeptidyl-peptidase whose deficiency causes accumulation of proteins in lysosomes of neurons leading to neuronal cell death. A rAAV2 vector expressing the human CLN2 cDNA was administered to 12 locations in the CNS of 10 children, enrolled in a Phase I clinical trial for Batten disease. Although this trial was not double-blinded and randomized, the data suggested, on the basis of a neurologic rating scale, that the rate of decline was significantly reduced in treated patients [46]. On the basis of promising preclinical studies [47], another clinical trial using the more efficient AAVrh10 serotype has been launched (http://www.abedia.com/wiley/record_detail.php?ID=1717).

Mucopolysaccharidosis type III (MPSIII), a disorder caused by the absence of one of the lysosomal enzymes required for the degradation of heparan sulfate (HS) which results in the accumulation of heparan sulfate oligosaccharides (HSOs) is also an interesting target for rAAV-mediated gene therapy. In MPSIIIB, α-*N*-acetylglucosaminidase (NaGlu) deficiency in particular is responsible for progressive mental neurodegeneration. Intracerebral stereotactic injections of AAV5 vectors coding for the missing enzyme reversed alterations of HS degradation, corrected pathology in neuronal cells and prevented neuroinflammation at the organ level in animal models [48]. Recently, a clinical trial using a rAAVrh10 vector has been launched for MPSIIIA, a deficiency in heparan-*N*-sulfamidase (http://www.abedia.com/wiley/record_detail.php?ID=318).

Parkinson's disease (PD) is also a good candidate for gene therapy since the main symptoms are caused by the progressive degeneration of a specific neuron population, the nigro-striatal dopaminergic (DA) neurons, located in a precise brain area, leading to the cardinal motor symptoms: bradykinesia, rigidity and tremor. Thanks to the discovery of the important role of dopamine depletion in PD, a breakthrough in the pharmacological treatment of PD was made in the 1960s, in the form of the oral administration of L-dopa, the precursor to dopamine [49]. Exogenous L-dopa is taken up into the remaining functional DA neurons where it is converted into dopamine by aromatic acid decarboxylase (AADC). With the ongoing loss of DA neurons, though, the uptake

of extracellular L-dopa declines and increasing doses of L-dopa need to be administered leading to important fluctuations (e.g. peak-dose dyskinesias). Continuous delivery of L-dopa directly to the striatum via gene therapy could be a significant improvement because L-dopa would reach only the clinically relevant target area [50]. A rAAV2 vector expressing AADC has been injected in PD patients' putamen in an attempt to reduce and stabilize the L-dopa dose necessary to alleviate the symptoms [51]. This combined treatment is predicted to provide more stable dopamine levels in the long-term than oral L-dopa treatment alone since AAV2-hAADC therapy results in expression of AADC in non-degenerating putaminal neurons [52], in contrast to nigral neurons that express endogenous AADC. Ten patients received bilateral intraputaminal infusions of 2 different doses of rAAV2-AADC (9×10^{10} and 3×10^{11} vg). Data based on PET imaging using a AADC-specific tracer [51] fluoro-L-tyrosine) [53] demonstrated stable transgene expression over 4 years confirming the preclinical data on the longevity of rAAV-mediated transgene expression [11]. This clinical trial further confirmed the safety and tolerability of rAAV intracerebral administration [54] but emphasized the need for a higher vector dose. The data showed dose-dependent improvements but even in the high-dose group in which the Unified Parkinson's disease Rating Scale (UPDRS) improved in all patients in the first 12 months, a slow deterioration was observed in subsequent years [53].

The motor abnormalities in PD are due to the overactivity of major output nuclei of the motor loop caused by the lack of dopamine in the striatum. Controlled inhibition of these excessively active nuclei can restore a normalized output to the cortex. Consistently, inhibiting the activity of the subthalamic nucleus (STN) by implanting adjustable electrodes provided impressive and immediate reversal of symptoms [55]. Using a similar paradigm, a rAAV2 vector expressing glutamic acid decarboxylase, the enzyme that synthesizes the inhibitory neurotransmitter GABA was injected in the STN. In preclinical studies, the vector transduced excitatory glutamatergic neurons which became inhibitory thereby reducing the STN activity [56]. A Phase I clinical trial demonstrated safety and tolerability of rAAV2 injections into the STN. Furthermore, 10 of 12

patients showed improvements in UPDRS at 12 months [57]. A subsequent double-blinded sham-surgery controled trial involving 16 patients injected with AAV2-GAD and 21 receiving control sham-surgery, confirmed the UPDRS improvements [58]. In this study, in which 23 controls and 22 treated patients were originally enrolled, in order to reinforce the statistical relevance of the data, the authors excluded from the analysis, the individuals for which identified technical failures occurred during the surgery.

However effective, these therapeutic strategies might be, though, they are only compensatory and are not expected to interfere with DA neuron cell death.

Providing neurotrophic support might constitute the first disease-interfering approach for PD. Glial cell line-derived neurotrophic factor (GDNF) and its analog Neurturin (NTN) were shown to protect and even in some instances restore DA neurons in most animal models (with the exception of alpha-synuclein overexpression [59]. Viral vectors expressing GDNF or NTN have been delivered in the striatum (Str) and the substantia nigra (SN) individually, and in both concurently. SN injections were efficiently protecting DA cell bodies but did not reverse motor symptoms due to the absence of enhanced striatal DA reinnervation [60] [61] [62]. In contrast, striatal injections protected both terminals and cell bodies and improve symptoms [61] [63] [64]. The fact that GDNF can be retrogradely transported from the striatum to the substantia nigra pars compacta [65] provides an interpretation for this observation.

A rAAV2 vector expressing NTN is currently being evaluated in Phase II clinical trials [66]. In a first trial, the virus was injected in the putamen (5.4×10^{11} vg per patient). At 12 months post-surgery, no statistical improvement in the UPDRS parameters could be established but in a subset of patients reaching 18 months post-surgery, several parameters were significantly improved [67, 68]. For 2 patients, post-mortem analysis revealed that NTN covered approximately 15% of the putamen. Contrary to data obtained with similar amounts of virus in monkey, though, very few NTN-positive cells were detected in the SN of the treated patients, suggesting a poor retrograde transport of NTN [69]. It should be noted that the enrolled patients were at a late PD stage and that

virus quantities were very limited for safety reasons. A second clinical trial has been launched using both putamen and SN as delivery area (as in Kordower's pioneer study, [63]) as well as a higher virus dose (2.4 x10^{12} vg per patient). Several preclinical [70] [71] [72] studies have described adverse effects related to uncontrolled dosage and off-target delivery of GDNF. In addition, a clinical trial using minipumps to deliver recombinant GDNF protein in patients' brain was interrupted due to the appearance of antibodies directed against GDNF [73]. Although none of these adverse effects have beed described so far in the AAV2-NTN gene therapy clinical trials, these observations raised the issue of the pharmacological aspects of rAAV-mediated gene delivery.

A variety of vectors are being designed and continuously improved to adapt to the needs of these very diverse situations. In some cases, localized (e.g GDNF) and cell-type specific (e.g. GAD) expression is required whereas in cases like MPSIII, brain-wide and efficient transgene expression is a prerequisite to success. In some cases, such with as the correction of enzymatic deficiencies, maximizing transgene expression is the ultimate goal whereas in other cases, such with many neuroprotective strategies, it will be necessary to regulate transgene expression in order to adapt the dose of the transgene product to the patient's needs and eventually terminate the treatment. The on-going development of better targeted and better regulated rAAV vectors is described in the following sections.

REGULATION OF TRANSGENE EXPRESSION

Several authors have reviewed the characteristics of an adequate system to regulate gene expression by exogenous drugs [74] [42] [75] [76]. An ideal regulatory system should harbour (a) a low basal activity in the uninduced state, (b) a high and inducer dose-dependent level of transgene expression in the induced state, (c) a rapid response to the administration or removal of the inducer, (d) a negligible toxicity, inflammatory response or immunogenicity associated with the regulatory elements, (e) absence of toxicity and good bioavailability of the inducing drug. The latter depends

on the target organ and mode of administration of the drug. In this section, we will only review regulatable systems used to control gene expression into the brain using rAAV vectors.

The constrained rAAV cloning capacity (~ 4.5 kb) limits the choice of regulatable systems. Drug-regulatable genetic systems usually comprise 2 elements : i) an inducible/repressible transcriptional promoter driving the expression of the transgene and ii) a genetically-engineered transactivator composed of a DNA-binding domain recognizing this promoter and interacting with the inducer, and an activator domain interacting with the cellular transcriptional machinery. Using two rAAV vectors to incorporate these 2 components separately requires that each cell is infected by both vectors at an optimal stoechiometry which is difficult to achieve *in vivo* [77] [78]. This has implications on the efficiency of transgene expression as the number of vector genomes rapidly decreases with distance from the injection site [26] [79]. Consistently the area covered by GDNF was considerably smaller despite a high vector dose ($4.12x10^{10}$ vg) when using a dual-component regulated rAAV [78] compared to single regulatable vector [72] [26] [80].

The Tet-system (Fig.1) is the most widely used technology for drug-dependent regulation of gene expression in eukaryotes. Since its conception by Gossen and Bujard in 1992, [81] various improvements have been made to the different components of the Tet-system. The Tet-Off system (Fig.1A), the first developed Tet-regulatable version, utilizes the *Escherichia coli* Tetracycline-repressor (TetR) protein fused to the activation domain of the herpes simplex virus type 1 (HSV1) VP16 transcription factor [81]. Being derived from a non-modified form of the TetR natural repressor, the Tet-Off system allows inhibition of transgene expression at a low inducer dose and has a low level of transgene expression in the non-induced state [82] [72]. However, the induction of expression *in vivo* is often slow and asynchronous because it requires complete removal of the inducer which depends on its half-life in the target organ [83, 84] [85] [82]. The half-life of elimination of doxycycline (Dox), a tetracycline analog widely used to control the Tet-system, is about 3 days [86]. Improvements made to the Tet-Off transactivator were focused on the VP16 activation domain to reduce its toxicity due to sequestering of transcriptional cellular factors [87].

A reverse transactivator (rtTA) which, unlike the Tet-Off system, responds to the presence of Dox by activating transgene expression [88] has been obtained by modification of 4 amino acids of the TetR domain by random mutagenesis, resulting in a "Tet-On" version of the Tet-system (Fig.1B). Modifications focused on the rtTA to increase its performance involve introduction of a nuclear localization signal [89] [90], codon usage optimization [91] [92] [93], removal of potential splice sites [91] and mutated activation domains [87] [91]. The most significant advances, however, came from the use of molecular evolution to identify improved rtTA mutants [94] [95].

Optimization of the *cis*-acting Tet-responsive promoter could also improve the Tet-system. Several modifications successfully reduced the basal activity of the Tet promoter [96] [97] [98] [99]. Usually, though, the decrease of the basal activity similarly affected the induced level of transgene expression. Interestingly, the latest developed Tet-promoter resulted in a low basal activity level while retaining a high activation potential [99].

Tet-regulatable rAAV vectors with different designs have been proposed, in order to avoid interferences with the transcriptional activity of the AAV ITR [100] and/or with the promoter used to express the Tet-transactivator [101] [102] [103] [104] [105] [72]. The size of the tetracycline-responsive *cis* elements and transactivator (2.1kb) allows the inclusion of many therapeutic and reporter genes with a size up to 2.4kb. Single vectors carrying the entire Tet-Off cassette have been used to regulate GFP [103] [104] [105] and GDNF [72] in healthy rat brain. In the latter case, an adverse effect of GDNF, weight loss, was shown to be tightly regulated by Dox [72]. Manfredsson and collaborators reported that the minimal Dox doses required to abrogate GDNF expression is 40 mg Dox/kg diet (corresponding to 2.4 mg/ kg) in SN and 100 mg Dox/kg diet (corresponding to 6 mg/ kg) in the striatum. The corresponding Dox serum levels were at least 8-fold lower than levels required for antimicrobial activity [106] and similar to those currently used as anti-inflammatory drug to treat rosacea [107], suggesting that clinically-acceptable Dox doses could be useful to regulate GDNF transgene expression in clinical trials.

Single rAAV using the Tet-On system were also described [101] [108] [80]. Using the rtTA2(S)M2 mutant developed by Urlinger, the quantity of Dox given to rats to achieve a biological effect was 600µg/ml in drinking water, equivalent to 70mg/kg for the GDNF transgene [108] [80] (Fig.2) and 3mg/kg for miRNA expression [101].

The constant antibiotics administration could lead to complications such as increased tolerance to tetracyclines or toxicity. Therefore, in order to minimize the period of treatment, the choice of an adequate version of the Tet-based system, will rely on the disease to be treated and the therapeutic strategy.

Due to the blood-brain barrier (BBB), the effective inducer dose required for the biological effect of a transgene delivered into the brain is less feasible than for one delivered to the peripheral organs. To overcome this difficulty, attempts have been made to optimize the genetic components of the Tet system or to modify the transactivator to interact with inducers having better pharmacokinetics properties (in particular, crossing the BBB more efficiently). The identification of alternative Tet system inducers could i) provide a better control of gene expression, ii) avoid the effect of long-term exposure to tetracyclines, i.e. selection of resistant bacteria and destabilization of bowel flora [109] toxicity including phototoxicity and accumulation in bones [110] [111] and iii) improve bioavailability in the brain thanks to more efficient passage through the BBB. Besides Dox, other tetracyclines are able to induce the tTA and rtTA. Methacycline [112], has an induction potency similar to Dox with a shorter half-life [113] which is an advantage for faster clearance. Minocycline is also a good inducer for the tTA and some versions of the rtTA [112] [114] and furthermore has the advantage to be anti-inflammatory in the brain [115] [116]. 4-epidoxycycline, a Dox metabolite without antibiotic activity which can induce the Tet-system [117] could also be an alternative. Chemically-modified tetracyclines (CMT) devoid of antibiotic activity [101] are also interesting but are not Tet-system inducers. Other inducers showing a higher affinity for rtTA2(S)M2 [91] than Dox on the basis of structural analysis while keeping the ability of Dox to cross the BBB have been discovered [118]. Finally, an alternative strategy to rapidly stop transgene

expression mediated by Tet-inducible vectors when the inducer's half-life is a limiting factor, is GR 33076X [119] a tetracycline antagonist able to competitively bind to the Tet transactivator. Notably, GR 33076X is not antibiotic and less toxic than other tetracyclines [119] [120].

The main disadvantage of the Tet system currently limiting its clinical use is the immune response elicited by the Tet transactivator. Indeed, after intramuscular delivery by plasmid-, adenovirus- [121] or AAV-mediated expression [122] [123] into non-human primates, a rapid loss of transgene expression correlating with a cellular immune response has been described. However, the immune reactions in the brain, an immune-privileged site, could be substantially less efficient from those in other organs, [124] [125] [126]. Indeed, tTA [127] and rtTA2(S)M2 do not elicit striatal immune response even when rats are pre-immunized [124]. However, the rodent immune system does not correlate directly with that of humans and the vast majority of the human population (60%) has been exposed to herpes simplex virus [128]. They may thus have circulating antibodies against the VP16 portion of the tTA/rtTA, which could block transgene expression. The use of immunologically-humanized mice could help predict the immune response to the Tet transactivator in patients [129]. Attemps to replace the viral VP16-derived activator domain by the human p65 activation domain of the NFκB complex, could help reducing the immunogenicity of the Tet transactivator [130]. In this respect, the use of autoregulatory vectors avoiding permanent expression of Tet-transactivators constitutes an improvement [102] [103] [131]. Epitope analysis and subsequent predictive design of new less immunogenic transactivators could help minimizing the deleterious effect of pre-existing HSV-1 seropositivity.

Another drug-inducible framework proposed for regulation AAV-mediated transgene expression in the brain is the rapamycin-inducible system [132]. However, until now this dual-vector system provides transgene expression levels that are much lower than constitutive promoters [78].

Methods to regulate transgene products posttranscriptionally have also been proposed. Although still in their infancy, it is worth citing: the regulation of protein stability through the binding of a small ligand, trimethoprim, to a destabilizing domain [133].

CELL TYPE-SPECIFICITY

Numerous AAV capsid variants, either naturally occuring (serotypes) or laboratory-engineered (by random mutagenesis or rationally-designed modifications), have been described [79] [134] [135] [136] [137] [138] [139] [140-143] [144] [145]. Capsid motifs are the key players in the interaction between viral particles and host cells mediating primary receptor attachment, secondary receptor-mediated cell entry and genome delivery to the nucleus, and as such, rAAV capsid variants are expected to differ in their cellular tropism. Interestingly, the variability between different serotypes is not evenly distributed along the capsid protein sequence but is higher in the domains that are displayed at the surface [146]. Thanks to the technology for trans-encapsidation of rAAV2 genomes into other serotype capsids [147], comparison between capsids without interferences due to the viral genome is possible.

Numerous comparisons between serotypes were performed in the CNS of mice [13] [148] [14], rats [34] [12] [149], cats [150] and non human primates [151] [152]. The majority of the tested serotypes or variants tested in rodents, mediate transgene expression mainly in neurons with the exception of AAVrh43 and rAAV4 which transduce glial cells [153] and ependymal cells [148] respectively, when injected in the striatum. In contrast, in monkeys a high proportion of reporter gene positive glial cells were observed with rAAV5 [151] and rAAV1 [154] .

In addition, different AAV serotypes transduce different neuronal subtypes. For example, in the rat hippocampus, rAAV2 targets dendate gyrus neurons whereas rAAV1 and 5 are more efficient in pyramidal neurons [12]. In the rat SN, rAAV2 mediates transgene expression exclusively in SN *pars compacta* [155] while rAAV1 and rAAV5 transduce *pars compacta* and

pars reticulata with similar efficiency[12]. Interestingly, the bb2 serotype transduces only medium spiny neurons in the rat striatum [153].

Other factors affecting rAAV cellular specificity include : the promoter, the age of the animals and the purity of the viral preparation. For example, rAAV8 using constitutive promoters almost exclusively mediate neuronal expression in adult brain. However, contaminants present in the stocks affected the virus' tropism resulting in a low propostion of astrocytes [34]. The mode of delivery can also determine the cell-type specificity of rAAV-mediated transgene expression. When injected in the brain parenchyma, rAAV9 directs transgene expression mainly in neurons [34] [35]. In contrast, intravascular rAAV9 delivery results in a variable percentage of astrocytes expressing the transgene depending on the age of the animal, the species and the structure of the rAAV genome. Indeed, when injected in the facial vein of new born and adult C57Bl/6 mice, respectively, mainly neurons or astrocytes expressed the transgene [31]. In addition, Gray et al. [41] reported that self-complementary AAV9 (a double-stranded form of AAV genome generated using a mutant ITR; see below) injected in the tail vein of adult BALB/c mice transduced twice as many neurons as astrocytes. This discrepancy might be explained by the injection site, the mice species or the genomic structure. Gray et al. further demonstrated that traditional single-stranded rAAV were far less efficient than self-complementary vectors. Finally, when the same vector was injected in the saphenous vein of non-human primate they observed a mainly astrocytic transduction [41]. Neuronal transduction was also reported by Duque and coll. in the spinal cord of adult mice after intravenous injection of self-complementary AAV9 in the temporal vein [32].

Differential rAAV cell-type specificity between fetal and adult brain was also reported. In rat fetal midbrain striatal grafts, rAAV1 allowed massive transduction of DA neurons whereas rAAV2 exclusively transduced non-DA neurons (Fig. 2) contrasting with rAAV2 tropism for adult midbrain DA neurons [155]. Similarly, rAAV1 but not rAAV5 encapsidating the same viral genome transduced fetal striatal neurons, whereas both very efficiently transduced adult striatum

[156]. Interestingly, enhanced glial gene delivery in the brain has been obtained by selecting AAV capsid variants through molecular evolution [5].

In order to restrict transgene expression in target cell populations, different cell type-specific promoters were tested. For example, using rAAV2, the neuron-specific enolase promoter mediated restricted expression into neurons [10] whereas with the cytomegalovirus (pCMV) promoter, a small proportion of astrocytes also expressed the reporter gene. Similar results were obtained with the human synapsin I promoter (phSYN) [157]. As expected, the tyrosine hydroxylase promoter allowed transgene expression restricted to DA neurons [158]. An elegant study using rAAV5-encapsidated bicistronic vectors comprising one expression cassette using a promoter sequence derived from the murine CMV (pmCMV) and another under the control of phSYN showed that pmCMV drives expression in cells located in the striosomes (possibly oligodendrocytes) whereas phSYN-driven expression was strictly neuronal [159]. In an experiment using rAAV8 and rAAVrh43 in combination with the GFAP astrocytic promoter, or the myelin basic protein (MBP) promoter active in oligodendrocytes Lawlor observed an expression restricted to astrocytes with GFAP promoter with both serotypes. With MBP promoter, in contrast, they observed reporter gene expression mostly in oligodendrocytes with rAAV8 and in both astrocytes and oligodendrocytes with rAAVrh43. Surprisingly, when rAAVrh43 pMBP was injected into the hippocampus, a weak expression of the reporter gene was only observed in a subpopulation of dentate gyrus neurons [153] indicating the necessity to take into account cell-specific promoters and capsid variants for targeting.

Viral or composite promoters thought to be ubiquituous can also affect the cell-type specificity of rAAV-mediated transgene expression. We showed that rAAV1 mediate differential specificities of transgene expression depending on whether the expression is driven by a pCMV promoter or a by Tet-ON cassette [26]. For example, in the midbrain, transgene expression was restricted to the DA regions (SNpc and VTA) with the TetON system but was widespread in the midbrain with pCMV. Promoter-related differential transduction has also been demonstrated in a

functional assay. In an epilepsy model, rAAV2 vectors expressing a NMDA receptor-1 (NR1) antisense under the control of pCMV and Tet-Off promoters caused an increase in seizures while pCMV-driven NR1 antisense expression lead to a significant reduction in seizures. Co-infusing these two vectors, each expressing a different reporter gene, demonstrated that they specify gene expression into different neuronal populations [131].

Intravascularly-administered rAAV6, rAAV8 and rAAV9 [58] are able to deliver genes to the brain. However, using this delivery mode, transgene expression is dramatically more efficient in the liver, spleen and heart than in the brain [41]. In order to restrict transgene expression to the CNS, Gao successfully repressed rAAV9 expression outside the CNS using a tissue-specific miRNA [160].

Another limiting step in rAAV-mediated gene delivery is the conversion of the single-stranded viral DNA into a transcriptionally-active double-strand DNA. The synthesis of the second strand complementary to the viral DNA is mediated by cellular factors which are present in limiting concentrations depending on the cell type [79]). Based on the discovery that (+) and (-) strand forms of AAV DNA are generated during the viral vector production [156], though, it has been proposed that when the genome copy number in a single cell is high enough, annealing of a sense sequence and an antisense sequence can occur. Based on the presence of dimeric head-to- head replicative forms, McCarty [161] has introduced a mutation within one of the ITRs which forces the encapsidation of dimers rather than monomers The two head-to-head halves of vector genomes called "self-complementary" (scAAV) can anneal and form a double-strand mediating faster and more efficient transgene expression in the brain [162]. It should be noted that, due to limited packaging capacity, the maximal genome size of scAAV is reduced by half compared to conventional vectors. ScAAV2 and scAAV1injected in the brain parenchyma mediate a faster onset of- and a stronger transgene expression than AAV2 but do not seem to alter the cellular tropism [163] [164]. Similarly, scAAV9 injected i.v. in the mice shows an efficacy similar to a 20-fold higher dose of AAV9 [41].

CONCLUSIONS

Various strategies for therapeutic gene delivery to the CNS have been explored. Aside from correction of recessive genetic defects [53] [165] [29] or gene silencing through RNA interference in autosomal dominant diseases [77] [6], gene transfer paradigms for diseases not having a clearly identified genetic origin have been explored. Interestingly, three different gene therapy approaches for sporadic PD have entered the clinics: converting L-dopa into dopamine through AADC gene delivery in the putamen [53]; synthesizing GABA through GAD gene delivery in the subthalamic nucleus [57] and providing neurotrophic support through Neurturin gene delivery in the nigro-striatal pathway [67]. It is worth citing another clinical trial for Parkinson's disease using a lentiviral vector which, like the AAV2-AADC gene therapy, encodes enzymes of the dopamine biosynthetic pathway. However, taking advantage of the larger cloning capacity of lentiviral vectors as compared to rAAV vectors, the 3 main enzymes required to achieve dopamine biosynthesis from tyrosine, i.e tyrosine hydroxylase, aromatic-L-acid decarboxylase (AADC) and GTP-cyclohydrolase I could be encoded in a single vector [166]. A clinical trial in which this vector is injected in the patients' putamen is ongoing (http://www.abedia.com/wiley/record_detail.php?ID=310).

In some cases, localized and cell-type specific expression is required. For example, the success of the rAAV2-GAD paradigm relies on the targeting of STN excitatory neurons to turn them into inhibitory neurons. In addition, off-target delivery might result in side effects. A clear demonstration was provided by Haberman and McCown showing that transferring a NMDA receptor anti-sense in different types of neurons may result in opposite behavioral effects [131]. Rationally-based targeting can be achieved by capsid engineering [136] [5] [167] or miRNA restricted-transgene expression [168]. In the case of motoneurons diseases, targeting can also be achieved through retrograde delivery [79] [129].

In other cases, in particular for enzymatic deficiencies, global and efficient transgene expression is a prerequisite to success. However, though global brain transduction is searched, cell-type specificity might still be a limitation. In leukodystrophies, for example, the missing enzyme must be supplied to oligodendrocytes [29]. Since the majority of gene therapy vectors, including rAAVs, transduce mostly neurons after intraparenchymal delivery in most brain regions [169] [12] [34] [155] [13], the derivation of vectors efficiently targeting oligodendrocytes is still a challenging issue. Interestingly, in regions that do not harbor neurons, such as the internal capsule or corpus callosum, glial cells are readily transduced [28] [27] [29]. On the other hand ,intravenous or intra-CSF delivery, results in astrocytic in addition to neuronal transduction [32] [31] [41]. A small degree of co-labeling between GFP and Olig1 (an marker for oligodendrocytes) is also mentioned in the last study [41].In contrast, in none of these reports were microglia transduced.

Pioneer clinical trials have established the safety and tolerability of rAAV2 delivery in the human brain at moderate doses. Therapeutic effects however, were modest, emphasizing the need for more efficient vectors and/or higher doses. More recently, clinical trials using AAVrh10 vectors for Batten disease (http://www.abedia.com/wiley/record_detail.php?ID=1717) and MPSIIIA (http://www.abedia.com/wiley/record_detail.php?ID=318) have been launched. Vectors deriving from other serotypes mediating efficient and widespread transgene expression are likely to be tested in patients in the near future. In addition, aside from the currently used stereotaxy-guided intraparenchymal delivery [170], new techniques for global brain transduction (by intravenous or intra-CSF injections) are very promising [171] [39]. These new vectors and delivery routes will require re-addressing the pharmacological aspects of rAAV-mediated gene transfer.

Adverse effects of uncontrolled or off-target delivery of neurotrophic factors have been described [71] [73] [42, 172]. With the launching of clinical trials for neuroprotective gene therapy with increasing vector doses [66], regulation of transgene expression will likely be required to avoid adverse events and, if necessary, terminate the treatment. Among the regulatable systems, the Tet-system is the most widely used and the best-developed [42]. However, important issues remain to

be solved. First, immune reactions to the VP16 portion of the Tet transactivator deriving from HSV are likely to arise in HSV-seropositive patients. Second, the inducer should be a clinically-approved molecule effective at a clinically-acceptable dose. The last developments of rAAV-based Tet-Off vectors, have shown regulation of an adverse effect of GDNF (weight loss) using a Dox dose which is below the approved anti-microbial dose [72]. However, therapeutical effects have not been addressed in this study. On the other hand, using a rAAV-tetON vector, Dox-dependent behavioral improvements have been demonstrated in a well-established rodent model of PD [80]. In this study, though, the Dox dose, by body mass was more than one order of magnitude higher than the approved dose for patients. The remaining challenges will be to characterize and eventually circumvent the immune reaction to the Tet transactivator as well as to obtain inducer-transactivator interactors fulfilling clinical requirements.

ACKNOWLEDGMENTS

The data of Fig. 1 and Fig. 3, were generated by Catherine Pythoud and Enni Lehtonen respectively. We thank Caleb McEntire for reading and proofreading the manuscript.

This work was supported by FNS Grant n° 31003A_127177 to ML and LT, by a grant from Association Française des Myopahties to LT and AC. TT was supported by Leonardo Mobility Fellowship (European Commission Lifelong Learning Program).

COMPETING INTERESTS

"All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

LEGENDS TO FIGURES

Figure 1.

A, Tet-Off system: in the absence of doxycycline the tTA transactivator binds to the tetO (tet operator) repeated sequence and activates transcription from the minimal promoter ($P_{hCMVmini}$) of human cytomegalovirus (hCMV). **B, Tet-On system**: in the presence of doxycycline the rtTA transactivator binds to the tetO repeated sequence and activates transcription from the minimal $P_{hCMVmini}$ promoter. P_{tet} is a fusion of seven repeated tetO sequence (7x42pbs) and the $P_{hCMVmini}$. The $P_{hCMVmini.}$ corresponds to the hCMV promoter without its enhancers sites. The (reverse) transactivator (r) tTA is composed of the (reverse) Tetracycline Repressor ((r)TetR) of the *Tn10 tetracycline resistance operon* of *Escherichia coli* and a portion of herpes simplex virus (HSV) protein 16 that functions as a potent activator of transcription.

Figure 2.

Regulation of GDNF transgene expression mediated by a single AAV-tetON tetracycline-inducible vector in the striatum in a partial rat model of PD.

A single injection of rAAV2/1-tetON-GDNF (3.5×10^8 vg/rat) was performed in the rat striatum. One month later, a 4-injection sites 6-hydroxydopamine lesion [61] was performed. Animals were continuously treated with doxycycline (A) (600 µg/l in drinking water)or remained untreated (B) and were sacrified one month post-lesioning.

Ten microns coronal sections were labeled with anti-GDNF antibodies using a peroxidase staining method [108].

Figure 3.

Differential cellular tropism of rAAV2/1 and rAAV2/2-mediated gene transfer into fetal ventral mesencephalon grafted in adult rat striatum.

The ventral mesencephali (VM) from 14-days embryos were dissected out and infected with rAAV vectors expressing eGFP reporter gene under the control of CMV promoter, trans-encapsidated into AAV1 (rAAV1-pCMV-eGFP) or AAV2 (rAAV2/2-pCMV-eGFP) capsids. Immediately after infection, VMs were dissociated into individual cells and animals were stereotactically infused into the right striatum with a cell suspension corresponding from the half of a VM per rat, as previously described [173] .

Four weeks posttransplantation, animals were sacrificed and 40-µm coronal brain sections were labeled using polyclonal rabbit anti-GFP antibodies (green fluorescence) and monoclonal anti-tyrosine hydroxylase antibodies (red fluorescence). Double-labeled cells appear yellow.

Scale bar : 100µm (10X pictures) and 50 µm (40X pictures).

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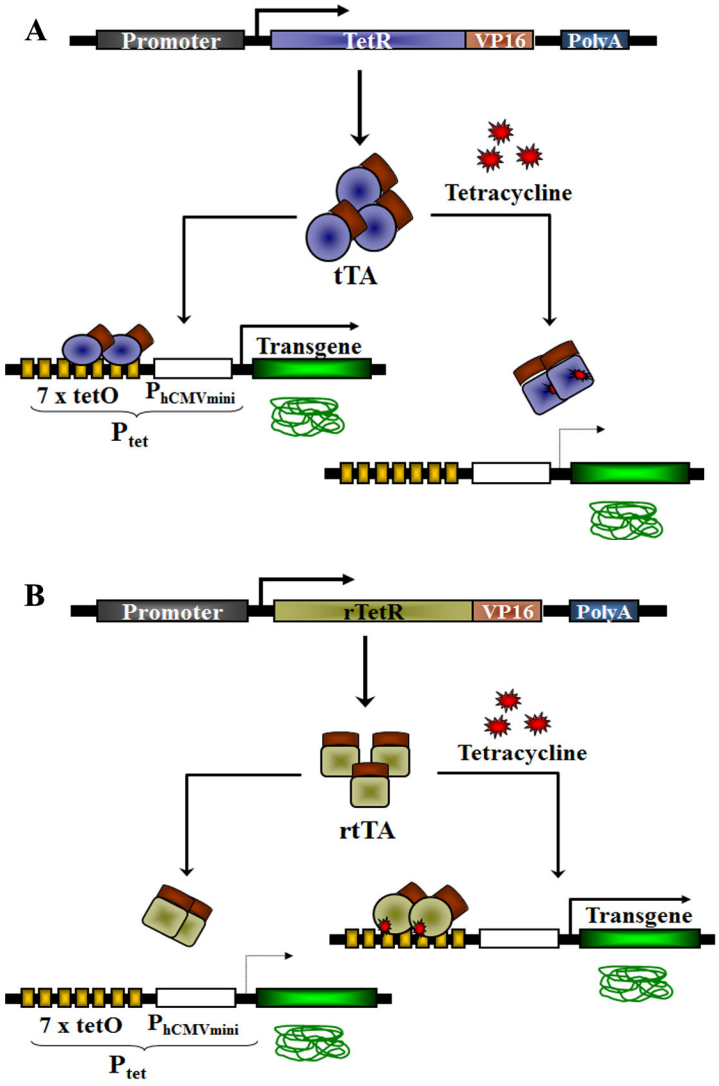

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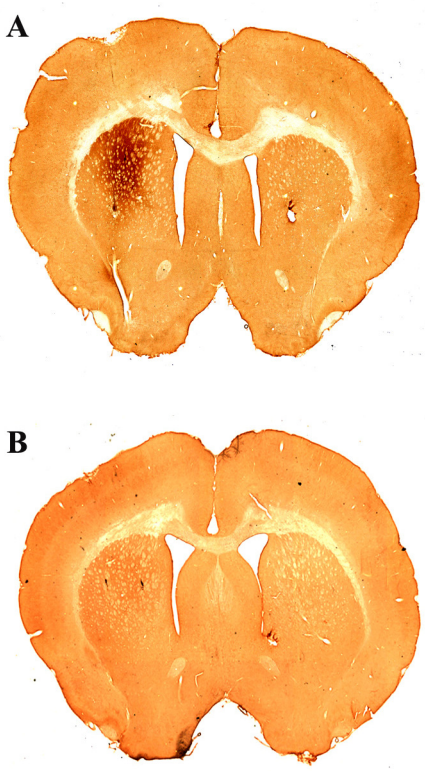

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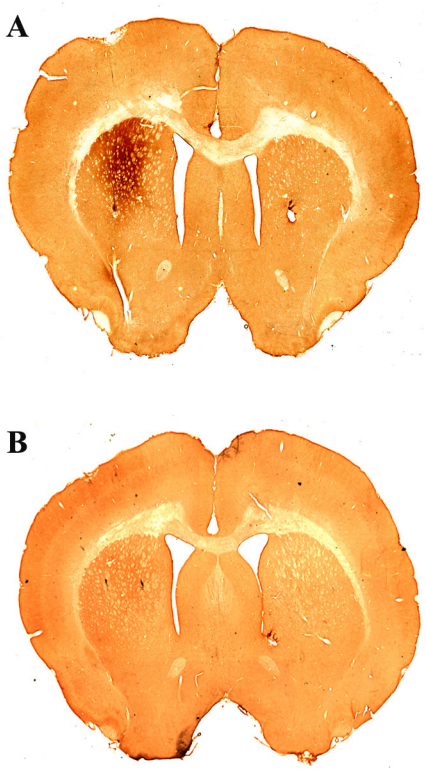

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