

Serveur Académique Lausannois SERVAL serval.unil.ch

Author Manuscript

Faculty of Biology and Medicine Publication

This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Published in final edited form as:

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](https://doi.org/10.1111/bcp.12065)

In the absence of a copyright statement, users should assume that standard copyright protection applies, unless the article contains an explicit statement to the contrary. In case of doubt, contact the journal publisher to verify the copyright status of an article.

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

A Chtarto^{1,2}, O.Bockstael^{1,2}, T Tshibangu³, O. Dewitte^{1,2}, M.Levivier³ and L.Tenenbaum^{3#}

¹Laboratory of Experimental Neurosurgery and ²Multidisciplinary Research Institute (I.R.I.B.H.M.), Free University of Brussels (ULB),

³Dpt of Clinical Neuroscience, CHUV, Lausanne

[#]Corresponding author : Laboratory of Cellular and Molecular Neurotherapy, Department of Clinical Neuroscience. Lausanne University Hospital, Switzerland

Tel: +41-21-3141048, E-mail: Liliane.Tenenbaum@chuv.ch

- **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 intracerebrospinal 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 transactivators; 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 μ l) 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 therapeutic 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 intraputaminous 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 controlled 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 concurrently. 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 (2.4×10^{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 been 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 stoichiometry 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 (4.12×10^{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]. Attempts to replace the viral VP16-derived activator domain by the human p65 activation domain of the NF κ B 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 occurring (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 dentate 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 proportion 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 ubiquitous 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 scAAV1 injected 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 Myopathies 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 ($P_{hCMV_{mini}}$) 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 $P_{hCMV_{mini}}$ promoter. P_{tet} is a fusion of seven repeated tetO sequence (7x42pbs) and the $P_{hCMV_{mini}}$. The $P_{hCMV_{mini}}$ 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 μ g/l in drinking water) or remained untreated (B) and were sacrificed 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- μ m 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 : 100 μ m (10X pictures) and 50 μ m (40X pictures).

REFERENCES

1. Samulski RJ, Berns KI, Tan M, Muzyczka N. Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proc Natl Acad Sci U S A* 1982;79(6):2077-81.
2. Laughlin CA, Tratschin JD, Coon H, Carter BJ. Cloning of infectious adeno-associated virus genomes in bacterial plasmids. *Gene* 1983;23(1):65-73.
3. Samulski RJ, Chang LS, Shenk T. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. *J Virol* 1989;63(9):3822-8.
4. Zolotukhin S, Potter M, Hauswirth WW, Guy J, Muzyczka N. A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. *J Virol* 1996;70(7):4646-54.
5. Koerber JT, Klimczak R, Jang JH, Dalkara D, Flannery JG, Schaffer DV. Molecular evolution of adeno-associated virus for enhanced glial gene delivery. *Mol Ther* 2009;17(12):2088-95.
6. Drouet V, Perrin V, Hassig R, Dufour N, Auregan G, Alves S, Bonvento G, Brouillet E, Luthi-Carter R, Hantraye P, Deglon N. Sustained effects of nonallele-specific Huntingtin silencing. *Ann Neurol* 2009;65(3):276-85.
7. Wu P, Phillips MI, Bui J, Terwilliger EF. Adeno-associated virus vector-mediated transgene integration into neurons and other nondividing cell targets. *J Virol* 1998;72(7):5919-26.
8. Schnepf BC, Clark KR, Klemanski DL, Pacak CA, Johnson PR. Genetic fate of recombinant adeno-associated virus vector genomes in muscle. *J Virol* 2003;77(6):3495-504.
9. Weinberg MS, Samulski RJ, McCown TJ. Adeno-associated virus (AAV) gene therapy for neurological disease. *Neuropharmacology* 2012.
10. Klein RL, Hamby ME, Gong Y, Hirko AC, Wang S, Hughes JA, King MA, Meyer EM. Dose and promoter effects of adeno-associated viral vector for green fluorescent protein expression in the rat brain. *Exp Neurol* 2002;176(1):66-74.
11. Hadaczek P, Eberling JL, Pivrotto P, Bringas J, Forsayeth J, Bankiewicz KS. Eight years of clinical improvement in MPTP-lesioned primates after gene therapy with AAV2-hAADC. *Mol Ther* 2010;18(8):1458-61.
12. Burger C, Gorbatyuk OS, Velardo MJ, Peden CS, Williams P, Zolotukhin S, Reier PJ, Mandel RJ, Muzyczka N. Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Mol Ther* 2004;10(2):302-17.
13. Taymans JM, Vandenberghe LH, Haute CV, Thiry I, Deroose CM, Mortelmans L, Wilson JM, Debyser Z, Baekelandt V. Comparative analysis of adeno-associated viral vector serotypes 1, 2, 5, 7, and 8 in mouse brain. *Hum Gene Ther* 2007;18(3):195-206.
14. Cearley CN, Wolfe JH. Transduction characteristics of adeno-associated virus vectors expressing cap serotypes 7, 8, 9, and Rh10 in the mouse brain. *Mol Ther* 2006;13(3):528-37.
15. Tenenbaum L, Lehtonen E, Monahan PE. Evaluation of risks related to the use of adeno-associated virus-based vectors. *Curr Gene Ther* 2003;3(6):545-65.
16. McCown TJ. Adeno-Associated Virus (AAV) Vectors in the CNS. *Curr Gene Ther* 2011;11(3):181-8.
17. Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J Infect Dis* 2009;199(3):381-90.
18. Calcedo R, Morizono H, Wang L, McCarter R, He J, Jones D, Batshaw ML, Wilson JM. Adeno-associated virus antibody profiles in newborns, children, and adolescents. *Clin Vaccine Immunol* 2011;18(9):1586-8.

19. Peden CS, Burger C, Muzyczka N, Mandel RJ. Circulating anti-wild-type adeno-associated virus type 2 (AAV2) antibodies inhibit recombinant AAV2 (rAAV2)-mediated, but not rAAV5-mediated, gene transfer in the brain. *J Virol* 2004;78(12):6344-59.
20. Treleaven CM, Tamsett TJ, Bu J, Fidler JA, Sardi SP, Hurlbut GD, Woodworth LA, Cheng SH, Passini MA, Shihabuddin LS, Dodge JC. Gene Transfer to the CNS Is Efficacious in Immune-primed Mice Harboring Physiologically Relevant Titers of Anti-AAV Antibodies. *Mol Ther* 2012;20(9):1713-23.
21. Boutin S, Monteilhet V, Veron P, Leborgne C, Benveniste O, Montus MF, Masurier C. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum Gene Ther* 2010;21(6):704-12.
22. Ciesielska A, Hadaczek P, Mittermeyer G, Zhou S, Wright JF, Bankiewicz KS, Forsayeth J. Cerebral Infusion of AAV9 Vector-encoding Non-self Proteins Can Elicit Cell-mediated Immune Responses. *Mol Ther* 2012.
23. Schnepf BC, Jensen RL, Chen CL, Johnson PR, Clark KR. Characterization of adeno-associated virus genomes isolated from human tissues. *J Virol* 2005;79(23):14793-803.
24. Bell P, Wang L, Lebherz C, Flieder DB, Bove MS, Wu D, Gao GP, Wilson JM, Wivel NA. No evidence for tumorigenesis of AAV vectors in a large-scale study in mice. *Mol Ther* 2005;12(2):299-306.
25. Rosas LE, Grieves JL, Zaraspe K, La Perle KM, Fu H, McCarty DM. Patterns of scAAV Vector Insertion Associated With Oncogenic Events in a Mouse Model for Genotoxicity. *Mol Ther* 2012.
26. Bockstael O, Chtarto A, Wakkinen J, Yang X, Melas C, Levivier M, Brotchi J, Tenenbaum L. Differential transgene expression profiles in rat brain, using rAAV2/1 vectors with tetracycline-inducible and cytomegalovirus promoters. *Hum Gene Ther* 2008;19(11):1293-305.
27. Chen H, McCarty DM, Bruce AT, Suzuki K, Suzuki K. Gene transfer and expression in oligodendrocytes under the control of myelin basic protein transcriptional control region mediated by adeno-associated virus. *Gene Ther* 1998;5(1):50-8.
28. Tenenbaum L, Jurysta F, Stathopoulos A, Puschban Z, Melas C, Hermens WT, Verhaagen J, Pichon B, Velu T, Levivier M. Tropism of AAV-2 vectors for neurons of the globus pallidus. *Neuroreport* 2000;11(10):2277-83.
29. Piguat F, Sondhi D, Piraud M, Fouquet F, Hackett NR, Ahouansou O, Vanier MT, Bieche I, Aubourg P, Crystal RG, Cartier N, Sevin C. Correction of Brain Oligodendrocytes by AAVrh.10 Intracerebral Gene Therapy in Metachromatic Leukodystrophy Mice. *Hum Gene Ther* 2012;23(8):903-14.
30. Cearley CN, Vandenberghe LH, Parente MK, Carnish ER, Wilson JM, Wolfe JH. Expanded repertoire of AAV vector serotypes mediate unique patterns of transduction in mouse brain. *Mol Ther* 2008;16(10):1710-8.
31. Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat Biotechnol* 2009;27(1):59-65.
32. Duque S, Joussemet B, Riviere C, Marais T, Dubreil L, Douar AM, Fyfe J, Moullier P, Colle MA, Barkats M. Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. *Mol Ther* 2009;17(7):1187-96.
33. Samaranch L, Salegio EA, San Sebastian W, Kells AP, Foust KD, Bringas JR, Lamarre C, Forsayeth J, Kaspar BK, Bankiewicz KS. Adeno-associated virus serotype 9 transduction in the central nervous system of nonhuman primates. *Hum Gene Ther* 2012;23(4):382-9.
34. Klein RL, Dayton RD, Tatom JB, Henderson KM, Henning PP. AAV8, 9, Rh10, Rh43 vector gene transfer in the rat brain: effects of serotype, promoter and purification method. *Mol Ther* 2008;16(1):89-96.

35. Xue YQ, Ma BF, Zhao LR, Tatom JB, Li B, Jiang LX, Klein RL, Duan WM. AAV9-mediated erythropoietin gene delivery into the brain protects nigral dopaminergic neurons in a rat model of Parkinson's disease. *Gene Ther* 2010;17(1):83-94.
36. Lowery RL, Zhang Y, Kelly EA, Lamantia CE, Harvey BK, Majewska AK. Rapid, long-term labeling of cells in the developing and adult rodent visual cortex using double-stranded adeno-associated viral vectors. *Dev Neurobiol* 2009;69(10):674-88.
37. Masamizu Y, Okada T, Kawasaki K, Ishibashi H, Yuasa S, Takeda S, Hasegawa I, Nakahara K. Local and retrograde gene transfer into primate neuronal pathways via adeno-associated virus serotype 8 and 9. *Neuroscience* 2011;193:249-58.
38. Mastakov MY, Baer K, Xu R, Fitzsimons H, During MJ. Combined injection of rAAV with mannitol enhances gene expression in the rat brain. *Mol Ther* 2001;3(2):225-32.
39. Fu H, Dirosario J, Killedar S, Zaraspe K, McCarty DM. Correction of neurological disease of mucopolysaccharidosis IIIB in adult mice by rAAV9 trans-blood-brain barrier gene delivery. *Mol Ther* 2011;19(6):1025-33.
40. Fu H, DiRosario J, Kang L, Muenzer J, McCarty DM. Restoration of central nervous system alpha-N-acetylglucosaminidase activity and therapeutic benefits in mucopolysaccharidosis IIIB mice by a single intracisternal recombinant adeno-associated viral type 2 vector delivery. *J Gene Med* 2010;12(7):624-33.
41. Gray SJ, Matagne V, Bachaboina L, Yadav S, Ojeda SR, Samulski RJ. Preclinical differences of intravascular AAV9 delivery to neurons and glia: a comparative study of adult mice and nonhuman primates. *Mol Ther* 2011;19(6):1058-69.
42. Manfredsson FP, Bloom DC, Mandel RJ. Regulated protein expression for in vivo gene therapy for neurological disorders: Progress, strategies, and issues. *Neurobiol Dis* 2012;48(2):212-21.
43. Leone P, Janson CG, Bilaniuk L, Wang Z, Sorgi F, Huang L, Matalon R, Kaul R, Zeng Z, Freese A, McPhee SW, Mee E, During MJ. Aspartoacylase gene transfer to the mammalian central nervous system with therapeutic implications for Canavan disease. *Ann Neurol* 2000;48(1):27-38.
44. Janson C, McPhee S, Bilaniuk L, Haselgrove J, Testaiuti M, Freese A, Wang DJ, Shera D, Hurh P, Rupin J, Saslow E, Goldfarb O, Goldberg M, Larijani G, Sharrar W, Liouterman L, Camp A, Kolodny E, Samulski J, Leone P. Clinical protocol. Gene therapy of Canavan disease: AAV-2 vector for neurosurgical delivery of aspartoacylase gene (ASPA) to the human brain. *Hum Gene Ther* 2002;13(11):1391-412.
45. McPhee SW, Janson CG, Li C, Samulski RJ, Camp AS, Francis J, Shera D, Lioutermann L, Feely M, Freese A, Leone P. Immune responses to AAV in a phase I study for Canavan disease. *J Gene Med* 2006;8(5):577-88.
46. Worgall S, Sondhi D, Hackett NR, Kosofsky B, Kekatpure MV, Neyzi N, Dyke JP, Ballon D, Heier L, Greenwald BM, Christos P, Mazumdar M, Souweidane MM, Kaplitt MG, Crystal RG. Treatment of late infantile neuronal ceroid lipofuscinosis by CNS administration of a serotype 2 adeno-associated virus expressing CLN2 cDNA. *Hum Gene Ther* 2008;19(5):463-74.
47. Sondhi D, Hackett NR, Peterson DA, Stratton J, Baad M, Travis KM, Wilson JM, Crystal RG. Enhanced survival of the LINCL mouse following CLN2 gene transfer using the rh.10 rhesus macaque-derived adeno-associated virus vector. *Mol Ther* 2007;15(3):481-91.
48. Cressant A, Desmaris N, Verot L, Brejot T, Froissart R, Vanier MT, Maire I, Heard JM. Improved behavior and neuropathology in the mouse model of Sanfilippo type IIIB disease after adeno-associated virus-mediated gene transfer in the striatum. *J Neurosci* 2004;24(45):10229-39.
49. Carlsson A. A half-century of neurotransmitter research: impact on neurology and psychiatry. Nobel lecture. *Biosci Rep* 2001;21(6):691-710.
50. Mandel RJ, Rendahl KG, Snyder RO, Leff SE. Progress in direct striatal delivery of L-dopa via gene therapy for treatment of Parkinson's disease using recombinant adeno-associated viral vectors. *Exp Neurol* 1999;159(1):47-64.

51. Eberling JL, Jagust WJ, Christine CW, Starr P, Larson P, Bankiewicz KS, Aminoff MJ. Results from a phase I safety trial of hAADC gene therapy for Parkinson disease. *Neurology* 2008;70(21):1980-3.
52. Sin M, Walker PD, Bouhamdan M, Quinn JP, Bannon MJ. Preferential expression of an AAV-2 construct in NOS-positive interneurons following intrastriatal injection. *Brain Res Mol Brain Res* 2005;141(1):74-82.
53. Mittermeyer G, Christine CW, Rosenbluth KH, Baker SL, Starr P, Larson P, Kaplan PL, Forsayeth J, Aminoff MJ, Bankiewicz KS. Long-term evaluation of a phase 1 study of AADC gene therapy for Parkinson's disease. *Hum Gene Ther* 2012;23(4):377-81.
54. Christine CW, Starr PA, Larson PS, Eberling JL, Jagust WJ, Hawkins RA, VanBrocklin HF, Wright JF, Bankiewicz KS, Aminoff MJ. Safety and tolerability of putaminal AADC gene therapy for Parkinson disease. *Neurology* 2009;73(20):1662-9.
55. Limousin P, Pollak P, Benazzouz A, Hoffmann D, Le Bas JF, Broussolle E, Perret JE, Benabid AL. Effect of parkinsonian signs and symptoms of bilateral subthalamic nucleus stimulation. *Lancet* 1995;345(8942):91-5.
56. Luo J, Kaplitt MG, Fitzsimons HL, Zuzga DS, Liu Y, Oshinsky ML, During MJ. Subthalamic GAD gene therapy in a Parkinson's disease rat model. *Science* 2002;298(5592):425-9.
57. Kaplitt MG, Feigin A, Tang C, Fitzsimons HL, Mattis P, Lawlor PA, Bland RJ, Young D, Strybing K, Eidelberg D, During MJ. Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. *Lancet* 2007;369(9579):2097-105.
58. LeWitt PA, Rezai AR, Leehey MA, Ojemann SG, Flaherty AW, Eskandar EN, Kostyk SK, Thomas K, Sarkar A, Siddiqui MS, Tatter SB, Schwalb JM, Poston KL, Henderson JM, Kurlan RM, Richard IH, Van Meter L, Sapan CV, During MJ, Kaplitt MG, Feigin A. AAV2-GAD gene therapy for advanced Parkinson's disease: a double-blind, sham-surgery controlled, randomised trial. *Lancet Neurol* 2011;10(4):309-19.
59. Lo Bianco C, Deglon N, Pralong W, Aebischer P. Lentiviral nigral delivery of GDNF does not prevent neurodegeneration in a genetic rat model of Parkinson's disease. *Neurobiol Dis* 2004;17(2):283-9.
60. Mandel RJ, Snyder RO, Leff SE. Recombinant adeno-associated viral vector-mediated glial cell line-derived neurotrophic factor gene transfer protects nigral dopamine neurons after onset of progressive degeneration in a rat model of Parkinson's disease. *Exp Neurol* 1999;160(1):205-14.
61. Kirik D, Rosenblad C, Bjorklund A, Mandel RJ. Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. *J Neurosci* 2000;20(12):4686-700.
62. Choi-Lundberg DL, Lin Q, Chang YN, Chiang YL, Hay CM, Mohajeri H, Davidson BL, Bohn MC. Dopaminergic neurons protected from degeneration by GDNF gene therapy. *Science* 1997;275(5301):838-41.
63. Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, McBride J, Chen EY, Palfi S, Roitberg BZ, Brown WD, Holden JE, Pyzalski R, Taylor MD, Carvey P, Ling Z, Trono D, Hantraye P, Deglon N, Aebischer P. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* 2000;290(5492):767-73.
64. Eslamboli A, Cummings RM, Ridley RM, Baker HF, Muzyczka N, Burger C, Mandel RJ, Kirik D, Annett LE. Recombinant adeno-associated viral vector (rAAV) delivery of GDNF provides protection against 6-OHDA lesion in the common marmoset monkey (*Callithrix jacchus*). *Exp Neurol* 2003;184(1):536-48.
65. Tomac A, Widenfalk J, Lin LF, Kohno T, Ebendal T, Hoffer BJ, Olson L. Retrograde axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult. *Proc Natl Acad Sci U S A* 1995;92(18):8274-8.
66. Bartus RT, Baumann TL, Brown L, Kruegel BR, Ostrove JM, Herzog CD. Advancing neurotrophic factors as treatments for age-related neurodegenerative diseases: developing and

demonstrating "clinical proof-of-concept" for AAV-neurturin (CERE-120) in Parkinson's disease. *Neurobiol Aging* 2012.

67. Marks WJ, Jr., Bartus RT, Siffert J, Davis CS, Lozano A, Boulis N, Vitek J, Stacy M, Turner D, Verhagen L, Bakay R, Watts R, Guthrie B, Jankovic J, Simpson R, Tagliati M, Alterman R, Stern M, Baltuch G, Starr PA, Larson PS, Ostrem JL, Nutt J, Kieburz K, Kordower JH, Olanow CW. Gene delivery of AAV2-neurturin for Parkinson's disease: a double-blind, randomised, controlled trial. *Lancet Neurol* 2010;9(12):1164-72.

68. Marks WJ, Jr., Ostrem JL, Verhagen L, Starr PA, Larson PS, Bakay RA, Taylor R, Cahn-Weiner DA, Stoessl AJ, Olanow CW, Bartus RT. Safety and tolerability of intraputamenal delivery of CERE-120 (adeno-associated virus serotype 2-neurturin) to patients with idiopathic Parkinson's disease: an open-label, phase I trial. *Lancet Neurol* 2008;7(5):400-8.

69. Bartus RT, Herzog CD, Chu Y, Wilson A, Brown L, Siffert J, Johnson EM, Jr., Olanow CW, Mufson EJ, Kordower JH. Bioactivity of AAV2-neurturin gene therapy (CERE-120): differences between Parkinson's disease and nonhuman primate brains. *Mov Disord* 2011;26(1):27-36.

70. Georgievska B, Kirik D, Bjorklund A. Aberrant sprouting and downregulation of tyrosine hydroxylase in lesioned nigrostriatal dopamine neurons induced by long-lasting overexpression of glial cell line derived neurotrophic factor in the striatum by lentiviral gene transfer. *Exp Neurol* 2002;177(2):461-74.

71. Hovland DN, Jr., Boyd RB, Butt MT, Engelhardt JA, Moxness MS, Ma MH, Emery MG, Ernst NB, Reed RP, Zeller JR, Gash DM, Masterman DM, Potter BM, Cosenza ME, Lightfoot RM. Six-month continuous intraputamenal infusion toxicity study of recombinant methionyl human glial cell line-derived neurotrophic factor (r-metHuGDNF) in rhesus monkeys. *Toxicol Pathol* 2007;35(5):676-92.

72. Manfredsson FP, Burger C, Rising AC, Zuobi-Hasona K, Sullivan LF, Lewin AS, Huang J, Piercefield E, Muzyczka N, Mandel RJ. Tight Long-term dynamic doxycycline responsive nigrostriatal GDNF using a single rAAV vector. *Mol Ther* 2009;17(11):1857-67.

73. Lang AE, Gill S, Patel NK, Lozano A, Nutt JG, Penn R, Brooks DJ, Hotton G, Moro E, Heywood P, Brodsky MA, Burchiel K, Kelly P, Dalvi A, Scott B, Stacy M, Turner D, Wooten VG, Elias WJ, Laws ER, Dhawan V, Stoessl AJ, Matcham J, Coffey RJ, Traub M. Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. *Ann Neurol* 2006;59(3):459-66.

74. Burcin MM, O'Malley BW, Tsai SY. A regulatory system for target gene expression. *Front Biosci* 1998;3:c1-7.

75. Stieger K, Belbellaa B, Le Guiner C, Moullier P, Rolling F. In vivo gene regulation using tetracycline-regulatable systems. *Adv Drug Deliv Rev* 2009;61(7-8):527-41.

76. Toniatti C, Bujard H, Cortese R, Ciliberto G. Gene therapy progress and prospects: transcription regulatory systems. *Gene Ther* 2004;11(8):649-57.

77. Davidson BL, McCray PB, Jr. Current prospects for RNA interference-based therapies. *Nat Rev Genet* 2011;12(5):329-40.

78. Hadaczek P, Beyer J, Kells A, Narrow W, Bowers W, Federoff HJ, Forsayeth J, Bankiewicz KS. Evaluation of an AAV2-based rapamycin-regulated glial cell line-derived neurotrophic factor (GDNF) expression vector system. *PLoS One* 2011;6(11):e27728.

79. Kaspar BK, Llado J, Sherkat N, Rothstein JD, Gage FH. Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. *Science* 2003;301(5634):839-42.

80. Yang X, Mertens B, Lehtonen E, Vercammen L, Bockstael O, Chtarto A, Levivier M, Brotchi J, Michotte Y, Baekelandt V, Sarre S, Tenenbaum L. Reversible neurochemical changes mediated by delayed intrastriatal glial cell line-derived neurotrophic factor gene delivery in a partial Parkinson's disease rat model. *J Gene Med* 2009;11(10):899-912.

81. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* 1992;89(12):5547-51.

82. Kistner A, Gossen M, Zimmermann F, Jerecic J, Ullmer C, Lubbert H, Bujard H. Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc Natl Acad Sci U S A* 1996;93(20):10933-8.
83. Corti O, Sanchez-Capelo A, Colin P, Hanoun N, Hamon M, Mallet J. Long-term doxycycline-controlled expression of human tyrosine hydroxylase after direct adenovirus-mediated gene transfer to a rat model of Parkinson's disease. *Proc Natl Acad Sci U S A* 1999;96(21):12120-5.
84. Corti O, Sabate O, Horellou P, Colin P, Dumas S, Buchet D, Buc-Caron MH, Mallet J. A single adenovirus vector mediates doxycycline-controlled expression of tyrosine hydroxylase in brain grafts of human neural progenitors. *Nat Biotechnol* 1999;17(4):349-54.
85. Harding TC, Geddes BJ, Murphy D, Knight D, Uney JB. Switching transgene expression in the brain using an adenoviral tetracycline-regulatable system. *Nat Biotechnol* 1998;16(6):553-5.
86. Maibach H. Second-generation tetracyclines, a dermatologic overview: clinical uses and pharmacology. *Cutis* 1991;48(5):411-7.
87. Baron U, Gossen M, Bujard H. Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. *Nucleic Acids Res* 1997;25(14):2723-9.
88. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. *Science* 1995;268(5218):1766-9.
89. Park HJ, RajBhandary UL. Tetracycline-regulated suppression of amber codons in mammalian cells. *Mol Cell Biol* 1998;18(8):4418-25.
90. Yoshida Y, Hamada H. Adenovirus-mediated inducible gene expression through tetracycline-controllable transactivator with nuclear localization signal. *Biochem Biophys Res Commun* 1997;230(2):426-30.
91. Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci U S A* 2000;97(14):7963-8.
92. Valencik ML, McDonald JA. Codon optimization markedly improves doxycycline regulated gene expression in the mouse heart. *Transgenic Res* 2001;10(3):269-75.
93. Wells KD, Foster JA, Moore K, Pursel VG, Wall RJ. Codon optimization, genetic insulation, and an rtTA reporter improve performance of the tetracycline switch. *Transgenic Res* 1999;8(5):371-81.
94. Das AT, Zhou X, Vink M, Klaver B, Verhoef K, Marzio G, Berkhout B. Viral evolution as a tool to improve the tetracycline-regulated gene expression system. *J Biol Chem* 2004;279(18):18776-82.
95. Zhou X, Vink M, Klaver B, Berkhout B, Das AT. Optimization of the Tet-On system for regulated gene expression through viral evolution. *Gene Ther* 2006;13(19):1382-90.
96. Agha-Mohammadi S, O'Malley M, Etemad A, Wang Z, Xiao X, Lotze MT. Second-generation tetracycline-regulatable promoter: repositioned tet operator elements optimize transactivator synergy while shorter minimal promoter offers tight basal leakiness. *J Gene Med* 2004;6(7):817-28.
97. Baron U, Freundlieb S, Gossen M, Bujard H. Co-regulation of two gene activities by tetracycline via a bidirectional promoter. *Nucleic Acids Res* 1995;23(17):3605-6.
98. Danke C, Grunz X, Wittmann J, Schmidt A, Agha-Mohammadi S, Kutsch O, Jack HM, Hillen W, Berens C. Adjusting transgene expression levels in lymphocytes with a set of inducible promoters. *J Gene Med* 2010;12(6):501-15.
99. Loew R, Heinz N, Hampf M, Bujard H, Gossen M. Improved Tet-responsive promoters with minimized background expression. *BMC Biotechnol* 2010;10:81.
100. Haberman RP, McCown TJ, Samulski RJ. Novel transcriptional regulatory signals in the adeno-associated virus terminal repeat A/D junction element. *J Virol* 2000;74(18):8732-9.
101. Chen Q, Xiong X, Lee TH, Liu Y, Sun QA, Wetsel W, Zhang X. Adeno-associated virus-mediated ILK gene silencing in the rat NAc core. *J Neurosci Methods* 2008;173(2):208-14.

102. Chtarto A, Bender HU, Hanemann CO, Kemp T, Lehtonen E, Levivier M, Brotchi J, Velu T, Tenenbaum L. Tetracycline-inducible transgene expression mediated by a single AAV vector. *Gene Ther* 2003;10(1):84-94.
103. Fitzsimons HL, McKenzie JM, During MJ. Insulators coupled to a minimal bidirectional tet cassette for tight regulation of rAAV-mediated gene transfer in the mammalian brain. *Gene Ther* 2001;8(22):1675-81.
104. Haberman RP, McCown TJ, Samulski RJ. Inducible long-term gene expression in brain with adeno-associated virus gene transfer. *Gene Ther* 1998;5(12):1604-11.
105. Jiang L, Rampalli S, George D, Press C, Bremer EG, O'Gorman MR, Bohn MC. Tight regulation from a single tet-off rAAV vector as demonstrated by flow cytometry and quantitative, real-time PCR. *Gene Ther* 2004;11(13):1057-67.
106. Ariza J, Bosilkovski M, Cascio A, Colmenero JD, Corbel MJ, Falagas ME, Memish ZA, Roushan MR, Rubinstein E, Sipsas NV, Solera J, Young EJ, Pappas G, International Society of C, Institute of Continuing Medical Education of I. Perspectives for the treatment of brucellosis in the 21st century: the Ioannina recommendations. *PLoS Med* 2007;4(12):e317.
107. Berman B, Perez OA, Zell D. Update on rosacea and anti-inflammatory-dose doxycycline. *Drugs Today (Barc)* 2007;43(1):27-34.
108. Chtarto A, Yang X, Bockstael O, Melas C, Blum D, Lehtonen E, Abeloos L, Jaspar JM, Levivier M, Brotchi J, Velu T, Tenenbaum L. Controlled delivery of glial cell line-derived neurotrophic factor by a single tetracycline-inducible AAV vector. *Exp Neurol* 2007;204(1):387-99.
109. Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 2001;65(2):232-60 ; second page, table of contents.
110. Sapadin AN, Fleischmajer R. Tetracyclines: nonantibiotic properties and their clinical implications. *J Am Acad Dermatol* 2006;54(2):258-65.
111. Rodriguez Hernandez H, Sanchez Anguiano LF, Quinones E. [Eradication of *Helicobacter pylori* in peptic ulcer and chronic gastritis. A randomized clinical trial]. *Rev Gastroenterol Mex* 1998;63(1):21-7.
112. Krueger C, Pflleiderer K, Hillen W, Berens C. Tetracycline derivatives: alternative effectors for Tet transregulators. *Biotechniques* 2004;37(4):546, 548, 550.
113. Agwuh KN, MacGowan A. Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylicyclines. *J Antimicrob Chemother* 2006;58(2):256-65.
114. Chtarto A, Tenenbaum L, Velu T, Brotchi J, Levivier M, Blum D. Minocycline-induced activation of tetracycline-responsive promoter. *Neurosci Lett* 2003;352(3):155-8.
115. Bantubungi K, Jacquard C, Greco A, Pintor A, Chtarto A, Tai K, Galas MC, Tenenbaum L, Deglon N, Popoli P, Minghetti L, Brouillet E, Brotchi J, Levivier M, Schiffmann SN, Blum D. Minocycline in phenotypic models of Huntington's disease. *Neurobiol Dis* 2005;18(1):206-17.
116. Blum D, Chtarto A, Tenenbaum L, Brotchi J, Levivier M. Clinical potential of minocycline for neurodegenerative disorders. *Neurobiol Dis* 2004;17(3):359-66.
117. Eger K, Hermes M, Uhlemann K, Rodewald S, Ortwein J, Brulport M, Bauer AW, Schormann W, Lupatsch F, Schiffer IB, Heimerdinger CK, Gebhard S, Spangenberg C, Prawitt D, Trost T, Zabel B, Sauer C, Tanner B, Kolbl H, Krugel U, Franke H, Illes P, Madaj-Sterba P, Bockamp EO, Beckers T, Hengstler JG. 4-Epidoxycycline: an alternative to doxycycline to control gene expression in conditional mouse models. *Biochem Biophys Res Commun* 2004;323(3):979-86.
118. Zhu P, Aller MI, Baron U, Cambridge S, Bausen M, Herb J, Sawinski J, Cetin A, Osten P, Nelson ML, Kugler S, Seeburg PH, Sprengel R, Hasan MT. Silencing and un-silencing of tetracycline-controlled genes in neurons. *PLoS One* 2007;2(6):e533.
119. Chrast-Balz J, Hooft van Huijsdijnen R. Bi-directional gene switching with the tetracycline repressor and a novel tetracycline antagonist. *Nucleic Acids Res* 1996;24(15):2900-4.

120. Love J, Allen GC, Gatz C, Thompson WF. Differential Top10 promoter regulation by six tetracycline analogues in plant cells. *J Exp Bot* 2002;53(376):1871-7.
121. Latta-Mahieu M, Rolland M, Caillet C, Wang M, Kennel P, Mahfouz I, Loquet I, Dedieu JF, Mahfoudi A, Trannoy E, Thuillier V. Gene transfer of a chimeric trans-activator is immunogenic and results in short-lived transgene expression. *Hum Gene Ther* 2002;13(13):1611-20.
122. Chenuaud P, Larcher T, Rabinowitz JE, Provost N, Joussemet B, Bujard H, Samulski RJ, Favre D, Moullier P. Optimal design of a single recombinant adeno-associated virus derived from serotypes 1 and 2 to achieve more tightly regulated transgene expression from nonhuman primate muscle. *Mol Ther* 2004;9(3):410-8.
123. Favre D, Blouin V, Provost N, Spisek R, Porrot F, Bohl D, Marme F, Cherel Y, Salvetti A, Hurtrel B, Heard JM, Riviere Y, Moullier P. Lack of an immune response against the tetracycline-dependent transactivator correlates with long-term doxycycline-regulated transgene expression in nonhuman primates after intramuscular injection of recombinant adeno-associated virus. *J Virol* 2002;76(22):11605-11.
124. Xiong W, Candolfi M, Kroeger KM, Puntel M, Mondkar S, Larocque D, Liu C, Curtin JF, Palmer D, Ng P, Lowenstein PR, Castro MG. Immunization against the transgene but not the TetON switch reduces expression from gutless adenoviral vectors in the brain. *Mol Ther* 2008;16(2):343-51.
125. Lowenstein PR, Mandel RJ, Xiong WD, Kroeger K, Castro MG. Immune responses to adenovirus and adeno-associated vectors used for gene therapy of brain diseases: the role of immunological synapses in understanding the cell biology of neuroimmune interactions. *Curr Gene Ther* 2007;7(5):347-60.
126. Streilein JW. Ocular immune privilege: therapeutic opportunities from an experiment of nature. *Nat Rev Immunol* 2003;3(11):879-89.
127. Han Y, Chang QA, Virag T, West NC, George D, Castro MG, Bohn MC. Lack of humoral immune response to the tetracycline (Tet) activator in rats injected intracranially with Tet-off rAAV vectors. *Gene Ther* 2010;17(5):616-25.
128. Xu F, Sternberg MR, Kottiri BJ, McQuillan GM, Lee FK, Nahmias AJ, Berman SM, Markowitz LE. Trends in herpes simplex virus type 1 and type 2 seroprevalence in the United States. *JAMA* 2006;296(8):964-73.
129. Towne C, Schneider BL, Kieran D, Redmond DE, Jr., Aebischer P. Efficient transduction of non-human primate motor neurons after intramuscular delivery of recombinant AAV serotype 6. *Gene Ther* 2010;17(1):141-6.
130. Burcin MM, Schiedner G, Kochanek S, Tsai SY, O'Malley BW. Adenovirus-mediated regulable target gene expression in vivo. *Proc Natl Acad Sci U S A* 1999;96(2):355-60.
131. Haberman R, Criswell H, Snowdy S, Ming Z, Breese G, Samulski R, McCown T. Therapeutic liabilities of in vivo viral vector tropism: adeno-associated virus vectors, NMDAR1 antisense, and focal seizure sensitivity. *Mol Ther* 2002;6(4):495-500.
132. Sanftner LM, Rivera VM, Suzuki BM, Feng L, Berk L, Zhou S, Forsayeth JR, Clackson T, Cunningham J. Dimerizer regulation of AADC expression and behavioral response in AAV-transduced 6-OHDA lesioned rats. *Mol Ther* 2006;13(1):167-74.
133. Iwamoto M, Bjorklund T, Lundberg C, Kirik D, Wandless TJ. A general chemical method to regulate protein stability in the mammalian central nervous system. *Chem Biol* 2010;17(9):981-8.
134. Shi W, Arnold GS, Bartlett JS. Insertional mutagenesis of the adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors targeted to alternative cell-surface receptors. *Hum Gene Ther* 2001;12(14):1697-711.
135. Bowles DE, McPhee SW, Li C, Gray SJ, Samulski JJ, Camp AS, Li J, Wang B, Monahan PE, Rabinowitz JE, Grieger JC, Govindasamy L, Agbandje-McKenna M, Xiao X, Samulski RJ. Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. *Mol Ther* 2012;20(2):443-55.

136. Asokan A, Schaffer DV, Samulski RJ. The AAV vector toolkit: poised at the clinical crossroads. *Mol Ther* 2012;20(4):699-708.
137. Grimm D, Lee JS, Wang L, Desai T, Akache B, Storm TA, Kay MA. In vitro and in vivo gene therapy vector evolution via multispecies interbreeding and retargeting of adeno-associated viruses. *J Virol* 2008;82(12):5887-911.
138. Gray SJ, Blake BL, Criswell HE, Nicolson SC, Samulski RJ, McCown TJ, Li W. Directed evolution of a novel adeno-associated virus (AAV) vector that crosses the seizure-compromised blood-brain barrier (BBB). *Mol Ther* 2010;18(3):570-8.
139. Johnson JS, Li C, DiPrimio N, Weinberg MS, McCown TJ, Samulski RJ. Mutagenesis of adeno-associated virus type 2 capsid protein VP1 uncovers new roles for basic amino acids in trafficking and cell-specific transduction. *J Virol* 2010;84(17):8888-902.
140. Girod A, Ried M, Wobus C, Lahm H, Leike K, Kleinschmidt J, Deleage G, Hallek M. Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. *Nat Med* 1999;5(12):1438.
141. Buning H, Ried MU, Perabo L, Gerner FM, Huttner NA, Enssle J, Hallek M. Receptor targeting of adeno-associated virus vectors. *Gene Ther* 2003;10(14):1142-51.
142. Perabo L, Endell J, King S, Lux K, Goldnau D, Hallek M, Buning H. Combinatorial engineering of a gene therapy vector: directed evolution of adeno-associated virus. *J Gene Med* 2006;8(2):155-62.
143. Boucas J, Lux K, Huber A, Schievenbusch S, von Freyend MJ, Perabo L, Quadt-Humme S, Odenthal M, Hallek M, Buning H. Engineering adeno-associated virus serotype 2-based targeting vectors using a new insertion site-position 453-and single point mutations. *J Gene Med* 2009;11(12):1103-13.
144. Klimczak RR, Koerber JT, Dalkara D, Flannery JG, Schaffer DV. A novel adeno-associated viral variant for efficient and selective intravitreal transduction of rat Muller cells. *PLoS One* 2009;4(10):e7467.
145. Peters-Silva H, Dinculescu A, Li Q, Deng WT, Pang JJ, Min SH, Chiodo V, Neeley AW, Govindasamy L, Bennett A, Agbandje-McKenna M, Zhong L, Li B, Jayandharan GR, Srivastava A, Lewin AS, Hauswirth WW. Novel properties of tyrosine-mutant AAV2 vectors in the mouse retina. *Mol Ther* 2011;19(2):293-301.
146. Gao G, Alvira MR, Somanathan S, Lu Y, Vandenberghe LH, Rux JJ, Calcedo R, Sanmiguel J, Abbas Z, Wilson JM. Adeno-associated viruses undergo substantial evolution in primates during natural infections. *Proc Natl Acad Sci U S A* 2003;100(10):6081-6.
147. Rabinowitz JE, Rolling F, Li C, Conrath H, Xiao W, Xiao X, Samulski RJ. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol* 2002;76(2):791-801.
148. Davidson BL, Stein CS, Heth JA, Martins I, Kotin RM, Derksen TA, Zabner J, Ghodsi A, Chiorini JA. Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. *Proc Natl Acad Sci U S A* 2000;97(7):3428-32.
149. McFarland NR, Lee JS, Hyman BT, McLean PJ. Comparison of transduction efficiency of recombinant AAV serotypes 1, 2, 5, and 8 in the rat nigrostriatal system. *J Neurochem* 2009;109(3):838-45.
150. Vite CH, Passini MA, Haskins ME, Wolfe JH. Adeno-associated virus vector-mediated transduction in the cat brain. *Gene Ther* 2003;10(22):1874-81.
151. Markakis EA, Vives KP, Bober J, Leichtle S, Leranath C, Beecham J, Elsworth JD, Roth RH, Samulski RJ, Redmond DE, Jr. Comparative transduction efficiency of AAV vector serotypes 1-6 in the substantia nigra and striatum of the primate brain. *Mol Ther* 2010;18(3):588-93.
152. Dodiya HB, Bjorklund T, Stansell J, 3rd, Mandel RJ, Kirik D, Kordower JH. Differential transduction following basal ganglia administration of distinct pseudotyped AAV capsid serotypes in nonhuman primates. *Mol Ther* 2010;18(3):579-87.

153. Lawlor PA, Bland RJ, Mouravlev A, Young D, During MJ. Efficient gene delivery and selective transduction of glial cells in the mammalian brain by AAV serotypes isolated from nonhuman primates. *Mol Ther* 2009;17(10):1692-702.
154. Hadaczek P, Forsayeth J, Mirek H, Munson K, Bringas J, Pivrotto P, McBride JL, Davidson BL, Bankiewicz KS. Transduction of nonhuman primate brain with adeno-associated virus serotype 1: vector trafficking and immune response. *Hum Gene Ther* 2009;20(3):225-37.
155. Klein RL, Meyer EM, Peel AL, Zolotukhin S, Meyers C, Muzyczka N, King MA. Neuron-specific transduction in the rat septohippocampal or nigrostriatal pathway by recombinant adeno-associated virus vectors. *Exp Neurol* 1998;150(2):183-94.
156. Nakai H, Storm TA, Kay MA. Recruitment of single-stranded recombinant adeno-associated virus vector genomes and intermolecular recombination are responsible for stable transduction of liver in vivo. *J Virol* 2000;74(20):9451-63.
157. Kugler S, Lingor P, Scholl U, Zolotukhin S, Bahr M. Differential transgene expression in brain cells in vivo and in vitro from AAV-2 vectors with small transcriptional control units. *Virology* 2003;311(1):89-95.
158. Oh MS, Hong SJ, Huh Y, Kim KS. Expression of transgenes in midbrain dopamine neurons using the tyrosine hydroxylase promoter. *Gene Ther* 2009;16(3):437-40.
159. Shevtsova Z, Malik JM, Michel U, Bahr M, Kugler S. Promoters and serotypes: targeting of adeno-associated virus vectors for gene transfer in the rat central nervous system in vitro and in vivo. *Exp Physiol* 2005;90(1):53-9.
160. Rahim AA, Wong AM, Hoefler K, Buckley SM, Mattar CN, Cheng SH, Chan JK, Cooper JD, Waddington SN. Intravenous administration of AAV2/9 to the fetal and neonatal mouse leads to differential targeting of CNS cell types and extensive transduction of the nervous system. *FASEB J* 2011;25(10):3505-18.
161. McCarty DM, Fu H, Monahan PE, Toulson CE, Naik P, Samulski RJ. Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo. *Gene Ther* 2003;10(26):2112-8.
162. McCarty DM, Monahan PE, Samulski RJ. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther* 2001;8(16):1248-54.
163. Fu H, Muenzer J, Samulski RJ, Breese G, Sifford J, Zeng X, McCarty DM. Self-complementary adeno-associated virus serotype 2 vector: global distribution and broad dispersion of AAV-mediated transgene expression in mouse brain. *Mol Ther* 2003;8(6):911-7.
164. Bockstael O, Melas C, Pythoud C, Levivier M, McCarty D, Samulski RJ, De Witte O, Tenenbaum L. Rapid transgene expression in multiple precursor cell types of adult rat subventricular zone mediated by adeno-associated type 1 vectors. *Hum Gene Ther* 2012;23(7):742-53.
165. Ciron C, Cressant A, Roux F, Raoul S, Cherel Y, Hantraye P, Deglon N, Schwartz B, Barkats M, Heard JM, Tardieu M, Moullier P, Colle MA. Human alpha-iduronidase gene transfer mediated by adeno-associated virus types 1, 2, and 5 in the brain of nonhuman primates: vector diffusion and biodistribution. *Hum Gene Ther* 2009;20(4):350-60.
166. Jarraya B, Boulet S, Ralph GS, Jan C, Bonvento G, Azzouz M, Miskin JE, Shin M, Delzescaux T, Drouot X, Herard AS, Day DM, Brouillet E, Kingsman SM, Hantraye P, Mitrophanous KA, Mazarakis ND, Palfi S. Dopamine gene therapy for Parkinson's disease in a nonhuman primate without associated dyskinesia. *Sci Transl Med* 2009;1(2):2ra4.
167. Chen H, McCarty DM, Bruce AT, Suzuki K. Oligodendrocyte-specific gene expression in mouse brain: use of a myelin-forming cell type-specific promoter in an adeno-associated virus. *J Neurosci Res* 1999;55(4):504-13.
168. Xie J, Xie Q, Zhang H, Ameres SL, Hung JH, Su Q, He R, Mu X, Seher Ahmed S, Park S, Kato H, Li C, Mueller C, Mello CC, Weng Z, Flotte TR, Zamore PD, Gao G. MicroRNA-regulated,

systemically delivered rAAV9: a step closer to CNS-restricted transgene expression. *Mol Ther* 2011;19(3):526-35.

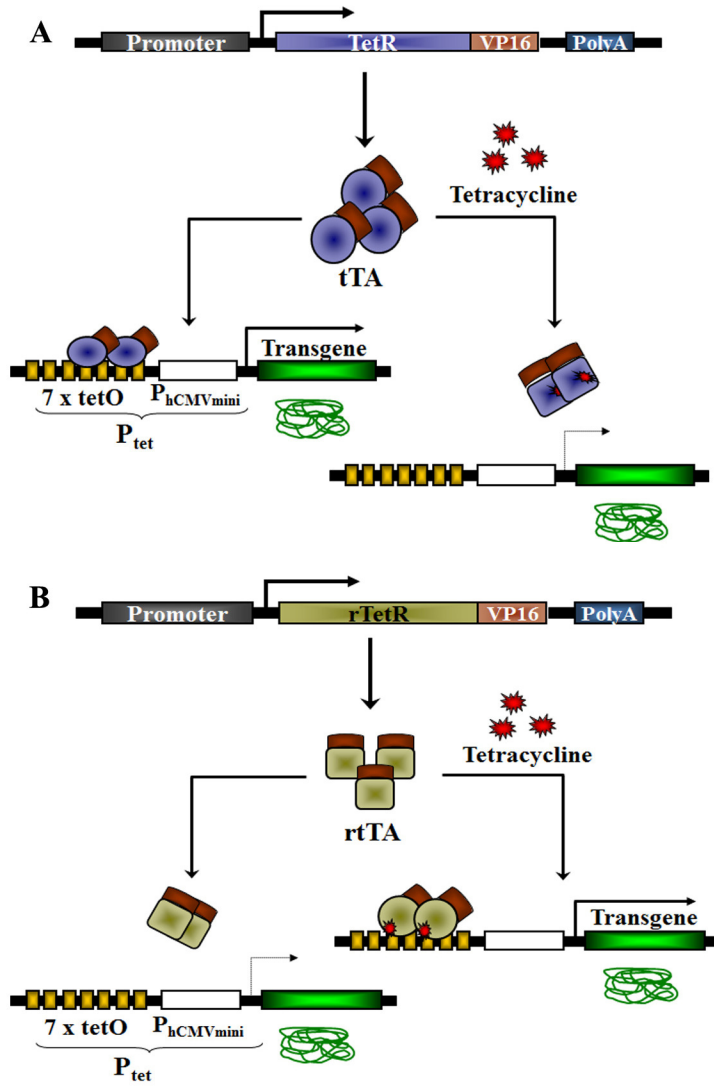
169. Bartlett JS, Samulski RJ, McCown TJ. Selective and rapid uptake of adeno-associated virus type 2 in brain. *Hum Gene Ther* 1998;9(8):1181-6.

170. Salegio EA, Samaranch L, Kells AP, Forsayeth J, Bankiewicz K. Guided delivery of adeno-associated viral vectors into the primate brain. *Adv Drug Deliv Rev* 2012;64(7):598-604.

171. Dayton RD, Wang DB, Klein RL. The advent of AAV9 expands applications for brain and spinal cord gene delivery. *Expert Opin Biol Ther* 2012;12(6):757-66.

172. Manfredsson FP, Tumer N, Erdos B, Landa T, Broxson CS, Sullivan LF, Rising AC, Foust KD, Zhang Y, Muzyczka N, Gorbatyuk OS, Scarpace PJ, Mandel RJ. Nigrostriatal rAAV-mediated GDNF overexpression induces robust weight loss in a rat model of age-related obesity. *Mol Ther* 2009;17(6):980-91.

173. Lehtonen E, Bonnaud F, Melas C, Lubansu A, Malgrange B, Chtarto A, Velu T, Brotchi J, Levivier M, Peschanski M, Tenenbaum L. AAV2 vectors mediate efficient and sustained transduction of rat embryonic ventral mesencephalon. *Neuroreport* 2002;13(12):1503-7.



chtarto_Figure_1.jpg



chtarto_Figure_2.jpg



chtarto_Figure3.jpg