Gene Therapy (2009) 16, 461–469

www.nature.com/gt

REVIEW

AAV for pain: steps towards clinical translation

AS Beutler and M Reinhardt
Department of Medicine (Hematology/Oncology), Mount Sinai School of Medicine, New York, NY, USA

Recombinant adeno-associated virus (rAAV) vectors consisting of self-complementary genomes and packaged in certain capsids can target primary sensory neurons efficiently and can control neuropathic pain long term by expressing opioid or non-opioid analgesic genes. This review examines the therapeutic potential of the approach in five sections: Pain control in oncology (including a discussion of cancer centers as translational pain research environment); vector biology; safety considerations and immunological lessons learned from rAAV clinical trials of other disorders; development of intrathecal rAAV therapy in rodent models of pain; and preclinical steps towards clinical translation of rAAV for pain. In the field of analgesic drug development, clinical validation of new approaches identified in rodents is currently a critical limiting step. Small-molecule therapeutics suitable as conventional drugs to probe novel targets in clinical trials are often unavailable. In this context, gene therapy could fill an important gap in the drug development process facilitating first-into-human trials of untested targeted treatments, each instantiated as a therapeutic gene.
Gene Therapy (2009) 16, 461–469; doi:10.1038/gt.2009.23; published online 5 March 2009

Keywords: *pain; dorsal root ganglion; AAV; endorphin; IL-10*

Introduction

Some recombinant adeno-associated virus (rAAV) vectors (that is, consisting of self-complementary genomes packaged in certain capsids) can target primary sensory neurons efficiently and control neuropathic pain long term by expressing opioid or non-opioid analgesic genes. Pain gene therapy with rAAV has to date been tested only in rodents. Yet, rAAV gene therapy of the retina and of other central nervous system (CNS) disorders has recently been tested in humans yielding promising results and thereby providing guidance, how this class of vector could be developed into a translational platform for clinical pain research. This review is divided into five sections. We will begin by offering a rationale for analgesic rAAV gene therapy focusing on the clinical challenge of intractable pain from advanced cancer, a likely focus of initial clinical trials. We will then provide an overview of rAAV vector biology referencing many of the reviews on the subject published in a recent special issue of this journal. We will then consider the safety of rAAV vectors and will provide a summary of recent rAAV clinical trials (for other CNS disorders) in that context. From there we will proceed by presenting our work on intrathecal (IT) rAAV vector development and studies in chronic neuropathic pain along with a discussion of earlier attempts by others and of the challenges that were encountered. Finally, we will propose a pathway of preclinical-to-clinical translation steps.

Correspondence: Dr AS Beutler, Departments of Oncology and Anesthesiology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA.
E-mail: beutler.andreas@mayo.edu
Received 6 January 2009; accepted 30 January 2009; published online 5 March 2009

'Intractable' pain from advanced cancer

Prevalence of chronic pain

Chronic pain from all causes is common. The National Institutes of Health states in a current program announcement: 'Pain is a critical national health problem. It is the most common reason for medical appointments, and costs this country over $100 billion each year in health care and lost productivity. Chronic pain affects more than 50 million Americans per year. Pain often results in disability and, even when not disabling, it has a profound effect on the quality of life.[1]' Among patients with cancer (unselected population) >60% suffer from chronic pain.[2–4] Pain treatment frequently fails even when the circumstances of care delivery are optimal, such as attentive, well-trained physicians; ready access to opioids; use of adjuvant analgesics; availability of patient-controlled analgesia; and evidence-based use of procedures like nerve blocks and IT pumps. Studies report rates of failure to control pain in cancer patients of 12–66%.[5–8] The lower analgesic failure rates (12–14%) apply to 'all comers'; the higher analgesic failure rates apply to patients with advanced solid tumors referred to pain specialists. Public interest in this health issue is considerable. 'Pain Control in Advanced Cancer' was ranked among the top 20 healthcare priorities by the Institute of Medicine in the 2003 report 'Priority Areas for National Action'.[9] For these areas, chronic pain, especially from advanced cancer, is a medical problem warranting research on novel agents, including highly experimental approaches such as gene therapy.

Failure of analgesic therapy

Opioid drugs like morphine are the mainstay of treatment for most severe chronic pain states, including

cancer pain. Their increased use has generally improved outcomes.[10–13] However, opioids fail in a significant number of patients due to side effects. Recent studies in cancer pain patients report failure rates of 12–66% for medical pain therapy.[5–8] To elucidate causes of pain treatment failure, Weiss *et al*.[8] studied the attitude of patients with severe pain (including cancer- and non-cancer pain states) towards their prescribed opioid doses. Of 514 patients treated for pain, 73% experienced moderate to severe pain. Yet, 60% of this group (that is, those with unrelieved pain) did not wish to increase their opioid dose. 10% even wished to reduce it. Major reasons given by patients for not wanting additional therapy with opioids were the mental side effects (sedation, cognitive failure, hallucinations) as well as the physical side effects (constipation, nausea) at the prescribed dose level. A 'Clinical Crossroads' discussion presented the dilemma in the words of a 44-year-old woman with lung cancer: 'So the biggest trade-off is that I'm in pain in order to stay lucid. I want to be awake and know everything that's going on, for as long as humanly possible. I do not want to die in pain. I do not want to be in pain. But I want to know what is going on around me.[14]' Here the pain has become 'intractable', because the available treatments fail to provide the kind of symptom control that would satisfy the patient. Symptom control is a main goal (requiring considerable time commitment) of most oncology practitioners.

Cancer center clinical research environment

Testing novel analgesics has thus far not been a focus of oncologists. Yet, the assessment of pain and symptom control is already common practice in most oncology clinical trials. Pain is frequently measured as secondary end point and/or to assess the toxicity of anti-neoplastic treatments. Thus, some clinical research expertise applicable to pain is available in cancer centers. More than a few cancer centers also have experience with gene therapy clinical trials, including that with the regulatory requirements imposed when recombinant DNA products are used in humans. Finally, patients choosing treatment at a major comprehensive cancer center are often attracted by clinical trial participation as a care option. Thus, novel phase I/II trials for pain, such as testing gene therapy, would seem to be a logical and realistic addition to the portfolio of 'experimental therapeutics' sections in oncology.

rAAV vectors

Wild-type versus recombinant AAV

Recombinant adeno-associated virus vectors are derived from a wild-type virus, AAV, which is non-pathogenic. No apparent ill effects have ever been associated with AAV even though the majority of humans have been exposed to AAV, as judged by serum neutralizing antibodies against at least one serotype.[15–18] It is therefore a common notion, that any derivative product should be at least as harmless.

Recombinant vectors are devoid of all AAV genes, that is, the *rep* and *cap* gene of the wild-type virus have been removed. The inverse terminal repeats (ITRs) are the only viral DNA sequences retained in the recombinant vector genome. It could therefore be argued, that rAAV should be even safer because *rep* and *cap* have been eliminated as a potential concern.

Favorable characteristics: targeting of non-dividing cells and long-term gene expression

Recombinant adeno-associated virus vectors can target non-dividing cells *in vivo* and express a transgene (therapeutic gene) long term. Our studies showed efficient targeting of primary sensory neurons and transgene expression for up to 4 months;[19,20] follow-up experiments found that transgene expression persisted undiminished for at least 15 months after gene transfer, the longest time point that we tested (unpublished).

Long-term gene expression by rAAV is such a universal finding in laboratory animals regardless of the organ targeted or the transgene used, that a finding of early cessation of vector activity (for example, Milligan *et al*., [21]) should raise concerns, whether the observed gene transfer was indeed due to rAAV or instead due to a contaminant (that should have been removed from the vector preparation) such as plasmid DNA.

Production

Preparation of highly pure, high-titer rAAV vectors is an advanced molecular biology task. It is generally more challenging than the production of most other viral vectors such as adenovirus or retroviruses. The proviral AAV plasmids must retain the ITRs, which can readily be lost through recombination. Two- or three-plasmid co-transfection of 293 (or similar) cells to provide the *rep* and *cap* genes, as well as adenoviral helper functions *in trans*, must be highly efficient (typically $\geqslant 50\%$), and the subsequent harvesting by freeze–thaw treatment and purification by repeat ultra-centrifugation in a cesium-chloride gradient must be optimized. We routinely achieve titers of $>10^{13}$ viral particles per ml allowing administration of 3×10^{10} particles in as little as <3 µl, if desired. Our standard amount of IT administered rAAV was 3×10^{9} in our initial study[19] and 3×10^{10} subsequently.[20] The higher amount became an option only as we grew to be more practiced with vector production. As we have yet to perform a definitive quantitative comparison, it is our impression that in the rat IT space, 3×10^{9} is around the minimum to achieve readily detectable gene expression in dorsal root ganglia (DRGs) and therapeutic efficacy in behavior models (when using self-complementary versions of serotype 1 or 8 vectors). 3×10^{10} provides an extra margin but probably less than a 10-fold increment in gene expression in this particular experimental paradigm. A typical rAAV preparation in our laboratory involves transfection of twenty 15-cm-diameter tissue culture dishes of cells, yielding $1–2 \times 10^{13}$ particles, which is sufficient material for experiments in several dozen rats. Experimentation in large animals, however, will stretch the approach such that a single preparation may only suffice for as few as one or two animals.

Manufacturing rAAV for clinical testing imposes substantial additional demands as reviewed by Wright[22] in the recent special AAV issue of this journal. Furthermore, clinical testing would require even larger vector quantities, because many doses are needed for quality control and a single batch has to last for an

entire trial. Production of such quantities by a small biotech company or an academically good manufacturing practices laboratory is possible, but with current methods being certainly demanding, it limits testing in patients to boutique-style clinical trials. Pharmaceutical scale production of vectors has not been achieved till date, but innovative methods have recently been reported, for example, the successful bioreactor-scale production with total yields potentially approaching the exa-(10^{18}) scale.[23–25] Whether such methods yield similar high-quality rAAV as do conventional methods, for example, a high ratio of packaged genomes to empty particles, will be critical for clinical progress in the rAAV field.

Capsid choice (pseudotyping) and the self-complementary rAAV variant

Recombinant adeno-associated virus vectors can be 'packaged' in capsids of many different serotypes. In all cases the packaged recombinant genome is derived from AAV2 (that is, the origin of the ITRs); hence the approach is referred to as 'pseudotyping'. The pseudotyped recombinant vectors are often designated rAAV2/1, rAAV2/5, rAAV2/8, *and so on*, referring to their hybrid origin. In this review, we simply refer to the pseudotyped vectors by the type of the capsid protein used, for example, rAAV8. The recombinant rAAV genome consists usually of single-stranded DNA as the genome of wild-type AAV. A deletion in the D-region of one of the ITRs of the proviral plasmids leads to efficient packaging of double-stranded rAAV, which are usually referred to as 'self-complementary' rAAV or sc-rAAV.[26] We have made extensive use of pseudotyping and of the sc-variant in our development of rAAV vectors for pain as described in detail below.

Recently, certain point mutations in the capsid protein of AAV2 were found to substantially increase transduction efficiency by improving viral trafficking to the nucleus;[27,28] the approach seems attractive and may warrant testing of such vectors in the IT space for transduction of DRG neurons.

Packaging capacity

A potential shortcoming of rAAV vectors is their limited packaging capacity of around 4.8 kB. After accounting for two ITRs, ~4.4 kB are left for regulatory elements and the transgene. Self-complementary vectors contain a third ITR and package two strands of the recombinant portion of the vector leaving only ~2.1 kB for the therapeutic expression cassette. We used the CMV (cytomegalovirus) promoter/enhancer and SV40 splice donor and acceptor sequences leaving less than 1 kB for the therapeutic cDNA. Although this has been sufficient to accommodate the therapeutic genes that we have used thus far, it creates a predicament for exploring larger therapeutic genes. Extended packaging capacities of up to 8.9 kB were recently reported with the use of serotype 5 capsids.[29] Although this serotype does not seem to be optimal in the IT space,[19] it raises the possibility that other capsids that target DRG neurons well could be found while accommodating larger therapeutic genes.

Safety considerations and clinical experience with rAAV vectors

Genomic integration

The wild-type AAV virus has long been known to integrate into a specific genomic locus in humans, AAVS1 on chromosome 19. Integration seems to be an essential step in the life cycle of wild-type AAV because it allows the virus to persist in a dormant state. Productive AAV infection requires a helper virus, most commonly adenovirus. rAAV vectors persist in targeted cells mainly in an episomal state. However, rAAV can also integrate into the genome. Integration in these instances seems to occur at random sites. This is an important difference from the wild-type virus, which requires *rep* for the site-specific integration mentioned above.[30] Random genomic integration of rAAV could, in principle, disrupt important host cell genes, such as tumor suppressors or oncogenes, and thereby act as carcinogen.

Recombinant adeno-associated virus can cause an increased incidence of liver tumors, when administered to newborn mice of certain susceptible strains.[31] Vector integration in tumors was shown. In at least some of the cases, integration occurred in a microRNA locus on mouse chromosome 12. This led to regional transcriptional activation of many small RNA genes with known growth regulatory properties, a plausible mechanism of carcinogenesis.[32] Another study found an increased incidence of liver tumors only with a specific transgene, but not with rAAV, administration *per se*.[33] Such findings seem to be limited to rAAV administration in newborn (as opposed to adult) mice; might occur only in specific, susceptible strains (such as knockout mice with lysosomal storage disease), in which liver tumors can even occur spontaneously; could have been favored by relatively high-vector doses (1.5×10^{11} particles per newborn mouse); and were thus far not noted in other organs or other species.

Serological immunity

Recombinant adeno-associated virus consistently induces serological immunity (that is, antibodies in the blood), if administered systemically in any laboratory animal tested or in humans.[17] Antibodies are often 'neutralizing', that is, counteracting the ability of rAAV to infect target cells. Serological immunity can also be induced, if rAAV is administered into the CNS of animals[34] or humans,[35–37] albeit the response seems to be milder and shorter lived. Whether systemic antibodies can interfere with transduction in the CNS and whether antibodies can be induced in the cerebrospinal fluid (CSF) will be discussed below in the sub-section *Immunity in the CNS*. Serological immunity can also be directed against a rAAV-encoded transgene product, especially if it is a 'foreign' protein, such as a clotting factor, in a patient with a truncating mutation. Regarding the development of pain therapy, this possibility should be born in mind in case an allogenic therapeutic gene is considered.

Cellular immunity: man versus mice

Recombinant adeno-associated virus escapes cellular immunity in all animal models tested to date, but seems

to induce it in humans, if administered outside the CNS.[38–40] Specifically, in a recent clinical trial, substantial transgene expression levels, achieved in two patients during the first month after portal vein infusion of AAV2, rapidly declined to undetectable levels at 8 weeks. This was accompanied by a moderate rise of liver transaminases indicative of a liver-directed inflammatory response. CD8+ lymphocytes specific for the AAV2 capsid protein (restricted by the patient's MHC (major histocompatiblity complex)) were isolated from both patients. The investigators therefore suggested that the loss of transgene production was caused by cellular immunity.[38,41–43] The notion that cellular immunity is directed against the capsid protein may seem counterintuitive, because the vector does not express the capsid gene; instead, the capsid protein present in the recombinant vector at the time of administration seems to persist for several weeks *in vivo*, marking targeted cells for immune-mediated cytotoxic destruction. The scientific quandary ironically derives from the fact that no such reaction seems to occur in any animal model. Therefore, rAAV researchers are left with minimum clinical data on cellular immunity and no good way to study the problem in the laboratory.[38]

Immunity in the CNS

Priming and execution of immune responses depends on the anatomic site involved, for example, effective antigen presentation by dendritic cells fosters immunity, whereas lack thereof or antigen presentation by cells expressing few 'co-stimulatory' molecules (such as B7.1 and 2) or pro-inflammatory cytokines (such as interleukin-2), may favor immune tolerance. The CNS seems to be the organ with the least proclivity to mediate induction of immunity, if exposed to an antigen, and a site, which in some instances can escape systemic immune surveillance over vectors.[44] This principle observation seems to be a generally holding one regarding gene vectors, for example, in certain instances immunogenic vectors, such as adenovirus, can be administered into the CNS without immune interference.[45] Certainly, such observations do not provide a 'free pass' because it is clear that strong immunity against vectors, for example, notoriously against adenovirus, is often induced after CNS delivery.[46] Vectors can drain from the brain to the neck lymph nodes at least in non-human primates.[47,48] In rodents, drainage to lymph nodes seems to occur after administration into the ventricles, but not following intraparenchymal injection.[45] Given the fact discussed above that rAAV does not induce cellular immunity in laboratory models, even if administered systemically, it is not surprising that it does not elicit cellular immunity, when administered into the CNS of animals either. The litmus test is therefore, what happens in humans, a question, for which a few clinical trials have recently furnished promising data, as discussed in the next subsection.

CNS clinical trials

Clinical trials of rAAV2 gene therapy in the forebrain and in the retina have been reported. Both sites will be considered together because the retina is developmentally a part of the CNS (that is, the diencephalon) containing neuron-like ganglion cells. (Also, the retina lies adjacent to a confined fluid-filled space, presenting similarity to the DRG-directed IT gene therapy considered in this review.) The CNS disorders addressed in these trials were Canavan disease,[49] Parkinson's disease,[35,36,50] and Batten disease.[37] The retina-directed trials were for Leber's congenital amaurosis (LCA).[51–54]

In the forebrain trials, vector administration was performed intracerebrally at one or multiple injection sites with vector doses of up to 3.2×10^{12} vector genomes per patient;[37] in LCA patients, injection was subretinally administered with vector doses up to 10^{11} vector genomes for one eye.[51] Overall, studies suggested safety and tolerability of rAAV in the CNS. The majority of patients experienced no major adverse events. Two patients with Batten disease developed refractory seizures; one of them died 59 days after vector administration in *status epilepticus*. As seizures are common in patients with this disease, it seems quite likely that the adverse events may have been unrelated to the rAAV administration. This conclusion was supported by the absence of inflammation in the patients' CSF.[37] In one of the LCA studies, a patient developed a hole in the macula after subretinal AAV administration. The authors argued that this was likely caused by the surgical procedure and not by an acute toxic reaction to the rAAV vector because no signs of inflammation or other retinal toxicity were observed.[51]

The finding of cellular immune toxicity in the hemophilia trial (discussed above) implied special safety concerns for the subsequent CNS trials. In LCA trials addressing this question,[51,52,54] no cellular immune response to the AAV capsid was detected. In two of the Parkinson's disease trials, evidence for persistence of transgene expression for more than 1 year was obtained by functional imaging,[35,36] further suggesting that no relevant cytotoxic immunity was mounted and that long-term gene expression in humans resembled the findings in laboratory models.

As none of the studies were randomized controlled trials, assessment of treatment efficacy remains difficult, a point clearly argued by the authors.[35] Nevertheless, it is tempting to take the PET imaging results correlating with positive trends in clinical Parkinson's disease scores as a promising sign for efficacy;[35,36] similarly, although each of the three LCA trials was small (three patients each) and none was blinded, clinical patient examinations were consistent with a therapeutic effect. Taken together, these trials provide converging evidence that rAAV vectors are well tolerated in the human CNS, express transgenes long term, and therefore have the potential to mediate the types of therapeutic responses that we have gotten to expect from these vectors in the laboratory.

Intrathecal rAAV in rodent models of pain

Rationale for vector delivery by the intrathecal route (lumbar puncture)

At its core, the promise of nervous system gene therapy (that is, *in vivo* gene transfer) is to improve upon traditional forms of drug delivery, for example, reaching the brain-side of the blood-brain barrier (BBB); providing a prolonged (or indefinite) drug/gene effect; targeting drug/gene activity to a desired anatomical site; reducing side effects; and freeing patients from repeat injections, external pumps and hazardous procedures. These goals may be most attainable by gene delivery through a

clinically established procedure that is widely available. Lumbar puncture (LP) is the only technique to access the nervous system safely at the bedside.

Pharmacological rationale for gene therapy targeting the DRG

Separation of wanted and unwanted opioid effects can be achieved on anatomic grounds: Untoward effects, such as sedation, are mediated by the forebrain, whereas the desired analgesic effect can be achieved by selectively enhancing opioid activity at the spinal 'pain gate'.

IT opioid administration is one such strategy. It is highly effective because μ-opioid receptors localized at the spinal level induce profound analgesia without marked effects. As analgesia after a single IT opioid administration lasts only a few hours, prolonged pain control requires the implantation of a pump and a permanent IT catheter. Although this approach has been shown to provide superior pain control in a randomized controlled clinical trial,[55] the method has not been adopted outside of few specialized medical centers fielding multi-specialty teams consisting of anesthesiologists, neurosurgeons and oncologists, who are able to service the implanted hardware and investigate acute complications, which might be related to the catheter.

In here lies the promise of gene therapy targeting the DRG. A single-dose, IT administration could mediate pain relief over a long time period, reduce side effects and improve quality of life by freeing patients from external pumps and hazardous procedures. In addition, certain gene products may not have a conventional drug equivalent, for example, certain larger proteins may not be available as a recombinant product or a small-molecule analog, but can be encoded and delivered as a therapeutic gene in a vector.

rAAV modifications to increase IT gene transfer efficacy

Conventional single-stranded rAAV2 vectors perform poorly after IT delivery in rats; expression can be detected only by highly sensitive methods like quantitative PCR even after administration of high vector doses. Confronted with this finding, we reasoned that rAAV2 could fail because of two roadblocks: Firstly, failure of target cells to take the vector up due to lack of compatible cell surface receptors, and secondly, second-strand DNA synthesis, which could be a limiting step especially in a quiescent tissue. Subsequent studies[19] showed that both issues needed to be addressed by vector modification to make rAAV effective in the IT space. Specifically, pseudotyping of rAAV with capsids of serotypes 1, 3 and 5 was tested alone or in combination with a modification of the ITR. The former alters vector tropism and the latter allows packaging of sc-rAAV vectors. Combining both types of modification led to the identification of sc-rAAV1 as a vector that performed superiorly in the IT space. IT delivery of 3×10^9 sc-rAAV1 particles per animal led to stable expression of enhanced green fluorescent protein (EGFP) for $\geqslant$3 months detectable by Western blotting, quantitative PCR, and in a blinded study by confocal microscopy. Expression was strongest in the cauda equina and the lower sections of the spinal cord, and only minimal in the forebrain.[19] Serotype 5 performed favorably in the same study but seemed to be substantially weaker than serotype 1. In a subsequent study, which will be discussed in detail below, we found that serotype 8 performed even better than any of the previously tested capsids and thereby became the basis of our current vectors.

Similar findings regarding the choice of serotype and the use of sc-vectors were reported after intraocular injection of AAV. AAV8 vectors yielded greater transduction efficiency than AAV2 and AAV5, and the self-complementary variants of AAV8 and AAV2 exhibited earlier onset and higher transgene production than the respective single-stranded vector.[56] Considering that findings pertaining to specific vector characteristics often seems to be species-specific, we found this similarity comforting and will follow any future clinical testing of sc-rAAV8 vectors in the retina with great interest.

Targeting of DRG neurons

The most noteworthy property of rAAV vector modified as above, that is, sc-rAAV1 and sc-rAAV8, was their remarkable ability to express the recombinant transgene highly effectively, and almost exclusively (>99%) in the primary sensory neurons. This was unexpected, because IT administration of other vectors fails to target neurons (for example, plasmids or adenovirus transduce meningeal fibroblasts). Primary sensory neurons are, perhaps, the ideal target for pain gene therapy. This point has been shown by a long series of studies with herpes simplex virus, a vector that targets sensory neurons if administered subcutaneously (reviewed elsewhere in this issue).

Microscopic examination of the brain, spinal cord, DRGs, nerve roots and meningeal linings 1 month after administration of sc-rAAV8 expressing the marker gene EGFP (under the control of the CMV promoter/enhancer) revealed strong specific EGFP fluorescence, exclusively in DRG neurons and their axons, and dendrites entering and exiting the DRG. Examination of the spinal cord showed EGFP fluorescence diagrammatically outlining the course of primary sensory neuron axons, which enter the spinal cord through the posterior nerve root, project into the posterior horn and form the *fasciculus gracilis* of the posterior column. Among DRG neurons, all immunohistochemical distinct subpopulations tested were found to be transduced, namely cells positively stained for nociceptive-neuron marker vanilloid receptor suptype 1 (TRPV1), for the small peptidergic-neuron markers substance P and calcitonin gene-related peptide and the non-peptidergic-neuron marker *griffonia simplicifolia* isolectin B4.

Testing in a rat neuropathy model

We chose the L5 spinal nerve ligation (SNL) rat model of neuropathic pain[57] to assess the efficacy of IT sc-rAAV8 for pain using two different known analgesic genes, namely prepro-beta-endorphin (ppβEP) and recombinant interleukin-10 (rIL-10). ppβEP is an artificial gene that has been developed earlier by us. It was found to induce secretion of βEP, which acts as μ-opioid receptor agonist.[58,59]

Additionally, one could consider the use of a transgene whose anti-allodynic activity may not rely on opioid receptor activation. As an example, we chose rIL-10, which is believed to exert its known analgesic activity in neuropathic pain through suppression of glial activation through its anti-inflammatory activity.[60]

IT administration of sc-rAAV8/ppβEP and of sc-rAAV8/rIL10 both led to a significant attenuation of allodynia in the SNL model. Therapeutic activity set in at 1-month post IT delivery and persisted until the predefined end point of the study at 3-month post IT delivery. In a subsequent experiment determining the expression kinetics of IT sc-rAAV8, the onset of expression occurred between 0.5 and 1 month. Hence, the delay in the onset of anti-allodynic activity was due to the gradual onset of transgene expression, that is, the type of delayed onset of activity that is universally observed with AAV vectors, as discussed in an earlier section.

Conclusions from IT sc-rAAV experiments in Rodents

The above discussed study[20] showed highly efficient and selective gene transfer into primary sensory neurons by administration of sc-rAAV8 vectors into the lumbar CSF. Transduction was selective for DRG neurons and did not affect other cells of the CNS. At the center of our study was the demonstration of efficacy in a chronic neuropathic pain model, an important functional outcome. Unlike earlier reports, the observed efficacy was long term, lasting for at least 3 months after a single administration of vectors. The injection technique was atraumatic, modeling an LP and thereby circumventing requirements for injections at multiple sites or for intraparenchymal injections into the CNS tissue. Furthermore, we administered the vector in a small volume of isotonic injectate, which is comparable to the volume and diluent of currently approved conventional IT drugs. The approach to IT gene transfer for pain presented here requires only a single standardized injection, feasible in patients by LP; confers long-term efficacy; accommodates different therapeutic genes; and uses a non-inflammatory vector with a unique safety record, rAAV, from which all viral genes have been removed and whose parent wild-type virus has never been associated with a clinical disease. Our observations in rodents raise the possibility that sc-rAAV8 through LP may be a candidate for developing clinically viable strategies for sensory neuron gene therapy for pain. In the remaining section we will therefore explore what questions relevant for clinical translation could be answered by further laboratory studies, and which other questions require special attention when devising an initial clinical study focused on establishing safety.

Preclinical steps towards clinical translation

Large animal models and toxicology

Up scaling from rodents to large animals will be as critical a step for the approaches outlined in this review as for any other gene therapy. Our studies thus far employed the rat. The distance the vector must diffuse (from the CSF) to reach target neuron populations (in the DRG) may be measured in tens of micrometer in the rat, whereas in larger animals, such as the dog, (and similarly in humans) this may be 100–200 μm or more.[61] Large molecules have been shown to have limited parenchymal diffusion and a similar limitation would be anticipated for viral particles. It is uncertain, how vectors traverse from the CSF to the DRG neuron. In one model, the viral particles would enter the posterior horn of the spinal cord along the entry path of the posterior spinal nerve (an area, where even large molecules can enter the cord more readily) and are then taken up by the axonal endings of the DRG neurons; such uptake might occur equally efficiently in large as well as in small animals. Only the appropriate experiments will allow us to answer these important questions.

Formal toxicology studies will be required to evaluate the distribution and expression of vector throughout the organism and to determine if there is any evidence for cytotoxicity, inflammation or other pathology in the CNS or in any peripheral tissue. Special emphasis will be on tissue in direct contact with the CSF, that is, meningeal fibroblasts, nerve roots, DRG and spinal morphology. This assessment will likely require the use of two species, for example, the rat and a large animal species. The dog has been widely used for the evaluation of IT delivered drugs, including for preclinical toxicology studies, leading to investigational new drug applications (for various small molecules and toxins) and would therefore be a logical consideration as a large animal species. Pigs, sheep or non-human primates would be alternatives.

Choice of vector for initial clinical testing

Although there are technical alternatives and potential improvements to explore over time (for example, as discussed in the next subsection), it is critical to decide, which vector and protocol should become the lead compound for the initial phase I trial, that is, a specific capsid, promoter, therapeutic gene, route of administration and dose range. On the basis of our completed experiments, we would presently consider sc-rAAV8 expressing human IL-10 under the control of the CMV promoter administering escalating doses of approximately 10^{10}–10^{13} packaged genomes IT in patients with intractable pain (that is, failing available analgesic treatments) from incurable cancer. We have tested the IT route extensively and thus have a solid foundation supporting this administration technique; it is also the simplest to perform in patients, because a LP is a safe bedside procedure. Such trial could establish the safety of vector administration into the CSF. (An offshoot could be that safety data may also help support other routes of administration to be considered later, for example, intraparenchymal injection directly into the DRG; in that case leakage of vector into the CSF could be a safety concern.) The current IT approach might also allow obtaining biochemical evidence of gene expression in patients *in vivo*, because CSF can be obtained safely and conveniently by LP, and in the case of the rat, allowed for detection of vector-encoded IL-10 by enzyme-linked immunosorbent assay.

Alternative therapeutic genes, serotypes and administration techniques

Opioids have been used as effective pain medication for millennia and continue to be the most important class of analgesics to date. Two principles of opioid therapy are relevant for its use in gene therapy of pain: first, opioids have a tight dose–effect relationship requiring titration of the dose in typical clinical practice. Second, opioids have no ceiling effect, that is, worsening symptoms can always be controlled by higher doses; however, dose increases

are limited by the occurrence of side effects (as discussed in section 1). The choice of an opioid-related therapeutic gene has to be made within this pharmacological framework. Pain gene therapy can modify the opioid system in two different ways: production of a secreted opioid ligand (such as ppβEP, described in section 4) or increased neuronal expression of an opioid receptor. An opioid ligand secreted at the spinal level (DRG and posterior horn of the spinal cord) can increase the therapeutic ratio, that is, high analgesic efficacy accompanied by little or no side effects (consistent with the second principle); however, with current vector technology, its activity could not be titrated over time (inconsistent with the first principle). Alternatively, an opioid receptor (as opposed to ligand) could be expressed. Ideally, this would have no effect except when opioid medications are given. Then it would increase the analgesic efficacy at the spinal level, in which the vector-encoded opioid receptor is expressed, allowing total doses to remain low and thereby side effects to be minimized. At this time, it seems not entirely clear, whether selective expression of one of the opioid receptors (for example, a splice forms of the μ-opioid receptor) would have the desired activity, if it occurs in a broad range of DRG neurons. Reports a few years ago suggested such a possibility;[62] further exploration of the concept using current rAAV vector technology may therefore be productive.

Development of alternative capsids is ongoing in the AAV field. Although serotype 8 has clearly been among the most effective for a variety of applications, it can be expected that it may ultimately be surpassed by others for various reasons, for example, packaging capacity, transduction efficacy, target cell specificity or immunological escape. Testing alternative capsids in the IT space will therefore be an ongoing goal.

In the context of clinical trials at specialized centers, alternative routes of spinally targeted vector administration could be considered, especially if they can be added on to (or randomized against) procedures that are undertaken as a matter of clinical routine. Radiofrequency ablation of DRGs (or a non-ablative local radio frequency-based DRG manipulation) is an approach that is sometimes performed out of a lack of alternative treatment options. It is supported only by scant clinical trial evidence and long-term outcomes are typically poor. It may therefore be ethically justifiable to offer an experimental alternative based on the same surgical approach, that is, percutaneous placement of a needle into (or close to) the DRG under fluoroscopic guidance. Patients requesting a radiofrequency procedure could be offered local DRG gene transfer as an alternative and, provided a proper informed consent process, could be randomized to gene transfer versus the radiofrequency procedure; the latter would serve effectively as sham-control.

Another approach of vector administration would be injection into the peripheral nerve at the level of the spinal root or further distally (ideally matching the neuroanatomical distribution of a given pain syndrome), given past reports of successful neuronal gene transfer of anterior horn fibers (that is, primary motor neurons) by peripheral rAAV delivery in mice,[63] investigation of an analogous approach directed at the sensory portion of peripheral nerves might be of interest.

Gene therapy as platform for translational pain research

Clinical validation, that is, testing in patients, is currently a critical limiting step in analgesic drug development. Novel molecular targets are typically discovered in rodents using molecular biology techniques leading to candidate proteins, peptides or gene therapeutics being tested in rodents. For instance, IL-10 has marked anti-allodynic activity in rat neuropathic pain models as several groups have shown;[60] it presumably acts through suppression of 'glial activation', an inflammation-like reaction. Considering pain syndromes in patients, it is entirely unclear if IL-10 might have any activity, while it also remains unclear if glial activation or a similar mechanism even occurs in humans (let alone whether it is causative or a mere epiphenomenon). Testing spinal IL-10 in humans is therefore a pressing clinical research question. Other candidate molecular targets and corresponding therapeutic protein candidates are currently being discovered at an increasing rate fueled by high-throughput massively parallel sequencing technology (ASB, unpublished). In most (practically all) of the newly discovered candidate analgesic targets, small molecule therapeutics suitable as conventional drugs are unavailable. It is unclear, if the high cost of small molecule identification (through screening of large chemical libraries) is warranted. Predicting analgesic efficacy in patients from rodent studies has provided mixed outcomes; for example, the correlation for opioid drugs is close but in other cases, like NK-receptor antagonists, rodent data failed to predict clinical results. Considering this quandary, gene therapy could fill an important gap in the drug development process facilitating first-into-human trials of novel targeted therapies, each instantiated as a therapeutic gene.

Acknowledgements

We thank Dr Stephen Russell for discussions. This work was supported by Research Grants K08NS046012 and 1R01NS063022 to ASB from the National Institute of Neurological Disorders and Stroke.

Conflict of interest

The authors declare no conflict of interest.

References

1 National Institutes of Health. Program Announcement (PA) Number: PA-07-282 'Mechanisms, Models, Measurement, & Management in Pain Research (R01)'. National Institutes of Health: Bethesda, MD, 2007.
2 Cleeland CS, Gonin R, Hatfield AK, Edmonson JH, Blum RH, Stewart JA *et al*. Pain and its treatment in outpatients with metastatic cancer. *N Engl J Med* 1994; **330**: 592–596.
3 Larue F, Colleau SM, Brasseur L, Cleeland CS. Multicentre study of cancer pain and its treatment in France. *BMJ* 1995; **310**: 1034–1037.
4 Mercadante S. Pain treatment and outcomes for patients with advanced cancer who receive follow-up care at home. *Cancer* 1999; **85**: 1849–1858.

5 Zech DF, Grond S, Lynch J, Hertel D, Lehmann KA. Validation of World Health Organization Guidelines for cancer pain relief: a 10-year prospective study. *Pain* 1995; **63**: 65–76.
6 Caraceni A, Portenoy RK. An international survey of cancer pain characteristics and syndromes. IASP Task Force on Cancer Pain. International Association for the Study of Pain. *Pain* 1999; **82**: 263–274.
7 Meuser T, Pietruck C, Radbruch L, Stute P, Lehmann KA, Grond S. Symptoms during cancer pain treatment following WHO-guidelines: a longitudinal follow-up study of symptom prevalence, severity and etiology. *Pain* 2001; **93**: 247–257.
8 Weiss SC, Emanuel LL, Fairclough DL, Emanuel EJ. Understanding the experience of pain in terminally ill patients. *Lancet* 2001; **357**: 1311–1315.
9 Adams K, Corrigan J. *Priority Areas for National Action*. National Academies Press: Washington, DC, 2003.
10 World Health Organization. *Cancer Pain Relief*. World Health Organization: Geneva, Switzerland, 1986.
11 Portenoy RK. Pharmacologic management of cancer pain. *Semin Oncol* 1995; **22**: 112–120.
12 Levy MH. Pharmacologic treatment of cancer pain. *N Engl J Med* 1996; **335**: 1124–1132.
13 Jacox A, Carr D, Payne R. Management of cancer pain: Clinical Practice Guideline Number 9—AHCPR publications 94-0592. Agency for Health Care Policy and Research, US Department of Health and Human Services, Public Health Service: Rockville, MD, 1994.
14 Foley K. A 44-year-old woman with severe pain at the end of life. *JAMA* 1999; **281**: 1937–1945.
15 Blacklow NR, Hoggan MD, Kapikian AZ, Austin JB, Rowe WP. Epidemiology of adenovirus-associated virus infection in a nursery population. *Am J Epidemiol* 1968; **88**: 368–378.
16 Blacklow NR, Hoggan MD, Rowe WP. Serologic evidence for human infection with adenovirus-associated viruses. *J Natl Cancer Inst* 1968; **40**: 319–327.
17 Chirmule N, Propert K, Magosin S, Qian Y, Qian R, Wilson J. Immune responses to adenovirus and adeno-associated virus in humans. *Gene Therapy* 1999; **6**: 1574–1583.
18 Erles K, Sebokova P, Schlehofer JR. Update on the prevalence of serum antibodies (IgG and IgM) to adeno- associated virus (AAV). *J Med Virol* 1999; **59**: 406–411.
19 Storek B, Harder NM, Banck MS, Wang C, McCarty DM, Janssen WG *et al*. Intrathecal long-term gene expression by self-complementary adeno-associated virus type 1 suitable for chronic pain studies in rats. *Mol Pain* 2006; **2**: 4.
20 Storek B, Reinhardt M, Wang C, Janssen WG, Harder NM, Banck MS *et al*. Sensory neuron targeting by self-complementary AAV8 via lumbar puncture for chronic pain. *Proc Natl Acad Sci USA* 2008; **105**: 1055–1060.
21 Milligan ED, Sloane EM, Langer SJ, Cruz PE, Chacur M, Spataro L *et al*. Controlling neuropathic pain by adeno-associated virus driven production of the anti-inflammatory cytokine, interleukin-10. *Mol Pain* 2005; **1**: 9.
22 Wright JF. Manufacturing and characterizing AAV-based vectors for use in clinical studies. *Gene Therapy* 2008; **15**: 840–848.
23 Cecchini S, Negrete A, Kotin RM. Toward exascale production of recombinant adeno-associated virus for gene transfer applications. *Gene Therapy* 2008; **15**: 823–830.
24 Negrete A, Kotin RM. Large-scale production of recombinant adeno-associated viral vectors. *Methods Mol Biol* 2008; **433**: 79–96.
25 Negrete A, Kotin RM. Strategies for manufacturing recombinant adeno-associated virus vectors for gene therapy applications exploiting baculovirus technology. *Brief Funct Genomic Proteomic* 2008; **7**: 303–311.
26 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 Therapy* 2003; **10**: 2112–2118.
27 Zhong L, Li B, Jayandharan G, Mah CS, Govindasamy L, Agbandje-McKenna M *et al*. Tyrosine-phosphorylation of AAV2 vectors and its consequences on viral intracellular trafficking and transgene expression. *Virology* 2008; **381**: 194–202.
28 Zhong L, Li B, Mah CS, Govindasamy L, Agbandje-McKenna M, Cooper M *et al*. Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. *Proc Natl Acad Sci USA* 2008; **105**: 7827–7832.
29 Allocca M, Doria M, Petrillo M, Colella P, Garcia-Hoyos M, Gibbs D *et al*. Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. *J Clin Invest* 2008; **118**: 1955–1964.
30 Smith RH. Adeno-associated virus integration: virus versus vector. *Gene Therapy* 2008; **15**: 817–822.
31 Donsante A, Vogler C, Muzyczka N, Crawford JM, Barker J, Flotte T *et al*. Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. *Gene Ther* 2001; **8**: 1343–1346.
32 Donsante A, Miller DG, Li Y, Vogler C, Brunt EM, Russell DW *et al*. AAV vector integration sites in mouse hepatocellular carcinoma. *Science* 2007; **317**: 477.
33 Bell P, Moscioni AD, McCarter RJ, Wu D, Gao G, Hoang A *et al*. Analysis of tumors arising in male B6C3F1 mice with and without AAV vector delivery to liver. *Mol Ther* 2006; **14**: 34–44.
34 Lo WD, Qu G, Sferra TJ, Clark R, Chen R, Johnson PR. Adeno-associated virus-mediated gene transfer to the brain: duration and modulation of expression. *Hum Gene Ther* 1999; **10**: 201–213.
35 Kaplitt MG, Feigin A, Tang C, Fitzsimons HL, Mattis P, Lawlor PA *et al*. 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**: 2097–2105.
36 Marks Jr WJ, Ostrem JL, Verhagen L, Starr PA, Larson PS, Bakay RA *et al*. Safety and tolerability of intraputaminal 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**: 400–408.
37 Worgall S, Sondhi D, Hackett NR, Kosofsky B, Kekatpure MV, Neyzi N *et al*. 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**: 463–474.
38 Herzog RW. Immune responses to AAV capsid: are mice not humans after all? *Mol Ther* 2007; **15**: 649–650.
39 Zaiss AK, Muruve DA. Immunity to adeno-associated virus vectors in animals and humans: a continued challenge. *Gene Therapy* 2008; **15**: 808–816.
40 Mingozzi F, High KA. Immune responses to AAV in clinical trials. *Curr Gene Ther* 2007; **7**: 316–324.
41 Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ *et al*. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* 2006; **12**: 342–347.
42 Mingozzi F, Maus MV, Hui DJ, Sabatino DE, Murphy SL, Rasko JE *et al*. CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nat Med* 2007; **13**: 419–422.
43 Li H, Murphy SL, Giles-Davis W, Edmonson S, Xiang Z, Li Y *et al*. Pre-existing AAV capsid-specific CD8+ T cells are unable to eliminate AAV-transduced hepatocytes. *Mol Ther* 2007; **15**: 792–800.
44 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**: 347–360.
45 Lowenstein PR, Kroeger K, Castro MG. Immunology of neurological gene therapy: how T cells modulate viral vector-

mediated therapeutic transgene expression through immunological synapses. *Neurotherapeutics* 2007; **4**: 715–724.
46 Thomas CE, Birkett D, Anozie I, Castro MG, Lowenstein PR. Acute direct adenoviral vector cytotoxicity and chronic, but not acute, inflammatory responses correlate with decreased vector-mediated transgene expression in the brain. *Mol Ther* 2001; **3**: 36–46.
47 Driesse MJ, Esandi MC, Kros JM, Avezaat CJ, Vecht C, Zurcher C *et al.* Intra-CSF administered recombinant adenovirus causes an immune response-mediated toxicity. *Gene Therapy* 2000; **7**: 1401–1409.
48 Driesse MJ, Kros JM, Avezaat CJ, Valerio D, Vecht CJ, Bout A *et al.* Distribution of recombinant adenovirus in the cerebrospinal fluid of nonhuman primates. *Hum Gene Ther* 1999; **10**: 2347–2354.
49 Janson C, McPhee S, Bilaniuk L, Haselgrove J, Testaiuti M, Freese A *et al.* 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**: 1391–1412.
50 Eberling JL, Jagust WJ, Christine CW, Starr P, Larson P, Bankiewicz KS *et al.* Results from a phase I safety trial of hAADC gene therapy for Parkinson disease. *Neurology* 2008; **70**: 1980–1983.
51 Bainbridge JW, Smith AJ, Barker SS, Robbie S, Henderson R, Balaggan K *et al.* Effect of gene therapy on visual function in Leber's congenital amaurosis. *N Engl J Med* 2008; **358**: 2231–2239.
52 Maguire AM, Simonelli F, Pierce EA, Pugh Jr EN, Mingozzi F, Bennicelli J *et al.* Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med* 2008; **358**: 2240–2248.
53 Cideciyan AV, Aleman TS, Boye SL, Schwartz SB, Kaushal S, Roman AJ *et al.* Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics. *Proc Natl Acad Sci USA* 2008; **105**: 15112–15117.
54 Hauswirth W, Aleman TS, Kaushal S, Cideciyan AV, Schwartz SB, Wang L *et al.* Phase I trial of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results. *Hum Gene Ther* (in press).
55 Smith TJ, Staats PS, Deer T, Stearns LJ, Rauck RL, Boortz-Marx RL *et al.* Randomized clinical trial of an implantable drug delivery system compared with comprehensive medical management for refractory cancer pain: impact on pain, drug-related toxicity, and survival. *J Clin Oncol* 2002; **20**: 4040–4049.
56 Natkunarajah M, Trittibach P, McIntosh J, Duran Y, Barker SE, Smith AJ *et al.* Assessment of ocular transduction using single-stranded and self-complementary recombinant adeno-associated virus serotype 2/8. *Gene Therapy* 2008; **15**: 463–467.
57 Chung JM, Kim HK, Chung K. Segmental spinal nerve ligation model of neuropathic pain. *Methods Mol Med* 2004; **99**: 35–45.
58 Beutler AS, Banck MS, Bach FW, Gage FH, Porreca F, Bilsky EJ *et al.* Retrovirus-mediated expression of an artificial beta-endorphin precursor in primary fibroblasts. *J Neurochem* 1995; **64**: 475–481.
59 Finegold AA, Mannes AJ, Iadarola MJ. A paracrine paradigm for *in vivo* gene therapy in the central nervous system: treatment of chronic pain. *Hum Gene Ther* 1999; **10**: 1251–1257.
60 Beutler AS, Banck MS, Walsh CE, Milligan ED. Intrathecal gene transfer by adeno-associated virus for pain. *Curr Opin Mol Ther* 2005; **7**: 431–439.
61 Allen JW, Mantyh PW, Horais K, Tozier N, Rogers SD, Ghilardi JR *et al.* Safety evaluation of intrathecal substance P-saporin, a targeted neurotoxin, in dogs. *Toxicol Sci* 2006; **91**: 286–298.
62 Xu Y, Gu Y, Xu GY, Wu P, Li GW, Huang LY. Adeno-associated viral transfer of opioid receptor gene to primary sensory neurons: a strategy to increase opioid antinociception. *Proc Natl Acad Sci USA* 2003; **100**: 6204–6209.
63 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**: 839–842.