Aspartoacylase Gene Transfer to the Mammalian Central Nervous System with Therapeutic Implications for Canavan Disease

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With the ultimate goal of developing safe and effective in vivo gene therapy for the treatment of Canavan disease and other neurological disorders, we developed a non–viral lipid-entrapped, polycation-condensed delivery system (LPD) for central nervous system gene transfer, in conjunction with adeno-associated virus (AAV)–based plasmids containing recombinant aspartoacylase (*ASPA*). The gene delivery system was tested in healthy rodents and primates, before proceeding to preliminary studies in 2 children with Canavan disease. Toxicity and expression testing was first carried out in human 293 cells, which demonstrated effective transduction of cells and high levels of functional ASPA activity. We performed in vivo toxicity and expression testing of LPD/pAAV*aspa* and LPD/pAAV*lac* in rodents, which demonstrated widespread gene expression for more than 10 months after intraventricular delivery, and local expression in deep brain nuclei and white matter tracts for more than 6 months after intraparenchymal injections, with no significant adverse effects. We also performed intraventricular delivery of LPD/pAAV*aspa* to 2 cynomologous monkeys, with 2 additional monkeys receiving LPD and saline controls. None of the monkeys demonstrated significant adverse effects, and at 1 month the 2 LPD/pAAV*aspa* monkeys were positive for human ASPA transcript by reverse transcriptase polymerase chain reaction of brain tissue punches. Finally, we performed the first in vivo gene transfer study for a human neurodegenerative disease in 2 children with Canavan disease to assess the in vivo toxicity and efficacy of *ASPA* gene delivery. Our results suggest that LPD/pAAV*aspa* is well tolerated in human subjects and is associated with biochemical, radiological, and clinical changes.

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Canavan disease is an autosomal recessive leukodystrophy resulting in spongiform degeneration of the brain and severe psychomotor retardation, which is generally apparent soon after birth in affected children. The cellular pathology in Canavan disease has yet to be fully explained but appears to be related to a deficiency of functional aspartoacylase (ASPA) in the white matter of the brain, leading to an excess of the substrate molecule *N*-acetyl-aspartate (NAA).[1,2] Current hypotheses for the etiology and progression of Canavan disease are related to osmotic, metabolic, or cytotoxic properties of excess NAA and its congeners such as *N*-acetyl-aspartic-glutamate (NAAG).[3] Although the normal physiological roles and exact pathological mechanisms of NAA are still uncertain, the key unifying principle in Canavan disease is the lack of ASPA, especially in the vicinity of the oligodendrocyte cell membrane.[4]

We reasoned that this genetically based, demyelinating disease is a natural model for optimizing global central nervous system (CNS) gene delivery owing to a presumptive single-gene etiology and a localization of pathology specifically to the brain. After demonstrating in vitro production of functional recombinant ASPA enzyme, we performed in vivo studies for assessment of

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gene expression in mammals. Expression of human *ASPA* and *lacZ* reporter constructs was directly measured by reverse transcriptase polymerase chain reaction (RT-PCR) or immunocytochemistry in rodents and monkeys. In 2 human subjects, it was necessary to use indirect measurements of *ASPA* expression because invasive measures for data collection (eg, serial brain biopsy) would be unethical in this setting. Subsequent to the first 2 human subjects who received a lipid-entrapped, polycation-condensed delivery system, LPD/pAAV*aspa*, a total of 14 children have enrolled in a larger phase I clinical trial using a modified delivery method, and their long-term clinical improvement is now being monitored. In this article, we report data from preclinical animal experiments and results from the first 2 human patients.

One problem with many previous gene transfer studies has been a dependence on cell culture and in vitro methods (which often involve nonphysiological conditions or toxic formulations) due to a lack of suitable animal models of disease. Until very recently, a knockout *ASPA* mouse was not available, and despite showing global *ASPA* or *lacZ* gene expression, it was not possible for us to demonstrate functional recovery in an animal model. Accordingly, human clinical trials have provided an important "proof-of-principle" approach to therapeutic gene delivery, following the demonstration of long-term safety and global CNS transgene expression in animals.

Protocols for gene transfer have used a range of delivery systems, both viral and nonviral. Neurotropic viral vectors afford a high efficiency of transgene expression in the brain, particularly as they have improved in recent years. However, most are naturally pathogenic or immunogenic, which has been an obstacle even when substantial portions of the native virion are deleted; minimal viral elements appear capable of initiating an inflammatory response with downregulation of transgene expression.[5–7] In the past, in vivo gene transfer to the mammalian brain has been obtained using herpes simplex virus,[8–15] adenovirus,[16–20] AAV,[21–26] and lentivirus.[27–29] These vary in their neurotropism, packaging capability, maximum titers obtainable, uptake by post-mitotic cells, stability of expression, and capacity for axonal transport. Some problems include the potential for deleterious recombination events, viral rearrangements and reversion to wild-type, or helper virus contamination. Moreover, many viral vectors do not diffuse well through brain parenchyma,[30] and safer, maximally deleted vectors can demonstrate altered tropism or more limited axonal transport. While the latest viral vectors show promise in terms of improved safety and high-level expression, continued safety concerns have limited the use of viruses for neuronal transfer in humans.

In theory, nonviral delivery systems such as plasmids offer a safer approach to CNS gene transfer. However, the efficiency of gene transfer has not been as high as with viral methods. Direct injection of plasmid DNA to the brain is very inefficient,[31] and although liposome-plasmid complexes are more efficient than naked plasmid DNA,[32] they are still relatively inefficient compared with viral vectors because of low uptake and transient expression.[33] Some limitations of liposome-plasmid complexes are that they tend to aggregate, diffuse poorly, and transduce inefficiently in the brain. Therefore, it was necessary to make substantial improvements in lipid formulation and plasmid construction to enhance *ASPA* expression.

The LPD system involves two components. To improve diffusion and transduction properties, DNA was condensed using poly-L-lysine or protamine sulfate and encapsulated with liposome to form nonaggregating 60- to 100-nm particles. The cationic polymers (poly-L-lysine or protamine) reduce the size of the complex, render it resistant to nucleases, and most likely also serve as a nuclear localization sequence.[34,35] Compared with other liposomal formulations, LPD is nontoxic in a wide variety of cell lines, offers superior protection of plasmid DNA against enzymatic digestion, is stable in vivo over time, and also gives high levels of gene expression in vivo in diverse tissues.[35–38] Moreover, DC-Chol/DOPE is not immunogenic compared with viral vectors, with no observable immune reaction[39–41] in treated humans. Recently, LPD has been shown to trigger cytokine release on administration to the lung, which may be responsible for transgene downregulation or apoptosis,[42] but this effect in the brain has not been described.

Because Canavan disease is a diffuse neurodegenerative disease, it appeared unlikely that gene transfer would be of much therapeutic benefit unless it was global rather than localized to a specific region of the brain. We tested intraventricular delivery as a means of maximizing the area accessible to LPD complexes. Various other approaches have been tried for global gene delivery to the CNS, including multiple stereotactic injections[21] and convection-enhanced infusion.[46] Based on this study, intraventricular administration is also possible for global, long-term gene delivery to the mammalian brain with a nonviral vector system.

Materials and Methods

Plasmid Construction and Characterization of Liposomes

Two expression plasmids (pAAVaspa and pAAVlac) were generated. To maximize expression in vivo, the plasmid vectors we used incorporated the 145 base pairs (bp) inverted terminal repeats (ITRs) from AAV, which have been shown to increase and prolong gene expression in cell culture compared with non-ITR plasmids.[43,44] Plasmid pSub201[47] was digested with *Xba*I to remove the AAV sequence with the

exception of the flanking 145 bp ITRs. A cytomegalovirus (CMV) promoter–*lacZ* gene–SV40-polyA cassette was isolated from plasmid pHCL[18] by digestion with *Spe*I and *Xba*I, and this fragment was inserted into *Xba*I-digested pSub201 to create pAAVlac. A second plasmid was created by digestion of pAAVlac with *Hind* III and *Xba*I to remove the lacZ gene and poly-A signal, followed by insertion of a *Hind* III-*Xba*I fragment from pREP4 (Invitrogen, Carlsbad, CA) containing a polylinker and SV40 poly-A signal. This plasmid was digested with *Nhe*I and *Not*I, followed by insertion of a human ASPA cDNA released from the pG-CMV-*aspa* plasmid[2] by *Xba*I-*Not*I digestion. The sequence fidelity of the final pAAVaspa construct was confirmed by sequencing on an ABI model 377 sequencer.

Using the pAAVlac construct, we screened commercial liposomal formulations in human 293 cells and rat glial 9L cells, including lipofectin and lipofectamine (Life Technologies, Rockville, MD), DOTAP (Boehringer Mannheim/Roche Diags, Indianapolis, IN), and TFX-50 (Promega, Heidelberg, Germany). We then obtained LPD (Leaf Huang, Pittsburgh, PA) for comparison. The liposome DC-Chol/DOPE is a mixture of a neutral lipid DOPE, dioleoyl phosphatidyl-ethanolamine, and a cationic liposome, DC-Chol (3b-[*N*-(*N'*,*N'*dimethyl-aminoethane)carbamoyl] cholesterol). The polycations poly-L-lysine (Sigma, St Louis, MO) and protamine sulfate (Eli Lilly, Indianapolis, IN) were incorporated into the DC-Chol/DOPE mixture with plasmid DNA as follows: 100 μg of pAAVlac or pAAVaspa (diluted in 200 μl of cell culture grade H_2O) was mixed with 8 μl of poly-L-lysine or protamine (10 mg/ml) and 30 μl of DC-Chol/DOPE liposomes. The resulting solution was briefly sonicated before to use.

Cell Culture for In Vitro LPD/pAAVaspa Expression

Human 293 cells were used to test expression levels of plasmid constructs. Because a monoclonal antibody to ASPA protein was not available, we used pAAVlac to determine maximum expression levels obtainable with LPD in vitro, using X-gal staining. Next, we wished to verify that we could generate physiologically active ASPA enzyme from pAAVaspa, so we transfected human 293 cells with pAAVaspa, harvested the cells according to a standard protocol, and subjected cell lysates to a functional test of ASPA enzyme activity.[1,48] Finally, to verify the independent contribution of ITRs in both plasmids, we generated a reporter construct (pAAVluc) that was identical to pAAVlac except that *lacZ* was replaced with a sensitive firefly luciferase reporter from the Promega pGL2 plasmid, and we tested this pAAVluc in vitro against a control pAAVluc plasmid with the ITRs removed. Luciferase activities were determined in a Wallac luminometer (Wallac/PE Life Science, Gaithersburg, MD) in 293 cells, with the pAAVlac plasmid as an additional negative control.

Vector Delivery and In Vivo Testing in Rodents and Primates

For toxicity and gene expression experiments, all rodent developmental stages were studied, including in utero. To assess toxicity, we intracranially injected 30 fetal or newborn and 175 adult male Fischer rats (280–300 g) with LPD (pAAVaspa or pAAVlac) or saline control. Animals were housed in approved animal facilities with food and water available ad libitum. Rats were anesthetized with ketamine and xylazine (80 mg/kg and 8 mg/kg) before surgery. We also optimized our delivery protocol by using intraperitoneal mannitol (1.5 g/kg) to lower brain interstitial pressure and increase tissue penetration[45] before intraventricular injection in animals or humans. LPD was delivered intracranially through a Hamilton glass microsyringe and Harvard precision pump with a 30-gauge needle connected to a Kopf stereotactic frame, at a flow rate of 0.5 to 2.0 μl/min. Injections were made to the lateral ventricle, caudate nucleus, or hippocampus using coordinates derived from a stereotactic atlas.[49] Rats were postsurgically followed for at least 3 days with daily weighing, measurement of rectal temperatures, and behavioral testing; after this time, rats were weighed at least twice weekly and were observed for coat quality (grooming behavior) and conjunctivitis (a sensitive stress indicator) as well as feeding and drinking behavior. Behavioral tests included assessment of open field and social behavior, rigidity and catatonia testing, and motor coordination using an inclined beam test. Rats were killed (depending on experimental group) for histochemical analysis after 3 days, 1 week, 2 weeks, 1 month, and other intervals up to 10 months. Samples of organs (lung, liver, testes) were collected for PCR analysis at the 1-month time point.

For primate studies of toxicity and gene expression, four adult male cynomologous monkeys (*Macaca fasciculari*) were chosen for their close phylogenetic relationship with humans. Two animals were randomly assigned to receive pAAVaspa, with the two remaining animals to receive pAAVlac or saline controls. A microchip identification device was subcutaneously implanted to uniquely identify each animal. The monkeys were anesthetized under isoflurane anesthesia, the head was placed in a stereotactic frame, a small burr hole was drilled, and 1 ml of LPD (prepared as above) was administered to the left lateral ventricle of each monkey. All monkeys were continually monitored for signs of abnormal behavior for 3 days after surgery and were checked twice daily afterward. Appetite and bowel movements were recorded at least once daily, and a thorough physical examination was conducted weekly. A full hematological screening was performed at days −3, +8, +15, and +28, including electrolytes, serum proteins and lipids, and cell counts. After 30 days of observation, the animals were euthanized and necropsies were performed, with histopathological examination of external and internal organs. Immediately on sacrifice, the brain was removed and sections of the left hemisphere were fresh frozen in liquid nitrogen; the remaining half of the ventricle and cortex (which included the needle track) was preserved in formalin for histopathological examination. Samples of adrenals, heart, lung, liver, kidneys, and testes were collected and frozen in liquid nitrogen for PCR analysis.

Immunocytochemistry and Histology of Rodent Brain

Rodent brain tissue was collected at 3 days, 1 week, 1 month, and 2, 3, 6, 9, and 12 months after LPD/pAAVaspa or LPD/pAAVlac delivery to quantify transgene expression and also to rule out immune reaction. For X-gal staining of whole brain slices, rats were deeply anesthetized with chloral

hydrate and transcardially perfused with ice-cold purified buffered saline (PBS), followed by 2% paraformaldehyde in PBS, with 5 mM EGTA added to eliminate staining due to endogenous enzymes. The brains were postfixed in 4% paraformaldehyde, followed by ascending solutions of 10%, 20%, and 30% sucrose/PBS, then sectioned and stained using hematoxylin and eosin (H&E) and X-gal as previously described.[10] For additional immunocytochemical analysis, sections were treated as previously described with antibodies[21] to GFAP, NF, and MAP2 (Chemicon, Temecula, CA), β-galactosidase (Promega), or CD4 and MHC-I (Pharmingen, San Diego, CA).

RT-PCR of Rodent and Primate Brain and Human Cerebrospinal Fluid

Because ASPA is endogenously expressed in the rat and monkey CNS, we used RT-PCR with primers specific to the human ASPA transcript to confirm expression of the transgene. The RT-PCR analysis was run under identical conditions for rodent and primate tissue. Sample mRNA was isolated from 1×1-mm brain tissue punches from rat frontal cortex, striatum, cerebellum, hippocampus, and midbrain; in primates, sample tissue was taken from fresh frozen brain sections. The polyadenylated mRNA was treated with deoxyribonuclease and reverse-transcribed.[10] The cDNA was amplified with the following primers in a 30-cycle PCR reaction: 5′-GACTGGAAACCACTGCATCCTG-3′ (sense primer corresponding to the 3′ end of the human *ASPA* gene) and 5′-TGTATCTTATCATGTCTGGATCCG-3′ (antisense primer to the SV40 poly-A sequence in the AAV vector). Following electrophoresis and transfer to a nylon membrane, the sequences were hybridized with a 551-bp probe corresponding to the entire length of the predicted PCR product. We also performed RT-PCR on cerebrospinal fluid (CSF) from the 2 human patients before and after surgery to assay gene expression. Because the antisense primer is specific to the vector sequence, we were assured that nonspecific detection of low levels of endogenous ASPA transcript was completely avoided.

Vector Delivery and Clinical Outcome Measures in 2 Human Subjects

Two children, aged 19 months (L.K.) and 24 months (A.M.), were selected to undergo CNS gene transfer. Both patients had close baseline radiological and biochemical tests, and neurodevelopmental tests were also within a similar range for both patients before gene transfer (8- to 16-week developmental equivalent). These patients were volunteered by their parents for the pilot study and were enrolled solely for that reason rather than for reasons particular to the study design. A.M. was homozygous for the E285A mutation, and L.K. was heterozygous for E285A and Y231X, which together constitute most mutations in patients from a Jewish background.[50] The pretest data from these 2 subjects and from other untreated Canavan disease patients, as well as a cohort of age-matched healthy reference patients, form a set of biochemical and radiological values for comparison.

For human neurosurgery in these patients, each child underwent general anesthesia, and systemic mannitol (1 g/kg administered intravenously) was used to increase interstitial permeability.[45] A burr hole was drilled above the lateral ventricle, and a catheter was inserted into the ventricular space, through which 5 ml of the LPD complex (80 μg plasmid DNA/ml) was directly passed. The surgical site was closed, and the patients were monitored until hospital discharge. The patients were assessed by multiple clinical outcomes measures, including proton spectroscopy of brain NAA levels; magnetic resonance (MR) brain imaging; somatosensory, visual, and brainstem auditory evoked potentials; RT-PCR and biochemical analysis of CSF; and regular neurological and psychometric assessment.

Results

Functional ASPA *and β-Galactosidase Are Efficiently Expressed In Vitro by LPD*

In vitro expression of ASPA enzyme was dependent on the relative concentrations of DNA, liposome, and plasmid. Under optimal conditions (see Materials and Methods), transfection efficiencies approaching 80% were obtained using LPD/pAAVlac in human 293 cells, based on X-gal staining. Compared with commercial liposomal formulations tested, the highest transfection efficiency in vitro was obtained using LPD with protamine, which also provided minimal cytotoxicity (Table 1).

With respect to the pAAV plasmid construct, work by other investigators suggests the superiority of AAV-based plasmids for high-level gene transfer,[43,44] and we confirmed ourselves that an AAV-based plasmid showed increased expression in vitro over non-ITR plasmid. The pAAVluc had a mean luciferase activity of 3×10^4 units (±3%), and the control (non-ITR)

Table 1. In Vitro Expression and Toxicity of Lipid Formulations in Human 293 Cells

Lipid Formulation	Relative LacZ Expression	Relative Cytotoxicity
LPD (protamine)	+++++	x
LPD (poly-lysine)	++++	x
Lipofectamine	++++	xxx
DOTAP	+++	x
Lipofectin	+++	xx
TFX-50	+++	xx

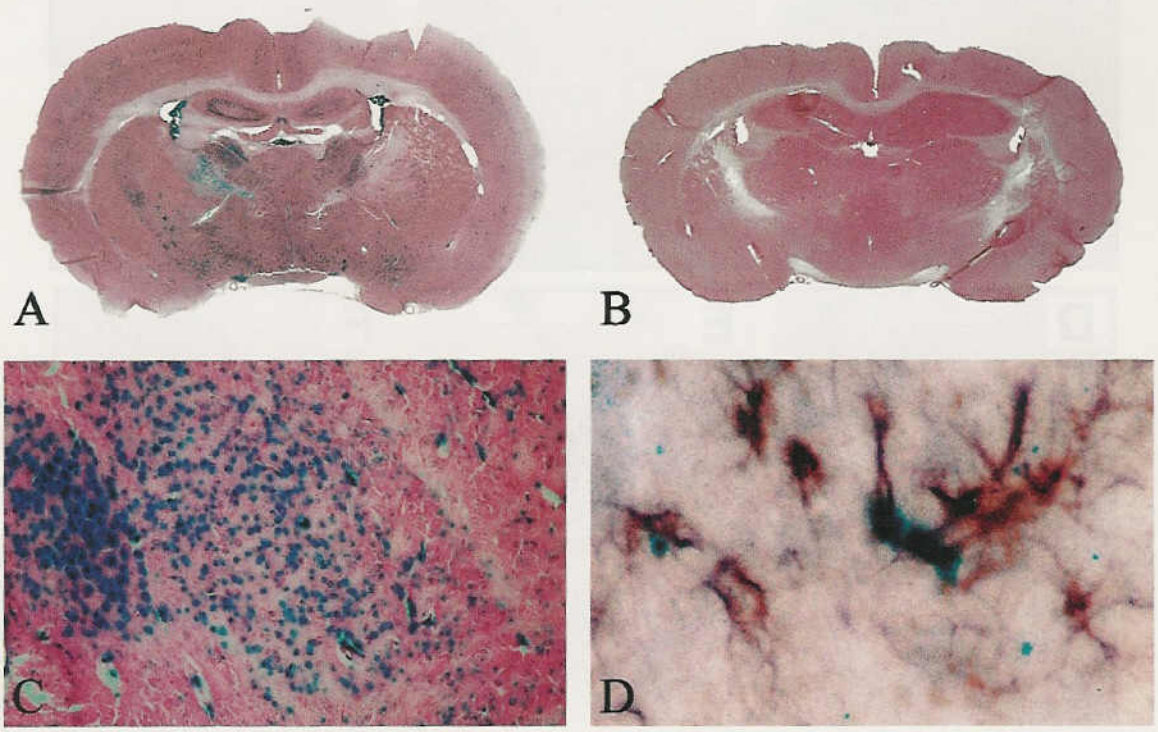
These in vitro studies were designed to assess the relative toxicity and expression of different lipid formulations. Cell counts and toxicities for each condition were transformed to range (rank order) data as shown. We found that most lipid formulations gave comparable expression in vitro, but the major difference was in cytotoxicity. Cell loss was measured by counting of viable cells upon Trypan blue exclusion. The formulations above were tested in triplicate on three separate occasions, with consistent results (total 56 values per condition). LPD-protamine showed a statistically significant difference in expression levels ($p < 0.05$) by the Kruskal-Wallis nonparametric test. Results obtained in 293 cells were repeated in rat glial 9L cells with equivalent expression and toxicity (data not shown).
+++ = 30–60% positive cells; ++++ = 50–70% positive cells; +++++ = 60–80% positive cells; x = ≤10% cell loss; xx = >20% cell loss; xxx = >30% cell loss.

had a mean activity of 9.8×10^3 units (±4%), indicating that the ITRs conferred an approximate 300% increase in expression in human 293 cells. Following in vitro expression/toxicity studies using LPD with pAAVlac and pAAVluc, 293 cells were then transfected with the LPD/pAAVaspa construct to verify the integrity of ASPA protein. Cultured 293 cells harvested at 60% confluence for ASPA enzyme activity showed mean and SEM levels of ASPA enzyme activity of 0.40 ± 0.12 mU/mg protein following LPD/pAAVaspa transduction. This compares to negligible ASPA activity for control LPD/pAAVlac cells and 1.04 and 0.04 mU/mg protein for bovine white and gray matter homogenate, respectively,[48] suggesting that transfected 293 cells can produce substantial quantities of functional ASPA as a percentage of total protein.

Widespread Expression of pAAVlac and pAAVaspa in Rodents and Primates

We tested expression of LPD/AAV-plasmid constructs in rats at both the RNA and the protein level. Because an antibody to human ASPA was not available, X-gal was used as a surrogate marker for transgene expression. After intraventricular injection of LPD/pAAVlac, β-galactosidase expression was detected starting at 3 days, and widespread expression was noted at 2 to 3 weeks, continuing to more than 10 months. Qualitatively, the staining was most intense in the vicinity of the ependymal and subependymal cells in the ventricular spaces, but it also appeared to a more limited degree in the corpus callosum, striatum and deep brain nuclei, and diffuse cortical and subcortical sites (Fig 1A–C). Within the ranges we tested, the number of cells positive for β-galactosidase was proportional to the volumes injected by the intraventricular and intraparenchymal routes. By the intraventricular route, we found linearly increasing expression of β-galactosidase in the range of 20- and 50-μl injectates. We performed semiquantitative cell counts on X-gal–stained sections from the striatum, cortex, and cerebellum, taking every fifth 20-μm coronal section from representative rat brains; we found that expression peaked at approximately 1 week for intraparenchymal animals and at 3 months for intraventricular animals. Thus, β-gal expression changed over time, with higher expression of intraventricularly delivered pAAVlac at 2 and 3 months compared with 2 weeks and persistent expression of β-gal out to 10 months. We directly compared the efficiency of intraventricular delivery with that of intraparenchymal delivery for global expression. After intraparenchymal striatal injection of 2 to 5 μl of LPD (containing 300 μg/ml pAAVlac), strong lacZ expres-

Fig 1. (A) X-gal staining of hematoxylin and eosin–prepared whole rat brain in coronal section (apparent magnification, ×4.5) 2 months after intraventricular injection of LPD/pAAVlac. (B) Control brain injected with purified buffered saline/vehicle, showing lack of staining to β-galactosidase. (C) Micrograph (apparent magnification, ×133) of lacZ-stained cells in rat striatum, 2 months after intraventricular injection of LPD/pAAVlac. (D) Double-labeling of astrocytes (apparent magnification, ×450) with antibodies to GFAP, which constituted over 60% of LPD-transduced cells by the intraparenchymal route.

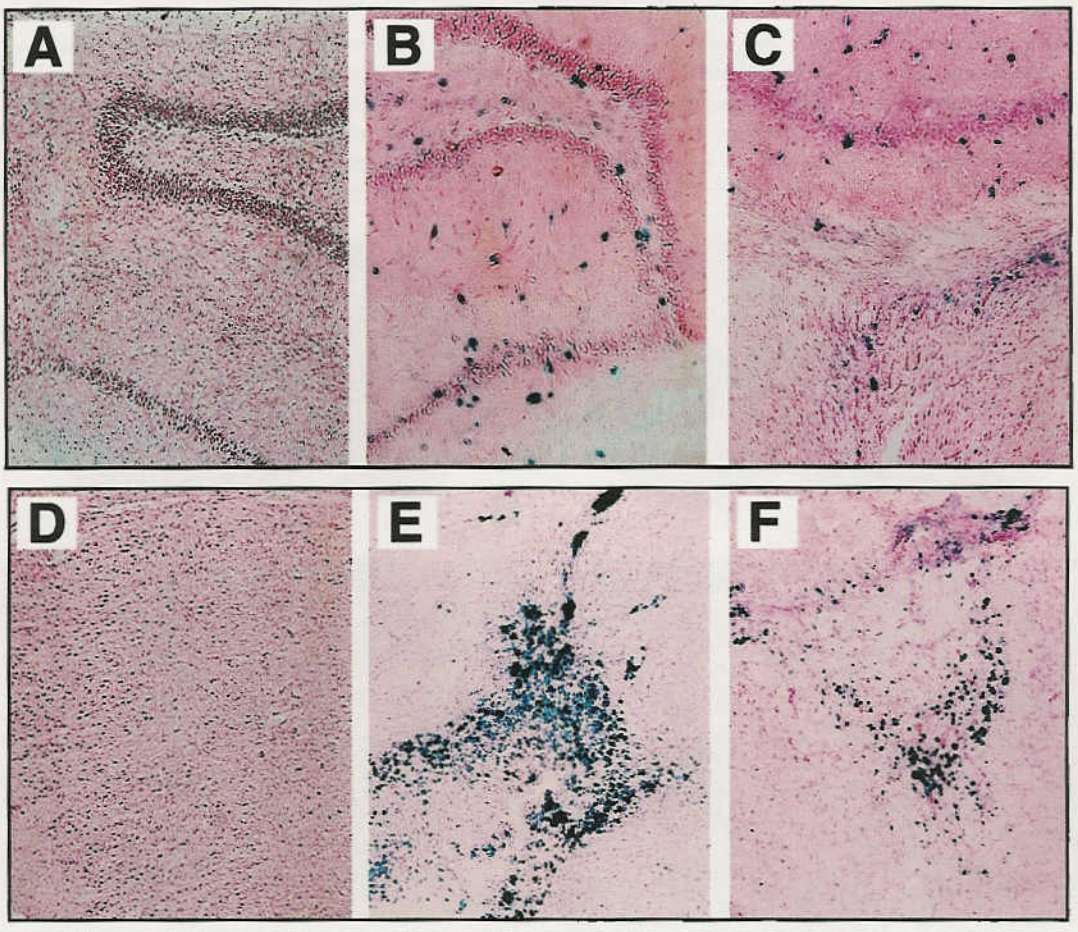


sion was noted in rats after 1 week, consisting of over 1×10^3 cells surrounding the needle track and along planes between deep brain nuclei and white matter tracts, which persisted for more than 6 months. However, the staining tended to be localized with a diffusion radius limited to about 2 to 3 mm around the needle track. By comparison, intraventricular injection of 50 μl LPD/pAAVlac gave up to 1×10^5 cells at the 2-month point, based on semiquantitative counting of tissue sections using the method of Szarijanni-Rethelyi.[55]

We also tested the efficacy of protamine sulfate versus poly-L-lysine transduction of rat brain cells. Consistent with published in vitro studies,[38,52] we found protamine to give 160% (±15%; $p < 0.05$) higher lacZ expression in vivo at 1 month after LPD/pAAVlac treatment by both intraventricular and intraparenchymal delivery (Fig 2) under the preparative conditions we used. Based on these results, the first two Canavan disease patients received high-molecular-weight poly-L-lysine, but all subsequent patients received protamine formulations. The difference in gene expression levels between poly-L-lysine and protamine formulations was most evident at the peak expression time points, although it persisted to the 10-month point.

To characterize the expression of the *lacZ* transgene among different cell types in rats, we performed immunocytochemistry for lacZ and either GFAP (glial marker) or MAP2 and NF (neuronal markers), which demonstrated lacZ double-labeling of astrocytes (see Fig 1D) and neurons following intraparenchymal delivery. Overall, most (>60%) lacZ-positive cells were astrocytes, with the remainder being neurons and other cells. Although we did not perform double-labeling to oligodendrocyte markers, we observed lacZ-positive cells that did not react to either astrocytic or neuronal markers and that were morphologically more similar to oligodendrocytes. All types of brain cells were considered potential target cells for gene transfer in humans. Indeed, our driving goal in humans was not to recreate

Fig 2. This composite (3 × 2) contains low-power brain sections of rats injected with PBS (control) and LPD/protamine or LPD/poly-L-lysine. The first row shows representative hippocampal sections, 1 month after intraventricular injection of 25 μl of PBS (A), LPD/protamine (B), and LPD/poly-L-lysine (C). The second row shows representative hippocampal sections, 1 month after intraparenchymal injection of 3 μl of PBS (D), LPD/protamine (E), and LPD/poly-L-lysine (F).



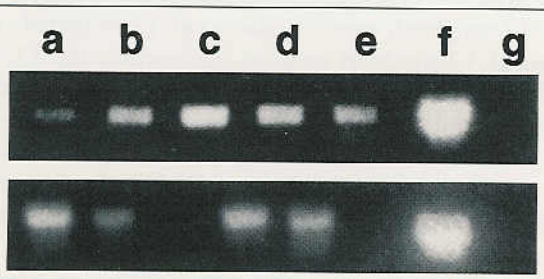
the precise physiological state of ASPA enzyme in vivo (which remains uncharacterized in humans), but rather to provide enough global expression in the brain to permit stabilization or reversal of phenotype.

More than 30 rats were also studied by RT-PCR analysis. RT-PCR results are from tissues collected at either 6 or 10 months, following intraventricular treatment with LPD/pAAVaspa at doses of 10 and 20 μl. At 6 months, all regions tested (cortex, cerebellum, midbrain, hippocampus, striatum) were positive for *ASPA* at the level of RT-PCR. At 10 months, however, the results were more variable, with some animals showing a loss in one or more brain areas, which did not demonstrate a clear pattern. Two representative animals are shown in a composite picture across five different brain areas at 6 and 10 months (Fig 3). In primates, we performed RT-PCR on periventricular and subcortical tissue (taken from sites 2 to 3 cm from the ependymal ventricular layer), which was positive for human ASPA transcript in both LPD/pAAVaspa monkeys at the 1-month time point. We could not detect ASPA in any peripheral tissues or organs by PCR in either rats or monkeys (see Materials and Methods), strongly suggesting that the vector did not escape the CNS.

Rodent and Primate Toxicology and Immune Response to Vector

There was no obvious histopathology or inflammatory cell infiltrate in rodent brain tissue after routine H&E and light microscopic evaluation in any of the animals treated with LPD/pAAVaspa or pAAVlac, by either the intraventricular or intraparenchymal route at multiple time points. However, we wanted to determine whether there was perhaps a lower-level immune reaction that would be detectable by other methods. Previous studies[53,54] had shown that adenoviral and herpes simplex vectors introduced to the brain resulted in inflammation with increased expression of multiple immunological markers, including MHC class I expression and T-cell infiltration. Therefore, we used OX-18 antibody, which reacts with a nonpolymorphic determinant of rat MHC class I antigen, and OX-35 antibody, which reacts with the CD4 antigen on T-helper cells, thymocytes, and activated macrophages or microglia, to perform immunocytochemistry on brain frozen sections.

Fig 3. RT-PCR from representative rats at two time points after intraventricular delivery of 20 μl of LPD/pAAVaspa. The first row represents the 6-month period, the second the 10-month period. The columns are as follows: (a) frontal cortex, (b) striatum, (c) substantia nigra, (d) hippocampus, (e) cerebellum, (f) positive control, (g) negative control.



In none of the intraventricularly treated animals were OX-35 immunoreactive cells identified, nor was there any neuronal MHC class I immunoreactivity. In the intraparenchymally treated animals sacrificed at 4 days, OX-18–positive and a small number of OX-35–positive cells were visible in the immediate vicinity of the needle track; however, this increase was seen in both saline-injected (control) and LPD animals and was negligible compared with the above studies using adenovirus or herpes simplex virus. At 1 and 3 months, there was no OX-18 or OX-35 staining in either group. These data suggest that LPD is minimally immunogenic compared with viral vectors. We also injected several groups of rats twice, to determine whether there was an immunological memory response in which inflammation would follow the second LPD vector challenge. We were also interested in seeing whether expression was boosted following redosing. However, we found no difference in immune markers or expression (which is itself a marker for an immune downregulatory response) after repeat dosage at 10 days or 1 month.

The rodent toxicity data we generated formed the basis for regulatory approval of a clinical trial in 2 humans. Following LPD delivery to more than 200 animals, there were no significant adverse events in any of them. Of the 175 adult rats we intracranially injected with LPD, 6 died overnight of complications of anesthesia or surgery, which were not specifically related to LPD. Of the remaining rats, none showed any post-treatment toxicity by multiple behavioral assessments. The animals maintained excellent coat quality and a complete absence of conjunctivitis. Feeding declined in all animals after surgery, but food and water intake was at normal levels within 48 hours. Even at the highest dose of 250 μl (>50% of the CSF volume), LPD was well tolerated and showed no difference in effect from high-dose saline (Table 2).

All monkeys survived a 1-month observation period with no clinically significant adverse events. After surgery the animals were hypoactive and had poor appetite, which normalized by day 3. Body weights dropped an average of 0.5 kg for all animals during the first week but began to increase after day 14 and had normalized by day 30. There was no febrile response, nor were there abnormalities in hematological and chemistry studies attributable to the test material. Likewise, physical examination and gross pathological examina-

Table 2. In Vivo Toxicity Testing

No. of Animals	Route of Administration	Volume (μl)	Duration	Vector	Formulation	Toxicity Outcome
80 adult (n = 4 × 20 groups)	Intracerebroventricular	10, 20 (single acute injection)	7 days; 1, 3, 6, 9 mo	β-galactosidase; *ASPA*	PL, PR	NT
40 adult (n = 2 × 20 groups)	Intraparenchymal	5, 12 (single acute injection)	3, 7 days; 2, 6, 8 mo	β-galactosidase; PBS/vehicle	PL, PR	NT
21 adult (n = 7 × 3 groups)	Intracerebroventricular	50[a]	2, 3 mo	β-galactosidase; *ASPA* PBS/vehicle	PL, PR	NT
14 adult (n = 7 × 2 groups)	Intraparenchymal	12[b]	40 days; 3 mo	β-galactosidase *ASPA*	PL, PR	NT
20 adult (n = 5 × 4 groups)	Intracerebroventricular	100,[a] 250	2 wk	β-galactosidase; *ASPA*	PL, PR	Mild[c]
30 newborn (n = 30)	Intracerebroventricular	10	1 mo	β-galactosidase	PL	NT

[a]Subset with repeated dose at 10 days.
[b]Subset with repeated dose at 30 days.
[c]All rats in the highest-dose group displayed some hypomotility (measured by open-field test and grooming behavior) during the 24 hours immediately after surgery. Within 48 hours, rectal temperature, food ingestion, and behavior were back to baseline levels.

NT = no toxicity; PL = poly-L-lysine; PR = protamine.

tion showed no effect from the test material, and no histopathology was detectable by light microscopy.

LPD/pAAVaspa Is Both Well Tolerated and Associated with Biochemical, Radiological, and Clinical Changes in Humans

The New Zealand Ministry of Health and the US Food and Drug Administration approved the export of LPD to New Zealand for the clinical portion of the study. LPD/pAAVaspa was well tolerated in both subjects, and there were no complications apart from mild postoperative fever and a transient CSF leak in one patient (A.M.). Moreover, the LPD/pAAVaspa gene transfer was associated with biochemical, radiological, and clinical changes.

Using ^{1}H nuclear magnetic resonance imaging, brain levels of NAA were noninvasively measured from the time of surgery to 1 year; after that point, other interventions were performed, which made it impossible to compare further posttreatment results until outcomes can be standardized to other patients. Before CNS gene transfer, the baseline NAA levels of L.K. and A.M were significantly elevated above the normal range throughout the brain (using multivoxel scanning) and were quantitated in the occipital lobe by single-voxel scanning at 15.4 and 15.6 mM, respectively. Because of an unanticipated system upgrade, a different scanner was used for follow-up studies, and data from the first scanner were recalibrated to standardize the data. At 1 month after surgery, NAA levels in the occipital region were measured at 14.5 and 12.8 mM, respectively.

Compared with a set of control values from 8 healthy age-matched patients and abnormal reference values from 5 untreated Canavan disease patients, the NAA levels in 1 child (L.K.) lay in the normal range for 12 months in both frontal and parietal lobes, although after about 2 months the occipital lobe showed no change from reference Canavan disease values. Conversely, the other child (A.M.) showed stabilization of occipital lobe NAA levels for 9 months but a reversion to reference Canavan disease levels in the frontal and parietal lobes after about 4 months. Figure 4 indicates that NAA levels in untreated controls are fairly stable and fall within the range of 12 to 19 mM across all regions tested. The variable results between L.K. and A.M. may be related to differences in regional diffusion of the vector, or alternatively can be explained by subtle differences in preexisting local brain pathology that were silent to imaging and neurological examination.

Subsequently, we also performed RT-PCR analysis on CSF samples obtained from both patients, using the vector-specific primers described earlier. The CSF samples were PCR amplified using primers that were specific for exogenous *ASPA* (see Materials and Methods). None of the samples were positive for *ASPA* before the original surgery, but samples from A.M. were still PCR-positive more than 1 year after LPD/pAAVaspa gene delivery. Samples from L.K. were PCR-negative for *ASPA* at the same time point. Of note, a decrease in RT-PCR signal in CSF is not necessarily a sign of decreased gene expression, because it may indicate uptake and spread of vector into cells in brain regions more distant from the CSF compartment.

Serial MRI images were obtained from patients before and after gene transfer. In L.K., we observed radiological changes in the white matter signal. MRI of the head was performed using sagittal T1-weighted, axial T2-weighted, axial FLAIR, axial T1-weighted, and coronal T2-weighted imaging. At baseline, the T1 signal showed an abnormal prolongation within the subcortical and deep white matter, and the T2 signal showed a reciprocal abnormal signal (hyperintensity) that was particularly evident on FLAIR. By 9 months after treatment, the subcortical white matter showed a decrease in the abnormal prolongation of the T1 signal; at 12 months, there was substantial loss of hypoin-

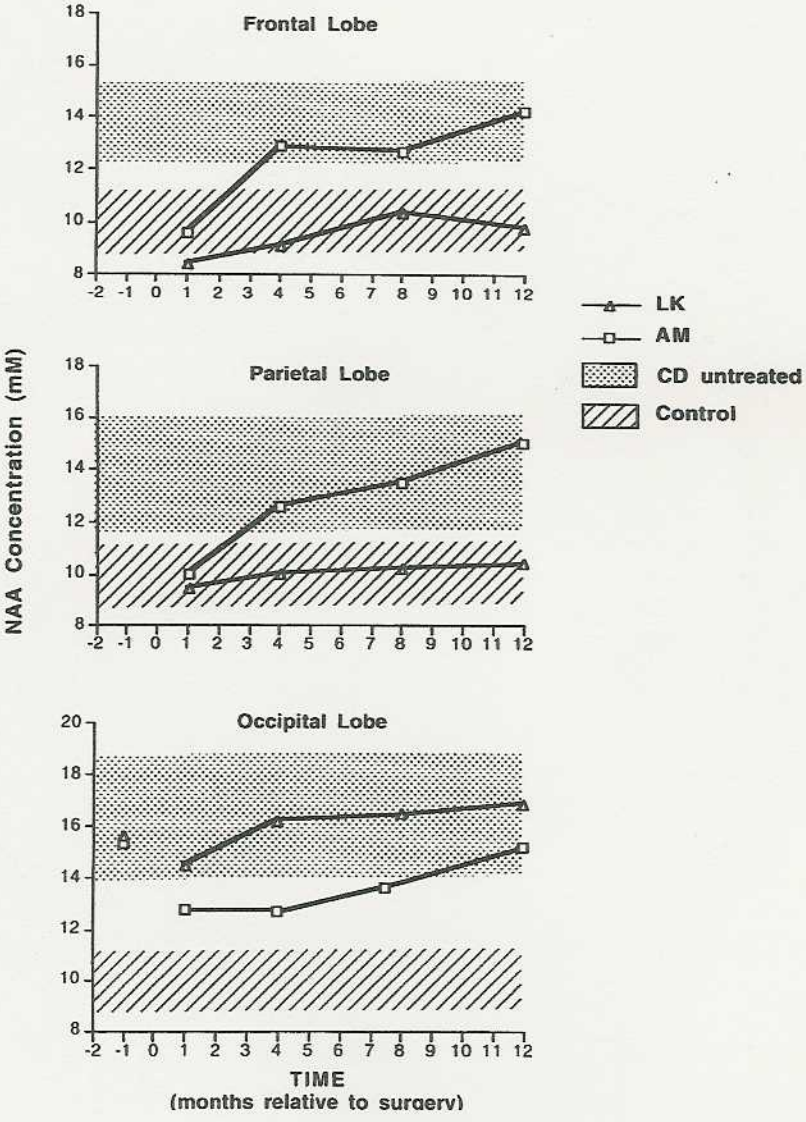


Fig 4. Diagram showing ^{1}H-NMR of brain NAA levels from 2 human patients (L.K. and A.M.) along with reference values from untreated Canavan disease patients and another group of age-matched normal controls, at periods from 1 month before to 12 months after delivery of LPD/pAAVaspa.

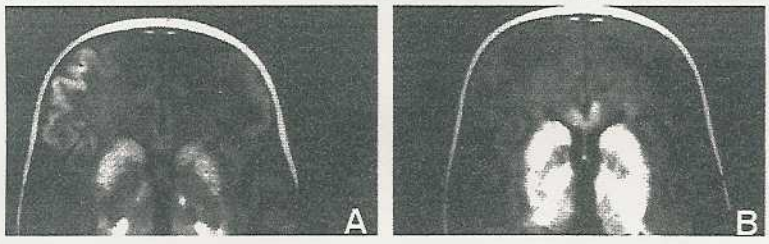


Fig 5. Representative axial T1-weighted MRI images of L.K. at baseline (A) and at 12 months after gene transfer (B).

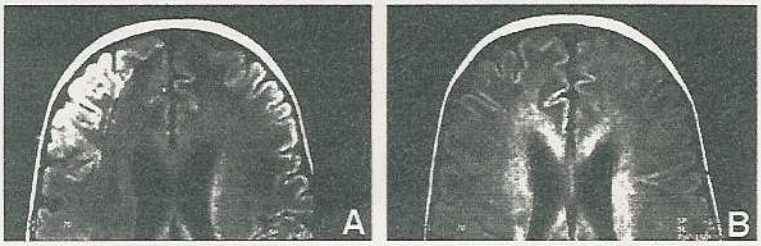


Fig 6. Representative coronal T1-weighted MRI images of L.K. at baseline (A) and at 12 months after gene transfer (B).

tensity on T1, suggesting new myelination of the corpus callosum as well as the basal ganglia and the posterior limb of the internal capsule (Figs 5 and 6), areas close to the ventricular delivery site and where we demonstrated efficient gene transduction in animal models. This myelination pattern was still only typical of a 4-month old infant, yet it was significantly improved from the baseline and 4-month studies, which had suggested a pattern more typical of a newborn. T2 signal at this time continued to show an abnormal hyperintensity consistent with spongiform pathology but with little interval change over the entire year after delivery. In patient A.M. and 5 untreated Canavan disease patients, there were neither positive nor negative changes in myelination visible on T1-weighted MRI over a 9- to 12-month interval, but the T2/FLAIR signal showed increased abnormal hyperintensity in some control patients. It is worth mentioning that in contrast to other neurodegenerative diseases such as multiple sclerosis, which can show great variability (often cyclic) in MRI images over a short period, untreated Canavan disease patients are not known to show improvement on serial imaging.

The biochemical and radiological changes in L.K. were associated with changes in neurological tests, although these are difficult to interpret outside of a larger trial. Before gene transfer, the visual evoked response (VER) demonstrated significant bilateral delay, and an ophthalmic examination had demonstrated marked deterioration of vision, with loss of fixation, amblyopia, exotropia, and nystagmus. At 2 months after surgery, the VER was normal and remained in the normal range at 6 and 12 months; moreover, fixation and exotropia had clinically improved and the amblyopia and nystagmus had completely resolved. A.M. was unable to cooperate sufficiently to obtain interpretable VER before surgery and at early time points (3 and 6 months) after surgery. At 12 months, cortical potentials were recorded that showed a bifid response, with an abnormal early 65-msec latency followed by a second deflection at 95 msec that was consistent with a normal VER. In a separate group of 5 untreated Canavan disease patients that were followed in parallel, we found that change in evoked potentials was only toward further pathological slowing of the visual, brainstem auditory, or somatosensory evoked potentials.

These 2 patients and 5 untreated controls were assessed with the Gesell-Provence Developmental Schedules (GPDS) and the Hawaii Early Learning Profile (HELP) by a pediatric neurologist. Before surgery (age 19 months) L.K. was assessed at a 8- to 12-week age equivalent. At 1 month after surgery, she had gained the ability to briefly hold the head unsupported, to turn from her side to her back, and to bear some weight on her legs, but her overall psychometric testing still indicated approximately a 3-month age equivalent. By 1 year after surgery (age 31 months), she demonstrated improved motor function (eg, reaching out with both hands), and her functional level in scattered skill development (in specific areas of cognition, expressive language, and social emotion) was the equivalent of a 18- to 24-month-old child. Five months after gene transfer, A.M. was assessed at a 12- to 16-week equivalent, but by the time of surgery, she had deteriorated to less than 12-week equivalent. By 1 month after surgery, she was reassessed at 12- to 16-week age equivalent, which was maintained at 2 months after surgery with advances in some areas, such as demonstrating a greater interest in her surroundings and increased social interactiveness. Her neurodevelopmental examination subsequently plateaued with only minor changes over a 12-month period. The other 5 Canavan disease patients assessed showed either no improvement or mild deterioration on psychometric tests. Yet the course of the disease can be variable, and a larger study is required to draw solid conclusions about any behavioral changes.

Discussion

We present evidence that LPD/pAAV is an effective delivery system to achieve nontoxic, widespread gene delivery in the rat CNS, when combined with systemic mannitol and administered by the intraventricular route. Using a nonhuman primate model, we also verified safety and gene expression in the CNS. These data were sufficient to support the first gene transfer study in 2 human subjects with Canavan disease. Although our preliminary human study was limited by a small sample size (n = 2), these first human subjects show some promising clinical findings that we plan to examine more fully in an ongoing trial in 14 patients.

The major limitation of CNS gene transfer remains the problem of global delivery and high-level expression in vivo. We found that diffusion of LPD in the rat brain was on the order of millimeters, and that expression of a *lacZ* reporter gene extended beyond the site of intraventricular or intraparenchymal delivery. Using injection volumes of 2 to 50 μl (at a concentration of 300 μg/ml of LPD) in the rat gave a transduction of 10^3 to 10^5 cells, based on cell counts in intraparenchymally and intraventricularly injected animals. In 2 human subjects treated with LPD/poly-L-lysine we used up to 500 times the volume used in most rodent studies, which was expected to transduce perhaps 10^6 to 10^7 cells, based on extrapolation from the available animal data. Even given a potentially lower transduction efficiency in humans, our hypothesis was that expression in perhaps as little as 10^5 cells would be sufficient to lower NAA regionally and to have an effect on the course of the disease. These data from 2 children with Canavan disease suggest that even a modest amount of transduction may lead to detectable clinical

changes, as regionally normalized NAA levels were associated with neurological and behavioral phenotypic improvements, both mild (A.M.) and more pronounced (L.K.). Yet the stated purpose of this trial was primarily to establish safety, and until a larger trial is completed we are unable to rigorously establish the efficacy of LPD delivery for Canavan disease.

In Canavan disease, both severe and mild phenotypes have been described for identical *ASPA* gene mutations, and specific mutations have not been correlated with a preponderance of mild or severe phenotype.[51] Therefore, while our patients had different mutations, it is unlikely that this fact alone explains the difference in their disease course. It seems equally likely that other factors may play a role, for example, the postsurgical CSF leak in A.M. may have compromised gene delivery and possibly led to less pronounced changes, and also the extent of brain damage may have been more variable than expected on the basis of pretesting between the 2 patients for reasons unrelated to the specific gene defect.

Recently, other investigators have used the intrathecal route for CNS gene expression in rats[22] and thereby achieved phenotypic correction of disease.[25] In previous work, we also demonstrated phenotypic correction in rats (and monkeys) after CNS delivery of AAV[21,24] vectors. Current work on a *ASPA* knockout mouse and rat models will help in clarifying the extent of phenotypic correction possible using different methods. Even if the *ASPA* knockout rescue paradigm is successful, it is important to bear in mind that the results may not be easily generalized to humans. Although global delivery is clearly attainable in rodent models, the human brain presents a much larger target. It remains to be seen whether true global penetration can be achieved in humans through the intraventricular route. Of course, it is possible that a more limited pattern of "global" expression is sufficient for partial phenotypic recovery, and infants are likely to be an ideal target group. With this end in view, we are currently following a larger cohort of children to determine whether the biochemical, radiological, and clinical changes we noted are replicated in all Canavan disease patients after LPD gene transfer.

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