Intracerebral adeno-associated virus-mediated gene transfer in rapidly progressive forms of metachromatic leukodystrophy

Caroline Sevin1, Abdellatif Benraiss1, Debby Van Dam2, Delphine Bonnin1, Guy Nagels3,4, Lucie Verot5, Ingrid Laurendeau6, Michel Vidaud6, Volkmar Gieselmann7, Marie Vanier5, Peter Paul De Deyn2,8, Patrick Aubourg1 and Nathalie Cartier1,*

1Institut National de la Santé et de la Recherche Médicale U561, Université Paris V, 75014 Paris, France, 2Laboratory of Neurochemistry and Behavior at Institute Born-Bunge, Department of Biomedical Sciences, University of Antwerp 1, Antwerp B-2610, Belgium, 3National MS Centre, Vanheylenstraat 16, 1820 Melsbroek, Belgium, 4Department of Neurology, University Hospital, Edegem, Belgium, 5INSERM, Fondation Gillet-Mérieux, Hôpital Lyon-Sud, 69310 Pierre-Benite, France, 6Laboratoire de Génétique Moléculaire-UPRES EA 3618, Faculté des Sciences Pharmaceutiques, Université Paris V, 75014 Paris, France, 7Institute of Physiological Chemistry, Rheinische Friedrich-Wilhelms-Universität, 53115 Bonn, Germany and 8Department of Neurology and Memory Clinic, Middelheim General Hospital, B-2020 Antwerp, Belgium

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Metachromatic leukodystrophy (MLD) is a neurodegenerative lysosomal disease caused by a defect of the enzyme arylsulfatase A (ARSA; EC 3.1.6.8) (1). Lack of ARSA activity results in an inability to degrade 3-O-sulfogalactosylceramide (sulfatide—Sulf) in glial cells and neurons (2). MLD is characterized by progressive demyelination in the central (CNS) and peripheral nervous systems (PNS). Demyelination is the main pathological finding, but substantial storage of Sulf in neurons also occurs, probably contributing substantially to the clinical phenotype (3,4). The prognosis of MLD is severe, with death resulting within a few years after the onset of neurological symptoms. The disease is usually classified on the basis of age at onset (1): the late infantile form manifests in the second year of life, the juvenile variants between years 4 and 12 and the adult form after 16 years of age. Currently, there is no specific treatment for the late infantile form of MLD, which is the most frequent (60%). Allogeneic hematopoietic cell transplantation (HCT) may delay...
or arrest cerebral demyelination in selected patients in very early stages of the juvenile or adult forms of the disease, but has no effect in the late infantile form (5).

The treatment of MLD is exceedingly difficult because the neurological deterioration occurs rapidly, particularly in infants with the late infantile form (1). Enzyme replacement therapies (ERTs) have been successful in preventing or reversing the systemic manifestations of several lysosomal disorders (6) by taking advantage of the cells’ ability to take up infused enzyme via the cation-independent mannose-6-phosphate receptors, which are present on the surface of virtually all cells (7,8). Intravenous injection of recombinant ARSA in MLD mice reduces the Sulf storage in peripheral nerves and, to some extent, unexpectedly in the brain (9). Although ERT has been found to be unsuitable for the treatment of CN5 lyosomal storage in humans, as the blood–brain barrier prevents the direct transfer of ARSA enzyme from the bloodstream, the results obtained in MLD mice suggest that this strategy could produce long-term benefits in MLD patients. However, the extent of metabolic correction would unlikely be sufficient to arrest the rapid cerebral demyelinating process that occurs in aggressive and devastating forms of MLD.

Treating neurodegeneration in MLD calls for strategies that not only bypass the physical blood–brain barrier, but also promote the rapid delivery of ARSA enzyme throughout the brain. In MLD mice, transplanting hematopoietic stem cells transduced with the ARSA gene has been shown to be more effective at preventing neurodegeneration than transplanting wild-type cells (10), probably through the overproduction of ARSA in CNS microglia. This strategy might have substantial advantage over conventional allogeneic HCT in MLD patients. However, the replacement of brain microglia that occurs after HCT is a slow process, and metabolic correction would occur too late to be effective in patients with rapidly progressive forms of MLD.

Given the rapidity of neurological deterioration in MLD, the direct delivery of ARSA enzyme by means of stereotactic injections of recombinant viral vectors could be a valuable therapeutic alternative. In vitro gene therapy of lysosomal storage disorders has been evaluated in several animal models (11). Importantly, such strategy could take advantage of the fact that the recombinant ARSA enzyme could be secreted by transduced CNS cells and taken up by distant cells, primarily via the mannose-6-phosphate receptors (7,8).

Toward this goal, we injected an adeno-associated virus serotype 5 (AAV5) vector encoding human ARSA cDNA into the brain of MLD mice, which lack ARSA activity and exhibit Sulf storage reminiscent to that found in MLD patients (12–16). AAV5 is a non-toxic virus that allows sustained, widespread and long-term expression of therapeutic genes in the CNS (17,18). We studied the diffusion of AAV5 vector and ARSA protein in the brain of treated mice and the long-term efficacy of vector injections on their phenotype. Our data show that AAV5-mediated gene therapy delivers functional ARSA rapidly and efficiently in the entire brain and prevents the accumulation of Sulf. Improved behavior, a near-complete disappearance of pathological signs in the brain, even in the white matter, and evidence for the in vivo secretion-recapture of ARSA all indicate that intracerebral AAV5-ARSA vector injections could provide an effective treatment for MLD patients.

RESULTS

Expression and secretion of functional ARSA in vitro

293T cells were transfected with an AAV5-ARSA or an AAV5-LacZ plasmid. ARSA activity above the levels of observed in untransfected or AAV5-LacZ-transfected 293T cells was detected in cell lysates and in the medium of AAV5-ARSA-transfected cells (Fig. 1A). Human MLD fibroblasts grown in the presence of medium from AAV5-ARSA-transfected 293T cells showed significantly increased ARSA activity (40% of the activity observed in wild-type fibroblasts), indicating that the recombinant ARSA was correctly secreted and endocytosed by cells in vitro (Fig. 1B). Recombinant ARSA correctly co-localized with Lamp2 in lysosomes (Fig. 1C).

Distribution of ARSA throughout the brain

Three-month-old MLD mice (n = 13) were injected with the AAV5-ARSA vector into the cerebellum and the internal capsules. These sites of vector injections were chosen to optimize ARSA delivery according to the brain pathology described in MLD mouse. Mice were sacrificed at 6 (n = 7) and 18 months (n = 6) of age, i.e. 3 and 15 months after injection, and compared with age-matched untreated MLD mice and wild-type mice. In treated MLD mice, the level of recombinant ARSA in the whole brain assessed by ELISA was four times greater than that in normal human brain (Fig. 2A). This level increased further up to eight times that of the normal human brain at 18 months of age (Fig. 2A). The highest levels of ARSA were found in sections close to the sites of injection (Fig. 2B and D), but significant ARSA expression was also detected in areas distant from injection sites (anterior part of the forebrain and brainstem; Fig. 2B and D). In the cervical spinal cord, the level of ARSA was 21.7 ± 2.4 ng ARSA/mg protein, i.e. 20% of that in human brain (Fig. 2B). No ARSA enzyme was detected in the liver, spleen or testis of treated mice (data not shown).

Immunohistochemical studies showed that vector-encoded ARSA protein had diffused into the brain and the cerebellum of treated MLD mice (Fig. 3). ARSA-positive cells were particularly abundant in areas surrounding the two injection sites (Fig. 3). In the cerebral cortex, ARSA expression was mainly restricted to layer IV (Fig. 4A), and to the CA3 area in the hippocampus (Fig. 4B). In the cerebellum, ARSA-positive cells were mainly present in the molecular layer, but positive cells were also observed in the granular and the Purkinje cell layers. ARSA-positive cells were also present in the white matter of the corpus callosum, fimbria and cerebellum (Fig. 4C and D). ARSA immunostaining revealed punctuated, perinuclear staining in neurons and astrocytes, arguing for the lysosomal localization of the enzyme (insets A and B in Fig. 3).

Morphological analysis and double labeling with specific markers showed that 80% of the cells expressing ARSA were neurons (Figs 3 and 5). Eighteen percent of ARSA-positive
cells expressed the astrocytic glial fibrillary acidic protein (GFAP) marker (Figs 4 and 5). Recombinant ARSA was also detected in $\sim 1\% - 2\%$ of microglial cells labeled with the tomato lectin. Many of these cells had small cell bodies.

Figure 1. (A) ARSA activity in cell lysate and medium of 293T cells, 72 h after transfection with AAV5-ARSA or AAV5-LacZ plasmids (mean ± SEM of 11 experiments). (B) ARSA activity in human MLD fibroblasts grown in the presence of medium from AAV5-ARSA or AAV5-LacZ-transfected 293T cells (mean ± SEM of six experiments). (C) Double immunostaining of AAV5-ARSA-transfected 293T cells with anti-hARSA and anti-Lamp2 antibodies showing the co-localization of ARSA and Lamp2 proteins in the lysosomal compartment. $^* P = 0.05$; **$P < 0.01$; ***$P < 0.001$.

Figure 2. ARSA levels analyzed by ELISA in the whole brain (A) and in serial brain sections (mean ± SEM of 14 samples) (B) of 6- and 18-month-old treated mice. (C) Logarithmic curve of the ratio between ARSA content and vector genome copy number according to the distance from injection sites. (D) ARSA levels (ng ARSA/mg protein, bold numbers) and vector genome copy number per 2$^\text{nd}$ genome (italic numbers) in brain sections of 18-month-old treated MLD mice ($n=3$). S2, S4 and S6 refer to brain coronal sections as indicated in B, C and D; *Injection sites in the internal capsules and the cerebellum; Cb: cerebellum; BS: brainstem; SC: spinal cord; HB: human brain; ***$P < 0.001$. 
with primary and secondary ramifications (Fig. 5), suggesting that they were resident microglia. Double immunostaining with anti-hARSA and anti-CNPase antibodies failed to detect ARSA expression in oligodendrocytes.

Widespread ARSA delivery results mostly from dispersal of the enzyme
To find out whether ARSA delivery resulted from dispersal of the vector or of the enzyme, we measured the number of vector genome copies and the amount of ARSA in serial coronal brain slices of 18-month-old treated MLD mice (Fig. 2D). The mean vector copy number was $19.4 \pm 7.4$ per $2n$ genome at the injection site in the internal capsules (S6) and $0.016 \pm 0.013$ copies per $2n$ genome at 4 mm from the injection site (S2). At the cerebellar injection site, the vector copy number was $1.4 \pm 0.6$ copies per $2n$ genome. AAV5-ARSA DNA was also detected in the brainstem and cervical spinal cord (Fig. 2D). No viral vector was detected in the liver, spleen or testis of treated mice, or in tissues of untreated MLD mice or wild-type mice (data not shown).

ARSA levels assessed by ELISA were significantly higher at the injection sites (Fig. 2B and D). We made the assumption that if ARSA could be secreted by transduced cells and endocytosed by untransduced cells, then the level of ARSA should decrease to a considerably lesser extent at a distance from the injection site than the vector copy number, and the ratio of ARSA concentration to vector copy number should increase significantly from the injection site to distant areas. In accordance with this hypothesis, the ratio of ARSA level/vector copy number was 2600 times higher at a distance than at the site of vector injection (Fig. 2C).

To confirm these findings, we determined the number of neurons and astrocytes expressing the human ARSA in the striatum and white matter, 3 and 15 months after injecting the AAV5-ARSA vector. In the striatum, the total number of neurons decreased slightly between 3 and 15 months after treatment ($1504 \pm 67$ and $1311 \pm 81$ mm$^{-2}$, NS), whereas the percentage of neurons expressing ARSA increased from 49.5 to 56% (Table 1). The number of astrocytes increased moderately in the oldest treated mice (from $104 \pm 9$ to $137 \pm 6$ mm$^{-2}$, $P = 0.01$), whereas the percentage of astrocytes expressing ARSA enzyme increased from 6 to 12% in the striatum, and from 4 to 14% in the white matter of the cerebellum (Table 1). As neurons are non-dividing cells, the increase of ARSA-positive neurons in the oldest treated mice strongly suggests that untransduced neurons were able to endocytose the ARSA secreted by adjacent transduced cells (neurons or astrocytes). However, we cannot rule out the possibility that the transduced neurons had some survival...
advantage over untransduced neurons. Similarly, the 2–3.5-fold increase in the percentage of astrocytes expressing ARSA indicates that many untransduced astrocytes were probably cross-corrected.

**ARSA delivery prevents Sulf storage**

Sulf storage is already detectable in MLD mice at the age of 3 months, and further increases with age thereafter (13). Using Alcian blue staining, this storage was absent, or at least markedly reduced, in all treated MLD mice, and was barely detectable in the white matter of treated mice in contrast to the staining observed in age-matched untreated mice (Fig. 6).

For biochemical evaluation of the Sulf storage, as brain slices contain variable proportions of gray and white matters, relating Sulf to galactosylceramide (GalC)—a major and almost exclusive component of the myelin sheath (19,20)—appeared to be the most informative expression of the data. Only minor fluctuations of the Sulf/GalC ratio occurred in wild-type mice between 3 and 18 months of age, whereas this ratio increased almost in a linear fashion in untreated affected mice, in agreement with the previous data (4). In treated mice, the Sulf/GalC ratio, which was already increased at the time of injection, did not increase further and remained stable during the entire study period (Fig. 7). These data indicate that the delivery of ARSA prevented further Sulf accumulation. The preservation of the Sulf/GalC ratio could also reflect a protective effect of ARSA delivery on oligodendrocytes, which is also supported by the disappearance of Alcian blue staining in these cells (Fig. 6B).

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**Table 1.** The percentage of neurons and astrocytes expressing ARSA increases with time

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<th>Neurons (%NeuN+/ASA+) in stratum</th>
<th>Astrocytes (%GFAP+/ASA+)</th>
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<td>6-month-old treated MLD mice (n = 4)</td>
<td>49.5</td>
<td>6</td>
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<tr>
<td>18-month-old treated MLD mice (n = 4)</td>
<td>56</td>
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A significant increase (P = 0.005) in ARSA-positive neurons is observed in the stratum; a 2–3.5-fold increase in ARSA-positive astrocytes is observed in the stratum and the white matter of the cerebellum.
Correction of brain pathology

**Glycolipid storage.** Periodic acid-Schiff (PAS)-reactive material reflecting lipid storage was detected in 18-month-old untreated animals, mainly in the cytoplasm of large swollen macrophages within the white matter (Fig. 8A), but was absent in both treated MLD mice and wild-type mice (Fig. 8A).

**Microglial activation and astrogliosis.** In the white and gray matters of 18-month-old untreated MLD mice, staining with tomato lectin revealed an increased number of the swollen amoeboid cells that characterize the activated microglia (12,13). A significant reduction in the number of amoeboid cells was observed in the treated mice, and most of the tomato lectin-positive cells displayed a ramified morphology suggestive of resting microglia (Fig. 8B). Severe astrogliosis observed in the white matter (corpus callosum, fimbria, cerebellar white matter), hippocampus and pons of old MLD mice was also markedly prevented in all brain areas (Fig. 8C).

**Neuronal degeneration.** Eighteen-month-old MLD mice display substantial loss of their Purkinje cells and a simplified dendritic architecture (15) (Fig. 8D). These abnormalities were significantly less marked in treated animals; the numbers of Purkinje cells were 7.2 ± 1.7, 14.1 ± 2.1 and 19.4 ± 2.2 cells/mm in the Purkinje cell layer of untreated MLD, treated MLD and wild-type mice, respectively. The number of Purkinje cells present in treated and wild-type animals did not differ significantly.

Fluoro-Jade B specifically stains degenerating neural cells (21). Brain sections from 18-month-old untreated MLD mice showed Fluoro-Jade B-positive cells scattered throughout the cerebral and cerebellar cortex, and the pons. In treated MLD mice.
mice, these positive cells were absent, as they were in wild-type animals (data not shown).

Lack of toxicity of the AAV5-ARSA vector. Hematoxilin–eosin staining of brain sections from 6- and 18-month-old treated mice failed to detect any sign of necrosis or astrocytic scarring along the needle tracts or in distant areas. Immunohistochemical staining with the lymphocyte marker CD45 revealed the presence of a few CD45-positive cells at the injection sites 3 months after injection. Fifteen months after injection, no CD45 staining was detected (data not shown).

Treated MLD mice are protected against neuromotor impairment

Prior to treatment, no significant differences in rotarod performance were observed between MLD and wild-type mice (effect genotype, $P = 0.086$; effect genotype × trial, $P = 0.782$). The treatment had a significant effect on rotarod performance at the age of 6 months (effect treatment group, $F_{2,111} = 6.407$, $P = 0.004$; effect treatment group × trial, $P = 0.364$). Untreated MLD mice performed significantly worse than wild-type mice (Tukey; $P = 0.005$), whereas treated MLD mice displayed the same level of performance as the control group ($P = 0.752$). Similar observations were made at the age of 9 months (effect treatment group, $F_{2,120} = 10.978$, $P < 0.001$; effect treatment group × trial, $F_{6,120} = 2.353$, $P = 0.035$), with post hoc analysis indicating that the treated mice performed better than the untreated MLD mice ($P = 0.007$), and displayed the same level of motor performance as wild-type mice ($P = 0.762$). At the age of 12 months, however, no differences were observed between the three groups (effect treatment group, $P = 0.247$; effect treatment group × trial, $P = 0.102$), whereas differences analogous to those observed between the treated and untreated animals at younger ages were noted in 15-month-old animals (two-way RM-ANOVA; effect treatment group, $F_{2,90} = 4.217$, $P = 0.024$; effect treatment group × trial, $P = 0.309$) and 18-month-old animals (effect treatment group, $F_{2,66} = 11.918$, $P < 0.001$; effect treatment group × trial, $P = 0.730$). Age-related changes in the rotarod performance of wild-type, treated and untreated MLD mice are depicted in Figure 9A.
Electrophysiology

The amplitude of waves recorded during the study of brainstem auditory evoked potentials (BAEPs) was severely decreased in 6-month-old untreated MLD mice. The latency of the I–V interpeak was no longer reliably measurable at 6 months, and BAEPs were completely abolished at 9 months of age (14). These electrophysiological parameters showed no improvement in treated MLD mice after the intracerebral injection of AAV5-ARSA into the internal capsules and cerebellum (data not shown).

In separate experiments, AAV5 vector was injected into the cochlear nuclei of 2-month-old MLD mice (n = 5; Fig. 9B) and significantly improved BAEPs (Fig. 9C and D). At 6 months of age, all the animals had good or intermediate BAEP responses (see Materials and Methods): 6/7 wild-type mice had good responses and one had intermediate responses; 0/4 untreated MLD mice and 4/5 treated MLD mice had good responses (P = 0.012, χ²-test). The analysis of the BAEP amplitude showed a significant effect for group (GLM: d.f. = 2, F = 24.758, overall P < 0.001). Wild-type mice had higher BAEP amplitudes than treated and untreated MLD mice (post hoc Tukey P < 0.001; Fig. 9D). However, the amplitude of BAEPs was on average higher in treated mice than in untreated ones, even though this difference was not statistically significant (post hoc Tukey P = 0.253; Fig. 9D).

DISCUSSION

In this study, we demonstrate that AAV5 vector-mediated ARSA gene transfer into the brain has the capacity to treat neuropathology throughout the entire brain of MLD mice, and to prevent neuromotor disability. Our study raises a number of issues that must be addressed to assess the clinical relevance of our results.

Delivery of ARSA into the brain

A striking observation was the induction of very high levels of ARSA through the entire brain of the treated mice, and not only in regions surrounding the injection sites. Intracerebral transfer of ARSA cDNA using a lentiviral vector resulted in more restricted distribution of the enzyme (22). Lower expression of the vector-encoded ARSA and differences between the sites of injection and the vector used could account for this discrepancy. MLD is characterized by demyelination and neuronal storage in the CNS (1,2,4). Intracerebral injection of the AAV5 vector resulted in the presence of ARSA enzyme in both the gray and white matters. In vitro studies with anti-Lamp2 antibodies showed that ARSA was correctly targeted to lysosomes, and immunohistochemistry of brain sections showed a punctuated staining of ARSA, which also suggested its localization within the lysosomes. As expected with the AAV5 vector, ARSA was mainly found in neurons, but up to 20% of astrocytes and 2% of microglial cells were also positive for the enzyme. As the transduction of astrocytes or microglia after intracerebral injections of AAV vectors is a rare event (17,18), cross-correction of these cells by transduced neurons probably occurred. We did not detect ARSA in oligodendrocytes with any certainty. For unknown reasons, these cells are refractory to gene transfer in vivo (23). However, the lack of Alcian blue staining in oligodendrocytes of treated animals and the absence of Sulf/GalC ratio deterioration suggest that ARSA delivery had a protective effect on these cells. Long-term studies did not reveal any harmful effect of ARSA overexpression in neurons or glial cells. ARSA expression was sustained over 15 months in the treated mouse brain and did not decrease with time.

Quantitative polymerase chain reaction (PCR) analysis of the vector genomes showed dispersal of the vector from the injection sites. The AAV5-ARSA vector was also detected in distant regions, such as the brainstem and the cervical spinal cord. However, vector dispersal could not account for the widespread delivery of ARSA enzyme throughout the entire brain. The ratio of ARSA levels to vector copy number demonstrated that the protein itself was spreading within the brain tissues. The possibility that ARSA was taken up by astrocytes, neurons and microglia was suggested by the disappearance of Sulf storage in areas devoid of vector genomes, and the increase over time of neurons and astrocytes expressing the recombinant enzyme. We did not determine how much of this uptake occurred via the mannose-6-phosphate receptors and how much via other internalization pathways. However, in vitro studies in MLD fibroblasts indicate that the entire process of enzyme release, transport and uptake was efficient. The difference in ARSA expression between astrocytes, microglia and oligodendrocytes suggests that these cells probably have differing abilities to endocytose-recombinant ARSA. We did not observe any greater expression of ARSA in brain regions that were synaptically connected to the two sites of injection; however, it is possible that the delivery of ARSA could also be mediated by the vesicular transport of the enzyme along axons originating from transduced neurons (24,25).

Disease correction

The MLD mouse does not show the severity of symptoms observed in humans and have a near-normal life expectancy. One major difference between the human and the mouse pathology is the absence of a clear demyelination process in the CNS and the PNS of MLD mice (12). For the CNS, one reason might be that Sulf accumulate by 8-fold in the brain of human MLD patients, whereas they accumulate by only 1.4-fold in the brain of MLD mouse (26). However, the accumulation of PAS-positive macrophages and the presence of microglial activation and severe astrogliosis in the CNS white matter of ARSA-deficient mice indicate a pathological reaction to Sulf accumulation. These data are of particular interest given the pathogenic role of activated microglia that triggers neuronal and oligodendrocyte death by cytokine release (27,28).

The complete disappearance of the pathology or the persistence of minimal lesions was observed in the brain of 18-month-old treated MLD mice, whereas the condition was severe in age-matched untreated mice. In the treated MLD mice, Sulf storage was absent or minimal, even in the white matter, and microglial activation and astrogliosis were prevented. PAS-reactive material reflecting lipid storage was also
absent in macrophages from the white matter of treated MLD mice. Delivery of ARSA also prevented both the appearance of dying cells (assessed using the Fluoro-Jade B marker) and the loss of Purkinje cells. The functionality of the ARSA delivered was also demonstrated by investigating the Sulf/GalC ratio, which remained unchanged from the time of treatment. An increase in this ratio is a marker of disease progression, and reflects both Sulf storage and demyelination (1,4,26).

MLD mice display motor coordination impairment during the rotarod test (16). ARSA delivery into the brain nearly completely prevented this neuromotor disability. Differences in performance between treated and age-matched wild-type mice may reflect involvement of the peripheral nerves that the intracerebral injection of vectors was not expected to target. This notion is supported by the rotarod data for MLD mice treated with ERT that show improvement of motor coordination (9). As expected, inner-ear defects leading to deafness in MLD mice were not corrected in treated mice, which displayed BAEP abnormalities similar to those in the untreated animals. Physiological barriers probably prevented the diffusion of ARSA enzyme from internal capsules and cerebellum into the acoustic ganglia and cochlear nuclei that are the site of early neurodegeneration (13,14). However, selected injections of AAV-ARSA vector in the cochlear nuclei of 2-month-old MLD mice did lead to a significant improvement of BAEPs at 6 months of age. These findings indicate that the delivery of ARSA into the cochlear nuclei probably prevented the neuronal degeneration of the acoustic ganglia and cochlear nuclei, allowing near-normal nerve conduction from the inner ear to the brainstem to be preserved.

CONCLUSION

A treatment for MLD is highly desirable. ERT could prevent peripheral nerve demyelination but this therapeutic approach needs further evaluation because nearly all patients with MLD develop severe PNS dysfunction in contrast to that observed in MLD mouse. This study provides evidence that, such as the transplantation of genetically corrected hematopoietic stem cells, intracerebral injection of AAV vector has the potential to alleviate the CNS disease in the MLD mouse model. Both methods are invasive. The lag time between the transplantation of genetically corrected hematopoietic stem cells and replacement of microglia expressing normal ARSA enzyme in the CNS might be a limitation of this approach in rapidly progressive form of MLD. The intracerebral delivery of ARSA might be effective more quickly in arresting the demyelination process and neuronal damage in the CNS. It is, however, difficult to extrapolate our results to already symptomatic patients, in particular those with infantile form of MLD. However, the slow disease evolution of the MLD mouse represents an advantage in the experimental setting to evaluate the potential of this therapeutic strategy to prevent the symptoms at a very early stage of the disease. Given also the obvious difference in brain size between mouse and human, extent of ARSA delivery mediated by intracerebral injections of AAV vector requires further evaluation in larger species animals before this therapeutic option can be proposed for use in MLD patients. Recent results attesting for improvement in clinical neurological signs and in brain myelination in a cat model of lysosomal storage disease after intracerebral injection of AAV vector suggest that such strategies could be envisaged in larger brain size (29).

MATERIALS AND METHODS

Production of AAV vector

Transfection of murine MLD fibroblasts with human ARSA cDNA leads to the complete restoration of ARSA activity (data not shown). Human ARSA cDNA was therefore used to construct the AAV vector.

Three PCR-generated fragments containing the entire sequences of the 0.6 kb murine phosphoglycerate kinase (PGK) promoter, the 1.6 kb hARSA cDNA and the 0.6 kb regulatory element of woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) were cloned from M48-ALD (30), pBEH HT14/CP8 and pTRIP-ΔU3-EF1Δ-ALD (31) plasmids, respectively, to generate the pAAV5/PGK-ARSA-WPRE plasmid, referred to as AAV5-ARSA.

AAV5-ARSA vector stocks were generated by the transient transfection of 293T cells (32) and purified using CsCl ultracentrifugation gradients. The final titers were $1.5 \times 10^{12}$ physical particles/ml (32).

Animal models

ARSA-deficient mice with a mixed 129sv/Ola genetic background were bred from homozygous founders (12). The animals were housed in a pathogen-free animal facility. The animal experiments were approved by the veterinary bureau of INSERM and the Animal Ethics Committee of the University of Antwerp, and were carried out in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-24, revised) and the European Communities Council Directive (86/609/EEC).

Direct injection of the AAV vector into the brain of MLD mice

Three-month-old MLD mice were anesthetized by the intraperitoneal injection of ketamine/xylazine (0.1/0.05 mg/g body weight) and positioned on a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). The animals were injected with 2 μl of viral preparation ($3 \times 10^{9}$ physical particles of AAV5-ARSA) using a 30-gauge blunt micropipette attached to a 10 μl Hamilton syringe (Reno, NV, USA) at a rate of 0.2 μl/min. The sites of the injections included the cerebellar vermis (stereotactic coordinates from the bregma: AP: $-6.48$ mm; ML: 0; DV: $-1.6$ mm), and the left and the right internal capsules (AP: $-1.2$ mm; ML: ±2.0 mm; DV: $-3.8$ mm) (33). Viral vector was also administered into the cochlear nuclei (AP: $-6.12$ mm; ML: ±2.2 mm; DV: $-3.12$ mm) of a separate group of 2-month-old MLD mice.

Behavior testing

The animals were housed in standard mouse cages under conventional laboratory conditions with ad libitum access to food.
and water, constant room temperature and humidity, and a 12/12 h light–dark cycle. Treated (n = 9–10) and untreated MLD (n = 16–25) and wild-type mice (n = 10–12) were tested before treatment and at the age of 6, 9, 12, 15 and 18 months. The experimenters were blind with regard to the genetic and treatment status of the animals.

Rotarod. Balance and motor coordination were evaluated on an accelerating rotarod apparatus (Ugo Basile, Italy). After two adaptation trials lasting up to 2 min each at a constant speed (4 rpm), mice were placed on the rotating rod for four test trials during which the rotation speed was gradually increased from 4 to 40 rpm. The time an animal was able to stay on the rod was measured up to a maximum of 5 min.

Statistics. Differences in rotarod performance were assessed using a two-way analysis of variance with correction for repeated measures (two-way RM-ANOVA). The treatment group (treated, untreated and wild-type) and either trial or time were considered as a source of variation. Tukey’s post hoc multiple comparisons were used to analyze age-dependent differences between treatment groups for specific parameters. Statistical analysis was performed using Sigmastat software (SPSS, Inc., Erkrath, Germany), with the level of probability set at 95%.

Electrophysiology

BAEPs were recorded using a Galileo–Sirius digital EEG-EP recorder. The mice were anesthetized by the intraperitoneal injection of pentobarbital (60 mg/kg). Their responses were recorded by a platinum needle electrode inserted subcutaneously behind each ear, and referenced to an electrode inserted behind the nose. Clicks lasting for 0.1 ms were delivered at a rate of 10 Hz and a sound level of 85 dB. Two runs were recorded and for each run, 2000 sweeps were averaged. This resulted in four traces of BAEPs per animal. For the mice injected in the cerebellum and internal capsules, BAEPs were recorded at 3 months of age (before treatment) and then at 6, 9, 12 and 15 months. The mice injected into the posterior cochlear nuclei were recorded at 6 months.

Analysis included a visual classification of BAEP morphology and measurement of amplitudes. BAEP morphology was visually classified as ‘good’ when all five peaks could be identified; ‘intermediate’ when some but not all the five peaks were identified; or ‘absent’ when no peaks were found. Amplitudes were measured for peaks I–V, relative to the lowest trough in the I–V complex. The highest of these amplitudes was used in the statistical analysis, as the ‘maximal amplitude’. Amplitudes were analyzed using the GLM model in SPSS, using ‘run’ (1 or 2), ‘side’ (left or right) and ‘group’ as factors.

Tissue processing

Anesthetized animals were transcardially infused with PBS eventually followed by 4% paraformaldehyde (PFA) in PBS. The brain, spinal cord, liver, spleen and testis were removed. For molecular and biochemical analyses, brain samples were placed on a mouse coronal brain matrix (World Precision Instrument Ltd, Stevenage, UK) and 1 mm thick coronal brain sections were prepared and stored at −80°C. For histological and immunohistochemical studies, the tissues were post-fixed in 4% PFA and then either cryoprotected in 30% sucrose, embedded in OCT (Tissu-tek®, Durham, NC, USA) at −55°C and cut into 10 μm sections in a cryostat for the immunohistochemical studies, or cut into 2 mm coronal sections for Sulf content determination.

Quantitative PCR

AAV5-ARSA vector genome copy numbers were measured by quantitative PCR in the brain, cerebellum, spinal cord, liver, spleen and testis of 18-month-old treated mice. The theoretical and practical aspects of quantitative PCR using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and the SYBR® Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems) have been described elsewhere (34). Vector WPRE and mouse genomic albumin (Alb) sequences (used as internal control) were simultaneously amplified, and each sample was expressed in terms of its Alb content. Quantitative values were obtained from the threshold cycle (Ct) value at which the increase of the signal associated with exponential growth of PCR product was first detected. Results, expressed as n-fold differences in the WPRE1 sequence copy number relative to the Alb gene were determined using the equation: 

\[ N_{\text{sample}} = 2^{\Delta C_{\text{t, sample}}}, \]

where the \( \Delta C_t \) value of the sample was determined by subtracting the \( C_t \) value of the WPRE1 sequence from the mean \( C_t \) value of the Alb gene. Final results were expressed as vector copy number per 2n genome. Samples were considered eligible for study if the Alb \( C_t \) value was ≤26. Samples with WPRE1 sequence \( C_t \) values >35 were scored WPRE1-negative.

The thermal cycling conditions comprised an initial denaturing step at 95°C for 10 min, and 50 cycles at 95°C for 15 s and 65°C for 1 min. Each sample was analyzed in duplicate, and samples with a coefficient of variation of \( C_t \) values higher than 1% were retested. The nucleotide sequences of primers are available upon request.

Determination of ARSA activity and expression

ARSA enzyme activity in vitro. Human embryonic kidney 293T cells (293T) were transfected with AAV5-ARSA or AAV5-LacZ plasmids using the Effectene transfection reagent (Qiagen, Courtaboeuf, France). Cell lysates and medium were assessed for protein concentration using the BioRad DC protein assay kit (Biorad Life Science, Hercules, CA, USA). ARSA activity was determined using the artificial p-nitro catechol sulfite substrate (pNCS, Sigma, Lyon, France) (35). MLD human fibroblasts were grown for 72 h in the medium of AAV5-ARSA- or AAV5-LacZ-transfected 293T cells, and assessed for ARSA activity using the same method.

Enzyme-linked immunosorbent assay. Tissues were homogenized in lysis buffer, sonicated (2 min in pulse mode 5 s/5 s, amplitude 25 Hz) and centrifuged. The supernatant was collected for determination of the protein content, and the concentration of recombinant ARSA was immunologically
measured by an indirect sandwich ELISA, as described (36). For each sample, the assay was performed in triplicate and the results were expressed as mean ± SEM of the three assays.

Analysis of Sulf and GalC
Total lipids were extracted from cerebral slices (7–50 mg) in chloroform–methanol–water 4:8:3 (v/v/v) as described (37). After evaporation, lipids redissolved in chloroform–methanol 1:2 (v/v) were subjected to alkaline saponification (37) and desalted by phase partition. Samples were applied to high-performance silica gel 60 thin-layer chromatography plates (Merck, Darmstadt, Germany) using a Camag linomat IV apparatus. Plates were developed in chloroform–methanol–water 65:25:4 (v/v/v). Detection with orcinol–sulfuric acid (Merck, Darmstadt, Germany) using a Camag linomat IV TLC scanner model II. Data are expressed as mean ± SD of Sulf/GalC ratios.

Histopathology

Standard histological procedures. Frozen brain sections were stained with hematoxilin–eosin and PAS according to a standard procedure. The presence of degenerating neurons was assessed with the marker Fluoro-Jade B (Histo-Chem, Jefferson, AR, USA) (21). Sulf storage was evaluated using Alcian blue staining as described (38).

Immunohistochemistry. For ARSA detection, cryosections (10 μm) of brain samples were incubated sequentially in: (a) PBS 4% PFA; (b) PBS 0.3% triton; (c) PBS 0.1% triton with 5% goat serum; (d) rabbit polyclonal anti-hARSA 1057 diluted in PBS 0.1% triton; (e) secondary anti-rabbit antibodies (Abcam, Cambridge, MA, USA) combined to FITC, Cy3 or peroxidase followed by dianimobenzidine revelation (Vector Laboratories, Burlingame, CA, USA).

Double immunostaining was performed using the following antibodies: anti-Lamp2 for lysosomes (H4B4, Developmental Studies Hybridoma Bank, Iowa City, IA, USA); anti-NeuN for neurons (Chemicon International, Temecula, CA, USA); anti-γ-glutamyl fibrillary acidic protein for astrocytes (GFAP, Abcam); anti-CNPase for oligodendrocytes (Sigma-Aldrich); tomato lectin for microglia (Sigma-Aldrich); anti-calbindin for Purkinje cells (Abcam). Vector-induced inflammatory response was assessed using anti-CD45 antibody (BD Biosciences Pharmingen, France).

Counts of cells. The number of neurons and astrocytes expressing ARSA was assessed in the striatum and in the cerebellar white matter. Quantification of Purkinje cells was performed using Histolab™ image analyzer software (Microvision Instruments, Paris, France). For each mouse, 10 brain sections were analyzed by two independent investigators.

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Conflict of Interest statement. None declared.

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