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ARTICLE

Axons Mediate the Distribution of Arylsulfatase A within the Mouse Hippocampus upon Gene Delivery

Tonia Luca,[1,*] Maria I. Givogri,[1,*] Laura Perani,[1] Francesca Galbiati,[1] Antonia Follenzi,[1] Luigi Naldini,[1,2] and Ernesto R. Bongarzone[1,†]

[1]*Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, and* [2]*Vita Salute San Raffaele University, Via Olgettina 58, Milan 20132, Italy*

[†]*To whom correspondence and reprint requests should be addressed. Fax: +39 02 264 34688. E-mail: bongarzone.ernesto@hsr.it.*

[*]*These authors contributed equally to this work.*

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Axonal transport of the lysosomal enzyme arylsulfatase A (ARSA) may be an additional mechanism of enzyme distribution after *in vivo* brain gene transfer in an animal model of metachromatic leukodystrophy (MLD). Direct molecular demonstration of the movement of this lysosomal enzyme within axonal networks was missing. We generated lentiviral vectors carrying the ARSA cDNA tagged with hemagglutinin or the green fluorescent protein and examined the subcellular localization and anatomical distribution of the tagged enzymes within the MLD hippocampus after *in vivo* lentiviral gene transfer. The use of tagged ARSA allowed direct real-time observation and tracking of axon–dendritic transport of the enzyme after lentiviral gene therapy. Tagged ARSA was expressed in transduced pyramidal, granule, and hilar neurons within the lentiviral-injected side and was robustly contained in vesicles within ipsilateral axon–dendritic processes as well as in vesicles associated with contralateral axons and commissural axons of the ventral hippocampal commissure. Axonal transport of tagged ARSA led to the correction of hippocampal defects in long-term treated MLD mice, which was accompanied by enzyme uptake in nontransduced contralateral neurons, enzyme accumulation within the lysosomal compartment, and clearance of sulfatide storage deposits in this region of the MLD brain. These results contribute to the understanding of the mechanisms of distribution of lysosomal enzymes within the mammalian brain after direct gene therapy, demonstrating the use of neural processes for enzyme transport.

Key Words: leukodystrophy, myelin, axonal transport, hippocampus, lentiviral vector

INTRODUCTION

Mutations of the gene encoding for the lysosomal enzyme arylsulfatase A (ARSA) cause inherited forms of metachromatic leukodystrophy (MLD). Loss of normal ARSA activity leads to the accumulation of sulfatides in lysosomes and eventually to cellular dysfunction. MLD patients show progressive dysmyelination of the central and peripheral nervous systems, presumably due to the death or dysfunction of oligodendrocytes and Schwann cells and deterioration of some neuronal populations [1]. There is no current successful strategy to treat central neurodegeneration of MLD individuals, who die prematurely from cumulative neurological impairments and peripheral insufficiency. Direct gene transfer to the CNS and transplant of neural stem cells constitute two of the most appealing and promising strategies to restore central neural function both electrophysiologically and structurally by providing an *in situ* source of normal ARSA and/or by enhancing directly neuroprotection and neuroregeneration. As an example of this, ARSA-deficient mice [2], an experimental model for human MLD, showed prevention of hippocampal dysfunction after an unilateral injection of lentiviral vectors carrying the human ARSA cDNA [3]. This approach led to sustained enzyme production within the hippocampal formation and improvement of hippocampal functions, constituting the first successful gene transfer treatment for MLD mice.

One major obstacle that direct gene therapy in the brain faces is how to broaden the extent of gene correction beyond the site of gene delivery. Lysosomal enzymes are primarily transported to the lysosomal compartment; however, a fraction of enzyme can be secreted to the extracellular milieu. After their release, these enzymes can

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diffuse limited distances and be taken up by neighboring cells by a mannose-6-phosphate receptor-mediated process [4,5]. This intercellular transfer of lysosomal enzymes, known also as "cross-correction," offers an additional advantage for viral-mediated gene transfer because it extends correction beyond the injection site. Yet, spatial limitations and the proximity of the cells producing and taking up the enzyme limit the extent of cross-correction by enzyme diffusion to the extracellular milieu, making this process likely significant for short-range but not for long-range enzyme correction. Recent findings using different lysosomal disease models after viral gene transfer have shown significant levels of correction in brain areas distant from and not in direct contact with the site of gene delivery [3,6,7]. These observations have shown that lysosomal enzymes can be transported through axons to distal axonal cell bodies, where they reverse the accumulated stored substrates in lysosomes. To study the dynamics of the axonal movement of a lysosomal enzyme, we designed experiments to study ARSA tagged with the hemagglutinin (HA) epitope or the green fluorescent protein (GFP).We generated new lentiviral vectors carrying the human ARSA cDNA tagged to either HA or GFP to enable real-time detection of the tagged enzyme distribution within neural tissue. Using these vectors, our results demonstrate that lysosomal ARSA is distributed globally within the MLD hippocampus by vesicle transport associated with the neural processes connecting with and projecting from the initial site of lentiviral gene transfer.

RESULTS

Validation of Tagged-ARSA Lentiviral Vectors

To allow *in situ* immunodetection of ARSA enzyme after lentiviral transduction, we generated tagged versions of the human ARSA protein by adding the HA epitope at the C-terminus or by fusion with eGFP as shown in Fig. 1A. The tagged cDNA was subcloned in self-inactivating lentiviral vectors immediately downstream of the intermediate early enhancer/promoter of the human cytomegalovirus (CMV). We first transduced HeLa cells with the different lentiviral vectors to obtain similar levels of integration for each vector (Fig. 1B) and then we compared the ARSA activity generated under each transduction condition after several *in vitro* passages (Fig. 1C). Five passages after initial trans-

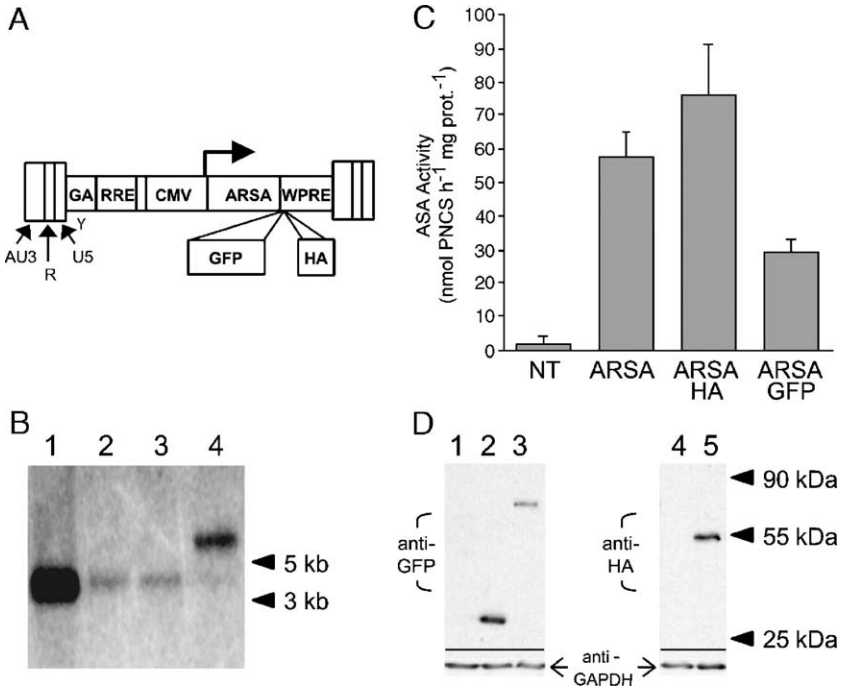

FIG. 1. Structure and *in vitro* validation of ARSA-tagged lentiviral vectors. (A) The cDNA for human ARSA was cloned under the control of the CMV promoter and tagged with GFP or HA epitope at the carboxy terminus. (B) HeLa cells stably incorporated the lentiviral provirion into their genome. Southern blot analysis of *Afl*II DNA digests using a WPRE probe revealed the presence of a 4.5-kb DNA fragment containing the ARSA-HA cDNA (lane 3) and a 5.4-kb DNA fragment corresponding to ARSA-GFP (lane 4). Lane 1, ARSA-HA lentiviral plasmid, and lane 2, HeLa cells transduced with nontagged ARSA lentiviral vectors. (C) HeLa cells showed a robust increase in ARSA activity after transduction with ARSA and ARSA-HA lentiviral vectors compared to transduction with ARSA-GFP and the background levels of nontransduced (NT) cells; $n = 3$, mean $\pm$ SD. (D) Recombinant tagged ARSA proteins showed the expected molecular size in immunoblot assays. Lanes 1 and 4, nontransduced HeLa protein extracts; lane 2, HeLa cells transduced with GFP-lentivirus; lane 3, HeLa cells transduced with ARSA-GFP lentivirus; and lane 5, HeLa cells transduced with ARSA-HA lentivirus. Blots were immunostained with anti-GFP antibodies (lanes 1 to 3) and anti-HA antibodies (lanes 4 and 5). Control for equal loading of proteins was done with anti-GAPDH antibodies.

duction, ARSA-GFP-, ARSA-HA-, and ARSA-transduced HeLa cells showed specific activities of 29 ± 4.2, 76.2 ± 15.1, and 57.5 ± 7.8 nmol *p*-nitrocatechol sulfate (PNCS)/h/mg protein, respectively, compared to the normal levels of untransduced HeLa cells (1.7 ± 2.4 nmol PNCS/h/mg protein). We confirmed that these lentiviral vectors led to the production of recombinant proteins with the expected molecular size (Fig. 1D) of about 80 kDa for ARSA-GFP, resulting from the fusion of GFP (28 kDa) to ARSA (51 kDa [8]), and about 51 kDa for ARSA-HA.

We performed double immunofluorescence confocal analysis using antibodies against Lamp1, a well-established marker for lysosomes, to evaluate if tagged ARSA was correctly targeted to the lysosomal compartment. Figs. 2A to 2C show that anti-HA antibodies detected ARSA-HA associated with vesicular profiles surrounding the nucleus of transduced HeLa cells (Fig. 2A). Most of this HA^+ vesicular profile was counterstained with anti-Lamp1 antibodies (Fig. 2B and the respective merged image in Fig. 2C, arrows), indicating that the recombinant enzyme was targeted to lysosomes. We obtained similar results with the ARSA-GFP lentiviral vector (seen as yellow dots in Fig. 2D). Neither ARSA-GFP nor ARSA-HA was observed associated with mitochondria (Fig. 2E). Additionally, ARSA-tagged proteins were associated with Lamp1-negative punctate structures in close vicinity to the plasma membrane, a pattern compatible with secretory vesicles and likely representing ARSA-HA in transport vesicles for extracellular secretion (Fig. 2C, arrowheads).

Lentiviral Delivery of ARSA Led to Significant Recovery of Enzyme Activity and Reduction of Neuropathology in Both Sides of the Hippocampus

To study the mode of ARSA distribution after lentiviral transduction, we injected unilaterally ARSA-tagged lentiviral vectors into the CA3, hilus, and dentate gyrus of the left hippocampus of mice and examined the mice 8 months later. To validate the capacity of the new vectors to reduce enzyme deficit and the associated histopathological defects in the MLD hippocampal formation, we acutely separated left (injected) and right (uninjected) hippocampi of ARSA-HA- or ARSA-GFP-treated MLD mice from surrounding tissue and processed it to measure ARSA activity 8 months after injection. The ARSA-HA vector led to almost twice the level of ARSA activity in the ipsilateral side compared to wild-type levels, while the ARSA-GFP vector showed incomplete recovery of ARSA activity (Fig. 3A, area I). Importantly, treatment with either vector allowed full (ARSA-HA) or less than half (ARSA-GFP) recovery of ARSA activity on the contralateral side (Fig. 3A, area II) with respect to normal values. The ipsilateral parietal cortex (Fig. 3A, area III) showed enzyme correction, although much less than that seen within the hippocampus. These results indicate that the ARSA-HA vector led to a more efficacious recovery of enzyme activity than the ARSA-GFP vector. Prevention of global neurodegeneration in the hippocampus by lentiviral gene transfer using a vector carrying untagged human ARSA has been previously shown in detail [3]. In this respect, the new HA-tagged vector was very efficient at reducing sulfatide accumulation as seen by

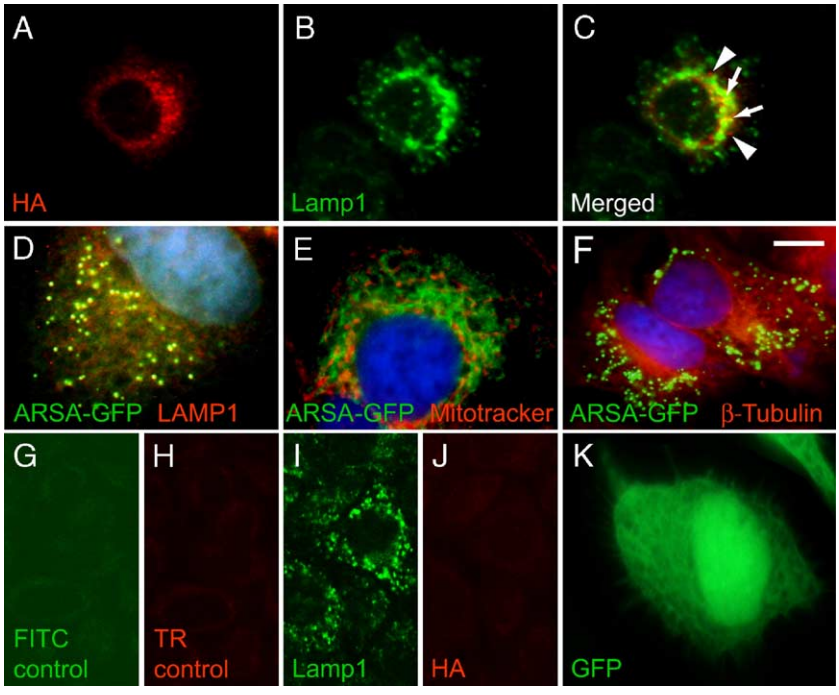

FIG. 2. Recombinant ARSA was targeted primarily to the lysosomal compartment of transduced cells. Confocal microscopy analysis of the subcellular distribution of (A to C) ARSA-HA or (D to F) ARSA-GFP recombinant enzyme in HeLa cells. ARSA-HA (A, in red) showed colocalization with anti-Lamp1 antibodies (B, in green, and arrows in C). Some ARSA-HA accumulated in vesicles not labeled with anti-Lamp1 (arrowheads in C), indicating enzyme likely fated for secretion. ARSA-GFP showed colocalization with Lamp1 (D, seen in yellow). Counterstaining of ARSA-GFP HeLa cells for mitochondria (E, in red) and cytoskeleton (F, in red) is also shown. (G and H) Controls for secondary antibodies. (I and J) Controls for specific staining of anti-Lamp1 (I, in green) and anti-HA (J, in red) antibodies performed on non-transduced HeLa cells. (K) Mock control for HeLa cells transduced with GFP-lentiviral vector. Bar: A–C, F, G–J, 10 μm; D, E, and K, 5 μm.

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Alcian blue staining of brain sections (Fig. 3B). We found that treatment of MLD mice with ARSA-HA lentiviral vector reduced sulfatide deposits in the ipsilateral as well as the contralateral hippocampus to about one-tenth of those seen in untreated MLD mice or in MLD mice treated with a mock GFP lentiviral vector (Figs. 3B to 3J).

Distribution of ARSA-HA within the Hippocampal Formation

The presence of the HA moiety permitted a very detailed and sensitive microscopic observation of the *in vivo* distribution of the tagged enzyme. We examined the bilateral distribution of HA-immunopositive cells 3 and 8 months after injection. Images showing biodistribution of the enzyme within the hippocampus are shown in Fig. 4. At both time points, the ipsilateral side showed numerous neuronal bodies containing ARSA-HA (Figs. 4A to 4H, 3 months; 4I to 4L, 8 months). Importantly, ARSA-HA-positive neuronal bodies were evident in the contralateral CA2 and CA3 layers 3 months after treatment (Figs. 4F and 4G, arrows) and within the contralateral hilus (Fig. 4H, arrow) as well as in the contralateral CA2 and CA3 layers and hilus 8 months after treatment (Figs. 4J and 4L). In most cases, the level of ARSA-HA expression observed on the contralateral side was reduced compared to the ipsilateral side, allowing a clear observation of the subcellular punctate pattern of the tagged enzyme, consistent with a lysosomal compartmentalized distribution of ARSA (Fig. 4F, inset). These results show that the correction of ARSA deficiency was not restricted to the ipsilateral side but was actively distributed to the contralateral side.

Axonal Transport of ARSA-HA within the Hippocampal Formation

To demonstrate the association of recombinant ARSA with axons within the hippocampus, we conducted double immunostaining with anti-HA and anti-neuro-

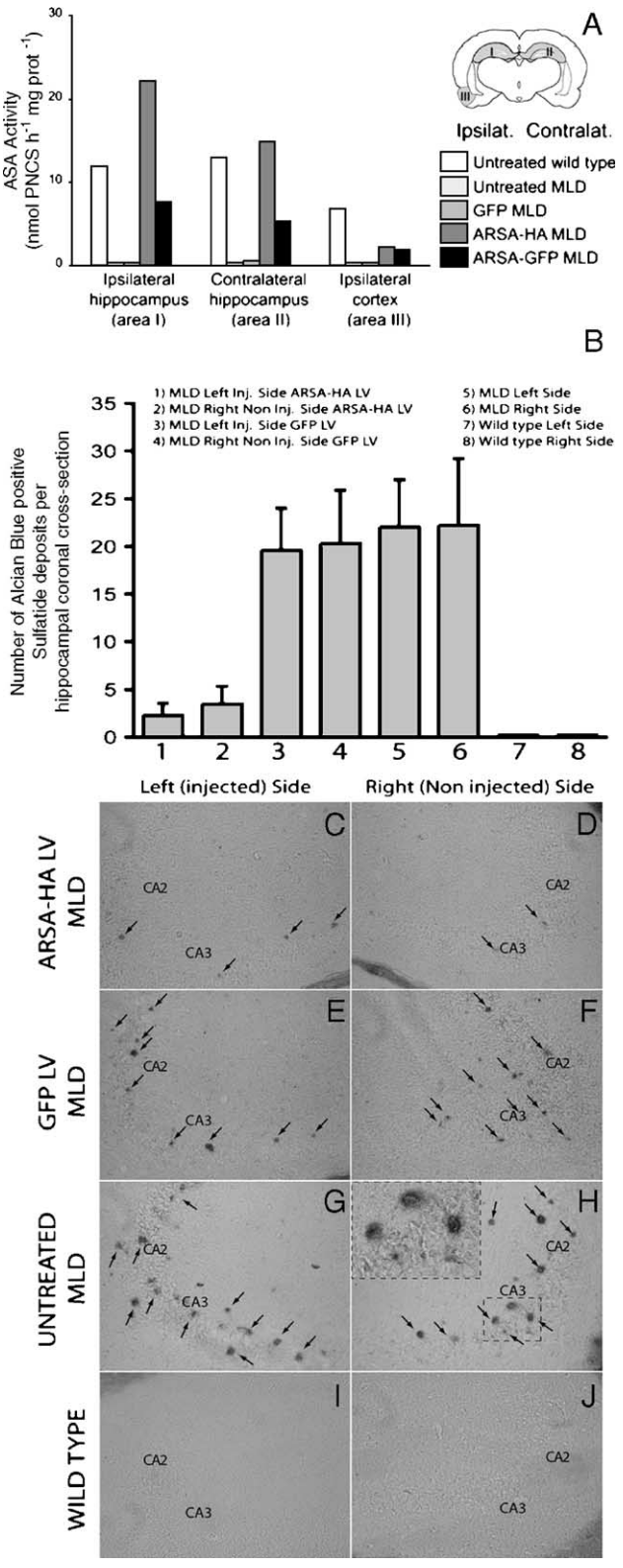

FIG. 3. Correction of sulfatide deposits in the MLD hippocampus after lentiviral gene transfer of ARSA-HA. (A) Enzyme activity was assessed in the ipsilateral (area I) and contralateral (area II) hippocampi as well as in the parietal cortex (area III) of MLD mice treated with either ARSA-HA or ARSA-GFP lentiviral vectors and compared to that of untreated healthy controls, untreated MLD mice, and mock-GFP-treated MLD mice. ARSA-HA-treated MLD mice showed correction of ARSA activity to levels superior to those obtained with the ARSA-GFP vector. Importantly, both vectors showed correction in both sides of the hippocampal formation, confirming that tagged ARSA molecules actively crossed the midline. (B to J) Hippocampal deposits of sulfatides were identified by Alcian blue staining of coronal sections of MLD brains treated with either ARSA-HA or GFP lentiviral vectors and compared to those from untreated healthy controls and untreated MLD. (B, C, D) ARSA-HA-treated MLD mice showed a significant reduction in the number of deposits contained in the entire cross section of either the ipsilateral or the contralateral hippocampus. In contrast, (B, E, F) GFP-treated MLD mice showed histopathological profiles similar to those observed in untreated MLD mice (B, G, H).

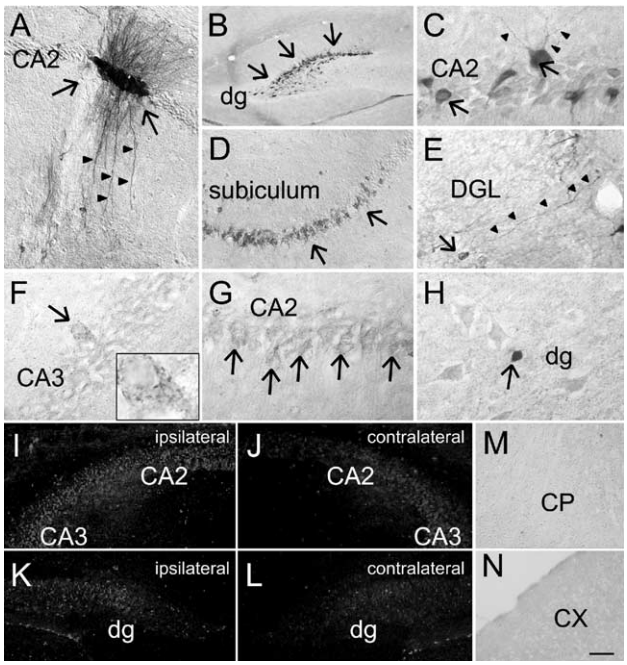

FIG. 4. Lentiviral vectors delivered ARSA-HA expression to several types of neurons within the hippocampus. ARSA-HA distribution was studied (A to H, M, and N) 3 and (I to L) 8 months after injection by immunohistochemical detection of the HA epitope. Hippocampal pyramidal neurons (A, C, and I), hilar neurons (B and K), subiculum medium-sized neurons (D), and granular neurons (E) in the ipsilateral side showed a significant sensitivity to lentiviral transduction. Significant accumulation of ARSA-HA was detected in non-transduced contralateral pyramidal (F, G, J) and hilar (H, L) neurons. Note the punctate pattern characteristic of lysosomal localization observed in contralateral pyramidal neurons (inset and arrow in F and arrows in G), while overexpression of the transgene in the ipsilateral side limited this visualization (arrows in C). ARSA-HA was clearly transported into the neuronal processes in most of the cells studied as evidenced by the abundant immunodetection of the enzyme in axonal and dendritic processes of pyramidal neurons (arrowheads in A and C) and granular neurons (arrowheads in E). ARSA-HA was not significantly evident in other regions such as caudate putamen (CP in M) and parietal cortex (CX in N). dg, dentate gyrus; DGL, dentate granular layer. Bar: A, B, D, E, and I to N, 50 μm; C and F to H, 25 μm.

filament M (NF-M) antibodies on coronal brain sections of 8-month ARSA-HA-treated MLD mice. Axonal profiles in the ipsilateral hilus were easily observed showing the presence of ARSA-HA protein (Fig. 5A) as evidenced by colocalization with anti-NF-M antibodies (Figs. 5B and 5C, arrows), in addition to axons that did not contain any detectable levels of ARSA-HA (Fig. 5C, arrowheads). ARSA-HA was detected in many axons in the contralateral hilus (Figs. 5D to 5F), indicative of axonal transport of ARSA-tagged molecules. Furthermore, we confirmed that ARSA-HA protein crossed the midline associated with commissural axonal connections within the hippocampal ventral commissure (Figs. 5G to 5I). We observed axonal association of ARSA-HA as punctate structures resembling transporting vesicles (Figs. 5A and 5D). To demonstrate conclusively the transport of lysosomal ARSA along axon–dendritic processes, we performed real-time confocal cinematography to detect the movement of ARSA-GFP within transduced hippocampal cells. We prepared brain slices containing lentiviral-transduced MLD hippocampi from MLD mice treated with a single injection of the ARSA-GFP lentiviral vector, maintained them *in vitro,* and scanned them every 7.5 min for up to 2 h. This technique enabled us to detect the progressive accumulation and intracellular transport of ARSA-GFP in transduced neurons (AVI movie in Fig. 8, online additional data). Fig. 5N (serial images) shows a serial reconstruction of the transport of ARSA-GFP within axon–dendritic processes (boxed and circled areas in Fig. 5N) in a transduced granule neuron.

Recombinant ARSA-HA Was Correctly Targeted to Lysosomes in Both Hippocampal Sides

The production of properly sorted ARSA after lentiviral transduction and the uptake of the enzyme by non-transduced cells is the basis for the rationale of direct long-term, long-distance spreading correction after gene therapy within the CNS. Therefore we correlated the presence of ARSA-HA molecules in lysosomes on both hippocampal sides 3 months after transduction. For better visualization of lysosomal targeting, Figs. 6A to 6C show the confocal analysis of a single transduced pyramidal neuron in the ipsilateral CA3 layer of an injected animal, double stained for HA/Lamp1. A punctate pattern of HA staining was observed within the cell body (Fig. 6A, arrow), colocalized with the signal visualized with anti-Lamp1 antibodies (Figs. 6B and 6C and inset). A confocal reconstruction (90° rotated) clearly showed that most of the HA signal colocalized with that from Lamp1 (Figs. 6A′ to 6C′). Similarly, Figs. 6D to 6F (arrow) depict a single pyramidal neuron located in the contralateral CA2 layer, which showed significant ARSA-HA within Lamp1-positive lysosomes.

Contralateral Cross-Correction Is Not Produced by Lentivirus Diffusion or Migration of Transduced Cells from the Ipsilateral Side

To examine whether the presence of ARSA-HA in contralateral neurons could be due to the diffusion of the lentiviral vector during the injection or the migration of transduced cells from the ipsilateral to the contralateral side, we evaluated by *in situ* hybridization the presence of the lentiviral sequences (and thus, the presence of transduced cells or their progeny) on both sides of the hippocampal formation. Many neurons within the ipsilateral hippocampal formation, including dentate gyrus (Figs. 7A and 7B), CA2 (Fig. 7C), CA3 (not shown), hilus (not shown), and subiculum (not shown), showed a strong signal from the hybridized woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) probe. In contrast, the contralateral hippocam-

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pus (Figs. 7D to 7F) or the fimbria (not shown) showed background staining. These results indicate that accumulation of enzyme in contralateral hippocampal cells was consequent to enzyme transport within the neuronal hippocampal circuitry and reuptake in nontransduced cells rather than to lentiviral diffusion or migration of transduced cells.

DISCUSSION

The present study focused on the biodistribution of ARSA-HA within the hippocampus of a mouse model for human MLD after a single unilateral injection of lentiviral vectors, aiding in the clarification of three fundamental aspects of gene transfer of this lysosomal enzyme to the CNS: we showed that axon–dendritic circuitry serves as a route for long-distance distribution of ARSA enzyme, we provided confirmatory data for the occurrence of *in vivo* cross-correction of lysosomal ARSA among neurons, and we identified neuronal populations sensitive to *in vivo* lentiviral gene transfer of ARSA within the mouse hippocampus.

Widespread Distribution of Lysosomal ARSA was Axon Mediated

We found a significant accumulation of ARSA activity on both sides of the MLD hippocampal formation after a single injection of ARSA-tagged lentiviral vectors. These results indicate that corrective ARSA molecules moved from the site of injection to the contralateral side of the hippocampus, primarily by axonal transport of the enzyme. Several lines of evidence presented in our study support this: First, if biodistribution of the tagged enzyme is due to axonal transport, then we should expect to find the presence of tagged enzyme associated with axonal projections crossing the midline. Indeed, we found abundant evidence of colocalization of ARSA-HA in axons within the ventral hippocampal commissure, a structure that crosses the midline and contains intrahippocampal axonal projections from pyramidal neurons on the CA2 and CA3 layers as well as axons from hilar neurons and some subicular neurons. Importantly, only subsets of commissural axons were shown to contain ARSA-HA, indicating that these HA-positive fibers were connected directly (either as afferents or as efferents) to the site of the injection.

Second, we observed abundant distribution of tagged enzyme within CA2 and CA3 layers at the site of the injection, where it is likely to have occurred by transport of Schaffer collaterals, which are known also to connect robustly ipsilateral to pyramidal cells in the CA1 layer of Ammon's horn [9–11].

Third, because hilar neurons send both ipsilateral associative and crossing (commissural) axons connecting to granular neurons in the dentate gyrus [12–14], the finding of ARSA-HA in axons both on the ipsilateral and on the contralateral hilus/dentate gyrus strongly supports axonal transport of the enzyme.

Fourth, CA1 pyramidal neurons are not connected to the contralateral side but rather they send efferent projections rostrally to the lateral septal nucleus via the septum and caudally to the subiculum and the entorhinal cortex [10,15]. We should then expect detectable expression of the transgene in the ipsilateral

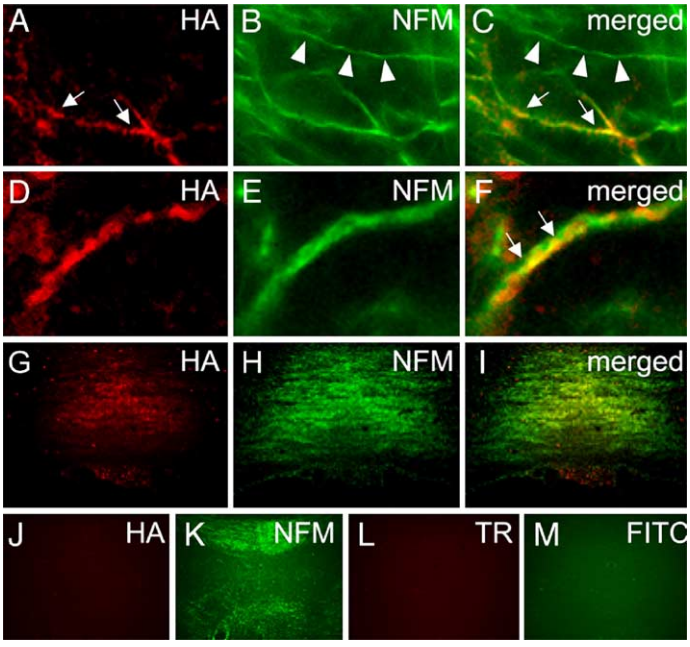

FIG. 5. Axonal association of ARSA-HA within the hippocampus of treated MLD mice. The presence of ARSA-HA in axons within the hippocampus of 8-month-treated MLD mice was evaluated by immunohistochemistry for HA (in red) and neurofilament M (NFM, in green) using confocal microscopy. Many axons were detected on (A to C) the ipsilateral and (D to F) the contralateral sides containing ARSA-HA (arrows in A, C, and F). Axonal transport of ARSA-HA was specific because several other axons showed a complete absence of ARSA-HA enzyme (arrowheads in B and C). Confocal analysis showed abundant presence of ARSA-HA in commissural axons within the anterior hippocampal commissure (G, H, and I), demonstrating that correction of enzyme deficiency on the contralateral side was due to midline crossing of ARSA-HA. (J and K) Controls for specific staining with anti-HA and anti-NFM antibodies on untreated MLD mice. (L and M) Controls for secondary antibodies. (N, serial images) Hippocampal slices from MLD mice were cultured after lentiviral ARSA-GFP transduction and consecutive confocal images were collected every 7.5 min for up to 1 h. The images show translocation of green fluorescent label within axons (boxed area) and dendrites (circled area) of a hippocampal granule neuron in the dentate gyrus.

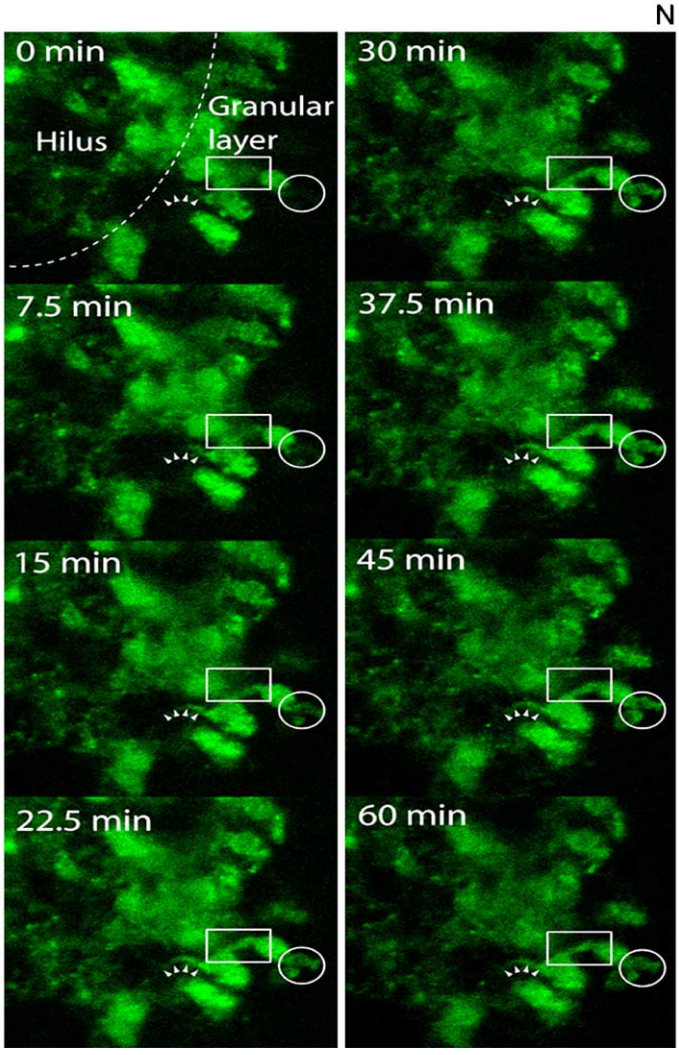

FIG. 5 (*continued*).

CA1 neurons due to direct exposure of these cells during the lentiviral transduction and also from the contribution of ipsilateral Schaffer collaterals from the neighboring CA2 and CA3 layers but little if any expression of ARSA-HA in the contralateral CA1 layer, as was found in our experiments.

Additionally, we observed a robust expression of the enzyme in CA2 and CA3 contralateral layers, which is attributable to the commissural projections of pyramidal neurons located in the ipsilateral CA3 layer [11]. ARSA distribution to the contralateral side was independent of the experimental setting used because long-term (8 months) ARSA-GFP-treated MLD mice showed a significant recovery of ARSA activity on both sides of the hippocampus, further supporting the movement of the lysosomal enzyme from the ipsilateral side through axonal networks, in agreement with our previous study using a lentiviral vector carrying human nontagged ARSA cDNA [3].

Furthermore, the use of this GFP-tagged form of ARSA allowed a unique opportunity to study the intracellular movement of the enzyme in neurons and within their cell processes in a real-time manner, demonstrating that this lysosomal enzyme is actively transported throughout neuronal cell processes. Our molecular approach using tagged vectors offers additional experimental tools to study the intracellular and intercellular trafficking of other lysosomal enzymes, in addition to ARSA. Previous work on the distribution of lysosomal enzymes after brain gene therapy has also indicated that lysosomal enzymes were axonally distributed [3,7,21]. Our work contributes to those studies, adding a level of molecular evidence that demonstrates this type of lysosomal enzyme transport.

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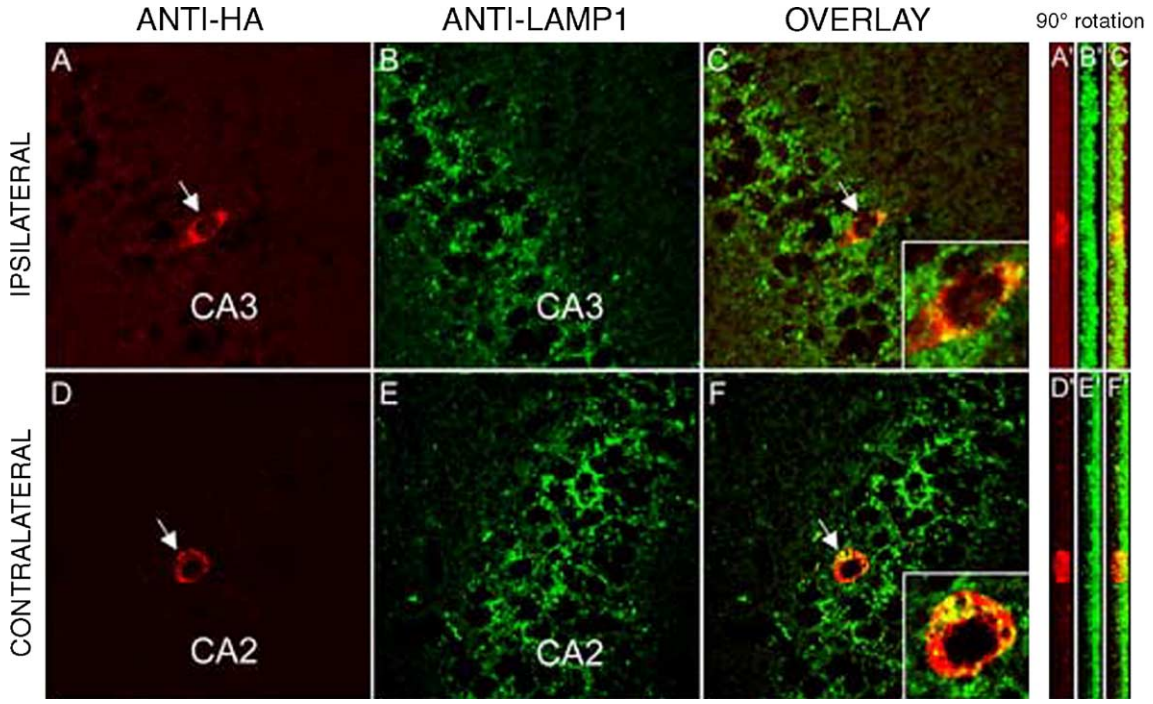

FIG. 6. Lysosomal accumulation of ARSA-HA within the hippocampus of treated MLD mice. The presence of ARSA-HA in lysosomes within the hippocampus of 3-month-treated MLD mice was evaluated by immunohistochemistry for HA (in red) and Lamp1 (in green) using confocal microscopy. For better visualization, A to C and A′ to C′ show a single pyramidal neuron expressing ARSA-HA on the ipsilateral side, which abundantly colocalized with Lamp1 (arrow in A and C and inset in C). Similarly, D to F and D′ to F′ show a single pyramidal neuron on the contralateral side, which accumulated ARSA-HA within Lamp1-positive vesicles (arrow in D and F and inset in F). Three-dimensional reconstruction analysis confirmed colocalization of ARSA-HA within the Lamp1 domain (A′ to C′ and D′ to F′).

Transport of Corrective ARSA to the Nontransduced Contralateral Hippocampus Led to Cross-Correction of Deficient Neurons

Lysosomal enzymes have the ability to be secreted to the extracellular milieu, where they can bind cation-independent mannose-6-phosphate receptors on the surface of neighboring cells, being internalized by endocytosis and transported to lysosomes of the cross-corrected cell [4,5,16–18]. Studies using direct lentiviral and AAV transduction of appropriate lysosomal enzymes pointed to the notion that long-distance correction of the deficiency was attributed to enzyme cross-correction [3,6,19–21]. Our study permitted us to address this issue by direct observation of ARSA-HA on the contralateral side of the treated MLD hippocampus. Our results show conclusively that internalized ARSA-HA not only was taken up in contralateral neurons but also, importantly, was targeted to lysosomes, constituting a proof of principle for the significant correction of neurodegeneration in this side of the hippocampus observed previously [3]. We conclude that this molecular mechanism accounts for most, if not all, of the correction and accumulation of ARSA-HA enzyme in the cell bodies of contralateral neurons and might contribute significantly to the spread of ARSA activity to those cells not transduced within the ipsilateral side.

Contralateral Correction of ARSA Does Not Appear to Rely on the Colonization of Migratory Transduced Cells

The hippocampal formation contains the dentate gyrus, one of the few postnatal sites of the brain where secondary neurogenesis continues to produce bona fide new neurons and glial cells over most of the adult mammalian life [22–24]. Because in this study hippocampal stem cells—among postmitotic neurons and glia—were exposed to the lentiviral vector on one side of the hippocampus, it is conceivable that they could spread ARSA activity to the contralateral side by migration and colonization of contralateral hippocampal structures. In contrast, *in situ* hybridization experiments did not reveal lentiviral-transduced cells on the contralateral side but only on the ipsilateral dentate gyrus, hilus, and CA layers. These results suggest that contralateral accumulation of ARSA was not generated by contribution of migratory ipsilateral progenitors and support a mechanism of axonal transport of the enzyme across the midline.

In Vivo Lentiviral Gene Transfer Conferred Long-Term and Nontoxic Expression of ARSA to Hippocampal Neurons

Lentiviral vectors have been shown to transduce dividing and nondividing cells efficiently [25–29]. Lentiviruses

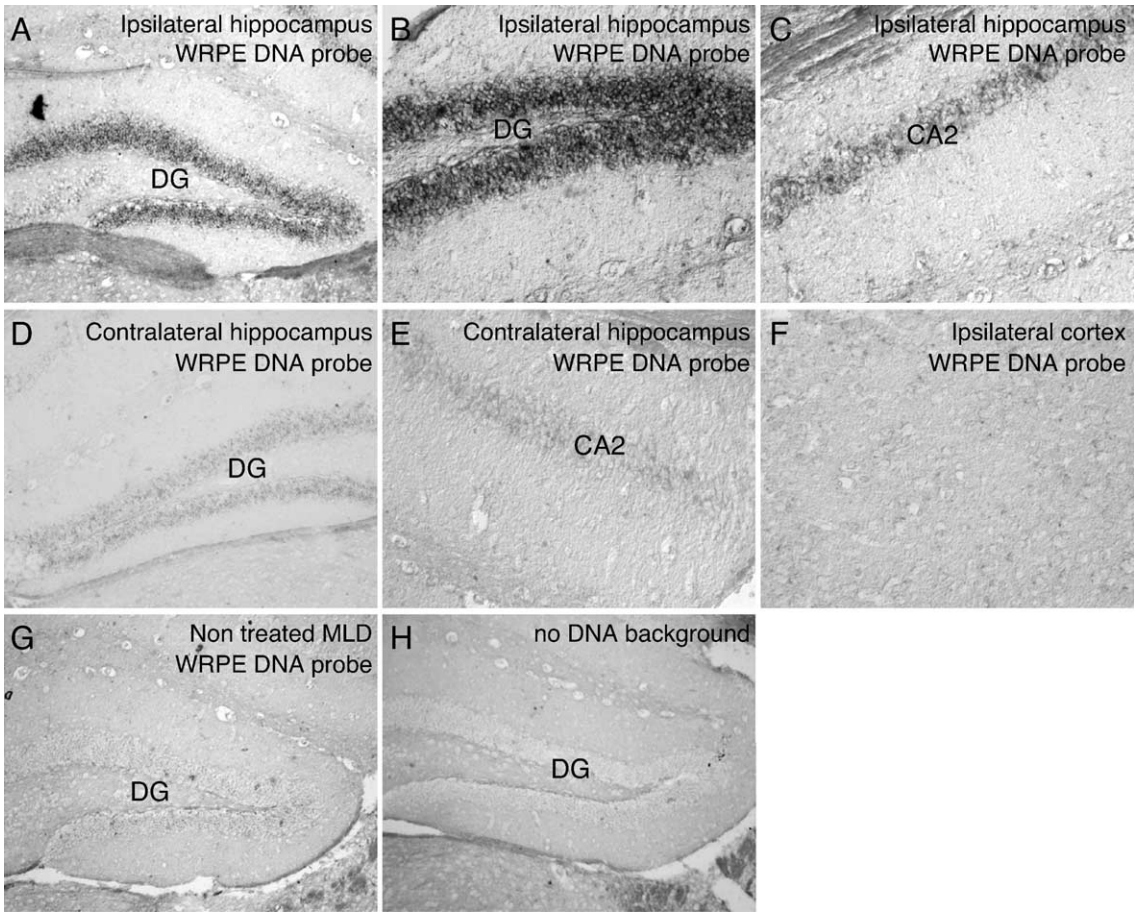

FIG. 7. *In situ* detection of lentiviral-transduced cells within the hippocampus of treated MLD mice. The presence of lentivirally transduced cells and their progeny genomes in the treated hippocampus was assessed by nonradioactive *in situ* hybridization of the WPRE sequence in samples taken 3 months after treatment. (A to C) The presence of abundant cells strongly positive for the WPRE probe within the dentate gyrus, hilus, and CA layers on the ipsilateral hippocampus of the treated MLD mouse is demonstrated. In contrast, (D to E) background and nonspecific probe binding are seen on the contralateral hippocampus. (G) Absence of transduced cells in the ipsilateral parietal cortex of a treated mouse and (H) negative control.

generated with the G-glycoprotein envelope from the vesicular stomatitis virus show a wide tropism and enhanced capacity to transduce neurons. Several groups have used lentiviral vectors for gene transfer of a variety of transgenes in neurons both *in vitro* and *in vivo*. This methodology has been successfully applied to deliver the expression of reporter genes such as GFP [30,31] or therapeutic genes [3,32–35]. We found that large- to medium-sized pyramidal neurons within the CA layers, hilar neurons, and granule neurons of the dentate gyrus and medium-sized pyramidal neurons in the subiculum are among the neurons that respond best to *in vivo* transduction by lentiviral vectors. It is noteworthy that our procedure of lentiviral-mediated gene transfer did not generate significant neurotoxicity and/or inflammation after long-term expression of tagged ARSA in these hippocampal neurons. We observed only an initial astrogliosis (during the first week after injection, data not shown) restricted to the injection site, which was not detected in long-term treated mice. These results stress the potential applicability of this gene transfer methodology for delivering the expression of therapeutic genes within the CNS with reduced or absent secondary inflammation.

In summary, this study presented molecular evidence for the mechanism governing the distribution of ARSA within the MLD hippocampus after lentiviral gene therapy. Our work demonstrates that active ARSA is distributed from the site of initial gene transfer by spreading along neuronal projections and reaching areas not exposed to the transducing vector, fostering long-range therapeutic correction. Our work and that from previous groups [3,6,19,21] confirmed that axonal transport is likely to be a general property of lysosomal enzymes and will likely aid in formulating efficient strategies for global correction of ARSA deficiency in the CNS of MLD individuals and in other lysosomal storage diseases. In addition, the availability of lentiviral vectors carrying tagged forms of lysosomal enzymes such as ARSA

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will allow studies of high molecular detail for tracking enzyme movement and the identification and mapping of other potential networks within the CNS to target for gene therapy of lysosomal storage diseases.

METHODS

Generation of tagged ARSA lentiviral vectors and transduction procedures. The cDNA encoding the human ARSA was tagged by PCR to the HA epitope or linked to eGFP as a fusion protein (Fig. 1) and then subcloned into a self-inactivating HIV-based vector, downstream of the immediate early enhancer/promoter of the human CMV, containing the posttranscriptional *cis*-acting regulatory element of the woodchuck hepatitis virus (WPRE). Lentiviral vectors were produced after transfection of 293T cells using a third-generation lentiviral system. Lentiviral preparations were concentrated by ultracentrifugation and used to transduce HeLa cells for *in vitro* validation. HeLa cells were grown in 10% fetal calf serum/DMEM and plated at a cell density of 35,000 cells/ml on 60-mm culture dishes 1 day before transduction. Cells were exposed to GFP, ARSA-GFP, or ARSA-HA lentiviral vectors (100 ng P24) overnight and then rinsed in fresh medium. Transduced cells were serially passed to attain long-term cultures (more than five passages) for lentiviral integration, enzyme activity, and subcellular distribution studies.

Southern blot. DNA was isolated from cell pellets and digested with *Afl*II, prior to electrophoresis in a 1.0% agarose gel. The integration of the lentivirus was then analyzed by Southern blot using a random-primed radiolabeled probe containing the WPRE sequence. The lentiviral vector carrying ARSA-HA was used as a positive control.

Immunoblot. Protein extracts were prepared from transduced and nontransduced cell pellets and were separated in 12% acrylamide gels and electrotransferred to nitrocellulose blots as described [36]. Membranes were blocked with 5% nonfat milk, 1% BSA in TBS and then incubated overnight with polyclonal antibodies against GFP (Chemicon, Temecula, CA, USA) or HA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or monoclonal anti-GAPDH (Chemicon). Immunocomplexes were detected with peroxidase-labeled secondary antibodies and visualized with the ECL kit (Pierce, Rockford, IL, USA).

ARSA activity assay. ARSA activity was measured as described [37] with the following modifications: tissue samples or cell pellets were homogenized in 0.1% Triton X-100 in the presence of protease inhibitors at 5000 rpm using a high-speed homogenizer. Samples were centrifuged at 20,000 rpm and the supernatant was filtered through a 0.45-μm filter. Protein content was brought to a final concentration of 1 μg/μl. Twenty micrograms of total protein was then incubated with 10 mM PNCS, 500 μM $Na_4P_2O_7$, 10% NaCl, 500 mM sodium acetate, pH 5.0, for 30 min at 37°C. Absorbance was read at 515 nm and the specific activity was calculated using a molar extinction coefficient of 11,400.

In vivo gene transfer. ARSA-null (MLD) mice [2] were used under protocols approved by the HSR Institutional Committee for Animal Care and Use. Eight-month-old MLD mice were anesthetized with avertin and a small hole was drilled through the skull. A 33-gauge blunt needle connected to a 5-μl Hamilton syringe was stereotactically implanted at the following coordinates: bregma −1.9, lateral 1.3, dorsoventral 2.1. Viral suspensions (80 ng P24/1 μl) were delivered over a period of 5 min. A total of 10 MLD mice received ARSA-HA viral injections while an additional group of 3 MLD mice received ARSA-GFP lentiviral injections. Controls included untreated MLD mice, MLD mice receiving GFP lentiviral injections, and untreated C57BL/6 mice (4 mice for each group). Tissue was collected 3 and 8 months postinjection. Mice were perfused and fixed with 4% paraformaldehyde and cryoprotected in 20% sucrose. Brains were sectioned at 30 μm for floating immunohistochemistry or 10 μm for *in situ* hybridization and histology. In other experiments, the left (injected) and right (uninjected) hippocampi as well as a sample of uninjected cerebral cortex were acutely dissected from 8-month-treated animals (wild type, untreated, mock GFP treated, ARSA-GFP treated, and ARSA-HA treated, two for each group) and processed for the determination of ARSA activity.

Confocal cinematography. MLD mice injected with the ARSA-GFP lentiviral vector were sacrificed 3 days after vector delivery. Brains were sectioned under sterile conditions and slices containing the entire hippocampal formation were maintained in 10% FCS DMEM. Slices were mounted on a special plastic dish containing a culture well with a polylysine-coated glass bottom (Cellbio) and fixed in place with 0.5% low-melting agarose. This method allowed tissue slices to be in place for up to 24 h. After identification of the area of interest, consecutive confocal imaging of ARSA-GFP fluorescence was performed every 7.5 min for up to 2 h. Digital images were collected using a Leica inverted confocal microscope connected with a humidity- and thermal-controlled chamber and processed using the NIH Scion Image software.

Immunohistology procedures. Floating sections were rinsed in PBS and quenched in methanol–oxygen peroxide. Sections were incubated with polyclonal anti-HA antiserum diluted 1:500 in 0.1% casein, 0.1% Triton X-100 and then stained using the biotin–streptavidin–peroxidase method using diaminobenzidine as a chromogenic substrate [36]. Alternatively, sections were stained with anti-HA, anti-neurofilament M, and anti-Lamp1 (Iowa Hybridoma Bank) and appropriate fluorescent secondary antibodies. After dehydration and clearing mounted DAB sections were photographed using a Zeiss Axiocam digital camera with Nomarsky optics. Fluorescent samples were observed by epifluorescence or confocal laser microscopy. Fixed cells were treated with 0.1% Triton/PBS and blocked in 5% BSA before appropriate cocktails of primary antibodies were added [38]. The following antibodies were used: rabbit polyclonal anti-HA, mouse monoclonal anti-LAMP1 (Iowa Hybridoma Bank), and mouse monoclonal anti-β-tubulin. Mitochondria were stained using the Mito-Tracker kit (Molecular Probes).

Storage deposit histology. Deposits of sulfatides were identified by histological staining with 1% Alcian blue. Three MLD mice were treated with ARSA-HA lentiviral vectors as described above and analyzed 5 months after the injection. Deposits were counted for each left (injected) and right (uninjected) hippocampus on coronal sections of the brain. We counted six consecutive coronal sections per brain and three brains per experimental condition. MLD injected with a GFP vector (three mice), untreated MLD (three mice), and healthy controls (three mice) served as controls for histopathology.

In situ hybridization. Nonradioactive digoxigenin *in situ* hybridization was performed using a DNA probe specific for the WPRE sequence of the lentiviral vector [39]. Sections were washed in PBS and treated with proteinase K (0.5 μg/ml) for 20 min. After prehybridization and preheating at 95°C, samples were hybridized with the denatured digoxigenin probe (0.5 ng in 100 μl per tissue section) in 50% formamide, 3× SSC, 1× Denhardt's, 10% dextran sulfate, 0.5 mg/ml ssDNA overnight at 42°C. After serial rinses in increasing stringency of SSC, samples were incubated with anti-digoxigenin antibodies (1:5000; Roche) for 1 h before color solution (X-phosphate/BCIP) was added as recommended by the manufacturer. Sections were then washed in PBS to terminate the color reaction and mounted.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymthe.2005.06.438.

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