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Oligodendroglial Progenitor Cell Therapy Limits Central Neurological Deficits in Mice with Metachromatic Leukodystrophy

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This work describes the first successful oligodendrocyte-based cell therapy for presymptomatic arylsulfatase A (ARSA) null neonate mice, a murine model for human metachromatic leukodystrophy (MLD). We found that oligodendrocyte progenitors (OLPs) engrafted and survived into adulthood when transplanted in the neonatal MLD brain. Transplanted cells integrated nondisruptively, did not produce tumors, and survived as proteolipid protein- and MBP-positive postmitotic myelinating oligodendrocytes (OLs) intermingled with endogenous MLD OLs within the adult MLD white matter. Transplanted MLD mice had reduced sulfatide accumulation in the CNS, increased brain ARSA activity, and full prevention of the electrophysiological and motor deficits that characterize untreated MLD mice. Our results provide direct evidence that healthy OLPs can tolerate the neurotoxic accumulation of sulfatides that evolves during the postnatal development of the MLD brain and contribute to OL cell replacement to limit the accumulation of sulfatides and the evolution of CNS defects in this lysosomal storage disease mouse model.

Key words: myelin; oligodendrocytes; migration; transplantation; leukodystrophy; demyelination

Introduction

Patients with metachromatic leukodystrophy (MLD), a genetic disease affecting the expression of arylsulfatase A (ARSA), show accumulation of sulfatides, oligodendrocyte (OL) dysfunction, loss of myelin, and progressive neurodegeneration (Gieselmann, 2003). Currently, transplantation of hematogenous stem cells has been used to limit clinical symptoms in MLD patients (Krivit et al., 1999a,b; Koc et al., 2002). However, any central remyelination therapy for MLD will face the fundamental problem of coping with global OL degeneration/dysfunction. Direct gene therapy to hippocampal neurons (Consiglio et al., 2001; Luca et al., 2005) and cerebellar neurons (C. Croci and E. Bongarzone, unpublished observations) of MLD mice led to a substantial reduction of neuronal degeneration, in association to long-range transport of lysosomal enzymes along axons (Neufeld and Fratantoni, 1970; Passini et al., 2002; Luca et al., 2005). However, direct gene therapy to restore ARSA activity of the entire population of OLs in the CNS remains a challenge. Alternatively, transplantation of migratory oligodendrocyte progenitor cells (OLPs), capable of nondisruptive integration within the population of endogenous OLs (i.e., mosaic distribution), might serve to therapeutically limit the progression of central degeneration and contribute to OL replacement.

OLPs have intrinsic and unique properties with respect to their differentiation potential in the brain and in vivo migration, making them appealing cells for central remyelination therapies of lysosomal storage diseases. OLPs are immature neural cells committed to the oligodendroglial lineage, which respond to several mitogens with increased cell proliferation (Barres et al., 1993; Calver et al., 1998; Redwine and Armstrong, 1998; Frost et al., 2003) and have a remarkable capacity for in vivo migration. Migration of OLPs depends on combined signals from chemoattractive cues such as PDGF-A (Baron et al., 2002), Notch signaling (Wang et al., 1998; Givogri et al., 2002, 2003), and interaction with extracellular matrix signals (Frost et al., 1996; Garcia et al., 2001). OLPs can be maintained for extended periods of time as proliferative precursors without significant loss of myelogenic potential. OLPs are graftable, particularly in the neonatal CNS of myelin mutants, where they respond remarkably well to endogenous cues with migration, integration, and long-term survival as well as the capacity to myelinate areas in which endogenous remyelination fails (Lubetzki et al., 1988; Lachapelle et al., 1990). Several elegant studies have demonstrated the therapeutic value...
of OLP transplantation in myelin mutants (Duncan et al., 1988; Learish et al., 1999; Yandava et al., 1999; Magy et al., 2003; Crag et al., 2004; Duncan, 2005). These studies have indicated that the outcome of cell therapy in severe mutants such as Jimpy, Shiverer, and Twitcher mice strongly relates to the extent of central and peripheral compromise and the accumulation of neurotoxic molecules such as the deposit of aberrant protein or toxic lipid metabolites. ARSA null mice display progressive sulfatide storage throughout the CNS, mild loss of myelin, and develop behavioral deficits in adulthood, constituting a murine model for late infantile/early adult forms of human MLD (Hess et al., 1996). MLD mice have not been evaluated as recipients of OLP-based central cell therapy. In this work, we successfully treated neonate MLD mice with healthy OLPs and limited the evolution of central deficits when transplanted MLD mice entered into adulthood.

Materials and Methods

Animals. Knock-out MLD (ARSA−/−) mice and the corresponding wild type (WT; ARSA+/+) animals were used throughout this study. MLD mice were maintained in the San Raffaele Scientific Institute animal research facility by crossing homozygous offspring from carrier ARSA−/− mice (Hess et al., 1996). All procedures were done under the protocols approved by the Animal Care and Use Committee of our institute in accordance with Italian law.

Preparation of OLP cultures from normal and MLD nervous tissue. OLP cells were prepared as described previously from primary glial cultures from MLD or wild-type animals (Gard and Pfeiffer, 1989; Louis et al., 1992; Bongarzone et al., 1996). Cells were grown in glial defined medium consisting of DMEM/F12 supplemented with 5 mg/mL insulin, 16.1 mg/L putrescine, 50 mg/L transferrin, 0.8 μg/mL sodium selenite, and 2.2 g/L sodium bicarbonate. OLPs were maintained in proliferative conditions by the addition of 30% of medium conditioned by B104 cells (a kind gift from Dr. J. De Vellis, University of California at Los Angeles, Los Angeles, CA) (Schubert et al., 1974; Louis et al., 1992; Luo and Miller, 1997).

In vitro genetic retroviral/lentiviral labeling and fluorescent labeling of OLPs. OLPs were grown to confluence in 75 cm² culture flask before cells were exposed to 10⁶ colony-forming units of either β-galactosidase-retroviral (bag cells; CRL-9560; American Type Culture Collection, Manassas, VA) or green fluorescent protein-lentiviral (GFP-LV) vector, supplemented with 8 mg/ml polybrene. Cells were left overnight in the incubator. Cells were rinsed with PBS and incubated with PKH26 red fluorescent cell linker kit (Sigma, St. Louis, MO) right before the injections by trypan blue vital staining (we also counted cell viability in the leftover of cell suspension after the injections).

Flow cytometry. MLD mice transplanted with GFP+ OLPs and untreated controls were killed 7 d after grafting and perfused with saline (three mice per group). Brains were dissected and mechanically dissociated. Cell suspension was filtered through a 40 μm cell strainer and cells centrifuged at 1500 rpm. Cell pellets were resuspended in fluorescence-activated cell sorting (FACS) solution (PBS without calcium and magnesium containing 2% FBS), and viable cells were counted using trypan blue. GFP+ cells were detected from an input of 10⁶ cells from whole-brain cell suspension using FACSScan (Becton Dickinson, Mountain View, CA).
**Table 1. In vitro phenotypic characterization of graftable progenitors**

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<tr>
<th>Cell marker</th>
<th>Proliferating in B104 CM</th>
<th>Differentiated in T3/1% FCS</th>
<th>Differentiated in 10% FCS</th>
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<tr>
<td>PSA-NCAM</td>
<td>+</td>
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<tr>
<td>PDGFαc</td>
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<td>A2B5</td>
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<td>O4</td>
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<td>MBP</td>
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<td>GFAP</td>
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<td>Tuj1</td>
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Progenitors were incubated with B104 conditioned medium (CM) to promote their proliferation or in low (1%) or high (10%) FCS to stimulate their differentiation. Low serum culture medium contained T3 hormone. Cells were then replated in three independent experiments. Tuj1, Class III β-tubulin.

**Figure 1. In vitro labeling of oligodendrocyte progenitors and detection of LacZ ARSA OLs in different regions of the neonate MLD brain after transplantation.** OLs were grown on coated coverslips in differentiating conditions for 3 d. A, OLs displayed multiple processes. OLs were transduced with β-gal retroviruses (LacZ-retro; B), GFP lentiviruses (C), or loaded with the cell membrane dye PKH26 (D). Neonate (P2) MLD and healthy pups received a single injection of β-gal OLs in their left ventricle. One day postinjection (dpi), OLs moved within the ventricular system (E, arrowheads), migrating latter (7 dpi) toward the cortex, thalamus, and olfactory bulb (F, arrowheads and arrows). At 7 dpi, OLs grafted in healthy controls remained primarily within the ventricular system (G) with few cells seen in the parenchyma. H1–H7, Serial coronal sections stained for β-gal and neutral red from an MLD mouse 1 d after surgery. Transplanted OLs were detected along the rostralcaudal axis and within the olfactory bulb (H1). RMS (H2), lateral ventricle (LV), chondria plexus, SVZ (H3 and its higher-magnification inset, H4, H5), third ventricle (H6), and fourth ventricle (H7). The arrows depict LacZ OLs that migrated within the brain parenchyma. n = 3 mice per group per time point. cc, Corpus callosum; ob or Olf. Bulb, olfactory bulb.

**Results**

**In vitro properties of the neural oligodendroglial progenitors**

Healthy OLs (i.e., with normal ARSA activity) were maintained at low density in proliferative conditions to favor the oligodendroglial phenotype, characterized by the expression of PSA-NCAM, PDGFαc,
and immunolabeling with the monoclonal antibody A2B5 (Table 1). More than 90% of the cells differentiated into mature cellular profiles associated with elaborated and delicate processes after incubation in the presence of 1% FCS and T3 (Fig. 1A, B, Table 1). Differentiated cells acquired the expression of mature cell markers for OLs such as those recognized by antibodies for PLP, MBP, and galactocerebrosides (O1) (Table 1). If cells were incubated in 10% FCS, most of them rapidly acquired the morphology of astrocytes expressing GFAP (Table 1). Proliferating OLs were easily transduced with a β-gal-retroviral vector (Fig. 1B) or a GFP-lentiviral vector (Fig. 1C) or alternatively, loaded with PKH26 dye, which specifically labels plasma membrane (Fig. 1D). None of these labeling methods affected the capacity of OLs to differentiate in vitro (evidenced by MBP and PLP gene expression analysis; data not shown) nor their in vitro survival and importantly allowed us to identify transplanted cells up to 1 year after the surgery, the longest time tested in this study.

OLPs migrated extensively within the MLD neonatal brain

We evaluated the in vivo capacity of β-gal+ OLs to survive and migrate within the brain of newborn MLD pups and their healthy controls. One day after the graft, we observed that β-gal+ cells distributed throughout the brain ventricular system, including the choroids plexus. However, β-gal+ cells migrated rapidly within the MLD brain parenchyma and less within the wild-type tissue (Fig. 1E, arrowheads). Many β-gal+ cells were seen exiting the ventricles 3 d after transplantation in the MLD brain, and by 7 d after grafting, LacZ+ cells were detected in different areas such as subcortical white matter, caudate–putamen, and olfactory bulbs (Fig. 1F, arrows and arrowheads). At this time point, wild-type recipients showed β-gal cells mostly associated to the ventricular system (Fig. 1G, but fewer β-gal+ cells were detected within the wild-type brain parenchyma. Coronal sections 1 d after the transplant allowed a more detailed localization of migratory β-gal+ cells within the MLD brain. We observed a large number of these cells lining on the walls of the ventricular system (Fig. 1H3,H4,H6). At this time point, many cells were found within the choroids plexus, whereas others migrated away from the ventricles and were seen within the brain parenchyma (Fig. 1 H2,H4,H5,H7, arrows). OLs were also detected in the subventricular zone (SVZ) (Fig. 1H3 and its inset), rostral migratory stream (RMS) (Fig. 1H2), and olfactory bulb (Fig. 1H1). In 1 year long-term experiments, grafted OLs were detected in the corpus callosum (see Fig. 4P, S), cortex (see Fig. 4P, Q, T), striatum (see Fig. 4U), and cerebellum (see Fig. 4R).

Survival of OLs is accompanied by early in vivo proliferation in the MLD brain

We conducted two transplantation experiments using either LacZ or GFP labeled OLs to evaluate the efficiency of the engraftment (i.e., the percentage of surviving grafted cells over time at 1, 7, 15, and 30 d after transplant). In the first experiment, we measured the activity of the reporter gene β-gal and correlated it with the β-gal activity of the input of cells at the moment of the graft as an indirect parameter of grafted cells remaining within the host tissue. Figure 2A shows that ~15% of the total input of β-gal activity remained in MLD brains 1 d after the grafting. However, whereas β-gal activity decreased to almost background levels in transplanted aged healthy controls, ~8% of LacZ activity was detected in the brain of transplanted MLD mice at 7, 15, and 30 d after surgery. In the second experiment, we analyzed the absolute number of GFP-positive cells at 7 d after transplantation by FACS of total brain cell suspension. Figure 2B shows an ex-

![Figure 2](image)

**Figure 2.** MLD brain promoted long-term survival and proliferation of OLs. *A,* To estimate the efficiency of the engraftment, β-galactosidase activity was measured in total brain homogenates from transplanted MLD and healthy mice and correlated to the total enzyme activity of the input of cells at the moment of the graft. The plot shows that after an initial phase of cell death, cells transplanted in MLD pups survived better than in healthy mice, and ~9% of the original amount of enzyme activity remained 1 month after surgery. **B, C,** We validated our findings on LacZ enzyme activity by FACS analysis of surviving GFP-positive cells in mice that received GFP+ OLs. **B,** An example of the cytometric analysis for the presence of GFP+ cells from 7 d postinjection transplanted MLD mice, whereas **C** shows the FACS analysis of an untreated control. A total of 10^6 cells from total brain cell suspensions were acquired. To evaluate whether grafted OLs continued to divide once transplanted in the neonatal brain, we performed pulses of BrdU to detect DNA synthesis. **D–R,** Coronal sections of transplanted mice displaying GFP+ OLs, counterstained with anti-BrdU antibodies (in red) and analyzed by confocal microscopy. Seven days after transplantation (**D–I**), GFP+ OLs transplanted in the MLD brain were detected dividing within the cortex (**D–F**) and the 90° rotation confocal reconstructions) and the SVZ (**G–I**). Fifteen days after the graft (**J–Q**), most of the double BrdU/GFP-positive cells (filled arrows) were primarily detected in the cortex (**J–L**) and the corpus callosum (**M–Q**). One year after the grafts (**P–R**), BrdU+ cells were in the SVZ (**P**) and the hippocampal granular layer (data not shown). Transplanted GFP+ cells were found in white matter areas such as the corpus callosum (**Q**) but did not immunolabel with anti-BrdU, indicating their withdrawal from the cell cycle (**R**). BrdU+ cells in the SVZ of an untreated 1-year-old MLD mouse. Scale bar: (**Q**) 25 μm, n = 3 mice per group per time point, dpt. Days post-transplantation; ypt, year post-transplantation; LV, lateral ventricle. Results are expressed as mean value ± SEM.
Our results indicated that MLD brain supported long-term migration and survival of OLPs, whereas healthy brain was significantly less permissive to transplanted cells. We used the agarose drop assay to test the capacity of brain-derived tissue to influence the migration of OLPs in vitro to examine whether MLD and healthy brain might influence differently OLP migration in an in vitro system. Exposure of OLPs to MLD brain slices showed reproducible migration out of the agarose drop in a 1 d assay (Fig. 3A, B). In contrast, migration of OLPs out of the agarose drop was reduced when cells were coincubated with healthy neonatal brain samples (Fig. 3A, C), in support of the findings of neonatal transplantation experiments.

Absence of ARSA activity does not influence the migration properties of MLD OLPs
To determine whether impairment in the migration of MLD OLPs contributes to the pathogenesis of MLD, we measured the migration capacity of ARSA−/− OLPs after transplantation in wild-type and MLD neonatal brains. In agamere action assays, we found that MLD OLPs behaved similarly to healthy OLPs (Fig. 3A). For the transplantation experiments, MLD OLPs were isolated from newborn knock-out mice and transduced with GFP-LV before grafting. Recipient mice were killed 7 and 15 d after transplantation to evaluate their migratory patterns. We found that ARSA−/− OLPs were able to migrate extensively in the MLD brain, with similar migration patterns to those of healthy OLPs (Fig. 3D–F). Grafted ARSA−/− OLPs migrated to the SVZ (Fig. 3D, E) and corpus callosum (Fig. 3F) between 7 and 15 d after the grafting. However, ARSA−/− OLPs migrated poorly and remained nearby the area of the graft in wild-type brains (Fig. 3G, H) without integration in white matter areas. ARSA−/− OLPs were detected 15 d after the graft (the longest time point in this experiment), adopting extensive cell processes and expressing MBP (data not shown), indicating their capacity to differentiate into mature OLs.

Grafted progenitors differentiated into mature myelinating OLs, expressed myelin genes, and integrated in myelinated areas of the MLD brain
Many LacZ+ OLs moved into the SVZ–RMS–olfactory bulb system where most of them expressed PDGFαR, an early marker for the oligodendroglial lineage 7 d after transplantation (Fig. 4A–C). Double-positive β-gal/PDGFrα precursors and β-gal/O4 OLs were also detected in other areas such as the hippocampal dentate gyrus, the wall of the third ventricle, corpus callosum, and external capsule of MLD mice (data not shown). At later time points (30 d after transplant), most of OLs were found in white matter areas such as the external capsule (Fig. 4F–H) coexpressing myelin spingolipids recognized by the monoclonal antibody O4. Figure 4I shows PKH26-loaded OLs connected to processes adopting a parallel orientation to axons within the corpus callosum, characteristic of interfascicular OLs. Sixty days after transplant, numerous β-gal+ cells residing within the corpus callo-
sum (Fig. 4J,K) coexpressed mature OL markers such as PLP (Fig. 4J) and MBP (Fig. 4K). Grafted cells (or their progeny) were detected 1 year after the transplant (Fig. 4P–S) using anti-β-Gal antibodies. 

β-Gal-positive cells showed delicate and elaborated processes, characteristic of myelinating OLs (Fig. 4Q–S). Untreated wild-type (Fig. 4N) or untreated MLD brains (Fig. 4O) showed no specific anti-β-Gal staining.

To study whether transplanted OLs were indeed forming myelin sheaths in the MLD brain, we used anti-MBP antibodies as a specific marker to visualize myelin sheaths and counted the number of MBP+/GFP+ myelin sheaths 1 year after transplants by double confocal microscopy. Figure 4, T and U, shows the presence of many double MBP+/GFP+ myelin sheaths in the cortex (Fig. 4T and its insets) and the striatum (Fig. 4U and its insets) amid endogenous myelin sheaths that are MBP positive but GFP negative. We estimated the abundance of myelin sheaths originated from transplanted GFP+ OLs by counting the number of MBP+/GFP+ sheaths per area. Figure 4V shows that ~27 and 15% of the MBP+ myelin sheaths in the cortex and striatum were derived from GFP+ OLs. Because of the high density of myelin sheaths in heavily myelinated structures such as the corpus callosum, we were unable to accurately count GFP+ myelin sheaths in this area. On the other hand, counting of myelinating cell bodies showed, for example, that ~5% of the population of OLs in the corpus callosum and the cortex derived from grafted OLs.

We did not find transplanted β-gal+ cells expressing NeuN, indicative that grafted OLs did not acquire a neuronal phenotype in vivo (Fig. 4L, compare cell pointed to by filled arrow to cell pointed to by open arrow). We rarely observed grafted cells expressing GFAP, and only in close association to blood vessels (Fig. 4M, arrow).

Reduction of astroglial and microglial reaction in the brain of transplanted MLD mice

MLD mice develop a mild astrogliosis over the evolution of the disease. This can be visualized by immunohistochemical analysis with anti-GFAP antibodies. Figure 5B shows that GFAP+ cellular profiles, characteristic of reactive astroglia, are more abundantly distributed within the corpus callosum as well as the cortex of 1-year-old untreated MLD brain compared with healthy brains (Fig. 5A). In contrast,
transplanted MLD mice showed a remarkable reduction of reactive astrocytes within the cortex (Fig. 5C).

MLD mice also develop signs of microglia activation and infiltration of macrophages, which can be studied by the level of immunoreactivity for MCP-1 (Fig. 5E, compare with background levels in healthy controls in D). Treatment with OLPs reduced the number of MCP-1-positive cells within the brain (Fig. 5F), indicating a less severe recruitment of macrophages in the CNS of treated MLD mice.

Diffusion of ARSA and in vivo cross-correction to endogenous neural cells reduced sulfatide deposits in transplanted MLD mice

Sulfatides accumulate progressively in MLD mice and are clearly detectable by Alcian blue staining after the first year of life. Microscopic examination of brain sections of untreated MLD mice revealed the presence of sulfatide deposits distributed abundantly throughout the brain, particularly in white matter areas. Figure 5 shows example pictures of Alcian blue-stained sulfatide deposits in diverse areas of the MLD brain such as corpus callosum (Fig. 5G), medial septal nuclei (Fig. 5I), and fimbria (Fig. 5K). In contrast, MLD mice that received OLP transplants showed a lower density of sulfatide deposits in these areas (Fig. 5H, J, L) as well as in other areas such as cortex, caudate–putamen, cerebellum, and spinal cord (Fig. 5M). Quantification of the density of Alcian blue spots per area showed that OLP transplants led to a reduction between 20 and 50% of sulfatide deposits in the MLD CNS (Fig. 5M). The reduction of sulfatide deposits was accompanied by an increase in ARSA activity. Enzyme activity in brain homogenates from 1-year-old treated MLD mice was 0.94 ± 0.28 nmol P-nitrocatechol sulfate (PNC) per milligram per hour compared with 3.04 ± 0.9 nmol PNC per milligram per hour in healthy brains (n = 5). These results indicate that neonatal transplants of OLPs in the brain of MLD mice led to ~31% reconstitution of the normal ARSA activity.

Immunohistochemical analysis using anti-ARSA antibodies allowed us to confirm that ARSA enzyme diffused to many areas of the brain in transplanted MLD mice (Fig. 6). ARSA was detected primarily as punctuated pattern in the cytoplasm of cells throughout the wild-type cortex (Fig. 6A, arrow), corpus callosum (Fig. 6D, arrow), striatum (Fig. 6G, arrow), basal ganglia (data not shown), and cerebellum (data not shown) and was absent in the brain of untreated MLD mice (Fig. 6B, E, F). In contrast, numerous ARSA-positive cells were seen in the cortex (Fig. 6C, arrow), corpus callosum (Fig. 6F, arrow), striatum (Fig. 6I, arrow), and fimbria (Fig. 6J, K, arrow), the vicinity of the ventricular areas (data not shown), and cerebellum (data not shown). Interestingly, we found that not all neural cells in the brain of transplanted MLD brains contained ARSA (Fig. 6, compare distribution of ARSA in the cortex of treated MLD mice in C with that of the corresponding area in the wild-type tissue in A). Figure 6K shows an example field from 1-year-treated MLD mice in with the fimbria clearly containing immunodetectable ARSA (Fig. 6, arrows) and cells that are not containing significant levels of ARSA (Fig. 6, arrowheads). Secretion, diffusion, and uptake of ARSA by deficient cells (cross-correction) are thought to play a central role in correction of the deficit of lysosomal enzymes in gene- and cell-based therapies. Our results confirmed that this process occurred in MLD mice transplanted with OLPs. Figure 6L–N shows an example field from 1-year-treated MLD cortex, in which a transplanted OLP (Fig. 6L, arrow), coimmunostained with anti-β-galactosidase and anti-ARSA antibodies, is in close proximity to an endogenous cell that took up the enzyme (anti-β-galactosidase negative; anti-ARSA positive).

Prevention of central electrophysiological and motor learning impairments in transplanted MLD mice

We then examined whether the engraftment of OLPs had a beneficial impact on central electrophysiology in the MLD mice by...
measuring MEPs. Central conduction time (CCT) and cortical MEP showed prolonged latencies in MLD mice after 1 year of age (Fig. 7A, black bars). Treatment with OLPs led to a complete normalization in the latency values for both CCT and cortical MEPs on MLD mice (Fig. 7A, gray bars).

Motor learning was evaluated in 1-year-old mice on an accelerating Rotarod apparatus. MLD mice were found severely impaired, whereas OLP-transplanted MLD mice showed a full prevention of their motor learning impairment. In fact, two-way ANOVA indicated a significant interaction between genotype and treatment ($F_{(2,25)} = 4.97; p < 0.05$). (Fig. 7B). In particular, whereas post hoc comparison revealed that MLD mice manifested a significant difference versus both WT and MLD-treated animals ($p < 0.05$ and $p < 0.01$, respectively), no statistical difference was seen between WT and treated mice ($p = 0.61$).

**Discussion**

We have examined the hypothesis that healthy OLPs transplanted in the brain of MLD pups at a time when myelination is not completed can migrate, integrate in white matter, and contribute to myelination, limiting the progression of central neurological deficits in this mutant. *In vitro* characterization confirmed the capacity of OLPs to generate mature MBP+ PLP+ OLs in T3/low serum (or astrocytes only in high serum). When OLPs were transplanted in the unmyelinated brain of MLD newborns, almost all of surviving cells became MBP+ PLP+ OLs. These results are consistent with other reports using similar preparations of OLPs in models of myelin disorders (Tontsch et al., 1994; Franklin and Blakemore, 1995; Learish et al. 1999; Espinosa de los Monteros et al. 2001; Crang et al., 2004).

Migration of grafted OLPs was significantly higher and targeted to white matter tracts when OLPs were intraventricularly grafted in neonate MLD pups. In contrast, a more reduced distribution of OLPs was observed when transplantation was performed in healthy pups and even when grafted in MLD mice 2 months after birth (M. Givogri and E. Bongarzone, unpublished observations). OLPs can migrate and survive better after transplantation in the brain of myelin mutants than in healthy animals (Gaillard et al., 1998; Bentlage et al., 1999). We found that $\sim 10\%$ of OLPs survived during the first month and migrated to prospective areas of myelination in the MLD brain. Furthermore, these cells appeared to contribute to the pool of adult OLs present after 1 year of...
treatment. In fact, ~4.5% of the total number of glial cells in the corpus callosum was derived from the transplant. Surviving OLs produced a substantial amount of myelin ranging from 15 to 27% of myelin sheaths. This indicates that surviving OLs were able to myelinate several internodes each, responding to myelogenic cues from the developing MLD brain.

Numerous factors might have contributed to the remarkable plasticity of the MLD brain to OLs. First, healthy OLs were grafted at a developmental time when the brain undergoes robust gliogenesis and there is intensive migration of glial progenitors in response to migratory cues (Levison et al., 1993, Luskin and McDermott, 1994; Zerlin et al., 1995). Recent data indicate that OLs originate from three different sites in the developing brain; Emx1-expressing progenitors in the cortex is the source for postnatal OLs (Kessaris et al., 2006). Our experiments transplanted OLs when this wave of Emx1 precursors are believed to be actively generating endogenous OLs; thus, it is feasible that grafted OLs have responded to the endogenous cues governing oligodendrogenesis during the first postnatal weeks. Several types of signals are expressed in the developing brain, which might have been preferentially recognized by grafted OLs, stimulating their migration in the MLD brain. OLs express members of the integrin family such as \( \alpha_\beta 1 \) and \( \alpha_\beta 1 \) integrins, which bind extracellular matrix molecules such as fibronectin, laminins, and vitronectin (Frost et al., 1996; Garcia et al., 2001). Integrins play a fundamental role in cell–cell interactions during homotypic migration of OLs (Milner et al., 1996; Jacques et al., 1998; Decker et al., 2000; Vitry et al., 2001). MLD pups showed slightly higher expression of laminin \( \beta 2 \) in the brain than controls, without significant differences in other extracellular matrix component members such as fibronectin and collagen, which might have facilitated OLs migration (Givogri and Bongarzone, unpublished observations). Interestingly, this migratory property was observed also \textit{in vitro}, where brain slices from MLD mice promoted a more efficient migration of OLs than brain slices from healthy mice. Furthermore, transplantation of MLD OLs showed that ARSA deficiency did not influence the migratory capacity of the mutant OLs. This result shows that the pathogenesis of MLD mice is not caused by deficient colonization of white matter territories by endogenous OLs (Yaghootfam et al., 2005) nor caused by a toxic effect from the MLD brain environment. Instead, it suggests that pathogenesis is caused by a cell-autonomous defect of ARSA deficiency after cell migration. Additional studies will investigate whether this cell autonomous defect is affecting both OLs and neurons in the MLD brain.

Second, transplants were done at a postnatal time when MLD pups had a delayed myelination (Yaghootfam et al., 2005). This condition likely facilitated transplanted OLs, carrying the selective advantage of a normal sphingolipid catabolism, to migrate and myelinate the MLD brain. Accumulation of sulfatides might be considered as a potential neurotoxic agent for neural cells, in particular leading to a progressive depletion of functional OLs and the consequent loss of myelin. However, the direct link between the accumulation of sulfatides and the loss of OLs and myelin in MLD individuals has not been unequivocally established yet. Furthermore, it is unclear whether the accumulation of sulfatides in perinatal OLs in the MLD mouse model, which might still be at a low concentration at the time of the transplantation, has an impact on their survival and/or differentiation. Some recent work elegantly illustrates the role of sphingolipids during oligodendroglial differentiation (Bansal et al., 1999; Hirahara et al., 2004; Yaghootfam et al., 2005). For example, absence of sulfatides stimulates a premature differentiation of OLs. Interestingly, Yaghootfam and coworkers showed that MLD mice have a significant delay in early postnatal myelination without a substantial compromise of ultimate myelination. Thus, the progressive accumulation of sulfatides might induce MLD OLs to persist for longer time as progenitors and delaying, but not blocking, their capacity to terminally differentiate and produce myelin. Under this scenario, the potential delay of endogenous MLD OLs to differentiate would give space to grafted OLs, the differentiation program of which is unaffected as a result of a normal sulfatide catabolism, to terminally differentiate and myelinate MLD axons, as we have seen in this study.

OLs also migrated within the MLD SVZ–RMS. Although neurogenesis and gliogenesis are primarily embryonic events, the postnatal mammalian brain retains areas such as the SVZ where a continuum production of new neuronal and glial progenitors is maintained throughout life (Doetsch et al., 1997). Cells born in the SVZ migrate along the RMS, a restricted area connecting the SVZ with the OB, where they replace dying neurons (Lois and Alvarez-Buylla, 1994). The adult SVZ is also a source of astrocytes and OLs, which migrate radially toward the cortex, particularly in response to brain insults (Goings et al., 2004; Gotts and Chesselet, 2005). Whether the MLD SVZ reacts to the progressive neuropathology by increasing the production of neuronal and glial progenitors for neurorepair is presently uncertain. BrdU proliferation experiments indicated that grafted OLs could home in the

Figure 7. MLD mice treated with OLs showed normalization of central electrophysiological parameters and of motor deficits. A, Electrophysiological measurement of central motor-evoked potentials (CMEPs) in transplanted mice showed significant protection of CCE and cMEPs 1 year after OLP transplantation. \( n = 7–10 \) mice per group. B, One-year-old MLD mice treated with OLs (\( n = 7 \)) or untreated (\( n = 11 \)) were compared with wild-type animals (\( n = 10 \)) on an accelerating Rotarod. Latencies were recorded over 3 days (3 trials each day). Means ± SEM of each day performances are indicated. Statistical analysis revealed motor recovery of the MLD transplanted animals compared with untreated MLD mice.
SVZ where they proliferated during the first weeks after transplantation but not in aged MLD mice. Together, these findings indicate that exogenous OLPs can be attracted to gliogenic sites in the brain, such as the SVZ, where mitogenic signals might stimulate their proliferation.

Sulfatide accumulation in MLD brain was reduced after transplantation of OLPs. Untreated mutants progressively accumulate sulfatides in the brain, which is accompanied by the appearance of neurological deficits in adulthood (Hess et al., 1996; Pernber et al., 2002; Luca et al., 2005). Sulfatides and other sphingolipids are important components of lipid rafts and have a fundamental role for the targeting and assembly of numerous proteins in myelin and neuronal plasma membranes (for review see Lee, 2001). Abnormal sulfatide metabolism might change raft composition, influencing on the biogenesis of plasma membranes, contributing to MLD neuropathogenesis. Then, reducing the endogenous levels of sulfatides after infusing cells with a normal expression of ARSA, capable of long distance migration and myelination of axons in the MLD brain, might have limited the evolution of neurological impairments in treated mice. We observed in fact a good correlation between the increase in ARSA activity in the brain, the reduction of sulfatide storage lesions, and the improved central electrophysiology and motor coordination. Previous studies demonstrated that gene transfer of ARSA to neural cells directly contribute to the reduction of sulfatides and to the prevention of central neurological deficits in MLD mice (Consiglio et al., 2001; Luca et al., 2005; Sevin et al., 2006). Extracellular secretion and receptor-mediated reuptake is a general property of lysosomal enzymes such as ARSA, contributing to cross-correction of enzyme deficiency in neighboring cells. In fact, we found that ARSA appeared to diffuse and accumulate in different areas of the treated brain in direct and indirect contact to the sites of initial transplantation. Furthermore, our results showed that endogenous cells can take up ARSA, constituting in vivo evidence of the process of cross-correction and that transplanted OLPs can serve as a source of enzyme. Our study and that of others contribute direct evidence to the notion of using neural progenitors as migratory vehicles to cross-correct endogenous cells in models of lysosomal storage disorders (Snyder et al., 1995; Matzner et al., 2000; Kondo et al., 2005). Therefore, the therapeutic benefits seen in this study may arise from both improved myelination by engrafted OLs and the cross-correction of ARSA deficiency in endogenous MLD neurons and glial cells.

Brain insults increase microgliosis and astrogliosis to remove cell debris and to contain secondary damage (for review, see Fawcett and Asher, 1999). Adult MLD mice show mild demyelination (Hess et al., 1996), likely consequent to OL death, and as expected, they show both microglial/astroglial responses. Introduction of healthy OLPs in developing MLD brains both reduced the presence of microglial cells, which contribute to cell death of neurons and myelinating cells (Wada et al., 2000; Omhi et al., 2003), as well as astrogliosis. These results likely represent an amelioration of OLs turnover in treated MLD mice, consequent to the establishment of a mosaic situation of grafted OLs intermingled with endogenous MLD OLs.

In summary, our results indicate that OLs have an intrinsic ability to populate the MLD brain, with appropriate migration, nontumorigenic integration in white matter, and differentiation into myelinating OLs. Our findings, analyzed within the context of other reports, present evidence for a potential mechanism of myelin pathogenesis in MLD CNS, in which sulfatides seem to interfere with terminal differentiation of OLs.

References


