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Efficacy of enzyme replacement therapy in an aggravated mouse model of metachromatic leukodystrophy declines with age

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Metachromatic leukodystrophy (MLD) is a lysosomal storage disease caused by a functional deficiency of arylsulfatase A (ASA). Previous studies in ASA-knockout mice suggested enzyme replacement therapy (ERT) to be a promising treatment option. The mild phenotype of ASA-knockout mice did, however, not allow to examine therapeutic responses of the severe neurological symptoms that dominate MLD. We, therefore, generated an aggravated MLD mouse model displaying progressive demyelination and reduced nerve conduction velocity (NCV) and treated it by weekly intravenous injections of 20 mg/kg recombinant human ASA for 16 weeks. To analyze the stage-dependent therapeutic effects, ERT was initiated in a presymptomatic, early and progressed disease stage, at age 4, 8 and 12 months, respectively. Brain sulfatide storage, NCV and behavioral alterations were improved only in early, but not in late, treated mice showing a clear age-dependent efficacy of treatment. Hematopoietic stem cell transplantation (HSCT) for late-onset variants is the only therapeutic option for MLD to date. ERT resembles a part of the HSCT rationale, which is based on ASA supply by donor cells. Beyond ERT, our results, therefore, corroborate the clinical observation that HSCT is only effective when performed in early stages of disease.

INTRODUCTION

The lysosomal enzyme arylsulfatase A (ASA, EC 3.1.6.1) catalyzes the hydrolytic desulfation of the acidic sphingolipid 3-*O*-sulfogalactosyl ceramide (sulfatide) and other sulfate esters (1). More than 100 different disease-causing mutations of the ASA gene have been identified to date. All of them reduce the ASA activity to levels that are no longer sufficient to maintain the equilibrium between the breakdown and the *de novo* synthesis of sulfatide. As a consequence, sulfatide accumulates in the lysosomal compartment of cells which synthesize sulfatide or ingest the lipid from outside. Accordingly, oligodendrocytes, Schwann cells, neurons, microglial cells and renal epithelial cells are among the most severely affected cell types. Lysosomal storage advances with age and eventually leads to the manifestation of a devastating lysosomal storage disease (LSD) called metachromatic leukodystrophy (MLD). The clinical picture of MLD is dominated by progressive and widespread demyelination of the central nervous system (CNS) and peripheral nervous system (PNS), deteriorating neurological symptoms and premature death. Currently, there is no established cure for the disease.

Hematopoietic stem cell transplantation (HSCT) has been performed in a number of patients. The therapy is based on the rationale that transplanted cells secrete ASA, which can be endocytosed by the deficient cells of the patient and thus correct the metabolic defect. Experience accumulated over the years has shown that this approach is not suited for patients who have already developed symptoms. It seems that in particular juvenile and adult patients in early stages of disease

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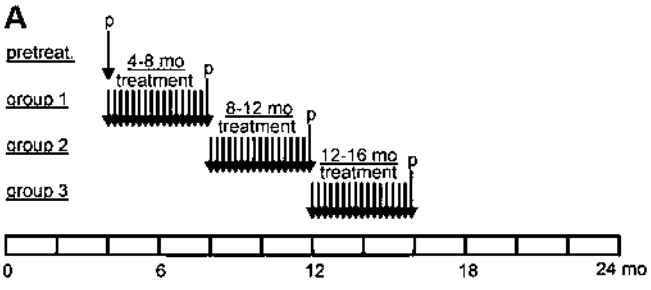
profit from transplantation (2). This argues that provision of enzyme from the transplanted cell is no longer effective after demyelination has started.

Preclinical studies in ASA-knockout (mASA−/−) mice provided a proof of concept for enzyme replacement therapy (ERT) of MLD by demonstrating that repeated intravenous injection of recombinant human ASA (rhASA) improves the nervous system histopathology and function (3,4). Importantly, ERT also reduced the sulfatide storage in the brain and spinal cord. The CNS effect was unexpected and challenged the consensus view that the blood–brain barrier is impermeable for lysosomal enzymes and neuronopathic LSDs are, therefore, untreatable by intravenous ERT (5). We could confirm transcytosis of rhASA across endothelial cells of brain capillaries in a cell culture model of the blood–brain barrier by demonstrating the receptor-dependent and receptor-independent transendothelial transfer routes for rhASA (6). However, transfer rates are very low both *in vivo* and *in vitro*, explaining the requirement of high enzyme doses (≥20 mg/kg) for corrective effects in the CNS (3,4).

Therapeutic efficacy of ERT does not only depend on the enzyme dose but also depends on the disease stage at which treatment is initiated. This has been shown, for example, for the visceral manifestations of the Pompe disease where the more robust responses are achieved the earlier in life ERT begins (7,8). Preclinical studies in mouse models of neuronopathic LSDs are in line with this view as the treatment of neonates causes an amelioration of the CNS pathology which is absent or less pronounced if treatment is started in elder animals (9–15). It has to be mentioned, however, that the higher efficacy of newborn treatment is rather due to the peculiarities of the blood–brain barrier in the first weeks of life than to the less progressed disease. In embryonic and perinatal stages, brain capillary endothelial cells express high levels of the insulin-like growth factor II/mannose 6-phosphate receptor, but drastically down-regulate its expression within the very first weeks after birth [for review, see Kornfeld (16)]. The receptor binds mannose 6-phosphate residues of soluble lysosomal enzymes and transcytosis them from the circulation to the brain parenchyma (6,12,13). Consequently, a much higher fraction of intravenously injected lysosomal enzymes is delivered to the CNS in neonates compared with elder animals. Actually, preclinical studies that systematically correlate therapeutic benefit to different disease stages after the postnatal down-regulation of the insulin-like growth factor II/mannose 6-phosphate receptor are not available.

One of the reasons why preclinical cross-age studies have not been done for MLD so far is the comparably mild phenotype of mASA−/− mice, which does not allow for credible predictions on therapeutic effects on the severe symptoms that dominate the clinical course of MLD (17). In particular, reduced nerve conduction velocity (NCV) and demyelination, two hallmarks of the human disease, are not detectable in conventional mASA−/− mice (17,18). To fill this gap and to assess the 'window of opportunity' for effective treatment of MLD, we used a novel double-transgenic mASA−/− strain with a demyelinating disease phenotype and immunotolerance to hASA. Age-specific effects of long-term and high-dose ERT were analyzed by starting treatment in a presymptomatic, an early clinical and an advanced clinical stage of the disease.



C

group	sub-group	geno-type	treatment drug	treatment duration	final age (mo)	n at start	deaths
pretreat.	a	MLD	none	---	4	3	0
	b	wildtype	none	---	4	3	0
1	a	MLD	rhASA	4-8 mo	8	14	1
	b	MLD	buffer	4-8 mo	8	13	0
	c	wildtype	none	---	8	12	0
2	a	MLD	rhASA	8-12 mo	12	14	0
	b	MLD	buffer	8-12 mo	12	12	0
	c	wildtype	none	---	12	12	0
3	a	MLD	rhASA	12-16 mo	16	12	3
	b	MLD	buffer	12-16 mo	16	12	1
	c	wildtype	none	---	16	12	1

Figure 1. Experimental set-up. (**A**) Scheme of the chronological order of treatments (short arrows) and final perfusion (p, long arrows) in the three treatment groups 1, 2 and 3 and pretreatment controls (pretreat.). (**B**) Schematic representation of the course of the disease in the MLD mouse model involved in the present study. (**C**) Subgroups and animal numbers as indicated.

Results from this study are particularly important to define inclusion and exclusion criteria for future clinical trials for MLD. They also support the notion that HSCT should not be performed in patients who have progressed already beyond the initial stages of disease.

RESULTS

Design of the study

MLD mice used in the present study were mASA−/− mice being double transgenic for the mouse cerebroside sulfotransferase (CST) and an active site mutant of hASA (hASAc69s). They combine the traits of their two parental lines PLP-CST/mASA−/− (18) and hASAc69s/mASA−/− (19), i.e. increased sulfatide synthesis in myelinating cells and immunotolerance to hASA. Treatment of MLD mice was started at age 4, 8 and 12 months, respectively (Fig. 1A). These ages were chosen because they resemble presymptomatic, early clinical and advanced stages of the disease, characterized by the absence of measurable neuronal dysfunctions (month 4), first signs of behavioral alterations and nerve conduction impairments (month 8) and more severe neurological symptoms (month 12), respectively (Fig. 1B). Each of the three age groups included three subgroups. Two subgroups were MLD mice that were treated either with 20 mg rhASA per kg body weight or, as a control, with

vehicle (mock treatment; Fig. 1C). The third subgroup comprised wild-type mice that were strain (C57BL/6 × 129/Ola) and age-matched to the rhASA- and mock-treated MLD mice. Each of the three subgroups consisted of 12–14 animals. Mice were perfused in the week after the 16th treatment, i.e. at an age of ~8, 12 and 16 months, respectively. For the evaluation of the pre-treatment storage of mice from group 1, lipid profiles of 4-month-old untreated MLD mice ($n = 3$) and wild-type controls ($n = 3$) were determined by thin-layer chromatography (TLC).

Side effects

Six of 119 mice (5%) died at different stages of the study (Fig. 1C). Casualties occurred among rhASA-treated MLD mice (four mice), placebo-treated MLD mice (one mouse) and untreated wild-type control animals (one mouse). Two of the five MLD mice had rectal prolapsis and dorsal wounds, respectively, and were killed due to ethical reasons. The remaining three MLD mice were found dead in their cages 2–4 days after treatment with no obvious relation to therapy. Most deaths were observed in the eldest age group (five mice). Also age-dependent alterations of the mean body weight were noticed. Although mice that were 12 and 8 months old at the start of treatment lost 16.2 and 4.4% of weight, respectively, young mice (4 months at start) gained 3.8% of weight on average (not shown). No weight differences between placebo and rhASA-treated MLD mice were detectable for any of the three age groups. Weight loss can therefore be ascribed to the stress induced by repeated injection, electrophysiological investigations and behavioral testings. Due to the genetic immunotolerization of the MLD mice, repeated intravenous injection of rhASA did not elicit anaphylactic reactions that have been described for immune-competent mASA−/− mice in previous studies (19).

Organ weights

The wet weights of the kidney, liver, brain and sciatic nerve were determined after termination. The liver and kidneys from mock-treated MLD mice were found to be enlarged compared with wild-type control organs. The differences to wild-type organs reached statistical significance at 8 months for the liver (Fig. 2A), and at 16 months for the kidney (Fig. 2B). ERT partially normalized the hepato- and nephromegaly and the liver and kidney lost 74 and 93% of their excess mass, respectively. In contrast to the liver and kidney, the brain tended to be smaller in mock-treated MLD mice compared with wild-type controls (Fig. 2C). The decrement was, however, only subtle, reached no statistical significance and no corrective effect of ERT could be detected. MLD mice show typical features of a hypertrophic neuropathy including thickening of peripheral nerves (18). Consequently, a significant increase in the wet weight of the sciatic nerve was seen for mock-treated MLD mice of all three age groups compared with age-matched wild-type controls (Fig. 2D). ERT did not reduce the nerve weight at any age.

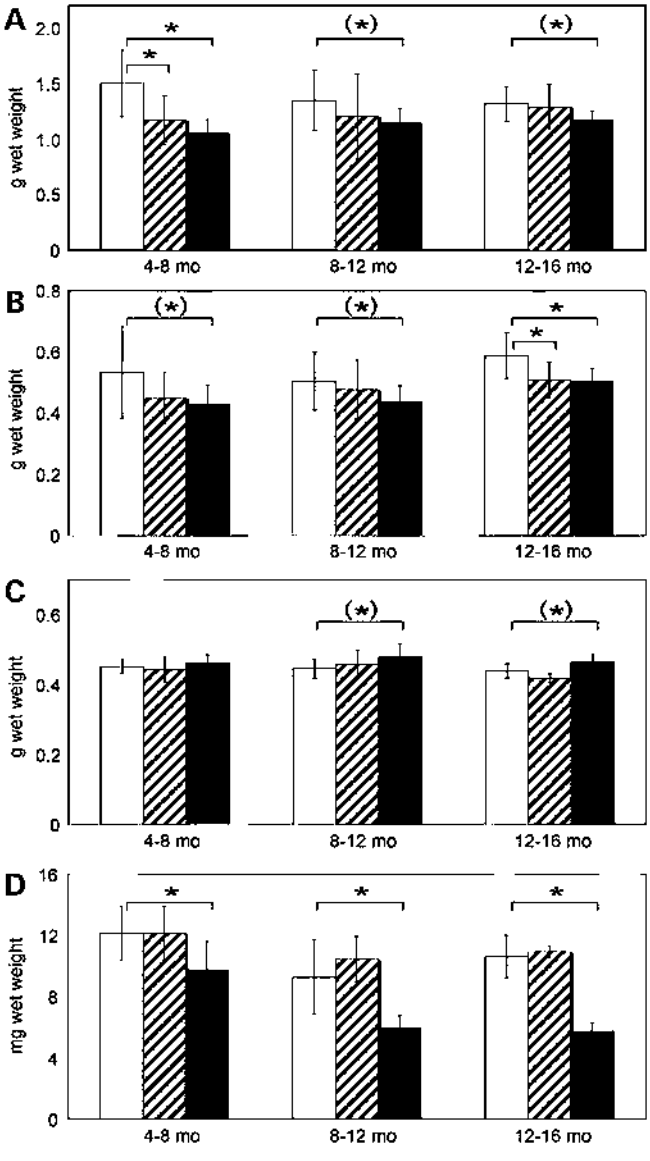


Figure 2. Organ and tissue wet weights. Weights of (**A**) the liver, (**B**) kidney, (**C**) brain and (**D**) sciatic nerve are compared between mock-treated MLD mice (open bars), rhASA-treated MLD mice (hatched bars) and wild-type control mice (closed bars). Bars represent the mean ± SD of $n = 5–7$ mice per subgroup. Age groups as indicated. Asterisks indicate statistically significant differences between subgroups ($P < 0.05$, student's t-test). Tendencies ($P < 0.15$) are indicated by asterisks in brackets.

Sulfatide storage in the kidney

Sulfatide storage in the kidney was first analyzed by histochemical staining of kidney slices with the cationic dye Alcian blue (20). As previously described for conventional mASA−/− mice (21), intense staining of the entire medulla and medullary rays was also detectable in the aggravated MLD mice of the present study (not shown). Within the medulla, epithelial cells of various renal tubule segments were filled up with storage granules and no clear increase in alcianophilic material was detectable between 8 and 16 months. Quantitative data obtained by TLC of lipid extracts

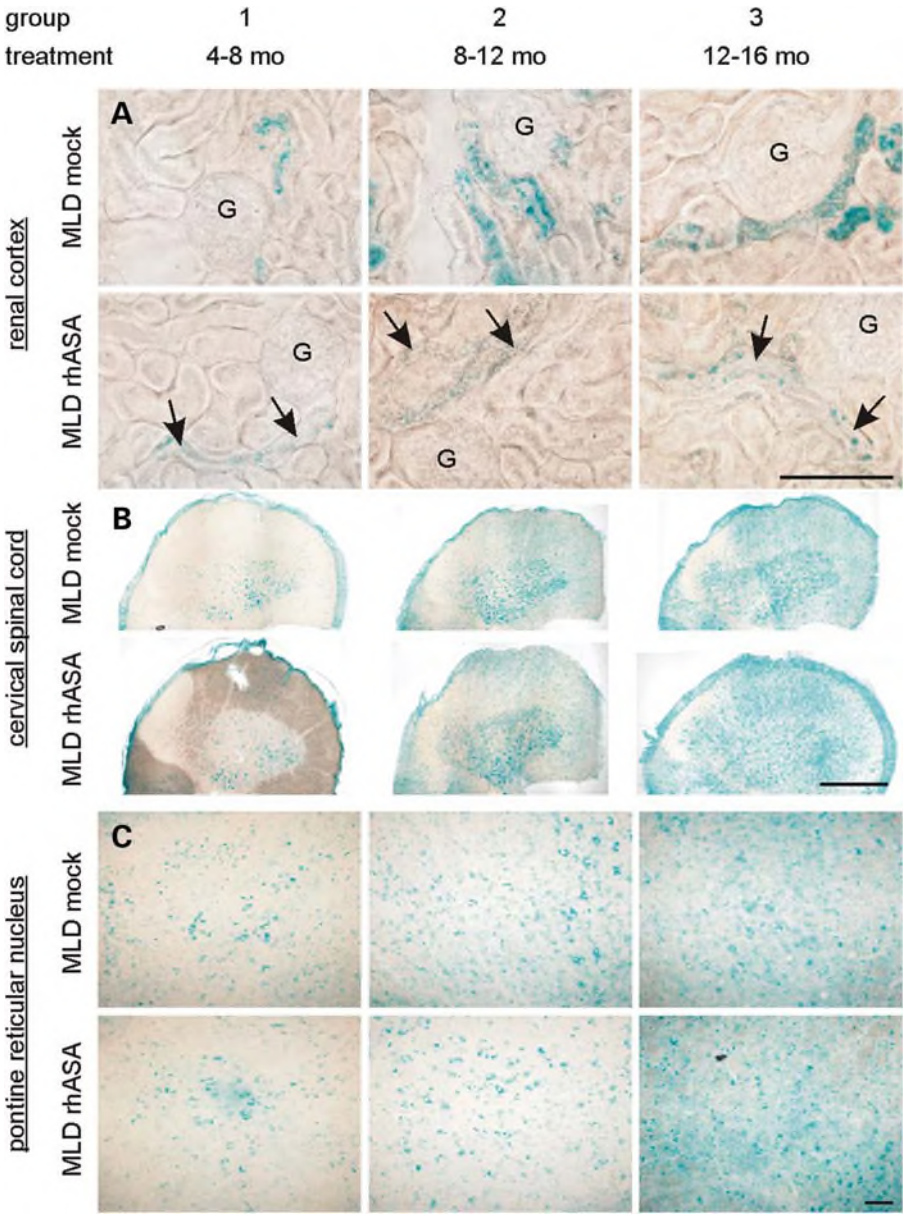


Figure 3. Histology of sulfatide storage as visualized by incubation of tissue sections with Alcian blue. (**A**) Renal cortex. G, glomerulus. Arrows in the lower panel point to TALs of the loop of Henle which are devoid of storage in rhASA-treated MLD mice, but filled with storage granules in mock-treated MLD mice (upper panel). (**B**) Cervical spinal cord. (**C**) Pons (reticular nucleus). The age groups and treatment periods are indicated at the very top. In all three subfigures, storage patterns of mock-treated MLD mice (upper panels) and rhASA-treated MLD mice (lower panels) are compared. Scale bars in (A), (B) and (C) represent 50, 500 and 100 μm, respectively.

from total kidney demonstrated, however, that sulfatide levels increased slowly from 1.7- to roughly 2.5-fold of normal between age 4 and 16 months (not shown). In contrast to the medulla, less alcianophilic material accumulated in the renal cortex and an age-dependent increase in storage was readily discernible on the histological level. The accumulation of storage material is shown for epithelial cells lining the thick ascending limbs (TALs) of the loops of Henle in the upper panel of Fig. 3A. TLC revealed that ERT reduced sulfatide storage in the total kidney of group 1, 2 and 3 mice by 26.6, 16.7 and 13.3%, respectively ($P < 0.05$ for each age group; Fig. 4A). In the histology of the medulla, the massive storage hampers the detection of minor differences in storage intensities and this decline could not be unambiguously demonstrated (not shown). Sulfatide clearance was, however, clearly visible in the cortex, where the alcianophilic material was almost entirely lost from TALs at all ages (Fig. 3A, lower panel).

Sulfatide storage in the CNS

The CNS was analyzed by Alcian blue staining of histological sections through the pons and cervical spinal cord. As described previously for conventional and aggravated

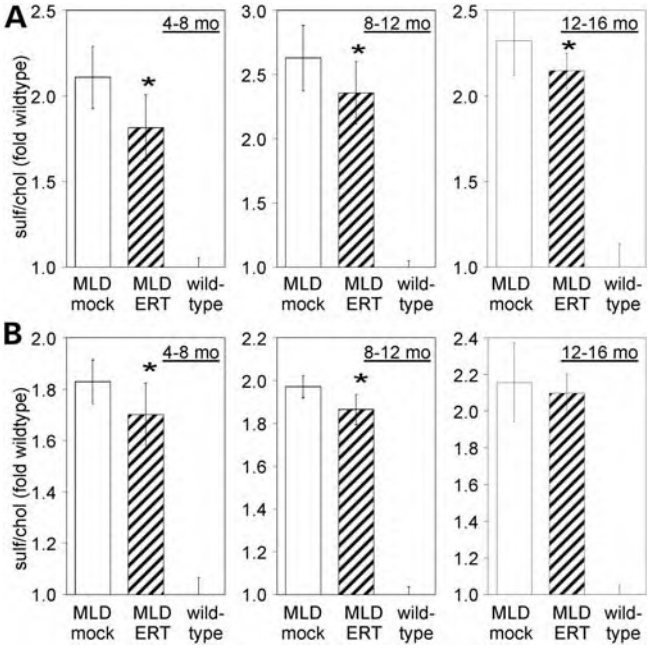


Figure 4. Sulfatide storage and sulfatide clearance. Sulfatide levels in (**A**) the kidney and (**B**) brain of mock-treated MLD mice (open bars), rhASA-treated MLD mice (scattered bars) and wild-type controls (whiskers only) were determined by TLC, normalized to cholesterol levels and expressed as multiples of the mean wild-type levels. Age groups as indicated. Bars represent the mean ± SD of $n = 5–7$ mice per subgroup. Asterisks indicate statistically significant differences between mock- and rhASA-treated MLD mice ($P < 0.05$, Student's *t*-test).

mASA−/− mice (18,22,23), storage was most prominent in microglial cells, oligodendrocytes and subpopulations of neuronal cells (Figs. 3B and C). The comparative analysis of 8, 12 and 16 months old mock-treated MLD mice indicated a progressive accumulation of alcianophilic material in the brain and spinal cord particularly in the microglial compartment confirming previous observations (18). TLC of lipid extracts from the total brain revealed an increase in sulfatide storage between age 4 and 16 months from 1.2- to 2.2-fold of normal (not shown). ERT did not result in a histologically detectable decline in storage material in the brain and spinal cord of MLD mice at any age (Fig. 3B and C, lower panels). TLC of total brain lipids indicated, however, a decline in storage levels by 15.4% in group 1 ($P = 0.023$) and by 11.0% in group 2 ($P = 0.005$). In group 3, no statistically significant decline in sulfatide storage was detectable ($P = 0.295$; Fig. 4B).

Electrophysiology

The nerve conduction properties of the PNS were determined by recording compound motor action potentials (CMAPs) in the foot muscles following the electrical stimulation of the tibial and the sciatic nerve. Specifically, the latencies, amplitudes and durations of the CMAP following proximal (sciatic nerve) or distal (tibial nerve) stimulation were measured. In addition, the F-wave latencies and NCVs were evaluated. Compared with the corresponding wild-type controls, the eight established electrophysiological parameters were consistently altered in mock-treated MLD mice of all age groups (Fig. 5). Thus, latencies [distal motor latency (dml), F-wave latency, latency after proximal stimulation] and CMAP duration (after distal and proximal stimulation) were significantly increased, whereas CMAP amplitudes and NCVs were significantly reduced in 8, 12 and 16 months old MLD mice. These alterations lead to a less compact CMAP. In accordance with the progressive nature of the disease, nerve conduction impairments were comparably mild in group 1 and severe in group 3. In the latter group, latencies and durations were prolonged by up to 97% and amplitudes and velocities were reduced by up to 86% and by 55%, respectively. For MLD mice of group 1, ERT significantly improved latencies, durations and amplitudes leading to a partial normalization of the disperse CMAPs observed in the mock-treated control group (Fig. 5A). Treatment of group 2 mice had no effect on the reduced NCV and other electromyographic changes (Fig. 5B). Also in group 3, no therapeutic effects could be determined (Fig. 5C).

Behavioral tests

To determine further effects of ERT on the nervous system function, mock-treated MLD mice, rhASA-treated MLD mice and wild-type controls were subjected to different behavioral tests in the week before the last treatment. As shown previously, conventional mASA−/− mice develop emotional deficits and coordination impairments (17,24). In the present study, comparisons between mock-treated MLD mice of the three age groups with age-matched wild-type controls allowed us to determine the progression of behavioral abnormalities developed by the aggravated MLD mouse model. Data are illustrated in Figure 6 (means) and Table 1 (*P*-values). The comparative analysis revealed that 12- and 16-month-old MLD mice were less anxious when tested on the elevated plus maze by showing a significantly higher percentage of counts in the open arms ($P < 0.01$ and $P < 0.001$, respectively). A tendency for an increased anxiety-related exploratory activity was already seen at 8 months ($P = 0.10$). Significant decline in swimming velocity was detectable in 12-month-old MLD mice for the first time ($P < 0.001$). The mean swimming velocity declined from 99 to 45% and to <32% of normal between age 8, 12 and 16 months, respectively. Experiments with an accelerating rotarod revealed a significant effect of the group on the drop latency already at 8 months as MLD mice fell down earlier than wild-type controls ($P = 0.05$). The drop latency declined continuously from 8 to 12 and to 16 months when MLD mice were almost unable to stay on the rod even at low rotating velocities. In addition, mock-treated MLD mice of all age groups did not perform significantly better in trial 4 compared with trial 1 indicating the absence of motor learning abilities at 8, 12 and 16 months. The walking endurance was measured in treadmill experiments in which mice were challenged to keep pace with a continuously moving belt. Although the treadmill performance was still normal at age 12 months, it was significantly reduced at 16 months.

Corrective effects of ERT on behavioral deficits can be determined by comparing rhASA- and mock-treated MLD mice. Tendencies for normalizations of the increased exploratory activity and the reduced swimming velocity were seen for 8- and 12-month-old MLD mice, respectively (Fig. 6A and B).

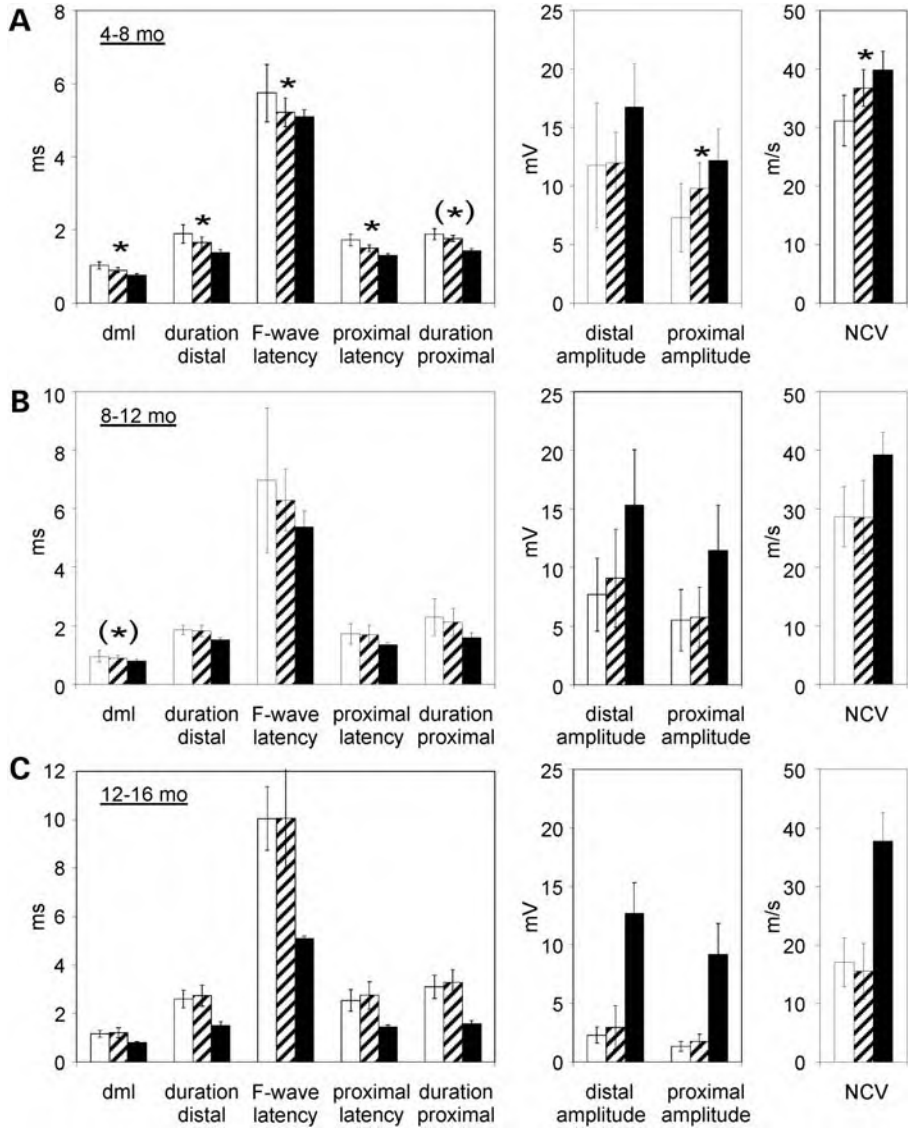


Figure 5. Neurography of the sciatic nerve. (**A**) Group 1 (8 months old at test), (**B**) group 2 (12 months old at test) and (**C**) group 3 (16 months old at test). CMAPs were elicited by proximal or distal stimulation of the sciatic nerve (see Material and Methods). The indicated electrophysiological parameters were compared between mock-treated MLD mice (open bars), rhASA-treated MLD mice (scattered bars) and wild-type controls (closed bars). dml, distal motor latency; NCV, nerve conduction velocity. Bars represent the mean ± SD of $n = 9–13$ mice per subgroup. Asterisks indicate statistically significant differences between mock- and rhASA-treated MLD mice ($P < 0.05$, Student's *t*-test). Tendencies ($P < 0.15$) are indicated by asterisks in parenthesis. Differences between mock-treated MLD mice and wild-type controls were significantly different for all 24 comparisons shown in the figure ($P < 0.05$, not indicated).

These improvements, however, did not reach statistical significance. In contrast to mock-treated MLD mice, rhASA-treated MLD mice displayed significant motor learning at age 8 months (difference between Trial 1 and 4: $P < 0.01$; Fig. 6C). Improvements of drop latencies and learning were, however, not detectable in elder animals. Beneficial effects of treatment on the poor treadmill performance shown by 16-month-old mock-treated MLD mice were undetectable as well (not shown).

DISCUSSION

MLD may be diagnosed in a presymptomatic stage if recognition of the disease in elder siblings prompts genetic testing of younger family members. Due to the lack of newborn screening programs, such an early diagnosis is, however, rare and MLD is usually not recognized before the onset of first or even progressed neurological symptoms. We started ERT of MLD mice in a presymptomatic, an early clinical and a progressed stage of the disease to evaluate the therapeutic outcome of medical intervention in these three case scenarios (Fig. 1A). The animals were from a novel double-transgenic mASA−/− strain with an aggravated disease phenotype and immunotolerance to hASA. In contrast to conventional mASA−/− mice, this strain shows a demyelination of the CNS and PNS, a progressive reduction in the NCV and severe neurological symptoms reminiscent of advanced stages of MLD (18). It therefore represents a more authentic

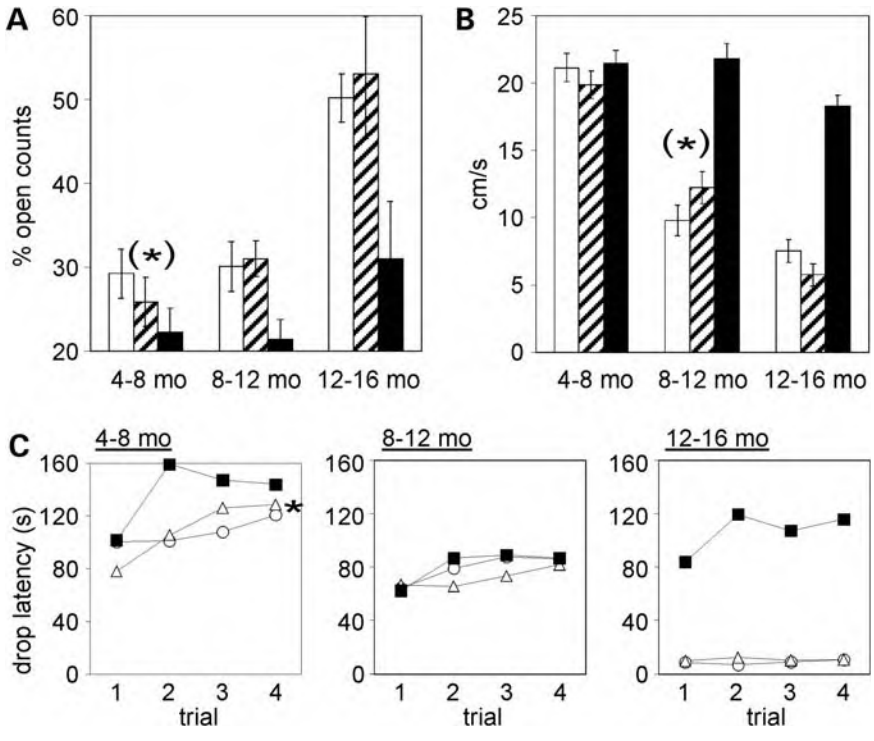


Figure 6. Behavioral tests. Mock-treated MLD mice (open bars and open circles), rhASA-treated MLD mice (hatched bars and open triangles) and wild-type control mice (closed bars and black squares) of the three indicated age groups are compared. (**A**) Elevated plus maze. The frequency of open arm entries (% open counts) is a measure for the anxiety-related exploratory activity. (**B**) Swimming velocity. (**C**) Rotarod performance. The mean time for which mice could stay on the rotating rod is indicated (drop latency). Mice were tested in four consecutive trials. Bars in (A) and (B) represent the mean ± SEM. Asterisks indicate statistically significant differences between mock-treated and rhASA-treated MLD mice ($P < 0.05$, ANOVA). Tendencies ($P < 0.15$) are indicated by asterisks in parenthesis. For differences between mock-treated MLD mice and wild-type controls, see Table 1.

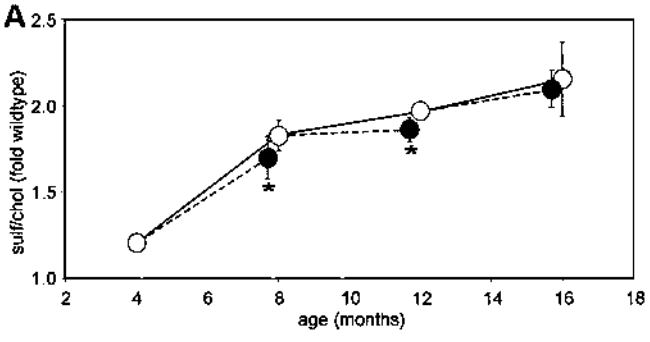
Table 1. Behavioral abnormalities of mock-treated MLD mice compared with wild-type controls

Group	1	2	3
Age at analysis (months)	8	12	16
Anxiety-related exploratory activity increased	(+)	+	++
Swimming velocity reduced	—	++	++
Rotarod performance reduced	+	(+)	++
Treadmill errors increased	n.d.	—	++

n.d., not determined. $n = 11–13$ per group (Fig. 1C).
++, $P \leq 0.01$.
+, $P \leq 0.05$.
(+), $P \leq 0.15$.
—, $P > 0.15$.

model of the human disease than conventional mASA−/− mice (17). Furthermore, due to the transgenic expression of an active site mutant of hASA, the mice do not develop detrimental immunological side effects when rhASA is repeatedly injected (25). Providentially, the course of the mouse disease is still comparably slow with first symptoms being detectable at age 6 months and demyelination and hind limb paralysis occurring around month 18 (Fig. 1B). Due to the immune tolerance to hASA and the combination of slow disease progression with severe end-stage disease, MLD mice are particularly advantageous to determine stage-dependent effects of long-term ERT. An rhASA dose of 20 mg/kg was chosen as lower doses failed to reduce the sulfatide storage in the CNS in the past (3,4). Treatment was conducted for 16 weeks so that mock-treated MLD mice of each age group could serve as pretreatment control for the subsequent age group.

Importantly, the CNS effect demonstrated for conventional mASA−/− mice (3,4) could be reproduced for the aggravated MLD mouse model used in the present study and up to 15.4% of the sulfatide stored as a consequence of ASA deficiency in the brain could be removed by ERT (Fig. 4B). This confirms the view that therapeutically relevant amounts of rhASA can cross the blood–brain barrier, reach the brain parenchyma and get access to the endosomal/lysosomal compartment of sulfatide-storing brain cells. On a percentage base, sulfatide clearance depended on age and was highest for the youngest age group (Fig. 4B). Figure 7A relates the natural course of sulfatide accumulation in MLD control mice to the extent of storage reduction. This comparison shows that ERT had not the potential to reduce brain storage at any age, but merely to counteract the continuous accumulation of sulfatide during the treatment period. Interestingly, even in the youngest age group, the percentage of storage reduction was substantially lower than previously reported for conventional mASA−/− mice. This is illustrated in Figure 7B that compares sulfatide clearance in the three age groups of the present study and two previously published studies in which conventional mASA−/− mice had been treated (3,4). In this meta-analysis of three independent investigations, a correlation between therapeutic efficacy and the level of sulfatide storage in the brains of mock-treated controls becomes



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group		1	2	3
treatment		4-8 mo	8-12 mo	12-16 mo
sulfatide clearance	kidney	++**	++**	+ **
	brain	++*	+ **	(+)
NCV improved		++**	-	-
behavioral improvements		++*/(*)	++/-(*)	-

Figure 7. Overview of therapeutic effects. (**A**) Sulfatide clearance from the brain of rhASA-treated MLD mice (closed circles) related to the kinetics of sulfatide accumulation in mock-treated MLD mice (open circles). Data points represent the mean ± SD of $n = 5–7$ mice per subgroup. Asterisks indicate statistically significant differences ($P < 0.05$). (**B**) Meta-analysis of sulfatide clearance from the total brain. The extent of storage reduction in rhASA-treated mice of group 1 (3-1), group 2 (3-2) and group 3 (3-3) was determined by TLC and related to storage levels in mock-treated MLD mice of the same age group. The diagram also shows data from two previously published ERT studies using conventional mASA−/− mice: 1, treatment with 52 doses of 50 mg/kg rhASA between age 1 and 6 months (4); 2, treatment with 4 doses of 20 mg/kg rhASA between age 11 and 12 months (3). Data points indicate the mean ± SD of $n = 3–7$ per subgroup. Storage reduction was statistically significant except for 3–3. (**C**) Age-dependent effects of ERT on sulfatide storage, NCV and behavior. Differences between the means of mock- and rhASA-treated MLD mice are indicated as follows: ++, >15%, +, 10–15%, (+), 3–10%; -, <3%. Asterisks indicate statistically significant differences: **, $P < 0.01$, *, $P < 0.05$, (*), (*), $P < 0.15$ (not significant).

evident. It can be concluded that the more sulfatide had accumulated at baseline the less storage material can be hydrolyzed. The aforementioned decline in sulfatide clearance with age may therefore be due to the progressive accumulation of sulfatide rather than a consequence of metabolic changes during aging. The molecular mechanism of sulfatide hydrolysis might provide a key to understand this correlation. The hydrolysis of sulfatide does not only depend on ASA, but also on the small glycoprotein saposin B (1,26). In the current view, saposin B binds and extracts sulfatide from lysosomal membranes and presents the sulfate group of the galactosyl moiety to the active site of ASA. Sulfatide molecules accumulating in MLD are deposited in water-insoluble lysosomal lipid aggregates. During the course of MLD, these aggregates grow in size. Although not yet proven, it is readily conceivable that the hydrophilic saposin B polypeptide cannot enter the hydrophobic core of the granules but rather acts on surface-bound lipid molecules. Because the surface area-to-volume ratio of a spheric structure is inversely proportional to its diameter, the percentage of surface-bound and therefore accessible sulfatide molecules decreases with storage advancing. Consequently, the same amount of saposin B and ASA will activate and hydrolyze an ever smaller proportion of stored sulfatide.

This model raises the question whether hydrolysis of preexisting sulfatide storage granules is possible at all. In the present study, this could not been unambiguously shown for the brain, but it was clearly demonstrated for the renal cortex (Fig. 3A). Due to the set-up and timing of the experiments, mock-treated MLD mice of groups 1 and 2 represent pretreatment controls for rhASA-treated MLD mice of groups 2 and 3, respectively. A comparison of sulfatide storage in TALs of rhASA-treated MLD mice and the corresponding pretreatment controls indicates that ERT not only halted further storage accumulation, but hydrolyzed preexisting storage granules. Despite an age-independent and almost complete hydrolysis of sulfatide storage granules in TALs, sulfatide levels in the total kidney were only partially diminished. Similar to the brain, an age-dependent decline in sulfatide storage from 26.6 to 13.3% was detectable (Fig. 4A), confirming the notion that the rate of sulfatide hydrolysis declines with the degree of sulfatide storage at baseline.

Similar to sulfatide clearance from the brain and kidney, the response of nervous system dysfunctions declined with age. In detail, reduced NCV and other electrophysiological changes are ameliorated only after early treatment (Fig. 5). Likewise, the reduced proactive anxiety, diminished swimming velocity and reduced rotarod performance profit by no means from late treatment (Fig. 6).

Figure 7C provides a tabular overview of the various therapeutic effects detected in the three age groups. It is clearly visible that treatment has to be started in a presymptomatic stage of the disease to efficiently address sulfatide storage, electrophysiological alterations and behavioral deficits. When treatment is started after manifestation of progressed nervous system dysfunctions (group 3), it is virtually of no benefit. Also, in an early stage when NCVs just begin to decline (group 2) treatment is of low efficacy as no restoration of the nerve conduction impairments can be achieved. This suggests that the drop of the NCV is due to secondary changes, such as the thickening of the nerve calibers (18), which cannot be readily reversed by the substitution of rhASA (Fig. 2D). From a clinical perspective, the motor function decline is an early event in the course of the most prevalent form of MLD (the late-infantile variant), because gait alterations typically lead to the recognition of the disease (1,2). In a recent study of 21 late-infantile patients, reduced

walking performance, as indicated by an instability when standing or walking without support, was detectable for the first time at a median age of 18 months (27,28). Conventional and aggravated ASA-knockout mice show corresponding early signs of motor incoordination and gait asynchrony at 6 months (18,23,24). Although it is difficult to relate the time course of motor deterioration of MLD patients to that of MLD mice, the robust therapeutic benefit only seen when mice are treated before the onset of gait alterations suggests that ERT of late-infantile MLD has to be started by age 18 months. While late-infantile MLD is characterized by a highly invariable and stereotypic disease course, juvenile MLD is more variable for age range and dynamics (28). Accordingly, the onset of gait disturbances was found to occur anytime between 30 months and 15 years with a median of 64.5 months (27,28). To address also the earlier onset variants within this group treatment should be initiated by age 2.5 years. Our data clearly suggest that earlier diagnosis and intervention is needed. Consequently, newborn screening would be mandatory to enable efficient treatment of MLD by ERT. The result of the study is reminiscent of HSCT studies done in the juvenile and adult variant of MLD, which showed that only patients treated before major cognitive or motor deficits occur may profit from transplantation (2). Our preclinical data may also explain the failure of a recent phase I/II clinical trial in which rhASA given every other week had no readily discernible effects on motor or cognitive function of late-infantile MLD patients (29). Nine of 13 children enrolled in this study had advanced disease at baseline, which may have prevented better therapeutic responses. Future clinical ERT trials should focus on patients who are still in a presymptomatic stage of the disease.

MATERIALS AND METHODS

Recombinant hASA

rhASA was isolated from the secretions of stably transfected Chinese hamster ovary suspension cells overexpressing the wild-type hASA using combinations of ion exchange, hydrophobic and size exclusion chromatography (30). A single production batch was stored in formulation buffer (3.2 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 27 mM glycine, 250 mM mannitol, pH 7.5) at 4°C and used for all experiments. The activity of this batch was remeasured in regular intervals (31) and found to be stable during the entire study period (not shown).

Mice

Double-transgenic mASA−/− mice with immunotolerance to hASA and supranormal rates of sulfatide synthesis were generated by a two-stage breeding protocol. First, the F1 offspring of matings between mASA−/− mice hemizygous for the hASAc69s transgene (19) were crossed. Breeding pairs with homozygocity for the hASAc69s transgene were identified on the basis of 100% transgenic offspring in the F2 generation. In the second step, mASA−/− males homozygous for the hASAc69s transgene were selected from the F2 generation and intercrossed with mASA−/− females being hemizygous for the PLP-CST transgene (18). Genotyping of the resulting pups was done by polymerase chain reaction on genomic mouse tail DNA using primer pairs specific for the wild-type and disrupted murine ASA allele, the CST transgene and the hASAc69s transgene, respectively. Around 50% of the pups had the desired genotype combination mASA−/− hASAc69s+/− PLP-CST+/− and were selected for the treatment experiments. For the sake of simplicity, these immunotolerant mASA−/− mice with an aggravated phenotype are designated as MLD mice throughout this study. Wild-type control mice were age- and strain-matched to the MLD mice (C57BL/6 × 129/Ola). Mice were kept on a 12-h-light and 12-h-dark schedule with *ad libitum* access to food and water and in accordance with the current German law on the protection of animals.

Treatment

Before treatment, mice were weighed, placed for 10 min under an infrared lamp and immobilized in a vertical cylinder-shaped restrainer. Treatment was done by intravenous bolus injection of 20 mg/kg rhASA into the tail vein. The rhASA solution was adjusted to a concentration that enabled us to inject <350 μl of solution per mouse. Treatment was repeated in weekly intervals for 16 weeks. Mock-treated MLD mice received 16 doses of 300-μl formulation buffer without rhASA. All animal experiments were approved by the state office for nature, environment and consumer protection of North-Rhine Westfalia, Germany (LANUV NRW reference number 8.87-50.10.35.08.027).

Behavioral studies

Mice were analyzed by behavioral tests in the week between the 15th and the 16th treatment. Anxiety-related exploration was measured in an elevated plus maze, which mice could freely explore for 10 min. The arena consisted of a plus-shaped maze with two arms (5 cm wide) closed by side walls and two arms without walls. Four infrared beams recording open and closed arm entries, and one recording the percentage of time per minute spent in the open arms were connected to a computerized activity logger. For the determination of swimming velocity, mice were placed at the same starting point in a circular pool (diameter 150 cm), filled with water (26°C). Swimming paths were recorded for 100 s using the EthoVision video-tracking equipment (Noldus Bv, Wageningen, the Netherlands). The software allowed for the calculation of the mean swimming velocity and other swimming parameters. Motor coordination and equilibrium were tested using an accelerating rotarod (MED Associates Inc., St Albans, VT, USA). Mice were first trained to maintain balance for 2 min at a constant speed of 4 rpm. This training trial was followed by four test trials, in which the rod accelerated from 4 to 40 rpm within 5 min. Consecutive trials were separated by a 10-min interval. Latency to falling off the rod was recorded up to 5 min.

Electrophysiological analysis

The nerve conduction of the sciatic nerve was studied under anesthesia 1 day after the 16th treatment as described (32).

In brief, the CMAP was recorded with two needle electrodes inserted into the foot muscles after the distal stimulation of the tibial nerve at the ankle or proximal stimulation of the sciatic nerve at the sciatic notch. The dml, F-wave latency, latency after proximal stimulation, CMAP duration as well as CMAP amplitude after distal and proximal stimulation were measured. Proximal and distal latency were used to calculate the NCV. Statistical analysis was performed using Student's *t*-test.

Histological, biochemical and morphometric investigations

Two days after the 16th treatment mice were deeply anesthetized and processed as described (3). In brief, mice were transcardially perfused for histological analyses with 6% glutaraldehyde and relevant tissues were dissected out. Subsequently, tissue slices (100 μm thick) were prepared with a Leica VT 1200S vibratome (Leica Microsystems, Wetzlar, Germany) and incubated with Alcian blue under conditions which warrant specific staining of sulfolipids (20). In the present study, histological sections through the pons [coronal 6 mm posterior to bregma according to Paxinos and Franklin (33)], cervical spinal cord (transversal in the region of cervical vertebra 1 and 2) and kidney (sectioned along the front plane) were analyzed. For TLC analysis of lipids, mice were transcardially perfused with phosphate-buffered saline (1× PBS), pH 7.4. Lipids were extracted from the brain and kidney, saponified, separated on silica gel 60 plates (Merck) and visualized by staining with phosphoric acid/$CuSO_4$ (3). Sulfatide and other lipids were quantified by densitometry scanning using lipid standards of known concentrations run on the same plate. Sulfatide levels were normalized to cholesterol levels and the sulfatide/cholesterol ratios of different experimental groups were compared. Percentage of storage reduction refers to the difference between mock-treated MLD mice and wild-type animals, which was regarded as 100%. Mice perfused with 1× PBS, pH 7.4, were also used to determine the wet weight of the sciatic nerve. To dissect the sciatic nerve, the muscles of the dorsal thigh were removed, the nerve was freed from fascia and cut proximal immediately at its exit from the vertebral column and distal immediately before the branching of the tibial nerve.

Conflict of Interest statement. None declared.

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