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Sensory-motor behavioral characterization of an animal model of Maroteaux-Lamy syndrome (or Mucopolysaccharidosis VI)

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Maroteaux-Lamy disease, also known as mucopolysaccharidosis (MPS) VI, is an MPS disorder caused by mutations in the *ARSB* gene encoding for the lysosomal enzyme arylsulfatase B (ARSB). Deficient *ARSB* activity leads to lysosomal accumulation of dermatan sulfate in a wide range of tissues and organs. There are various animal models of MPS VI that have been well characterized from a biochemical and morphological point of view. In this study, we report the sensory-motor characterization of MPS VI rats carrying homozygous null *ARSB* mutations. We show that adult MPS VI rats are specifically impaired in vertical activity and motor endurance. All together, these data are consistent with biochemical findings that show a major impairment in connective tissues, such as joints and bones. The behavioral abnormalities of MPS VI rats represent fundamental endpoints for studies aimed at testing the pre-clinical safety and efficacy of novel therapeutic approaches for MPS VI.

Mucopolysaccharidoses (MPS) are a group of lysosomal storage disorders caused by deficiency of enzymes that are responsible for catalyzing the degradation of glycosaminoglycans (GAGs). MPS VI, also known as Maroteaux-Lamy disease, is one of the MPS disorders with autosomal recessive inheritance and is caused by mutations in the *ARSB* gene encoding for the lysosomal enzyme arylsulfatase B (ARSB). *ARSB* mutations result in defective ARSB activity which leads to lysosomal accumulation of dermatan sulfate and chondroitin sulfate in a wide range of tissues and organs. Because the extent and timing of the damage in different tissues is variable, as is the case with all MPS disorders, MPS VI is a clinically heterogeneous disease in terms of the extent and rate of progression of organ impairment¹. Despite such heterogeneity, classic features of MPS VI patients include dwarfism/growth retardation, progressive skeletal (dystosis multiplex) and joint deformities, upper airway obstruction, aortal and mitral valvular dysfunction, spinal cord compression, hepatomegaly, and corneal clouding¹. As a consequence, if not treated, MPS VI patients may ultimately become wheelchair-bound or bedridden due to skeletal, joint and cardiopulmonary defects. Unlike most other MPS disorders, such as MPS I, II, III and multiple sulfatase deficiency (MSD), neurodegeneration and the associated cognitive impairment is generally absent in MPS VI patients. Mental retardation has been rarely reported² and is generally associated with meningeal thickening and hydrocephalus^{2,3}. Therefore, cognitive impairment is not prominent in MPS VI pathology.

Although, bone marrow transplantation and enzyme replacement therapy (ERT) clearly ameliorate the clinical phenotype of MPS VI patients⁴⁻⁹, there is no cure for MPS VI or any other MPS. Preclinical basic research on spontaneous animal models of MPS VI in cats, dogs, and rats, as well as a knockout model in mice created with gene targeting, is being undertaken with the expectation that it will provide an effective novel and resolute therapy for this disorder¹⁰⁻¹⁵.

Characterization of the mutant enzymes and genes in these animal models has proceeded in much the same way as in human MPS VI, and the associated biochemistry and pathology recapitulates the human counterpart^{13,16,17}. MPS VI animal models present increased urinary secretion of dermatan sulfate, develop malformed skull, vertebrae, ribs, pelvis, and long bones, and possess various other symptoms due to alterations of connective tissues: growth retardation, facial dysmorphism, and dysostosis multiplex. GAGs accumulations have been found



in all organs examined in animal models (liver, spleen, heart, cornea etc.); however, aortal and mitral valvular dysfunction and hepatomegaly have been reported only in MPS VI humans, cats and mice^{15,18}. Unlike other animal models of MPS^{19–26}, however, MPS VI animals have never been fully characterized from a behavioral point of view.

Recently, we analyzed the motor activity of MPS VI cats and have generated a behavioral score sheet that is sensitive enough to detect the beneficial effects of gene therapy on MPS VI cat behavior¹¹. In MPS VI rats we showed that they are impaired in performing the rotarod task and that performance in this task positively correlates with circulating levels of ARSB while negatively correlating with biological markers of inflammatory processes¹². More importantly, we found that although low to moderate levels (6–11% of normal) of circulating ARSB were enough to reduce storage and inflammation in the visceral organs, to ameliorate skull abnormalities, and to reduce urinary GAG excretion, much higher levels ($\geq 50\%$ of normal) were required to rescue abnormalities of the long bones and motor activity in the rotarod¹¹. These results highlight the necessity of addressing the efficacy of novel therapies also at the behavioral level.

In this study we expand on our initial observations by systematically characterizing the sensorimotor behavioral phenotype of MPS VI rats. For this purpose, adult normal (NR) and affected (AF) MPS VI rats underwent a series of behavioral tests designed to assess motor function, which is the main behavioral issue potentially associated with MPS VI in humans.

Results

There were only two measures (body weight and distance in the open field) where we found a significant interaction between sex and genotype; therefore, for all the other behavioral measures we pooled male and female rats in the same group.

The body weight of adult MPS VI rats was significantly lower ($F_{1/23} = 22.215$; $p < 0.0001$) than that of control rat (Fig. 1). However, this effect was more evident in males [NR(8) = 387 ± 19 ; AF(5) = 268 ± 23] than in females [NR(7) = 233 ± 4 ; AF(7) = 205 ± 11] leading to a gender ($F_{1/23} = 48269$; $p < 0.0001$) and a genotype \times gender significant interaction ($F_{1/23} = 8.792$; $p = 0.007$).

Motor activity in the home cage was tested by singularly housing the animals in an activity wheel for 24 hrs. Figure 2 shows the number of rotation made by each animal during this period, divided into 3 hr time intervals. Both groups showed a time-dependent activity level ($F_{7/119} = 9.474$; $p < 0.0001$), as is evidenced by their high activity at the earliest time point and an overall bell-shaped curve, with peaks during the dark phase of the light/dark day cycle. Interestingly, affected rats were entirely comparable to normal rats with regards to the shape of their activity curve, which suggests a normal circadian rhythm and response to novelty. However, the level

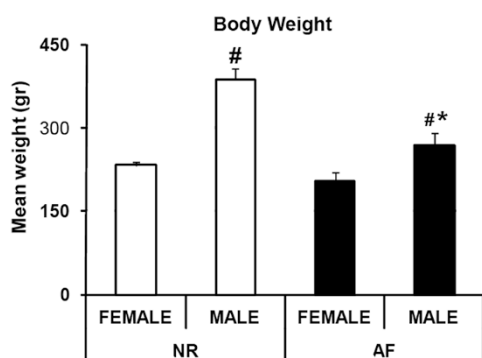


Figure 1 | Mean body weight of MPS VI rats. Mean \pm SEM body weight in female and male normal (NR) and affected (AF) rats. # = p -value ≤ 0.05 male vs female within genotype, * = p -value ≤ 0.05 AF vs NR within sex.

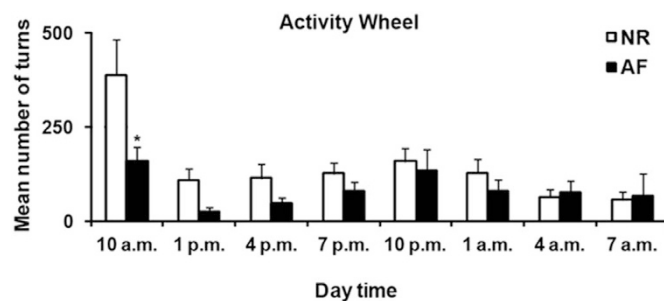


Figure 2 | Wheel running in the home cage of MPS VI rats across 24 hrs. Mean number of turns made by normal (NR) and affected (AF) rats when singularly housed for 24 hrs in a cage containing a wheel. Results are expressed as mean \pm SEM. * = p -value ≤ 0.05 AF vs NR, within time interval.

of motor activity was significantly different between the two groups ($F_{7/119} = 2.816$; $p = 0.009$). In particular, affected rats were much less active than control rats ($p = 0.04$) at the first time point, which suggests either an impaired reaction to novelty or an impaired ability to reach such a high level of motor activity elicited by being placed in a novel home cage.

Exploratory behavior in response to novelty was tested in a standard open field apparatus for 30 minutes. As shown in figure 3A, there was a sex \times group dependent effect ($F_{1/24} = 4.97$; $p = 0.03$), which demonstrates that while normal females were more active than normal males, the opposite occurred in affected animals. Time-interval (5 min) analysis confirmed that there was an initially high level of exploration in the novel cage that decreased over time for all groups ($F_{5/120} = 16.57$; $p < 0.0001$), independently of the genotype or sex ($F_{5/120} = 1.87$; $p = \text{n.s.}$) (Fig. 3B). This suggests that affected animals had normal activation and habituation reactions to novelty. Affected rats showed a non-significant reduction in maximal speed ($F_{1/26} = 2.4$; $p = \text{n.s.}$ - Fig. 3C) and an increase in immobility time ($F_{1/26} = 1.5$; $p = \text{n.s.}$ - Fig. 3D). Vertical activity was generally affected in MPS VI rats; although the decrease in leaning time was not significant ($F_{1/26} = 1.6$; $p = \text{n.s.}$ - Fig. 3E), a dramatic and significant reduction of rearing time was observed in affected animals ($F_{1/26} = 7.4$; $p = 0.01$ - Fig. 3F). We did not find any significant increase in self-scratching (or self grooming, data not shown) behavior (Fig. 3G).

We also analyzed the percentage of time spent in the central quadrant of the open field, which is considered an index of anxiety, but there were no significant differences between the two groups (data not shown).

A completely unexpected result was that affected animals had a significantly ($F_{1/22} = 7.06$; $p = 0.01$) higher (Fig. 3H), rather than lower, thermal threshold as compared to normal animals (Fig. 3I); suggesting that their pain sensitivity was reduced.

Various tests were used to measure muscular strength and resistance in affected rats. We used the grip strength task to measure forelimb strength and found no difference between normal and affected animals (Fig. 4A). The latency to fall off both a wire, while hanging with all four paws up-side down ($F_{1/24} = 4.3$; $p < 0.05$ - Fig. 4B), and a steel, while hanging with the forelimbs ($F_{1/17} = 6.3$; $p < 0.05$ - Fig. 4C), was significantly affected in MPS VI animals compared to normal rats. This suggests that muscular endurance, rather than muscular strength, *per se*, was affected in MPS VI rats.

Motor coordination and motor learning were tested during the rotarod task. In order to estimate the animals' ability to learn the task, we trained them for 5 consecutive days. We found a significant effect in the number of training days ($F_{4/92} = 10.62$; $p < 0.05$). Affected animals were significantly impaired from the very first day ($F_{1/23} = 10.15$; $p < 0.05$). Interestingly, however, post-hoc analysis revealed that both normal ($p < 0.0001$) and affected ($p < 0.05$) animals

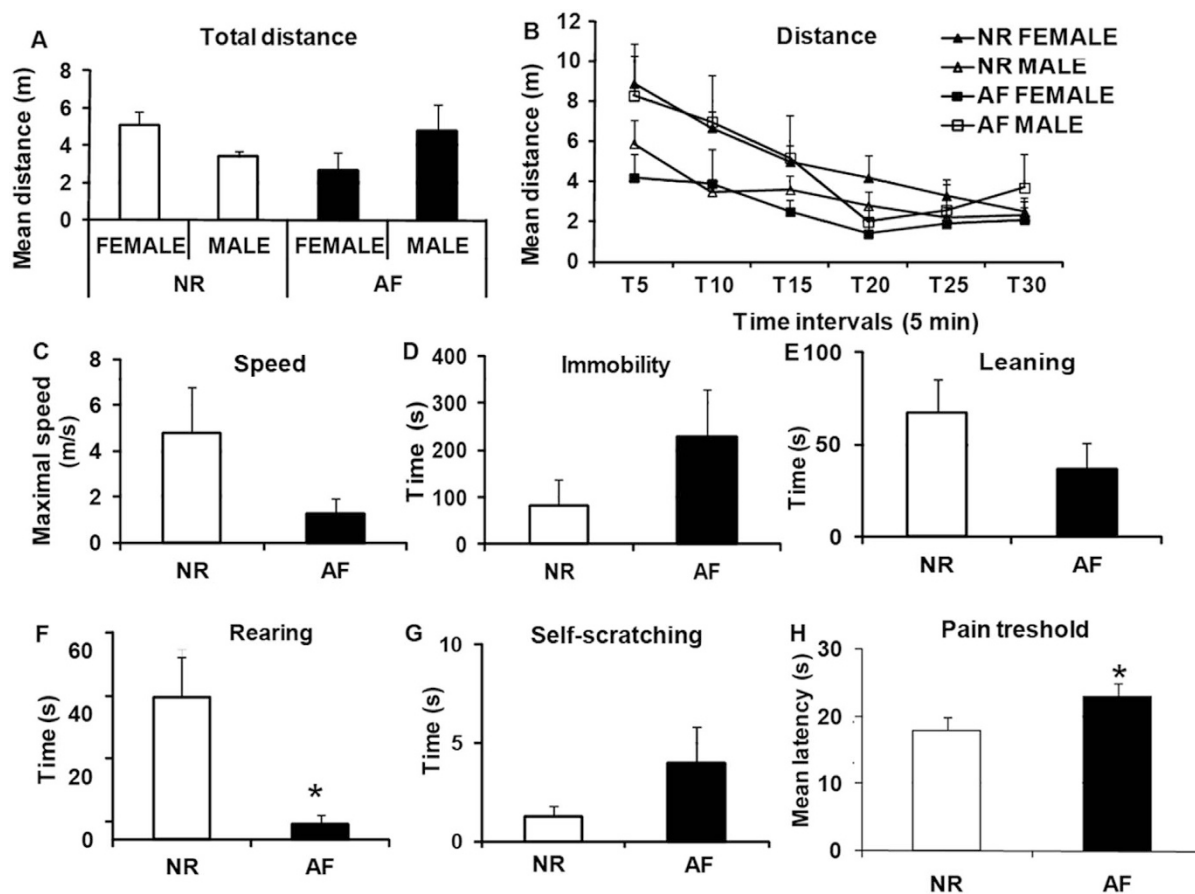


Figure 3 | Exploratory activity and pain sensitivity in MPS VI rats. Total (A) and time interval (B) walking distance, maximal speed (C), immobility time (D), leaning time, rearing frequency (F) and self-scratching time (G) in 30 min recording in an open field for normal (NR) and affected (AF) rats. Paw withdrawal latency in contact with a hot plate in NR and AF adult rats, cut off 30 sec (H). Results are expressed as mean \pm SEM. * = p-value \leq 0.05 AF vs NR.

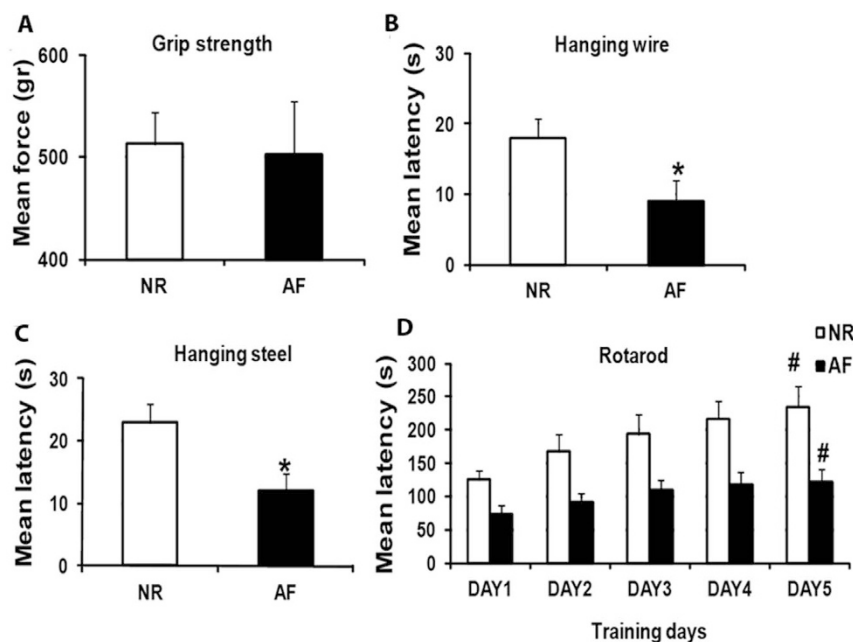


Figure 4 | Neuromuscular ability in MPS VI rats. Forelimb grip force (gr) (A), latency (s) to fall off a grid (B), a steel (C) or an accelerating rod across days (D) in normal (NR) and affected (AF) rats. Results are expressed as mean \pm SEM. * = p-value \leq 0.05 AF vs NR. # = p-value \leq 0.05 DAY5 vs DAY1, within genotype.



improved their performance over time (Fig. 4D), which suggests that motor learning ability was unaffected in MPS VI animals. We also tried to test visuo-spatial learning ability of some of these animals in the water maze task (supplementary methods). The required motor ability associated with the water maze task was evidently very demanding for MPS VI affected rats (Supplementary results). Therefore, only 4 affected rats were tested in the visual version of the water maze task and 3 were tested in the hidden version. Consistently with the minor histopathological changes in the central nervous system of these animals that we have previously reported and with the lack of primary neural functional impairment in MPS VI subjects^{2,3,27,28}, these tested animals showed no major learning deficits as compared to control animals (supplementary results, and supplementary Figure 1). Nevertheless, the low number of animals tested, and the general motor impairment observed in MPS VI affected rats limit any conclusive interpretation of behavioural changes selectively related to cognitive functions.

Tissue GAG storage and inflammation in adult MPS VI rats. To confirm the presence of GAG storage and inflammation, representative tissues (kidney, spleen) and knee joints were collected from MPS VI and control rats at the end of the study.

GAG levels were measured in kidney (NR = 5.8 ± 0.45 vs AF = 12.26 ± 2.1) and spleen (NR = 6.34 ± 0.55 vs AF = 10.2 ± 0.95) tissues using the quantitative dimethyl-methylene blue method, which revealed significant GAG storage in both tissues (kidney: $t8 = -4.5$; $p = 0.001$; spleen: $t7 = -3.75$; $p = 0.007$), and were analyzed as previously reported¹². Consistently, and as was previously described¹², CD68+ activated macrophages were found to accumulate in both spleen and kidney samples from MPS VI rats, which confirms the presence of inflammatory processes in these tissues (Fig. 5A). Similarly, haematoxylin and eosin (H&E) stained histological sections of knee joints from MPS VI, but not from NR animals, showed extensive vacuolization in cortical bone osteocytes (Fig. 5B, full arrowheads) and in chondrocytes of the articular surface of the femur (Fig. 5B, empty arrowheads) and tibia (data not shown). The presence of cells distended with material that appears clear after H&E staining has been associated with lysosomal storage that is lost in tissue processing in bone-cartilage tissues from MPS animal models^{12,29}. CD68+ cells, representing activated macrophages and/or osteoclasts also accumulated in the subchondral region of MPS VI femur (Fig. 5A, arrows) and tibia (data not shown).

Discussion

The aim of this study was to analyze, in detail, the behavior phenotype of MPS VI rats. We demonstrated that MPS VI rats have

impaired vertical exploratory ability, reduced hanging strength and motor endurance, and an increased thermal threshold induced by thermal stimuli, but retained unaffected motor learning abilities.

We previously showed that MPS VI rats are strongly impaired in the rotarod task, and they fall off the rod much earlier than normal animals¹². The ability to run on the rotarod in accelerating mode is dependent not only on motor ability but also on pulmonary capacity, which is seriously affected in MPS VI. Therefore, the rotarod, unlike all the other motor tasks used in this study, may also be sensitive to this other aspect of the pathology and thus, have major predictive validity to test novel therapeutic strategies¹².

In this study, we show for the first time the motor learning curve of the rotarod task, and prove that MPS VI animals do improve their performance across training days, which suggests that their motor learning ability is intact.

Cartilage and bone are the main sites of MPS VI pathology, which leads to poor bone growth and joint motility, and therefore significantly hinders an affected child's autonomy in dressing, moving and performing simple, everyday life actions. Cartilage and bone defects have been identified in all species affected by MPS VI^{4,12,16,30-32}. Moreover, the production of pro-inflammatory cytokines³³ and the presence of CD68+ macrophages and/or osteoclasts have been reported in the cartilage and bone of MPS VI rats¹², and are presented in Figure 5 of our study. Due to the almost complete lack of similar studies in animal models of MPS VI, and although most of the other animal models of MPS are also characterized by cartilage and bone abnormalities, we will discuss the results of this study in relation to various animal models of cartilage, joint and neuropathic alterations, such as arthritis and spinal cord injury^{34,35}. Indeed, animal models of osteoarthritis, unlike other MPS models, share with MPS VI not only the same pathological changes in bone, joint and cartilage, but also the lack of central nervous system abnormalities, which makes more straightforward the interpretation of the observed behavioral deficits in terms of peripheral alterations^{19,22,23,36,37}.

We expected that motor behavior would be significantly affected in adult MPS VI rats. However, when periodically observed in their home cage, MPS VI rats evidently are not motionless. Therefore, to unravel possible motor defects we subjected them to different behavioral conditions known to elicit different kinds of exploratory behaviors. Adult MPS VI rats were generally minimally impaired in novel cage exploration. They presented reduced maximal speed, increased motionless time, and reduced distance traveled, but none of these effects were statistically significant. This suggests that the affected rats' walking ability, unlike that of human patients^{8,9,38,39} was relatively preserved at this age. However, affected animals were extremely impaired in vertical activity, especially when forced to stand on

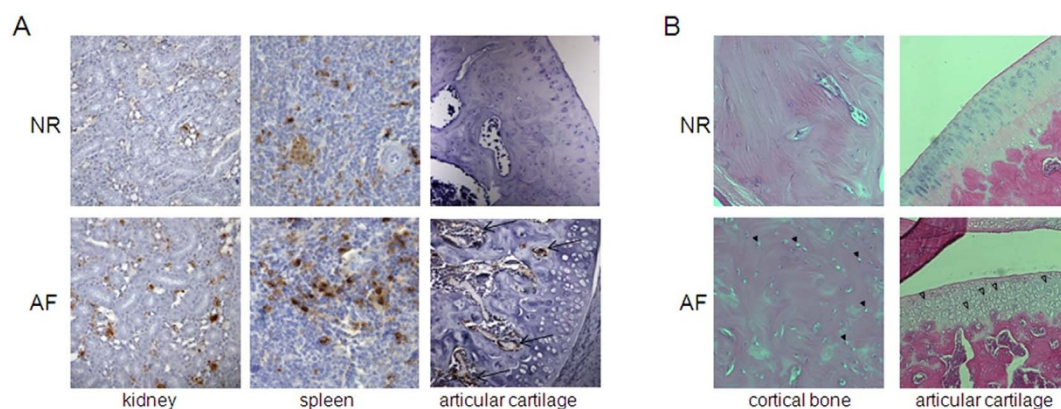


Figure 5 | Accumulation of GAGs and CD68+ cells in tissues from MPS VI rats. Immunohistochemical CD68 staining was performed on paraffin cross sections from the kidney, spleen and articular surface of the femur of NR and AF rats (A). (B) Hematoxylin and eosin staining on paraffin cross sections of cortical bone and articular cartilage from the femur of NR and AF rats. Full arrowheads: cortical bone osteocytes. Empty arrowheads: articular cartilage chondrocytes. Representative pictures from animals in each group are shown. Magnification 40x.



their hindlimbs without any forelimb support (rearing), and it has been shown to be one of the most affected behavioral parameters in animal models of osteoarthritis^{34,35}. This might be due to the fact that rearing is a behavioral pattern that mostly relies on joint flexibility and endurance. Accordingly, when we tested muscular strength in MPS VI affected rats we found that their “passive” forelimb grip strength was not impaired. However, when the animals had to counteract the force of gravity while hanging onto something, a strong and consistent impairment was found, which suggests that muscular endurance is affected in MPS VI rats. This impairment, in our opinion, is due to the impairment in the joints, ligaments and tendons all of which are affected in MPS VI subjects^{4,40}.

In animal models of experimental arthritis, it has been suggested that some parameters of gait or endurance analysis may represent a good measure for pain, while others may be more influenced by mechanical joint deformation as indicated by cartilage and bone destruction³⁴. The changes we observed in hanging tasks and in rearing behavior do not seem to be correlated with pain sensitivity in these animals. Indeed, we found an interesting and, entirely unexpected result relative to pain sensitivity in MPS VI affected rats. Chronic, diffuse joint inflammation occurs in MPS VI subjects; inflammation is known to be responsible for the sensitization of peripheral sensory neurons, leading to spontaneous pain and invalidating pain hypersensitivity^{34,41–45}. Accordingly, decreased thermal threshold has been amply reported in animal models of spinal cord injury, or arthritis^{34,41–43,45}. In contrast, we found an increase, rather than a decrease, in the thermal threshold of affected animals, despite the fact that they showed clear signs of inflammation. The increased latency to withdraw the hind paw in this task may be secondary to motor impairment; the fact that a similar deficit was found in younger animals (2 months old) in the absence of any other motor impairment (data not shown), suggests that this was not the case. Similarly, we did not find any significant increase in scratching behavior in our MPS VI rats, which has been suggested to be a sign of chronic pain in arthritic rats⁴⁶. This result needs to be further confirmed through additional pain sensitivity tasks in future studies; nevertheless, based on this experimental evidence we tried to translate this unexpected result to human subjects. In the Management Guidelines for Mucopolysaccharidosis VI is stated that “In patients with MPS VI, spontaneous reporting of typical complaints of pain and paresthesia is rare,”⁴⁷ although they show carpal tunnel syndrome. The only study we could find in the literature measuring perceived pain in MPS VI patients reported moderate levels of pain, an average score of 50 in a scale from 0 = no pain to 100 = maximal pain, measured using an analogue scale based on the Health Assessment Questionnaire (HAQ)³⁸. While waiting for further experimental evidence on the nociceptive phenotype associated with MPS VI, we speculate that GAGs accumulation may affect pain transmission itself, as a result of nerve compression or myelopathy that have both been shown to affect MPS VI subjects^{47,48}.

The preservation of behavioral functionality is an increasing challenge in the treatment of MPS patients and its maintenance should be defined as an objective to be reached by classical and novel therapies. The effects of ERT in MPS VI patients are generally evaluated only on the 6–12 min walking test or climb test, and it is generally reported to improve these behavioral functions as well as urinary GAG levels^{7,9,38,49}. Nevertheless, when more detailed behavioral analysis is performed, using for instance joint motility scores, some of the therapeutic limits of ERT are generally unrevealed^{6,38}. These results highlight the importance of addressing the efficacy of novel therapies on different behavioral parameters in both clinical and pre-clinical research. In this study, by reporting the first detailed sensory-motor behavioural characterization of animal model of Maroteaux-Lamy disease, we provide experimental evidence that is crucial for future *in vivo* studies, aimed at testing the therapeutic and side effects of novel approaches, whose efficacy has been suggested by *in vitro* or single

tissue organs studies. Finally, these results may also be relevant for all the other forms of MPS, affecting about 4 per 100,000 live births, as they all share common histopathological phenotypes in most of the organs and tissue (excluding the brain).

Methods

Ethics statement. Every possible effort was made to minimize animal suffering. All procedures were approved by the “Ministero della Salute” Committee Rome, Italy for “Good Animal experimental Activities”. The investigation conforms to the European Commission Directive 86/609/EEC.

Subjects and tissues collection. MPS VI rats were obtained from Dr. Tetsuo Kunieda (Okayama University, Okayama, Japan) and maintained at the Cardarelli Hospital’s Animal House (Naples, Italy). Imported MPS affected, homozygous rats were initially bred with wild type, Wistar rats (Harlan, S. Pietro al Natisone, Italy) upon arrival to expand the colony and obtain heterozygous animals. Heterozygous animals were then bred a second time with Wistar rats. All animals used in this study were obtained by subsequent breeding of the heterozygous rats produced in this way, allowing for the production of normal, heterozygous and affected offspring. Genotype analysis was performed by polymerase chain reaction on genomic DNA as previously described⁵⁰, and the presence of mutation was detected through sequencing (PRIMM, Naples, Italy). Rats were housed on a 12 h light-dark cycle with lights on 7.00 a.m.–7.00 p.m. Food and water were available *ad libitum*. Female and male adult rats (about 5–6 months old; 15 normal and 13 affected), belonging to the same colony used for previous studies^{12,51}, were used for the behavioral analysis. Animals of the two groups were matched on age and we used littermates when possible. We started with a total number of 28 animals (15 NR and 13 AF rats), and most of these animals were used as a control for another study on gene therapy¹². Only 11 animals were littermates, with similar or different genotypes and genders, therefore, litter was not a factor in the statistical analysis. In general, the same animals were used for the different behavioral tasks, which were performed within 3 consecutive weeks; however, as detailed below there were varying numbers of animals in the different tests because of lost data, animals, and/or particular conditions linked to the specific task. The test order was as follows. The open field (15 NR and 13 AF) was performed on the first testing day; after the open field one AF animal (rat number 6648) died. Animals had 2–3 days of rest, and then they were subjected to the rotarod task for 5 days, after which rats had at least 2–3 days rest. During this period one affected animal died. Then the hanging wire test was performed, followed the day after by the grip-strength and the hot plate tasks, performed in this order distanced by 3 hr in the same day. 2 AF and 4 NR rats were sacrificed for initial GAG assay, while the remaining rats were subjected to the hanging steel task in the two following days and then to the activity wheel cages. The collection of 24 hr spontaneous activity data was made by housing AF and NR animals individually in the two activity wheel cages available. This required 10 days to test all animals. As long as some of the AF and NR rats completed this task they were subjected to the visuo-spatial learning task, which was performed only on a small number of animals because of frequent animals floating (see supplementary results). The behavioral testing room had constant sound (classical music) and a light background, and animals were tested during their light phase, between 9.00 am and 6.00 pm. Before each behavioral task, animals were acclimatized to the testing room for at least 30 min.

Rats used for histological analysis were sacrificed (6–7 months of age) by cardiac PBS perfusion and the kidney, spleen and knee joints were collected.

Kidney and spleen samples were frozen in dry ice (for GAG quantitative assays) or fixed in methacarn solution (30% chloroform, 60% methanol, 10% acetic acid) for 24 h for staining with anti-CD68 antibodies. Knee joints were fixed in 10% formalin (Sigma-Aldrich, Milan, Italy) for 24 hrs.

Apparatus and behavioral procedures.

Spontaneous activity in the home cage. 11 NR and 9 AF rats were subjected to a wheel cage, which is a normal breeding cage with a wheel connected to a multifunctional printer, which records the number of turns at different time points. Animals were placed in the activity cage at 9–10.30 a.m. and removed 24 hrs later. The total number of wheel turns every 3 hrs was recorded and used for statistical analysis. One NR rat was excluded due to a data collection error.

Exploratory behavior in the open field. General exploratory activity was measured in an activity cage (Ugo Basile, Italy). At the beginning of the measurement session, NR (n = 7 female, n = 8 male) and AF (n = 7 female, n = 6 male) rats were released from the centre of the activity cage and tested for 30 min. During this time, a video-tracking system (Any-Maze, Stoelting, USA), connected to a video camera calculated the mean distance travelled (m), the maximal speed (m/s), and the time spent in the center and in the periphery of the field. The percentage of time spent in the center of the arena in the first five minutes was calculated and considered as an index of anxiety. Vertical activity, distinguished by leaning (standing on the hind limbs with both forelimbs on the wall) and rearing (standing on the hind limbs with no support for the forelimbs) time, and stereotyped behaviors, distinguished by self-grooming (grooming directed to the rat’s body) time and self-scratching time, were manually recorded off-line by a trained observer kept blind to the genotype of the rats.

Thermal sensitivity test. Thermal pain sensitivity was tested using a hot plate apparatus (Ugo Basile, Italy), heated to 52°C. NR (n = 15) and AF (n = 11) animals were



placed on the plate and the latency to lick one of their hind paws or to jump was recorded, with a cut-off time of 30 sec. Three consecutive trials were performed (ITI = about 15 min). Data of one NR and one AF rats were lost.

Neuromuscular tests. Forelimb muscular strength was tested using the grip strength meter (Ugo Basile, Italy). NR (n = 15) and AF (n = 11) rats were suspended by the tail while they hang onto a trapezium with both forelimbs. The maximal force applied in grams was measured in continuous modality for 3 consecutive intervals (ITI = about 15–30 min). The mean value between the three measurements, minus the body weight of the animal on that day, was used for statistical analysis. One AF animal was excluded because it was not able to hang on the trapezium.

Neuromuscular endurance was tested using the hanging wire and the hanging steel tests. For the hanging wire test NR (n = 15) and AF (n = 11) rats were placed on a wire cage lid and the lid was gently waved, causing the rat to grip the wire. The lid was then turned upside down 50 cm above a sawdust-covered cage. Latency to fall off the grid was recorded, with a cut-off time of 30 s, and used for statistical analysis. For the hanging steel test (11 NR and 9 AF), the front paws of the rats were placed on a horizontal wire (2 mm in diameter) 50 cm above a sawdust-covered cage. The trial was repeated twice on 2 consecutive days. Latency to fall off the steel was recorded, with a cut-off time of 120 s, and used for statistical analysis. One AF animal was excluded because it was not able to hang on the steel.

Motor learning test. Motor coordination and learning were tested using the rotarod apparatus for rats (Ugo Basile, Italy) with 4 rods. Two NR animals had to be excluded from testing due to an animal handling error, specifically an accidental flooding of their waiting cage, the night before the first training day. On the first day NR (13) and AF (n = 12) rats were gently placed on each rod set at a steady, slow speed of 4 rpm, and trained to remain on the rod for 60 sec. After this habituation trial, each animal was submitted to 4 trials per day for 5 consecutive days, with an intertrial interval of 15 min. The trial started when all rats moved in the right direction. The rotarod was set at increasing speeds ranging from 5 to 40 rpm over 5 min, and rats were left on the rod for an additional 3 min. The rats' latency to fall off the rod within this period was recorded. The daily mean latency was used for statistical analysis.

Statistical analysis. A preliminary statistical analysis was made using genotype (2 levels: Normal, Affected) and sex (2 levels: male, female) as between factors. If no significant interaction between sex and genotype was revealed in this preliminary analysis, the sex variable was removed from our statistical analysis. Although we used littermates when possible, no more than one genotype from each litter was used in general. This did not allow for a reliable statistical evaluation of the effects of litters. One-way ANOVA, with genotype as the between group factor, was used to analyze body weight, distance travelled, maximal speed, learning, rearing, self-grooming and self-scratching time in the open field, mean licking latency on the hot plate, grip-strength, latency to fall off the grid and the mean latency to fall off the steel in the hanging tasks. A two-way ANOVA for repeated measures was applied, with the same between group factor using testing days, trials or time intervals as repeated measures for activity in the wheel cage (8 levels: T1–T8), and latency to fall of the rod across days (5 levels: Day1–Day5). The Duncan post hoc test was used when appropriate and the statistical significance was set at $p < 0.05$. For the comparison of tissue GAG levels, an unpaired *t*-test between the various data sets was performed using the *t*-test for independent groups comparison. A significance of $p \leq 0.05$ is indicated by a single asterisk in the figures.

Quantitative analysis of GAG accumulation in tissues. 250 µg of protein extracts from spleen (6 NR and 3 AF) and kidney (7 NR and 3 AF) were used for the GAG assay, as previously described. The GAG concentrations were determined using the dermatan sulfate standard curve (Sigma-Aldrich, Milan, Italy). Tissue GAGs are expressed as µg GAG/mg protein.

Anti-CD68 immunohistochemistry. Formalin fixed knee joints were decalcified in 8% formic acid (Sigma-Aldrich, Milan, Italy). All tissues were dehydrated, embedded in paraffin, and sectioned into 7 µm sections. For the CD68 staining, knee joint sections were rehydrated and digested for 1 h at room temperature with 0.05% hyaluronidase (Sigma-Aldrich, Milan, Italy). Bone and tissue sections were incubated for 1 h with blocking solution (1× PBS, 0.5% Tween-20, 0.1% bovine serum albumin and 10% fetal bovine serum, GIBCO BRL–Invitrogen, Gaithersburg, MD, USA) and incubated overnight with a polyclonal anti-CD68 antibody (1 : 300 diluted, Serotec, Oxford, UK). After washing, sections were incubated for 1 h with biotinylated secondary anti-rabbit IgG (Vector laboratory, CA, USA). The reaction was developed using the Vectastained Elite ABC–Peroxidase Kit (Vector laboratory, CA, USA), followed by a 30 min DAB staining (Vector laboratory, CA, USA). Finally, sections were counterstained with hematoxylin (Sigma-Aldrich, Milan, Italy) and mounted with Eukitt (Kaltel, Padova, Italy).

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Author contributions

E.D.L., G.C. and P.S. wrote the main manuscript text, and analyzed data. E.D.L., A.A., A.T. and G.C. conceived and designed and F.R., R.M. and G.C. performed the experiments. G.C. and P.S. prepared figures. All authors reviewed the manuscript.

Additional information

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