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Evidence of a progressive motor dysfunction in Mucopolysaccharidosis type I mice

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Abstract

Mucopolysaccharidosis (MPS) type I (Hurler syndrome) is a lysosomal storage disorder characterized by deficiency of alpha-L-iduronidase (IDUA), intracellular storage of glycosaminoglycans (GAGs) and progressive neurological pathology. The MPS I mouse model provides an opportunity to study the pathophysiology of this disorder and to determine the efficacy of novel therapies. Previous work has demonstrated a series of abnormalities in MPS I mice behavior, but so far some important brain functions have not been addressed. Therefore, in the present study we aimed to determine if MPS I mice have motor abnormalities, and at what age they become detectable. MPS I and normal male mice from 2 to 8 months of age were tested in open-field for locomotor activity, hindlimb gait analysis and hang wire performance. We were able to detect a progressive reduction in the crossings and rearings in the open field test and in the hang wire test in MPS I mice from 4 months, as well as a reduction in the gait length at 8 months. Histological examination of 8-month old mice cortex and cerebellum revealed storage of GAGs in Purkinje cells and neuroinflammation, evidenced by GFAP immunostaining. However TUNEL staining was negative, suggesting that death does not occur. Our findings suggest that MPS I mice have a progressive motor dysfunction, which is not caused by loss of neuron cells but might be related to a neuroinflammatory process.

Keywords

mucopolysaccharidosis type I; hang wire; open field; gait; neuroinflammation

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1. Introduction

Mucopolysaccharidosis type I (MPS I, OMIM #607014, #607015, and #607016) is an autosomal recessive lysosomal disorder due to deficiency of α-L-iduronidase (IDUA, EC 3.2.1.76) that results in storage of partially degraded heparan sulphate and dermatan sulphate glycosaminoglycans (GAGs) within the lysosomes. Patients with the severe form of the disease (Hurler syndrome) have progressive central nervous system (CNS) deterioration, and symptoms that include mental dullness and hypoactivity [1].

The MPS I animal models are a remarkable tool to study disease pathogenesis and treatment options [2, 3, 4]. Behavioral studies have been performed in two MPS I mouse models, the Neufeld model [2] and the Clarke model [5]. Both were created by disruption of exon 6 of the mouse *Idua* gene, resulting in mice with the severe form of the disease. Previous studies in these mice have shown impairment in both aversive and non-aversive memory tasks [6, 7] and acoustic startle behaviors [8]. These mice also show cognitive dysfunction evaluated by the Morris water maze test in some [9] but not all [8] studies. Nevertheless, important aspects of brain function such as motor activity and anxiety showed conflicting results in these studies [6, 8].

In addition, previous work reported activation of glial cells in the cortex [2] and storage of undegraded material, which include glycosaminoglycans and GM3 ganglioside [10]. Also, it is possible that oxidative stress may play a role in the process [11]. However, the mechanism of neurological deficit in MPS I mice are not fully understood.

A better understanding of the abnormalities in these mice and the age of onset of the clinical manifestations is imperative to study mechanisms by which disease occurs as well as to evaluate treatments. Therefore we conducted a study in MPS I mice from 2 to 8 months to evaluate if they develop motor deficits and anxiety and, if so, at what age these alterations can be detectable.

2. Materials and Methods

2.1 Animals

All animal studies were approved by our local ethics committee and complied with National Guidelines on Animal Care. MPS I mice on a C57BL/6 background (kindly donated by Dr Elizabeth Neufeld, UCLA) were used [2]. The MPS I mice carry a disrupted version of the *Idua* gene that contains the neomycin resistance gene inserted in the opposite orientation in exon 6 and could be detected by PCR after DNA extraction from ear tissue, using the following primers: Forward 5′-GAGACTTGGAATGAACCAGAC-3′ and ReverseA 5′-ATAGGGGTATCCTTGAACTC-3′ and ReverseB 5′-GTTCTTCTGAGGGGATCGG-3′. Normal mice amplify a sequence of 514 base pairs (bp) and MPS I mice amplify a 321 bp fragment. Heterozygous mice were used for breeding.

Male Idua−/− mice (referred to as the MPS I group) and their littermate controls (Idua−/+ and Idua+/+, referred to as the Normal group) were the subjects for these experiments. Heterozygous mice had no differences in behavior studies compared to Idua+/+ mice (supplementary figure 1). At 3 weeks of age, offspring were separated from the dam, genotyped and housed (2-5 per cage) by gender. Animals were maintained in conventional housing under a 12 h light/12 h dark cycle with controlled temperature (19 ± 1°C) and humidity (50 ± 10%). All male mice born from January 2009 to March 2010 were submitted to behavioral tests blindly for genotype. Behavioral tests were conducted every other month for 4 test periods beginning at 2 months of age. The number of mice in each test and at each time point varied due to death (because of MPS I disease) sacrifice (for other purposes, such

as histological analysis) or availability of equipments, and it is indicated in each figure. Some mice were analyzed at more than one time point, while others were analyzed only once. No differences were found between mice that were tested at more than 1 time point from those that were submitted to a single test (data not shown). Mice from the same litter were analyzed at the same day. At each time point, each test was performed with a 24h interval from the previous one, in the following order: open field, elevated plus maze, hang wire and gait test. Mice were weighed at 21 days, 2, 4, 6 and 8 months.

2.2 Urinary GAGs measurement

Urine was collected at 1, 2, 4, 6 and 8 months and GAG measurement was performed using the Dimethyl blue assay based on our previous study [12] with small modifications. Briefly, 25 uL of urine was mixed with 2 mL of freshly prepared dimethyl blue solution (Dymethyl blue 0.3 mol/L with 2 mol/L Tris) and absorbance was read at 530 nm. Creatinine was measured spectrophotometrically using the picric acid method [13]. Results were expressed as μg GAGs/mg creatinine.

2.3 Elevated plus maze

Anxiety was analyzed by the elevated plus maze test [14]. It consisted of two open arms and two enclosed arms, 65 cm length × 5 cm wide and walls of 15 cm height with an open roof, assayed so that the two arms were opposite to each other, resulting in a plus shape. The maze was elevated to a height of 50 cm and mice were placed in the center of the maze, facing one of the open arms. Percent time spent on open arms (time spent in open arm/test duration) X 100 and percent open arm entries (open arm entries/total number of entries) X 100 were calculated. An arm entry was defined as presence of all four feet of the animal into one arm [14]. The apparatus was thoroughly cleaned with 70% ethanol after removal of each mouse. In this test, animals with a reduction in anxiety spend more time on the open (unprotected) arms.

2.4 Open field test

Locomotor and exploratory activities were assessed using an open field test [6]. The test consisted of a square arena (52 × 52 cm) with 60 cm high walls. The floor was divided into 16 squares by parallel and intersecting lines, obtaining four centered squares and 12 peripheral squares. Mice were placed in one of the corners of the open field and (a) crossings (total number of times the animal crossed a line with the 4 paws), and (b) exploratory behavior (rearings) were observed during 5 min for both control and MPS I animals. The percentage of crossings in the center squares compared to the total crossings and number of fecal pellets were also analyzed as additional tests for anxiety.

2.5 Gait analysis

Hind-limb gait was determined with a footprint test, as previously described for the MPS IIIA mice [15]. Animals had their paws painted with non-toxic ink and were placed at the beginning of a well-lit runway approximately 50 cm long and 12 cm wide, with walls of 15 cm height, and allowed to move towards the end of the runway. Once dried, the average distance between footprints was determined for both left and right hind-limbs and gait length for each limb was determined. In addition, a line perpendicular to the direction of movement from one foot to the other (supplementary figure 2) was drawn, to determine gait width. The average of at least three lengths and five widths was taken for each run. The first and last two strides were discarded due to alterations in speed in the beginning and the end of the runway.

2.6 Hang wire test

This test was used as a measure of neuromuscular strength. Animals were placed by the forelimbs on a stainless steel bar (30 cm length, 2 mm in diameter, and elevated 30 cm from the surface) at a point midway between the supports and observed for 60 s. Three consecutive trials were performed, with a 60s interval between trials. The amount of time spent hanging was recorded and scored from 1 to 5 (5 as the best) according to the following scheme: 1, hung onto the bar with two forepaws; 2, in addition to 1, attempted to climb onto the bar; 3, hung onto the bar with two forepaws and one or both hind paws; 4, hung onto the bar with all four paws with tail wrapped around the bar; 5, escaped to one of the supports located on the edges of the apparatus [16, 17].

2.7 Histological analysis

Two and eight-month normal and MPS I mice (n=3-5 each group) were anesthetized with ketamine and xylazine and perfused with phosphate buffer saline solution. Brains were collected; the cortex and cerebellum were isolated and fixed in buffered formalin. Thin cross sections (4 μm) were submitted to routine histological processing, stained with toluidine blue and analyzed for storage in Purkinje cells (cerebellum), cortical neurons and glial cells (cortex). Immunohistochemistry for glial fibrillary acidic protein (GFAP) was performed using specific antibody (Dako Cytomation, Polyclonal Rabbit anti-GFAP) and a secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase. Slides were analyzed by a researcher blinded to the groups, counting positive cells in 5 high-power fields (40X). TUNEL staining was performed to analyze cell death using the kit ApopTag® (Millipore, USA) according to fabricant instructions. Sections were counterstained wit hematoxylin. A small intestine biopsy was used as a positive control.

2.8 Statistical Analysis

All values were expressed as mean ± SD. Behavioral data was analyzed using Student's *t*-test; significance level was chosen at $P < 0.05$. All statistical analyses were carried out by using SigmaStat software version 11.0.

3. Results

3.1 General aspects

The MPS I mice showed no abnormalities at birth. From the fourth week on, the facial bones started to appear abnormal, with the MPS I mice having a broader face than the normal mice, Although the body weight was not different from normal mice at weaning (day 21), the MPS I mice weighed significantly more than the normal mice from 2 months on (Supplemetary figure 3A). GAG excretion was significantly higher in the urine from MPS I mice by 1 month of age (Supplemetary figure 3B). From 4 months on, MPS I mice started to become hypoactive and docile (showing no aggressive behavior), being easier to catch in the cage, compared to normal mice.

3.2 Anxiety tests

We could not observe differences between normal and MPS I mice in any of the anxiety tests that were performed, which included the elevated plus maze test (figure 1), the number of crossings to the central squares and the number of fecal pellets on the open field test (data not shown).

3.3 Open field test

MPS I and normal mice had no detectable differences at 2 months in an open field test. However, the number of crossings and rearings at 4 months in *Idua* knockout mice were

reduced by 33% and 25%, respectively compared with normal mice ($p<0.05$). The reduction in both parameters was progressive and were reduced 53% and 59% at 8 months ($p<0.01$, figure 2).

3.4 Hang wire

Mice were evaluated in the hang wire test based on the time spent hanging on the wire as well as how they performed the test, using a score previously published [17]. Alterations in the time spent on the wire could be observed in MPS I mice at the second and third trials performed at 4 months as well as at most trials at 6 and 8 months, as shown in figure 3. MPS I mice usually fell because of difficulties in hanging onto the wire, while the few normal mice that fell, usually did in attempting to climb to one of the edges (supplementary videos 1 and 2). Therefore, we decided to score the performance in some animals, based on a previous report [17]. When the score (ranked from 1 to 5, with 5 as the best) was analyzed, we could observe consistent differences from 6 months-old. At this time, normal mice had an average score of 4.2 ± 0.6 on the first trial, and 3.7 ± 0.9 on the third one. Both were superior to scores obtained by MPS I mice (3.2 ± 1.3, $p<0.05$ and 1.5 ± 0.8 $p<0.05$ respectively). Those differences were even more accentuated at 8 months (4.0 ± 0.7 and 3.8 ± 0.7 for Normal vs 2.2 ± 1.7 and 1.3 ± 0.7 for MPS I in the first and third trials, with $p <0.05$). At 6 and 8 months, we could observe that both MPS I and normal mice appear to fatigue with the repeat testing, spending less time hanging on the wire in the third trial ($p =0.09$ for Normal and $p=0.01$ for MPS I at 6 months and $p=0.01$ for both Normal and MPS I mice at 8 months).

3.5 Gait analysis

The average gait length and width are shown in figure 4. MPS I mice had no alterations in gait width at any of the ages analyzed. However, a reduction in gait length was observed in MPS I mice at 8 months ($p<0.01$). Independent analysis from left and right paws showed similar results (reduction in 19% and 15% of gait length, respectively), and thus confirmed the consistency of the analysis.

3.6 Histological analysis

Since differences were noted in all motor tests at 8-months, animals were sacrificed at this age, and cortex and cerebellum were isolated and processed for histological analysis. Toluidine blue staining revealed storage of undegraded GAGs in Purkinje cells, shown as white vacuoles inside the cytoplasm (Supplementary figure 4B), and also on neurons and glial cells in the cortex (Supplementary figure 4D). Normal mice did not have those features (Supplemetary figures 4A and C). TUNEL staining was negative in normal and MPS I mice (figure 5), suggesting that there is no significant apoptosis in the brain.

Some mice were sacrificed at 2-months to evaluate GFAP content. At this time MPS I mice had 1.4-fold as many GFAP-positive cells in cerebellum as did normal mice, ($p= 0.16$) and 2-fold as many positive cells in the cortex ($p<0.05$). At 8 months, the values were higher at 2.2-fold normal in the cerebellum and 5.7-fold normal in the cortex ($p<0.05$ in both cases). Positive cells were located throughout the brain structures analyzed, indicating activation of glial cells and neuroinflammation in both cortex and cerebellum, which seems to get more accentuated as the disease progresses (figure 6).

4. Discussion

Previous studies have shown behavioral abnormalities in MPS I mice, with special emphasis on memory impairment [6, 7, 8]. However, characterization of the motor deficits and anxiety in this model has shown contradictory results. The present study sought to expand upon

these initial observations describing motor dysfunction in the MPS I model, trying to establish the time-course of the development of these abnormalities.

The open field test was used here as a measure of locomotor and exploratory activities. In the study published by Reolon *et al* [6] in the Neufeld model, MPS I mice analyzed at 5 to 7 months of age only showed differences in rearing. On the other hand a study performed by Pan *et al* [8] in the Clarke model described that MPS I mice were hypoactive from early stages in life, being noticed as early as 2 months. We were able to clearly demonstrate that MPS I mice are less active than normal mice from 4 months-old onwards, evidencing a motor dysfunction in MPS I mice. The difference found between our results and the previous work on the same animal model [6] might be due to differences in the size of the apparatus, since we used a bigger arena. However our results are similar to those found by Pan in the Clarke model [8]. The only discrepancy between the results is the time when the abnormalities could be detected (2 months in the previous study versus 4 months in our study) and it might be related to the higher number of animals analyzed in the previous work.

The hang wire test is not only a measure of neuromuscular strength and fine motor skills, but also of cerebellar function [16]. The MPS I mice showed difficulty in holding onto the wire and fell much more quickly from 6 months onwards, although some differences were noted at 4 months. Alterations in gait are usually associated with abnormalities in cerebellar function [16], although they could also reflect skeletal abnormalities. Gait analysis was abnormal at 8 months, which is about the time MPS I mice start to die. This is an interesting finding, since cerebellar function has never been studied in severe MPS I patients, although other lysosomal disorders are known to develop cerebellum-related problems [1]. Possibly other brain abnormalities such as mental retardation may underlie the ataxia phenotype and/or the patients die before developing it. As improvements verified after enzyme replacement therapy and hematopoietic stem cell transplantation are leading patients to better quality of life [18] and a possible increase in life spam [19], it is possible that impairment in cerebellar function may develop in these patients and could be clinically relevant.

Previous studies reported motor abnormalities in MPS IIIA mice [13, 20, 21], a disease resulting from sulfaminidase (EC 3.10.1.1) deficiency, which blocks exclusively the degradation of heparan sulphate. Two of these studies showed that MPS IIIA mice exhibit hyperactivity [13, 21], while a third study [20] reported hypoactive behavior. MPS IIIA patients usually develop hyperactive behavior and then progress to a vegetative state, which is not found in MPS I patients or mice. Whether this difference is due to the presence of undegraded dermatan sulphate in MPS I or because of the step at which heparan sulphate degradation is blocked still remains unknown [22].

Post-mortem studies in MPS I mice have shown widespread accumulation of undegraded GAGs in the brain [23]. Here, we confirm those findings, showing storage in the cortex and cerebellum, two brain regions responsible for motor function. In addition, increased expression of GFAP in these areas evidences a neuroinflammatory process which seem to progress with time and can contribute to the neurological deficit observed [24]. The activation of glial cells has been previously shown in the MPS I mouse cortex [2], but this was never described to occur in the cerebellum. However, storage and inflammation are not leading to cell death, as shown by TUNEL staining. Therefore, the behavioral deficits found in MPS I mice seem not to be due to cell death, but more likely to happen because of a neuronal dysfunction. These results raise the question whether blocking inflammation could prevent or delay SNC disease in MPS I mice and humans, as recently described for MPS IIIA mice [25].

In summary, these findings and the behavioral analysis suggest that motor deficits are present in this animal model, while anxiety is not altered. It is worth noticing that MPS I mice develop a progressive multisystemic disease which includes bone and joint alterations [23, 26], and that could be influencing motor tasks. However, the battery of tests applied here strongly suggest that MPS I mice have impaired motor skills. Hopefully these tests can provide additional methods for measuring functional improvement after administration of novel therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MPS	mucopolysaccharidosis
IDUA	alpha-L-iduronidase
GAG	glycosaminoglycans
GFAP	glial fibrilary acid protein
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

6. References

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Highlights

- Mucopolysaccharidosis type I (MPS I) mice develop impaired motor skills.
- MPS I mice have storage of undegraded material in neurons and glial cells.
- Neurological deficits are not due to cell apoptosis.
- Neuroinflammation was observed, raising the possibility of neuronal dysfunction.

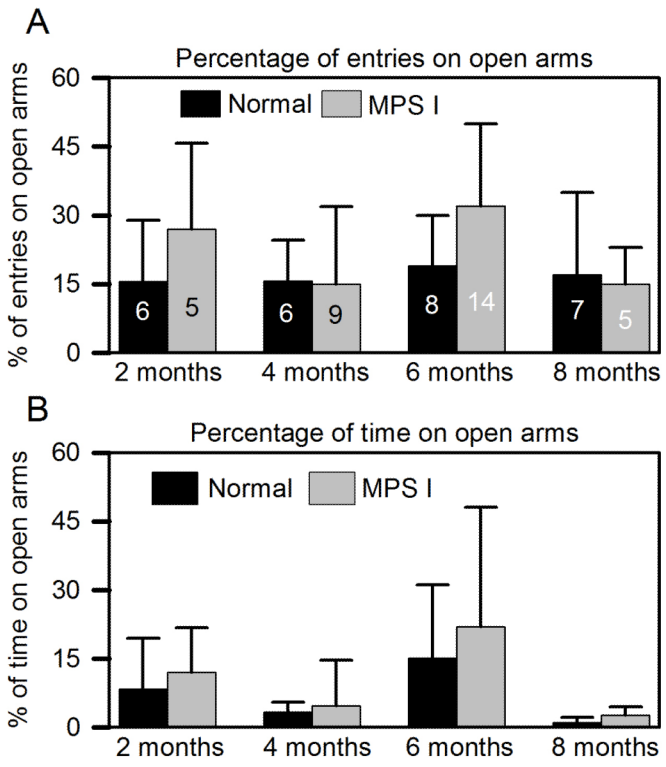

Figure 1.

Plus maze test. Mice were placed in the center of an apparatus which had 2 arms with walls (protected environment) and 2 arms without walls (unprotected environment), to evaluate anxiety. A) The percentage of entries in the open arms and B) the percentage of time spent in the open arms were recorded during 5 minutes. No differences were observed between normal and MPS I mice from 2 to 8 months. The number of mice at each time point is indicated in the bars.

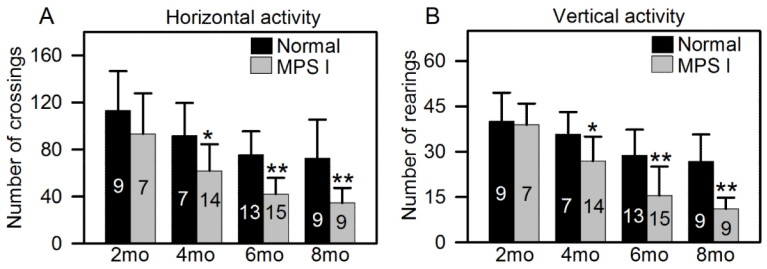

Figure 2.

Results from open field test. MPS I and normal mice were submitted to the test for 5 minutes. A) The number of crossings from one region to another is a measure of locomotor activity (panel A), and the number of rearings (panel B) is a measure of exploratory behavior. Groups were compared at each age. The number of animals can be seen in the bars. Statistical analysis was performed using Student's t test. *$p<0.05$ and ** $p<0.01$. Abbreviations: mo-months.

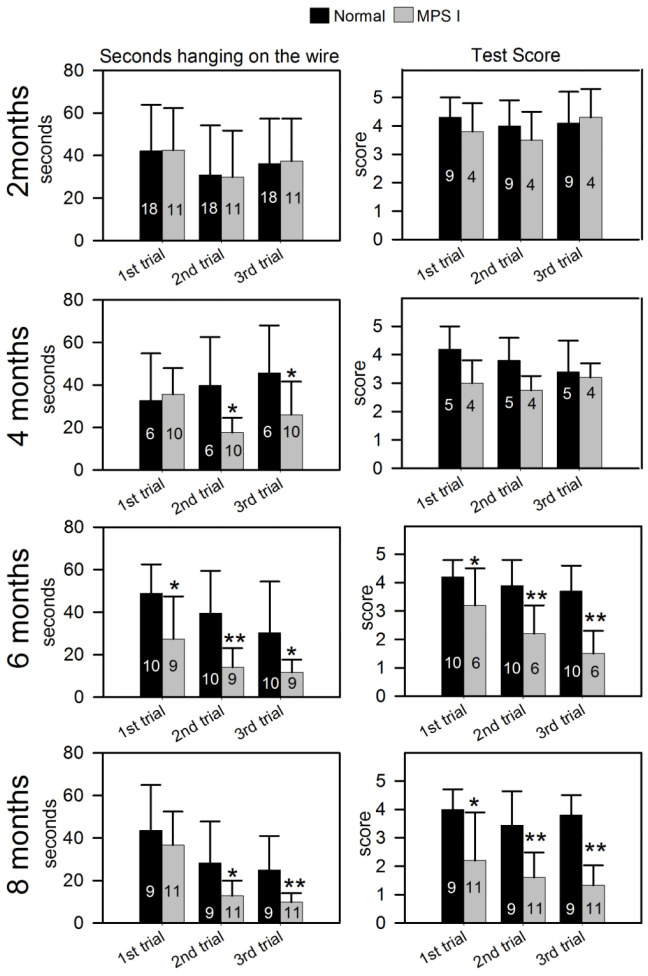

Figure 3.

Results from hang wire test. This test was used to evaluate motor coordination and muscular strength. The animals were placed in a suspended wire, and observation was carried for up to 60 seconds, in 3 consecutive trials analyzing A) time spent hanging on the wire and B) scoring the performance (score from 1 to 5, with 1, hung onto the bar with two forepaws; 2, in addition to 1, attempted to climb onto the bar; 3, hung onto the bar with two forepaws and one or both hind paws; 4, hung onto the bar with all four paws with tail wrapped around the bar; 5, escaped to one of the supports). The number of mice analyzed at each time point is indicated in the bars. *$p<0.05$, Student's t test.

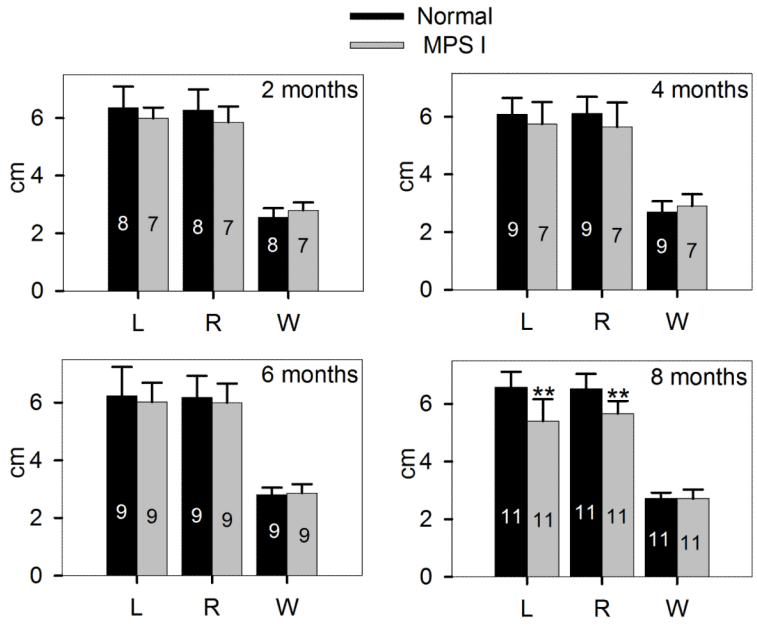

Figure 4.

Gait analysis was performed. The animals had their paws painted with ink and were allowed to walk on a runway with a blank paper on the floor. The length of their right (R) and left (L) steps and the width (W) were measured as described in the materials and methods session. The number of animals at each time point is indicated in the bars. Statistics were performed by Student's t test. **p<0.01 compared to normal.

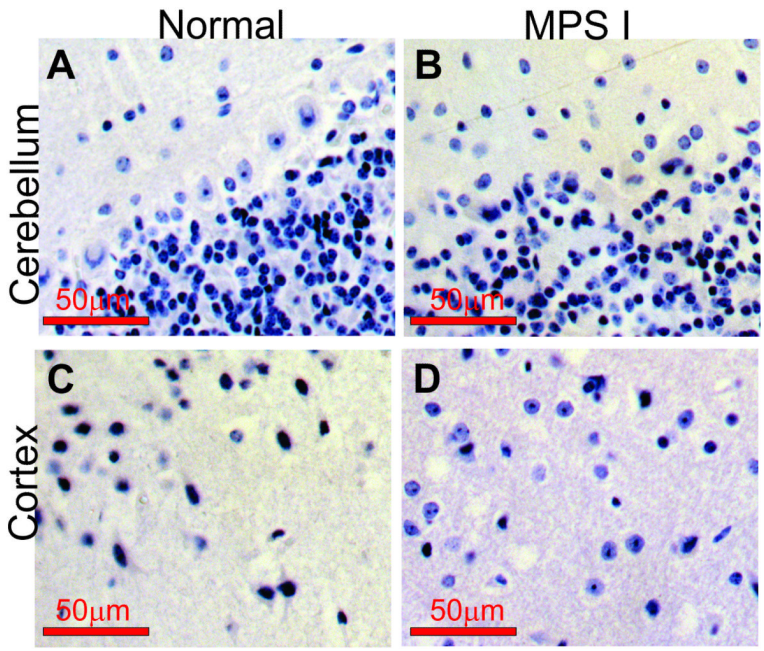

Figure 5.
TUNEL staining in 8-month old mice. Sections were tested for TUNEL staining. A) Normal mouse cerebellum. B) MPS I mouse cerebellum. C) Normal mouse cortex. D) MPS I mouse cortex. There was no evidence of TUNEL positive cells in the groups analyzed. Positive control for the TUNEL stain can be seen in the supplementary files.

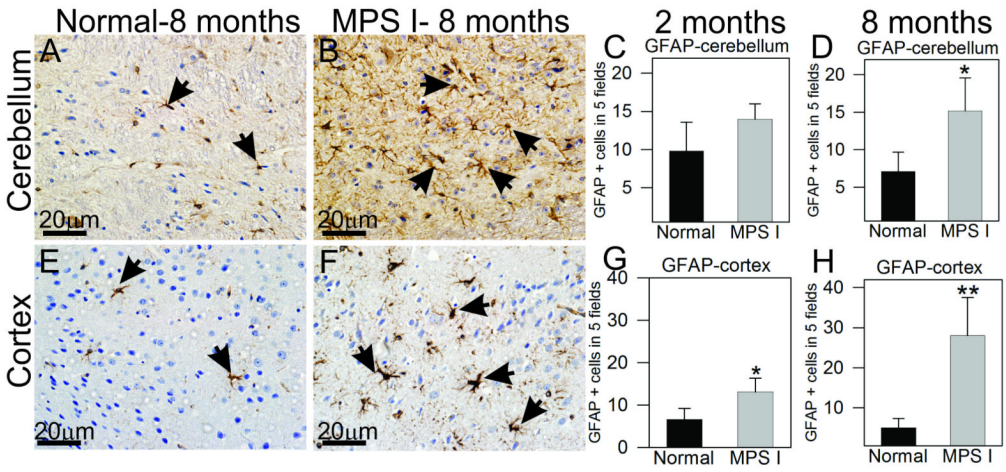

Figure 6.

Glial fibrilary acidic protein (GFAP) immunohistochemistry. Representative sections from 8-month normal (A and E) and MPS I (B and F) mouse brains. Number of positive cells was counted in 5 fields (40X magnification) in mouse cerebellum and cortex at 2 months (panel C and G) and 8 months (D and H). Arrows indicate some cells positive for GFAP immunohistochemistry, evidencing glial activation in MPS I mice. * $p < 0.05$; ** $p < 0.01$ Student t test. N= 3-5/group.