Progressive neurologic and somatic disease in a novel mouse model of human mucopolysaccharidosis type IIIC

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ABSTRACT

Mucopolysaccharidosis type IIIC (MPSIIIC) is a severe lysosomal storage disease caused by deficiency in activity of the transmembrane enzyme heparan-α-glucosaminide N-acetyltransferase (HGSNAT) that catalyses the N-acetylation of α-glucosamine residues of heparan sulfate. Enzyme deficiency causes abnormal substrate accumulation in lysosomes, leading to progressive and severe neurodegeneration, somatic pathology and early death. There is no cure for MPSIIIC, and development of new therapies is challenging because of the unfeasibility of cross-correction. In this study, we generated a new mouse model of MPSIIIC by targeted disruption of the Hgsnat gene. Successful targeting left LacZ expression under control of the Hgsnat promoter, allowing investigation into sites of endogenous expression, which was particularly prominent in the CNS, but was also detectable in peripheral organs. Signs of CNS storage pathology, including glycosaminoglycan accumulation, lysosomal distension, lysosomal dysfunction and neuroinflammation were detected in 2-month-old animals and progressed with age. Glycosaminoglycan accumulation and ultrastructural changes were also observed in most somatic organs, but lysosomal pathology seemed most severe in liver. Furthermore, HGSNAT-deficient mice had altered locomotor and exploratory activity and shortened lifespan. Hence, this animal model recapitulates human MPSIIIC and provides a useful tool for the study of disease physiopathology and the development of new therapeutic approaches.

KEY WORDS: Lysosomal storage disease, MPSIIIC, HGSNAT, Animal model, Neurodegeneration

INTRODUCTION

Mucopolysaccharidosis type III, or Sanfilippo syndrome, encompasses a group of rare lysosomal storage diseases (LSDs) inherited in an autosomal recessive manner and caused by mutations in any of the enzymes specifically involved in the catabolism of the glycosaminoglycan (GAG) heparan sulfate (HS) (Valstar et al., 2008), leading to the accumulation of HS in lysosomes of cells of all tissues and organs. Sanfilippo syndrome is classified into four different subtypes based on the deficient enzyme: MPSIIIA (OMIM#252900) is caused by N-sulfoglucosamine sulfohydrolase (EC 3.10.1.1) deficiency (Kresse and Neufeld, 1972); MPSIIIB (OMIM#252920) by α-N-acetylgalactosaminidase (EC 3.2.1.78) deficiency (figura and Kresse, 1972); MPSIIIC (OMIM#252930) by heparan-α-glucosaminidase N-acetyltransferase (HGSNAT, EC 2.3.1.78) deficiency (Klein et al., 1978); and MPSIIID (OMIM#252940) by the result of the N-acetyl-glucosamine-6-sulfatase (EC 3.1.6.14) deficiency (Kresse et al., 1980). MPSIIIA is the most common type of Sanfilippo syndrome in north-west Europe and Australia, and type B is the most frequent type in south-east Europe and Brazil, whereas MPSIIIC and D seem to be much rarer (Coelho et al., 1997; Meikle et al., 1999; Valstar et al., 2008).

All subtypes of Sanfilippo syndrome show similar clinical symptoms, and manifest primarily as progressive neurodegenerative diseases, but MPSIIIC seems to have a milder clinical course although the severity of the disease is highly variable, even within families (Delgadoillo et al., 2013; Héron et al., 2011; Ruijter et al., 2008; van de Kamp et al., 1981). After a few months of normal growth, developmental delay and behavioral problems appear as the first signs and symptoms in affected individuals (Héron et al., 2011; Malm and Månsson, 2010; Ruijter et al., 2008). Later on, the disease slowly progresses to neurological deterioration characterized by loss of speech that precedes loss of motor function (Delgadoillo et al., 2013; Héron et al., 2011; Malm and Månsson, 2010; Ruijter et al., 2008). During this period, affected individuals show severe behavioral problems with hyperactivity that worsens as a result of sleep disturbances (Delgadoillo et al., 2013; Malm and Månsson, 2010; Ruijter et al., 2008). Somatic disease in MPSIIIC, although mild, is characterized by a wide range of clinical manifestations such as recurrent ear-nose-throat infections, diarrhea, mild facial dysmorphisms and visceral organomegaly, such as mild hepatomegaly (Delgadoillo et al., 2013; Héron et al., 2011; Ruijter et al., 2008). The largest study on a cohort of individuals with MPSIIIC performed to date was carried out in the Netherlands and showed longer survival for Sanfilippo syndrome subtype C than for subtypes A or B, with death occurring at a median age of 34 years, ranging from 25 to 48 (Ruijter et al., 2008). These data are supported by another study in a smaller cohort from France in which individuals with MPSIIIC lived significantly longer than individuals affected by MPSIII A or MPSIII B (Héron et al., 2011). Currently there is no cure for MPSIIIC and existing treatments are aimed solely at controlling the symptoms of the disease in order to improve the quality of life of patients and their families.
HGSNAT is a lysosomal transmembrane enzyme whose function is to N-acetylate the terminal α-glucosamine residues of intralysosomal HS prior to its hydrolysis by α-N-acetylglucosaminidase (Klein et al., 1978). The HGSNAT gene is composed of 18 exons and is located on chromosome 8p11.1 (Fan et al., 2006; Hřebíček et al., 2006). The cDNA encodes a product of 635 amino acids in length, which contains 11 transmembrane domains, up to five consensus asparagine-linked glycosylation sites, a cleavable N-terminal signal peptide of 30 amino acids, and a short eight-amino acid C-terminal domain facing the cytosol (Fan et al., 2006, 2011; Hřebíček et al., 2006). At present, 66 HGSNAT mutations have been identified, which include 37 missense mutations, 14 splicing mutations, five small deletions, two gross deletions, five small insertions, one small indel mutation, one gross insertion and/or duplication and one complex rearrangement (https://portal.biobase-international.com/hgmd/pro/gene.php?gene=HGSNAT), and some rare polymorphisms that have no effect on the activity, processing and targeting of the enzyme (Canals et al., 2011; Feldhammer et al., 2009a). Although the spectrum of mutations in individuals with MPSIIIC shows high heterogeneity, some of the identified mutations have high frequency within certain populations, such as p.R344C and p.S518F in the Netherlands (Ruijter et al., 2008), c.S25dupT in Portugal (Coutinho et al., 2008; Feldhammer et al., 2009b), c.852–1G>A in Spanish (Canals et al., 2011) and c.234+1G>A in Spanish and Moroccan individuals with MPSIIIC (Canals et al., 2011; Hřebíček et al., 2006; Ruijter et al., 2008), suggesting geographical founder effects. Other mutations, e.g. p.R384*, c.23411G>A, c.49311G>A, p.R344H and p.S541L, show a relatively high incidence among individuals with MPSIIIC from different geographical origins, suggesting a founder effect of an ancient mutation (Fan et al., 2006; Fedele et al., 2007; Feldhammer et al., 2009b; Hřebíček et al., 2006). Despite the identification of several MPSIIIC-causing mutations, it has proven difficult to establish a clear genotype-phenotype correlation, except e.g. for mutations p.G262R and p.S539C from the majority of individuals with MPSIIIC are affected by at least one missense mutation in or adjacent to transmembrane domains of HGSNAT, interfering with the proper folding of the enzyme (Feldhammer et al., 2009a,b).

Several naturally occurring or engineered small and large animal models of Sanfilippo syndrome have been described in the past few years (Bbaumik et al., 1999; Ellinwood et al., 2003; Fischer et al., 1998; Li et al., 1999; Thompson et al., 1992; Yogalingam et al., 2002), including a rodent model of MPSIIIC (Martins et al., 2015). These animals have proven invaluable to study the ethiopathology of MPSIII, and have been used to establish proof-of-concept for the development of new therapeutic approaches (Crawley et al., 2011; Duncan et al., 2015; Ellinwood et al., 2011; Fu et al., 2011; Haugrigot et al., 2013; Langford-Smith et al., 2012; Murrey et al., 2014; Ribera et al., 2015; Sorrentino et al., 2013). Herein, we report a novel murine model of MPSIIIC that recapitulates the human disease at biochemical, histological and functional level, with disease affecting not only the brain but also somatic organs.

RESULTS

Generation of Hgsnat knockout mouse to develop an animal model for MPSIIIC

Embryonic stem cells (ESCs) with targeted disruption of the Hgsnat gene were obtained and microinjected into C57BL/6J blastocysts using standard methods. In these cells, successful homologous recombination of the targeting construct results in expression of the LacZ gene encoding bacterial β-galactosidase under the control of the endogenous Hgsnat promoter from the targeted alleles (Fig. S1). Correct targeting was confirmed by Southern blot (data not shown) and by specific PCR that yielded different band patterns for wild-type (WT) (Hgsnat/−/−), heterozygous (Hgsnat/−/+ ) or homozygous (Hgsnat/++ ) knockout mice (Fig. 1A). There was no indication of embryonic lethality and at birth HGSNAT-deficient mice were viable and completely indistinguishable from WT and heterozygous littermates. No statistical differences were detected in the body weight at any of the ages analyzed (Fig. S2).

No Hgsnat transcripts could be detected in the kidney of homozygous Hgsnat/−/− male mice by northern blot analysis with a probe specific for murine Hgsnat (Fig. 1B). This observation was later confirmed by qRT-PCR in samples obtained from brain and liver (Fig. 1C). Accordingly, HGSNAT enzymatic activity was practically undetectable in brain and liver of Hgsnat/−/− mice (Fig. 1D). Heterozygous mice showed reduced Hgsnat expression and HGSNAT activity (Fig. 1B-D). This lower than normal enzymatic activity in Hgsnat/−/− mice did not alter the content of GAGs in tissues (data not shown), indicating that half of normal HGSNAT activity is enough to support normal catabolism of GAGs. Similar results were obtained in females (Fig. S5A,B).

Fig. 1. Generation of Hgsnat knockout mouse. (A) Genotyping of wild-type (WT), heterozygous (Hgsnat/−/+ ) and homozygous (Hgsnat/++ ) mice was performed by PCR analysis of genomic DNA. The location within the gene locus of the primers used is indicated in Fig. S1. The bands of 395 and 329 bp correspond to WT and mutant alleles, respectively. (B) Representative northern blot performed on total RNA extracted from the kidney of WT, Hgsnat/−/− and Hgsnat/−/+ male mice and hybridized with a probe specific for Hgsnat mRNA. (C) Hgsnat mRNA expression analyzed by qRT-PCR in brain and liver of the same cohorts with primers and probes as listed in Materials and Methods; n=4 animals/group. AU, arbitrary units. (D) HGSNAT activity in brain and liver of the same cohorts; n=5-7 animals/group. Results are shown as mean±s.e.m.; ***P<0.001 versus WT by one-way ANOVA followed by Dunnett post-test.
Taken together these results demonstrated efficient disruption of the Hgsnat gene.

**Study of Hgsnat gene expression in the CNS and somatic organs**

The design of the targeting construct allows for analysis of the sites of endogenous Hgsnat expression through detection of $\beta$-galactosidase activity. *In toto* X-gal staining of the encephalon of Hgsnat$^{-/-}$ mice showed widespread and strong blue signal of the reaction product throughout the forebrain and midbrain (Fig. S3, upper panel and A-C). In general, the pattern of expression was homogeneous and diffuse, e.g. in the neocortex, the piriform lobe and thalamus (Fig. S3A-C), except for the hippocampus in which the signal was particularly intense in CA1 and CA3 cells, with lesser expression detected in the dentate gyrus (Fig. S3B). Lower but detectable levels of LacZ expression were observed in the cerebellum, with Purkinje cells evidencing the most intense signal in this structure (Fig. S3D). The widespread expression of the Hgsnat gene throughout the CNS was in agreement with the fact that the brain is the most affected organ in Sanfilippo syndrome type C (Kurihara et al., 1996; Martin et al., 1979).

We then evaluated *in toto* $\beta$-galactosidase activity in somatic organs (Fig. S4). The vast majority of organs analyzed showed expression of Hgsnat. In the kidney, the cortex stained more than the medulla; in the medullar region, positivity was associated with tubular structures that converged in the renal pelvis, namely papillary ducts (Fig. S4A). Very strong staining was observed in the ureter and, to a lesser degree, in the urinary bladder (Fig. S4A,B). In contrast, the lungs were practically negative, with minimal staining of the lobar bronchus (Fig. S4C). When the whole heart was incubated in X-gal, ventricles showed very weak and diffuse signal whereas atriums were strongly positive, and so was the pulmonary trunk (Fig. S4D). When the heart was sectioned to allow visualization of inner structures, a diffuse staining was observed in the inner surface of the myocardium of the left ventricle, particularly in the papillary muscles (Fig. S4E,F). In the transversal section of the atrium, the tricuspid valve stained strongly positive (Fig. S4G). The staining of the liver was particularly intense in the hepatocytes, with lesser expression detected in the bile ducts (Fig. S4A). The parenchyma of the spleen was negative to X-gal staining, and the only structures that stained positive were identified as arterial blood vessels (Fig. S4K). Similarly, muscle fibers of the gastrocnemius muscle seemed negative for LacZ expression but muscular arterioles were markedly positive (Fig. S4L). When X-gal staining was performed on paraffin-embedded sections of the gastrocnemius muscle, a strong blue signal was observed in the arterial tunica media of these vessels (Fig. S4M). Finally, in the reproductive tract, in females the oviduct stood out as the most positive structure, although the follicles inside the ovary were also fairly positive (Fig. S4N); in testicles, the seminiferous tubules stained weakly with X-gal (Fig. S4O).

**CNS lysosomal pathology in HGSNAT-deficient mice**

Neurological alterations are one of the main features of MPSIIIC disease (Héron et al., 2011; Malm and Månsson, 2010; Ruijter et al., 2008). Therefore, we set out to investigate the presence of lysosomal pathology in the brain of mice with HGSNAT deficiency. Animals of 2, 12 and 22 months of age were analyzed to evaluate the age of onset and the progression of the lysosomal pathology affecting the CNS. HGSNAT-deficient male and female mice showed a progressive increase in GAG accumulation in all brain regions analyzed, ranging from 4% to 80% over values observed in healthy littermates (Fig. 2A; Fig. S5C). In both sexes, the accumulation was particularly noticeable in the most frontal portions of the brain (Sections I-III; Fig. 2A; Fig. S5C), in which the excessive storage of GAG was readily detectable as early as 2 months of age. Consistent with the observed accumulation of GAGs, immunohistochemical analysis of brain sections with an antibody reactive to the lysosomal marker lysosomal associated membrane protein 2 (LAMP2) used as indicator of the size of the lysosomal compartment revealed a statistically significant increase in LAMP2+ area in all regions analyzed in the CNS of both Hgsnat$^{-/-}$ male and female mice (Fig. 2B; Fig. S5D). Furthermore, signal intensity increased with age in both sexes, suggesting the distension of the CNS lysosomal compartment worsened as animals grew.

This result was further confirmed by ultrastructural analysis of the encephalon by transmission electron microscopy, which showed an increase in the size of the lysosomal compartment in the cerebral cortex and cerebellum of HGSNAT-deficient mice (Fig. 3A). At 5 months of age, Hgsnat$^{-/-}$ animals but not WT littermates had large electronlucent vacuoles that seemed to be lysosomes loaded with GAGs in the cytoplasm of perineuronal glial cells opposed to neurons of the cerebral cortex (Fig. 3A, left panels). The juxtaposed neurons had few or no discernable storage lesions at this age (Fig. 3A, left panels). In the cerebellum, electronlucent vesicles of smaller size were detected in the cytoplasm of Purkinje cells in Hgsnat$^{-/-}$ mice (Fig. 3A, right panels). The content of these vesicles seemed different from that observed in cortical perineuronal glial cells (Fig. 3A, right panels). Furthermore, the cellular pathology observed in Purkinje cells from HGSNAT-deficient mice affected not only lysosomes but also mitochondria, which showed significant cristae loss and disorganization of their inner membrane system (Fig. 3B). Taken together, these data demonstrate the presence of progressive storage disease in the CNS of HGSNAT-deficient mice starting from an early age.

**Secondary lysosomal pathology in the CNS of HGSNAT-deficient mice**

In LSDs, the loss of activity of a specific lysosomal enzyme results in the perturbation of normal lysosomal homeostasis, characterized, amongst other changes, by secondary alteration of other enzymatic activities and accumulation of additional compounds (Sardiello et al., 2009). In the brain of HGSNAT-deficient mice, we observed changes in the activities of several lysosomal enzymes, such as IDS, SGSH, NAGLU, GALNS, GUSB and $\beta$-HEXO (Fig. 3C, Fig. S6). These changes were detectable as early as 2 months of age and remained altered in older mice (Fig. 3C; Fig. S6).

**Neuroinflammation in the brain of mice deficient in HGSNAT**

There is plenty of evidence from animal models (Martins et al., 2015; Ohmi et al., 2003; Wilkinson et al., 2012) and humans (Hamano et al., 2008; Kurihara et al., 1996; Tamagawa et al., 1985) indicating that there is activation of glial cells in all forms of Sanfilippo syndrome. To investigate if the deficiency in HGSNAT also caused a neuroinflammatory response in our animal model, we stained brain sections from 2-, 12- and 22-month-old mice with an anti-GFAP antibody that specifically recognizes glial fibrillary acidic protein expressed in activated astrocytes (Eng and Ghirnikar, 1994) and with the lectin BSI-B4, which under our staining protocol binds to activated microglia (Ribera et al., 2015; Streit and Kreutzberg, 1987). At the early age of 2 months, Hgsnat$^{-/-}$ mice of both sexes already showed marked astrocitosis in all brain regions analyzed (Fig. 4A,B; Fig. S7A,B). The increase in GFAP+ signal remained statistically significant at later time points in all
Fig. 2. CNS storage pathology in Hgsnat−/− mice. (A) GAG content in different brain regions (sections I-V illustrated in the diagrams above the plot) in WT and Hgsnat−/− male mice at 2, 12 and 22 months of age; n=4-5 animals/group. Absolute values of CNS GAG content in WT animals are listed in Table S1. (B) Analysis of the lysosomal compartment by LAMP2 immunostaining in different brain regions of the same cohorts of animals as in A. HGSNAT-deficient animals showed a progressive increase in the size of the lysosomal compartment with age. Insets show a magnified region of the main panel. Histograms below the images depict the corresponding quantification of LAMP2+ signal for each brain region in each group; n=4-5 animals/group. Results are shown as mean±s.e.m.; *P<0.05, **P<0.01 and ***P<0.001 versus WT by two-tailed t-test for statistical comparison of HGSNAT-deficient mice with their respective controls. Scale bars: 100 µm; insets, 20 µm.
Fig. 3. Ultrastructural analysis and evaluation of lysosomal homeostasis in the CNS of HGSNAT-deficient mice. (A) Transmission electron microscopy of samples from the frontal cortex (left panels) and cerebellum (right panels) of 5-month-old WT and Hgsnat<sup>−/−</sup> male mice. Large electrondense vacuoles (red arrows) were detected in perineuronal glial cells (1) associated with neurons (2) of the frontal cortex in HGSNAT-deficient male mice, whereas smaller vesicles with seemingly different content were observed in cerebellar Purkinje cells. (B) Representative images of mitochondria found in Purkinje cells of WT and Hgsnat<sup>−/−</sup> mice. The mitochondrion from a Hgsnat<sup>−/−</sup> Purkinje cell shows loss of normal mitochondrial structure and reduced cristae (asterisks). (C) Percentage of WT activity for a range of lysosomal enzymes analyzed in brain extracts from WT and Hgsnat<sup>−/−</sup> male animals at 2, 12, and 22 months. Hgsnat<sup>−/−</sup> mice showed altered activities of iduronate-2-sulfatase (IDS), N-sulphoglucosamine sulphohydrolase (SGSH), α-N-acetylglucosaminidase (NAGLU), N-acetylgalactosamine-6-sulfatase (GALNS), β-glucuronidase (GUSB) and β-hexosaminidase (β-HEXO); n=4-5 animals/group. Absolute values of CNS enzymatic activities in WT animals are listed in Table S2. Results are shown as mean±s.e.m.; *P<0.05, **P<0.01 and ***P<0.001 by two-tailed t-test for statistical comparison of HGSNAT-deficient mice with their respective controls. Scale bars: 2 µm in A, 100 µm in B.
regions, except for the superior colliculus in which significance was lost in very old animals (Fig. 4A,B; Fig. S7A,B). Similarly, BSI-B4 staining evidenced strong infiltration with activated microglia in the brains of Hgsnat−/− male and female mice, starting at 2 months and worsening with age (Fig. 4C,D; Fig. S7C,D). Quantification of signal intensity showed statistically significant increases in the BSI-B4+ area in Hgsnat−/− animals in all brain regions at all ages (Fig. 4C,D; Fig. S7C,D). Moreover, in the case of BSI-B4 staining, the progression with age was much more apparent than for GFAP staining. These results provide further evidence of progressive pathology in the CNS of HGSNAT-deficient mice.

Loss of HGSNAT activity causes somatic pathology in mice

Lack of HGSNAT activity (Fig. 1D; Fig. S5B) led to the accumulation of GAGs in peripheral organs of male and female mice as early as 2 months of age (Fig. 5A; Fig. S8A). The build-up of undegraded GAGs was most noticeable in liver, spleen and kidney (Fig. 5A; Fig. S8A) and, in most organs, was progressive, showing a considerable increase particularly between 2 and 12 months of age (Fig. 5A; Fig. S8A). This excessive somatic accumulation of GAGs was accompanied by an expansion of the lysosomal compartment in male and female Hgsnat−/− mice, as detected by immunostaining of liver, heart, lung and urinary bladder tissue sections with an antibody against the lysosomal marker lysosomal associated membrane protein 1 (LAMP1) (Fig. 5B; Fig. S8B). As observed for GAG content, in both sexes the size of the lysosomal compartment seemed to increase with age.

At ultrastructural level, the pattern of enlargement of the lysosomal compartment in HGSNAT-deficient mice varied depending on the cell type and organ. In the liver, whereas hepatocytes showed a large number of small electrolucent vacuoles in their cytoplasm, Kupffer cells had few but very distended vesicles (Fig. 6A). Conversely, cells from the proximal tubules of the kidney showed vesicles of a size similar to those observed in hepatocytes (Fig. 6A). Ciliated cells from the bronchial tube of the lung and cells interspersed amongst myocardial cells of the cardiac ventricle had relatively large storage vesicles, which did not seem to be present in other cells of the parenchyma of these structures (Fig. 6A). Very similar observations were made in both sexes (Fig. S9). The excessive storage of undegraded substrates can give rise to organomegaly, a common feature of LSDs, including MPSIIIC (Bartsocas et al., 1979; Héron et al., 2011; Huh et al., 2013; Ruijter et al., 2008). Hgsnat−/− mice did not show any organomegaly at 2 months of age, but organ weight increased with age to an evident hepato- and splenomegaly in older mice (Fig. S10A,B). Taken together, these results provide evidence of severe progressive storage pathology in peripheral organs of Hgsnat−/− mice of both sexes.

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**Fig. 4. Astrocytosis and progressive infiltration with activated microglia in the brain of Hgsnat−/− mice.** (A) Representative images of the immunostaining of brain sections with an antibody specific for the astrocyte marker GFAP. WT and Hgsnat−/− male mice were analyzed at 2, 12 and 22 months of age. Insets show a magnified region of the main panel. (B) Histograms represent the percentage GFAP+ area in each brain region analyzed; n=4-5 animals/group. (C) Representative images of the analysis of microgliosis by staining with BSI-B4 lectin in brain sections from the same cohorts. Microgliosis worsened with age in Hgsnat−/− mice. (D) Histograms depict the quantification of BSI-B4+ area in each brain region analyzed; n=3-5 animals/group. Results are shown as mean±s.e.m.; **P<0.01, ***P<0.001 by two-tailed t-test for statistical comparison of HGSNAT-deficient mice with their respective controls. Scale bars: 50 µm; insets, 20 µm. Ctx, Cortex; S., Superior; m, months.
As previously observed in the CNS, the lack of HGSNAT activity in liver of knockout mice resulted in secondary alteration of the activity of lysosomal enzymes involved not only in the degradation of GAGs (i.e. IDUA, IDS, SGSH, NAGLU, GALNS and GUSB) but also in the degradation of other macromolecules (i.e. β-HEXO), indicating a general alteration of normal lysosomal homeostasis (Fig. 6B; Fig. S10C). In most cases the activity of these enzymes was increased by several fold over the levels observed in healthy WT animals from an early age, and the increment was particularly noticeable for β-HEXO (Fig. 6B; Fig. S10C).

Finally, to assess general health status, we measured several biochemical parameters, including glucose, aspartate aminotransferase...
(AST), alanine aminotransferase (ALT), total bilirubin, creatinine and urea in sera obtained from 2-, 12- and 22-month-old animals. Of these, ALT and urea showed statistical differences from the values observed in WT animals (Fig. S10D), suggesting a mild degree of liver and kidney pathology. Taken together, these data demonstrate that Hgsnat−/− mice have established disease in somatic organs as early as 2 months of age, with increased intracellular GAG storage, evident lysosomal distension and perturbation of normal lysosomal homeostasis, all of which could affect organ functionality.

**Behavioral deficits and shortened lifespan in Hgsnat−/− mice**

Behavioral disturbances are a hallmark of progressive neurological diseases such as MPSIIIC (Delgadillo et al., 2013; Héron et al., 2011; Malm and Månsson, 2010; Ruijter et al., 2008). To test if the behavior of Hgsnat−/− mice was altered by a lack of HGSNAT, naïve Hgsnat−/− males of 2, 12 and 22 months of age were subjected to the open field test, which evaluates the general locomotor and exploratory activity of mice in surroundings that are unknown to them (Bailey and Crawley, 2009). At all ages tested,
Hgsnat<sup>−/−</sup> mice crossed fewer lines and travelled less distance than WT littermates during the first three minutes of the recording period, which combined with the observation that Hgsnat<sup>−/−</sup> mice spent more time resting, suggested hypoaactive behavior (Fig. 7A-C). Hgsnat<sup>−/−</sup> mice also took longer to reach the center of the arena, known to be most stressful zone, and did so on fewer occasions, spending more time at the border (Fig. 7D-F). Indeed, at 2 months of age, 28.5% of Hgsnat<sup>−/−</sup> mice failed to enter the center of the arena in the first 3 min of recording, whilst all WT did. At 12 and 22 months the percentage of Hgsnat<sup>−/−</sup> mice that took longer than 3 min to enter the center of the arena for the first time increased to 34% and 68.4%, respectively. Only one of the tested WT animals failed to visit the center during the first 3 min at 12 months of age, increasing to 25% in 22-month old animals. Taken together, these results suggest that Hgsnat<sup>−/−</sup> mice suffer from anxiety. When the data corresponding to the recording over a period of 15 min was analyzed, a few of the statistical differences observed during the first 3 min at 2 and 12 months of age disappeared, including those that are more representative of locomotor activity, such as total resting time. This observation argues in favor of anxiety being a strong component of the behavior observed during the first 3 min of the test. In animals aged 22 months, the observations made at 15 min resembled those made after 3 min of recording, evidencing profound decline in motor functions at this age, which is compatible with late stage neurodegeneration. These behavioral alterations were in line with those previously observed by several other groups in two different mouse models of Sanfilippo disease (Fraldi et al., 2007; Haurigot et al., 2013; Hemsley and Hopwood, 2005; Lau et al., 2008; Li et al., 1999; McIntyre et al., 2010, 2014; Ribera et al., 2015).

Finally, the analysis of survival showed a highly statistically significant reduction in the lifespan of Hgsnat<sup>−/−</sup> male mice, with a median survival of 21.2 months compared with 24.6 months for WT siblings (P<0.0001; Fig. 7G). Similarly, HGSNAT-deficient females were shorter-lived than WT counterparts (19.7 months and 23 months, respectively, P=0.0008; Fig. S11).

**MPSIIIC progresses more slowly than MPSIIIA and MPSIIIB in rodents**

HGSNAT-deficient males (MPSIIIC males) showed behavioral alterations that reproduced the observations we and others have previously reported in two other mouse models of Sanfilippo disease, MPSIIIA and MPSIIIB mice (Fu et al., 2007; Haurigot et al., 2013; Hemsley and Hopwood, 2005; Hemsley et al., 2009; Lau et al., 2008; Li et al., 1999; Ribera et al., 2015). The main difference between the MPSIIIC model and the MPSIIIA and MPSIIIB models was the...
progression of the behavioral disturbances; at 22 months of age the degree of behavioral changes in HGSNAT knockout mice was the same as in 6-month-old MPSIIIA or MPSIIIB mice (Haurigot et al., 2013; Ribera et al., 2015). Similarly, the lifespan of HGSNAT-deficient males and females was shortened but not as dramatically as that of MPSIIIA and MPSIIIB mice housed in the same animal facility (Haurigot et al., 2013; Ribera et al., 2015; Ruzo et al., 2012a,b). To investigate the reasons for the slower progression of neurological disease in the MPSIIIC model, we compared GAG content in different regions of the encephalon of MPSIIIA, MPSIIIB and MPSIIIC male mice of the same age (5-6 months) (Fig. 8A). In all regions analyzed, the accumulation of GAGs was less pronounced in MPSIIIC mice than in MPSIIIA or MPSIIIB mice, a decrease that reached statistical significance in most cases, particularly in the most caudal regions (Sections III, IV and V in Fig. 8A). In agreement with this, lysosomal-integrated membrane protein 2 (LIMP2) immunostaining indicated less expansion of the lysosomal compartment in MPSIIIC mice (Fig. 8B). Interestingly, this milder CNS storage disease observed in MPSIIIC mice reflected in less infiltration by activated microglia in the brain of these animals (Fig. 8C). This was particularly evident when MPSIIIC mice were compared with MPSIIIB mice. It should be noticed that whereas MPSIIIB and MPSIIIC animals are full knockouts, MPSIIIA mice arouse from a spontaneous point mutation and have a small percentage of residual activity (Bhattacharyya et al., 2001; Bhaumik et al., 1999; Haurigot et al., 2013; Ruzo et al., 2012a,b).

**DISCUSSION**

Here we describe a novel animal model with targeted disruption of the Hgsnat gene. To generate this model we used ESC from the European Conditional Mouse Mutagenesis Program (EUCOMM) resource, which has extensively been used to generate hundreds of knockout mouse mutants for analysis of mammalian gene function (Hrabé de Angelis et al., 2015).

HGSNAT-deficient mice showed progressive accumulation of GAGs and expansion of the lysosomal compartment throughout the

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**Fig. 8.** Comparison of GAG accumulation, CNS lysosomal pathology and brain inflammation in different murine models of MPSIII disease. (A) GAG content analyzed in different brain regions (sections I-V illustrated in the diagrams above the plot) from MPSIIIA, MPSIIIB and MPSIIIC mice at 5-6 months of age; n=4-5 animals/group. Absolute values of CNS GAG content in WT animals from different MPSIII colonies are listed in Table S3. (B) Analysis of the size of the lysosomal compartment through LIMP2 immunostaining and quantification of LIMP2+ area in different brain regions in the same cohorts of animals as in A; n=3-5 animals/group. (C) Analysis of microgliosis by staining with lectin BSI-B4 in brain tissue sections obtained from the same experimental groups. Histograms depict the quantification of positive area in each brain region analyzed; n=3-5 animals/group. Results are shown as means±s.e.m.; *P<0.05, **P<0.01 and ***P<0.001 by one-way ANOVA followed by Tukey post-test.
enencephalon. At ultrastructural level, and in agreement with observations made in MPSIIIA and MPSIIIB models (Bhaumik et al., 1999; Haurigot et al., 2013; Jolly et al., 2007; Ribera et al., 2015; Ruzo et al., 2012a), cortical neurons showed minimal signs of storage whereas perineuronal glial cells juxtaposed to them had large intracytoplasmic vacuoles. Purkinje neurons showed vacuoles of smaller size in their somas, filled with material of different aspect from that observed in cortical perineuronal glial cells. Post-mortem analyses of human and animal brains have shown secondary storage in LSDs; GM2 and GM3 gangliosides accumulate, for example, in MPSIII (Constantopoulos and Dekaban, 1978; Constantopoulos et al., 1980; Tamagawa et al., 1985). Storage of these compounds in cellular compartments different from those that accumulate GAGs, and sometimes even in different cells types (Jolly et al., 2007; Martins et al., 2015; McGlynn et al., 2004), could account for the dissimilar aspect of the storage vesicles found in glia and neurons. In addition, a proportion of the mitochondria of Purkinje cells showed structural abnormalities. The nature of mitochondrial alterations is not completely understood but a recent study has postulated that pathological GM storage in these organelles might be involved (Martins et al., 2015).

The changes in the activity of lysosomal hydrolases reflected the perturbation of normal lysosomal physiology by pathological storage of GAGs, in marked similarity to what we previously observed in MPSIIIB rodent brains (Ribera et al., 2015). This boost in lysosomal function is believed to be the consequence of the activation of the transcription factor EB (TFEB), a master regulator of lysosomal physiology, in an attempt to counteract pathological storage (Sardiello et al., 2009). In LSDs, TFEB translocates to the nucleus and binds to the coordinated lysosomal expression and regulation (CLEAR) element in the promoter of a number of genes that control lysosomal biogenesis and enzymatic activities (Ribera et al., 2015; Sardiello et al., 2009; Settembre et al., 2013). In contrast to this coordinated upregulation of lysosomal hydrolases, the activity of GALNS was decreased in the brain of HGSNAT-deficient mice, possibly resulting from direct inhibition by accumulated compounds. In vitro studies have suggested that GALNS is inhibited by HS (Rowan et al., 2013), and keratan sulfate, one of the substrates of GALNS, is increased in the serum of Sanfilippo animal models (Rowan et al., 2013) and humans with Sanfilippo syndrome (Tomatsu et al., 2005).

The brains of HGSNAT-deficient mice also showed astrocytosis and microgliosis from an early age. This glial response, a hallmark of human Sanfilippo syndrome (Hamano et al., 2008; Kurihara et al., 1996; Tamagawa et al., 1985), is believed to be at the epicenter of disease pathology. In particular, microgliosis, which can be detected as soon as 10 days after birth in MPSIII mice (Ausseil et al., 2008), seems to participate in neurodegeneration through multiple pathological mechanisms including, but not limited to, the secretion of cytokines and the production of reactive oxygen species (Archer et al., 2014; Ausseil et al., 2008; Hamano et al., 2008; Villani et al., 2007; Wilkinson et al., 2012).

At the somatic level, we found hepatomegaly and progressive accumulation of GAGs in all organs analyzed from HGSNAT-deficient mice, but most noticeably in liver, spleen and kidney as well as biochemical signs of modest liver and kidney damage. At all ages, GAG accumulation in the liver and spleen seemed to be slightly less pronounced in females, which otherwise showed very similar CNS and somatic phenotypes to HGSNAT-deficient males. The alteration of lysosomal hydrolases was even more pronounced in liver than in brain, but followed the same pattern. At the ultrastructural level, storage lesions seemed to affect specific cell types within each organ. For example, ciliated cells in the bronchus and cells interspersed in the myocardium had large quantities of cytoplasmic storage vesicles but club cells or cardiomyocytes did not.

HGSNAT-deficient males showed behavioral alterations characterized by anxiety in younger animals and hypoactivity in later stages of the disease. These observations reproduced those we and others have previously reported in MPSIIIA and MPSIIIB mice (Fu et al., 2007; Haurigot et al., 2013; Hemsley and Hopwood, 2005; Hemsley et al., 2009; Lau et al., 2008; Li et al., 1999; Ribera et al., 2015). The main difference between those models was the progression of the behavioral disturbances; at 22 months of age the degree of behavioral changes in HGSNAT knockout mice was the same as in 6-month-old MPSIIIA or MPSIIIB mice (Haurigot et al., 2013; Ribera et al., 2015), likely because of milder CNS storage disease observed in the former. GAG storage, the expansion of the lysosomal compartment and microgliosis were all less pronounced in the brains of adult MPSIIIC mice compared with age-matched animals from our MPSIIIA and MPSIIIB colonies. Similarly, the lifespan of HGSNAT-deficient males and females was shortened but not as dramatically as that of MPSIIIA and MPSIIIB mice housed in the same animal facility (Haurigot et al., 2013; Ribera et al., 2015; Ruzo et al., 2012a,b). These observations support the notion of slower progression of MPSIIIC in rodents when compared with MPSIIIA and MPSIIIB diseases, and are in agreement with the less aggressive clinical course attributed to MPSIIIC in humans (Héron et al., 2011; Malm and Månsson, 2010; Ruijter et al., 2008, 2008; Valstar et al., 2008; van de Kamp et al., 1981). In a study of a large cohort of French individuals affected by different forms of MPSIII, individuals with MPSIIIC were found to live significantly longer than individuals with MPSIIIA or MPSIIIB (Héron et al., 2011). Ruijter et al. (2008) estimated the median survival of Dutch individuals with MPSIIIC to be 34 years, ranging from 25 to 48, whereas the median age at death of individuals with MPSIIIA from Germany has been reported to be 15.2 years (range: 8.5-25.5 years) (Meyer et al., 2007). Indeed, there are several cases in the literature of individuals with MPSIIIC living into their fourth and fifth decades (Berger-Plantinga et al., 2004; Héron et al., 2011; Kurihara et al., 1996; Ruijter et al., 2008).

Interestingly, Martins et al. (2015) described hyperactivity and reduced anxiety in the open field test in another recently published mouse model of MPSIIIC, generated by the gene trap technology. There are important differences in the experimental procedures followed by each group that could account for this discrepancy. First, we followed the most standard open field protocol by which analysis is performed during the first few minutes of recording, before habituation to the environment occurs (Fraldi et al., 2007; Haurigot et al., 2013; Hemsley and Hopwood, 2005; Lau et al., 2008; Li et al., 1999; McIntyre et al., 2010, 2014; Ribera et al., 2015). Under these conditions, MPSIIIA and MPSIIIB animals have been described to be hypoactive (Fraldi et al., 2007; Haurigot et al., 2013; Hemsley and Hopwood, 2005; Lau et al., 2008; Li et al., 1999; McIntyre et al., 2010, 2014; Ribera et al., 2015), hyperactivity having been described only temporarily in very young (3-week-old) MPSIIIA mice that turn hypoactive by 6 weeks of age (Hemsley and Hopwood, 2005). Moreover, when we analyzed the behavior over a period of 15 min, a time at which habituation to the environment has already begun, younger HGSNAT-deficient mice showed identical behavior to WT animals, but older mice continued to show hypoactivity, indicating loss of motor functions. In contrast to this protocol, Martins et al. reported the total outcome after 60 min of recording, and this could have influenced the type of behavior evidenced by the test. Secondly, in their manuscript, Martins et al.
only show the results of the open field test performed in females, in which statistical significance was reached at 6 and 8 month of age but disappeared as animals grew older (Martins et al., 2015). The authors stated that the same trend was observed in male mice, although in this case statistical difference was reached only at 8 months of age for most parameters. Conversely, we performed the open field test in HGSNAT-deficient males, as in our experience with different murine models of MPSIII the results obtained in females can vary considerably owing to the influence of cyclic sexual hormones. In both mixed and congenic MPSIIIA mouse strains, altered locomotor open field activity manifested mostly in male mice (Hemsley and Hopwood, 2005; Lau et al., 2008), an observation that authors attributed to the neuroprotective effect of estrogens (Hemsley and Hopwood, 2005). In addition, despite having almost identical genetic background, the mouse model described by Martins and colleagues (C57BL/6NCrl) showed a considerably reduced lifespan in comparison with ours (C57BL/6NTac), with most MPSIIIC-affected animals dead by the age of 70 weeks (~14 months), further emphasizing the impact that differences in housing conditions, diet and experimental protocols can have on the phenotype described.

Two previous studies analyzed the profile of HGSNAT expression in human samples by northern blot (Fan et al., 2006; Hrebíček et al., 2006). Although both studies used the same commercial source of polyA+ RNA extracted from various human tissues, the results were somewhat different. Whereas Fan et al. (2006) found high levels of expression in heart, skeletal muscle, liver and kidney, with moderate expression in brain, small intestine, colon, spleen, and placenta and low levels in other organs, Hrebíček et al. (2006) reported that heart, leucocytes, lung, placenta and liver were the sites with the highest expression, with the lowest expression being observed in the brain. Taking advantage of the design of our targeting construct, in which the reporter bacterial protein β-galactosidase is expressed under the control of the endogenous Hgnsat promoter in the recombined allele, we performed a thorough anatomical description of the sites of Hgnsat expression in mice. In toto incubation of tissues and organs in X-gal solution revealed the encephalon as the organ most readily stained. Whereas coronal sections of the encephalon showed intense blue staining after only 4 h of incubation, most somatic organs needed an overnight incubation to become positive. This staining pattern is compatible with a disease in which the lack of enzymatic activity results mostly in neurological manifestations, but contradicts previous studies in WT mice in which the highest levels of HGSNAT activity were reported for spleen and lungs, and brain showed much lower activity (~sixfold lower than spleen – and in the same range as liver (Martins et al., 2015). Of note was the finding that splenocytes were negative for β-galactosidase activity and only blood vessels stained blue in the spleen. This was somewhat unexpected as splenomegaly is a frequent sign of Sanfilippo syndrome (Valstar et al., 2008, 2010a,b), and HGSNAT-deficient mice accumulated large amounts of GAGs in this organ (sixfold higher than WT). To a lesser degree, a similar observation was made in the liver. Given that hepatomegaly is not an uncommon finding in affected individuals (Buhrman et al., 2014; Cleary and Wraith, 1993; Héron et al., 2011; Huh et al., 2013; Rujiter et al., 2008; Valstar et al., 2008) we expected significant staining of this organ, almost in the same range as that observed in the brain. However, after an overnight incubation in X-gal solution there was only faint staining of hepatocytes and the only intensely blue structures were identified as blood vessels. Despite this weak LacZ expression in hepatocytes, the content of GAGs in the liver of HGSNAT-deficient animals was almost tenfold higher than in WT littersmates, and at ultrastructural level, hepatocytes and, specially, Kupffer cells, showed a large number of electronolucent vesicles, an observation previously made in subjects affected by MPSIIIC (Kurihara et al., 1996). All tissues analyzed were cut across to ensure penetration of the X-gal reagent. Although technical limitations cannot be completely ruled out, these apparent discrepancies suggest intriguing pathophysiological mechanisms of disease. It might be the case that under normal circumstances the liver and spleen produce enough HGSNAT protein to cope with the normal turnover of GAGs in these tissues. However, in a disease state these organs might participate in the clearance of GAGs from the circulation, as GAG levels are considerably increased in the blood and urine from individuals with all forms of MPS (de Ruijter et al., 2012, 2013; Pievani et al., 2015; Rowan et al., 2013; Sifuentes et al., 2007; Tomatsu et al., 2005). That macrophages might buffer storage overload by uptake of GAGs from the interstitial space or blood was proposed a few years ago to justify the presence of large storage vesicles in glial cells juxtaposed to neurons with very little evidence of storage disease, or the presence of foamy macrophages in almost all tissues of MPS animal models (Jolly et al., 2007). Alternatively, the rate of turnover of GAGs in liver and spleen might be higher than in other tissues, hence the lack of enzymatic activity has a much bigger impact in terms of substrate accumulation. Finally, another plausible explanation to such a pronounced increase in GAG content in organs that do not seem to express Hgnsat at high levels could be that the accumulation of HS and derivatives causes inhibition of lysosomal enzymes involved in the degradation of other GAGs. In other words, the GAG quantified by the unspecific Bllyscan method is a GAG different from HS. As mentioned before, a recent report suggested that in Sanfilippo syndrome, the enzyme GALNS might be inhibited by HS and heparin (Rowan et al., 2013) resulting in secondary accumulation of keratan sulfate in humans (Tomatsu et al., 2005). Supporting this hypothesis we found decreased activity of GALNS in the liver of HGSNAT-deficient mice.

In summary, the disruption of the Hgnsat gene in mice led to appearance of a wide range of signs of CNS and somatic pathology that recapitulate human MPSIIIC at biochemical, histological and functional level (Delgadillo et al., 2013; Héron et al., 2011; Huh et al., 2013; Kurihara et al., 1996; Malm and Månsson, 2010; Martin et al., 1979; Turki et al., 1989). This new animal model should prove a valuable tool for the study of the pathophysiology of the disease as well as for the development of novel therapeutic approaches. Although mice fully deficient in HGSNAT represent the most severe phenotype of MPSIIIC disease, any evidence of therapeutic efficacy demonstrated in this model would be encouraging for clinical translation to human individuals with a broader clinical spectrum.

MATERIALS AND METHODS

Animals

C57BL/6N-A/a ESC clones carrying a reporter (LacZ gene) tagged insertion in the murine Hgnsat gene [Hgnsat(LacZemplrEUCOMM(Wtsi))](http://www.mousephenotype.org) available through the International Mouse Phenotyping Consortium (IMPC, www.mousephenotype.org) were obtained. The clone EPD0485_4_B08 was microinjected in C57BL/6J blastocytes in the Transgenic Animal Unit of the Center of Animal Biotechnology and Gene Therapy (CBATEG) at Universitat Autonoma de Barcelona (UAB), and the resulting male chimeras were bred with C57BL/6NtaC females to generate Hgnsat knockout offspring, which were 100% C57BL/6NtaC. Genotyping was performed by PCR analysis using both locus-specific and LacZ cassette-specific primers Hgnsat-Fw: 5′-ACA AAT ACC TTG TTC CAT GCC GCC A-3′; Internal-Rev: 5′-GCC ACC CAA CTG ACC TTG GGC-3′; Hgnsat-Rev: 5′-ACT GCA CCT CTC CAG CTA GA-3′. Bands of 395 or...
329 bp were obtained for WT or knockout alleles, respectively. Homozygous mutant mice (Hgsnat−/− mice) were obtained by mating heterozygous littermates. Mice were fed ad libitum with standard diet (Harlan-Teklad) and maintained under a light-dark cycle of 12 h. All experimental procedures were approved by the Ethics Committee for Animal and Human Experimentation of UAB.

**Sample collection**
Mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine), blood was extracted by cardiac puncture, and animals were intracardially perfused with 12 ml PBS to clear remaining blood from tissues. Brain and somatic tissues were collected and kept at −80°C or fixed in formalin. For the analysis of LacZ expression, animals were anaesthetized with intraperitoneal injection of 500 IU of heparin (Hospira) prior to anesthesia.

**RNA analysis**
For northern blot analysis, total RNA was obtained from kidneys homogenized in TriPure Isolation Reagent (Roche) and purified following manufacturer’s instructions. 20 µg of RNA were electrophoresed in 1% denaturing agarose gels, transferred to nylon membranes, and blots were hybridized with 32P-labeled Hgsnat cDNA probe labeled by random oligopriming (GE Healthcare). For qRT-PCR analysis, total RNA from brain or liver was purified using RNaseasy Mini Kit (Qiagen), and 1 µg was retrotranscribed with Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative PCR was performed in a Light Cycler® 480 (Roche) using LightCycler® 480 SybrGreen 1 Master (Roche) and primers specific for Hgsnat (forward: 5′-CGG CGT TCT TCT GCG-3′; reverse: 5′-GTT CGG CCA CAG TCA GTC CG-3′) and normalized to the expression of mouse Rplp0 gene (forward: 5′-GGC CCT ACA CTC TCG CTT T-3′; reverse: 5′-TGC CAG GAC GCG CTT GT-3′).

**X-gal staining**
Tissues were dissected and fixed in 4% paraformaldehyde (Sigma) for 1.5 h and incubated protected from light at 37°C in X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution [0.4 mg/ml X-gal (Sigma), 200 mM potassium ferricyanide, 200 mM potassium ferrocyanate, 1 M MgCl2 diluted in PBS]. Coronal sections of the encephalon were incubated for 4 h, whereas sagittal sections and somatic tissues were incubated overnight. To stop the reaction, X-gal solution was removed and tissues were washed twice with PBS. Samples were kept in 10% neutral buffered formalin at 4°C until images were captured under a SMZ1000 zoom stereomicroscope (Nikon).

**Activity of lysosomal enzymes**
Brain and liver samples were sonicated in 250 and 500 µl of water, respectively. Homogenized tissues were clarified by centrifugation and, after washing with PBS. Samples were kept in 10% neutral buffered formalin at 4°C until images were captured under a SMZ1000 zoom stereomicroscope (Nikon).

**Activity of enzymes**
Acetylgalactosaminidase (GUSB), activity was assayed in 15 µg (brain) or 5 µg (liver) of protein incubated for 1 h with 2 mM 4-methylumbelliferyl-β-D-glucuronide (Sigma) at pH 4.8 and 37°C. After stopping each enzymatic reaction by increasing the pH, the released fluorescence was measured with a FLx800 fluorimeter (BioTek Instruments). Results were calculated as nanomoles of substrate cleaved per hour per mg of protein quantified by Bradford assay (Bio-Rad), and then presented as a percentage of the activity of the WT, which was set to 100%.

**GAG quantification**
Tissues were weighed and digested overnight in 0.2 mg/ml proteinase K solution. Extracts were centrifuged and supernatants were clarified by filtration in 0.22 µm microporous membrane-containing filters (Ultrafree MC, Millipore). GAG content was determined using the Blysan sulfated glycosaminoglycan kit (Biocolor) using chondroitin 4-sulfate as standard and was normalized to wet tissue weight. Owing to the inherent technical variability of the assay, the GAG content of WT animals at each age was set to 1 to allow comparison of data obtained at different points in time.

**Histology**
Formalin-fixed, paraffin-embedded tissue sections were incubated overnight at 4°C with rat anti-LAMP1 (SC-19992, Santa Cruz Biotechnology, 1:100), rat anti-LAMP2 (ab13524, Abcam, 1:500), rabbit anti-LIMP2 (NB400; Novus Biologicals, 1:100), rabbit anti-GFP (Z0334, Dako Cytomation, 1:1000) and BSI-B4 lectin (L5391, Sigma, 1:100). For bright-field immunostaining, biotinylated rabbit anti-rat IgG (E0467, Dako, 1:300) and biotinylated goat anti-rabbit IgG (31820, Vector Laboratories, 1:300) were used as secondary antibodies. Bright-field sections were stained with 3,3-diaminobenzidine (Sigma) and counterstained with haematoxylin.

**Electron microscopy**
Frontal cortex, cerebellum, liver, lung, heart and kidney were processed for transmission electron microscopy (Nikon). LAMP2, L2MP, GFP and BSI-B4 signals were quantified with the NIS-Elements Advanced Research 2.20 software (Nikon) in 3-5 20× images of each brain region of each animal using the same signal threshold settings for all images. The percentage positive area of each image was then calculated.

**Open field test**
Animals were always analyzed between 9:00 A.M. and 2:00 P.M., to minimize influence of circadian cycles. After placing the mouse in the corner of a brightly lit chamber (41×41×30 cm) divided in three squared concentric regions (center, 14×14 cm; periphery, 27×27 cm; and border, 41×41 cm), movements were recorded with a video-tracking system (SmartJunior v3, Panlab). Motor and exploratory activities were evaluated during the first three minutes, except for latency to center, which was recorded over 15 min, as several MPSIIIC mice failed to enter the arena during the first 3 min of the test.

**Statistical analysis**
Results are expressed as mean±s.e.m. Statistical comparisons were made with either unpaired t-test two-tailed or one-way analysis of variance (ANOVA). Multiple comparisons were made using Dunnett or Tukey post-tests. Statistical significance was considered if P<0.05. The Kaplan–Meier estimate was applied to survival analysis, followed by log-rank test for comparisons.

**Acknowledgements**
We thank Marta Moya, Ángel Vázquez, Verónica Meigarejo, Lorena Noya and David Ramos for technical assistance.

**Competing interests**
The authors declare no competing or financial interests.
Author contributions
S. Marcó, V.H. and F.B. designed the study, analyzed the results and wrote the paper. A.P. generated the animal model. J.R. performed the ultrastructural studies. S. Marcó, C.R., S. Motas, A.R., M.G., M.M., P.V., C.S.M., V.S., X.S. and J.B. generated reagents and performed experiments. All authors reviewed the results and approved the final version of the manuscript.

Funding
This work was supported by funding from: Plan Nacional (F+D+I) from the Ministerio de Economia y Competitividad [grant numbers SAF2014-54866-R and INNPACTO IPT-2012-0772-300000], Generalitat de Catalunya [grant number 2014SGR-1669] and Institucio Catalana de Recerca i Estudis Avancats Academia Award to F.B., Laboratorios ESTEO S.A., Spain, and from the European Union through the Regional Development Fund (ERDF) and the Seventh Framework Programme [project INFRAFRONTIER3]. A.R. received a predoctoral fellowship from Ministerio de Ciencia e Innovación and S. Marcó, S. Motas and V.S. from Generalitat de Catalunya, Spain.

Supplementary information
Supplementary information available online at http://dmm.biologists.org/lookup/doi/10.1242/dmm.025171.supplemental

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