

Neuroinflammation, mitochondrial defects and neurodegeneration in mucopolysaccharidosis III type C mouse model

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Severe progressive neurological paediatric disease mucopolysaccharidosis III type C is caused by mutations in the *HGSNAT* gene leading to deficiency of acetyl-CoA: α -glucosaminide *N*-acetyltransferase involved in the lysosomal catabolism of heparan sulphate. To understand the pathophysiology of the disease we generated a mouse model of mucopolysaccharidosis III type C by germline inactivation of the *Hgsnat* gene. At 6–8 months mice showed hyperactivity, and reduced anxiety. Cognitive memory decline was detected at 10 months and at 12–13 months mice showed signs of unbalanced hesitant walk and urinary retention. Lysosomal accumulation of heparan sulphate was observed in hepatocytes, splenic sinus endothelium, cerebral microglia, liver Kupffer cells, fibroblasts and pericytes. Starting from 5 months, brain neurons showed enlarged, structurally abnormal mitochondria, impaired mitochondrial energy metabolism, and storage of densely packed autofluorescent material, gangliosides, lysozyme, phosphorylated tau, and amyloid- β . Taken together, our data demonstrate for the first time that deficiency of acetyl-CoA: α -glucosaminide *N*-acetyltransferase causes lysosomal accumulation of heparan sulphate in microglial cells followed by their activation and cytokine release. They also show mitochondrial dysfunction in the neurons and neuronal loss explaining why mucopolysaccharidosis III type C manifests primarily as a neurodegenerative disease.

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Abbreviation: MPS = mucopolysaccharidosis

Introduction

Mucopolysaccharidosis III or Sanfilippo syndrome is a metabolic genetic disease caused by lysosomal accumulation of heparan sulphate (Neufeld and Muenzer, 2001; Valstar *et al.*, 2008). The disease includes four allelic subtypes caused by the genetic deficiencies of heparan N-sulphatase (MPS III type A; MIM #252900), α -N-acetylglucosaminidase (MPS III type B; MIM #252920), heparan sulphate acetyl-CoA: α -glucosaminide N-acetyltransferase or HGSNAT (MPS III type C; MIM #252930), and N-acetylglucosamine 6-sulphatase (MPS III type D; MIM #252940).

The majority of MPS IIIC patients have severe clinical manifestations with onset in early childhood. They rapidly develop progressive and severe neurological deterioration causing hyperactivity, sleep disorders, loss of speech, behavioural abnormalities, neuropsychiatric problems, mental retardation, hearing loss, visceral manifestations, such as mild hepatomegaly, mild dysostosis multiplex, mild coarse facies, and hypertrichosis (Bartsocas *et al.*, 1979). Most patients die before adulthood but some survive to the fourth decade with progressive dementia and retinitis pigmentosa (Neufeld and Muenzer, 2001; Ruijter *et al.*, 2008; Valstar *et al.*, 2008). The birth prevalence of MPS IIIC in Portugal and The Netherlands is 0.12 and 0.21 per 100 000, respectively (Poorthuis *et al.*, 1999; Pinto *et al.*, 2004).

HGSNAT transfers an acetyl group from cytoplasmic acetyl-CoA to terminal N-glucosamine residues of heparan sulphate within the lysosomes (Klein *et al.*, 1978). To date, more than 80 HGSNAT mutations have been identified including 18 missense which all result in misfolding of the mutant enzyme (Feldhammer *et al.*, 2009).

Important insights into the physiological mechanism of MPS III have been obtained from studying animal models of the disease. Spontaneous avian (Aronovich *et al.*, 2001) and canine (Aronovich *et al.*, 2000; Ellinwood *et al.*, 2003) models of MPS IIIA and B and a caprine model of MPS IIID (Ginsberg *et al.*, 1999) have been described, but the majority of data have been obtained by studying a knockout mouse model of MPS IIIB (Li *et al.*, 1999) and a spontaneous mouse model of MPS IIIA (Bhaumik *et al.*, 1999). Several mechanisms potentially underlying the neurodegenerative process in MPS III were described. First, in both mouse models microglia cells are activated through interaction of their Toll-like receptor 4 (TLR4) with

under-degraded heparan sulphate fragments, as are astrocytes and the entire immune system in the brain (Ohmi *et al.*, 2003; Villani *et al.*, 2007; Ausseil *et al.*, 2008; DiRosario *et al.*, 2009). At the same time, inhibition of the TLR4 signalling pathway in MPS IIIB mouse while delaying brain inflammation did not stop progression of the neurodegenerative process (Ausseil *et al.*, 2008). Second, storage of G_{M2} and G_{M3} gangliosides occurring in brain cerebral cortical and cerebellar neurons (McGlynn *et al.*, 2004; Crawley *et al.*, 2006) was previously suggested to cause neuronal apoptosis in Tay-Sachs and Sandhoff diseases where G_{M2} ganglioside is accumulated due to genetic defects of hexosaminidases A and B, respectively (Huang *et al.*, 1997; Wada *et al.*, 2000). Interestingly, recently described double-mutant MPS IIIA and IIIB mice with a knockout GalNAc transferase (*Galnt3*) crucial for the synthesis of gangliosides have significantly reduced lifespan and increased neurodegeneration as compared to the corresponding single-mutant MPS IIIA and MPS IIIB mice, suggesting that absence of gangliosides may also be deleterious in MPS III (Mohammed *et al.*, 2012). Third, neurodegeneration could be caused by protein aggregates (Ginsberg *et al.*, 1999; Hamano *et al.*, 2008; Ohmi *et al.*, 2009) detected in neurons of medial entorhinal cortex and the dentate gyrus and linked to impaired autophagy and/or increased extralysosomal level of heparan sulphate proteoglycans, glypicans (Ohmi *et al.*, 2011). Fourth, stored heparan sulphate fragments, which are in excess and abnormally sulphated in MPS IIIA and IIIB (Wilkinson *et al.*, 2012), can cause adverse signalling reactions in brain neurons inducing, in particular, overexpression of GM130 protein and subsequent alterations of the Golgi ribbon architecture (Roy *et al.*, 2012), enhanced proliferation and outgrowth of neuritis (Hocquemiller *et al.*, 2010) as well as alteration of neural cell migration (Bruyère *et al.*, 2014) all potentially contributing to neuropathology.

However, it still remains to be determined which of the above mechanisms are important for the development of neuronal dysfunction and, therefore should be the target for the pharmaceutical intervention along with finding the ways to restore deficient HGSNAT activity in the patients' cells. It is also not understood whether the pathophysiological mechanism in all four subtypes of MPS III is the same despite different genetic and biochemical defects and severity of the clinical manifestation.

In the current work we report the generation of the first animal model of the MPS IIIC by inactivation of the *Hgsnat* gene in mice and present new pathological and mechanistic findings explaining the brain disease progression by the neuronal loss associated with mitochondrial dysfunction.

Materials and methods

Animals

Generation of C57Bl6 mice with targeted disruption of *Hgsnat* gene was performed at the Texas Institute for Genomic Medicine as previously described (Zambrowicz *et al.*, 2003; Hansen *et al.*, 2008). The heterozygous mice were crossed to C57BL/6NCrl strain distributed by Charles River Quebec or bred to each other and the litter genotyped by PCR as described below. The *Hgsnat-Geo* mice were compared with the appropriate age and sex-matching wild-type controls. All mice were bred and maintained at the Canadian Council on Animal Care (CCAC)-accredited animal facilities of the CHU Ste-Justine Research Centre according to the CCAC guidelines. Mice were housed in an enriched environment with continuous access to food and water, under constant temperature and humidity, on a 12-h light/dark cycle. Approval for the animal experimentation was granted by the Animal Care and Use Committee of the CHU Ste-Justine.

The genotypes of mice were determined using genomic DNA extracted from the clipped tail tip (see [Supplementary material](#) for sequences of primers and PCR conditions). A 463 and 297 bp fragments were amplified separately in wild-type and *Hgsnat-Geo* homozygous mice, respectively, whereas both fragments were amplified in mice heterozygous for the *Hgsnat-Geo* allele.

Quantification of mouse *Hgsnat* mRNA and cytokines in mouse brain tissues was performed using a Stratagene Mx3000P[®] QPCR System (see [Supplementary material](#) for sequences of primers and PCR conditions). Total RNA was isolated from cultured cells or mouse tissues using the TRIzol[®] reagent (Invitrogen) according to the manufacturer's protocol and reverse-transcribed to cDNA using random primers and SuperScript[®] III reverse transcriptase (Invitrogen). *RPLP32* mRNA was used as a reference control.

Neurological and behavioural examination of mice

The motor performance of mice was evaluated using a simplified neurological examination as previously described (Lema *et al.*, 2004). The rotating rod motor coordination test was performed using an accelerating 5-line Rotarod treadmill for mice (3 cm in diameter). Animals (eight per age per genotype) were briefly trained at 4 rpm and tested using an accelerating mode (from 4 to 40 rpm over 5 min). Three trials separated by 20 min rest intervals were performed on three consecutive days. The open field test was performed by individually placing 2, 4, 6, 8 and 10-month-old *Hgsnat-Geo*, or wild-type mice into the centre of an arena (40 cm length × 40 cm width × 50 cm height, white Plexiglas). Mouse behaviour was

recorded for 1 h and analysed using Top Scan software version 2.0 (Clever Sys. Inc). The path length, rapid exploratory behaviour (speed > 100 mm/s), immobility (speed < 0.05 mm/s) as well as frequency, path length, duration and speed in the central (25%) area of the cage were analysed. The open field test was performed 1 h into the mouse light cycle (Langford-Smith *et al.*, 2011). A 30-min room adjustment period was implemented before the start of each test. All experiments were performed with a naïve group of 6–10 animals by the same investigator (L.G.).

The 5, 7, 8 and 10-month-old mice (six per age per genotype) were subjected to the Morris Water Maze test for spatial learning, essentially as described by Seyrantepe *et al.* (2010). During a 3-day habituation period, mice were required to swim to a visible platform located in a selected quadrant of a circular (1.4 m in diameter and 34 cm high) tank filled with water (18 ± 1°C). The escape latencies were measured and visual and motor acuity as well as motivation were tested. On the fourth day, the water was made opaque with an inert paint, the platform was moved to a different quadrant and submerged (1 cm), the visual wall cues were switched and 5 days of hidden-platform testing ensued. Mice were given three trials of 90 s to find the platform (the maximum intertrial interval was 45 min). On Days 1 and 4, mice that could not find the platform in the allotted time were guided to and allowed to stay on it for 10 s. On Day 9, following hidden platform testing, all mice were given a 60 s probe trial in which the percentages of time spent and distance travelled in the target quadrant (no longer containing a platform), as well as the number of crossings over the previous location of the hidden platform were recorded, along with swimming speed. Escape latencies were acquired with the 2020 Plus tracking system and Water 2020 software (Ganz FC62D video camera; HVS Image). All experiments were started at the same time every day and performed by the same investigator (C.M.).

Analysis of glycosaminoglycans and gangliosides in mouse tissues

Total glycosaminoglycans and lipids were extracted from brain and liver tissues as previously described (Ausseil *et al.*, 2008). Briefly, frozen tissues were homogenized in water (10% v/w). Lipids were extracted by addition of two volumes of methanol and one volume of chloroform to one volume of the homogenate. After 10 min centrifugation at 1000g the organic phase was collected, evaporated and used to analyse gangliosides. The pellet was resuspended in 100 mM sodium acetate buffer pH 5.5, containing 5 mM cysteine, 5 mM EDTA and 1 mg/ml of papain, digested overnight at 65°C and cleared by centrifugation at 2500g for 15 min. For the analysis of total glycosaminoglycans, 100 µl of the supernatant was added to 2.5 ml of dimethylmethylene blue reagent (Whitley *et al.*, 1989) and the absorbance at 535 nm was measured. glycosaminoglycans concentration (µg per mg of dried pellet) was calculated using purified heparan sulphate standard.

Sphingolipids were extracted from the lipid fraction in the presence of deuterium-labelled standards [*N*-stearoyl (D³)-monosialoganglioside G_{M2} and *N*-stearoyl (D³)-monosialoganglioside G_{M3}, Matreya] by saponification (Kyrklund, 1987) and then fractionated and desalted using reverse-phase Bond

Elut[®] C18 columns. Samples were analysed by direct flow injection at a rate of 200 μ l/min on a triple-quadrupole mass spectrometer (API 3200 MS/MS; Sciex Applied Biosystems) in the negative ionization mode using the multiple reactions monitoring (MRM) method. G_{M2} and G_{M3} species were measured separately. The concentrations of individual molecular species were calculated by comparing with the corresponding internal standards. Total G_{M2} and G_{M3} concentrations (pmol per mg of dried pellet) were calculated as sums of the concentrations of all molecular species.

Lysosomal enzyme assays

N-acetyltransferase, β -galactosidase, α -galactosidase and β -hexosaminidase activities in cellular and tissue homogenates were assayed using the corresponding fluorogenic 4-methylumbelliferyl glycoside substrates as previously described (Seyran-tepe *et al.*, 2008). Protein concentration was measured using a Bio-Rad Bradford kit.

Analysis of LC3 in the mouse brain tissues by western blot

Total brains were homogenized in five volumes of 250 mM sucrose buffer containing 50 mM Tris-HCl pH 7.4, 1 mM EDTA and protease inhibitor cocktail using a Dounce homogenizer. Nuclei were cleared by centrifugation at 500g for 10 min at 4°C. Supernatants were mixed with an equal volume of sucrose buffer containing 1% Triton[™] X-100 and incubated for 1 h at 4°C. Resulting lysates were centrifuged for 15 min at 13 000g and supernatants separated by SDS-PAGE on 15% gels. Western blot analyses were performed according to standard protocols using anti-LC3 (Sigma, 1:2000) and 12G10 anti- α -tubulin antibody (DSHB, 1:15 000). Equal protein loading was assured by Ponceau S staining. Signals were quantified using ImageQuant software.

Tissue processing for morphological studies

Animals were deeply anaesthetized with sodium pentobarbital and perfused via intracardiac catheter with phosphate-buffered saline, pH 7.4 (PBS) followed by 4% paraformaldehyde in PBS. Brains and visceral organs were removed and immersed in 4% paraformaldehyde in PBS overnight. Tissues for light microscopy were trimmed, dehydrated with an ethanol series followed by acetone, acetone-xylene mixture and xylene and then embedded in paraffin. Tissues for fluorescent confocal microscopy were treated sequentially in 10%, 20% and 30% sucrose in PBS overnight at 4°C and embedded in O.C.T. compound before freezing at -80°C . Tissues for electron microscopy were trimmed into small cubes, post-fixed with 1% osmium tetroxide, dehydrated with an ethanol series and propylene oxide and embedded in Durcupan[®]-Epon[®] mixture.

Histopathology and immunohistochemistry

Paraffin-embedded tissues (4- μ m thick sections) were rehydrated with isopropyl alcohol, and then with 96%, 70%,

and 60% ethanol. The sections were stained with either haematoxylin and eosin, or periodic acid-Schiff technique (PAS), and a set of primary antibodies. In the latter case the sections were treated with 1% NaN_3 and 0.3% H_2O_2 for 10 min to inactivate endogenous peroxidase, blocked with 5% bovine foetal serum in PBS for 30 min (both at room temperature), and incubated with primary antibodies diluted in 5% bovine serum albumin in PBS overnight at 4°C. After staining with primary and secondary antibodies (see [Supplementary material](#) for the list of antibodies and their dilutions) sections were analysed and photographed using a Nikon E800 equipped with Olympus digital camera (DP70). Autofluorescence of neurons was analysed in unstained sections using fluorescence filter BV-2 A (Ex 400-440 nm/DM 455/BA 470).

For fluorescent confocal microscopy 40- μ m thick sagittal sections were cut from O.C.T.-embedded frozen brains using a CM3050 S Microtome (Leica). The sections were treated with 1% Triton[™] X-100, blocked with 10% goat serum in PBS and incubated overnight at 4°C with primary antibodies in 3% goat serum, 0.1% Triton[™] X-100 in PBS followed by secondary antibodies. The slides were mounted with Vectashield[®] mounting medium and analysed using a LSM510 Meta Laser inverted confocal microscope (Zeiss, $\times 63$ oil objective, N.A. 1.4). Images were processed and quantified using the LSM image browser software (Zeiss) and Photoshop (Adobe).

Electron microscopy

Semi-thin sections were cut and stained with Toluidine blue and viewed by light microscopy. The regions of interest for electron microscopy were selected and ultrathin sections were cut and mounted on 200 mesh copper grids. Sections were double contrasted with uranyl acetate and lead nitrate and then analysed using a transmission electron microscope (JEOL 1200 EX).

Analyses of mitochondrial energy metabolism in mouse brain

Frozen brain tissues were homogenized at 4°C in 20 volumes of 50 mM Tris-HCl buffer pH 7.4, containing 150 mM KCl, 2 mM EDTA and 0.2 μ g/ml aprotinin. Mitochondria were isolated from the homogenate by differential centrifugation as described (Makinen and Lee, 1968). The activities of the mitochondrial enzymes, NADH: ubiquinone oxidoreductase (NQR, complex I), succinate:CoQ reductase (SQR, complex II), ubiquinol:cytochrome *c* oxidoreductase (QCCR, complex III), cytochrome *c* oxidase (COX, complex IV), NADH:cytochrome *c* reductase (NCCR, complex I + III), succinate:cytochrome *c* reductase (SCCR, complex II + III) and citrate synthase (CS) were measured as described (Srere, 1969; Rustin *et al.*, 1994). Pyruvate dehydrogenase activity was determined as described (Dudkova *et al.*, 1995). Protein concentration was measured according to Lowry *et al.* (1951).

Total content of coenzyme Q10 in brain homogenate was determined as described (Mosca *et al.*, 2002) with minor modifications. Brain homogenate (100 μ l) was diluted with 100 μ l Milli-Q[®] H_2O . The mixture was supplemented with 50 μ l of 1,4-benzoquinone solution (2 mg/ml) and vortexed for 10 s.

After 10 min, 1 ml of n-propanol was added. The mixture was vortexed for 10 s and centrifuged at 10 000 rpm for 2 min. The supernatant (50 μ l) was analysed by HPLC using the Supelcosil LC 18 column (Supelco) eluted by ethanol/methanol (70/30 v/v) mixture at a flow rate of 1 ml/min. UV detection was performed at 275 nm. Results were expressed as pmol of Q10 per mg of total protein.

Quantitative analysis of neuronal density in the mouse brain cortex

Four mice (two male, two female) were studied for each age and genotype. For each brain, three sagittal sections, cut at 1.44, 1.68 and 1.92 mm lateral from bregma, (Paxinos and Franklin, 2013) were simultaneously stained with anti-NeuN antibodies as described above. For each section, two images of the S1 somatosensory cortex (S1Tr and S1HL for 1.44 and S1Tr and S1FL for 1.68 and 1.92 sections, respectively) were acquired using Nikon Eclipse E600 Epifluorescence Microscope (magnification $\times 200$), Zeiss AxioCam 506 digital camera with the same settings for all sections. The acquired images were analysed using Adobe Photoshop CS5 (Adobe Systems Inc). In each image, two rectangular fields with the area of 1.92 mm² were defined, on the right and left extremities of the photographed areas. The numbers of NeuN-stained neuronal nuclei for each field (12 fields for each mouse) were counted by two non-biased observers blinded for the genotype, age and sex of the animals.

Results

Mice homozygous for *Hgsnat-Geo* allele show deficient *Hgsnat* mRNA and activity in tissues

A functional knockout of the *Hgsnat* locus in C57Bl/6N mice was generated using gene trap technology as previously described (Zambrowicz *et al.*, 2003; Hansen *et al.*, 2008). A selectable marker β -geo, a functional fusion between the β -galactosidase-encoding gene and the *neo* gene from Tn5 encoding an aminoglycoside 3'-phosphotransferase, was inserted into the intron 7 of the *Hgsnat* gene leading to splicing of exon 7 into the β -geo cassette to generate a fusion protein containing *Hgsnat* amino acid sequence encoded by the exons 1–7 followed by aminoglycoside 3'-phosphotransferase and β -galactosidase (Fig. 1A). The *Btk* exon in the trap construct contained termination codons in all reading frames to prevent translation of the downstream *Hgsnat* exons (Fig. 1A).

Mice homozygous for *Hgsnat-Geo* allele (*Hgsnat-Geo* mice) were viable and born in the frequency expected from Mendelian inheritance (26 of the first 100) indicating that disruption of the *Hgsnat* gene did not cause embryonic lethality. Mice showed normal growth, were fertile and indistinguishable from wild-type or heterozygous animals until the age of 11 months.

Both expression of *Hgsnat* mRNA measured by real-time quantitative PCR (Fig. 1B) and the level of HGSNAT activity measured with fluorogenic substrate Muf- β -D-glucosaminide (Fig. 1C) in tissues and primary cultures of skin fibroblasts from the homozygous animals were reduced to 0.6–1.5% of that in wild-type mice (below or close to detection limit of the method) confirming efficiency of the splicing outcome and validating the model biochemically. In the heterozygous mouse tissues HGSNAT activity was reduced to $\sim 50\%$ of wild-type (Fig. 1C). In contrast, β -galactosidase activity measured in liver, kidney, brain, muscle and lung tissues of the *Hgsnat-Geo* mice at pH 7.5 was significantly increased as compared to wild-type mice (not shown) indicating that the *Hgsnat-Geo* fusion protein is produced but does not have *N*-acetyltransferase activity, which was expected because it is missing $\sim 60\%$ of the *Hgsnat* sequence including its predicted active site residue, His269 and 9 of 11 transmembrane domains presumably forming the AcCoA-transporting channel (Durand *et al.*, 2010).

Activities of other lysosomal glycosidases measured in the tissues of homozygous *Hgsnat-Geo* mice were either similar or higher than those in wild-type mice and progressively increased with age of the animals suggesting augmented production of lysosomes previously described for the mouse models of other subtypes of MPS III and linked to lysosomal storage phenotype (Bhaumik *et al.*, 1999; Li *et al.*, 1999). In the *Hgsnat-Geo* mice the highest increase was observed in liver tissues where α -galactosidase activity was induced ~ 5 -fold and β -hexosaminidase activity ~ 20 -fold by the age of 6 months (Fig. 1D). β -Hexosaminidase activity was also increased 2–3-fold in the brain, lungs and kidney suggesting that lysosomal storage occurs also in these tissues.

Hgsnat-Geo mice show reduced longevity, progressive behavioural changes and learning impairment

No visible signs of illness were observed in the homozygous *Hgsnat-Geo* mice until the age of 11–12 months, when they showed weight loss and abnormal gait. About 30% of animals at this age showed spasticity of hind limbs and loss of coordination in a balance test (Supplementary Video 1). At ~ 65 weeks of age mice presented signs of urinary retention resulting in abdominal distension absent in human patients but present in mouse models of both MPS IIIA and MPS IIIB (Bhaumik *et al.*, 1999; Li *et al.*, 1999) and had to be euthanized (Fig. 2A). At all ages, heterozygous mice were clinically undistinguishable from their wild-type siblings. No signs of skeletal abnormalities such as scoliosis reported previously in human patients or shortened skull with a 'blunt' snout described in several MPS mouse models including those of MPS IIIA (Bhaumik *et al.*, 1999) were detected in the homozygous *Hgsnat-Geo* mice by X-ray analysis (Supplementary Fig. 1). Similarly, we did not

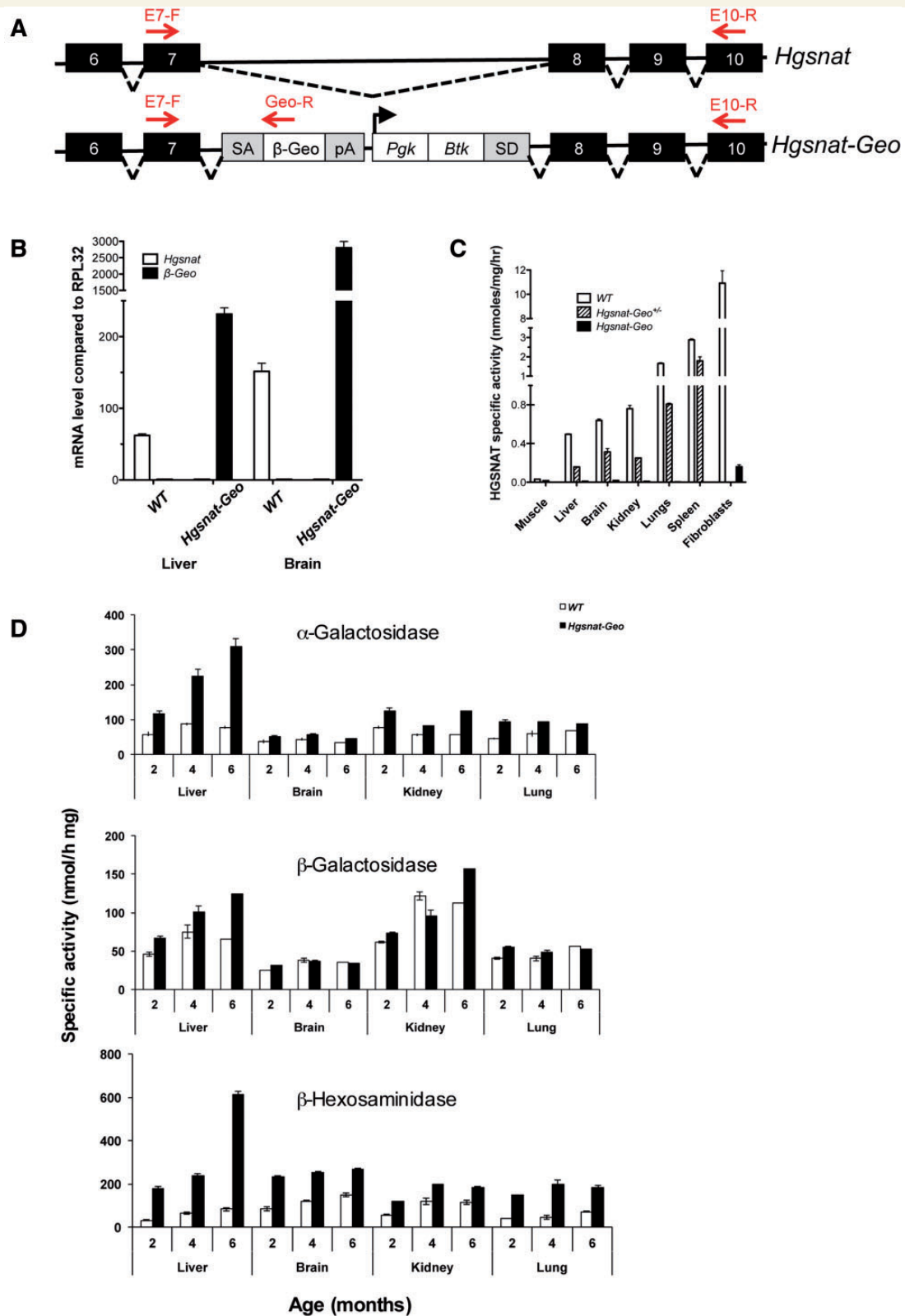


Figure 1 Targeting the *Hgsnat* gene in mice. (A) Strategy for producing the targeted disruption of the *Hgsnat* gene. The gene trap vector used by Texas A&M Institute for Genomic Medicine contains a splice acceptor site (SA) upstream of a β -galactosidase/neomycin phosphotransferase (*Geo*) fusion gene followed by polyadenylation sequence (pA), *PGK* and *BTK* genes and splice donor site (SD) inserted into intron 7 of the *Hgsnat* gene as confirmed by genomic sequencing. Hence, the downstream exons 8–13 in the gene trap transcript were replaced by the *Geo* sequence. Exons in the mouse *Hgsnat* gene are shown as black numbered boxes and *Geo*-*PGK*-*BTK* cassette, as white boxes. Primers (E7-R, *Geo*-R, and E10-F) were used to measure the expression of the wild-type *Hgsnat* and of the targeted *Hgsnat-Geo* alleles by real time quantitative PCR.

(continued)

detect any signs of femoral head necrosis also reported for human MPS III patients (de Ruijter *et al.*, 2013).

Neurological assessment (gait, posture, avoidance response, righting reflex, horizontal bar test, inverted wire screen test, all conducted on the group of 10 *Hgsnat-Geo* and 10 wild-type mice by the same examiner blinded for the mouse genotype) was performed at 2 months, when MPS IIIA and B mice still show normal behaviour and then repeated every 2 months with a naïve group of animals. Also every 2 months starting from the age of 4 months we studied motor activity using a motor coordination test performed on an accelerating Rotarod treadmill for mice. Neither neurological assessment nor the accelerating Rotarod test (Supplementary Fig. 2) revealed any signs of neuromuscular pathology in HGSNAT-deficient mice up to the age of 10 months.

In contrast, open field test performed 1 h into their light cycle showed definite signs of increased activity and reduced anxiety including higher than average speed and distance travelled, increased frequency of crossing the central field and central distance travelled, as well as decreased frequency and duration of periods of immobility (Fig. 2B and Supplementary Video 2). The same trend was observed for both male and female mice but for female mice the differences with the control group became significant already at the age of 6 months whereas, for male mice, for most studied parameters the significant difference was observed only at 8 months. By the age of 10 months the signs of hyperactive behaviour diminished resembling the trend observed in human MPS III patients (Fig. 2B).

Hippocampal function was assessed at 5, 7, 8 and 10 months using the Morris Water Maze test to measure memory and spatial learning capability. To make sure that HGSNAT-deficient mice had no visual or motor deficits that could affect the outcome of the test, we assessed their ability to reach a visible platform in a 3-day pre-training test. We found that there were no differences between the mutant and wild-type groups as HGSNAT-deficient and wild-type mice of all age groups showed similar escape latencies in the visible platform testing (Fig. 2C). Learning progress of mutant mice at the ages of 5, 7 and 8 months was similar to that of their wild-type counterparts, as shown in Fig. 2C for the 8-month-old group. In contrast, at the age of 10 months, HGSNAT-deficient mice needed

significantly more time to find the hidden platform compared to their wild-type counterparts, and to both wild-type and mutant 8-month-old mice, suggesting impaired spatial learning. Additionally, in the probe trial on Day 9 when the platform was removed, HGSNAT-deficient mice spent significantly less time and travelled less distance than wild-type mice in the target quadrant where the platform used to be located, which indicated a decline of spatial memory (Fig. 2C). Moreover, 10-month-old mutant mice displayed lack of precision with fewer platform crossings compared to wild-type counterparts, and to younger wild-type and HGSNAT-deficient mice (Fig. 2C).

Pathological changes in tissues

Pathological examination of homozygous *Hgsnat-Geo* mice performed at the ages of 2, 4, 6, 10 and 11–12 months did not reveal any gross changes in the visceral organs and brains, except for the strikingly reduced abdominal fat detected at 11–12 months. The same animals usually also had a largely distended bladder filled with 1–2 ml of urine, consistent with urinary retention (Supplementary Fig. 3). Microscopic examination however revealed multiple pathological changes in numerous tissues and organs already detectable at 2 months and progressively increasing with age.

Hepatocytes in the centrolobular and intermediate zones showed microvacuolization of cytoplasm, which became more prominent with age (Supplementary Fig. 4A). Immunostaining with antibody against cathepsin D (CTSD) detected expanded lysosomal system in hepatocytes compatible with lysosomal storage starting from 2 months (Supplementary Fig. 4B). Kupffer cells are increased in number starting from 2 months and become enlarged and strongly stained for CTSD at later stages, showing signs of transformation into foam cells (Supplementary Fig. 4A–C). Besides Kupffer cells, liver sinusoids contained a sparse mixed inflammatory infiltrate. High-resolution analysis of liver tissues by electron microscopy revealed massive accumulation of vacuoles, either electron-lucent or containing a fine sparse material characteristic of glycosaminoglycan storage in the cytoplasm of hepatocytes and less prominent storage of this type in Kupffer and Ito cells (Supplementary Fig. 4E–H).

Figure 1 Continued

(B) Real time quantitative PCR shows that expression of the *Hgsnat* mRNA in brain and liver tissues of mice homozygous for the *Hgsnat-Geo* allele is reduced to 0.6 and 1.6% of that in the tissues of their wild-type (WT) siblings. Total RNA was extracted from tissues of 4-month-old wild-type and *Hgsnat-Geo* mice and analysed for *Hgsnat* expression by real time quantitative PCR. The values were normalized for the level of control *RPL32* mRNA. Data are expressed as means (\pm SD) of experiments performed with six mice for each genotype. (C) Deficient HGSNAT activity in tissues of mice homozygous for *Hgsnat-Geo* allele. HGSNAT activity was measured with fluorogenic substrate, Muf- β -D-glucosaminide in the tissues of 4-month-old wild-type, *Hgsnat-Geo*^{+/-} and *Hgsnat-Geo* mice or in cultured skin fibroblasts of wild-type and *Hgsnat-Geo* mice. Data are expressed as means (\pm SD) of independent experiments performed with tissues of six mice for each genotype or with three different cell cultures. (D) Lysosomal hydrolases are increased in tissues of *Hgsnat-Geo* mice. Acidic α -galactosidase, β -galactosidase and total β -hexosaminidase activity were measured in the tissues of 1, 4 and 6 month-old *Hgsnat-Geo* mice and their wild-type siblings. Data are expressed as means (\pm SD) of experiments performed with three mice for each genotype.

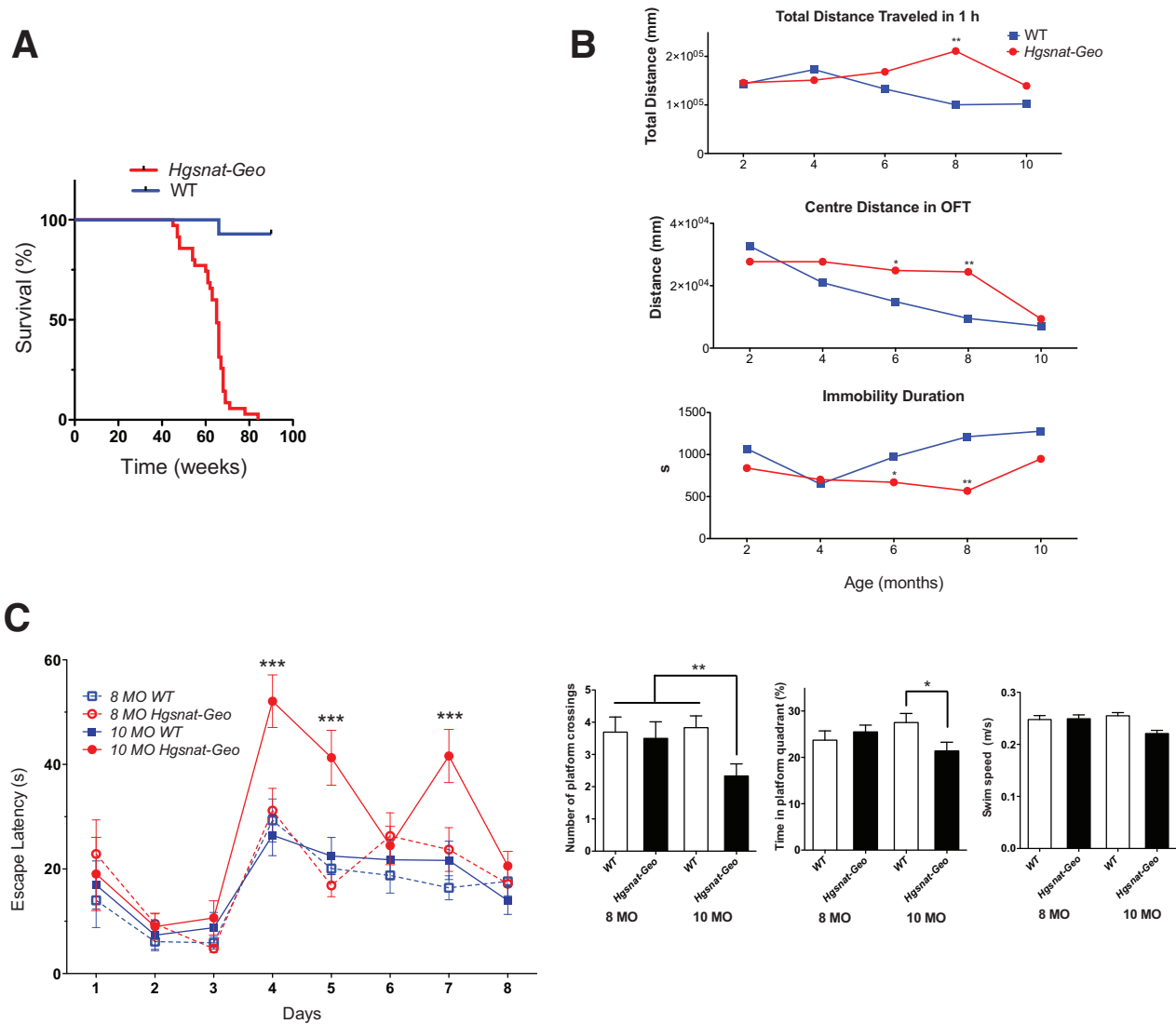


Figure 2 *Hgsnat-Geo* mice have shorter life span and show signs of hyperactivity between the ages of 6 and 8 months and learning impairment at the age of 10 months. **(A)** Kaplan-Meier plot showing survival of *Hgsnat-Geo* mice ($n = 50$) and their wild-type (WT) counterparts ($n = 70$). By the age of 70 weeks the vast majority of *Hgsnat-Geo* mice died or had to be euthanized on veterinarian request due to urinary retention. **(B)** Six and 8-month-old *Hgsnat-Geo* female mice show signs of hyperactivity and reduced anxiety compared to wild-type mice as detected by the open field test (OFT) performed 1 h into their light cycle. Increased activity (total distance travelled) was detected starting from ~5 months of age, with significant hyperactivity at 8 months. Reduced anxiety (increased centre activity) was detected starting from ~3 months, with significant difference with wild-type at 6 and 8 months. At 6 and 8 months *Hgsnat-Geo* mice also spent significantly less time immobile as compared to wild-type mice. P -value was calculated by two-way ANOVA ($*P < 0.05$, $***P < 0.01$). From six (2, 4 and 6 month-old) to 10 (8 and 10 month-old) naive mice were studied per age/per genotype. **(C)** *Hgsnat-Geo* mice showed impaired performance in the spatial memory-based Morris Water Maze test at 10 months (MO). All mice showed similar average latencies on Days 1–3 of visible platform testing. Whereas 8-month-old *Hgsnat-Geo* mice had latencies in the hidden platform testing similar to those of their wild-type counterparts (Days 4–8), 10-month-old mutant mice were significantly impaired in this spatial learning test. During the removed platform probe trial on Day 9, *Hgsnat-Geo* mice displayed reduced time in the target quadrant as compared to their wild-type siblings. Numbers of passes over the previous location of the hidden platform were also reduced. Swim speed was comparable among all groups. Six mice were studied for each group. P -value for escape latency was calculated by two-way ANOVA ($***P < 0.001$) and for the number of platform crossing and time in platform quadrant, by t -test ($*P < 0.05$, $**P < 0.01$).

In the spleen, storage dominated in splenic sinus endothelial cells giving them an appearance resembling that of foam cells (Supplementary Fig. 5). Population of macrophages in red pulp represented a mixture of small dendritic macrophages and slightly enlarged round cells containing

autofluorescent lipopigment and corresponding to phagocytic phenotype. Macrophages in white pulp revealed mostly an inconspicuous stellate appearance. In general, there was no convincing presence in spleen tissue of macrophages with storage material even at the most advanced

stages of disease (surviving 16-month-old mice). CTSD immunostaining was compatible with lysosomal storage in splenic sinus endothelial cells and was increased starting from 2 months (Supplementary Fig. 5A). Electron-lucent storage vacuoles in splenic sinus endothelial cells were also detected by electron microscopy (Supplementary Fig. 5I).

Quantification of NeuN-stained neurons in the two selected areas of somatosensory cortex showed that neuronal loss in homozygous *Hgsnat-Geo* mice actually occurs starting from the age of 6 months and continues throughout life resulting, by the age of 12 months, in the >30% reduction of neuronal density in these areas (Fig. 3A). In the cerebellar cortex, Purkinje cell loss was massive in the anterior lobe with lobules I, II and III most severely affected and lobules IX and X less affected at 12 months of age (Supplementary Fig. 6). The number of GFAP-positive astrocytes and CD68-positive microglia cells became augmented starting from 4 months in all studied brain areas of the *Hgsnat-Geo* mice as compared with wild-type mice and was further increased with age (shown for somatosensory cortex in Fig. 3B).

The most striking phenomenon observed in all parts of the brain was a coarse vacuolization of the cytoplasm of multiple CD68-positive cells giving them foam cell-like appearance (Fig. 3C). These cells dispersed in grey and less so in white matter, were frequently found adjacent to neurons suggesting that they represent perineuronal microglia. Individual macrophage-like CD68-positive cells with cytoplasmic vacuoles were also detected in perivascular areas. Microglia cells containing storage materials were most prominent in caudatoputamen and in brain cortex. They were first detected in 2-month-old mice, thus preceding pathological changes in neurons and become more frequent at later stages. Contrary to microglial cells, neurons were mostly featured by finely granular appearance of their perikarya without marked distension. The granular material accumulated in these neurons with a variable intensity was strongly PAS-positive (Fig. 3D) and displayed intense autofluorescence (Fig. 3E). Immunostaining with antibodies against CTSD (Fig. 3F) revealed increased number and size of lysosomes in neurons of mutant mice. Neurons strongly stained with CTSD were found in brain cortex, thalamus, hypothalamus, amygdala, midbrain, pons and cerebellum (Supplementary Fig. 7). Accumulation of PAS-positive and autofluorescent materials was most prominent in neurons present in deep cortical layers, in hippocampus and in cerebellum. Neurons in caudatoputamen showed little involvement. Incipient neuronal storage was detectable in 5-month-old mice and widespread yet irregular neuronal storage was present in 12-month-old animals. No signs of lysosomal storage were detected in vascular endothelial cells (data not shown).

Electron microscopy performed in brain cortex confirmed that neurons and microglial cells had two distinct types of storage (Fig. 4). Cortical microglia displayed prominent accumulation of electron-lucent storage vacuoles or those

with a sparse fine content. In contrast, neuronal pathology was dominated by progressive lysosomal storage of electron-dense heterogeneous ceroid-like material and early detectable mitochondrial structural abnormalities. Mitochondria were pleomorphic and increased in number with many of them swollen with disorganized or reduced inner membranes. Storage in microglia was not accompanied by any structural mitochondrial changes. Storage compartments in neurons contained heterogeneous, granular and/or lamellar material, occasionally with lipid droplets. At the same time, electron microscopy of neurons did not reveal lysosomes containing classical zebra-bodies, but some storage deposits contained structures resembling degenerated mitochondria. At the most advanced stages of the disease (14–16 months), ultrastructural pattern in neuronal lysosomes was dominated by massive accumulation of closely packed fibrillary deposits often resembling storage material of a rectilinear and/or fingerprint type in neuronal ceroid lipofuscinoses (Fig. 4).

Other studied mouse organs and tissues stayed generally unaffected even at 12 months of age except for urinary bladders, which were markedly distended with thin walls. A sparse presence of cells strongly stained for CTSD and presumably having lysosomal storage was detected in lamina, muscularis propria and adventitia. Normally arranged cardiomyocytes without regressive changes were observed in the heart. There was no fibrosis in the interstitium and interstitial elements did not show signs of lysosomal storage at the optical level. Similarly, lysosomal storage was not detectable in alveolar septa in lungs. Population of septal and alveolar macrophages was rich, with focally increased phagocytic activity and undistinguishable from that seen in wild-type. Respiratory bronchial epithelium displayed increased staining for CTSD suggestive of lysosomal storage. In kidney, increased immunostaining for CTSD was detected in glomeruli and in epithelial cells of distal tubules and collecting ducts (Supplementary Fig. 5E). Storage of electron-lucent vacuoles in fibroblasts, vascular pericytes and rarely in vascular endothelial cells was detectable at the ultrastructural level (Supplementary Fig. 5J–L).

Lysosomal glycosaminoglycans and ganglioside storage in *Hgsnat-Geo* mice

To investigate if HGSNAT deficiency in mouse tissues resulted in impairment of heparan sulphate catabolism, total glycosaminoglycan in mouse brain and liver tissues were measured at the age of 2, 4, 6, 8, 10 and 12 months (Fig. 5A). In both tissues, glycosaminoglycan levels were slightly increasing with age while remaining significantly higher than in the wild-type mice (on average 2-fold increase in brain and almost 10-fold increase in liver at 12 months). At the same time, total glycosaminoglycan in

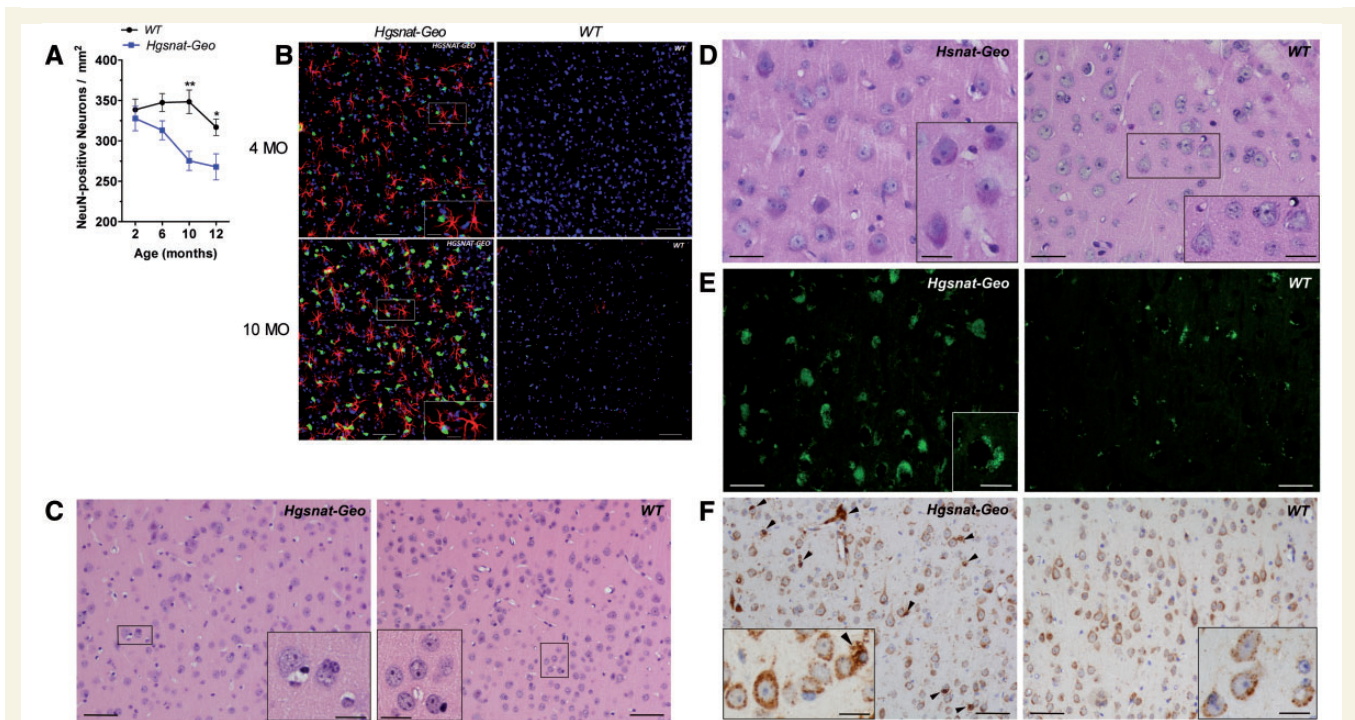


Figure 3 Pathological changes in CNS of *Hgsnat-Geo* mice. (A) Progressive loss of neurons in somatosensory cortex. NeuN-positive neurons were counted by two observers blinded for the mouse genotype in two similar fields on three sagittal sections (cut 1.44, 1.68 and 1.92 mm from bregma) of S1 somatosensory cortex of each mouse; four (two male, two female) mice were studied for each age and each genotype. Two-way (age and genotype) ANOVA was used to test differences between the mouse groups: significant differences between the genotypes at a given age in Bonferroni post-test ($*P < 0.05$, $**P < 0.01$) are shown. (B) Increased numbers of GFAP-positive astrocytes and C68-positive microglia are detected in the somatosensory cortex of *Hgsnat-Geo* mice. Somatosensory cortex (layer V) of 4- and 10-month-old *Hgsnat-Geo* mouse and wild-type littermate control (WT); slides stained with chicken polyclonal antibodies against GFAP (AbCam ab4674, 1/600) and antibodies against mouse CD68 (MCA 1957, 1/400), followed by DyLightTM 549-conjugated goat anti-chicken IgG (Jackson ImmunoResearch Laboratories 103-505-155, 1/50) and Alexa Fluor[®] 488-conjugated goat anti-rat IgG (Invitrogen, 1/400) antibodies. The nuclei were stained with DRAQ5TM (Thermo Scientific 62254, 1/1000). Scale bars = 50 μ m; inserts = 20 μ m. (C) Presence in cortical grey matter of microglial cells with vacuolized cytoplasm and foam-like appearance. Microglial cells showing storage are either dispersed or adjacent to neurons (shown in details in the insert). Somatosensory cortex (layer V) of 12-month-old *Hgsnat-Geo* mouse and wild-type littermate control (WT); haematoxylin and eosin stain. Scale bars = 100 μ m; inserts = 30 μ m. (D) Accumulation of PAS-positive granular material is detectable in perikarya of multiple neurons in the brain of *Hgsnat-Geo* mouse. Microglia show presence of storage and foam-like appearance. Brain of wild-type littermate control displays only discrete PAS-positive deposits corresponding with age pigment. Inserts show detailed views of neurons and microglia. Somatosensory cortex (layer V) of 12-month-old *Hgsnat-Geo* and wild-type mice. Scale bars = 50 μ m; inserts = 30 μ m. (E) A massive accumulation of granular autofluorescent ceroid is widely present in cortical neurons of 12-month-old *Hgsnat-Geo* mouse. Insert shows a detailed view of a neuron with ceroid deposits in the perikaryon. Brain of wild-type littermate control contains a small amount of autofluorescent lipofuscin in individual neurons. Somatosensory cortex (layer V) of 12-month-old *Hgsnat-Geo* mouse. Scale bars = 100 μ m; insert = 30 μ m. (F) Increased CTSD immunostaining comparable with lysosomal storage in microglia and neurons of 12-month-old *Hgsnat-Geo* mouse. Dispersed, perineuronal or perivascular microglia is strongly positive for cathepsin D (marked by arrowheads). Lysosomal system in neurons is irregularly activated and shows a coarsely granular appearance. Neuronal perikarya are not markedly distended in contrast to microglia. Inserts show detailed views of both cell types. Somatosensory cortex (layer V) of 12-month-old *Hgsnat-Geo* and wild-type mice. Scale bars = 100 μ m, insert = 30 μ m. Panels show representative images of at least 30 studied for four *Hgsnat-Geo* and four wild-type mice. MO = months.

brain tissues of *Hgsnat-Geo* mice at all ages was significantly lower than in MPS IIIB mice.

Glycosaminoglycan accumulation at the cellular level was studied by immunohistochemistry using monoclonal 10E4 antibody specific against a native heparan sulphate epitope (David *et al.*, 1992), which detected increased staining of cytoplasmic LAMP1-positive organelles in multiple cells throughout the somatosensory cortex and all regions of the hippocampus. The lysosomal heparan sulphate storage was detected in multiple NeuN-positive neurons, but even

more intense 10E4 staining was detected in the cells negative for NeuN, but positive for the markers of activated microglia cells: Alexa Fluor[®] 568-labelled isolectin B4 (ILB4), and CD68 (Fig. 5B).

Tandem mass spectrometry demonstrated \sim 2-fold increased levels of G_{M2} and G_{M3} gangliosides in the brain tissues of 4-month-old *Hgsnat-Geo* mice as compared to their wild-type siblings (Fig. 5C), consistent with previously reported storage of these lipids in the mouse models of MPS IIIA and B (Bhaumik *et al.*, 1999; Li *et al.*, 1999).

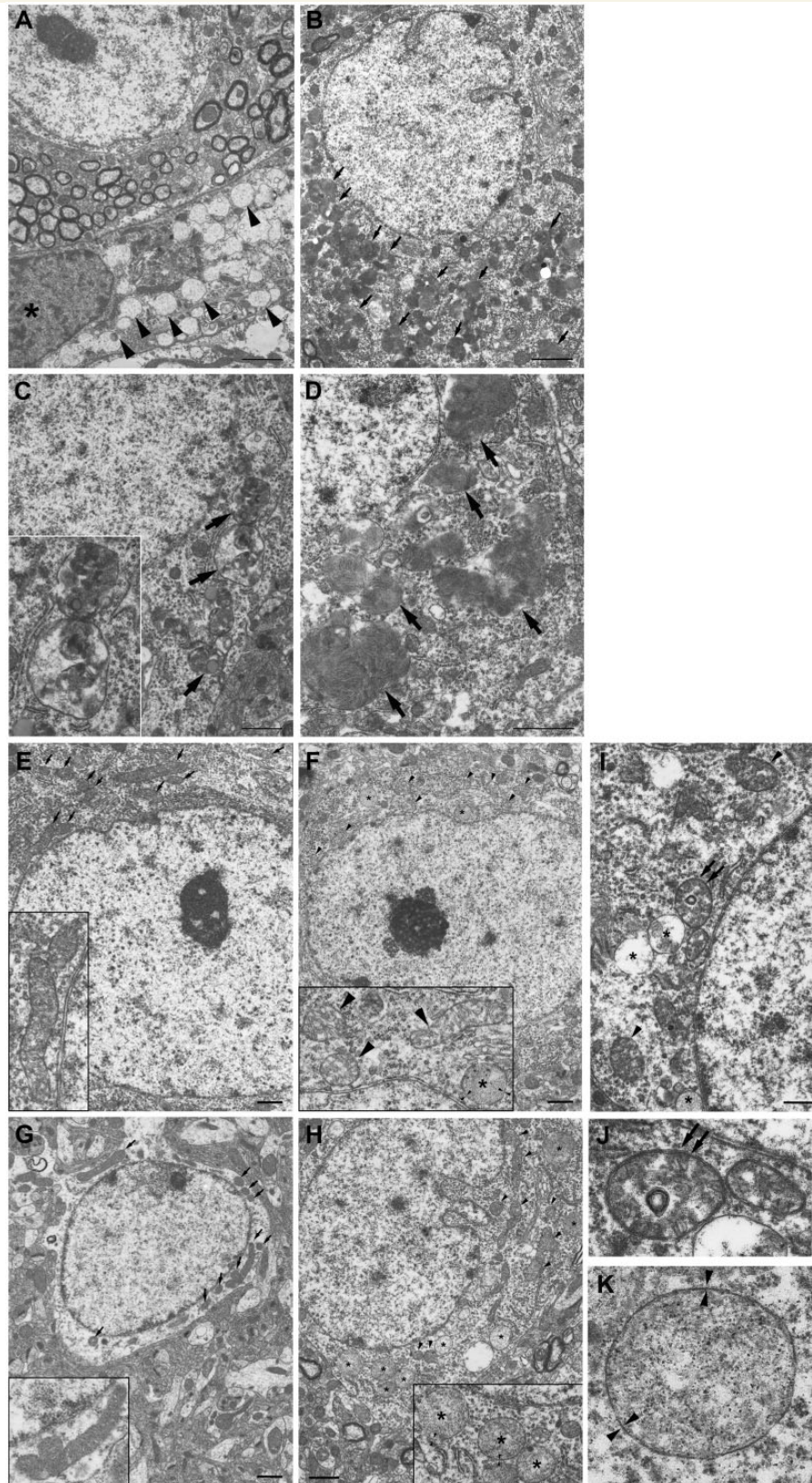


Figure 4 Ultrastructural pathology in the brain of *Hgsnat-Geo* mice. (A) Storage pattern in microglia detected at 5 months. Massive accumulation of vacuoles with single limiting membranes and a sparse fine content in the cytoplasm of a cortical microglial cell is compatible with lysosomal glycosaminoglycan storage. Lysosomes containing storage materials are marked by arrowheads. The microglial cell (nucleus is marked by an asterisk) is in a close proximity to a cortical brain neuron. Scale bar = 2 μ m. (B) Lysosomal system in a cortical neuron at 12 months is expanded and massively overloaded by electron dense material (marked by arrows). Scale bar = 2 μ m. (C) High magnification micrograph of a

(continued)

Accumulation of gangliosides in brain tissues was further studied by immunohistochemistry using the human-mouse chimeric monoclonal antibody, KM966 (Nakamura *et al.*, 1994) specific to G_{M2} ganglioside (Huang *et al.*, 1997) and mouse monoclonal antibody specific to G_{M3} ganglioside. Both gangliosides, almost undetectable in the brain of wild-type mice (Supplementary Fig. 8), were highly present in the brains of *Hgsnat-Geo* mice (Fig. 5D). The ganglioside storage was observed in most areas of the brain, including the cerebellum, but was more prominent in deep layers of cortex and hippocampus (Fig. 5D and Supplementary Fig. 8).

All ganglioside-accumulating cells were recognized by the anti-NeuN antibody and were not stained with ILB4 indicating that they are neurons but not microglial cells (Fig. 5D). In contrast to glycosaminoglycans accumulation, both the number of ganglioside-positive granules in the hippocampal and cerebellar neurons and their size were dramatically increasing between the ages of 8 and 12 months (Supplementary Fig. 8). Intriguingly, when the tissue sections were co-stained for both G_{M3} and G_{M2} gangliosides, we detected only modest level of co-localization indicating that the corresponding storage granules tend to segregate from each other (Fig. 5D). A similar pattern was previously observed in MPS IIIA and B mouse models (McGlynn *et al.*, 2004). Also, we observed only partial co-localization between the stored gangliosides and lysosomal marker LAMP1 (Fig. 5D), which could indicate that the gangliosides are accumulated in the compartments having both lysosomal and non-lysosomal origin.

When we, however, co-stained the cells for gangliosides and the mitochondrial marker cytochrome *c* oxidase subunit 4 (COX4I1) we detected a high degree of co-localization (Fig. 5D) suggesting that some of the storage granules could appear in the result of impaired mitophagy also consistent with the results of the electron microscopy analysis of neurons (as described above). COX4I1 staining also revealed that the mitochondrial network is less organized and the ratio between the fused and single mitochondria is

reduced in the tissues of the mutant mice (Supplementary Fig. 9).

Brain tissues of *Hgsnat-Geo* mice show altered autophagy, impaired proteolysis and accumulation of misfolded proteins

Impaired autophagy associated with decreased lysosomal and proteosomal proteolysis was found to be a characteristic feature of cells in many lysosomal disorders (Bifsha *et al.*, 2007; Settembre *et al.*, 2013). To test whether it is also the case for liver and brain tissues of HGSNAT-deficient mice, we have analysed the relative abundance of the two forms of light chain three protein (LC3, now known as MAP1LC3A). During the formation of the autophagosome a cytosolic form of LC3 (LC3-I) is cleaved and conjugated with phosphatidylethanolamine to form the LC3-phosphatidylethanolamine conjugate (LC3-II) tightly associated with the autophagosomal membranes, so the amount of LC3-II or the presence of LC3-positive punctate in the cytoplasm reflects the existence of autophagosomes. In liver tissues of *Hgsnat-Geo* mice the increased levels of the LC3-II were detected at all ages indicating increased autophagosomal genesis or decreased macroautophagic flux (Supplementary Fig. 10). In the brain, the increase of LC3-II was also detected, but only at the age of 6 months and older (Fig. 6A).

The results of western blot were consistent with the results of immunohistochemistry that showed presence of LC3 in the cytoplasm of medial entorhinal cortex neurons at 10 months of age (Fig. 6B). We also detected drastically increased neuronal levels of SCMAS (subunit C of mitochondrial ATP synthase) aggregates and ubiquitin suggestive of mitophagy and a general impairment of proteolysis. We also detected increased levels of O-GlcNAc-modified proteins, an indication of the endoplasmic reticulum stress

Figure 4 Continued

cortical neuron at 12 months demonstrates a storage compartment (marked by arrows) containing ceroid-like granular and/or lamellar material packed with a variable density as well as occasional lipid droplets. Insert shows a detailed view of storage deposits. Scale bar = 1 μ m. (D) Storage of densely packed fibrillary material (marked by arrows) strongly resembling rectilinear or fingerprint-like ceroid deposits in neuronal lysosomes at advanced stages of the disease. Brain cortex of 16-month-old *Hgsnat-Geo* mouse. Scale bar = 1 μ m. Mitochondrial population in neurons of wild-type mice at the age of 5 (E) and 12 months (G) is relatively uniform and is composed of normally shaped mitochondria (marked by arrows) with largely regular cristae (details are shown in the inserts). Scale bar = 1 μ m. Mitochondria in a neuron of *Hgsnat-Geo* mouse at the age of 5 months (F) are relatively numerous and pleiomorphic with some of them revealing incipient swelling (marked by arrowheads). Oedematous mitochondria with largely dissolved cristae (marked by asterisks) but with remnants of double membranes (marked by double arrowheads) were detected occasionally. A detailed view of incipient mitochondrial abnormalities is shown in the insert. Scale bar = 1 μ m. At the age of 12 months (H), mitochondria in neurons of *Hgsnat-Geo* mice are clearly pleiomorphic with many of them displaying swelling and disorganization of their inner membranes (marked by arrowheads). Substantially oedematous mitochondria with largely dissolved cristae are marked by asterisks. Insert shows a detailed view of highly oedematous mitochondria with remnants of double membranes (marked by double arrowheads). Scale bar = 1 μ m. (I) A higher magnification demonstrates structural mitochondrial abnormalities in a neuron of *Hgsnat-Geo* mouse at 12 months. Mitochondria are swollen with disorganized (arrowheads) or even dissolved (asterisks) cristae and occasionally contain circular formations (marked by double arrows). Scale bar = 500 nm. (J) A detailed view of a mitochondrion with inner membranes forming circular structures. (K) A detailed view of a highly oedematous mitochondrion with dissolved cristae but with detectable remnants of double membranes (marked by double arrowheads). Panels show representative images of at least 30 studied for 3 *Hgsnat-Geo* and 3 wild-type mice for each age.

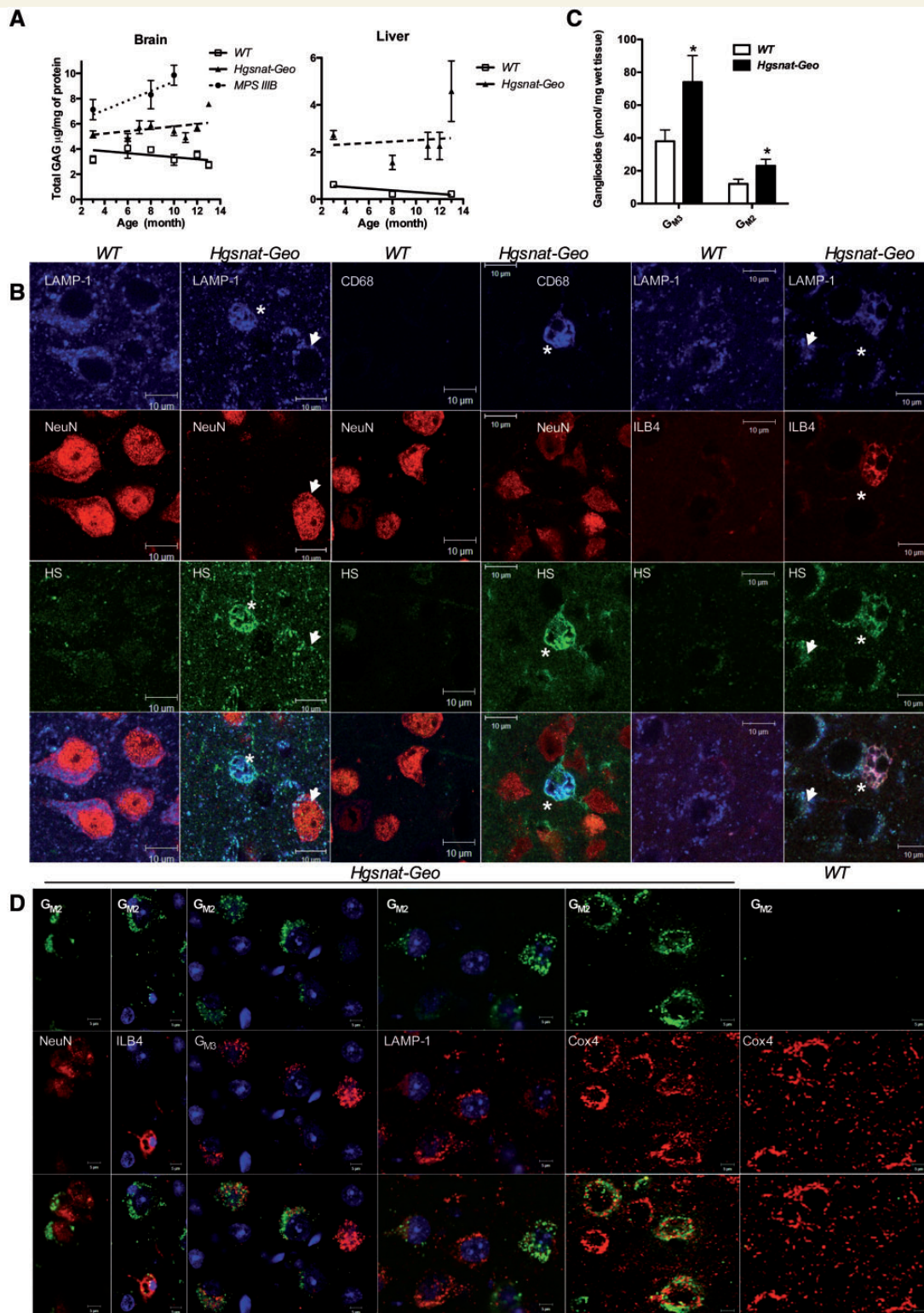


Figure 5 Accumulation of primary and secondary storage materials in tissues of *Hgsnat-Geo* mice. (A) Total glycosaminoglycans were measured in the whole brain tissues of *Hgsnat-Geo* mice, α -N-acetylglucosaminidase-deficient (MPS IIIB) mice and their corresponding wild-type controls as well as in liver tissues of *Hgsnat-Geo* and wild-type mice. The data show means (\pm SD) of individual measurements performed with 15 mice for each age and genotype. (B) Intralysosomal accumulation of heparan sulphate in the brain cells of *Hgsnat-Geo* mice. Sagittal brain sections (40- μ m thick) of 4-month-old wild-type and *Hgsnat-Geo* mice were stained with anti-heparan sulphate and anti-LAMP1 antibodies and either antibody against neuronal marker NeuN or microglial marker, isolectin B4 (ILB4) or with antibodies against heparan sulphate, NeuN and CD68. Somatosensory cortex of wild-type mouse does not show heparan sulphate (green) staining while that of *Hgsnat-Geo* mouse contains multiple neurons (arrowheads) or microglia (asterisks) with lysosomal accumulation of heparan sulphate. Foamy microglia (negative for neuronal

(continued)

often associated with impaired cellular proteolysis (Chatham and Marchase, 2010) (Fig. 6C).

Recent studies have demonstrated that neurons in certain brain areas of MPS IIIB and MPS IIIA mice, primarily dentate gyrus and medial entorhinal cortex involved in learning and memory have increased levels of protein markers of Alzheimer disease and other tauopathies leading to dementia such as lysozyme, hyperphosphorylated tau, phosphorylated tau kinase, GSK3B, and amyloid- β (Ohmi et al., 2009, 2011). All of these markers are also increased in the brains of HGSNAT-deficient mice (Supplementary Fig. 11) although their levels are somewhat lower than those in the MPS IIIA and MPS IIIB mice of similar age (Ohmi et al., 2011).

Mitochondrial energy metabolism is compromised in brain tissues of *Hgsnat-Geo* mice

To verify whether mitochondrial energy metabolism is affected in the brain cells of *Hgsnat-Geo* mice we measured activities of several mitochondrial respiratory chain enzymes including NADH:CoQ reductase (NQR, complex I), succinate:CoQ reductase (SQR, complex II), cytochrome *c* oxidase (COX, complex IV), NADH:cytochrome *c* reductase (NCCR, complex I + III), succinate:cytochrome *c* reductase (SCCR, complex II + III) in the isolated brain mitochondria. We also measured activities of pyruvate dehydrogenase complex and citrate synthase.

Activities of complex IV (COX) and complex II (SQR) enzymes were significantly lower in *Hgsnat-Geo* mice than in the corresponding wild-type controls at the ages of 8 and 12 months and 8 months, respectively (Fig. 7A). No significant differences in activities of enzymes of complex I, complex I + III, complex III, and complex II + III of respiratory chain as well as pyruvate dehydrogenase and control mitochondrial enzyme citrate synthase were found between *Hgsnat-Geo* and wild-type mice. However, when we analysed the activities of individual enzymes as a function of mouse age, we found that the activities of complex II (SQR), complex II + III (SCCR) and citrate synthase in *Hgsnat-Geo* mice decreased significantly with age, whereas no dependence was detected for the wild-type animals (Fig. 7B).

Consistent with the gradual reduction of the activities of respiratory chain enzymes was the observed decrease of the total content of coenzyme Q10 in the brain tissues of *Hgsnat-Geo* mice. At the age of 4 months the levels of

Q10 in the wild-type and HGSNAT-deficient mice were the same, but in the wild-type mice of older age Q10 stayed at the same level; in the brains of *Hgsnat-Geo* mice it showed a negative correlation with age and at the age of 12 months became significantly lower than that in wild-type mice (Fig. 7).

Progressive neuroinflammation in *Hgsnat-Geo* mice

Chronic progressive neuroinflammation is well documented in mouse models of lysosomal neurodegenerative diseases including those of MPS IIIA and B, which are characterized by activation of resident microglia and astrocytes, infiltration of leucocytes from the periphery and production of the inflammatory cytokines (Ohmi et al., 2003; Ausseil et al., 2008; Wilkinson et al., 2012). Two-fold increased expression of inflammation markers, MIP1 α (CCL3) and TNFA, in the brains of homozygous *Hgsnat-Geo* mice was detected as early as 10 days after birth. The levels of these cytokines further increased with age reaching the maximum at 8 months of age (Fig. 8A). At the same time, IL1B and TGFB1 were not significantly increased as compared with wild-type controls. Similarly increased expression of MIP1 α (CCL3) and TNFA cytokines was detected also in the brain tissues of MPS IIIA mice, whereas MPS IIIB mice had ~2-fold higher levels at all ages.

Consistent with high expression level of cytokines brain tissues of HGSNAT-deficient mice showed increased levels of activated microglia cells detected by isolectin B4 staining (Fig. 8B) or antibodies against CD68 (Fig. 3B), whereas almost no staining was detected in matching controls. Besides, significantly higher amount of astrocytes stained with anti-GFAP antibodies was detected in the somatosensory cortex of mutant mice starting from the age of 4 months (Fig. 3B).

Signs consistent with an immune reaction were detected also in the liver. Early activation of Kupffer cells preceding the substantial storage and increased presence in liver sinusoids of inflammatory cells was detectable in *Hgsnat-Geo* mice but not in wild-type mice (Supplementary Fig. 4).

Discussion

Altogether our data demonstrate that HGSNAT deficiency in mice results in lysosomal accumulation of heparan

Figure 5 Continued

marker, NeuN, but positive for ILB4 or CD68) show on average an increased staining for heparan sulphate compared to neurons. (C) Levels of G_{M3} and G_{M2} gangliosides are increased in the total brain extracts of 4-month-old *Hgsnat-Geo* mice as compared to their wild-type siblings. Total lipids from brain tissue homogenates were extracted with chloroform/methanol 1:1 mixture and analysed by tandem mass spectroscopy. (D) Accumulation of gangliosides in cortical neurons of *Hgsnat-Geo* mice. Forty μ m-thick sagittal brain sections of 8-month-old wild-type and *Hgsnat-Geo* mice were stained with anti-G_{M2} antibodies and either microglial marker, isolectin B4 or antibodies against neuronal marker NeuN, G_{M3} ganglioside, LAMP1 and mitochondrial marker, cytochrome *c* oxidase subunit 4 (Cox4, now known as COX4I1). Slides were studied on a Zeiss LSM510 inverted confocal microscope. Panels show representative images of at least 30 studied for three *Hgsnat-Geo* and three wild-type mice for each age.

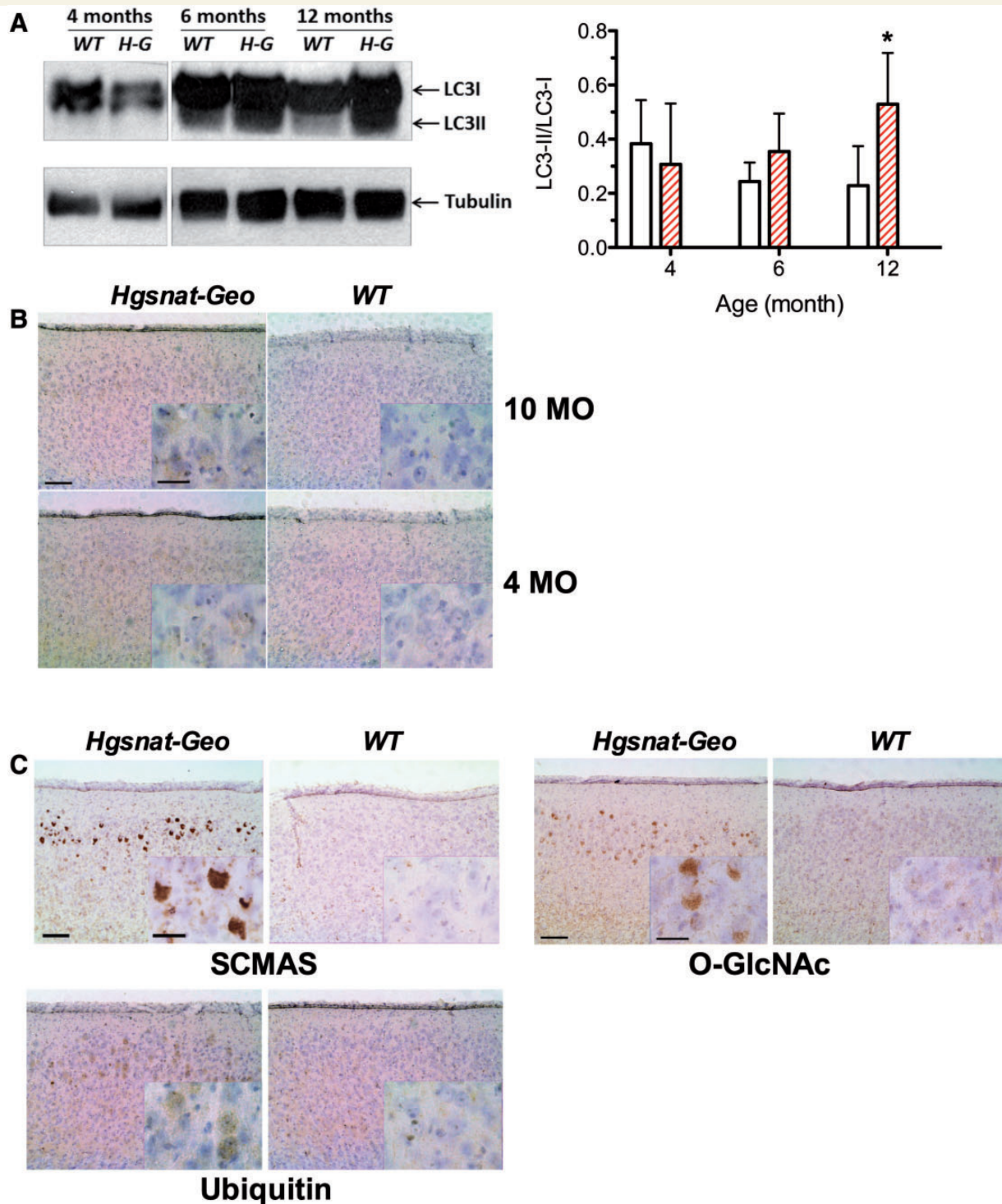


Figure 6 Brain tissues of *Hgsnat-Geo* mice show altered autophagy and lysosomal proteolysis. **(A)** Increased levels of LC3-II were detected in the brain tissues of *Hgsnat-Geo* (H-G) mice at the age of 6 and 10 months but not at the age of 4 months by western blot. Total mouse brains were homogenized in 250 mM sucrose buffer and after removal of nuclei proteins were solubilized with 1% Triton™ X-100. Protein extracts were analysed by western blotting using antibodies specific for mouse LC3 and β -tubulin as a loading control. Panel shows representative data of three independent experiments performed with three *Hgsnat-Geo* and three wild-type mice for each age. Inset graph shows ratios (means and SD) of signal intensities for LC3-II and LC3-I estimated with ImageQuant software. * $P < 0.05$ in unpaired two-tailed t-test. **(B)** LC3 staining was present in the cytoplasm of medial entorhinal cortex neurons of 10-month-old but not of 4-month-old *Hgsnat-Geo* mice. **(C)** Signs of impaired lysosomal proteolysis and endoplasmic reticulum stress in the neurons of *Hgsnat-Geo* mice. SCMAS-positive aggregates, O-GlcNAc-modified proteins and increased staining for ubiquitin were detected in medial entorhinal cortex neurons of 10-month-old *Hgsnat-Geo* mice. Scale bars = 100 μ m; insert = 30 μ m.

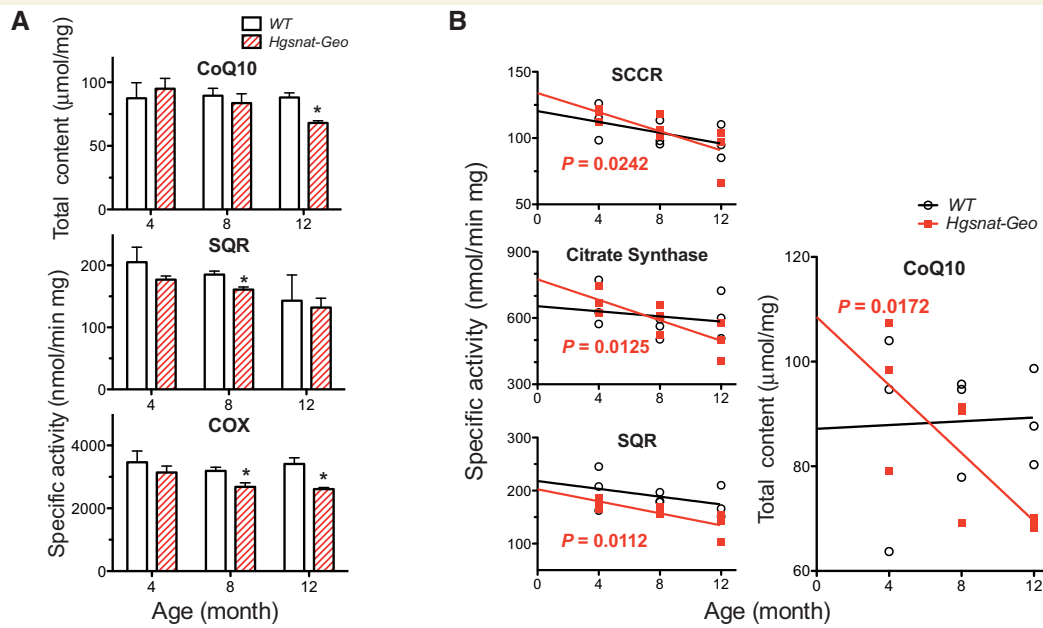


Figure 7 Partial impairment of mitochondrial oxidative phosphorylation system in the brains of *Hgsnat-Geo* mice. (A) Activity of succinate: CoQ reductase (SQR, complex II) and cytochrome c oxidase (COX, complex IV) in isolated brain mitochondria of *Hgsnat-Geo* mice are reduced as compared with age-matching wild-type controls. Total content of coenzyme Q10 in brain homogenate of *Hgsnat-Geo* mice is reduced as compared with wild-type controls. The data show means (\pm SD) of individual measurements. Three mice were analysed for each age and each genotype. * $P < 0.05$ in unpaired two-tailed *t*-test. **(B)** Correlation between the age and activities of succinate: CoQ reductase (SQR), succinate: cytochrome c reductase (SCCR), citrate synthase (CS), and total coenzyme Q10 content in the brain tissues of *Hgsnat-Geo* and wild-type mice was analysed by the linear regression method. For all parameters in the tissues of *Hgsnat-Geo* but not of wild-type mice the slopes of linear regression lines showed a significant deviation from zero ($P < 0.05$). Three mice were analysed for each age and each genotype.

sulphate, in multiple cell types, including brain neurons and microglia. In brain neurons heparan sulphate accumulation is accompanied by secondary accumulation of gangliosides and increased lysozyme, phosphorylated tau, GSK3B, and amyloid- β levels. Signs of general inflammation in the brain including activation of astrocytes, microglia and cytokine production, previously reported for other MPS mouse models are clearly present from the very early age. At the same time a lower level of glycosaminoglycans storage as compared to the MPS IIIB mouse model results in a reduced level of brain inflammation, later onset and slower progression of the disease.

The main organs affected at the optical and ultrastructural levels are the brain, liver and spleen. Lysosomal storage consistent with accumulation of undegraded glycosaminoglycans developed early in hepatocytes, splenic sinus endothelium, and cerebral microglia, and to a lesser extent in liver Kupffer and Ito cells, in fibroblasts and in perivascular cells. In peripheral tissues, storage in epithelial and mesenchymal cells, except for hepatocytes occurred later and with a lesser intensity as compared to mouse MPS IIIA and MPS IIIB models (Bhaumik *et al.*, 1999; Li *et al.*, 1999). Presence of macrophages with storage materials in visceral organs was limited, except for liver Kupffer cells, which were activated and developed storage phenotype with age.

In the brain, substantial heparan sulphate storage in microglia was found as early as at 2 months and seems to be the initial pathological event more evident at the age of 2–4 months then pathological changes in neurons, and possibly explained by high phagocytic activity of microglia causing an extracellular substrate burden. The storage was further increased with age in all examined brain regions. Foam microglial cells were frequently in close contact with neurons. Storage vacuoles in microglia had a uniform electron-lucent appearance different from ceroid-type accumulation in neurons. The apparently different storage patterns detected in microglia and neurons by electron microscopy could be also attributed to the loss of glycosaminoglycans during fixation of the cells; however, further immunohistochemical analysis confirmed that lysosomal storage of heparan sulphate in microglia exceeded that in neurons, whereas gangliosides accumulated mainly in neurons.

Neuronal pathology was also featured by cytoplasmic accumulation of densely packed material in neuronal perikarya, which was likely a source of strong autofluorescence and positive immunostaining for SCMAS. Storage of granular autofluorescent material restricted to individual neurons at 5 months was detected throughout the whole neuronal population at 12 months, but even at this advanced stage, neurons showed variations in the amount

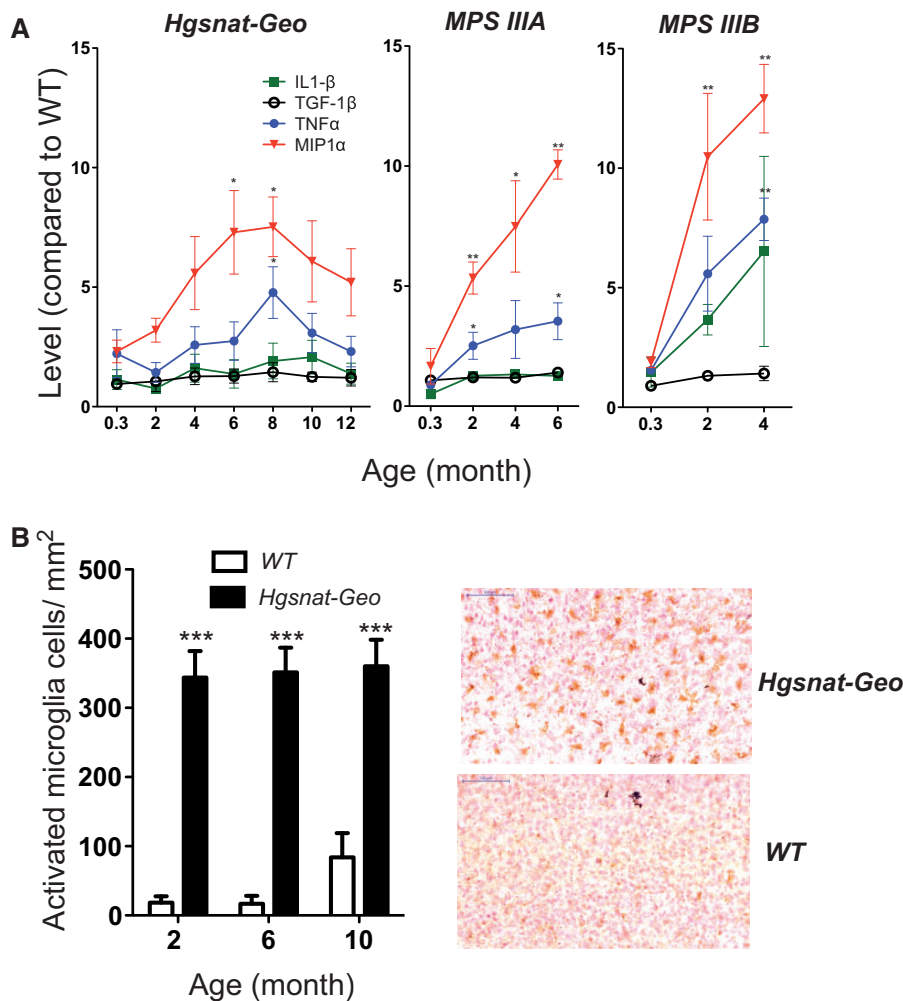


Figure 8 Brain inflammation in *Hgsnat-Geo* mice. (A) Total brain tissues of *Hgsnat-Geo* mice show progressively increased expression of inflammation markers, $MIP1\alpha$ (CCL3) and $TNFA$ similar to those in MPS IIIA, but lower than those in MPS IIIB mouse models. Total RNA was isolated from whole mouse brain, reverse-transcribed to cDNA and quantification of cytokines was performed by real time quantitative PCR. The data show ratios of the cytokine levels in *Hgsnat-Geo* mice and those in wild-type (WT) controls (both normalized for the content of $RLP32$ mRNA). Data show mean values (\pm SD). At least three mice were analysed for each age, sex and genotype. Two-way ANOVA was used to test differences between the mouse groups: significant differences between the mean values in Bonferroni post-test ($*P < 0.05$, $**P < 0.001$) are shown. (B) Increased number of activated microglial cells in brain cortex of *Hgsnat-Geo* mice. Sagittal brain sections (40- μ m thick) of wild-type and *Hgsnat-Geo* mice were stained with horseradish peroxidase-conjugated microglial marker isolectin B4/DAB and counter-stained with haematoxylin, as described (Wilkinson *et al.*, 2012). The total number of microglial cells was counted for three adjacent 0.25 mm² sections of somatosensory cortex. Data show mean values (\pm SD). Two mice were analysed for each age, sex and genotype. $**P < 0.001$ in unpaired two-tailed t-test.

of stored materials between brain regions and between cells within a region. Similar heterogeneous storage character was reported for the other MPS disease mouse models (McGlynn *et al.*, 2004). Neuronal loss was not a dominant feature at the early stage of the disease but it became significant at 10 months and further progressed with age. At the electron microscopy level, neuronal pathology was characterized by a combination of early detectable structural alterations in mitochondria, progressive lysosomal storage of heterogeneous material and massive lysosomal accumulation of deposits resembling those detected in neuronal ceroid lipofuscinoses. Similar ‘fingerprint-like’

structures were previously detected in MPS IIIB mouse and identified as SCMAS aggregates (Ryazantsev *et al.*, 2007). The mitochondrial network in neurons was disorganized and the partial impairment of OXPHOS enzymes (complex II, II + III and IV) in brain tissues was detected by enzymatic assays and immunohistochemical analysis, while mitochondrial compartment in age-matching controls did not reveal any abnormalities.

Taken together, our data characterize MPS IIIC as a neurodegenerative disorder with dominant lysosomal and mitochondrial alterations in neurons. Furthermore, our findings suggest that autophagy, namely mitophagy,

represents a substantial source for accumulation of undegraded materials in the lysosomal system of neurons.

The precise sequence of events that starts with the accumulation of heparan sulphate and leads to a widespread brain pathology and neuronal death is yet to be determined, however, our results allow hypothesizing that the malfunction and loss of neurons can be at least partially mediated by pathological changes in their mitochondrial system. We speculate that the disease starts with accumulation of primary storage materials (mostly heparan sulphate and heparan sulphate-derived oligosaccharides) as documented by electron microscopy analysis and immunohistochemistry with heparan sulphate-specific antibodies. These materials released presumably by exocytosis of lysosomes are known to induce general inflammation reactions in the brain by activating TLR receptors of microglia cells, resulting in release of multifunctional cytokines such as TNFA and MIP1 α (CCL3), known to cause mitochondrial damage through formation of reactive oxygen species and oxidative stress (Baregamian *et al.*, 2009; Chen *et al.*, 2010; Chuang *et al.*, 2012; Vitner *et al.*, 2012) eventually leading to neuronal death observed in the somatosensory cortex. In the cerebellar cortex, Purkinje cell degeneration typically observed in other lysosomal storage disorders (Sarna *et al.*, 2001; Macauley *et al.*, 2008) was also present. Although neurodegenerative disease can develop even without neuronal loss, it is important to mention that neuronal death is observed in MPS III patients (Hamano *et al.*, 2008). Ganglioside accumulation detected in neurons starting from 2 months is probably of non-lysosomal origin. It could be caused by altered Golgi function as described previously for MPS IIIB mouse model (Roy *et al.*, 2012). Stored lipids and gangliosides can also be partially of mitochondrial origin due to mitophagy and impaired catabolism of autophagosomal content. Indeed, our data define accumulation of densely packed material displaying a strong autofluorescence as a determining feature in brain neurons of MPS IIIC mice at the advanced stage of the disease. As these granules are SCMAS-positive and their ultrastructural pattern strongly resembles that in neuronal ceroid lipofuscinoses, we speculate that they are derived from autophagosomes. Impairment of autophagosome-lysosome fusion can also result in accumulation of deformed and dysfunctional mitochondria otherwise eliminated through autophagy and lysosomal catabolism (de Pablo-Latorre *et al.*, 2012; Osellame *et al.*, 2013). Our data suggest that autophagic alterations may be involved in the neuropathological changes in *Hgsnat-Geo* mouse, nevertheless it remains to be determined whether autophagy is impaired or in contrast increased, as the detected LC3-II accumulation can reflect both increased autophagy or defective proteolysis following formation of the autophagosome.

Together, our data validate *Hgsnat-Geo* mouse as an animal model relatively well matching the phenotype of human MPS IIIC. MPS IIIC stands out among lysosomal diseases as it is the only one caused by a deficiency of not a hydrolase but a transferase. It is also one of rare disorders

involving defects of an integral membrane protein untreatable by enzyme replacement or haematopoietic stem cell therapy. This makes the *Hgsnat-Geo* mouse a valuable model for experimental evaluation of the efficacy of novel therapeutic strategies that can be potentially applied for such disorders, including gene therapy, substrate deprivation therapy or novel methods of enzyme delivery. Further, our model can provide an important opportunity to better understand underlying pathogenic mechanisms of the disease, particularly regarding the CNS, given that human material is highly limited due to the relative rarity of MPS IIIC. Finally, the mechanism of brain disease in the MPS IIIC mouse model involving mitochondrial dysfunction and brain inflammation may represent a common phenomenon for metabolic neurodegenerative diseases. It also justifies future studies to determine if the mitochondrial defects in MPS IIIC cells can be at least partially rescued by known anti-oxidative drugs.

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Supplementary material

Supplementary material is available at *Brain* online.

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