BRAIN
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# Astrocytic glycogen accumulation drives the pathophysiology of neurodegeneration in Lafora disease

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The hallmark of Lafora disease, a fatal neurodegenerative disorder, is the accumulation of intracellular glycogen aggregates called Lafora bodies. Until recently, it was widely believed that brain Lafora bodies were present exclusively in neurons and thus that Lafora disease pathology derived from their accumulation in this cell population. However, recent evidence indicates that Lafora bodies are also present in astrocytes.

To define the role of astrocytic Lafora bodies in Lafora disease pathology, we deleted glycogen synthase specifically from astrocytes in a mouse model of the disease (malin$^{KO}$). Strikingly, blocking glycogen synthesis in astrocytes—thus impeding Lafora bodies accumulation in this cell type—prevented the increase in neurodegeneration markers, autophagy impairment, and metabolic changes characteristic of the malin$^{KO}$ model. Conversely, mice that over-accumulate glycogen in astrocytes showed an increase in these markers.

These results unveil the deleterious consequences of the deregulation of glycogen metabolism in astrocytes and change the perspective that Lafora disease is caused solely by alterations in neurons.

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Abbreviations: CAL = corpora amylacea-like bodies; MGS = muscle glycogen synthase; nLBs = neuronal Lafora bodies

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## Introduction

Glycogen, a branched polymer of glucose, is the only energy reservoir of the brain and it plays important roles in this organ.[1,2] Recent work has demonstrated that astrocytes and neurons have an active glycogen metabolism that is key to their normal functioning.[1–5] However, in some conditions, glycogen accumulates abnormally in the brain.[6,7] The most striking example of this is Lafora disease, a neurodegenerative condition that presents as a severe progressive myoclonus epilepsy characterized by the presence of glycogen aggregates, called Lafora bodies. Lafora bodies are intracellular, aberrant glycogen aggregates that accumulate in several tissues, including the brain. Patients with Lafora disease initially present with myoclonic jerks followed by epileptic seizures resistant to treatment. This phase is followed by rapid neurodegeneration, dementia and finally death within 5–10 years after onset.[8,9]

Lafora disease is caused by mutations in one of two genes: *EPM2A* encoding laforin, a dual-specificity phosphatase, and *EPM2B* encoding malin, an E3 ubiquitin ligase. Mutations in either gene cause the same disease, thus indicating that the two proteins form a functional complex.[10–12] Blocking brain glycogen synthesis in mouse models of Lafora disease prevents the progression of the disease.[13–15] This observation thus demonstrates that the accumulation of glycogen aggregates underlies the aetiopathology of all aspects of the disease.

Until recently, it was widely assumed that glycogen aggregates in the brains of patients with Lafora disease and animal models accumulate exclusively in neurons[8,16] and thus neuronal Lafora bodies were considered to be solely responsible for Lafora disease pathophysiology. In this regard, we demonstrated that glycogen accumulation in neurons induces their death by apoptosis.[17] However, there is a growing body of evidence showing that Lafora bodies are also present in astrocytes. In 2011, we reported the presence of glycogen aggregates in both neurons and astrocytes of the malin knockout mouse model (malin$^{KO}$).[18] More recently, we[19] and others[20] further described the presence of these aggregates in astrocytes of Lafora disease mouse models, by means of co-localization with cell type-specific markers. We classified the glycogen aggregates in the brain into two types: astrocytic corpora amylacea-like bodies (CAL) and neuronal Lafora bodies (nLBs). The former are analogous to corpora amylacea, which are glycogen aggregates that normally accumulate in aged brains.[21] In this regard, CAL: (i) are polymorphic; (ii) are found grouped in clusters; and (iii) co-localize with astrocytic markers. In contrast, nLBs: (i) are round and normally larger than CAL; (ii) co-localize with neuronal markers; and (iii) are not present in clusters but are isolated in the form of a single aggregate close to the neuronal nucleus.[19]

To determine which cell type contributes to the different Lafora disease pathophysiologies, we generated a malin$^{KO}$ mouse model in which the enzyme responsible for glycogen synthesis in the brain, namely the muscle isoform of glycogen synthase (MGS), is specifically ablated from astrocytes (malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$). Our results show that most glycogen aggregates observed in the brains of malin$^{KO}$ mice are not present in the malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ animals, thereby proving that these aggregates accumulate in astrocytes. Furthermore, the latter animals did not show an increase in neurodegeneration markers, autophagy impairment, or the metabolic changes typical of the malin$^{KO}$ mouse model, indicating that these symptoms are a consequence of the accumulation of astrocytic CAL. To corroborate these results, we generated mice that over-accumulate glycogen specifically in astrocytes. These animals also showed an increase in neurodegeneration markers. In contrast, the epileptic phenotype was not corrected in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice, indicating that the accumulation of astrocytic Lafora bodies is not the main driver of this symptom. Taken together, these results demonstrate the contribution of astrocytes to the pathology of Lafora disease and have important implications regarding the role of astrocytic metabolism in neurodegenerative diseases.

## Materials and methods

### Generation of malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ and 9A-MGS$^{Gfap}$ mice

To generate a malin$^{KO}$ mouse that is unable to accumulate glycogen in astrocytes (malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$), we took advantage of our MGS conditional knockout model, which is based on Cre-Lox technology.[1] The ablation of MGS specifically in astrocytes (MGS$^{Gfap\text{-}KO}$) was achieved by crossing this conditional knockout with mice expressing Cre recombinase under the control of a *Gfap* promoter (Gfap-Cre mice).[5] The resulting mouse was then crossed with malin$^{KO}$, the descendants were genotyped for the three alleles involved (malin, MGS and Cre), and the suitable genotypes were intercrossed to generate all the experimental groups. To generate mice that over-accumulate glycogen in astrocytes (9A-MGS$^{Gfap}$ mice), we took advantage of our mouse model conditionally expressing a non-inactivatable form of MGS (9A-MGS).[17] Again, we combined this animal with Gfap-Cre mice to drive the expression of 9A-MGS specifically to astrocytes.

### Animal studies

All procedures were approved by the Barcelona Science Park's Animal Experimentation Committee and were carried out following Spanish (BOE 34/11370-421, 2013) and European Union (2010/63/EU) regulations, and The National Institutes of Health guidelines for the care and use of laboratory animals. Mice were maintained in collective cages (up to five animals per cage) on a 12/12 h light/dark cycle under specific pathogen-free conditions in the Animal Research Center at the Barcelona Science Park. Animals were allowed access *ad libitum* to commercial mouse chow and water. After weaning at 3 weeks of age, tail clippings were taken for genotyping by quantitative PCR (performed by Transnetyx).

### Biochemical analyses

Mice were deeply anaesthetized and then decapitated, and the brain was quickly removed and placed in liquid nitrogen. Whole brains were then pulverized in liquid nitrogen. For western blot analyses, fractions of the powder were weighed and homogenated in 10 volumes of ice-cold buffer containing 25 mM Tris-HCl (pH 7.4), 25 mM NaCl, 1% Triton$^{TM}$ X-100, 0.1% SDS, 0.5 mM EGTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM NaF, 25 nM okadaic acid and a protease inhibitor cocktail tablet (Roche). Homogenates were loaded in 10% acrylamide gels for SDS-PAGE and transferred to Immobilon membranes (Millipore). Antibodies against MGS (from Cell Signaling), laforin (a gift from Dr Santiago Rodríguez de Córdoba) and p62 (from Progen) were used. Proteins were detected by the ECL method (Immobilon Western Chemiluminescent HRP Substrate, Millipore). Loading control of the western blot membrane was performed using the Revert$^{TM}$ total protein stain. For glycogen measurements, fractions of the brain powder were boiled in 30% KOH for 15 min and glycogen was determined by an amyloglucosidase-based assay, as previously described.[3] For quantitative (q)PCR, total RNA was isolated from fractions of brain powder using TRIzol® reagent (Life Technologies), purified with an RNeasy® Mini Kit (Qiagen) and treated with DNase I (Qiagen) to degrade genomic DNA. Reverse

transcription was performed using qScript cDNA Synthesis Kit (Quanta Biosciences). Quantitative PCR was performed using a QuantStudio™ 6 Flex (Applied Biosystems). The following mouse-specific SYBR® Green sets of primers (Sigma) were used: *C3* (forward: 5′-TCCTGAACTGGTCAACATGG-3′; reverse: 5′-AAACTGGG CAGCACGTATTC-3′); *Ccl2* (forward: 5′-AGGTGTCCCAAAGA AGCTGTAG-3′; reverse: 5′-TCTGGACCCATTCCTTCTTG-3′); *Lcn2* (forward: 5′-CAGAAGGCAGCTTTACGATG-3′; reverse: 5′-CCTGG AGCTTGGAACAAATG-3′); *Cxcl10* (forward: 5′-CCGTCATTTTC TGCCTCATC-3′; reverse: 5′-CTCGCAGGGATGATTTCAAG-3′) and β-actin (Actβ, *Actb*), used as a housekeeping gene (forward: 5′-ACTGAGCTGCGTTTTACACC-3′; reverse: 5′-AGCCATGCCAATGTT GTCTC-3′). Samples were run as triplicates. For representation of the results, dCt was calculated as Ct (Actβ) – Ct (gene of interest), and average dCt from control hippocampus was used to calculate ddCT. Results are expressed as $2^{ddCt}$ in relative units.

## Histology

Animals were anaesthetized and perfused transcardiacally with PBS containing 4% of paraformaldehyde. Brains were removed, postfixed overnight with PBS 4% paraformaldehyde and embedded in paraffin. Paraffin coronal brain sections (3-μm thick) were cut using a Leica microtome. Periodic acid-Schiff (PAS) staining was carried out using an Artisan Link Pro machine (AR16592-2 kit, Artisan, Dako, Agilent). Immunostainings were performed using primary antibodies against MGS (ref. 3886 from Cell Signaling), GFAP (ref. MAB360, from Merck Millipore), and Iba1 (ref. 019-19741 from Wako). The secondary antibodies used were Goat Anti-Mouse (P0447, Dako, Agilent) or a BrightVision Poly-HRP-Anti Rabbit IgG (Immunologic, DPVR-110HRP). Antibody complexes were revealed with 3,3′-diaminobenzidine (K3468, Dako) with the same time exposure per antibody. Brightfield images were acquired with a NanoZoomer-2.0 HT C9600 digital scanner (Hamamatsu) equipped with a 20× objective. All images were visualized with the NDP.view 2 software (Hamamatsu, Photonics, France). QuPAth software was used to perform image analysis for quantifying the area of GFAP- or Iba1-positive pixels[22] in haematoxylin-counterstained sections. The region of interest (hippocampus area) was selected manually. Results are the percentage of the positive area following the equation: % of positive pixels = positive pixels × 100 / (positive + negative pixels).

## Assessment of kainate-induced epilepsy

Animals were placed in individual cages and administered three consecutive intraperitoneal injections of kainate (8 mg/kg per dose, 24 mg/kg total), one every 30 min from the onset of the experiment in order to induce convulsive non-lethal seizures. Seizure stages after kainate injections were evaluated as described previously.[23–25] After the first kainate injections, the animals developed hypoactivity and immobility (Stages I–II). After successive injections, hyperactivity (Stage III) and scratching (Stage IV) were often observed. Some animals progressed to a loss of balance control (Stage V) and further chronic whole-body convulsions (Stage VI). Extreme behavioural manifestations such as uncontrolled hopping or 'popcorn behaviour' and continuous seizures (more than 1 min without control of body movement) were included in Stage VI. All behavioural assessments were performed blind to the experimental group (genotype) *in situ*, and were also recorded and reanalysed blind to the first analysis. Analysis consisted of recording the time spent until the onset of the first seizure, the number of seizures per animal and the time spent in each stage by each animal.

## Gas chromatography-mass spectrometry quantitation of metabolites

Brain metabolomics analysis was performed based on our previously established methods.[26–28] Twenty milligrams of each pulverized tissue were extracted in 1 ml of 50% methanol containing 20 μM L-norvaline as an internal procedural control and separated into polar (aqueous layer) and insoluble fractions by 15 000 rpm centrifugation at 4°C for 10 min. The pellet was subsequently washed three times with 50% methanol and once with 100% methanol to remove polar contaminants. Polar fractions were dried at $10^{-3}$ mBar using a SpeedVac (Thermo) and stored at –80°C until derivatization steps were performed. Hydrolysis of the protein/glycogen fraction was performed by first resuspending the dried pellet in $ddH_2O$, followed by the addition of equal part 6 M HCl. Samples were vortexed thoroughly and incubated at 95°C for 2 h. Hydrolysis was quenched with 100% methanol with 40 μM L-norvaline, samples incubated on ice for 30 min, and the supernatant collected after centrifugation at 15 000 rpm at 4°C for 10 min. The collected supernatant was subsequently dried by vacuum centrifuge at $10^{-3}$ mBar.

Dried polar and glycogen samples were derivatized by the addition of 20 mg/ml methoxyamine hydrochloride in pyridine and incubated for 1.5 h at 30°C. *N*-methyl-trimethylsilyl-trifluoroacetamide (MSTFA) was then sequentially added to samples, followed by an incubation time of 30 min at 37°C with thorough mixing between addition of solvents. The mixture was then transferred to a v-shaped amber glass chromatography vial and analysed by gas-chromatography mass spectrometry (GC-MS). An Agilent 7800B gas chromatograph coupled to a 5977B mass spectrometry detector was used for this study. GC-MS protocols were similar to those described previously,[25,26] except a modified temperature gradient was used for GC: the initial temperature was 130°C, held for 4 min, rising at 6°C/min to 243°C, then rising at 60°C/min to 280°C, and held for 2 min. The electron ionization (EI) energy was set to 70 eV. Scan (m/z : 50–800) and full scan modes were used for metabolomics analysis. Mass spectra were translated to relative metabolite abundance using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) software matched to the FiehnLib metabolomics library (available through Agilent) for retention time and fragmentation pattern. Quantitation was performed using the software Mnova (Mestrelab) with a primary ion and at least two or more matching qualifying ions. Relative abundance was corrected for recovery using the L-norvaline standard and adjusted to protein input.

## Data collection and statistical analysis

For the biochemical analyses, image quantification and behavioural assessment of seizure susceptibility after kainate administration, computed results were processed for statistical analysis with PRISM 8.0 (GraphPAD Software, San Diego, USA). Data are presented as the mean ± standard error of the mean (SEM). Normality of the distributions was checked via the Shapiro-Wilk test. All the tests performed were two-sided. Statistical significance of differences between groups was inferred by Student's *t*-test or two-way ANOVA, followed by the Bonferroni *post hoc* comparison for all pairwise multiple comparison procedures. Statistical significance was set at $P < 0.05$.

## Data availability

Raw data and images are available upon request from the corresponding author.

## Results

### Accumulation of glycogen is prevented in young malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice

To generate a mouse model of Lafora disease that is unable to accumulate glycogen specifically in astrocytes, we bred malin$^{KO}$ mice[13,18] with an astrocyte-specific knockout of MGS[5] to obtain malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ animals. We examined the accumulation of glycogen in the brains of young (3-month-old) malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ animals, an age at which malin$^{KO}$ mice already show a great number of Lafora bodies in this organ. To this end, PAS staining and MGS immunostaining were performed in coronal brain sections, and total brain glycogen content was biochemically quantified. A considerable number of glycogen aggregates were observed in malin$^{KO}$ brains after PAS staining and MGS immunostaining, as previously reported.[13] In contrast, malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ animals showed a dramatic reduction in the number of aggregates (Fig. 1A). The biochemical determination of glycogen content also revealed an increase in the brains of malin$^{KO}$ mice ($P < 0.0001$) while glycogen levels in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ brains were lower than those of wild-type controls ($P < 0.0001$) (Fig. 1B).

### Malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice accumulate neuronal Lafora bodies but not corpora amylacea-like bodies

We previously reported that most aggregates that accumulate in malin$^{KO}$ hippocampi correspond to astrocytic CAL, although some nLBs were detected in the CA2-CA3 region.[19] As expected, the hippocampi of malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ brains contained nLBs but were devoid of CAL (Fig. 2, top panels). Likewise, nLBs, but not CAL, were present in the cortices of malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ animals (Fig. 2, middle panels). The cerebella of malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice were almost completely free of the glycogen aggregates typical of malin$^{KO}$ cerebella, thereby indicating that these aggregates were mainly astrocytic CALs (Fig. 2, bottom panels). These results confirm that most brain Lafora bodies are in fact astrocytic and that they correspond to the CAL morphology.[19]

### Western blot analyses of brain extracts

We next analysed the levels of MGS and laforin in brain extracts of the different groups by western blot. MGS and laforin were increased in the brains of young malin$^{KO}$ animals ($P = 0.0056$ and $P = 0.0407$, respectively), reproducing the results previously reported for older animals.[13,18] In contrast, in the brains of young malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice, MGS levels were decreased and laforin showed normal levels ($P = 0.0002$ and $P = 0.7056$, respectively). These findings thus indicate that the accumulation of these proteins in malin$^{KO}$ brains takes place mainly in astrocytes (Fig. 3). In older malin$^{KO}$ brains, the autophagy adaptor p62 is increased, which has been described as an indicator of autophagy impairment in these animals.[29] We found that young malin$^{KO}$ animals also presented an increase in p62 levels in the brain ($P = 0.0002$). Importantly, p62 levels were normalized in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ animals ($P < 0.0001$ with respect malin$^{KO}$), thereby indicating again that this increase was due mainly to the astrocytic population (Fig. 3).

### Assessment of pathology in aged malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$

The results described thus far established that most Lafora bodies in malin$^{KO}$ brains accumulate in astrocytes. We next analysed whether these astrocytic Lafora bodies contribute to the pathology of Lafora disease. Three-month-old malin$^{KO}$ animals do not show signs of brain damage, which appear at older ages.[18,30] We therefore used 11-month-old animals from the different groups to dissect the specific contribution of glycogen accumulation in astrocytes to the pathology of the disease. The analysis of this process in the brains of these aged groups reproduced the results obtained with younger animals (Supplementary Fig. 1). Old malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice presented nLBs, but not CALs (Supplementary Fig. 1A), and low levels of brain glycogen, while malin$^{KO}$ brains showed a clear increase in glycogen with respect to controls (Supplementary Fig. 1B). Western blot analyses also revealed increased levels of MGS, laforin and p62 in malin$^{KO}$ brains. In contrast, these parameters were reduced in the brains of malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ animals (Supplementary Fig. 1C).

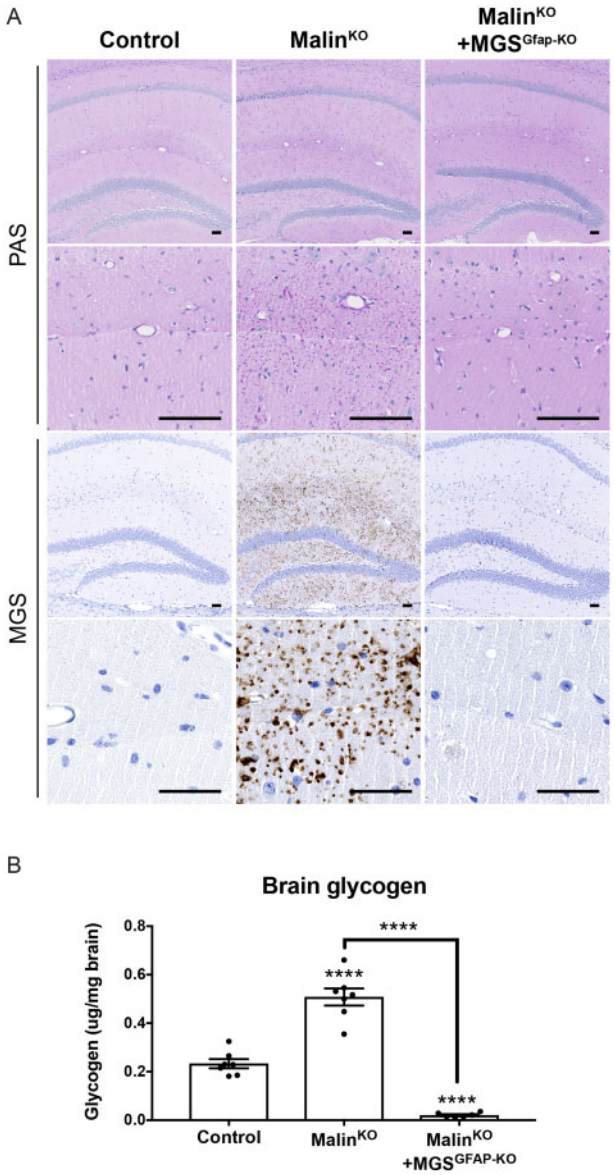


**Figure 1 Accumulation of glycogen in the brains of 3-month-old mice.** Lafora bodies are abundant in malin$^{KO}$ hippocampi but greatly diminished in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ hippocampi. (**A**) Low and high power photomicrographs illustrating the distribution of Lafora bodies after periodic acid-Schiff (PAS) staining and MGS immunostaining of the hippocampi of 3-month-old littermates from the different groups. Scale bar = 100 μm. (**B**) Glycogen content of total brain. Control ($n = 7$), malin$^{KO}$ ($n = 7$), malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ ($n = 6$). Data are expressed as mean ± SEM. ****$P < 0.0001$.

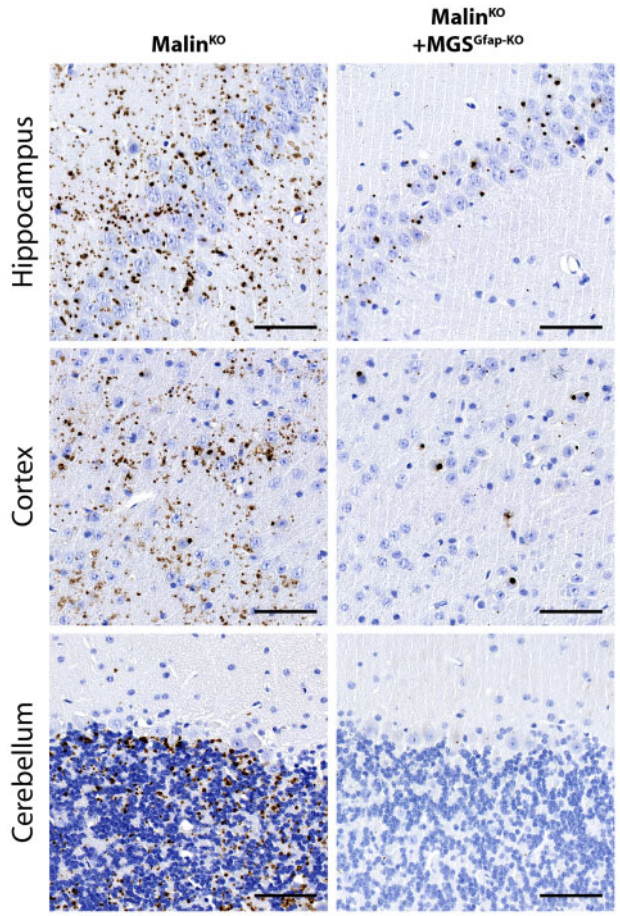


**Figure 2 Malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ brains contain nLBs but not CAL.** MGS immunostainings of the CA3-CA2 region of the hippocampus (*top*), the cortex (*middle*) and the cerebellum (*bottom*) of 3-month-old mice. Scale bar = 100 μm.

Histological markers, namely GFAP for astrogliosis and Iba1 for microgliosis, have been widely used to assess brain damage in Lafora disease mouse models.[13,14,30–32] The analysis of these markers showed increased staining in aged malin$^{KO}$ brains [GFAP ($P < 0.0001$), Iba1 ($P = 0.0024$)], as we previously described.[9] The increase was particularly prominent in the hippocampus, a region that accumulates numerous CAL.[4] Strikingly, the levels of these markers were normalized in the brains of malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice [GFAP ($P = 0.2993$), Iba1 ($P = 0.8521$)] (Fig. 4A and B). In line with these histological markers, the upregulation of genes encoding mediators of the inflammatory response has also been reported in mouse models of Lafora disease.[30,31]The analysis of some of these genes, namely *C3*, *Ccl2*, *Lcn2* and *Cxcl10*, by qPCR confirmed the upregulation of their expression in malin$^{KO}$ brains ($P = 0.0006$, $P = 0.0051$, $P = 0.0083$ and $P = 0.0015$, respectively). Consistent with the histological results, the expression of these genes was similar to normal levels in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ brains ($P = 0.1098$, $P = 0.0325$, $P = 0.2041$ and $P = 0.3590$, respectively) (Fig. 4C). Cumulatively, these results indicate that astrogliosis and microgliosis in malin$^{KO}$ brains are mainly a result of the accumulation of glycogen in astrocytes.

## Glycogen overaccumulation in astrocytes *per se* induces astrogliosis and neuroinflammation

After the above results, we next examined whether the overaccumulation of glycogen in astrocytes *per se* induces the changes in brain damage markers that we described in malin$^{KO}$ brains. To this end, we took advantage of our animal model, in which a non-inactivatable form of MGS (9A-MGS) can be expressed in a cell-type-specific manner.[17] We crossed this mouse model with Gfap-Cre mice to induce the accumulation of glycogen in astrocytes (9A-MGS$^{Gfap}$). PAS staining, MGS immunostaining and biochemical determination of glycogen in the brains of 3-month-old 9A-MGS$^{Gfap}$ mice revealed a conspicuous accumulation of glycogen ($P < 0.0001$) (Fig. 5A and B). Western blot analyses in total brain homogenates of these mice revealed that MGS overexpression was accompanied by an increase in laforin, thereby mimicking the results obtained in the brains of malin$^{KO}$ mice. Also, the autophagy marker p62 showed increased levels in the brains of 9A-MGS$^{Gfap}$ mice (Fig. 5C).

We analysed the presence of GFAP and Iba1 in these brains. Interestingly, both markers were dramatically increased in 9A-MGS$^{Gfap}$ animals when compared to control littermates ($P = 0.0016$ and $P = 0.0005$, respectively) (Fig. 6A and B). These results indicate that the accumulation of glycogen in astrocytes *per se* induces astrogliosis and microgliosis. The inflammatory phenotype in the 9A-MGS$^{Gfap}$ model was again confirmed by qPCR analysis of genes involved in the inflammatory response [*C3* ($P < 0.0001$), *Ccl2* ($P = 0.0027$), *Lcn2* ($P < 0.0001$) and *Cxcl10* ($P < 0.0001$)] (Fig. 6C). Taken together, the results obtained with the 9A-MGS$^{Gfap}$ mice confirm that the overaccumulation of glycogen in astrocytes *per se* induces brain damage.

## Analysis of susceptibility to kainate-induced epilepsy in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$

The accumulation of Lafora bodies in malin$^{KO}$ brains also leads to increased susceptibility to kainate-induced epilepsy,[13,18] which is in accordance with early Lafora disease symptoms. To analyse the contribution of astrocytic Lafora bodies to this epileptic phenotype, we measured the susceptibility of malin$^{KO}$ and malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ animals to kainate-induced seizure. Mice received three consecutive kainate injections (8 mg/kg, i.p. every 30 min) and were video-recorded for 180 min after the first injection to monitor their behaviour (i.e. epileptic events). Mice from the three groups reached similar severity stages. However, an increased proportion of malin$^{KO}$ and malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ animals reached the highest severity stages (Stages V and VI, Fig. 7A). Analysis of the time spent in each stage revealed that malin$^{KO}$ mice spent significantly less time in stages I to IV ($P = 0.035$) and more time in stage VI ($P = 0.051$) when compared to control animals, indicating an increased susceptibility to kainate-induced seizures. Malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ animals were positioned in-between the two groups, not being significantly different from either control or malin$^{KO}$ animals in any stage (Fig. 7B). Similarly, when we analysed the number of seizures per animal correlated by time, we found significant differences between control and malin$^{KO}$ animals after the third kainate administration ($P = 0.002$), with malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ animals again showing an intermediate response in-between the two genotypes (Fig. 7C). In summary, malin$^{KO}$ mice were more susceptible to kainate-induced epilepsy with respect to control littermates, as previously reported. This increase was not significantly reduced in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice. This observation thus indicates that astrocytic Lafora bodies are not the main driver of the increase in the susceptibility to kainate-induced epilepsy in malin$^{KO}$ animals.

## Metabolomic profiles

Neurophysiological impairments are difficult to detect in Lafora disease mouse models,[12,33,34] and recent work demonstrated that

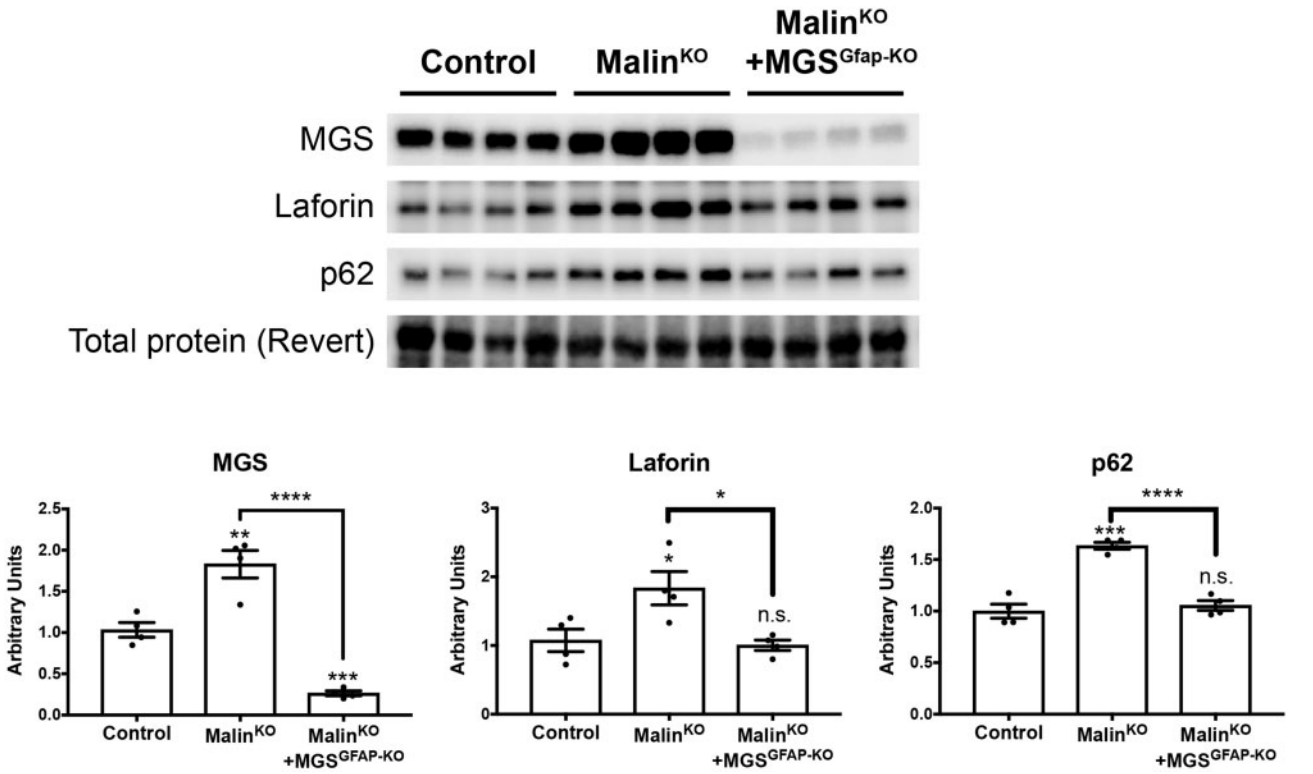


**Figure 3 Accumulation of proteins is prevented in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ brains.** Western blotting for MGS, laforin and p62 of total brain homogenates of 3-month-old mice. (**A**) Representative blots. (**B**) Quantification of the bands normalized to Revert. Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; n.s. = non-significant. $n = 4$ animals per group. Revert was used as loading control.

Lafora disease causes changes in the cerebral metabolome.[26] Therefore, we used the Lafora disease brain metabolic signature to assess disease status. To this end, we used GC-MS to define the cerebral metabolome of malin$^{KO}$, malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ and control animals. We performed targeted metabolomics analysis of central carbon metabolites involved in glycolysis, the pentose phosphate pathway (PPP), the TCA cycle, and amino acid metabolism. Using this information-rich metabolomics dataset, we performed supervised clustering analysis to assess the overall metabolic profiles. In line with our hypothesis, distinct clustering was observed between malin$^{KO}$ animals and control animals (Fig. 8A). Importantly, the malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice clustered and partially overlapped with the control animals, demonstrating a partial normalization of brain metabolism. Corroborating these findings, similar trends were observed using Ward hierarchical cluster analysis, where malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ more closely clustered with control rather than malin$^{KO}$ mice (Fig. 8B). Additionally, metabolites used to generate these analyses were related to glycogen metabolism, glycolysis, TCA cycle, and amino acids (Fig. 8B). Taken together, these data strongly suggest that astrocytic Lafora bodies are the main contributors to the metabolic alterations present in malin$^{KO}$ mice.

## Discussion

Lafora disease is a devastating condition for which there is no current treatment. Demonstration that the accumulation of glycogen in the nervous system underlies the pathophysiology of the disease identified glycogen synthase as a druggable target and opened up the possibility of designing potential treatments.[26,35] Traditionally, it was considered that under normal conditions glycogen is present exclusively in astrocytes,[36] while in Lafora disease, Lafora bodies accumulate exclusively in neurons.[16] These two concepts represented a paradox that was difficult to understand. However, we first demonstrated that neurons have an active glycogen metabolism,[3,4] which explains why they can accumulate aberrant glycogen in conditions like Lafora disease. Also, we[13,19] and others[20] showed that Lafora bodies are not exclusive to neurons and that astrocytes also accumulate these aggregates in Lafora disease. Here we reveal that the accumulation of glycogen aggregates in astrocytes contributes to the pathophysiology of Lafora disease.

The analysis of the Lafora bodies present in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ brains demonstrates that the vast majority of aggregates accumulate in astrocytes, thereby confirming our previous indirect results obtained by co-localization with neuronal and astrocytic markers.[19] Furthermore, biochemical quantification showing low levels of glycogen in the brains of malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice also indicates that neuronal Lafora bodies represent a very small fraction of the glycogen accumulated in malin$^{KO}$ brains. Lafora bodies have traditionally been classified into type I, type II and type III subtypes. The first type accounts for the largest proportion of total Lafora bodies, they affect the neuropil and they are granular, polymorphic, 'dust-like' particles. In contrast, type II Lafora bodies and the more rare type III are bigger and spherical and they are always located in the neuronal perikaryon.[16,37] Based on these characteristics, type I Lafora bodies correspond to CAL, and type II and III to nLBs.[19] It has been hypothesized that type II Lafora bodies are formed only when the production of type I Lafora bodies is insufficient to contain the polyglucosans generated.[16] In light of our results, this hypothesis can be discarded, since type I and type II

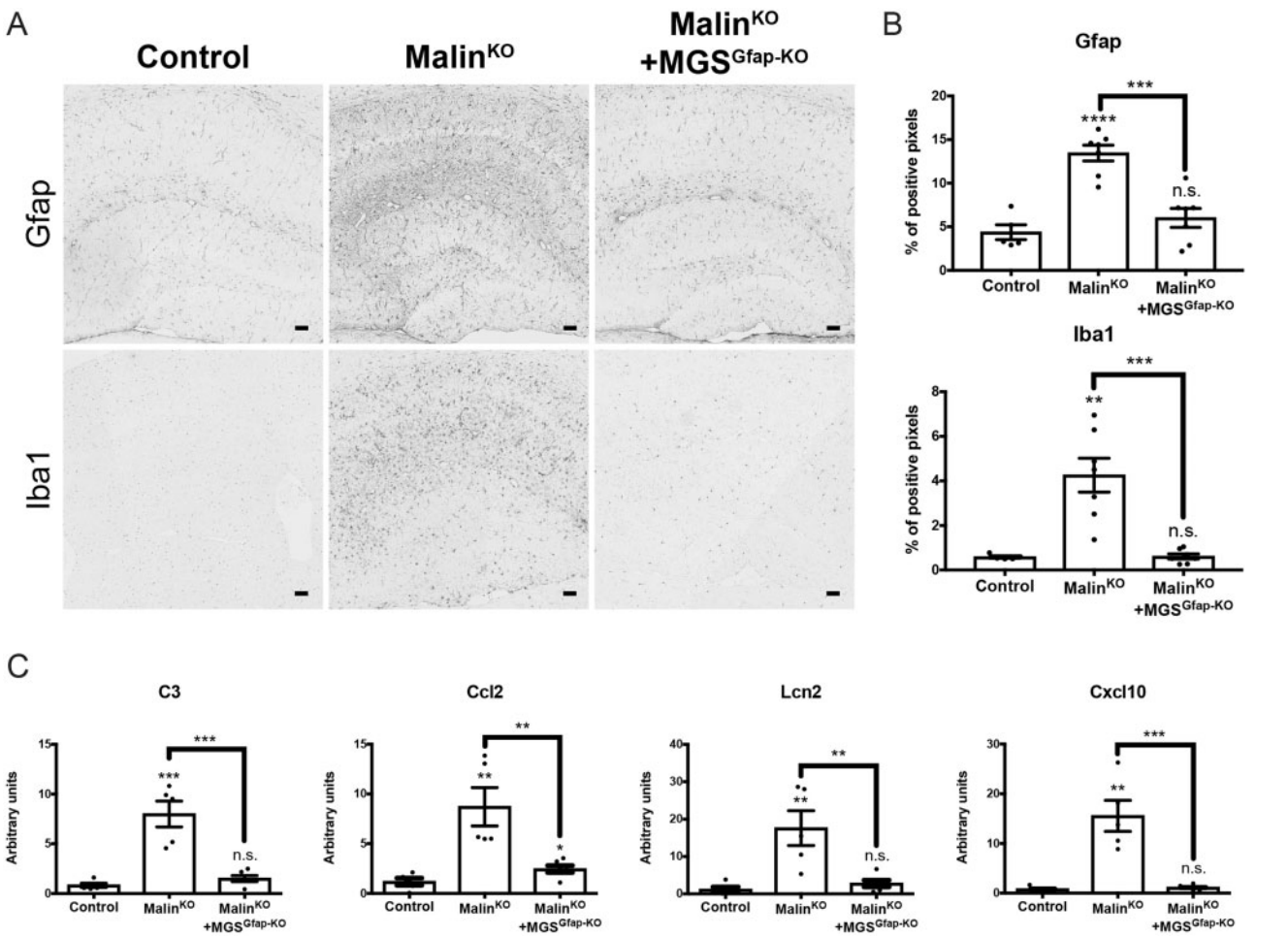


**Figure 4 Analysis of brain damage in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice.** Neuroinflammation markers are increased in the brains of old malin$^{KO}$ mice but normalized in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice. (**A**) GFAP and Iba1 immunostainings of hippocampi from the different groups. (**B**) Quantification of hippocampal area of the immunostainings. Data are expressed as mean ± SEM of percentage of positive pixels [control ($n = 5$), malin$^{KO}$ ($n = 7$), malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ ($n = 7$)]. (**C**) Quantitative PCR analysis of genes involved in the inflammatory response. Data are expressed as mean ± SEM of $2^{\Delta\Delta Ct}$ in relative units for each genotype analysed [control ($n = 5$), malin$^{KO}$ ($n = 5$), malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ ($n = 6$)]. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; n.s. = non-significant.

Lafora bodies are in fact generated in different cell types, i.e. type I Lafora bodies in astrocytes and type II and III in neurons. Along the same line, our results demonstrate that astrocytic Lafora bodies are formed in the astrocytes themselves and do not derive from neurons. Likewise, they show that neurons generate Lafora bodies and do not import them from astrocytes.

Neuropathology has classically been dominated by neuron-centric views, in which pathology is focused on the survival or death of neurons. However, astrocytes are emerging as a central element of neuropathology. Astrocytes play essential roles in brain function,[38,39] and astrocytic dysfunction has been shown to contribute to the pathology of disorders like Alzheimer's disease,[40] Huntington's disease,[41] Parkinson's disease[42,43] and amyotrophic lateral sclerosis.[44] However, whether astrocytic dysfunction reflects cell-autonomous mechanisms in these diseases is not yet clear. Neuroinflammation is one of the most important traits in the pathophysiology of Lafora disease.[30] Here we show that neuroinflammation is corrected in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice, as shown by GFAP and Iba1 immunostaining, as well as by the expression of genes encoding mediators of the inflammatory response. In line with these observations, 9A-MGS$^{Gfap}$ animals showed clear signs of neuroinflammation. Although in this model the accumulation of glycogen in astrocytes is very high, and thus it does not faithfully mimic Lafora disease, these results indicate that the overaccumulation of glycogen in astrocytes *per se* induces this pathophysiological trait. Furthermore, autophagy markers were also normalized in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ brains and altered in 9A-MGS$^{Gfap}$ brains. These findings indicate that dysfunctions in autophagy, which had been proposed as the underlying cause of Lafora disease,[11,45] also occur mainly in astrocytes. Similarly, our metabolomics analyses indicated that the metabolic alterations characteristic of malin$^{KO}$ brains are partially corrected in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ brains. These results demonstrate not only that the impairment of astrocytic function plays a role in the pathophysiology of Lafora disease but that it is a primary role, resulting from the accumulation of Lafora bodies in the astrocytes themselves, and not a secondary role derived of the accumulation of neuronal Lafora bodies. Our findings unveil astrocytes as a key target in the treatment of Lafora disease and make Lafora disease one of the few examples of neurodegenerative diseases in which a cell-autonomous dysfunction of astrocytes has been demonstrated.

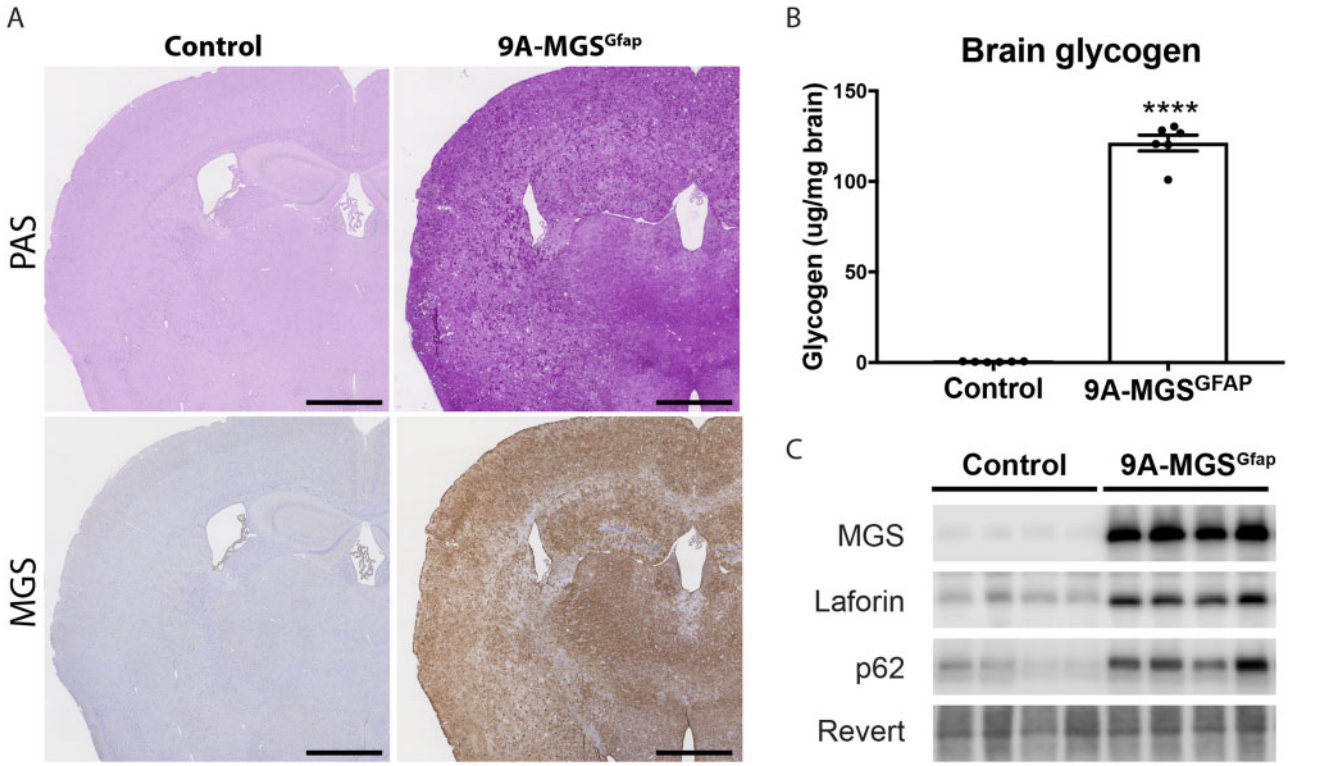


**Figure 5 Accumulation of glycogen in 9A-MGS$^{Gfap}$ mice.** (**A**) PAS staining and MGS immunostaining of 9A-MGS$^{Gfap}$ in consecutive serial coronal sections. (**B**) Glycogen content of total brain ($n = 6$ animals per group). Data are expressed as mean ± SEM. $^{****}P < 0.0001$. (**C**) Western blotting for MGS, laforin and p62 of total brain homogenates. Revert was used as loading control.

Targeted metabolomics analyses and supervised clustering analysis revealed rescue of a wide range of interconnecting metabolic pathways. These include central carbon metabolic pathways (hexose and lactate), amino acid metabolism (glycine and cysteine), and lipid metabolism (lactamide). Thus, these data suggest a change in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ brain metabolism towards control metabolism. Brain metabolism is the biochemistry that underlines brain physiology, and shifts within these pathways towards control levels suggest partial rescue of the diseased phenotype. However, the total pooled metabolites analysed here only represent a change in metabolism. Isotopic tracing is required to obtain detailed flux data such as substrate utilization and direction of change for each individual pathway.

The many roles of astrocytes in the regulation of neuronal excitability make them key players in the pathogenesis of epilepsy.[46,47] Furthermore, neuroinflammation, which is rescued in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice, has been shown to play a role in epileptogenesis.[48] For these reasons, we examined whether the accumulation of Lafora bodies in astrocytes also underlies the epileptic phenotype of Lafora disease. However, susceptibility to kainate-induced epilepsy was not rescued in malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ mice. Indeed, these mice showed only a minor improvement in the number and severity of seizures, which could be due to the absence of neuroinflammation. These results therefore strongly suggest that astrocytic Lafora bodies are not the main contributors to the increased susceptibility to kainate-induced epilepsy, and thus this pathological trait would be more attributable to neurons. In this regard, deletion of PPP1R3D in the mouse model of Lafora disease leads to reduced nLBs and a trend towards decreased susceptibility to kainate-induced seizures.[49]

The results presented here are consistent with those reported for adult polyglucosan body disease (APBD), in which accumulation of abnormal glycogen in astrocytes is sufficient to cause the disease.[50] Interestingly, patients with APBD share symptoms with Lafora disease, like dementia, but they do not present an epileptic phenotype. These data again suggest that the Lafora disease epilepsy derives from the accumulation of aberrant glycogen in neurons.

The accumulation of aberrant glycogen in astrocytes is not exclusive to Lafora disease and APBD. Ageing and some neurodegenerative conditions (including Alzheimer's, Huntington's, Parkinson's and Pick's diseases) also lead to the appearance of astrocytic glycogen aggregates known as corpora amylacea.[51,52] Thus, the results presented here will impact beyond Lafora disease and open up the question of whether glycogen accumulation in astrocytes contributes to the cognitive decline associated with ageing and to the pathology of these other neurodegenerative diseases.

In summary, our results shed new light on the pathophysiological bases of Lafora disease, moving it from a neurocentric perspective to one in which astrocytes are also key players. Based on the results presented here, we propose that the accumulation of CAL in astrocytes underlies autophagy impairment, metabolic alterations and neuroinflammation, while epilepsy is mainly the result of nLB-induced neuronal dysfunction. By expanding a growing body of evidence supporting the involvement of glial cells in neurodegenerative

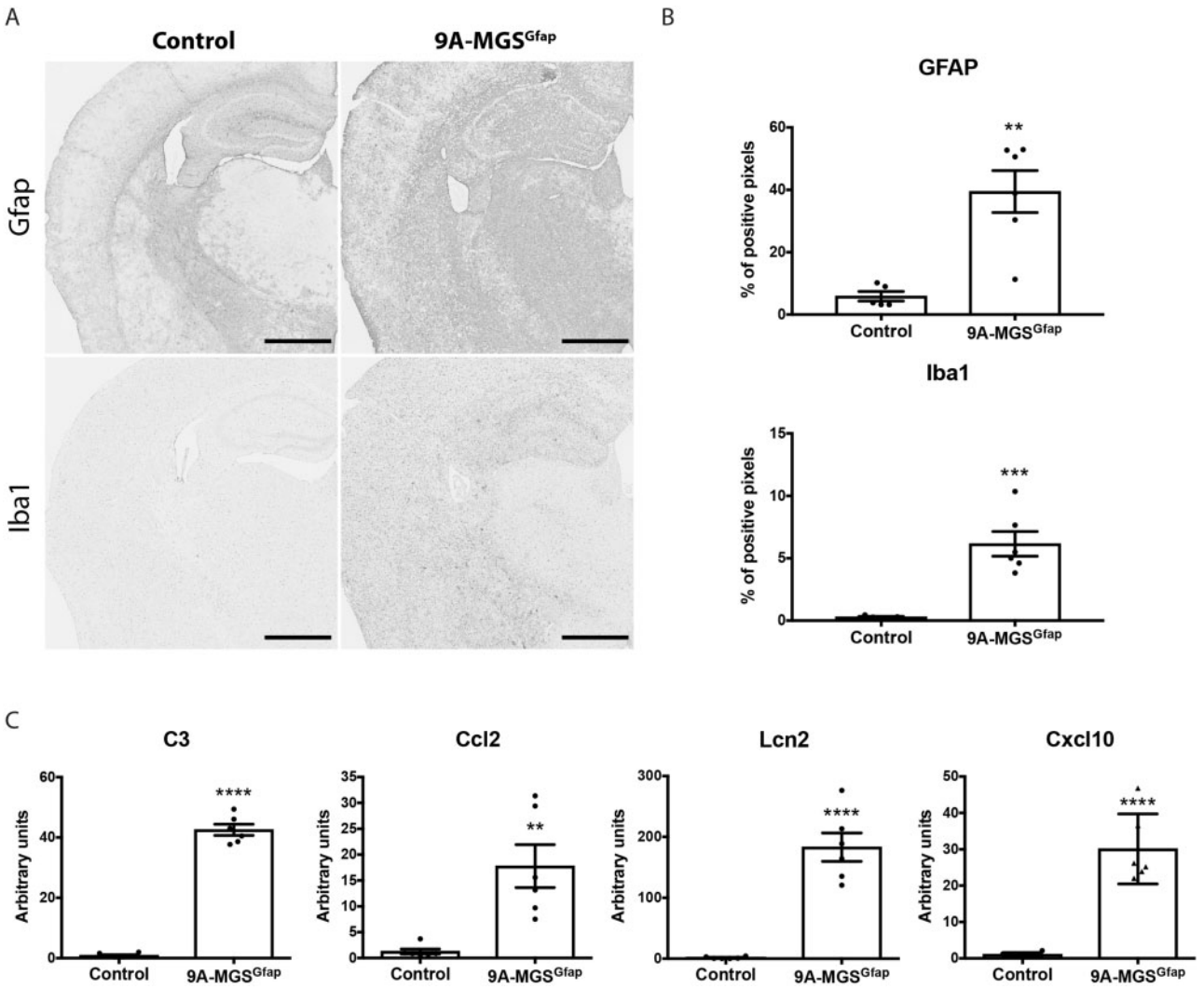


**Figure 6 Analysis of brain damage in 9A-MGS$^{Gfap}$ mice.** Neuroinflammation markers are increased in the brains of 9A-MGS$^{Gfap}$ mice. (**A**) GFAP and Iba1 immunostainings of hippocampi. (**B**) Quantification of the hippocampal area of the immunostainings. Data are expressed as mean ± SEM of percentage of positive pixels [control ($n$ = 5), 9A-MGS$^{Gfap}$ ($n$ = 6)]. (**C**) Quantitative PCR analysis of genes involved in the inflammatory response. Data are expressed as mean ± SEM of $2^{\Delta\Delta Ct}$ in relative units for each genotype analysed ($n$ = 6 animals per group). $^{**}P < 0.01$. $^{***}P < 0.001$. $^{****}P < 0.0001$.

diseases, our results have important implications for the design of treatments for Lafora disease and other disorders.

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## Competing interests

The authors report no competing interests.

## Supplementary material

Supplementary material is available at *Brain* online.

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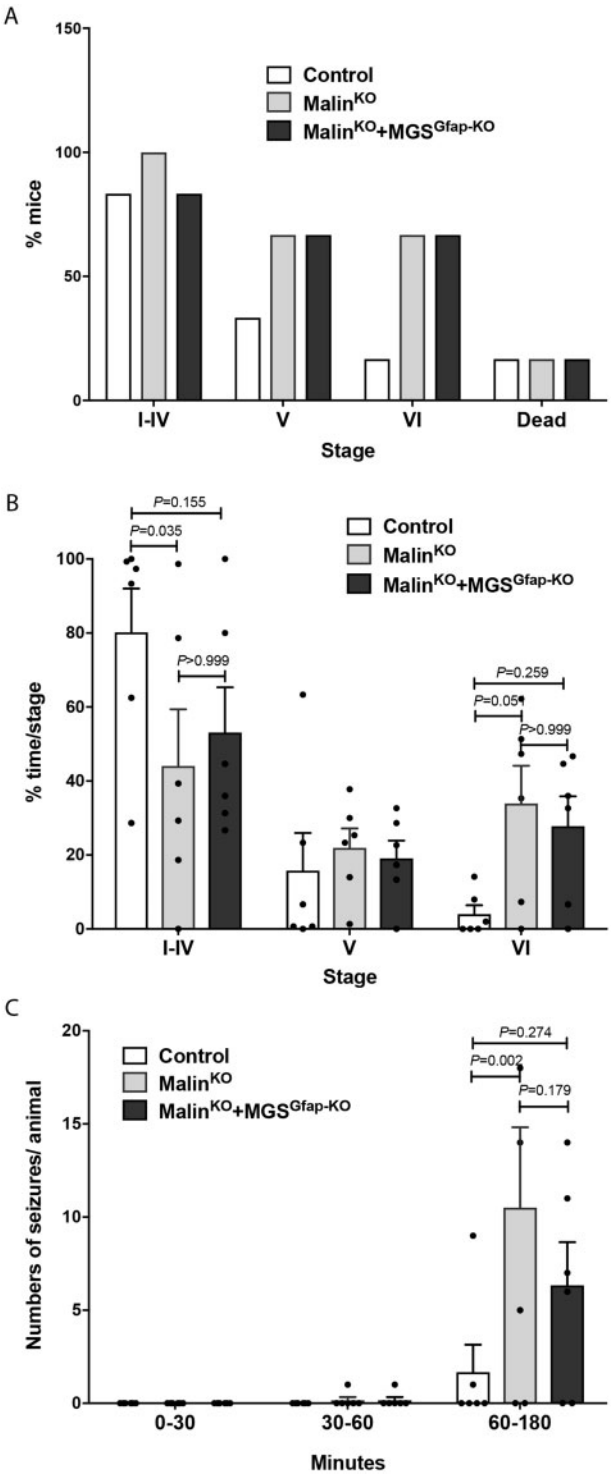


**Figure 7 Study of kainate-induced epilepsy.** Eleven-month-old mice were subjected to three kainate injections (8 mg/kg every 30 min), and epileptic responses were analysed for 180 min after the first injection. (**A**) Percentage of mice reaching seizure Stages I to VI and kainate-induced mortality. (**B**) Percentage of time spent in each stage during the course of the experiment. Data are expressed as average ± SEM. $n = 6$ mice/group. Two-way ANOVA *P*-values display *post hoc* Bonferroni differences. (**C**) Number of seizures experienced per animal divided by time segments after the first, second and third kainate injections. Data are expressed as average ± SEM. $n = 6$ animals per group. Two-way ANOVA *P*-values display *post hoc* Bonferroni differences.

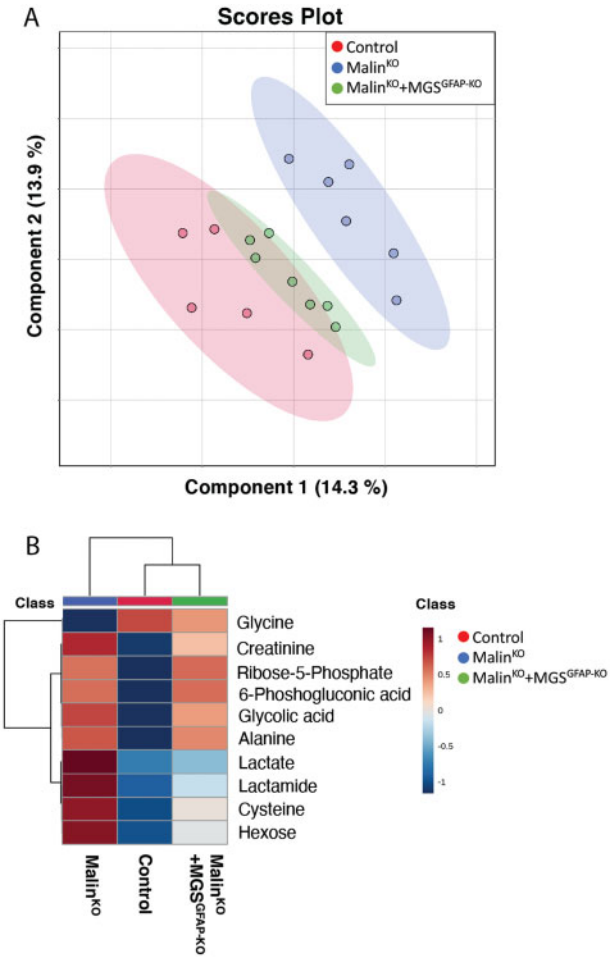


**Figure 8 Metabolic profiles.** (**A**) Sparse partial least squares-discriminant analysis (sPLS-DA) multivariate analysis of control (red), malin$^{KO}$ (blue) and malin$^{KO}$ + MGS$^{Gfap\text{-}KO}$ (green) brains. (**B**) Heat map clustering showing the 10 most affected metabolites from sPLS-DA multivariate analysis.

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