Glycogen accumulation underlies neurodegeneration and autophagy impairment in Lafora disease

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Lafora disease is a fatal neurodegenerative condition characterized by the accumulation of abnormal glycogen inclusions known as Lafora bodies. It is an autosomal recessive disorder caused by mutations in either the laforin or malin gene. To study whether glycogen is primarily responsible for the neurodegeneration in Lafora disease, we generated malin knockout mice with impaired (totally or partially) glycogen synthesis. These animals did not show the increase in markers of neurodegeneration, the impairments in electrophysiological properties of hippocampal synapses, nor the susceptibility to kainate-induced epilepsy seen in the malin knockout model. Interestingly, the autophagy impairment that has been described in malin knockout animals was also rescued in this double knockout model. Conversely, two other mouse models in which glycogen is over-accumulated in the brain independently of the lack of malin showed impairment in autophagy. Our findings reveal that glycogen accumulation accounts for the neurodegeneration and functional consequences seen in the malin knockout model, as well as the impaired autophagy. These results identify the regulation of glycogen synthesis as a key target for the treatment of Lafora disease.

INTRODUCTION

Glycogen is a branched polymer of glucose that constitutes the sole carbohydrate reserve in mammals. It is synthesized by glycogen synthase (GS), the only mammalian enzyme able to polymerize glucose. The most glycogenic tissues are the liver and the skeletal muscle, but essentially every other tissue stores some glycogen. Within the brain, glycogen is found mainly in astrocytes (1), although neurons have the machinery for its synthesis (2). However, glycogen synthesis in neurons has to be tightly controlled, because an over-accumulation of this polysaccharide induces neuronal apoptosis (3).

Lafora disease (LD, OMIM #254780) is a fatal neurodegenerative condition that starts as myoclonus epilepsy and proceeds to rapid cognitive deterioration and death. It is characterized by the accumulation of aberrant glycogen, in the form of the so-called Lafora bodies (LBs), in several cell types, including neurons. LD is caused by mutations in two genes: NHLRC1, which encodes malin, an E3-ubiquitin ligase, and EPM2A, which encodes laforin, a serine-threonine phosphatase. Patients carrying loss-of-function mutations in either of these two genes are indistinguishable. Both enzymes interact functionally to promote the degradation of GS and protein targeting to glycogen (PTG), a scaffolding molecule for protein phosphatase 1 (PP1), which promotes GS dephosphorylation and thus activation (2).

The causal role of glycogen accumulation in neurodegeneration in LD remains controversial, since the malin–laforin complex has been described to have additional functions to that of the regulation of glycogen synthesis. In this regard, it has been proposed that the complex participates in the control of autophagy, a process by which damaged components of the...
cell are sequestered and degraded in the lysosome. In fact, knockouts (KO) of malin and laforin present autophagy impairment (4–6). Thus, this defect in autophagy could be at the basis of the neurodegeneration seen in LD, and therefore the accumulation of glycogen could be a mere epiphenomenon.

We had previously generated a malin KO mouse model (malinKO) that reproduces LD (7). To study whether the accumulation of glycogen is primarily responsible for the neurodegeneration and autophagy seen in this mutant mouse (and therefore, in LD), we have generated and studied malinKO mice with impaired (totally or partially) glycogen synthesis. In addition, we have generated and examined several mouse models in which we force glycogen over-accumulation without manipulating malin.

Our findings reveal that glycogen accumulation is responsible for the neurodegeneration and functional consequences seen in the malinKO model, as well as for the impaired autophagy. These results identify the regulation of glycogen synthesis as a key target for the treatment of LD.

RESULTS

Generation of a malin and GS double KO model

LD is characterized by the accumulation of LBs in many tissues and organs. However, the main manifestations of the disease are neurological. The isoform of GS that is expressed in the brain is muscle glycogen synthase (MGS) (8, 9). In order to generate a malinKO that is unable to accumulate glycogen in the brain, and given that MGS constitutive KO shows high embryonic lethality (10), we generated a double KO model in which malin is deleted in all tissues, while MGS is specifically deleted in the brain (malinKO + MGSKO). For this purpose we took advantage of our MGS conditional KO model, which is based on Cre-Lox technology (8). The ablation of MGS specifically in the brain was achieved by expressing Cre Recombinase under the control of a nestin promoter (11). This nestin–Cre shows gamete leakiness (12) and thus recombination in some cases also took place in gametes, which resulted in the deletion of the conditional gene in these cells. As a result we also obtained malinKO animals that were heterozygous for MGS (MalinKO + MGS het).

All experiments were conducted with 11-month-old animals, the age at which we described the accumulation of LBs, neurodegeneration and functional impairments in the malinKO model (7).

Analysis of LB accumulation

As we previously described (7) malinKO mice accumulated LB in the brain. As expected, malinKO + MGSKO brains showed no LB accumulation, since these animals cannot synthesize glycogen in the brain (Fig. 1A). Interestingly, malinKO + MGS het brains showed much less accumulation of glycogen than the malinKO. We attribute this result to the fact that MGS het animals showed less MGS expression than the controls (Supplementary Material, Fig. S1), thus indicating that GYS1, the gene encoding MGS, is haploinsufficient. Accordingly, glycogen content was less prominent in the malinKO + MGS het than in the malinKO brains, while malinKO + MGSKO brains were devoid of glycogen (Fig. 1B).

The accumulation of MGS and laforin, which is characteristic of malinKO models (7), was also reverted in the malinKO + MGSKO brains, and partially reverted in those of the malinKO + MGS het animals (Fig. 1C). As expected, total GS activity was not altered in the malinKO animals (7), was halved in the malinKO + MGS het ones and was undetectable in malinKO + MGSKO group (Supplementary Material, Fig. S2).

Analysis of neurodegeneration

In the hippocampus, neuronal death/damage is accompanied by the activation of astrocytes and microglia (13–15). Therefore, to analyze whether the decrease in the accumulation of glycogen was accompanied by a reversion in the neurodegenerative phenotype, we measured the extent of neurodegeneration in brain slices by immunostaining for glial fibrillary acidic protein (GFAP), a marker of astrocytes, and for Iba1, a marker of activated microglia. MalinKO brains showed an increase in the staining for both markers with respect to the controls, which was indicative of neurodegeneration. However, the neurodegeneration was not present in malinKO + MGSKO brains, since they showed a similar staining to that of the controls. Furthermore, in the malinKO + MGS het animals the increase in staining was more moderate than in the malinKO mice (Fig. 2), thus showing that a reduction of about 50% of GS suffices to attenuate neurodegeneration.

Electrophysiological analyses

We next studied input/output curves and long-term potentiation (LTP) evoked at the hippocampal CA3–CA1 synapse of the animal models, as we had previously shown that this parameter is altered in malinKO animals (7). However, since MGS and laforin animals present impairment in the experimental induction of LTP and other electrophysiological parameters (8), we excluded the malinKO + MGSKO group from the analysis.

Animals were chronically implanted with stimulating electrodes in the hippocampal Schaffer collaterals and with a recording electrode in the ipsilateral pyramidal CA1 area (Supplementary Material, Fig. S3). LTP was then analyzed in all the groups after a high-frequency stimulus (HFS). The LTP evoked in the control group was similar to that previously reported in the same synapse in behaving mice (16). The malinKO animals presented a significantly larger LTP during the first recording session after HFS than the control group, as previously described (7) (Fig. 3A and B). Importantly, the malinKO + MGS het group presented an LTP equivalent to that of the control group (Fig. 3C) indicating that the alteration had been rescued in this model. Quantification of the records showed that malinKO animals presented a larger LTP than the other two groups (Fig. 3D).

Susceptibility to kainate-induced epilepsy

We then checked the susceptibility of the mouse models to a single injection (8 mg/kg, i.p.) of kainic acid. We detected a noticeable difference in the percentage of animals presenting spontaneous seizures and in the intensity of the same. The malinKO animals presented the highest incidence of hippocampal seizures (84%), accompanied on occasions (two out of six) by myoclonus. The other groups showed seizures of very much lower amplitude and duration than those observed in the
malin$^\text{KO}$ animals (control: 40%; malin$^\text{KO}$ + MGS$^\text{Het}$: 40%) (Fig. 4A), indicating again that impeding glycogen accumulation prevented neurological impairment.

Train stimulation of Schaffer collaterals evoked long-lasting after-discharges in 100% of the malin$^\text{KO}$ mice. Again, the other groups presented much milder seizure responses following train stimulation of the CA3–CA1 synapse (control: 40%; malin$^\text{KO}$ + MGS$^\text{Het}$: 40%) (Fig. 4B).

On the whole, malin$^\text{KO}$ animals showed a large propensity to generate hippocampal seizures, a feature that was not observed
in malin\(^{\text{KO}}\) + MGS\(^{\text{het}}\) animals, indicating again that a reduction in glycogen accumulation prevented neurological impairment.

**Analysis of autophagy**

Glycogen accumulation in the malin\(^{\text{KO}}\) model has been suggested to be caused by impaired autophagy (4). According to this hypothesis, the lack of malin would lead to autophagy impairment, which would result in the accumulation of glycogen. However, it has also been proposed that autophagy impairment in mouse models of LD is caused by the LBs themselves (6, 17). To address this controversy, we measured p62, a widely used marker to study autophagic flux (18). Western blot revealed an increase in p62 in malin\(^{\text{KO}}\) brains, indicating impaired autophagy (Fig. 5A). Remarkably, malin\(^{\text{KO}}\) + MGS\(^{\text{het}}\) animals did not show this increase. The impairment in autophagy was also partially rescued in the malin\(^{\text{KO}}\) + MGS\(^{\text{KO}}\) brains. These observations suggest that the autophagy impairment is a consequence and not the cause of glycogen accumulation. Similar results were obtained in western blots against NBR1, another marker of autophagy flux (19, 20). These results were confirmed by immunostaining of p62 in the brains of the animal groups (Fig. 5B).

**Analysis of autophagy in other models of glycogen accumulation**

To further analyze the causative effect of glycogen accumulation on impaired autophagy, we generated other mouse models in which glycogen is accumulated, independently of the lack of malin. In the first model, PTG is constitutively overexpressed in all tissues, including the brain (PTGOE). These animals showed the accumulation of polyglucosan bodies in the brain, similar to but less abundant than that observed in the malin\(^{\text{KO}}\) group (Fig. 6). We also used our previously described model in which a non-inactivatable form of MGS, 9A-MGS, is specifically overexpressed in the brain (9A-MGS\(^{\text{OE}}\)). In this second model,
glycogen is dramatically over-accumulated in the brain (3). Western blot analyses showed accumulation of p62 in these two models (Fig. 7), indicating that the accumulation of glycogen per se can cause autophagy impairment.

Furthermore, we crossed the PTG\textsuperscript{OE} with the malin\textsuperscript{KO} strains to generate a new model (malin\textsuperscript{KO} + PTG\textsuperscript{OE}) in which the accumulation of glycogen in the brain (Fig. 6), but also in the heart and skeletal muscle (Supplementary Material, Fig. S4), was dramatically enhanced. The increase in p62 in the brains of malin\textsuperscript{KO} + PTG\textsuperscript{OE} mice was higher than that detected in malin\textsuperscript{KO} mice, in concordance with the higher accumulation of glycogen in this organ (Fig. 7).

DISCUSSION

Our results settle the controversy regarding whether glycogen is the direct cause of the neurodegeneration and functional impairments seen in LD. We show that glycogen accumulation is, indeed, the cause of both, since a malin-deficient mouse that cannot synthesize glycogen in the brain did not show the increase in markers of neurodegeneration, the impairments in LTP of hippocampal synapses nor the susceptibility to kainate-induced epilepsy seen in the malin\textsuperscript{KO} model. These results are in concordance with our previous findings demonstrating that glycogen accumulation per se drives neuronal death (3). Furthermore, we also demonstrate that by only knocking out one of the alleles of the \textit{GYS1} gene the phenotype was partially rescued. This finding has important implications for the treatment of LD, as it implies that partial inhibition of GS activity may be sufficient to prevent the progression of the disease.

This paper completes and extends two other recent reports exploring the contribution of glycogen to the pathophysiology of LD. In the first (21), it is demonstrated that the deletion of PTG prevents the manifestation of LD in the malin\textsuperscript{–/–} model. These animals showed reduced glycogen accumulation; however, since PTG is a regulatory protein of PP1, which has many targets, it could not be excluded that the observed effects were due to the dephosphorylation of other targets. The second report (22) shows that the double laforin-MGS KO animal is devoid of LBs and also offers some hints that the neurological alterations inherent to LD are rescued. In the present study not only do we show that the malin-MGS double KO is devoid of LBs, but we have performed complete analyses that unequivocally demonstrate that neurodegeneration is rescued in these animals. Neurodegeneration markers are clearly decreased in malin\textsuperscript{KO} + MGS\textsuperscript{het} and malin\textsuperscript{KO} + MGS\textsuperscript{KO} mice when compared with malin\textsuperscript{KO} KO counterparts. With respect to the electrophysiological properties of hippocampal pyramidal synapses in KO animals, of note is the observation of larger and longer-lasting LTP in these animals that is not present in the malin\textsuperscript{KO} + MGS\textsuperscript{het} group. Furthermore, in the kainate test malin\textsuperscript{KO} mice presented a larger susceptibility to hippocampal seizures after kainic acid administration, while the malin\textsuperscript{KO} + MGS\textsuperscript{het} group showed no difference from the control group. This observation thus indicates that the malin\textsuperscript{KO} mice were rescued from their neurological dysfunctions when GS was diminished.

Another relevant finding is that autophagy impairment is rescued in the malin\textsuperscript{KO} + MGS\textsuperscript{KO} animals, and partially rescued in the malin\textsuperscript{KO} + MGS\textsuperscript{het}. These results demonstrate that the
accumulation of glycogen is not a consequence of autophagy impairment, but rather the cause of it, and thus provide an answer to whether autophagy precedes glycogen accumulation or vice versa. To reinforce this idea, we also show that other models of glycogen accumulation in which malin levels have not been modified (namely the 9A-MGS\textsuperscript{OE} and the PTG\textsuperscript{OE} models) show impaired autophagy. A similar conclusion was reached by Puri et al.\textsuperscript{6}. These authors show that loss of laforin does not alter the brain levels of key autophagy players, and thus conclude that defects in autophagic induction contribute only marginally to the increase in p62 in this model. They propose that impairments at later stages of the process, such as phagosome–lysosome fusion, are responsible for the increased levels of autophagic substrates described in the LD mouse models. However, all together, we cannot exclude the possibility that the impaired autophagy induced by the accumulation of glycogen is the cause of the neurodegeneration in the malin\textsuperscript{KO} animal. In fact, loss of autophagy has been shown to cause neurodegeneration in mice (23, 24). Nevertheless, we can conclude that without glycogen accumulation there is no alteration of autophagy in LD.

Another interesting observation is that laforin does not accumulate in malin\textsuperscript{KO} + MGS\textsuperscript{KO} brains, in contrast to other models in which malin is unaltered but glycogen overaccumulates (PTG\textsuperscript{OE} and 9A-MGS\textsuperscript{OE}). According to these results, the accumulation of laforin in the malin\textsuperscript{KO} animal would result from the accumulation of glycogen, and not because laforin is a direct target of malin, as previously suggested (25). The presence of a carbohydrate binding domain in Laforin may explain this behavior.

One of the most promising findings of this study is that the malin\textsuperscript{KO} + MGS\textsuperscript{het} animals are also largely freed from the neurodegeneration and functional impairments that characterize the malin\textsuperscript{KO} mode, indicating that the impact of the disease could be greatly diminished by only partially decreasing the activity of GS. These results might have implications for other diseases in which glycogen accumulates in the brain (i.e. adult polyglucosan body disease, OMIM #263570). We therefore conclude that GS inhibition may provide a potential treatment for LD and other glycogenoses.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Antibodies against MGS (from Cell Signalling), actin (from Sigma), GFAP (from Dako), Iba1 (from Wako), p62 (from Progen), NBR1 (from Abnova) and laforin (a gift from Dr Santiago Rodriguez de Córdoba) were used in this study.
Generation of the animal models

For the generation of the malinKO + MGSKO model, malinKO mice (7) were crossed with brain-specific MGSKO animals (8). The descendants were genotyped for the three alleles involved (malin, MGS and Nestin Cre), and the suitable genotypes were intercrossed to generate the double KO and the other experimental groups. As the Nestin Cre shows gamete leakiness (12), we

Figure 5. Analysis of autophagy markers. Accumulation of p62 and NBR1 in the malinKO brains was dependent on the expression of MGS. (A) Brain extracts from 11-month-old mice were analyzed by western blot with antibodies against p62 and NBR1. Actin was used as loading control. (B) Immunostaining with antibodies against p62 of the hippocampus and cerebellum of 11-month-old littermates from the different groups. Hoechst (blue) was used for nuclear staining. Scale bar = 100 μm.
also obtained animals that were heterozygous for MGS (malin\(\text{KO} + \text{MGS}\text{het}\)). Animals overexpressing active MGS specifically in the brain (9A-MGSOE) were generated as described in (3). Animals constitutively overexpressing PTG (PTGOE) were generated using a similar strategy. Briefly, the PTG cDNA was placed under the control of the ubiquitous CAG promoter. This expression cassette was introduced into the Hprt locus in the X chromosome by homologous recombination, thus avoiding the uncontrolled outcomes of random integration. This PTG-overexpressing model was mated with malin\(\text{KO}\) mice, and the resulting animals were crossed again with malin KO animals to generate the malin\(\text{KO} + \text{PTGOE}\) model.

**Animal studies**

All procedures were approved by the Barcelona Science Park’s Animal Experimentation Committee and were carried out in accordance with the European Community Council Directive and National Institutes of Health guidelines for the care and use of laboratory animals. Mice were maintained on a 12/12 h light/dark cycle under specific pathogen-free conditions and allowed free access to a standard chow diet and water and in the Animal Research Center at the Barcelona Science Park. After weaning at 3 weeks of age, tail clippings were taken for genotyping by qPCR (performed by Transnetyx).

**Biochemical analysis**

Mice were deeply anesthetized (sodium thiopental, 0.25 mg/g of animal) and decapitated with a guillotine. The brain was quickly removed and dropped in liquid nitrogen, this process taking , 1 min. Whole brains were then pulverized in liquid nitrogen. Fractions of the powder were weighted and homogenated in 10 volumes of ice-cold homogenization buffer containing 10 mM Tris–HCl (pH 7), 150 mM KF, 15 mM EDTA, 15 mM 2-mercaptoethanol, 0.6 M sucrose, 25 mM okadaic acid, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, 1 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride. They were then homogenized (Polytron) at 4°C. Total homogenates were used for western blotting and the determination of GS activity. For glycogen measurements, frozen brain aliquots were boiled in 30% KOH for 15 min and glycogen was determined by an amyloglucosidase-based assay as described in (26). Due to the high turnover of glycogen post-mortem, part of the glycogen might have been degraded during the isolation process and thus the levels of glycogen of all groups could be underestimated.

**Histology**

Animals were anesthetized and perfused transcardially with phosphate buffered saline (PBS) containing 4% of paraformaldehyde (PF). Brains were removed, postfixed overnight with PBS 4% PF and either embedded in paraffin or cryoprotected with PBS 30% sucrose, and then frozen. To obtain sections of
the frozen tissues, brains were sectioned coronally at 30 μm, distributed in 10 series, and maintained at −20°C in PBS 30% glycerol 30% ethylene glycol for the free-floating processing. For PAS staining, paraffin sections were cut at 5 μm. Images were obtained with a Nikon Eclipse E800 microscope equipped with an Olympus DP72 camera.

**Immunohistochemistry**

For the immunodetection of antigens, sections were blocked for 2 h at RT with PBS containing 10% of normal goat serum (NGS) and 0.2% of gelatin. Primary antibodies were incubated overnight at 4°C with PBS with 5% of NGS. Sequential incubation with biotinylated secondary antibodies and streptavidin–HRP was also performed in PBS 5% NGS. Antibodies were visualized by reaction using 0.03% diamobenzidine, 0.002% H2O2 and 0.05% nickel ammonium sulfate. Sections were then dehydrated and mounted (Eukitt). For immunohistofluorescence, dye-labelled secondary antibodies and Hoechst 33342 were incubated for 2 h at RT in PBS-5% NGS and mounted in Mowiol.

**Electrophysiological tests**

Methodological information regarding the input/output curves, the electroencephalographic (EEG) recordings, the kainate injection and the recording of seizure activities can be found in Supporting Information Methods.

**Statistical analysis**

Data are expressed as mean ± S.E.M. Statistical significance was determined by the unpaired Student’s t-test using GraphPad Prism software (version 5; GraphPad Software, Inc.). Statistical significance was assumed at P ≤ 0.05.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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