Inhibiting Glycogen Synthesis Prevents Lafora Disease in a Mouse Model

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Lafora disease (LD) is a fatal progressive myoclonus epilepsy characterized neuropathologically by aggregates of abnormally structured glycogen and proteins (Lafora bodies [LBs]), and neurodegeneration. Whether LBs could be prevented by inhibiting glycogen synthesis and whether they are pathogenic remain uncertain. We genetically eliminated brain glycogen synthesis in LD mice. This resulted in long-term prevention of LB formation, neurodegeneration, and seizure susceptibility. This study establishes that glycogen synthesis is requisite for LB formation and that LBs are pathogenic. It opens a therapeutic window for potential treatments in LD with known and future small molecule inhibitors of glycogen synthesis.

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Lafora disease (LD) is the major teenage-onset progressive myoclonus epilepsy (PME). Insidious cognitive decline and escalating myoclonic, visual, convulsive, and other seizures follow an initial decade of normal development. Within a few years, seizures are intractable, myoclonic absences are near constant, and a disinhibited dementia has set in. A vegetative state with continuous myoclonus characterizes the final stage, and most patients die in status epilepticus before age 30 years.[1]

The neuropathology of LD is characterized by progressive formation and growth of Lafora bodies (LBs) in neuronal somata and processes, and by neurodegeneration.[1] LBs are composed of aggregates of a variety of proteins and an abnormal form of glycogen that lacks the branching and spherical structure of normal glycogen essential to its solubility. The abnormal glycogen, called polyglucosan, makes up >70% of an LB.[2] Whether LBs are pathogenic or are a mere epiphenotype remains uncertain.

LD is caused by loss of function of either of 2 interacting enzymes, malin, a ubiquitin E3 ligase, and laforin, a phosphatase.[3] Malin regulates the amount of laforin, and laforin regulates glycogen phosphorylation. The latter is essential to normal glycogen structure, through mechanisms that remain poorly defined.[3–5] Based mostly on cell culture experiments, several additional functions, unrelated to glycogen metabolism, have been tentatively attributed to laforin and malin, including tau kinase dephosphorylation, Wnt signaling regulation, and others.[6–9] It is possible that loss of 1 or more of these functions, rather than effects on glycogen metabolism and LB formation, underlie the neurodegeneration and PME of LD.

PTG is an adaptor protein that mediates dephosphorylation of the glycogen synthesizing (glycogen synthase [GS]) and degrading (glycogen phosphorylase [GP]) enzymes by the pleiotropic phosphatase PP1, which activates GS, inactivates GP, and thus increases glycogen production.[10] We recently hypothesized that, malstructured though they are, polyglucosans are glycogen, and reducing glycogen synthesis might reduce LB formation, which, if LBs cause the disease, might prevent LD. As a test of this hypothesis, we removed PTG from the laforin-deficient mouse model of LD (laforin knockout [LKO]) by crossing PTG knockout mice with the LD mice. This resulted in a drastic reduction of LBs and rescued the neurodegeneration of the LD mice.[11] Although these results supported the view that LBs are pathogenic, there remained the possibility that PTG has adaptor or other functions outside of glycogen metabolism and that its removal prevented neurodegeneration through pathways unrelated to glycogen metabolism and LBs. Furthermore, the study was of short duration and did not assess whether the correction of the

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neurodegeneration is maintained. To address these 2 issues, we removed GS itself from LKO mice and found that the absence of glycogen synthesis alone was sufficient to prevent LB formation, neurodegeneration, and seizure susceptibility, and to do so long-term.

Materials and Methods

Mice

All animal procedures were approved by the Toronto Centre for Phenogenomics and Ball State University animal care committees. The LD mouse model in this study is the previously described[11,12] *Epm2a*$^{-/-}$ LKO model (mixed C57BL/6J and 129Sv/J).

Mice and humans have 2 GS isoforms, GYS2 expressed in liver, and GYS1 in most other tissues, including brain. Extrahepatic glycogen is not essential to murine life postnatally; Gys1 knockout (*Gys1*$^{-/-}$) results in perinatal lethality in 90% of cases, but 10% survive birth, and these thereafter thrive, having normal exercise capacity.[13–15] To obtain laforin–GS double knockout (DKO) mice, we crossed *Epm2a*$^{-/-}$ mice with *Gys1*$^{-/-}$ mice (mixed C57BL/6J and 129Sv/J). Double heterozygotes (*Epm2a*$^{+/-}$/*Gys1*$^{+/-}$) resulting from this mating were then crossed with *Epm2a*$^{-/-}$ mice. Resulting *Epm2a*$^{-/-}$/*Gys1*$^{+/-}$ offspring were intercrossed to generate *Epm2a*$^{-/-}$/*Gys1*$^{+/+}$ (LKO) and *Epm2a*$^{-/-}$/*Gys1*$^{-/-}$ (DKO) experimental mice, and *Epm2a*$^{+/-}$/*Gys1*$^{+/-}$ offspring were intercrossed to generate *Epm2a*$^{+/+}$/*Gys1*$^{+/+}$ (wild-type [WT]) controls. DKO mice continued to have the 90% perinatal lethality of *Gys1*$^{-/-}$ mice, but the 10% birth survivors were healthy, had normal lifespans, and were studied here at age 20 to 26 months.

Seizure Susceptibility Measurements

Seizure susceptibility was assessed by the response to kainic acid injected intraperitoneally. An 8 mg/kg dose, previously shown to distinguish LD mice from WT,[16] was used. Seizures were scored based on a modified Racine scale[17]: stage 0 = no change; stage 1 = immobility, head bobbing; stage 2 = myoclonic jerk within 5 minutes of injection; stage 3 = forelimb clonus and rearing, tail shaking; stage 4 = convulsive seizure within 90 minutes of injection, continuous rearing and falling; and stage 5 = mortality.

Pathology

For light microscopy, mice were sacrificed by cervical dislocation, and tissues were fixed in 10% formalin. Periodic acid–Schiff staining following diastase pretreatment was used to stain LBs and assess LB load, and glial fibrillary acidic protein immunostaining was used to assess the extent of gliosis, as previously described.[11] For electron microscopy (EM), mice were perfused through the left ventricle of the heart with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH = 7.4), with subsequent processing as described previously.[11]

Results

Laforin and GS DKO Mice Have No LBs, Neurodegeneration, or Gliosis

LKO mice had the previously documented[11,12] profuse amounts of LBs in brain and skeletal muscle. DKO mice, like WT, had none (Fig 1A–F). LKO mice had the previously described increased astrocytes and gliosis.[11,12] DKO mice did not and were no different from WT (see Fig 1G–I). Neurodegeneration in LKO mice does not involve apoptosis or necrosis; it is instead characterized, as observed under EM, by loss of neuronal cytoplasmic fullness, shrinking and darkening of neurons, nuclear and cytoplasmic membrane retraction, and loss of synaptic architecture and contacts at the retracted cell membranes.[11,12] The LKO mice of this study showed these features, whereas the DKO mice did not and were no different from their WT controls (Fig 2).

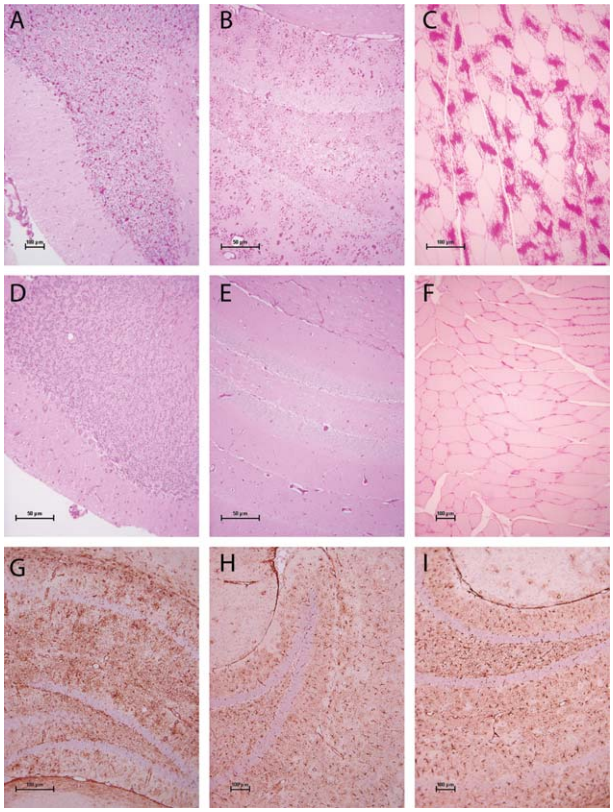

FIGURE 1: Laforin knockout (LKO) mice lacking glycogen synthase (double knockout [DKO] mice) have no Lafora bodies (LBs) and no gliosis. (A–C) LBs in the cerebellum (A), hippocampus (B), and skeletal muscle (C) of LKO mice. (D–F) Corresponding brain regions and muscle in DKO mice. (G–I) Glial fibrillary acidic protein staining in the hippocampus of LKO (G), DKO (H), and wild-type (WT; I) mice. Note the increase in astrocytes (gliosis) in the LKO mice; DKO mice are similar to WT. Scale bars: B, D, E = 50 μm; A, C, F–I = 100 μm.

DKO Mice Have No Increased Seizure Susceptibility

LD mouse models closely recapitulate the pathology of LD, including widespread LB formation and neurodegeneration, but only modestly reproduce the clinical features of the disease. Their behavioral and epileptic abnormalities are mild, and are highly affected by genetic

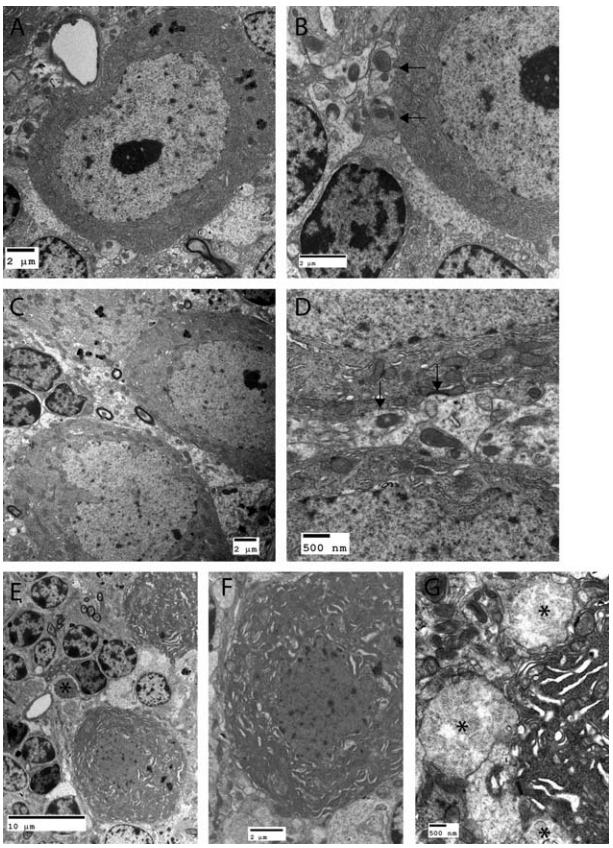

FIGURE 2: Double knockout (DKO) mice do not exhibit the neurodegeneration seen in laforin knockout (LKO) mice. (A, B) Representative low- and high-power electron micrographs of a cerebellar Purkinje cell from a wild-type (WT) mouse. Note the smooth linear cell contour and normal synaptic contacts with the cell membrane *(arrows)*. (C, D) Corresponding images from a DKO mouse. Purkinje cells exhibit healthy cytoplasms, smooth linear cell membranes, and normal synaptic contacts similar to WT. (E, F) Purkinje cells from an LKO mouse. Note the darkened amorphous cytoplasms, wrinkled plasma membranes, and disturbed synaptic contacts; asterisk in E indicates a Lafora body (LB). (G) Part of a Purkinje cell from an LKO mouse with multiple surrounding LBs *(asterisks)*. Scale bars: A, C, F-I = 100 μm; B, D, E = 50 μm.

background. Small differences between LKO mice and WT were originally reported in anxiety (startle response), memory (passive avoidance), balance (rotorod), and muscle strength tests.[12] We were unable to replicate these results in the current LKO mice bred into the genetic background of the *Gys1*$^{-/-}$ mice (the LKO mice were no different from WT in this background; data not shown). Spontaneous convulsive seizures do occur in LKO mice, but their frequency is too low to use as a reliable outcome measure. Finally, myoclonus was a distinguishing feature between LKO mice and WT controls in our previous study in which LKO mice were bred into the PTG knockout background,[11] but this was no longer the case in the present study in the *Gys1*$^{-/-}$ background.

A recent study demonstrated that sensitivity to seizure induction by kainic acid clearly distinguishes LD mice from WT.[16] We found this to apply to our present mice in the *Gys1*$^{-/-}$ genetic background. We therefore utilized this test to determine whether DKO mice were susceptible to seizures. Whereas LKO mice were highly sensitive to kainic acid seizure induction, DKO mice were not, and were no different from WT (Fig 3).

Discussion

Lafora, 102 years ago, identified LBs in the brains of patients with a PME, the genetics, neurological, and neuropathological features of which he described in nearly comprehensive detail. His and subsequent human pathological studies showed that LBs are present in profuse quantities in the neuropils of patients with active LD, often completely replacing the cytoplasms of countless neuronal processes.[1] These observations long suggested a pathogenic role for LBs in the disease. The present work corroborates this, establishing that, at least in the disease's standard mouse model, generation of polyglucosans and LBs are necessary for the neurodegeneration and seizure susceptibility; when polyglucosan generation and LB formation are absent, LKO mice no longer exhibit neurodegeneration and are no longer sensitive to kainic acid–induced seizures, even at advanced age.

The demonstration that LB formation is dependent on glycogen synthesis opens crucial therapeutic possibilities for LD. Humans who have total absence of hepatic (glycogenosis 0a)[18] or extrahepatic (glycogenosis 0b)[19] GS (ie, GYS2 or GYS1, respectively) are healthy except for osteopenia in the former and a late childhood

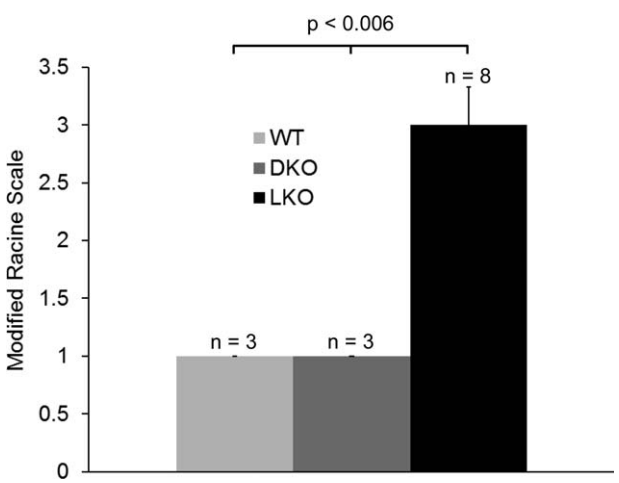

FIGURE 3: Laforin knockout (LKO) mice exhibit a much more severe epileptic response to kainic acid than wild-type (WT) mice. Double knockout (DKO) mice are similar to WT. There were 3 to 8 mice per genotype (age = 20–26 months). Data are shown as means ± standard error of the mean; significance was calculated using an unpaired Student *t* test.

cardiomyopathy in the latter; their parents, with 50% GS activities, are completely healthy. Whereas in the present study murine extrahepatic GS was removed completely for the purpose of unambiguously establishing the critical role of glycogen synthesis in LB formation, in our preceding study GS activity had only been partially reduced (by approximately 30%) through removal of PTG, which inhibits GS by promoting its phosphorylation. In that study, there was no cardiac or other disease, and LBs, neurodegeneration, and myoclonus were still eliminated,[11] suggesting that partial GS reduction might suffice to prevent LD. There are a number of compounds that are known to partially inhibit GS, including sirolimus, which is in clinical use in another neurological disease (tuberous sclerosis), and which leads to GS phosphorylation and glycogen reduction by ~30% in certain tissues tested (brain not yet tested).[20] These and new GS inhibitors that can be identified through high-throughput small molecule screens could prove useful in halting LD early in its course, or preclinically, and prevent its devastating progression.

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Authorship

B.A.P. and J.T. contributed equally to this work.

Potential Conflicts of Interest

Nothing to report.

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