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Original Article

ORIGINAL ARTICLE

# Suppression of leptin signaling reduces polyglucosan inclusions and seizure susceptibility in a mouse model for Lafora disease

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## Abstract

Lafora disease (LD) represents a fatal form of neurodegenerative disorder characterized by the presence of abnormally large number of polyglucosan bodies—called the Lafora bodies—in neurons and other tissues of the affected patients. The disease is caused by defects in the *EPM2A* gene coding for a protein phosphatase (laforin) or the *NHLRC1* gene coding for an ubiquitin ligase (malin). Studies have shown that inhibition of glycogen synthesis in the brain could prevent the formation of Lafora bodies in the neurons and reduce seizure susceptibility in laforin-deficient mouse, an established animal model for LD. Since increased glucose uptake is thought to underlie increased glycogen in LD, and since the adipocyte hormone leptin is known to positively regulate the glucose uptake in neurons, we reasoned that blocking leptin signaling might reduce the neuronal glucose uptake and ameliorate the LD pathology. We demonstrate here that mice that were deficient for both laforin and leptin receptor showed a reduction in the glycogen level, Lafora bodies and gliosis in the brain, and displayed reduced susceptibility to induced seizures as compared to animals that were deficient only for laforin. Thus, blocking leptin signaling could be a one of the effective therapeutic strategies in LD.

## Introduction

Lafora disease (LD) is an adolescent onset, autosomal recessive and fatal form of disorder characterized by progressive myoclonus epilepsy (OMIM #254780ID). Besides the epileptic seizures, which also include absence, tonic-clonic and occipital seizures, the patients also develop ataxia, dementia and show cognitive impairment (1). Affected patients die at around 10 years after the onset, often due to the respiratory failure (2). LD is caused by defects in the *EPM2A* gene coding for the protein phosphatase laforin or the *NHLRC1* gene coding for ubiquitin ligase malin. A major pathological hallmark of LD is the presence of glycogen-rich inclusion bodies in multiple tissues including the neurons (3). These inclusions, called Lafora bodies, are composed of lesser branched glycogen, and are rich in phosphate (4–7). Animal models of LD, developed by the targeted deletion of the *Epm2a* or the *Nhlrc1* gene, exhibited most of the disease defining symptoms including the epilepsy, Lafora bodies and neurodegeneration (5,8). While the mechanism that triggers the epileptic episodes in LD is to yet to elucidated, studies have shown that reducing the glycogen levels in the brain protect the neurons from degeneration and the seizure onset in the LD animal models (9–11). In these studies, the gene coding for the glycogen synthase (GS) or the R5/PTG gene coding for one of the glycogen targeting subunits of type 1 protein phosphatase that regulates the GS, was inactivated in the laforin or malin-deficient mouse to bring-down or to prevent the formation of Lafora bodies in the neurons (9–11).

The laforin-deficient mouse—an established animal model for Lafora disease—is known to have abnormally higher levels of glycogen in brain and other tissues in addition to the Lafora bodies (8). Since increased glucose uptake is thought to underlie increased glycogen in LD (12), we reasoned that reducing the cellular glucose uptake might ameliorate the LD pathology.

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Leptin, an adipocyte hormone, is known to enhance the cellular glucose uptake (13–16), and, conversely, loss of leptin receptor is known to result in impaired glucose uptake (17). Thus, blocking the leptin signaling in the laforin deficient mouse might lower the brain glycogen level and associated pathology. To test this possibility we have created a double mutant mouse, by crossing the laforin deficient LD mouse with the leptin receptor mutant (db/db) and have characterized the LD phenotype in the double mutant. We show here that loss of leptin receptors in the LD brain reduces the glycogen level, Lafora bodies, gliosis and susceptibility to induced seizures.

## Results

### Laforin-deficient mice lacking the leptin receptor are obese with reduced brain glycogen levels

We have shown earlier that the LD proteins laforin and malin regulate glucose uptake by modulating the localization of glucose transporters on the plasma membrane (12). Therefore, we reasoned that whether reducing glucose uptake in LD mice would ameliorate the disease pathophysiology. To test this, we have created a double mutant, by crossing the laforin deficient mouse with the leptin receptor mutant (db/db) and have characterized the LD phenotype in the double mutant. For the study, the adult animals of the 4–5 month age groups were selected, as Lafora bodies are significantly more prominent in this age group (8) and the LD mice of this age group are highly susceptible to induced seizures (18).

The double mutant mice were obese, and were comparable in size and body weight to the db/db mutant animals (Fig. 1A and B). Similarly, the double mutants showed ∼4-fold increase in the blood glucose level, both in fed and unfed conditions, as compared to laforin mutant, and the values were comparable to the db/db animals, suggesting that double mutants did become diabetic (Fig. 1C). To test if the glucose uptake is compromised in the double mutants, as known in the db/db single mutant (13–16) the glucose uptake was measured using the glucose tolerance test. Consistent with an earlier report (19), the laforin-deficient mice showed normal insulin sensitivity and glucose disposal while the double mutants displayed insulin-insensitivity for all the time points tested and were comparable to that of the db/db mice (Supplementary Material, Fig. S1A and B). Thus, the double mutant appeared to have compromised glucose uptake as was known for the db/db mutants (20,21). We next measured the glycogen level in the double mutants using an established method (11,22). The brain tissues of the laforin-deficient mice show a 4-fold increase in the glycogen level while the value for the db/db mutant was comparable to that of the wild-type animals (Fig. 1D). The double mutants however showed a 2-fold reduction in the glycogen content as compared to the laforin-deficient animals (Fig.1D). The glycogen level in the double mutants however was higher as compared to the wild-type or the db/db single mutant animals (Fig. 1D). Intriguingly, the muscle tissue of the double mutants did not show such a reduction in the glycogen level (Supplementary Material, Fig. S2A), suggesting that the observed difference in the glycogen level in the double mutant was restricted to the brain tissue.

### Laforin-deficient mice lacking the leptin receptor display decreased Lafora bodies and reduced gliosis in the brain

Having seen a reduction in the brain glycogen level in the double mutants, we next wanted to evaluate the presence of Lafora bodies in the brain. The Lafora bodies are aggregates of abnormally branched glycogen, resistant to amylase and can be easily visualized using Periodic acid–Schiff (PAS) staining (23). The double mutants showed significant reduction in the PAS-positive Lafora bodies as compared to the laforin-deficient mice at multiple regions of the brain (Fig. 2A and B). As was observed for the glycogen, the muscle tissue from the double mutant however did not show any reduction in the size and/or distribution for the PAS-positive Lafora bodies (Supplementary Material, Fig. S2B). The wild type and db/db mutants, on the other hand, did not show Lafora bodies in the muscle or in the brain tissues (Supplementary Material, Figs S2B and S3). Besides being glycogen-rich inclusions, the Lafora bodies are also known to recruit few proteins, notable among them are the ubiquitin, p62 and glycogen synthase (GS) (11,24–26). As expected, the double mutants showed considerable reduction in the p62 or GS-positive inclusions in the brain as compared to the brains sections of the laforin-deficient mice (Fig. 3A–D). However, there was no change in the total level of glycogen synthase in the brain tissue when measured using immunoblot (Supplementary Material, Fig. S4).

Lafora bodies are known to be neurotoxic, and therefore gliosis is widely observed in the Lafora disease mouse models (10,11,24). To check if the reduction in the Lafora bodies is associated with reduced gliosis as well in the double mutant, we looked at the distribution of glial fibrillary acidic protein (Gfap), an established marker of gliosis. As shown in Figure 4A, immunostaining showed a significant reduction in the number of Gfap-positive astrocytes in the brains sections of the double mutants as compared to the laforin-deficient mice, and were comparable to the wild-type or the db/db animals (Fig. 4B and Supplementary Material, Fig. S5). We also found that the *Gfap* transcript levels were higher in the laforin-deficient mouse brain and while in the double mutant the *Gfap* transcript level was reduced and was comparable to the wild-type (Fig. 4B). We also looked at the distribution of 1ba1 immunoreactive microglia which is reported to be at higher levels in the LD mouse model (11,27). A show in Figure 4C and D, the double mutant showed a significant reduction in the number of 1ba1-positive microglial cells as compared with the laforin-deficient mice. Similar difference was also observed for the *Iba1* at the transcript level (Fig. 4D). As reported earlier (27), the laforin-deficient mouse brain showed a significant increase in the Iba1 positive cells (Fig. 4D and Supplementary Material, Fig. S5) and also at the transcript level (Fig. 4D). Taken together these results suggest that the double mutants display a significant reduction in Lafora bodies and neurodegeneration as compared to the laforin-deficient single mutant animals.

### Laforin-deficient mice lacking the leptin receptor display reduced susceptibility to induced seizures

Spontaneous seizures are known to occur in the laforin-deficient mice (8). However these symptoms are obvious in the 1-year-old animals, but at lower frequencies and that not all the animals develop them either (8,28). The laforin-deficient mice were known to show increased susceptibility to induced seizures—both for pentylenetetrazole and kainic acid, and this approach is being used to distinguish the seizure phenotypes in LD mouse models (9,11,18). We therefore next tested if the double mutants have altered seizure threshold as compared to the laforin-deficient single mutants. We have used a subconvulsive dose (25 mg/kg body weight) of pentylenetetrazole (PTZ) reported for the seizure induction in the laforin-deficient

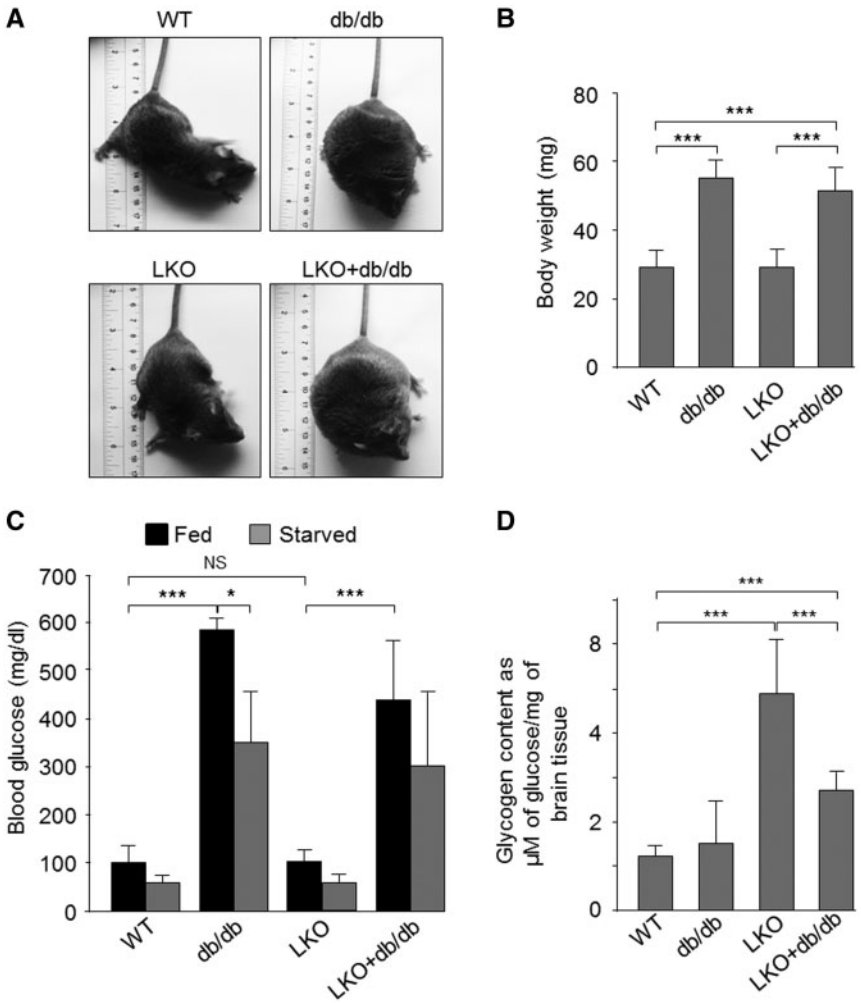


**Figure 1.** Loss of leptin signaling reduces brain glycogen level in the laforin-deficient mouse: (**A**) Representative images showing the wild-type (WT), leptin receptor mutant (db/db), Laforin knock-out (LKO) and the double mutant (LKO + db/db) animals, with the ruler to appreciate the relative size differences. (**B**) Bar diagram showing the average body weight of WT ($N = 23$), db/db ($N = 8$), LKO ($N = 28$), LKO + db/db ($N = 10$) animals (t-test; ***$P < 0.001$). (**C**) Bar diagram showing blood glucose level in the fed and in the overnight-starved conditions for the animal bearing the genotype as indicated. The db/db and LKO + db/db mice had higher blood glucose levels both in fed and in starved conditions as compared with the WT mice. For the LKO animals, the blood glucose levels were comparable to WT (N for WT = 8; db/db = 3; LKO = 8; LKO + db/db = 3; t-test; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; N.S, not significant). (**D**) Bar diagram showing the reduced levels of glycogen in the brain tissue (presented as μM glucose release per 1 mg of brain tissue) of the double mutant as compared to laforin knock-out mice (N for WT = 14; db/db = 6; LKO = 8; LKO + db/db = 8) (t-test; ***$P < 0.001$).

mice (18) and scored for the seizure latency and duration. As reported earlier, the laforin-deficient animals showed increased susceptibility to PTZ-induced seizures as compared to their wild-type littermates (Fig. 5). Specifically, we found that the duration of the partial clonus and myoclonic seizures were significantly higher in the laforin knock-out mice as compared to wild-type littermates. However, the double mutants exhibited a significantly reduced frequency for both partial clonus and myoclonic seizures as compared to the laforin knock-out mice (Fig. 5A and B). The double mutants however did not show any significant difference in the latency for these seizures as compared to the laforin-deficient mice (data not shown).

## Discussion

Lafora disease (LD) is one of the most severe forms of progressive myoclonus epileptic disorders and is characterized by the accumulation of Lafora bodies in the neurons (3). Abnormal form of glycogen (polyglucosan) is one of the principal components of these Lafora bodies (7). Besides, LD tissues, including the brain, are also known to have higher levels of glycogen (7,8,29,30). The mechanism behind the formation of abnormally branched and hyperphosphorylated glycogen as Lafora bodies and/or the increased levels of glycogen in LD is yet to be fully understood. However studies on the LD animal models suggest that inhibiting the glycogen synthesis could ameliorate the disease pathology, including the epileptic seizures in LD (9–11). Administration of leptin is known to enhance the glucose uptake in the brain (13–16) and conversely the leptin-deficient mice exhibited reduced glucose uptake (17). Since the loss of laforin results in increased glucose uptake (12), we reasoned that blocking leptin signaling would bring-down the glucose uptake, and hence the Lafora bodies. We demonstrate here that the loss of leptin receptor in the laforin-deficient mouse led to a significant reduction in the glycogen level, Lafora bodies and gliosis in the brain and reduced susceptibility to induced seizure.

Leptin, secreted by the white adipose tissue, is known to act via brain to activate the catabolic pathway and to inhibit the

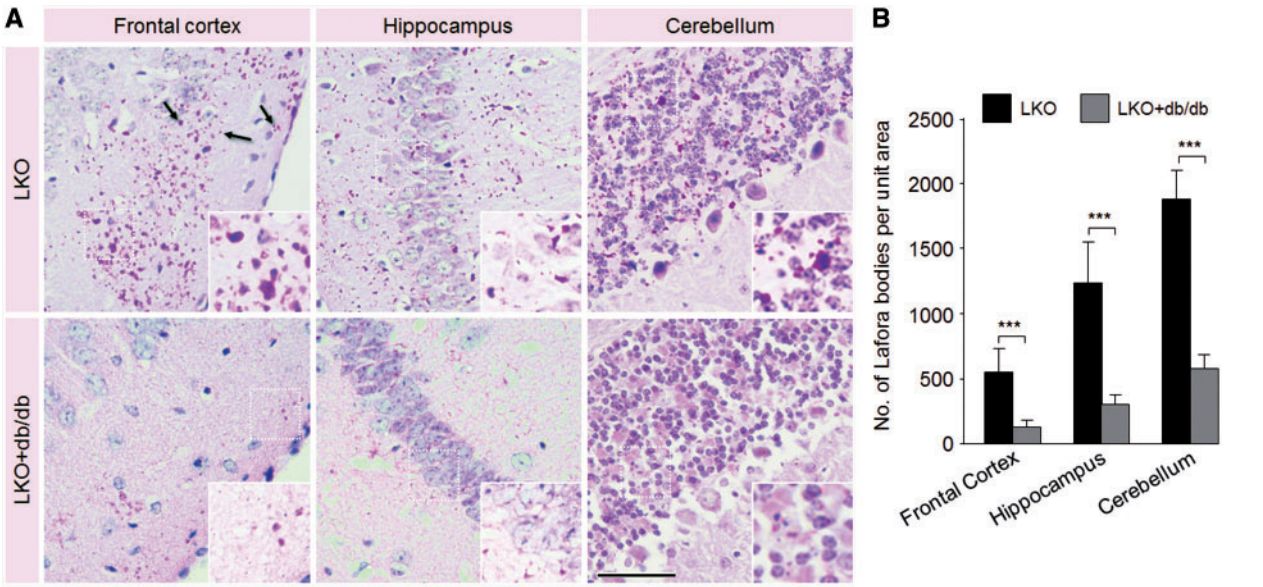


**Figure 2.** Loss of leptin signaling in laforin-deficient mouse reduces Lafora bodies in the laforin-deficient mouse brain: (**A**) Representative images showing the distribution of PAS+ Lafora bodies (identified by black color arrows) in the indicated regions of the brain tissues of the animals with the genotype as identified. Note the reduction in the number as well size of the Lafora bodies in the double mutant in the three regions of the brain shown (scale bar = 50 μM). The insets in each image represent the magnified view of the area denoted by the dotted lines. The absence of PAS+ inclusions for the wild-type and db/db mutant brains is shown in the Supplementary Material, Figure S3. (**B**) Bar diagram showing the reduction in the number of PAS+ Lafora bodies in the double mutant as compared with the laforin-deficient mouse for the three regions analyzed (N = 6 for LKO, and 8 for LKO + db/db) (*t*-test; ***$P < 0.001$).

anabolic pathway and the feeding behavior (31). Amongst the other physiological functions, leptin is known to increase cellular glucose uptake, especially in the brain (13–16), by regulating the expression levels of glucose transporters (32). Consistent with these observations we find that effect of loss of leptin signaling in the laforin-deficient mouse appears to be restricted to the brain tissue; while there was a significant reduction in the glycogen levels and Lafora bodies in the brain tissue of the LD mouse lacking the leptin receptor, the muscle tissue did not show any such difference. Intriguingly, the double mutants also showed a significant reduction in the Gfap and Iba1 reactivity, indicating a reduction in the reactive gliosis and inflammation. A recent study has demonstrated the increased inflammatory response in LD mouse models (27), and that the treatment with sodium selenite, an element with anti-oxidant and anti-inflammatory functions, reduced the gliosis and seizure susceptibility in the LD animals but without affecting the Lafora bodies or the glycogen content (33). Intriguingly, leptin is also known to promote astrogenesis (34). Thus, the current observations that the loss of leptin signaling in laforin-deficient mice associates with reduced seizure susceptibility could be due to the reduced glycogen content in neurons, or the reduced gliosis or a combination of both. The earlier reports that inhibiting glycogen synthesis in LD animal models reduces gliosis also suggest a direct link between glycogen and inflammatory response in the brain tissue (9–11).

While much progress has been made in understanding the possible reasons behind the abnormal glycogen accumulation in LD and on the role for laforin and malin in the glycogen synthesis (12,35,36), the molecular mechanism behind the epileptic seizures in LD has remained an enigma. Inhibiting the glycogen synthesis is known to reduce the seizure onset and seizures susceptibility (9–11), and the same has been observed in the present study. However the causal relationship between the glycogen and the seizure phenotype could not be explained. Nonetheless, our observations that blocking the leptin signaling could ameliorate the LD pathology in the brain and could partially rescue the seizure susceptibility in the LD model suggest the potential of leptin antagonists in LD therapy. Since effective leptin antagonists are available (37), it would be interesting to test them in the LD animal models.

## Materials and Methods

### Generation of double mutant animals

Animals were maintained according to the ethical guidelines stipulated by the CPCSEA, Govt. of India, and the study protocol was approved by the animal ethics committee of the institute. The laforin-deficient mouse line, established by the targeted deletion of the last exon of the *Epm2a* gene and reported in an earlier study (8), and the db/db mouse line deficient for the leptin receptor (21) were used for the study. Animals that were heterozygous for the *Epm2a* null allele (LKO/+) and the leptin receptor (db/+) were crossed to obtain animals that were double homozygous mutants (LKO/LKO; db/db), double heterozygous for the two genes (LKO/+; db/+) or the heterozygous for one of the two gene defects (LKO/+ or db/+). All animals were coded, genotyped and the genotype was not revealed to the user. All the experiments were performed on animals belonging to the 4–5 month age group. Genotyping was carried out as reported earlier (38), and the primers used and PCR conditions are listed in Supplementary Material, Table S1.

### Glycogen estimation

Biochemical estimation of glycogen was performed as described earlier (12). Briefly, 25 mg of the tissue was boiled at 100 °C in 10% (w/v) of 30% KOH (20 min). One hundred microliter (i.e. equivalent to 10 mg of the tissue) of the lysate was spotted on a

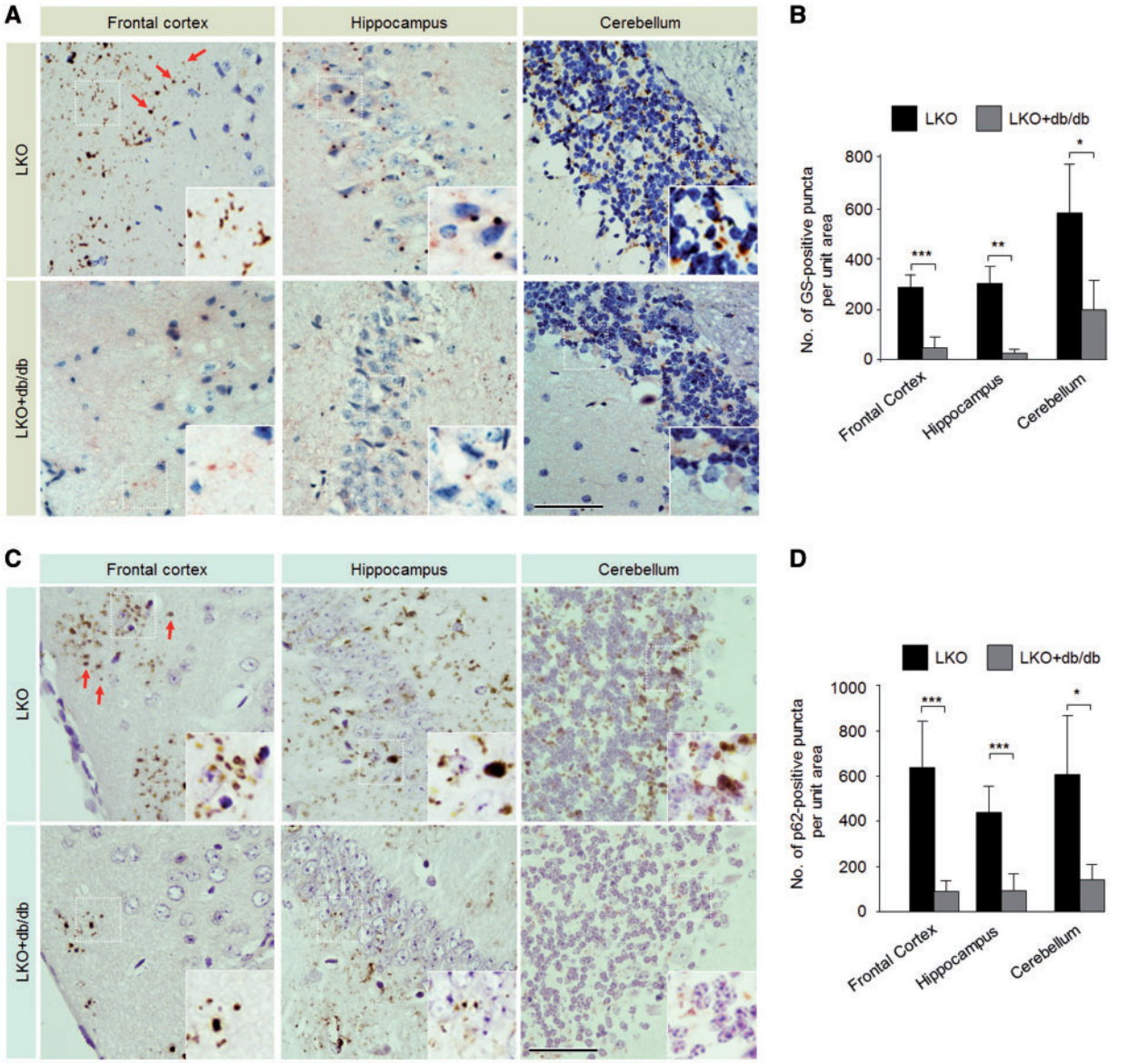


**Figure 3.** Loss of leptin signaling in laforin-deficient mouse reduces the number of glycogen synthase-positive and p62-positive granules in the brain: (**A**) Representative images of the brain sections showing glycogen synthase-positive granules (identified by red color arrows) in the frontal cortex, hippocampus and cerebellar regions of the LKO and LKO + db/db animals, as indicated (scale bar, 50 μM). The insets in each image represent the magnified view of the area denoted by the dotted lines. (**B**) Bar diagram showing a significant reduction in the number of glycogen synthase-positive granules in the brain regions of the double mutant as compared with the laforin-deficient mouse ($N = 3$ for LKO + db/db and 5 for LKO) (t-test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (**C**) Representative images of the brain sections from the LKO and LKO + db/db animals were immunostained with an antibody against the p62 protein. The images represent the distribution of the immune-positive granules (identified by red color arrows) in the hippocampal, frontal cortex and cerebellum regions. Note the reduction in the p62-positive granules in LKO + db/db as compared to LKO (scale bar = 50 μM). (**D**) Bar diagram showing a significant reduction in the number of p62 positive granules in all the three different regions of the double mutants (LKO + db/db) as compared to laforin deficient mouse (LKO) [$N = 5$ (LKO + db/db) and 7 (LKO)] (t-test; $^{*}P < 0.05$; $^{***}P < 0.001$).

filter paper (Whatman paper 31-ET CHR), washed in 66% ethanol (3 washes of 10 min each and first wash in chilled 66% ethanol) and dried over-night at 37 °C. The dried filter paper was incubated in 0.5 mg/ml of amyloglucosidase, dissolved in 0.2 M sodium acetate (pH 4.8), for 1 h and the glucose released was measured using a glucose estimation kit (ERBA Diagnostic Mannheim Gmbh Ltd.). The value thus obtained was represented, in figures, as released glucose (μM) per 1 mg protein of the tissue.

## Periodic acid–Schiff (PAS) staining

The PAS staining was performed as described earlier (8,24). Briefly, 5 μ thin tissue sections were deparaffinised and hydrated using gradient alcohol series. Sections were oxidized using 1 mg/ml of hydrogen peroxide for 15–20 min, washed with distilled water and then treated with the Schiff's reagent for 30 min. The section were counterstained with hematoxylin and visualized under a light microscope.

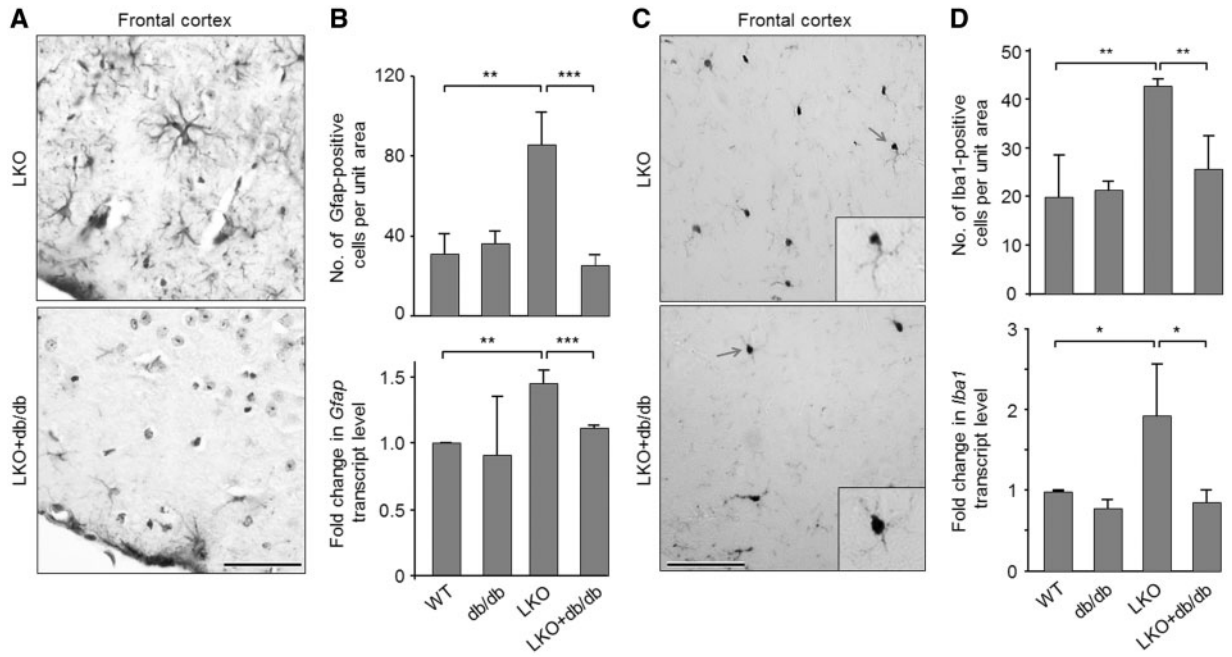


**Figure 4.** Reduced gliosis in the double mutant mouse. (**A**) Representative images of the brain from the LKO and LKO + db/db animals showing the distribution of Gfap-positive cells in the frontal cortex region. Note the reduced immunoreactivity for Gfap in the LKO + db/db animal as compared with the LKO animal (scale bar, 50 μM). (**B**) Bar diagram showing the distribution of Gfap-positive cells, as measured by image analysis (upper panel) ($N = 3$; *t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$), and the fold change in the level of *Gfap* transcripts, normalized to the *β-actin* transcript (lower panel) ($N = 3$; *t*-test, $^{**}P < 0.01$; $^{***}P < 0.001$), in the brain tissues of the animals with indicated genotype. (**C**) Representative images of the brain from the LKO and the LKO + db/db animals showing the Iba1-positive cells in the frontal cortex region. The inset represents the magnified view of the Iba1-positive cell identified by the red arrow in each image (scale bar, 50 μM). (**D**) Bar diagram showing the distribution of the Iba1-positive cells, as measured by image analysis (upper panel) ($N = 4$; *t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$), and the fold change in the level of *Iba1* transcripts, normalized with the β-actin gene (lower panel) ($N = 4$; *t*-test, $^{*}P < 0.05$), in the brain tissues of the animals with indicated genotype.

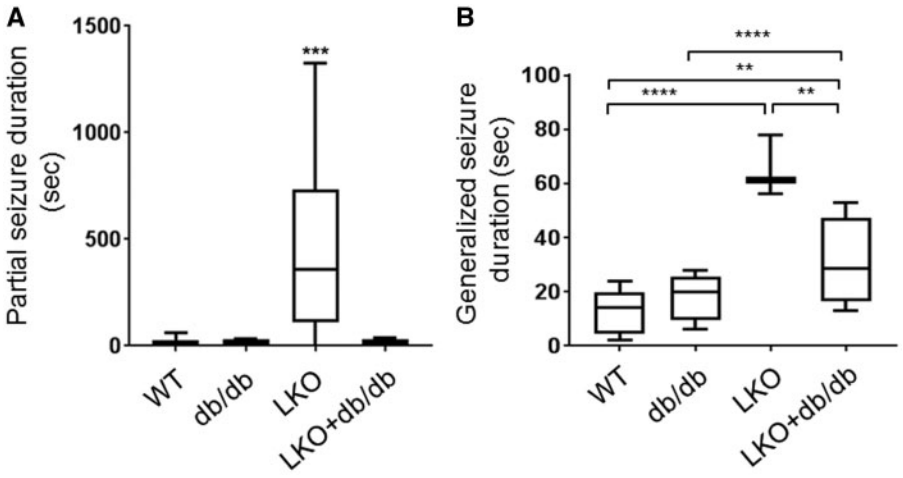


**Figure 5.** Reduced susceptibility for induced seizures in the double mutant. Box plot showing the duration of induced partial seizures (**A**) and generalized seizures (**B**) observed in animals with indicated genotype. The duration of the partial seizures and generalized seizures was at the maximum in the LKO and a significant reduction was observed in LKO + db/db as compared to LKO (*N* for WT = 12, LKO = 8, LKO + db/db = 6 and db/db = 6; one-way ANOVA; *P*-value ***, 0.0001; **, 0.0030).

## Immunohistochemistry

Immunohistochemistry on paraffin embedded sections was performed as described earlier (8,24). Briefly, the paraformaldehyde-fixed and wax embedded sections (5 μ thick) were deparaffinised, hydrated and processed for antigen retrieval, as reported. The sections were incubated overnight at 4 °C with a primary antibody. After washing, the sections were reacted with a HRP-tagged secondary antibody and the signals were developed using the DAB kit (Genei Laboratories Pvt Ltd, India).

## Image analysis

The images were taken using the Nikon ECLIPSE 80i microscope and the quantification of the immunoreactive signals was done using the ImagePro software of Media Cybernetics. For calculating the number of Gfap-positive cells, PAS+ Lafora bodies, and the p62- or GS- positive puncta, images taken using a 40× objective lens (image area = 222 μm × 314 μm) were used. For Iba1-positive cells, images taken using a 20× objective lens (image area = 445 μm × 627 μm) were used. For all calculations, 4 to 5

images per region per section of an animal, and 3-to-7 animals per genotype were analyzed. The value of the signal for each section was taken, and the mean was calculated using raw data from all the sets. Statistical significance was calculated using Student's *t*-test (GraphPad).

### Glucose tolerance test (GTT) and insulin tolerance test (ITT)

For the GTT, the animals were starved for 16 h. At the end of 16 h, glucose (20 mg/kg body weight) was injected and the blood glucose level was monitored at 15, 30, 60, 90 and 120 min post-injection, using a glucometer (LifeScan Inc). For the ITT, animals were starved for 4 h, and insulin (Humulin, Eli Lilly) was injected at 0.5 u/kg of dose. Blood glucose level was monitored at 0, 15, 30, 60, 90 and 120 min post-injection using a glucometer (LifeScan Inc).

### Induced-seizures scoring

Laforin knock-out mice are known to be more susceptible for pentylenetetrazol (PTZ)-induced seizures (18). To induce seizure, 4-to-5 month-old animals were injected with sub-convulsive dose (25 mg/Kg body weight) of PTZ (18) and the animals were recorded for 30 min using a video camera. The seizure onset, duration and severity were scored by a person who was blind for the genotype of the animal. For the seizure score, jerk in any part of the body (head, tail or limbs) was counted as partial seizures and the loss of upright posture was considered as generalized seizures (39). The duration of individual seizure types in the 30 min recorded period was calculated. Statistical significance in the seizure scores was calculated using one-way ANOVA.

### RNA isolation and real-time PCR

RNA isolation and quantitative real-time PCR were carried out as described (38) and the primers used for the PCR are given in Supplementary Material, Table S1. Fold change has been represented in the form of bar graph and significance was calculated using Student's *t*-test.

### Immunoblotting

Tissue samples were processed for immunoblotting as reported earlier (38). Briefly, the tissue lysates were prepared in 1× RIPA lysis buffer, resolved on a SDS-PAGE, and transferred onto Nitrocellulose membrane for immunoblotting. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) was used to develop the signal on an X-ray film. The band intensities were quantified using ImageJ software as reported earlier (12,40,41).

### Antibodies used

The following primary antibodies were used in the present study: anti-GFAP (#3670, CST; IHC: 1:50 dilution), anti-GS (# 3893, CST; IHC, 1:50 dilution; IB, 1:1000 dilution), anti-p62 (PW9860, Enzo; IHC, 1:100 dilution) and anti-Iba1 (ab178846, Abcam; IHC, 1:50 dilution). The HRP-tagged secondary antibodies were from Jackson Immunoresearch.

## Supplementary Material

Supplementary Material is available at *HMG* online.

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*Conflict of Interest statement*. None declared.

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