Lafora bodies and neurological defects in malin-deficient mice correlate with impaired autophagy

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Lafora disease (LD), a fatal neurodegenerative disorder characterized by the presence of intracellular inclusions called Lafora bodies (LBs), is caused by loss-of-function mutations in laforin or malin. Previous studies suggested a role of these proteins in the regulation of glycogen biosynthesis, in glycogen dephosphorylation and in the modulation of the intracellular proteolytic systems. However, the contribution of each of these processes to LD pathogenesis is unclear. We have generated a malin-deficient (Epm2b−/−) mouse with a phenotype similar to that of LD patients. By 3–6 months of age, Epm2b−/− mice present neurological and behavioral abnormalities that correlate with a massive presence of LBs in the cortex, hippocampus and cerebellum. Sixteen-day-old Epm2b−/− mice, without detectable LBs, show an impairment of macroautophagy (hereafter called autophagy), which remains compromised in adult animals. These data demonstrate similarities between the Epm2a−/− and Epm2b−/− mice that provide further insights into LD pathogenesis. They illustrate that the dysfunction of autophagy is a consequence of the lack of laforin–malin complexes and a common feature of both mouse models of LD. Because this dysfunction precedes other pathological manifestations, we propose that decreased autophagy plays a primary role in the formation of LBs and it is critical in LD pathogenesis.

INTRODUCTION

Lafora progressive myoclonus epilepsy [Lafora disease (LD)] is a fatal autosomal recessive neurodegenerative disorder characterized by the presence of glycogen-like intracellular inclusions called Lafora bodies (LBs) (1–5). LD manifests during adolescence with generalized tonic–clonic seizures, myoclonus, absences, drop attacks or partial visual seizures.

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As the disease progresses, a progressive dementia with apraxia, aphasia and visual loss ensues, leading patients to a vegetative state and death, usually within the first decade from onset of the disease (6). Up to date, LD-causing mutations have been identified in two genes, EPM2A (7,8) and EPM2B (9), encoding respectively laforin, a dual-specificity protein phosphatase with a functional carbohydrate-binding domain (10,11), and malin, an E3 ubiquitin ligase (12–14). EPM2A and EPM2B are mutated in 60 and 40% of LD cases, respectively (12,15,16), although there is also evidence for a third very minor locus (17). Patients carrying homozygous loss-of-function mutations in laforin or malin are indistinguishable, suggesting that both proteins work together in the same physiological pathway. The functional characterization of LD-associated EPM2A and EPM2B mutations has illustrated that the enzymatic activities of laforin and malin are critical in the pathogenesis of LD (18). Similarly, these assays have identified ID-associated mutations that exclusively affect the formation of laforin–malin complexes (19,20) or complexes of laforin with PTG (protein targeting to glycogen; a regulatory subunit of protein phosphatase-1 that enhances glycogen accumulation) (21), indicating that, in addition to the laforin and malin enzymatic activities, their protein–protein interactions are also critical in LD pathogenesis. Despite these advances, it is still unclear how laforin and malin loss-of-function mutations are related with the disease. We and others have shown that the laforin–malin complex causes specific ubiquitination and proteasome-dependent degradation of proteins involved in the regulation of glycogen biosynthesis (13,19,20,22,23). Recent studies carried out by Turnbull et al. (24) have shown that down-modulation of glycogen synthesis by PTG depletion reduces LB formation and eliminates neuronal loss and myoclonic epilepsy, supporting a role for glycogen dysregulation in LD pathogenesis. In addition, others have shown that laforin acts as a glycogen phosphatase and proposed that, by suppressing excessive glycogen phosphorylation, laforin prevents the formation of the phosphorylated, poorly branched and aggregate-prone glycogen polymers that comprise LB (25–28). While LD could be an error of carbohydrate metabolism, other studies in cultured mammalian cells, or in the Epm2a−/− mouse model developed by Ganesh et al. (29), have also lead to the proposal that defects in the intracellular proteolysis system affecting protein clearance, autophagy and the ubiquitin–proteasome system may also contribute to LD pathogenesis (30–34). In an attempt to further understand LB formation and LD pathogenesis, different Epm2b−/− mouse models have recently been generated. Like Epm2a−/− mice, these mice show LBs and elevated glycogen levels in the brain, heart and skeletal muscle with no detected differences in levels or activity of the proteins implicated in glycogen metabolism at 3 or 6 months of age (35,36). No changes in glycogen synthase activity were also recently reported by Valles-Ortega et al. (37) in Epm2b−/− mice of 11 months of age, although they indicated an accumulation of the muscular isoforms of glycogen synthase. Based on the analysis of glycogen phosphorylation, it was suggested that LBs in the Epm2b−/− mice are a consequence of the lack of glycogen phosphatase activity of laforin (36). These animals, however, were not studied regarding a putative defect in the intracellular proteolysis system that may affect protein and glycogen clearance.

Here we report the generation of a novel Epm2b−/− mouse strain and show that these malin-deficient mice present a phenotype similar to that of LD patients. Our data also illustrate close similarities between the Epm2a−/− and Epm2b−/− mice, including impaired autophagy and neurological defects similar to those characterizing LD patients, which provide additional insights into LD pathogenesis.

RESULTS

Distribution of LBs in central nervous system and other tissues in Epm2b−/− mice

Our targeting strategy to generate Epm2b knock-out mouse is described in the Supplementary Material and summarized in Supplementary Material, Figure S1. Our Epm2b−/− mice breed normally and show the accumulation of LBs characteristic of LD. To evaluate the distribution of LBs in the Epm2b−/− mice, sections of different tissues from mice at P16 and 3 and 5 months were stained with Periodic acid Schiff (PAS), with and without treatment with diastase, and compared with equivalent sections from wild-type animals. PAS is a histochemical staining used to detect structures containing a high proportion of carbohydrate macromolecules, such as glycogen, glycoproteins or proteoglycans. Diastase is an enzyme that digests free glycogen from a histological section. LBs formed by abnormally branched glycogen are stained by PAS and are diastase resistant. LBs were never detected in tissues from wild-type animals at any of the analyzed ages (Fig. 1) and were also undetectable in any tissue from Epm2b−/− mice at P16, but were plentiful in the brain, heart and skeletal muscle at 3 mo and increased progressively with time (Fig. 1). They were particularly abundant in the heart (data not shown) and specific areas of the brain, including the olfactory bulb, hippocampus, superior and inferior colliculi, preoptic area, lateral geniculate nucleus, cerebellum and pontine nuclei (Fig. 1). Other central nervous system (CNS) areas like the neuretina and the cortex (Supplementary Material, Fig. S2) were initially characterized by fewer LBs, which however increased with time. LBs appeared mostly localized to interneurons, such as the granule cells of the cerebellum, rather than in excitatory projection neurons, i.e. Purkinje cells (Fig. 1). Similarly, in the cortex, LBs were clustered in interneuron-enriched layers as well as in the molecular layer (Supplementary Material, Fig. S2). In the rectus femoris skeletal muscle, LBs were only observed in the glycolytic type II fibers (data not shown).

Utrastructurally, LBs in both Epm2a−/− and Epm2b−/− appeared as non-membrane bound conglomerates of granular material and branching filaments of ~50 to 90 Å, with different shapes (Fig. 2A–D). In the myocardium and skeletal muscle, they were always located within the sarcoplasm of myocytes. The size of LBs in tissue from Epm2b−/− mice was, however, consistently larger than that observed in Epm2a−/− mice (Fig. 2A–D), suggesting a greater deposition of polyglucosan in the former. Indeed, up to 16 LBs as large as 8.7 µm in diameter were observed in a single myocyte of 5–6-month-old Epm2b−/− mice (Fig. 2E and F), whereas they were fewer and smaller in age-matched Epm2a−/− mice (Fig. 2A). Sarcomeres were displaced by LBs, but otherwise
no specific alterations of the contractile apparatus were observed. LBs were generally surrounded by a clear granular area and mitochondria, which frequently showed varying degrees of swelling of the matrix chamber (Fig. 2E and F).

In the CNS, large LBs were found in the neuronal perikarya displacing the nucleus, whereas LBs of smaller size were often observed within processes (Supplementary Material, Fig. S3), likely corresponding to the numerous small PAS-positive granules observed by light microscopy in the different brain areas (Fig. 1 and Supplementary Material Fig. S2). Other ultrastructural changes observed in the CNS include pronounced cytoplasmic retraction, dissolution of rough endoplasmic reticulum and mitochondrial alterations in some neurons and increased neuronal lipofuscin in otherwise normal neurons (Supplementary Material, Fig. S3). These changes were not related to the presence of LBs within the neuron. Irregular, small LBs were seen in smooth muscle cells associated with the vessel and in the periphery of capillaries (Supplementary Material, Fig. S3).

Laforin accumulates in Epm2b−/− mice

Mutational and functional data have shown that laforin and malin form a complex and that formation of this complex influences the levels of both laforin and malin. Laforin stabilizes and prevents degradation of malin and, consequently, malin levels decrease in the absence of laforin (13,19). On the other side, laforin, being a target of malin, accumulates in the absence of malin (13). In agreement with these early data and consistently with previous analysis in other Epm2b−/− mice (35–37), we observed that the levels of laforin were augmented in the brain of Epm2b−/− mice (Fig. 3A). To determine whether laforin was in the soluble fraction or forming part of insoluble aggregates, we subjected tissue extracts to a high spin centrifugation (100 000 g for 90 min at 4°C) and analyzed by western blot the presence of laforin in the supernatant (soluble fraction) and pellet (insoluble fraction).

At P16, when no LBs are detected, laforin is mostly present in the soluble fraction of all examined tissues in Epm2b−/− mice. This was particularly remarkable in the brain (Fig. 3). As mice age, laforin is increasingly found in the insoluble fraction, most probably bound to the polyglucosans present in this fraction (Fig. 3B). It is interesting to remark that laforin in the insoluble fraction could not be released by amylase digestion. Early data have also shown that laforin and glycogen synthase (MGS) colocalize intracellularly at sites of active glycogen synthesis (11,21). Consistent with all these data, and in agreement with a recent report (37) analysis of MGS in the Epm2b−/− mice shows that brain MGS also translocates in the Epm2b−/− mice from the soluble to the insoluble fraction in coincidence with the appearance of LBs. This translocation decreases significantly the levels of MGS in the soluble fraction (Fig. 3C).

Figure 1. LBs in the brain of Epm2b−/− mice. Frontal sections of different brain regions (as indicated in the panels) from Epm2b−/− and wild-type mice at 3 and 5 mo as indicated in the panels were stained with PAS and counterstained with hematoxylin. Note the abundant accumulation of LBs compared with wild-type animals, where PAS staining was only occasionally observed around blood vessels (arrow) and their rapid increase with age. CA1, Cornu Ammonis 1; ml, molecular layer; Pcl, Purkinje cell layer; gl, granule cell layer. Scale bar: 30 μm.
Laforin colocalizes with ubiquitin and MGS at two distinct polyglucosan structures

Consistent with the experiments described above, immunolocalization of laforin in thin consecutive sections from hippocampus and heart revealed a patchy distribution that closely resembled that of PAS-positive LBs, suggesting that laforin accumulates in these structures (Fig. 4A–D). This accumulation was further confirmed by colocalization studies (data not shown). Laforin staining also reproduces the two different types of LBs revealed by PAS staining (Fig. 1), large round and uniform structures and small and irregular ones. Detailed analysis of these LBs with confocal microscopy demonstrates that the small and irregular type of LBs also stain for ubiquitin, the ER chaperone GRP78/Bip that often localizes to protein aggregates (38) and the polyubiquitin-binding protein p62/SQSTM1 (Fig. 4E–G, and Fig. 5A–C), but not for MGS (Fig. 4H–J). In contrast, the large LBs are strongly positive for MGS (Fig. 4H–J) and p62/SQSTM1 (Fig. 5C), but not for ubiquitin or GRP78/Bip (Fig. 4E–G and Fig. 5A and B). Confocal z-stack reconstruction of two adjacent LBs present in the brainstem and representing the two different inclusions demonstrates a uniform and completely overlapping staining for laforin, ubiquitin and GRP78/Bip in the small and irregular LBs but lack of colocalization in the large LBs (Fig. 5A and B). In contrast, in the large and well-defined LBs, MGS localizes mostly to the surface of the LB, whereas laforin is found also in the inner part sometimes forming a strongly positive core, as illustrated by the confocal analysis of several representative individual LBs (Fig. 5D). Notably, p62 was widely distributed in the brain, in large

Figure 2. LBs in myocardium of Epm2a−/− and Epm2b−/− mice. Electron micrographs from the myocardium of Epm2a−/− (A and B) and Epm2b−/− mice (C–F) at 5 mo, showing LBs (asterisks) within the contractile apparatus of a myofiber. (B) and (D) are magnified sections of (A) and (C) to illustrate the fine structure of LBs with a dense central core and peripheral branching filaments (arrows) in both Epm2a−/− and Epm2b−/− mice. LBs are scarce and measure ~0.8 μm in Epm2a−/− mice (A) but are very numerous (E) with variable sizes ranging from <1 μm to ~8 μm in Epm2b−/− mice. LBs are often surrounded by a clear halo and mitochondria (mt) (C–F).

Figure 3. Western blot analysis of laforin in brain tissues of Epm2b−/−, Epm2a−/− and control mice. (A) Western blot of total extracts of brainstem (Bs), cortex (Cx) and hippocampus (Hp) from P16 Epm2b−/−, Epm2a−/− and control mice. Note the accumulation of laforin in Epm2b−/− in the three tissues. Tubulin was used as a loading control. (B) Western blot of total extracts (T), soluble (S) and insoluble (P) fractions of cortex (Cx), brainstem (Bs) and hippocampus (Hp) obtained as described in Materials and Methods from P16 and 3 mo Epm2b−/− mice. Note that laforin is not detected in the soluble fraction of 3 mo Epm2b−/− mice but accumulates in the insoluble fraction. (C) Western blot of total extracts (T), soluble (S) and insoluble (P) fractions of cortex from Epm2b−/− mice to determine the location of glyco- gen synthase (MGS). Total levels of MGS were identical in P16 and 3 mo-old Epm2b−/− mice. However, MGS is distributed differently in the soluble and insoluble fractions, being mostly insoluble in 3 mo-old animals, when LBs are present. Tubulin was used as a loading control.
endogenous LC3-II, a marker that directly correlates with the number of autophagosomes (39), in the brains of P16 and 3 mo Epm2b−/− mice (Fig. 6A and B). This reduction follows a pattern identical to that of Epm2a−/− mice of similar ages, which suggest that autophagy is also impaired in Epm2b−/− mice. Impairment of autophagy was confirmed in the brains of P16 and 3 mo old Epm2b−/− mice by the presence of increased levels of p62/SQSTM1 (Fig. 6C), an autophagy marker that accumulates upon autophagy dysfunction and serves to study autophagic flux (40). In contrast, neither the three proteolytic activities of the proteasome (Fig. 6D and data not shown) nor the levels of proteasome subunits (data not shown) nor the levels of ubiquitinated proteins (Fig. 6E) were significantly different in brains from Epm2a−/− and Epm2b−/− mice, when compared with age-matched control mice. Therefore, our results suggest that autophagy is the main proteolytic system affected in the brain of LD mouse models.

To confirm these findings, we measured the degradation of radio-labeled proteins in mouse embryonic fibroblasts (MEFs) from Epm2b−/− and control mice under starvation conditions to induce autophagy. Total and autophagy-dependent (i.e. methyladenine-sensitive) degradation of long-lived proteins is decreased in MEFs from Epm2b−/− mice (Fig. 7A). In agreement with these results, endogenous LC3-II levels, under high (Krebs–Henseleit medium, H) and low (full medium, L) proteolysis, were reduced in MEFs from Epm2b−/− in comparison to control MEFs. These differences in the levels of LC3-II were also observed in experiments in vitro and ex vivo performed in the presence of bafilomycin A1, a lysosomal inhibitor (Fig. 7B and Supplementary Material Fig. S4). As a whole, these data strongly suggest that the decrease levels of LC3-II in Epm2b−/− mice are a consequence of an impaired autophagosome formation and not the result of increased clearance. As an additional measurement of autophagy conditions, we transfected MEFs with an enhanced green fluorescence protein (EGFP)-LC3 construct. We observed lower amounts of EGFP-LC3 dots in MEFs from Epm2b−/− mice (Fig. 7C). Cumulatively, these results indicate that, as in the case of mice lacking laforin (33), malin knock-out mice present compromised autophagy.

We have recently reported that autophagy dysfunction present in Epm2a−/− mice could, in part, be related to the activation of the mammalian target of rapamycin (mTOR) pathway (33). Thus, we checked whether the mTOR pathway was affected also in Epm2b−/− mice by comparing the levels of phospho-Thr389 p70S6K, a direct target of the mTOR kinase, in extracts of whole brain from 3 mo old Epm2b−/− mice, with those in control animals. No differences could be detected (data not shown). To confirm these findings, we repeat these experiments in the hypothalamus of Epm2b−/− and Epm2a−/− mice under basal and insulin-treated conditions. We chose the hypothalamus because this part of the brain responds best to insulin treatment (41,42) and insulin is a positive effector of the Akt-mTOR pathway. As shown in Supplementary Material, Figure S5, the levels of pT389-p70S6K and pS473-Akt (an indicator of insulin signaling) were similar in Epm2b−/− and control mice under basal or insulin-treated conditions, but elevated in Epm2a−/− mice upon insulin treatment, as we reported.
previously (33). A different behavior between Epm2b−/− and Epm2a−/− mice in the activation of the Akt-mTOR pathway was also observed when we analyzed the insulin signaling pathway in liver extracts or MEFs from these animals (Supplementary Material, Fig. S6). These results suggest that autophagy dysfunction in Epm2b−/− mice follows an mTOR-independent mechanism.

Neurological and behavioral abnormalities in the Epm2b−/− mice

Spontaneous motor activity and coordination as well as balance of Epm2b−/− mice were significantly impaired when compared with those of age-matched controls (Fig. 8A and B). Upon tail suspension test, a high percentage of Epm2b−/− animals were abnormally clasping (Fig. 8C), further reflecting important neurological abnormalities. Moreover, the object recognition task demonstrated a marked decrease in the episodic memory of Epm2b−/− mice compared with that of control animals (Fig. 8D). Cortical recordings of electroencephalographic (EEG) activity showed spontaneous low frequency spike, spike-wave and poly spike-wave complexes with or without correlated myoclonic jerks (Fig. 8E). Epileptiform activity was observed at the age of 3 mo and thereafter. Additionally, Epm2b−/− mice presented myoclonic jerks with no EEG correlates, while intermittent light stimulation did not cause any epileptiform EEG activity or convulsive movements (data not shown). These neurological alterations correlate well with the abundant accumulation of LBs in cerebral cortex, hippocampus, cerebellum and brainstem, suggesting that the lack of laforin or malin

Figure 5. Confocal analysis of proteins associated with LBs in Epm2b−/− mice. (A) Confocal z-stack slices of the same pair of LBs immunostained with anti-laforin (green) and (A) anti-ubiquitin (Ubq, red); (B) Grp78/bip (red) and (C) p62 (red). Laforin and ubiquitin colocalize with Grp78/Bip and p62 in the small irregular LB (arrow). Large round LBs are stained for both laforin and p62 (arrowhead). Note that p62 accumulates also outside of LBs (star). (D) Mid-views of the overlay of different examples of large LBs stained with anti-laforin (green) and anti-glycogen synthase (MGS, red) antibodies to show the predominantly peripheral distribution of MGS. Scale bars: 10 μm.
in these areas could cause neuronal degeneration and/or brain damage that lead to cognitive and behavioral deterioration.

**DISCUSSION**

Analyses in $Epm2b^{-/-}$ mice have provided us with novel data regarding pathogenic mechanisms and LB formation in LD that advance significantly previous work. Consistent with the observation that LD patients with mutations in either laforin or malin present identical phenotypes, we show here that both $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice present impaired autophagy and illustrate in $Epm2b^{-/-}$ mice neurological and behavior abnormalities, correlating with the presence of LBs in different brain regions which are similar to those found in $Epm2a^{-/-}$ mice. Importantly, we also show that autophagy is impaired in both $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice before LBs are detected and that autophagy remains compromised in adult animals and during LBs development. *In vivo* studies to measure autophagic flux in brains are technically difficult. To circumvent this problem, we measure autophagic flux in brain homogenates (20 mg protein) from P16 and 3 mo control, $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice using the Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay. Values are means ± SEM. (E) Total brain lysates (75 µg protein) from brains of P16 and 3 mo control, $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice were analyzed by western blot using antibodies that recognize ubiquitinated proteins and actin. The positions of molecular-mass markers and their size in kDa are indicated on the right. Poly-ub, polyubiquitinated chains.

**Figure 6.** Analysis of autophagic and proteasomal activities in the brain of $Epm2b^{-/-}$ mice. (A) Representative western blots using anti-LC3 or anti-actin antibodies with lysates (40 µg protein) from brains of P16 and 3 mo-old control, $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice. (B) The LC3-II bands from at least three different experiments similar to those shown above were densitometered and normalized to the corresponding actin bands. Data are expressed in % relative to control mice. Values are means ± SEM ($**P < 0.01, ***P < 0.001$). (C) Total brain homogenates (30 µg protein) from P16 and 3 mo control and $Epm2b^{-/-}$ mice were analyzed by western blot with anti-p62 or anti-actin antibodies. Densitometric analysis from three different experiments is shown on the right. Values are means ± SEM ($**P < 0.01$). (D) Proteasome activities were measured in brain homogenates (20 mg protein) from P16 and 3 mo control, $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice using the Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay. Values are means ± SEM. (E) Total brain lysates (75 µg protein) from brains of P16 and 3 mo control, $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice were analyzed by western blot using antibodies that recognize ubiquitinated proteins and actin. The positions of molecular-mass markers and their size in kDa are indicated on the right. Poly-ub, polyubiquitinated chains.
complex causes specific ubiquitination and proteasome-dependent degradation of proteins involved in the regulation of glycogen biosynthesis (13,19,20,22,23). Now we demonstrate that the lack of the laforin–malin complex, as a consequence of loss of either laforin (33) or malin (this report), impairs autophagy. Based on these novel data and on previous reports, we propose that the combination of both, impaired autophagy and glycogen dysregulation, results in the formation of LBs, leading to neurological and behavior abnormalities. This process is likely exacerbated by uncontrolled glycogen hyperphosphorylation.

Our detailed analyses of Epm2b−/− mice demonstrate two different types of LBs that suggest distinct stages of maturation. Formation of LBs likely initiates as small and irregular

Figure 7. Loss of malin decreases the degradation of long-lived proteins by autophagy and decreases autophagosome formation. (A) Intracellular protein degradation (total) and the amount of protein degraded by autophagy (MA) were calculated as described in Materials and Methods in MEFs from control (black histograms) and Epm2b−/− (white histograms) mice. (B) Representative blots, using anti-LC3 or anti-actin, of MEFs lysates from control and Epm2b−/− mice, incubated for 2 h with (left) or without (right) bafilomycin A1 (400 nM) under conditions of high (H, Krebs_Henseleit medium) and low (L, full medium) proteolysis. The histograms below show the densitometric quantification of LC3-II bands from three different experiments normalized to the corresponding actin bands. Upper gels: low exposure (exp.), lower gels: high exposure. Values are expressed in % relative to MEFs from control mice, incubated under conditions of high proteolysis with bafilomycin A1, and are means ± SEM (**P, 0.001). (C) MEFs, transfected with 0.5 μg EGFP-LC3 for 6 h, were fixed and analyzed for EGFP-LC3 vesicles at 24 h post-transfection. The number of fluorescent dots per cell (on the right) in Epm2b−/− MEFs (white histogram), is lower than in control MEFs (black histogram).
PAS-positive inclusions that stain positive for laforin and ubiquitin. This is consistent with an early defect in autophagy, which would enhance the accumulation of glycogen and ubiquitinated proteins (43–45). The large LBs with a smooth and well-defined round morphology are predominantly present in older animals and likely correspond to mature LBs. These structures show an intense, but asymmetric, staining for laforin and MGS. The presence of MGS and absence of ubiquitin and GRP78/Bip in the large LBs may be an indication of a remodeling during the maturation of the LBs.

Previous studies have shown that during formation of LBs, there is an accumulation of glycogen that progressively becomes more phosphorylated and insoluble, and suggested that LBs are mainly a consequence of the lack of the glycogen phosphatase activity of laforin (25–28). This hypothesis is supported by elegant experiments showing that in vitro laforin alone can de-phosphorylate phosphorylated glycogen (26,27) and that laforin complements in plants the deficiency of Sex4, which has a well-established role as a starch phosphatase (25,28). Interestingly, it has been noted by Turnbull et al. (35) that glycogen phosphorylation in Epm2b<sup>−/−</sup> animals does not reach significant levels until 3 months of age and that, in any case, it is considerably reduced compared with that of Epm2a<sup>−/−</sup> mice. It is at this age (3 months) when Epm2b<sup>−/−</sup> mice start to show abundant LBs and when most laforin is sequestered, bound to them.

One possibility to explain these differences in glycogen phosphorylation between Epm2b<sup>−/−</sup> and Epm2a<sup>−/−</sup> is

![Figure 8](https://example.com/figure8.png)

**Figure 8.** Neurological and behavioral abnormalities in Epm2b<sup>−/−</sup> mice. (A) Patterns of accumulated motor activity in 4–6 mo-old C57BL/6J and Epm2b<sup>−/−</sup> mice measured as surface displacements (a), rearing (b) and stereotyped movements (c). Quantitative data represent mean ± SEM. **P < 0.001 versus control Student’s t-test (n = 12–17 in each group). (B) Rotarod-based analysis of motor coordination performance of 4–6 and 9–13 mo-old control and Epm2b<sup>−/−</sup> mice (n = 12–16). The mean latencies (time to fall off the rotarod) of young (trials 3–4) and aged (trial 3) Epm2b<sup>−/−</sup> mice were significantly lower than those of controls. **P < 0.05, Student’s t-test. (C) Percentage of 4–6 and 9–13 mo-old C57BL/6J and Epm2b<sup>−/−</sup> mice showing normal posture (0), partially altered (1) or high abnormal (2) stereotypical clasping of the hind-limb upon tail suspension (n = 8–16). The frequent hind-limb clasping of Epm2b<sup>−/−</sup> mice worsens with age. (D) Assessment of memory performance with the object recognition task in 4–6 and 9–13 mo-old control and Epm2b<sup>−/−</sup> mice (n = 11–16). The discrimination index (DI) of Epm2b<sup>−/−</sup> mice is poor and decreases with age, indicating deficits in the retention of episodic memory. Values are expressed as mean ± SEM. Student’s t-test. **P < 0.01. (E) Determination of the cortical EEG activity. (a) Intracranial recording of basal EEG activity (6–7 Hz) in control mice. (b) Spontaneous polyspike-wave complex (arrowhead) corresponding to a muscular jerk in a 3 mo-old Epm2b<sup>−/−</sup> mouse. Spontaneous spike (c) (open arrowhead) and spike-wave complexes (d) (arrows) corresponding to myoclonic jerks in a 14 mo-old Epm2b<sup>−/−</sup> mouse.
that in \textit{Epm2b}\textsuperscript{−/−} mice glycogen phosphorylation is prevented by the presence of elevated levels of soluble laforin. In this case, impaired autophagy would be the primary cause of the accumulation of polyglucosans in young \textit{Epm2b}\textsuperscript{−/−} mice. Later on, however, glycogen phosphorylation may play a role exacerbating the accumulation of polyglucosans in \textit{Epm2b}\textsuperscript{−/−} mice. Since laforin in these mice is progressively mobilized to the LBs, with age these animals could become deficient of both malin and laforin. Consistent with the idea that the loss of both proteins accelerates the formation of LBs, we have observed that in the double \textit{Epm2a}\textsuperscript{−/−}; \textit{Epm2b}\textsuperscript{−/−} mice, LBs develop earlier than in the \textit{Epm2b}\textsuperscript{−/−} mice (LD-T, SRDeC and PB, unpublished data). Our data reinforce the concept that the lack of laforin–malin complexes is critical in LD pathogenesis. We have previously shown that the ability of laforin to stimulate autophagy is not saturated in cells, and that overexpression of laforin induces autophagy and enhances autophagy substrate clearance (33). This is not the case of our \textit{P16 Epm2b}\textsuperscript{−/−} mice, which present impaired autophagy, despite the presence of elevated levels of soluble laforin. These observations indicate that the impairment of autophagy in the \textit{Epm2a}\textsuperscript{−/−} and \textit{Epm2b}\textsuperscript{−/−} mice is a consequence of the lack of laforin–malin complexes and not just a consequence of a laforin deficiency. In this respect, our early report indicating that autophagy dysfunction in \textit{Epm2a}\textsuperscript{−/−} mice could be in part explained by the activation of the mTOR pathway (33) is in apparent conflict with our failure to demonstrate activation of the mTOR pathway in \textit{Epm2b}\textsuperscript{−/−} mice. Additional experiments are needed to determine the precise molecular mechanisms underlying autophagy impairment in \textit{Epm2a}\textsuperscript{−/−} and \textit{Epm2b}\textsuperscript{−/−} mice. At present, we can only suggest that autophagy dysfunction in these animals is likely due to an altered mTOR-independent mechanism (46). Because \textit{Epm2b}\textsuperscript{−/−} mice express laforin without mTOR activation, the mTOR activation observed in \textit{Epm2a}\textsuperscript{−/−} mice may be exclusively related to the lack of laforin.

The massive presence of LBs in the cortex, hippocampus and cerebellum of \textit{Epm2b}\textsuperscript{−/−} mice at 3–6 months of age very likely explains the behavioral and neurological defects observed in these animals, which well mimic those characterizing LD patients. Our \textit{Epm2b}\textsuperscript{−/−} mice show significant motor coordination and activity impairments, hindlimb clasp- ing, memory deficits and epileptiform activity. Episodic memory deficits and hindlimb claspig get worse with age and are more significant in \textit{Epm2b}\textsuperscript{−/−} mice at 9–13 mo, probably accounting for the increased accumulation of LBs in the cortex/hippocampus and in the cerebellum and motor nuclei of the brainstem, respectively. Epileptiform activity was observed in \textit{Epm2b}\textsuperscript{−/−} mice regardless of the age, although deeper analysis of video-EEG activity is currently being performed and will be reported elsewhere. Notably, these behavioral defects are more pronounced than those reported in a similar \textit{Epm2b}\textsuperscript{−/−} mouse line by Valles-Ortega \textit{et al.} (37), who did not observe sign of cerebellar ataxia, associative memory deficits or alterations in the basal EEG activity, although reported a tendency to hippocampal seizures, following an epileptogenic stimulus. Difference in the genetic strain or in the number of backcrosses may explain this milder phenotype. Our data instead demonstrate that \textit{Epm2b}\textsuperscript{−/−} mice have similar motor and memory impairments as those reported in \textit{Epm2a}\textsuperscript{−/−} (29), although motor alterations of young \textit{Epm2b}\textsuperscript{−/−} mice are slightly enhanced.

In conclusion, our data provide a significant advance in the understanding of the pathogenic mechanisms in LD. Further work should delineate the biochemical pathways that, altered by the loss of laforin–malin complexes, result in impaired autophagy. Additional studies should also define whether impaired autophagy has, in addition to LB formation, other manifestations in the CNS of \textit{Epm2a}\textsuperscript{−/−} and \textit{Epm2b}\textsuperscript{−/−} mice. The postulated role of autophagy in synaptogenesis and neural plasticity (47,48) are obvious targets for future analyses.

**MATERIALS AND METHODS**

**Generation of \textit{Epm2b} knock-out mice**

A detailed description of the procedures that ended in the generation of the initial \textit{Epm2b}\textsuperscript{−/−} mice is provided in Supplementary Materials. All experiments included in this report were performed exclusively with animals derived from the mating of these first founder mice. Mice were maintained at the Centro de Investigaciones Biológicas (CIB-CSIC, Madrid) on a light/dark 12:12 h cycle under constant temperature (23°C) with free access to water and regular chow diet (65% carbohydrate, 11% fat, 24% protein). All animal care and procedures used in this study were in accordance with the Declaration of Helsinki principles and the guidelines from the Institutional Animal Care and Use Committee, and approved by the CIB-CSIC ethical review board.

**Antibodies and other reagents**

Primary antibodies used in this study were the following: anti-glycogen synthase (1:1000 dilution; Cell Signalling Technology); anti-tubulin and anti-actin (1:4000 and 1:5000, respectively; Sigma); anti-ubiquitin and anti-p62 (1:1000 each; Enzo-Biomol Exeter, UK); anti-LC3 (1:1000, Nano- tools); mouse monoclonal antibody 3.3.5, which recognizes human and mouse laforin (generated in our laboratory immunizing \textit{Epm2a}\textsuperscript{−/−} mice with recombinant human laforin); and anti Grp78/Bip (1:500; Stressgen). The anti-p62 antibodies used for immunohistochemistry were a polyclonal antibody generated in guinea-pig (1:100; Progen). Primary antibodies were detected with the following reagents: anti-mouse or anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibody (1:5000 or 1:250000, Dako); biotin-anti-rabbit (1:500; Jackson Immuno Research); anti-mouse-Alexa488 (1:2000, Molecular Probes); Cy3-tyramide (PerkinElmer Life Sciences Inc.); Cy3-anti-ginea-pig (1:600; Jackson Immuno Research); Mach4 (Bio CARE Medical); and DAB [0.5 mg/ml in phosphate-buffered saline (PBS); Sigma]. Other reagents were: fetal calf serum (Invitrogen), BSA (Sigma), Triton X-100 (Sigma), Tween-20 (Sigma), Periodic acid (Panreac), Schiff reagent (Merck), Hematoxylin (Merck), DEPEX (BDH) and Enhanced Chemiluminiscence substrate (ECL, GE Healthcare).
Histological procedures

Epm2b−/− and control C57BL/6J mice were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate buffer 0.2 M pH 7.3 and thereafter post-fixed for 3 h at room temperature (RT). Tissue was embedded in paraffin and sectioned at 10 μm with a microtome (Leica). For PAS-diastase staining, sections were deparaffinized, rehydrated and incubated with 0.1% fresh diastase (Sigma) for 30 min at 57°C. After washing in distilled water, sections were incubated with 1% periodic acid for 15 min, followed by an additional 15 min incubation with Schiff reagent. Sections were counterstained with hematoxylin, dehydrated and mounted in DEPEX. For electron microscopy (EM) studies, mice were anesthetized and perfused with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After fixation, tiny square blocks of the heart, liver and selected areas of the CNS (brain cortex, hippocampus, basal ganglia, brainstem and cerebellum) were washed in cacodylate buffer and treated with 2% osmium tetraoxide. After progressive dehydration, the blocks were routinely embedded in Epon (EMBed-812, Electron Microscopy Sciences, USA). Semi-thin sections were stained with toluidine blue. Ultrathin sections were cut using an ultramicrotome (Leica Ultracut UCT, Reichert, USA), mounted on copper grids, contrasted with uranyl acetate and lead citrate, examined under a Philips CM-100 transmission electron microscope and photo-documented with a Veleta camera (Item System).

Immunohistochemistry and confocal microscopy

Epm2b−/− and control mice were transcardially perfused with 4% PFA and processed as above. The sections were deparaffinized, rehydrated and boiled at 115°C for 2 min in 10 mM citrate buffer using a decloaking chamber (Biocare Medical) for antigen retrieval. Immunohistochemistry was performed with standard protocols. The Mach4 reagent or biotinylated secondary antibodies followed by streptavidin peroxidase were used to detect primary antibodies. When fluorescent secondary antibodies (mouse-Alexa488 and Cy3-tyramide) were used, tissue was fixed as above, washed in PBS, incubated in a 30% sucrose/PBS solution, embedded and frozen in a 7.5% gelatin in 15% sucrose solution, sectioned at 10 μm with a cryostat and processed for antigen retrieval. Staining was performed using standard protocols, and analyzed and photographed with a Leica confocal microscope (TCS SP5II).

Western blotting

Tissue homogenates and cell lysates were prepared in the radio immunoprecipitation assay buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF) containing 1 mM sodium orthovanadate, 50 mM NaF, aprotinin and benzamidine, 2 μg/ml each. Protein content was determined using the Bradford method (Bio-Rad). Comparable amounts of proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis on polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Blocking was performed in 5% non-fat milk for 1 h at RT. Blots were probed with indicated antibodies in TRIS buffered saline and Tween 20 (50 mM Tris–HCl, 150 mM NaCl, pH 7.5 with 0.1% Tween 20) plus 5% non-fat milk. Primary antibodies were detected with antimouse or anti-rabbit IgG-HRP and visualized using ECL detection kit.

Analysis of autophagy

To label long-lived proteins, C57BL/6J (control) and Epm2b−/− MEFs were incubated for 48 h in complete fresh medium with 2 μCi/ml [3H]valine (Hartmann Analytic GmbH). Cells were chased for 24 h at 37°C in fresh full medium containing 10 mM l-valine to degrade short-lived proteins (49). Cells were then incubated for 4 h under high proteolysis conditions in the Krebs–Henseleit medium containing 10 mM Hepes, pH 7.4 and 10 mM l-valine. Protein degradation was analysed 1 h later, to ensure maximal effects of the various additions, at intervals of 1.5 h, for a period of only 3 h, to avoid possible secondary effects. Protein degradation was determined by measuring the net release of trichloroacetic acid-soluble radioactivity from the labeled cells into the culture medium and expressed as percentage of protein degraded in 1 h, and the contribution of macroautophagy was calculated using 10 mM 3-methyladenine (Sigma-Aldrich) as previously described (49). Endogenous LC3-II levels, which directly correlate with autophagosome numbers (39), were detected with anti-LC3 antibody. Actin was used to normalize the assays. To assess autophagic flux, LC3-II was measured in cells treated with or without 400 mM bafilomycin A1 for 2 h (50). To analyze autophagosome formation, control and Epm2b−/− MEFs were transfected with 0.5 μg EGFP-LC3 for 6 h. After 24 h, cells were incubated for 2 h under high proteolysis conditions and fixed for analysis of EGFP-LC3 vesicles. Approximately 50 EGFP-positive cells were randomly selected and the number of fluorescent dots per cell was counted.

Analysis of proteasome activity

Twenty micrograms of soluble protein extracts were incubated with the Promega Proteasome-Glo Assay Reagent (Promega Bioscience, Madison, WI, USA) for 10 min. The chymotrypsin-like proteasome activity was detected as the relative light unit generated from the cleaved substrate in the reagent and represented as fold increase in chemiluminescence during 10 min. Luminescence generated from each reaction condition was detected with a Wallac 1420 VICTOR luminometer.

Neurological and behavioral analysis

Two groups of animals, considered representative samples of young adult and aged mice, were analyzed for each experiment (group 1: 4–6 months; group 2: 9–13 months). Experiments were approved by the Animal Research Committee of the IIS-FJD. Analysis of spontaneous motor activity, neuromuscular abnormalities, claspng responses and episodic memory retention in Epm2b−/− mutant mice were performed as detailed in the Supplementary Materials. For
video-EEG recordings, custom-made stainless steel screw electrodes were fixed to the skull of anesthetized animals (Domtor, Ketolar and sterile water, 1:1:5:1.5). Two monopolar electrodes were implanted symmetrically over the frontal cortex in front of bregma, whereas two ground and reference electrodes were positioned posterior to lambda. The pins of the electrodes were attached to a plastic pedestal (Plastic One Inc.), the headset was fixed in place and the wound closed with dental cement (Selectaplus CN, Dentsply DeTrey GmbH, Dreireich, Germany). After a 4-day recovery period, synchronized video-EEG activity was recorded for up to 12 h for a total duration of 1–4 days per mouse, using a computer-based system (Natus Neurwork EEG, Natus Medical Inc, San Carlos, CA, USA), while mice were freely moving. After each recording session, the entire EEG record was inspected for electrographic seizure activity and corresponding segments of video were reviewed for behavioral correlation.

Statistical analyses

Values are given as means ± SEM of at least three independent experiments. Differences between groups were analyzed by two-tailed Student’s t-tests (Graph-Pad Prism 2.0). Statistical significance was considered at *P < 0.05, **P < 0.01 and ***P < 0.001, as indicated in each case.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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