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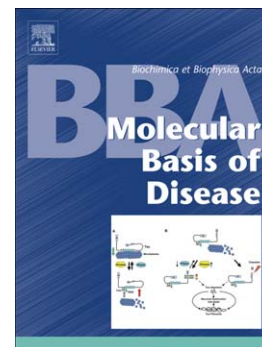
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Homeostasis of the astrocytic glutamate transporter GLT-1 is altered in mouse models of Lafora disease.

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Running title: GLT-1 function is altered in Lafora disease

ABSTRACT

Lafora disease (LD, OMIM 254780) is a fatal rare disorder characterized by epilepsy and neurodegeneration. Although in recent years a lot of information has been gained on the molecular basis of the neurodegeneration that accompanies LD, the molecular basis of epilepsy is poorly understood. Here, we present evidence indicating that the homeostasis of glutamate transporter GLT-1 (EAAT2) is compromised in mouse models of LD. Our results indicate that primary astrocytes from LD mice have reduced capacity of glutamate transport, probably because they present a reduction in the levels of the glutamate transporter at the plasma membrane. On the other hand, the overexpression in cellular models of laforin and malin, the two proteins related to LD, results in an accumulation of GLT-1 (EAAT2) at the plasma membrane and in a severe reduction of the ubiquitination of the transporter. All these results suggest that the laforin/malin complex slows down the endocytic recycling of the GLT-1 (EAAT2) transporter. Since, defects in the function of this transporter lead to excitotoxicity and epilepsy, we suggest that the epilepsy that accompanies LD could be due, at least in part, to deficiencies in the function of the GLT-1 (EAAT2) transporter.

Highlights:

- GLT-1 glutamate transporter is reduced at the membrane of astrocytes from LD mice.
- Glutamate transport is also reduced in primary astrocytes from LD mice.
- Overexpression of laforin and malin impairs ubiquitination of GLT-1 transporter.
- The laforin/malin complex impairs the endocytic recycling of the GLT-1 transporter.

Key words: Lafora disease, epilepsy, glutamate transport, ubiquitination, endocytosis, protein recycling, EAAT2, GLT-1.

Abbreviations: EAAT2, excitatory amino acid transporter 2; GLT-1, glutamate transporter 1; LD, Lafora disease; IHC, immunohistochemistry; IHF, immunohistofluorescence; WGA, wheat germ agglutinin.

1.- INTRODUCTION

Lafora progressive myoclonus epilepsy (Lafora disease, LD, OMIM 254780) is a fatal rare autosomal recessive neurodegenerative disorder with an onset when the patients are around 6 to 19 years of age. It is characterized by generalized multiple types of seizures, myoclonus and the accumulation of insoluble polyglucosan inclusions, called Lafora bodies (LBs), in the cytoplasm of neurons and other cells in peripheral tissues. As the disease progresses, patients present a rapid progressive dementia concomitant with an amplification of seizures leading to death within a decade after the first symptoms [1][2]. Up to now, two main causative genes have been identified, *EPM2A* which encodes the dual specificity phosphatase laforin and *EPM2B* which encodes the E3-ubiquitin ligase malin. Although both proteins might have independent functions, it is now well known that laforin and malin form a functional complex, explaining why patients with mutations in the genes encoding either laforin or malin are neurologically and histologically indistinguishable [3]. It has been recently described that laforin and malin participate in several processes, such as glycogen biosynthesis [4][5] or regulation of cellular protein homeostasis (proteostasis) (see [6] for review). However, in spite of the advances in the understanding of the molecular basis of LD, the mechanisms that lead to epilepsy are poorly known. Only recently it has been suggested that inhibitory GABAergic neurons could be affected in LD [7][8], yielding some preliminary insights into how epilepsy develops in LD patients.

In this work we present evidence indicating that the homeostasis of glutamate transporter GLT-1, called EAAT2 (Excitatory AminoAcid Transporter 2) in humans, is affected in mouse primary astrocytes lacking either *EPM2A* or *EPM2B* genes. Glutamate is the main excitatory neurotransmitter in the central nervous system and it is implicated in important physiological and pathological processes. It is also well known the importance of the homeostasis of this neurotransmitter in a healthy brain: when glutamate is released in the synaptic cleft, it must be rapidly taken up by different transporters in order to prevent glutamate receptors overstimulation, as this can lead to neuronal injury and death in a process known as excitotoxicity [9]. GLT-1 (EAAT2) transporter is responsible of up to 90% of glutamate uptake, and it is expressed mainly in perisynaptic astrocytes [10][11][12][13]. This transporter is incorporated into the astrocytic plasma membrane by exocytosis in a Ca^{2+} dependent manner [14], and recycled by endocytosis upon PKC activation and Nedd4.2-mediated ubiquitination [15][16][17]. Other groups have also described the sumoylation at the C-terminal tail as a mechanism of regulation of EAAT2 localization, since EAAT2 sumoylation retains the transporter in intracellular compartments [18]. However, the mechanisms regulating EAAT2 trafficking are still far from being understood.

There is an increasing evidence of the importance of EAAT2 dysfunction in different neurological disorders. Defects in the clearance of glutamate from the synaptic cleft by

EAAT2 appear in a wide variety of neurodegenerative diseases, ranging from Alzheimer's disease [19], to amyotrophic lateral sclerosis (ALS) [20], including epilepsy [21]. Dysfunctions in the regulation of the subcellular localization of EAAT2 may conduct to altered glutamate uptake and excitotoxicity [13][22], and this could be responsible for the establishment of these and other neurological disorders. Thus, understanding the molecular elements participating in the homeostasis of EAAT2 becomes an interesting goal to achieve. In this work we present evidence indicating that the subcellular location of GLT-1 (EAAT2) transporter is altered in Lafora disease cellular and mouse models and that laforin and malin affects the endocytic recycling of this transporter.

2.- MATERIALS AND METHODS

2.1.- Ethic statement, animal care, mice and husbandry

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Consejo Superior de Investigaciones Cientificas (CSIC, Spain). All mouse procedures were approved by the animal committee of the Instituto de Biomedicina de Valencia-CSIC [Permit Number: INTRA12 (IBV-4)]. All efforts were made to minimize animal suffering. To eliminate the effect of differences in the genetic background of the animals, we backcrossed *Epm2a*^{-/-} and *Epm2b*^{-/-} mice (with a mixed background 129sv:C57BL/6) previously described [23][24] with control C57BL/6JRccHsd mice obtained from Harlan laboratories (Barcelona, Spain), ten times to obtain homozygous *Epm2a*^{-/-} and *Epm2b*^{-/-} in a pure background. Mice were maintained in the IBV-CSIC facility on a 12/12 light/dark cycle under constant temperature (23°C) with food and water provided *ad libitum*.

2.2.- Mammalian cell cultures

Human neuroblastoma SH-SY5Y cells [kindly provided by Dr. Domingo Baretino (Institute of Biomedicine of Valencia, CSIC, Valencia, Spain)] were grown in Dulbecco's Modified Medium/Nutrient Mixture F-12 (Life Technology, Madrid, Spain) supplemented with 15% inactivated fetal bovine serum (FBS) (Invitrogen, Madrid, Spain), 1% L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Human embryonic kidney (HEK293) cells were grown in Dulbecco's Modified Medium (Lonza, Barcelona, Spain), supplemented with 10% inactivated FBS, 1% L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Both cell types were grown in a humidified atmosphere at 37°C and 5% CO₂.

2.3.- Mouse primary astrocyte cell cultures

Mouse primary astrocyte cell cultures from control, *Epm2a*^{-/-} and *Epm2b*^{-/-} mice were obtained from newborn mice cortices as previously described [25]. Cells were grown in Dulbecco's modified Eagle's medium (Lonza, Barcelona, Spain) containing 20% of inactivated fetal bovine serum (FBS), supplemented with 1% L-glutamine, 1% glucose, 1% fungizone, 100 units/ml penicillin and 100 µg/ml streptomycin, in a humidified atmosphere at 37°C with 5% CO₂. After the first week, FBS was reduced to 10%. The purity of astrocytes was confirmed by immunofluorescence using anti-GFAP (an astrocyte marker; Sigma-Aldrich, Madrid, Spain), anti-Neurotrophin 3 (a neuron marker; Chemicon International, Madrid, Spain) and anti-Iba-1 (a microglia marker; Wako Chemicals, Richmond, USA).

2.4.- Flow cytometry analyses

Mouse primary astrocytes and human neuroblastoma SH-SY5Y cells were plated in 60 mm diameter culture dishes and grown in the corresponding media described above in a humidified atmosphere at 37°C with 5% CO₂. Cells were transfected with the indicated plasmids and 24 hours after transfection, they were washed twice with cold PBS, incubated with anti-EAAT2 antibodies (Santa Cruz Biotechnology, Madrid, Spain) at 4°C for 30 minutes, washed twice with cold PBS and incubated with phycoerythrin-conjugated goat anti-mouse antibodies (Santa Cruz Biotechnology, Madrid, Spain) for 30 min at 4°C. Cells were washed again twice with cold PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes. Cells were finally detached with trypsin for 5 minutes at 37°C and suspended. Cell bound fluorescence was measured in a BD FACSCanto flow cytometer (BD Biosciences, Madrid, Spain) and analyzed by the software provided by the manufacturer.

2.5.- Glutamate uptake

Mouse primary astrocyte cell cultures from control, *Epm2a*^{-/-} and *Epm2b*^{-/-} mice were seeded 48h before the assay at a density of 2×10^5 cells/well in a 48 wells plate and grown in Dulbecco's modified Eagle's medium (Lonza, Barcelona, Spain), supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 10% of inactivated fetal bovine serum (Lonza, Barcelona, Spain) in a humidified atmosphere at 37°C with 5% CO₂. Cells were washed twice with PBS at 37°C and then incubated with L-glutamate [$2 \mu\text{Ci/ml}$ L-[³H]glutamate (PerkinElmer, Madrid Spain)] in PBS at 37°C for 10 min. Incubations were carried out in triplicate. For each assay, parallel determinations were performed in a sodium-free PBS (NaCl was iso-osmotically substituted by choline chloride). Assays were stopped by aspiration followed by two washes with ice-cold PBS or sodium-free PBS respectively. Cells were lysed in 0.2 M NaOH and accumulated radioactivity measured in a liquid scintillation counter (PerkinElmer Liquid Scintillation Analyzer Tri-Carb 2800TR). Glutamate uptake was expressed as the fold change in radioactivity incorporation between cells treated with PBS

and cells treated with sodium-free PBS, as sodium is required for the GLT-1 mediated co-transport of glutamate. Each experiment was repeated at least three times.

2.6.- Immunohistochemistry and immunohistofluorescence analyses.

Male mice of 12 months of age were sacrificed by cervical dislocation. Brain was recovered and the two hemispheres separated, one conserved at -80°C for processing for western blot analyses, and the other hemisphere fixed in 4% paraformaldehyde in phosphate buffer saline (PBS) and dehydrated for posterior histological analyses. Dehydrated tissues were embedded in paraffin and sectioned at 4 µm. For immunohistochemistry analyses (IHC), sections were deparaffined, rehydrated, and warmed at 95°C for 10 min in 10 mM citrate buffer for antigen retrieval. Sections were blocked in blocking buffer (1% BSA – 5% FBS, in PBS) and incubated overnight at 4°C with primary anti-Nedd4 (Abcam, Cambridge, UK) antibody diluted at 1/200 in blocking buffer. After three washes of 10 min in PBS, sections were incubated for one hour at room temperature with biotin-conjugated rabbit secondary antibody diluted at 1/250 in blocking buffer, washed three times with PBS for 5 min, incubated with the immunopure ABC kit (Fisher scientific, Madrid, Spain) for 30 min in darkness, washed three times in PBS for 5 min and developed using metal enhanced dab method (Fisher scientific, Madrid, Spain). IHC sections were finally counterstained with haematoxylin (Sigma, Madrid, Spain), dehydrated and mounted in DPX (Merck, Germany). Images were acquired with a DM6000 Leica Microscope and analyzed with Image J software (NIH, Bethesda, MD).

For immunohistofluorescence analyses (IHF), sections were deparaffined, rehydrated, blocked with sodium borohydrate (1mg/mL) for 40 minutes to avoid autofluorescence and warmed at 95°C for 10 minutes in 10 mM citrate buffer for antigen retrieval. Sections were blocked in blocking buffer (1% BSA - 5%-FBS) and incubated overnight at 4°C with anti-EAAT2 antibody (Santa Cruz Biotechnology, Madrid, Spain), anti-glutamine synthase (Sigma, Madrid, Spain) or anti-GFAP (Sigma, Madrid, Spain), as indicated in each case. After three washes of 10 minutes in PBS, sections were incubated with Alexa Fluor 488-conjugated anti-mouse (Invitrogen, Madrid, Spain), Alexa Fluor 594-conjugated anti-rabbit (Invitrogen, Madrid, Spain), Texas Red-conjugated anti-mouse (Invitrogen, Madrid, Spain) or Alexa Fluor 488-conjugated anti-rabbit (Invitrogen, Madrid, Spain) for 45 minutes at room temperature in darkness. After two 10-minutes PBS washes, sections were incubated with DAPI 0.2 µg/mL (Sigma, Madrid, Spain) in PBS for ten minutes, washed again three times in PBS and mounted using Aqua-Poly/Mount coverslip medium (Polysciences, Inc. Eppelheim, Germany). Images were acquired with a Leica TCS SP8 confocal microscope (Leica, Wetzlar, Germany) and were processed with ImageJ software (NIH, Bethesda, MD).

2.7.- Immunofluorescence analyses

Mouse primary astrocyte cultures were grown on 12-wells plates containing coverslips during 24 hours. Cells were washed twice with cold phosphate saline buffer (PBS) at 4°C. Then, they were incubated with anti-EAAT2 antibody (Santa Cruz Biotechnology, Madrid, Spain) 30 minutes at 4°C. Cells were then washed twice with cold PBS and incubated with TexasRed-conjugated anti-mouse antibodies (Invitrogen, Madrid, Spain) for 30 minutes at 4°C. Cells were washed again with cold PBS and incubated with Wheat Germ Agglutinin (WGA) Alexa Fluor 488 conjugate (Invitrogen, Madrid, Spain) during 20 minutes at 4°C. After two washes with cold PBS, cells were finally fixed with 4% paraformaldehyde (PFA) in PBS for 20 minutes, washed three times in PBS and mounted on slides using Aqua-Poly/Mount coverslip medium (Polysciences, Inc. Eppelheim, Germany). Images were acquired with a Leica TCS SP8 confocal microscope (Leica, Wetzlar, Germany) and were processed with ImageJ software (NIH, Bethesda, MD).

2.8.- Plasmids

pCMV-6xHis-ubiquitin, pHA-malin, pFlag-malin, pHA-laforin and pFlag-laforin plasmids have been previously described [26]. pCMV-HA-Nedd4.2 plasmid was from Dr. Lynne Yenush (Instituto de Biología Molecular y Celular de Plantas, CSIC, Valencia, Spain). pcDNA3-EAAT2 plasmid was a generous gift from Dr. Peter Dodd (School of Chemistry and Molecular Biosciences, University of Queensland, Queensland, Australia).

2.9.- Western blot analyses

Mouse brain homogenates were lysed in RIPA buffer [20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1% NP-40; 1 mM Na_3VO_4 and complete protease inhibitor cocktail (Roche Diagnostics, Barcelona, Spain)] for 20 min at 4°C and centrifuged at 10,000 g for 15 min. Cell culture extracts were prepared using lysis buffer [25 mM TrisHCl pH 7.4, 15 mM EDTA, 50 mM NaF, 0.6 M sucrose, 15 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1% nonidet P40, 10 mM NaCl, 1 mM PMSF, and a complete protease inhibitor mixture (Roche Diagnostics, Barcelona, Spain)]. Cells were lysed by repeated passage through 24Gx5/8" needle and whole lysates were centrifuged at 10,000 x g for 15 min. Supernatants were collected and 30 µg of total protein subjected to SDS-PAGE, transferred into nitrocellulose blotting membranes (GE Healthcare Life Sciences, Barcelona, Spain) and incubated with the appropriated antibodies: anti-EAAT2 from Santa Cruz Biotech (Barcelona, Spain); anti-Nedd4.2 and anti-Nedd4 from Abcam (Cambridge, UK), anti-actin, anti-HA and anti-Flag (Sigma, Madrid, Spain). After that, they were revealed using goat anti-mouse IRDye 680LT (LI-COR Biosciences, Germany) or goat anti-rabbit IRDye 800CW (LI-COR Biosciences, Germany) and images obtained with the

Odyssey Infrared Imaging System (LI-COR Biosciences, Germany). The results were analyzed using the software Image Studio Lite version 3.1 (LI-COR Biosciences, Germany).

2.10.- Analysis of ubiquitination

HEK293 cells were co-transfected with plasmids pcDNA3-EAAT2, pCMV-6xHis-ubiquitin and, when indicated, with pFlag-malin and pFlag-laforin, or pCMV-HA-Nedd4.2, using X-treme GENE transfection reagent, according to the manufacturer's instructions (Roche Diagnostics, Barcelona, Spain). After 18 h of transfection, cells were lysed in guanidinium hydrochloride to inhibit the action of deubiquitinases and ubiquitinated proteins purified by metal affinity chromatography as in [27]. Bound proteins and clarified extracts were analyzed by immunoblotting with the appropriate antibodies.

2.11.- Nedd4-2 and Nedd4 degradation assay

HEK293 cells were transfected with pFlag-laforin and pFlag-malin plasmids using X-treme GENE transfection reagent (Roche Diagnostics, Barcelona, Spain). 24 hours after transfection, 350 μ M cycloheximide (Sigma, Madrid, Spain) was added to stop protein synthesis. At the indicated times (0 to 24 hours), cells were removed and lysed to analyze the amount of endogenous Nedd4-2 and Nedd4 relative to actin by SDS-PAGE and Western blot.

2.12.- qRT-PCR analyses

Dissected brain biopsies or primary astrocyte cultures were frozen in liquid nitrogen and stored at -80°C . Thawed samples were homogenized in 2 ml TRIPURE reagent (Roche Diagnostics, Mannheim, Germany) and total RNA was isolated according to the manufacturer's instructions. The concentration of the total RNA was determined using Thermo NanoDrop 2000 (Thermo Fisher Scientific inc., Waltham, USA). First strand cDNA was synthesized from 1 μ g of total RNA using random hexamer and expand reverse transcriptase (Roche Diagnostics, Mannheim, Germany). cDNA was used as a template for real-time PCR. PCR primers and fluorogenic TaqMan probe sets for each gene were designed using Universal probe library Service (Roche Molecular Biochemicals) to meet all TaqMan design guidelines (Universal probe #103 and suggested primers for *SLC1A2* gene). Each PCR was carried out in a final volume of 25 μ l of PCR Master Mix (Applied Biosystems), containing 200 nM of each primer and 40 nM of specific fluorescent probe. The cycle conditions were 20 s at 95°C for initial denaturing, followed by 35 cycles of 95°C for 3 s and 60°C for 30 s in the 7500 Fast Real-time PCR system (Applied biosystems). GAPDH was used as an internal standard. Each reaction was done in triplicate from at least three

independent experiments. The relative amount of each mRNA was calculated using the second derivative comparative Ct method.

2.13.- Statistical analysis

Data are expressed as means with standard deviation. Statistical significance of differences between the groups was evaluated by a paired Student's t-test with two-tailed distribution or by an ANOVA test in case of having more groups. The significance has been considered at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, as indicated in each case.

3.- RESULTS

3.1.- LD mice present a marked reactive astrogliosis in different brain areas.

We have recently described that *Epm2b*^{-/-} mice present a clear reactive astrogliosis in different areas of the hippocampus [28]. These results were in agreement with previous reports [29]. In order to check whether reactive astrogliosis was also present in *Epm2a*^{-/-} mice we carried out an immunohistofluorescence analysis of hippocampal regions of both *Epm2a*^{-/-} and *Epm2b*^{-/-} mice of 12 months of age and compared them with healthy control animals. As shown in Fig. 1, both *Epm2a*^{-/-} and *Epm2b*^{-/-} mice presented an increased GFAP (Glial Fibrillary Acidic Protein) related staining, a recognized marker of reactive astrogliosis, in comparison with control animals. However, we observed no differences in the staining when we used anti-glutamine synthase (GS) antibodies (a general astrocyte marker) in the assay. Thus, we conclude that although there is no evident change in the number of total astrocytes in the studied sections, there is an increase in the number of astrocytes that have become reactive in *Epm2a*^{-/-} and *Epm2b*^{-/-} mouse brains. Similar results were obtained in the cortex and other brain sections (not shown). Therefore, both *Epm2a*^{-/-} and *Epm2b*^{-/-} mice present reactive astrogliosis in different brain areas.

3.2.- Astrocytes from LD mice present decreased levels of GLT-1 at the plasma membrane.

Since reactive astrogliosis may change the physiological properties of affected astrocytes, and since one of the hallmarks of Lafora disease is epilepsy, we planned to study the functionality of different astrocytic channels and/or transporters related to epileptogenesis. We started the analysis with GLT-1 (EAAT2 in humans) glutamate transporter, since it is responsible of up to 90% of glutamate uptake [10][11][12][13]. We analyzed the expression of the *SLC1A2* gene encoding GLT-1 transporter and the total protein levels of this protein in different areas of the brain (hippocampus and cortex) of 12 month old mice by qRT-PCR, Western blotting and immunohistofluorescence. However, we

found no statistical differences between control and *Epm2a*^{-/-} or *Epm2b*^{-/-} mice (Supplementary Fig. S1), suggesting that expression of GLT-1 is not affected in the brain of old LD mice in spite of the reactive astrogliosis present (Fig. 1).

Then, we analyzed the ability of primary astrocytes to transport glutamate and found that astrocytes from both *Epm2a*^{-/-} and *Epm2b*^{-/-} mice had lower capacity of glutamate uptake in comparison to primary astrocytes from control mice (Fig. 2). These results suggested that astrocytes from LD mice have reduced capacity for glutamate transport. We analyzed next whether there were differences in the levels of GLT-1 transporter, but again we found similar levels both at the level of the expression of corresponding *SLC1A2* gene (Fig. 3A) and total protein (Fig. 3B) in the primary astrocytes from LD mice in comparison to healthy animals. Since GLT-1 is a protein mainly located at the plasma membrane of the astrocytes, being its localization regulated by ubiquitin-dependent endocytosis [15][16][17][30], we decided to study whether the localization of this transporter was affected in primary astrocytes from LD mouse models. With this aim, we stained them with anti-EAAT2 antibodies and analyzed them by flow cytometry under native conditions, to label only the protein that was present at the outer surface of the plasma membrane. As observed in Fig. 3C, the relative fluorescence at the membrane surface was diminished in primary astrocytes from *Epm2a*^{-/-} and *Epm2b*^{-/-} mice respect to control mice, with a mean reduction of around 80% in the case of *Epm2a*^{-/-} and around 64% in the case of *Epm2b*^{-/-} mice. Primary astrocytes were also analyzed by immunofluorescence using anti-EAAT2 antibodies and wheat germ agglutinin (WGA, a plasma membrane marker) under native conditions. As observed in Fig. 3D, astrocytes from control mice presented a GLT-1-related fluorescence at the outer surface of the plasma membrane that co-localized with the wheat germ agglutinin marker. However, we observed a clear decrease in the fluorescence associated to GLT-1 protein in the case of the astrocytes from *Epm2a*^{-/-} and *Epm2b*^{-/-} in comparison to control mice. All these results indicate that astrocytes from LD mice present a decrease in the levels of GLT-1 at the plasma membrane and that this decrease is not due to altered total GLT-1 protein levels.

3.3.- Overexpression of laforin and malin increases the levels of EAAT2 at the plasma membrane.

Since the absence of laforin or malin produced a decrease in the levels of GLT-1 at the plasma membrane in the astrocytes from LD mice, we decided to study the effect of the overexpression of laforin and malin on the subcellular localization of EAAT2, the human version of mouse GLT-1. In these studies we used human neuroblastoma SH-SY5Y cells, as they retain the ability to express endogenous EAAT2 transporter. SH-SY5Y cells were transfected with plasmids expressing laforin and malin and the levels of endogenous EAAT2

analyzed by different techniques. First, we confirmed by Western blotting that laforin and malin were overexpressed in SH-SY5Y cells (Fig. 4A) and observed that this overexpression did not alter the total levels of endogenous EAAT2 present in the cells (Fig. 4A). Second, we labelled with anti-EAAT2 antibodies the transfected cells and analyzed them by flow cytometry under native conditions, to label only the proteins that were present at the outer surface of the plasma membrane. As shown in Fig. 4B, SH-SY5Y cells expressing laforin and malin showed an increase in the fluorescence associated to EAAT2 at the surface of the plasma membrane (around 3.5 fold increase) respect to cells transfected with empty plasmids (mock cells). Taking together, all these results indicate that the overexpression of laforin and malin increases the levels of EAAT2 at the plasma membrane, without affecting the total cellular level of the protein.

3.4.- Overexpression of laforin and malin prevents the ubiquitination of EAAT2.

The fact that the levels of GLT-1 (EAAT2) at the plasma membrane were changing depending on the presence or not of laforin and malin suggested a possible involvement of these proteins on the trafficking/recycling of the GLT-1 (EAAT2) transporter. As indicated above, the plasma membrane localization of mouse GLT-1 transporter is regulated by ubiquitin-dependent endocytosis [15][16][17][30]. Since laforin and malin form a functional complex in which laforin recruits specific substrates to be ubiquitinated by the E3-ubiquitin ligase activity of malin, we studied whether the overexpression of laforin and malin affected the ubiquitination status of GLT-1 (EAAT2) transporter. To carry out these experiments we used human embryonic kidney (HEK293) cells transfected with a plasmid expressing human EAAT2 (since the endogenous levels of this transporter are quite low in these cells), a plasmid expressing a modified form of ubiquitin with an N-terminal tag of 6xHis (to allow the purification of ubiquitinated proteins) and plasmids encoding laforin and malin. Twenty-four hours after transfection cells were lysed in guanidinium hydrochloride (to preserve ubiquitination of proteins by inhibiting the action of deubiquitinating enzymes) and ubiquitinated proteins recovered by metal affinity chromatography. Purified proteins were analyzed by Western blotting using anti-EAAT2 antibodies. As shown in Fig. 5, under regular conditions EAAT2 showed a clear pattern of ubiquitination (lane 2) that did not change when laforin alone was expressed (lane 3). However, when laforin and malin were expressed together, we observed a dramatic decrease in the recovery of ubiquitinated forms of EAAT2 (lane 5). This decrease was also observed in cells expressing malin alone (lane 4), probably because HEK293 cells contained enough endogenous laforin to allow malin to form the functional complex. These results indicated that, first, EAAT2 was not a direct ubiquitination substrate of the laforin/malin complex and second, that the regular ubiquitination of EAAT2 was prevented by the action of the laforin/malin complex. Since cells overexpressing laforin

and malin contained higher levels of EAAT2 at the plasma membrane (Fig. 4; see above), the decrease in the ubiquitination status of EAAT2 by the overexpression of laforin and malin could be the reason why the transporter gets accumulated at the plasma membrane, due probably to an endocytic recycling failure of the transporter.

It has been described that the E3-ubiquitin ligase Nedd4.2 participates in the ubiquitination of EAAT2, regulating cell surface turnover of the transporter [16][17]. We overexpressed Nedd4.2 in HEK293 cells in order to improve the ubiquitination of EAAT2, but we did not observe major differences respect to cells transfected with an empty plasmid (Fig. 5, compare lanes 3 and 6), probably because HEK293 cells may have enough endogenous Nedd4.2 protein to allow a maximal ubiquitination. However, in cells co-expressing Nedd4.2 and the laforin/malin complex we did not recover ubiquitinated forms of EAAT2 (Fig. 5, lane 7), indicating that the laforin/malin complex either inactivated Nedd4.2 and other alternative E3-ubiquitin ligases responsible for the modification of EAAT2, or affected alternative adaptor proteins that linked the action of these E3-ubiquitin ligases to EAAT2.

In order to explore whether Nedd4.2 could be a direct substrate of the laforin/malin complex, we analyzed if this complex could ubiquitinate this protein. However, when we performed these ubiquitination experiments in HEK293 cells we observed that although laforin and malin auto-ubiquitinated themselves (indicating that the complex was active), we did not observe any laforin/malin mediated ubiquitination of Nedd4.2, either when overexpressed as a fusion protein (HA-Nedd4.2) or when the endogenous levels of Nedd4.2 were analyzed in the same way (Supplementary Fig. S2A). We extended our analysis to Nedd4, the closest functional homologue of Nedd4.2, with similar negative results (Supplementary Fig. S2A). Next we studied whether laforin and malin could indirectly affect the half-life of Nedd4.2 or Nedd4. HEK293 cells were transfected with plasmids expressing laforin and malin, and then treated with cycloheximide to inhibit protein synthesis. As shown in Supplementary Fig. S2B, the presence of the laforin/malin complex did not affect significantly the half-life of endogenous Nedd4.2 and Nedd4 proteins present in HEK293 cells.

However, when we analyzed by immunohistochemistry different regions of the hippocampus of *Epm2a*^{-/-}, *Epm2b*^{-/-} and control mice of 12 months of age using specific anti-Nedd4 antibodies, we observed an increased staining in the samples corresponding to LD mice respect to similar sections from control animals (Fig. 6A) (we repeated the same experiment with anti-Nedd4.2 antibodies but they were not adequate for IHC techniques). Nevertheless, when we analyzed by Western blotting the levels of endogenous Nedd4.2 and Nedd4 in brain extracts from control and LD mice, we did not observe major differences (Fig. 6B). These results could suggest a putative negative correlation between the laforin/malin complex and the levels of Nedd4 in some particular neural cells. Alternatively, the

laforin/malin complex might affect indirectly the subcellular localization of the GLT-1 (EAAT2) transporter by regulating other components of the EAAT2 ubiquitination reaction (i.e., E3-ubiquitin ligase adaptors).

4.- DISCUSSION

In recent years, several works have been published explaining part of the molecular basis of the dysfunctions present in Lafora disease (LD) (see [6][31][32] for review). However, we have poor information about the molecular determinants that are responsible for the epilepsy that accompanies the disease. In this work we present evidence indicating that the homeostasis of the GLT-1 (EAAT2) transporter is compromised in mouse models of LD. GLT-1 (EAAT2) is a protein mainly located at the plasma membrane of the astrocytes, being its localization regulated by ubiquitin-dependent endocytosis [15][16][17][30]. Our results indicate that although the total protein levels of this transporter do not change in primary astrocytes from LD mouse models in comparison to astrocytes from control mice, the presence of GLT-1 (EAAT2) at the plasma membrane is reduced in primary astrocytes from LD mice. We also show that primary astrocytes from these mice have reduced capacity of glutamate transport, what is consistent with the reduced levels of the GLT-1 transporter in their plasma membrane. On the other hand, the overexpression of laforin and malin results in a severe reduction of ubiquitination of EAAT2 and a consequent accumulation of the transporter at the plasma membrane. Taking all these results together we suggest that the laforin/malin complex affects the endocytic recycling of the GLT-1 (EAAT2) transporter.

It is known that GLT-1 (EAAT2) is constitutively endocytosed into the recycling endosome via a clathrin-dependent pathway, a process that is dependent on the ubiquitination of the C-terminal domain of the transporter by the E3-ubiquitin ligase Nedd4.2 [16][17]. Since the laforin/malin complex reduces the ubiquitination of EAAT2, even in cells overexpressing Nedd4.2, one possibility is that the complex affects the activity of Nedd4.2. In this sense, although we found that the laforin/malin complex did not ubiquitinate Nedd4.2 or its closest functional homologue Nedd4, we observed that in the absence of laforin or malin, Nedd4 was accumulated in specific neural cells of the hippocampus of LD mice. Therefore it seems that there is a negative correlation between the laforin/malin complex and the levels of Nedd4 in these cells.

Another possibility is that the laforin/malin complex could affect the endocytic recycling of GLT-1 (EAAT2) transporter by an indirect mechanism. It has been described that ubiquitination of substrates by Nedd4.2 is mediated by the binding of the E3-ubiquitin ligase to PPXY structural motifs present in its corresponding substrates [33][34]. In substrates that do not contain a PPXY motif, Nedd4.2 binds to specific adaptors that bridge the binding of

the E3-ubiquitin ligase to the substrate [35][36][37]. Since GLT-1 (EAAT2) transporter does not bear PPXY structural motifs [38], this implies the need for intermediate proteins to mediate the interaction of Nedd4.2 with GLT-1 (EAAT2). Perhaps the laforin/malin complex could affect the stability/activity of the intermediate adaptor, affecting in this way the overall functionality of the Nedd4.2-adaptor system.

In any case our results indicate that primary astrocytes from LD mice have lower ability for glutamate transport, probably because they have reduced levels of GLT-1 at the plasma membrane. Since defects in the function of this transporter lead to excitotoxicity and epilepsy [13][21][22], we suggest that the neuronal death and epilepsy that accompanies LD could be due, at least in part, to deficiencies in the function of the GLT-1 (EAAT2) transporter. Therefore, EAAT2 could be a good target to develop a treatment for epilepsy in LD.

Furthermore, defects in glutamate uptake could also contribute to other alterations observed in LD patients. The entry of glutamate mediated by EAAT2 transporter is essential to maintain high levels of intracellular glutamate to allow the uptake of cystine into the cells by the cystine/glutamate antiporter. As cystine is a precursor of the synthesis of glutathione, then glutamate uptake is essential for the synthesis of this metabolite, which plays an important role in redox detoxification [39]. For this reason, impairment of glutamate uptake could slow the synthesis of glutathione and favor oxidative damage in LD, what would be in agreement with the conditions of increased oxidative stress that have been recently described in this disease [40].

5.- CONCLUSIONS

In this work we present evidence indicating that glutamate transport is impaired in primary astrocytes from LD mice. This is probably due to altered homeostasis of the glutamate transporter GLT-1 (EAAT2) at the plasma membrane. Since defects in the function of this transporter lead to excitotoxicity and epilepsy, we suggest that the epilepsy that accompanies LD could be due, at least in part, to deficiencies in the function of the GLT-1 (EAAT2) transporter.

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7.- AUTHOR CONTRIBUTION STATEMENT

C.M-B., A.B. and R.V. carried out the experimental work. C.M-B., A.B., R.V. and P.S. analyzed the data. C.M-B. and P.S. wrote the paper.

8.- DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1: GFAP expression is increased in LD mice, indicating reactive astrogliosis. Hippocampal regions from 12 months old control, *Epm2a*^{-/-} and *Epm2b*^{-/-} mice were analyzed by immunohistofluorescence using anti-glutamine synthase (GS, in green, upper panels) and anti-GFAP (in red, middle panels) as primary antibodies and Alexa Fluor-488 conjugated anti-rabbit and Texas red-conjugated anti-mouse as secondary antibodies. A merged analysis of both fluorescences is presented in the lower panels. A representative picture of the analysis of samples from three different mice of each type is presented. White bar: 50 μ m.

Figure 2: Glutamate uptake is impaired in primary astrocytes from LD mice. Glutamate uptake was carried out as described in Materials and Methods. As GLT-1 co-transporters glutamate and Na⁺, results are expressed as the ratio between the glutamate uptake in a buffer containing Na⁺ and the glutamate uptake in an osmotically-equivalent buffer lacking Na⁺. Values are the mean of 5 different experiments (bars indicate standard deviation, asterisks denote significant differences comparing the corresponding groups with values from control astrocytes; *** $p < 0.001$ using ANOVA test).

Figure 3: Levels of GLT-1 transporter are decreased at the plasma membrane of primary astrocytes from LD mice. A) qRT-PCR analysis of the expression of *SLC1A2* gene encoding the GLT-1 glutamate transporter. qRT-PCR was performed in at least three independent samples of primary astrocytes from control and LD mice, as described in Materials and Methods; bars indicate standard deviation. B) Western blot analyses of the total levels of GLT-1 in primary astrocytes. Crude extracts (30 μ g) from primary astrocytes from control and LD mice were prepared and analyzed by immunoblotting using anti-EAAT2 and anti-actin (loading control) antibodies. No differences were observed in the total amount of GLT-1 in the different samples. C) Analysis of the levels of GLT-1/EAAT2 transporter at the plasma membrane by flow cytometry. Primary astrocytes from control and LD mice were analyzed under native conditions by flow cytometry, as described in Materials and Methods, using mouse anti-EAAT2 and phycoerythrin-conjugated goat anti-mouse antibodies. Graphics represent the levels of phycoerythrin (PE)-dependent fluorescence respect to the number of cells (count); the vertical line separates the PE-dependent fluorescence (P9) from unspecific cellular signals (this threshold was determined using cells without anti-EAAT2 antibody). PE-dependent fluorescence levels in the samples were referred to the levels found in the primary astrocytes from control mice (100%). Flow cytometry analyses were repeated three times and the mean relative fluorescence of each type of primary astrocytes is indicated in the bottom panel (bars indicate standard deviation, asterisks denote significant differences

comparing the corresponding groups with values from control astrocytes; *** $p < 0.001$ using ANOVA test). D) Similar samples of these cells were also analyzed by immunofluorescence using anti-EAAT2 antibodies and Wheat Germ Agglutinin (WGA) Alexa Fluor 488 conjugate (to mark plasma membrane). Fluorescence of the two markers was merged to demonstrate the co-localization of the signals.

Figure 4: Protein levels of EAAT2 at the plasma membrane are increased in cells overexpressing laforin and malin. A) Human neuroblastoma SH-SY5Y cells were transfected with plasmids expressing Flag-laforin and Flag-malin. Crude extracts (30 μ g) were prepared and the total levels of endogenous EAAT2 and actin analyzed by Western blotting. Similar crude extracts were also analyzed to demonstrate the overexpression of laforin and malin (anti-Flag antibody). B) SH-SY5Y cells transfected with plasmids expressing laforin and malin or with empty plasmids (mock cells) were analyzed under native conditions by flow cytometry, as described in Materials and Methods, using mouse anti-EAAT2 and phycoerythrin-conjugated goat anti-mouse antibodies. Graphics represent the levels of phycoerythrin (PE)-dependent fluorescence respect to the number of cells (count); the vertical line separates the PE-dependent fluorescence (P2) from unspecific cellular signals (this threshold was determined using cells without anti-EAAT2 antibody). PE-dependent fluorescence levels in the samples were referred to the levels found in the mock cells (1x). Flow cytometry analyses were repeated three times and the mean relative fluorescence indicated (lower panel; bar indicate standard deviation, asterisks denote significant differences comparing the corresponding groups; * $p < 0.05$ using a paired Student's t-test).

Figure 5: Ubiquitination of EAAT2 is decreased in cells overexpressing laforin and malin. HEK293 cells were transfected with the indicated plasmids. Ubiquitinated proteins were recovered by metal-affinity chromatography as indicated in Materials and Methods. Proteins bound to the metal-affinity column were analyzed by Western blotting using anti-EAAT2 antibodies. Crude extracts (CE, 30 μ g) were also analyzed by Western blotting using the indicated antibodies: anti-EAAT2, anti-Flag (to detect Flag-laforin and Flag-malin), anti-HA (to detect HA-Nedd4.2) and anti-actin. The fuzzy bands in the CE are probably due to the presence of 6 M guanidinium hydrochloride in the extracts. Ubiquitinated forms of EAAT2 are indicated.

Figure 6: Hippocampal regions of LD mice show higher levels of Nedd4. A) Hippocampal regions (cornu ammonis 1; CA1) from control, *Epm2a*^{-/-} and *Epm2b*^{-/-} mice of 12 months of age were analyzed by immunohistochemistry using anti-Nedd4 antibodies, as indicated in Materials and Methods. A representative picture of the analysis of samples from

three different mice of each type is presented. B) Brain crude extracts (30 μ g) from control and LD mice of 12 months of age were analyzed by Western blotting using anti-Nedd4, anti-Nedd4.2 and anti-actin antibodies.

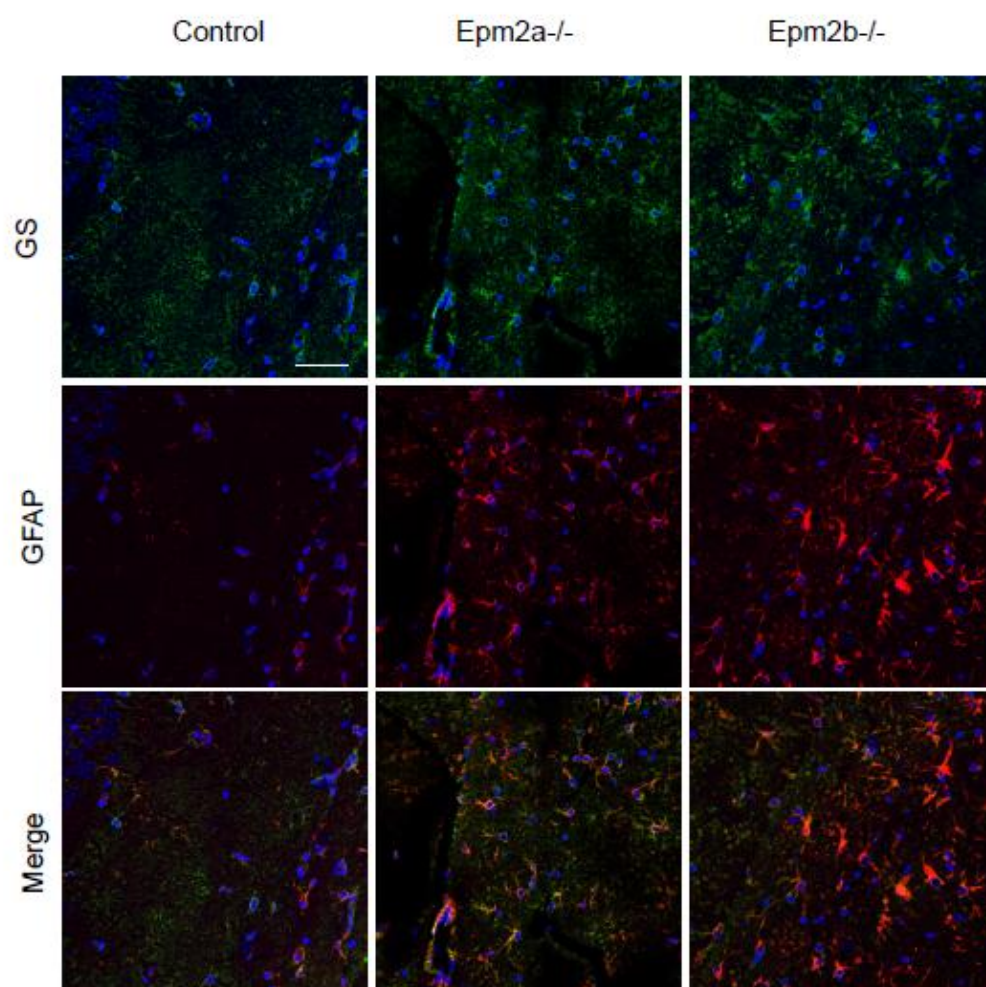


Fig. 1

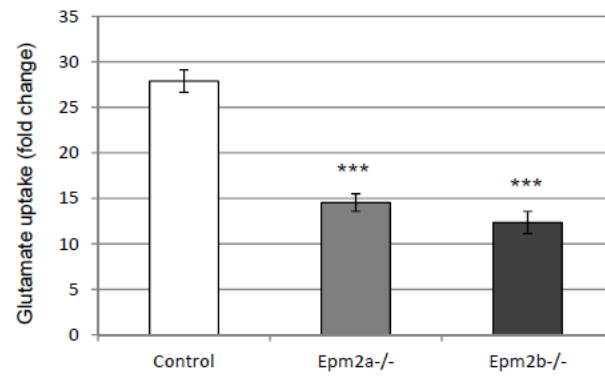


Fig. 2

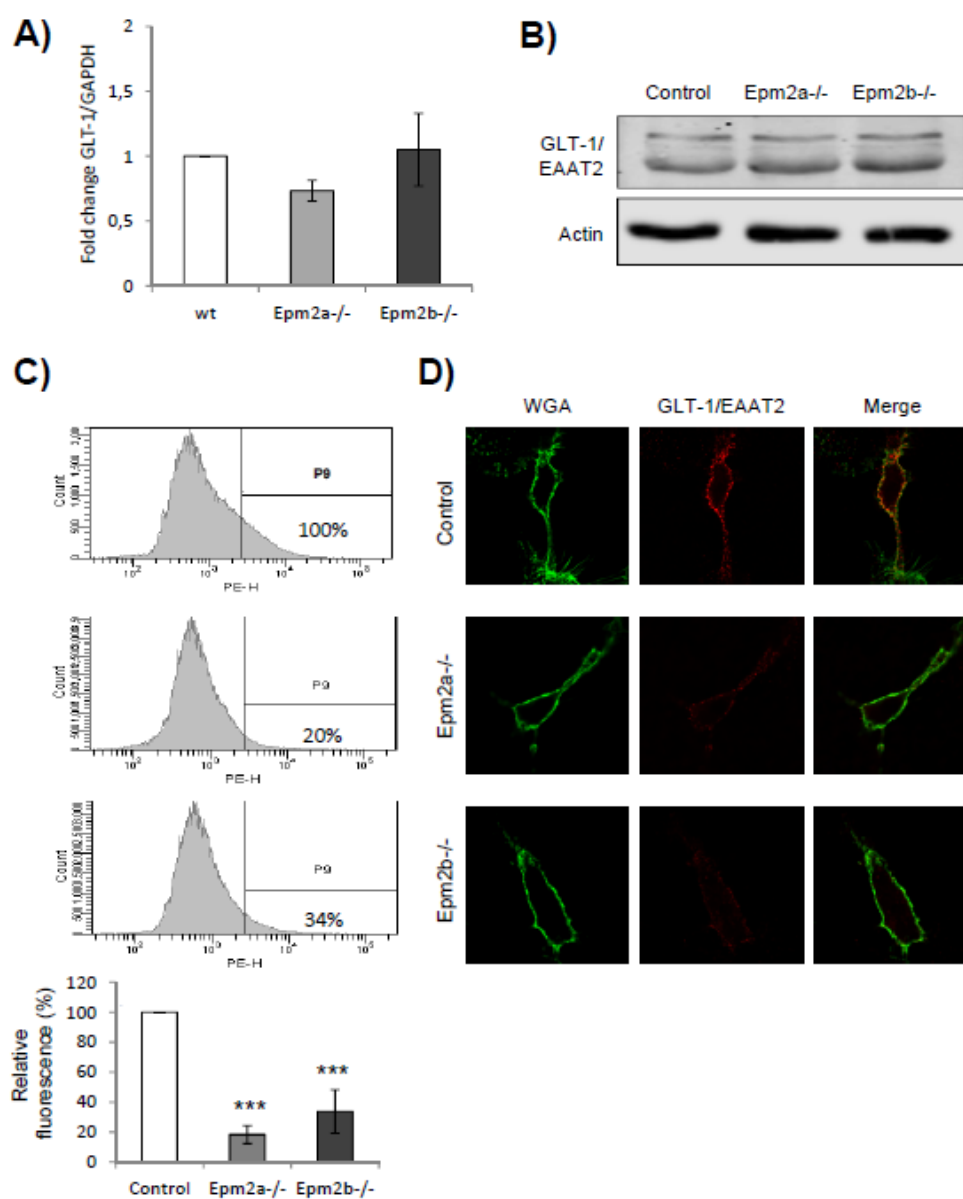


Fig. 3

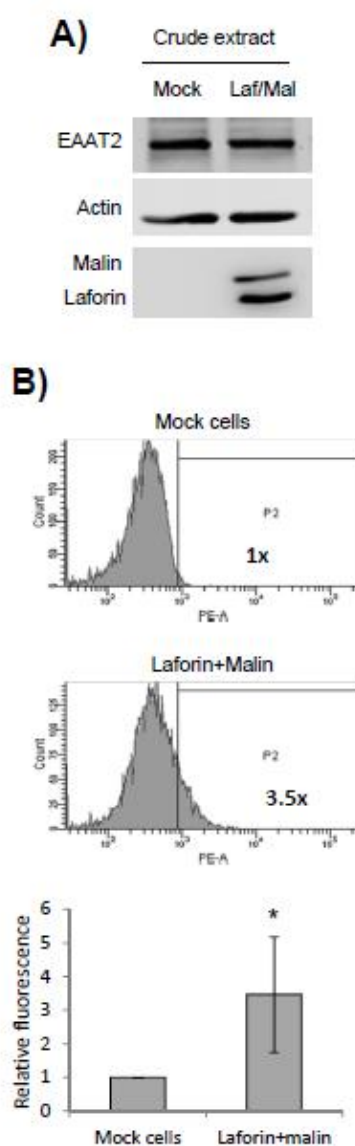


Fig. 4

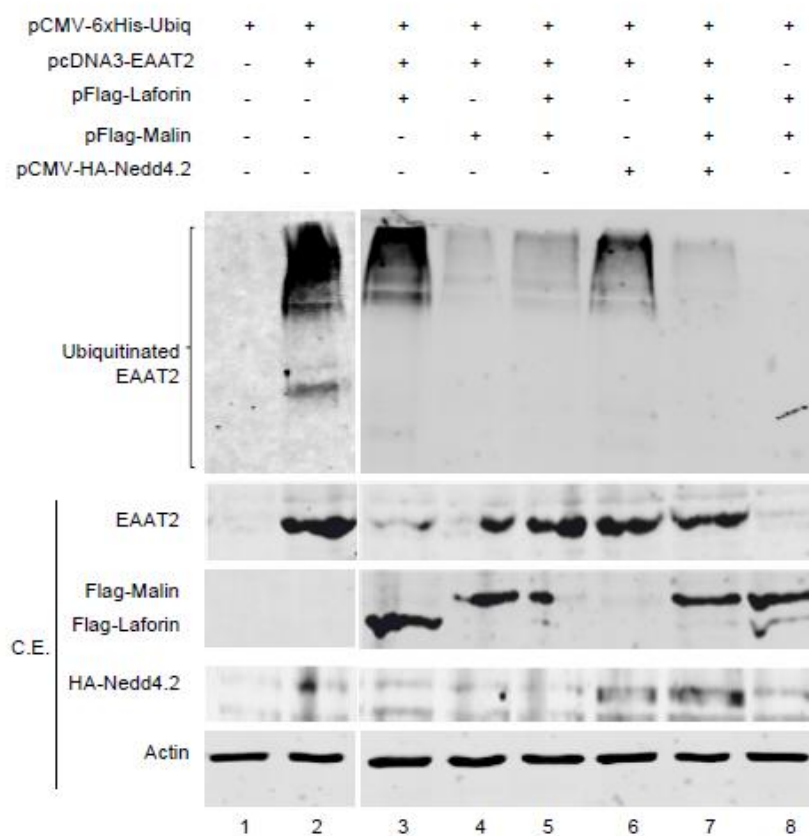


Fig. 5

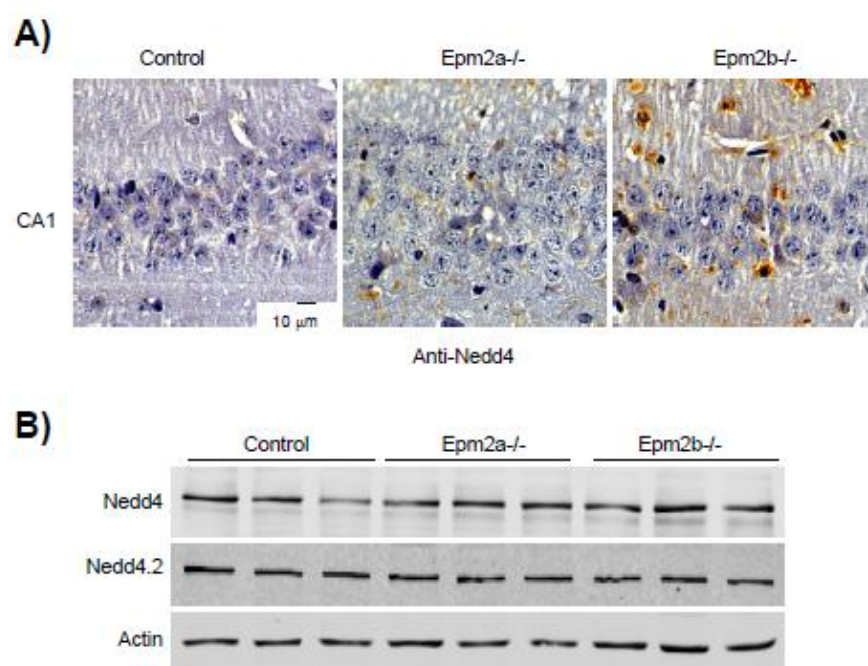


Fig. 6

Highlights:

- GLT-1 glutamate transporter is reduced at the membrane of astrocytes from LD mice.
- Glutamate transport is also reduced in primary astrocytes from LD mice.
- Overexpression of laforin and malin impairs ubiquitination of GLT-1 transporter.
- The laforin/malin complex impairs the endocytic recycling of the GLT-1 transporter.