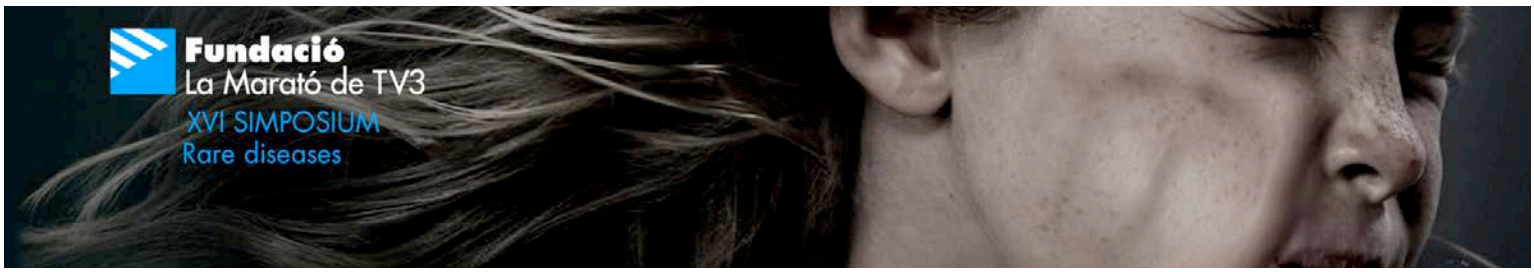

Lafora progressive myoclonus epilepsy: physiopathological basis of the disease and therapeutic approaches.

Dr Pascual Sanz Bigorra
Centro de Investigación Biomédica en Red de Enfermedades Raras, CIBERER, and Instituto de Biomedicina de Valencia, CSIC, Valencia.

Dr Erwin Knecht Roberto
Centro de Investigación Príncipe Felipe, Valencia

Dr Santiago Rodriguez de Córdoba
Centro de Investigaciones Biológicas, CSIC, Madrid

1. Project Summary

A. Project objectives

Lafora disease is an autonomous recessive neurodegenerative disorder caused by mutations in either the EPM2A gene, which encodes the dual-specificity phosphatase laforin, or the EPM2B gene, which encodes the E3-ubiquitin ligase malin. These proteins form a functional complex that regulates glycogen synthesis and possibly alternative important cellular processes such as cellular proliferation, apoptosis, endoplasmic reticulum stress, energetic metabolism and differentiation. The main objective of the present project is to gain information on the biological functions of laforin and malin, and also to identify putative therapeutic targets of the disease. To reach these goals, both cellular and animal models of the disease have been used.

B. Main accomplishments

Lafora disease presents a dysfunction of the regulation of intracellular proteolysis, mainly mediated by autophagy. We have demonstrated the involvement of different cellular components that participate in this pathway as the E2-conjugase UBE2N and the protein kinase p38. Other observed alterations of the ubiquitin-proteasome system, endocytosis and mitochondrial function (with the concomitant increase in oxidative stress) could be secondary to the reported main autophagy defect.

These results identify cellular protein homeostasis (protein folding and intracellular proteolysis: proteostasis) as a putative pharmacological target. In this sense, we report the use of different compounds that improve either intracellular proteolysis [by increasing autophagy (rapamycin and trehalose treatment)] or protein folding (by the use of chemical chaperones as 4-phenylbutyrate), or the use of activators of AMP-activated protein kinase (AMPK; a cellular energy sensor with neuroprotective properties) (metformin), as candidates to ameliorate neurological symptoms in mouse models of the disease. Only in the case of 4-

phenylbutyrate and metformin did we observe a beneficial effect on the neurological alterations that are present in Lafora disease mouse models.

C. Translational applicability

Our results indicate that improving the conditions of cellular protein homeostasis (proteostasis) has a beneficial effect on the neurological symptoms of the disease. Since the active compounds (4-phenylbutyrate and metformin) are already being used in clinical practice of other more common diseases, their use to treat Lafora disease patients could be straightforward. In parallel, these compounds could be used as a starting point to generate more potent compounds to be used in the treatment of Lafora disease patients.

2. Results

A. Molecular basis of Lafora disease: mechanism of action of the laforin-malin complex.

Previous results in the group indicated that laforin and malin formed a functional complex with E3-ubiquitin ligase activity. In this project we demonstrate that in order to be functional the complex needs to interact with the E2-conjugase UBE2N. Binding to this enzyme determines the topology of the ubiquitin chains that the laforin-malin complex is able to attach to its corresponding substrates, the K63-linked ubiquitin chains being the main product of the reaction.

We also observed that laforin forms dimers, residue Cys329 (at the C-terminus) being involved in this process. In addition we have observed that the phosphatase activity of laforin is not necessary for obtaining a functional laforin-malin complex. These results suggest that laforin would play a role in recognizing the specific substrates but the phosphatase activity would not be important in this process. The recognized substrates would then be modified by the E3-ubiquitin ligase activity of malin.

B. Pathological alterations in Lafora disease:

B.1) Alterations in cellular protein homeostasis (proteostasis): Lafora disease is characterized by the accumulation of polyglucosans (poorly branched glycogen-like polysaccharides) in neurons and other cellular types in peripheral tissues. In this project we have demonstrated that the laforin-malin complex, in addition to its role in the regulation of glycogen synthesis, participates in the regulation of several processes aimed to maintain cellular protein homeostasis (proteostasis). In this sense, we have shown that in cellular and animal models of disease there is an impairment of intracellular proteolysis (mediated by both the ubiquitin-proteasome system and autophagy) which alters misfolded protein turnover. These alterations trigger the unfolded protein response at the endoplasmic reticulum, increasing cellular sensitivity to conditions of endoplasmic reticulum stress.

The impairment in autophagy also affects the function of related pathways such as fluid phase- and receptor-mediated endocytosis, which are also deficient in cellular and animal models of the disease.

B.2) Alterations in the regulation of oxidative stress: Possibly as a consequence of the alterations described above, mitochondria functionality is also impaired, which leads to an increase in the production of reactive oxygen species (ROS). Mitochondria dysfunction, and the fact that the activities of some of the enzymes involved in the detoxification of ROS are diminished, leads to conditions of oxidative stress, which, if maintained, could eventually end in cell death.

B.3) Alterations in neuronal plasticity: By studying the pyramidal neurons in the CA1 zone of the hippocampus of the brain of animal models of the disease we have observed differences in the pattern of organization of the corresponding dendrites. We also observed an increased neuronal excitability in the brain of these animals, which could be explained by the aberrant dendrite organization. This higher excitability is not due to differences in the AMPAR/GABAR-mediated transmission ratio, which is similar to control animals. However, the brain of

animal models of disease showed longer axon initial segments (AIS), which correlates with the higher neuronal excitability.

C. Pharmacological approaches in Lafora disease:

C.1) Improvement of autophagy: Since as described above in the animal models of Lafora disease there was an impairment of protein degradation by the autophagy-lysosomal pathway, we subjected these animals to a combined treatment of temsirolimus [a rapamycin analogue with capacity to inhibit the mTOR pathway (a negative regulator of autophagy)] and trehalose (an mTOR-independent activator of autophagy). However, in spite of a clear inhibition of the mTOR pathway, we did not observe any beneficial effect of these compounds either in terms of neurological markers (number of Lafora bodies, appearance of reactive gliosis) or the behavioral tests performed.

C.2) Improvement of cellular protein homeostasis (proteostasis): We also treated the animal models of disease with compounds that improved cellular proteostasis. We used a molecular chaperone (4-phenylbutyrate) and an activator of the AMP-activated protein kinase, a cellular energy sensor with neuroprotective properties (metformin). After two months of treatment we observed in the treated animals a reduction in the number of Lafora bodies in different areas of the brain, a reduction of neuronal loss and a reduction of the reactive gliosis. In addition, treated animals performed better in the behavioral tests we carried out.

3. Relevance and possible implications

Lafora progressive myoclonus epilepsy is a neurological disorder characterized by neurodegeneration, epilepsy and the accumulation of polyglucosans (Lafora bodies) in the brain of the affected patients. It has been described that the two proteins that are related to the disease (laforin and malin) form a functional complex that regulates glycogen synthesis in neurons and other tissues. In the

absence of a functional laforin-malin complex, glycogen synthesis is increased, leading to the appearance of polyglucosan granules (Lafora bodies). However, it is still not clear whether the accumulation of Lafora bodies is the direct cause of the disease, or whether the key factor that triggers the disease is involved in alternative pathways.

The results obtained during this project indicate that the laforin-malin complex participates in cellular protein homeostasis (proteostasis), regulating different key processes such as the response to misfolded proteins and intracellular proteolysis mediated by the ubiquitin-proteasome system and autophagy. Therefore our results have allowed the identification of different therapeutic targets non-related to the regulation of glycogen synthesis. We have also designed and assayed different strategies aimed to improve the functionality of the proteostatic targets. We have found that improvement of proteostasis ameliorates both the neuronal parameters and the behavioral tests performed. Therefore, restoration of proteostasis emerges as a therapeutic target in the development of possible treatments of Lafora disease.

In addition, since the compounds we have used in the project (4-phenylbutyrate and metformin) are regularly being used in clinical practice for the treatment of other more common diseases, their use in the treatment of Lafora disease could be straightforward.

These compounds could also be considered as leading compounds in the design of novel compounds with strong properties. In addition, the combination of these products or the introduction of antioxidants (aimed to alleviate oxidative stress) could give even better results.

4. Literature generated

Criado, O., Aguado, C., Gayarre, J., Duran-Trio, L., Garcia-Cabrero, A.M., Vernia, S., San Millan, B., Heredia, M., Romá-Mateo, C., Mouron, S., Dominguez, M., Navarro, C., Serratosa, J.M., Sanchez, M., Sanz, P., Bovolenta, P., Knecht, E. and Rodriguez de Cordoba, S. "Lafora bodies and neurological defects in malin-deficient mice correlates with impaired autophagy". Hum. Mol. Genet. 21, 1521-1533 (2012).

Knecht, E., Criado, O., Aguado, C., Gayarre, J., Duran, L., Garcia-Cabrero, A.M., Vernia, S., San Millán, B., Heredia, M., Romá-Mateo, C., Mouron, S., Juana-López, L., Domínguez, M., Navarro, C., Serratosa, J.M., Sanchez, M., Sanz, P., Bovolenta, P. and Rodriguez de Cordoba, S. "Malin knockout mice support a primary role of autophagy in the pathogenesis of Lafora disease". Autophagy, 8, 701-703 (2012).

Garcia-Cabrero, AM., Marinas, A., Guerrero, R., Rodriguez de Córdoba, S., Serratosa, JM. and Sanchez, M.P. "Laforin and malin deletions in mice produce similar neurologic impairments. J. Neuropathology Exp. Neurol. 71: 413-421 (2012).

Ghislat G, Patron M, Rizzuto R, Knecht E. "Withdrawal of essential amino acids increases autophagy by a pathway involving Ca2+/calmodulin-dependent kinase kinase-β (CaMKK-β)". J Biol Chem**.** 287, 38625-38636. (2012).

Roma-Mateo, C., Sanz, P*., Gentry, M.S. "Deciphering the role of malin in the Lafora progressive myoclonus epilepsy". IUBMB Life, 64, 801-808 (2012).

Gentry, M.S., Romá-Mateo, C., Sanz, P. "Laforin, a protein with many faces: glucan phosphatase, adaptor protein, et alii" FEBS Journal, 280, 525-537 (2013).

Moruno-Manchón JF, Pérez-Jiménez E, Knecht E. "Glucose induces autophagy under starvation conditions by a p38 MAPK-dependent pathway". Biochem. J. 449:497-506 (2013).

Sanchez-Martin, P., Raththagala, M., Bridges, T.M., Husodo, S., Gentry, M.S., Sanz, P. and Romá-Mateo, C. “Dimerization of the glucan phosphatase laforin requires the participation of cysteine 329”. PLoS One 8: e69523 (2013).

Rubio-Villena, C., Garcia-Gimeno, M.A. and Sanz, P. “Glycogenic activity of R6, a protein phosphatase 1 regulatory subunit, is modulated by the laforin-malin complex”. Int. J. Biochem. Cell Biol. 45: 1479-1488 (2013).

Gayarre J, Duran-Trío L, Criado Garcia O, Aguado C, Juana-López L, Crespo I, Knecht E, Bovolenta P, Rodríguez de Córdoba S. “The phosphatase activity of laforin is dispensable to rescue *Epm2a* $^{-/-}$ mice from Lafora disease”. Brain 137:806-818 (2014).

Roma-Mateo, C., Aguado, C., Garcia-Gimenez, J.L., Ibañez-Cabellos, S., Seco-Cervera, M., Pallardó, F.V., Knecht, E. and Sanz, P. “Increased oxidative stress and impairment of antioxidant systems in Lafora disease models”. Mol Neurobiol. PMID: 24838580 (2015).

Romá-Mateo, C., Aguado, C., Garcia-Gimenez, J.L., Knecht, E., Sanz, P., Pallardó, F.V. “Oxidative stress, a new hallmark in the pathophysiology of Lafora progressive myoclonus epilepsy”. Free Rad. Biol. Med. PMID: 25680286 (2015).

Berthier, A., Payá, M., Garcia-Cabrero, A.M., Ballester, M.I., Heredia, M., Serratosa, J.M., Sanchez, M.P., Sanz, P. “Pharmacological interventions to ameliorate neuropathological symptoms in a mouse model of Lafora disease”. Mol. Neurobiol, PMID: 25627694 (2015).