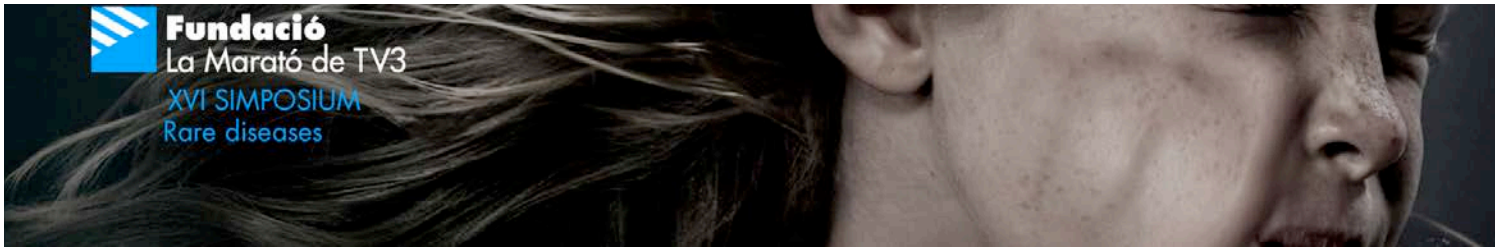
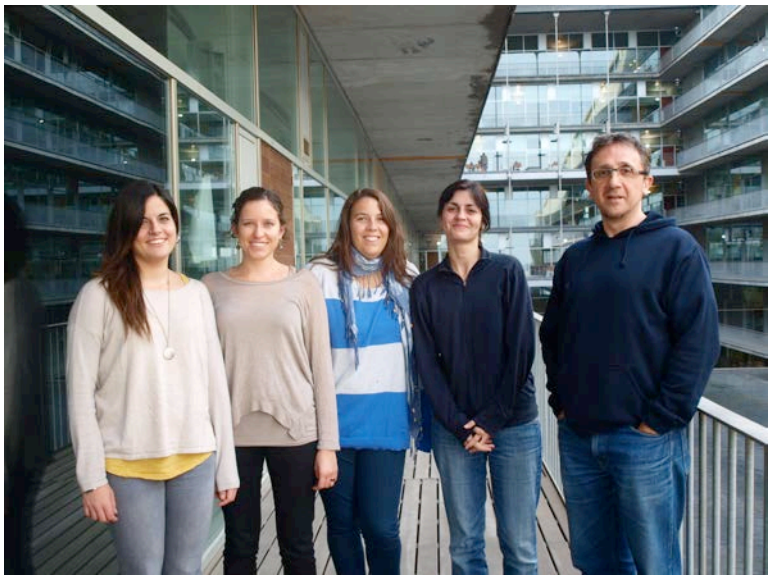


Study of the role of nitro-oxidative stress in the formation of oligomer and fibers of Aß and toxicity in skeletal muscle myopathy with type 2 inclusion bodies

Dr Francisco J. Muñoz López
Universitat Pompeu Fabra (Barcelona)



1. Summary

Myopathy type 2 inclusion bodies (formerly IBM2 and now called GNE myopathy) is a disease caused by the mutation of the GNE gene, which codes for the enzyme that catalyzes the first two reactions in the production of sialic acid. Degeneration of skeletal muscles, progressive atrophy and finally very severe disability occur by unknown mechanisms. However, there is much evidences of the intracellular accumulation of beta-amyloid (Aß) peptide in skeletal muscle. In this project we studied the mechanisms leading to intracellular accumulation and aggregation of the peptides Aß1-42 and Aß1-40 and skeletal muscle cells and their effect on cell viability. This study had the following objectives: i) to study the accumulation and intracellular Aß aggregation and its effect on cytotoxicity; ii) to study the production of nitro-oxidative stress by different Aß peptides and their aggregates in skeletal muscle cells; iii) to study the expression of BACE1 (the key enzyme in the production of Aß) and the production of Aß by SAPKs (stress activated protein kinase); iv) study of the genes involved in the cytotoxicity of Aß by its overexpression in yeasts.

The biological material was cultured myoblast cell lines (and myotubes when they are differentiated) C2C12 (mouse myoblast cell line) and human myoblast cells with a mutation in the GNE and unmutated gene; samples of human muscle tissue of a patient with GNE myopathy and a control donor; and yeasts of the species *Saccharomyces cerevisiae*.

Our results demonstrate that in GNE myopathy there is cellular stress that increases BACE1 expression and triggers the production of Aß. This is secreted but quickly binds to the asialylated GM1 favoring its endocytosis by clathrin. It follows the lysosomal pathway where it starts to aggregate due to the acid pH and produces oxidative stress. This stress increases BACE1 activity through SAPKs (MAPK and c-jun-38) and induces cell damage that prevents the activation of Akt, promotes GSK3ß activation and triggers apoptosis. Finally, with all the genes

identified as key cellular targets in the protection and toxicity of amyloid, our group will continue to study the role of these proteins and their protective functions.

2. Results of the project

2.1. Intracellular amyloid in myocytes from GEN myopathy patients and its effects on cell viability

Using myocytes from GNE myopathy patients and healthy individuals we found an increase in intracellular concentration of Aß in the cells from GNE myopathy patients. This correlates with increased production of Aß (increased activity of BACE1) and an increase in clathrin-mediated endocytosis. Interestingly, we found that the intracellular Aß in the cells of GNE myopathy patients is aggregated in the form of oligomers, mainly dimers and trimers, which are the most toxic states in Aß aggregation. We have consequently found that intracellular Aß1-42 induces apoptosis through activation of caspase-3 and bax, and reduced levels of Bcl-2 and mitochondrial membrane potential. These results were also demonstrated in samples of human muscle tissue of a patient with GNE myopathy and a donor control. Finally, myocytes from GNE myopathy patients demonstrated a greater proapoptotic state than controls in terms of mitochondrial membrane potential and of annexin V expression.

2.2. Intracellular effects of the induction of nitro-oxidative stress by Aß

We have found that the production of free radicals is triggered by Aß oligomers. We studied the increase in calcium in the mitochondria by the action of Aß oligomers and we found that treatment with Aß oligomers induces increased mitochondrial calcium. The role of calcium in Aß toxicity is very important considering that the calcium would be able to activate nitric oxide production by mitochondrial NO synthase. Oxidative stress occurs when the superoxide anion is in the presence of NO and peroxynitrite is generated, which is responsible for the

protein nitrotyrosination, mainly the triosephosphate isomerase (TPI), a key enzyme in glucose metabolism. We studied the nitrotyrosination of Aß and its effect on aggregation and we found that it stabilizes the oligomers, the most toxic forms. We also expressed stably in myocytes an inert peptide with a similar size to Aß (flit) and wild type Aß finding that Aß expressing cells showed significantly decreased viability compared to those that expressed flit, demonstrating that it was a specific effect of Aß.

2.3. Effect on the activation of intracellular kinases that mediate stress and BACE1 expression

Aß aggregates produce nitro-oxidative stress and in this objective we studied the effects of this stress on BACE1 expression via activation of intracellular JNK and p38 MAP, routes that are known to be activated by cellular stress. We obtained that Aß induces nuclear migration of c-jun and the appearance of cells positive for p38 MAPK. This activation augments BACE1 transcription and its expression, which results in increased production of extracellular Aß. In addition we studied the Akt survival pathway and we found that myocytes from GNE myopathy patients showed an inhibition of Akt phosphorylation, which means deprotection against injury induced by Aß. On the other hand the inactivation of the enzyme GSK3-beta by serine-9 phosphorylation was analyzed. This enzyme is downstream of the activation of Akt, which phosphorylates GSK3-beta to prevent its activation, which is considered negative for the cells. In these experiments the Aß decreased phosphorylation of GSK3-beta, which means impaired transcription of Tcf-lef protective genes for beta-catenin and probably increases BACE1 translation.

2.4. Study of genes that contribute to cell survival or Aß-induced toxicity in yeast

Aß was overexpressed in yeast to identify mutated genes affecting amyloid induced toxicity. We studied 6,000 genes and their role in cell survival and toxicity of Aß. After the experimental study 150 genes were significantly identified to

increase cell survival or to contribute to the toxicity of Aß. The most relevant genes are listed below and grouped in different cellular pathways and functions:

- Antioxidants: SOD1, catalase and glutathione reductase (protective).
- Mitochondrial respiratory chain: cytochrome C oxidase (protective).
- Endocytosis: clathrin (increased toxicity) and flippase (protective).
- Via the pentose-phosphate: ribulose 5-p-3-epimerase (protective).
- Krebs cycle: enzymes involved in the production of citrate, malate and Acetyl-CoA (protective).
- Glycolysis: triosephosphate isomerase (protective)
- Gluconeogenesis: phosphor enol carboxyl pyruvate kinase (protective)
- Synthesis of sphingolipids: ceramidase IDC1 (increased toxicity)
- Glycosylation: mannosyltransferase (protective)
- Protein synthesis: diphthamide synthetase (increased toxicity)
- Folate synthesis: tetrahydrofolate synthase (protective)
- Calcium homeostasis: orthologs of SERCA and DIRK-1 mammalian (increased toxicity)

Following identification of potential targets for inducing protection against damage of Aß in myocytes, different compounds were tested. First, given the presence of nitro-oxidative stress and the protective role of SOD1, catalase and glutathione reductase, protection experiments were performed with different antioxidants (trolox, reduced glutathione and n-acetylcysteine). However, no significant protection was obtained with these compounds. Regarding glycolysis and the protective role of triosephosphate isomerase, a different set of experiments was performed showing that its functional integrity is essential to avoid Aß toxicity of. Finally, the protective role of the folate pathway was tested with different compounds. Cell protection was obtained using vitamin B12, which is closely related to the role of folate and is proposed to be neuroprotective in Alzheimer's disease, a disease also due to aggregation of Aß.

3. Relevance and possible implications

The results obtained allow a better understanding of this disease, in which, although increased vacuolization of skeletal muscle cells with a concomitant presence of intracellular aggregates of Aß was originally described, it was not established that a deficiency in the GNE gene, which prevents sialylation of proteins, induces the disease.

Our work opens the possibility of these therapeutic targets: i) the **inhibition of BACE1**, the key enzyme in amyloid production, preventing the aggregation of amyloid and subsequent intracellular cytotoxicity; ii) considering that the toxicity of amyloid is directly dependent on their aggregation into oligomers, treatment with **intracellular aggregation inhibitors** would be essential; iii) promoting the **sialylation of biomolecules**; iv) inhibition of GSK-3ß would also be essential to induce cellular protection; v) treatment with **drugs that act on the paths connected with the genes identified** as being able to increase cell survival, the folate path being one of them, and those associated with the modulation of intracellular calcium.

4. Publications generated

4.1. Published articles

- Pacheco C, Morales CN, Ramírez AE, Muñoz FJ, Gallegos SS, Caviedes PA, Aguayo L, Opazo C. Extracellular α-synuclein alters synaptic transmission in brain neurons by perforating the neuronal plasma membrane. J Neurochem, 132:731-41, 2015

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ischemic stroke impairs thrombolysis and promotes neuronal death. Biochim Biophys Acta Mol Basis Dis, 1852:421-8, 2015

-Vicario-Orri E, Opazo C, Muñoz FJ. The Pathophysiology of Axonal Transport in Alzheimer's Disease. J Alzheimers Dis, 43:1097-113, 2015

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- Ramos-Fernandez E, Palomer E, ILL-Raga G, Tajes M, Bosch-Morató M, Guivernau B, Román-Dégano I, Nuñez L, Paez A, Alameda F, Elosúa R, Boada M, Valverde MA, Muñoz FJ. Posttranslational nitro-glycative modifications of albumin in Alzheimer’s disease: implications in cytotoxicity and amyloid ß-peptide aggregation. J. Alzheimer Dis, 40:643-57, 2014.

- Muñoz FJ, Godoy JA, Cerpa W, Poblete IM, Huidobro-Toro JP, Inestrosa NC. Wnt-5a increases NO and modulates NMDA receptor in rat hippocampal neurons. Biochem Biophys Res Commun 444:189-94, 2014.

- Marín T, Contreras P, Castro JF, Chamorro D, Balboa E, Bosch-Morató M, Muñoz FJ, Alvarez AR, Zanlungo S. Vitamin E Dietary Supplementation Improves Neurological Symptoms and Decreases c-Abl/p73 Activation in Niemann-Pick C Mice. Nutrients 6:3000-3017, 2014.

- Tajes M, ILL-Raga G, Palomer E, Ramos-Fernandez E, Guix FX, Bosch-Morató M, Guivernau B, Jiménez-Conde J, Ois A, Pérez-Asensio F, Reyes-Navarro M, Caballo C, Galán AM, Alameda F, Escolar G, Opazo C, Planas A, Roquer J, Valverde MA and Muñoz FJ. Nitro-oxidative stress after neuronal ischemia induces protein nitrotyrosination and cell death. Oxid. Med. Cell. Longev, 2013:826143, 2013.

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4.2. Articles in preparation

- “Increased amyloid ß-peptide uptake due to hyposialyation induces apoptosis in GNE Myopathy”

- “Identification of modulators of Aβ1-42 toxicity by genome-wide analysis of *Saccharomyces cerevisiae*”

4.3. Communications to Congresses

- 4 communications at BBVA Foundation-IRB Barcelona. Barcelona Biomed Conferences: Amyloid-β and Alzheimer's Disease: From Fundamental Principles to Therapeutic Strategies. Barcelona. July 2014.

- 4 communications at 3rd IRB Barcelona PhD Student Symposium. The Clock of Life: cellular and molecular processes of development, ageing and disease. Barcelona. November 2013.

- 2 communications at The 8th International Congress on Vascular Dementia, Athens (Greece). October 2013.

- 3 communications at The 11th international conference on Alzheimer's and Parkinson's disease. Florence (Italy). March 2013.

- 1 communication at CHILEAN SOCIETY FOR CELL BIOLOGY - XXVI ANNUAL MEETING, Puerto Varas (Chile). October 2012.

- 2 communications at BASIC AND CLINICAL RESEARCH: Present and Future of Alzheimer's Research. Madrid. September, 2011.

- 2 communications at International Bunsen Discussion Meeting on "Structure of Amyloid Fibrils and Mechanism of Amyloid Formation". Halle (Germany). August 2011.