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ROLE OF NMDA RECEPTORS PHOSPHORYLATION IN ALZHEIMER'S DISEASE AETIOLOGY: FROM PROTEOMICS TO IN VIVO MODELS

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1. Summary

Glutamate is the main excitatory amino acid in the brain, and glutamatergic neurotransmission is pivotal in neuronal processes (neurogenesis, synaptogenesis, neuron survival, synaptic plasticity) underlying brain functions (memory, cognition, motor functions). Functionally, glutamate release binds and activates glutamate receptors (metabotropic and ionotropic subtypes), allowing synaptic transmission. The activation of ionotropic glutamate receptors (iGluRs) allows ion influx required for synaptic processes, and their disturbance impairs synaptic communication and leads to cognitive dysfunction. In particular, the *N*-methyl-D-Aspartate receptor (NMDAR) is a key element in memory and cognition processes, and is currently the unique target for the FDA-approved drug memantine treatment of Alzheimer's disease (AD). Importantly, NMDAR synaptic location and biophysical properties are modulated by post-translational mechanisms. Based on these, our project aimed to identify the NMDAR phosphopattern, towards the identification of novel therapeutic targets (phosphosites, kinases, phosphatases) and to elucidate NMDAR-mediated synaptopathy mechanisms in AD. The working hypothesis of our proposal was based on a well-established role of NMDARs subcellular distribution in their dichotomic behaviour, which can either promote neuronal activation and cell survival or, on the contrary, induce the activation of pro-apoptotic signalling pathways.

To address this hypothesis, we performed an untargeted Mass Spectrometry analysis of subsynaptic compartments of AD biopsies of brain regions (hippocampus and entorhinal cortex) affected in the initial stages of AD, as well as in samples from Ts65Dn mouse model. Indeed, Ts65Dn is a trisomic mouse model of Down syndrome (DS), a neurological condition with early onset of AD histopathological hallmarks and cognitive deficits that recapitulate AD-like alterations. Phosphoenrichment-coupled mass spectrometry analysis revealed specific subsynaptic- and trisomy-associated iGluR phosphorylation signature, concomitant with differential subsynaptic kinase and phosphatase composition of Ts65Dn hippocampal subsynaptic compartments. Furthermore, biochemical data were used to build up a genotype-kinome-iGluR phosphopattern matrix in the different subsynaptic compartments. Overall, our results provide a precise profile of iGluR phosphopattern alterations in the glutamatergic synapse of the Ts65Dn mouse model and support their contribution to DS- and AD-associated synaptopathy conditions. The alteration of iGluR phosphoresidues in Ts65Dn

hippocampi, together with the kinase/phosphatase signature, identified potential novel therapeutic targets for the treatment of glutamatergic dysfunctions in DS and, potentially, in AD.

Further, we performed a hypothesis-driven analysis of DYRK1A-mediated phosphorylation of GluN2A subunit at serine residue 1048. Indeed, previous studies have proposed a candidate role of DYRK1A overexpression in AD and DS aetiology. At the beginning of this project, our group identified the phosphorylation of GluN2A by DYRK1A. Interestingly, during the project we have generated an anti-phospho GluN2A(pS1048) polyclonal antibody and determined, following a targeted biochemical and histological analysis, the increased levels of phosphorylated GluN2A(pS1048) subunit in the postsynaptic fractions of both AD and Ts65Dn samples. In order to evaluate the potential pathogenic impact of the increased phosphorylation levels of this post-translational modification, we conducted *in vitro* experiments and showed that GluN2A(pS1048) overexpression (transient transfection of its phosphomimetic form in primary neuronal cultures) leads to synaptic dysfunction. Overall these data strongly support the association between aberrant NMDAR phosphopattern (in particular, an increase of GluN2ApS1048 levels) and synaptic dysfunction. Currently, in order to functionally evaluate the pathological relevance of the aforementioned phosphopattern changes of the NMDAR, we have generated an in vivo mouse model constitutively expressing the phosphomimetic GluN2A(S1048D) subunit. This mouse model, still under phenotypic behavioural assessment, will definitely make it possible to assess the contribution of NMDAR phosphopattern alteration in neuronal dysfunction and in the cognitive deficits associated with AD, as well as future drug screening.

In summary, this project provided valuable molecular insights on the synaptopathy changes associated with DS and AD, allowing the identification of NMDAR phosphosites and the related kinases / phosphatases for future drug screening. In the next section, we describe the main scientific achievements of the project (illustrated on the last page of this document).

2. Results

Specific objective 1. Characterisation of the phosphoproteome profile of synaptic and extrasynaptic NMDA receptor-associated protein complexes (NRC) in early stages of Alzheimer's disease and in murine models of AD.

We have established a robust, highly fine and reproducible method for the subsynaptic fractioning of brain regions from both mouse and human post-mortem samples. Semi-quantitative Proteomic analysis of postsynaptic and extrasynaptic fractions of the hippocampus of trisomic and control mice has been completed, showing an alteration of the protein content ant the associated gene functions, related with synaptic transmission (Fig.1A).

Characterisation of the phosphoproteomic profile, with particular focus on the ionotropic glutamate receptors of trisomic mice, a model of AD. This study allowed the most exhaustive defined repertoire of iGluR phosphopattern in a single animal model, as well as the identification of putative kinases/phosphatases dysregulated in this animal model, representing drug targets (Fig.1B).

Identification of alterations on the kinome, phosphatases and phosphorylation signature of Ts65Dn mouse model of synaptopathy, which represent potential pharmacologic targets (Fig.1C).

Production and validation of anti-GluN2ApSer1048 antibodies, showing the synaptic presence of this phosphoevent in hippocampal synapses (Fig.2A).
Identification of significant increased levels of GluN2A(pSer1048) and DYRK1A levels in the hippocampus and entorhinal cortex of AD biopsies, in a clinical stage- dependent manner (Fig.2B).

Currently, a comparative analysis of the Ts65Dn *vs*. AD phosphoproteome is performed, towards the identification of potential biochemical signature commonalities and differences between AD and Ts65Dn.

Specific objective 2. Generation of molecular tools and in vivo models to express phosphomodulated GluN subunits of NMDARs.

Generation of GFP-GluN2A wildtype and phosphomimetic lentiviral particles for *in vitro* and *in vivo* delivery of mutant GluN2A constructs.

Validation of *in vitro* infectivity of Lv-GluN2A viral particles, in primary neuronal cultures.

Generation of knock-in GluN2A(S1048D) mouse model using CRIPSR-Cas9 technology. Obtaining founder mice and genomic analysis of off-target sequences in the genomic DNA of founder mice. Colony amplification at Northwestern University (Chicago, USA) Animal Facilities.

Specific objective 3. Evaluate the functional impact of NMDAR phosphoproteomic modulation in mouse models of Alzheimer's disease

Characterization of glial activation (GFAP- and Iba1-positive cells) in the adult Ts65Dn mouse model (4- and 9-months old mice).

The overexpression of GluN2A(S1048E) phosphomimetic in primary neuronal cultures affects neuronal morphology and function. Indeed, the overexpression is associated with an abnormal increase in spine density (thin-shape spines, Fig.3A). GluN2A(S1048E) overexpression in primary neuronal cultures increases NMDAR-mediated EPSCs, with potential deleterious (excitotoxicity) effects. Further, glycine-induced chemical LTP synaptic plasticity process is impaired as a consequence of GluN2A(S1048E) overexpression. Overall these data support the pathogenic impact of increased levels of GluN2A(pS1048), associated with synaptopathy conditions, and define the NMDAR as a target for further therapeutic interventions in AD (Fig.3B). Currently, we are starting the phenotyping of the animal model KI-GluN2A(S1048D), with particular interest in the cognitive phenotype. This analysis will be followed by the evaluation of pharmacological benefits of drugs targeting the NMDAR.

3. Relevance and potential future outcomes

The project entitled "Role of NMDA receptors phosphorylation in Alzheimer's disease aetiology: from proteomics to *in vivo* models" was designed with the overarching goal to decipher the impact of NMDAR phosphopattern in AD pathophysiology. At the end of

these 3+1 years of extensive work, several relevant findings have been generated that support the pathogenic impact of aberrant NMDAR phosphopattern in synaptic function. These robust preclinical data open new therapeutic avenues and will be the object of ongoing and future research lines with potential clinical applicability. In the following paragraphs, we summarize the main relevance and possible clinical applications derived from this project.

We have delivered an extensive and comprehensive integration of phosphoproteomic and proteomic alterations in a mouse model of Down syndrome, a genetic condition with early-onset AD. These data represent the most complete report describing the phosphoproteomic alterations of ionotropic glutamate receptors, and we are currently expanding these findings to the complete synaptic phosphoproteome. Further, the systems biology approach combined to proteomic findings allowed the identification of potential therapeutic targets (protein kinases and phosphatases) for future therapeutic interventions.

Besides the untargeted analysis of phosphoproteomic alterations, we have developed an experimental pipeline to evaluate the impact of discrete (hypothesis-driven) phosphoresidues of the NMDA receptor in synaptic dysfunction. In this line, we have characterised the physiological role of the phosphorylation of serine residue at position 1048 of the GluN2A subunit of the NMDAR. More importantly, in the context of synaptopathy conditions, we have shown that the increased levels of phosphorylated GluN2A(pSer1048) subunits (resulting from the overexpression of DYRK1A, a protein kinase associated with Alzheimer's disease and Down syndrome) is sufficient to affect glutamatergic transmission and affect synaptic morphology and plasticity. Overall, these findings provide strong evidence of the involvement of glutamatergic neurotransmission alteration in AD condition, and define these excitatory synapses as a therapeutic target for AD therapy.

In relation with the glutamatergic synapses alterations observed in synaptopathy conditions, during the project we have generated molecular and biological tools for the pharmacological screening of FDA-approved drugs targeting the glutamatergic synapse. These tools are currently used in our laboratory and, together with the current research lines, we will pursue the characterisation of the KI-Grin2A(S1048D)

mouse model phenotypic alterations and the potential benefit of therapeutic interventions.

Overall, the project achievements are placing the glutamatergic synapse in the core of the synaptopathy conditions and strongly defining the relevance of this neuronal structure / system for ongoing and future therapeutic strategies.

4. Bibliographic production

Publications (bold characters indicate the members of the team directly benefited by the Fundació La Marató TV3 grant)**:**

Gómez de Salazar M., **Grau C.**, **Locubiche S**. (…) and **Altafaj X**. "Increased phosphorylation of GluN2A subunit at serine residue 1048 is associated with synaptopathy conditions". In preparation for *The Journal of Neuroscience.*
Soto D., Olivella M., **Grau C**. (…) **Gómez de Salazar M**. (…) and **Altafaj X.** "GRIN2B loss-of-function variant triggering NMDA receptor hypofunctionality in pediatric encephalopathy is attenuated by L-serine diet". *Science Signaling*, under second revision.

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nitric oxide-Heme-Regulated eIF2α kinase in cortical neurons. *Oncotarget*. 2016 Sep 13;7(37):58876-58892.

Poster presentations (bold characters indicate the members of the team directly benefited by the Fundació La Marató TV3 grant)**:**

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Soto D, Olivella M, Grau C, Armstrong J, Alcón C, Gómez de Salazar M, Gratacòs-Batlle E, Ramos-Vicente D, Gasull X, Fernández-Dueñas V, Ciruela F, Bayés À, Sindreu C, López-Sala A, García-Cazorla À, Altafaj X. "Rett-like severe encephalopathy caused by a de novo GRIN2B mutation is attenuated by D-serine dietary supplement". Gordon Research Conference "Excitatory synapses and brain function". Les Diablerets, Switzerland, June 2017. Poster.

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M Gómez de Salazar, C Grau, K Arató, JM Fernández-Fernández, P Sanchís, D Soto, A Valderrama, C Sindreu, C Fillat, I Ferrer, S de la Luna, X Altafaj. "DYRK1A, a novel regulator of NMDA receptors: implications for Down syndrome and Alzheimer's disease". Federation of European Societies of Neuroscience (FENS) Meeting. Copenhagen, Denmark. July 2016.

Doctoral Thesis and students internships

Macarena Gómez de Salazar obtained her PhD degree in September 2018, exclusively as a result of this action. Dr. Gómez de Salazar was with the grant funded by Fundació La Marató TV3 and her doctoral thesis was fully focused to address the objectives of this project.

This project also benefited from the contribution of internship students, mostly from the "Neuroscience Master program" (UB) that pursued their academic research career as PhD students, either in our research group or in external universities. The students that received training and participated in this project were: A. Santos (2017), currently in her second PhD year; S. Locubiche (2018), applicant to PhD fellowships; A. Amelianchik (2016), PhD student at Cornell University (NY, USA); P. Sanchís (2015), PhD student at UAB.

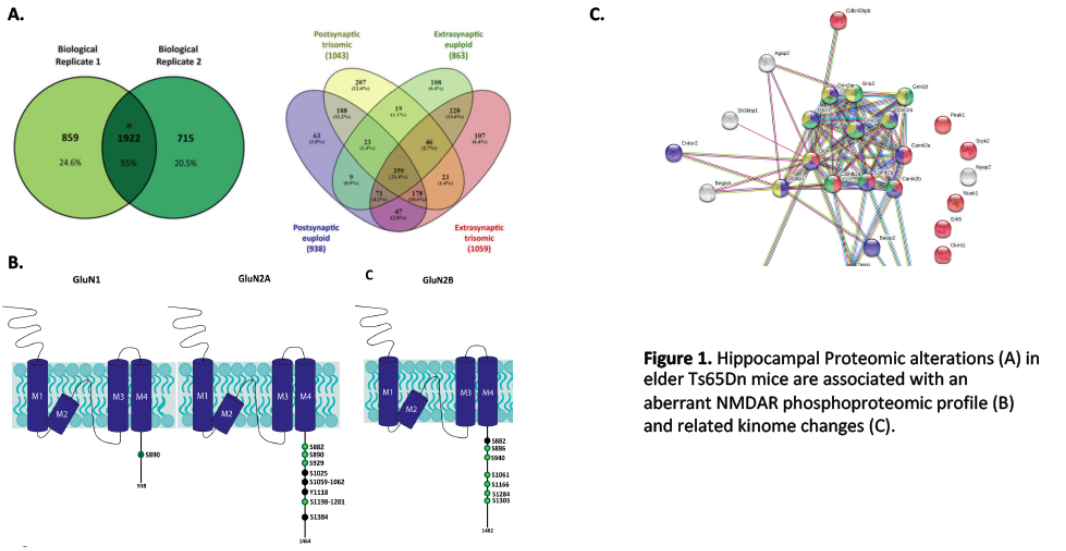

Figure 1. Hippocampal Proteomic alterations (A) in elder Ts65Dn mice are associated with an aberrant NMDAR phosphoproteomic profile (B) and related kinome changes (C).

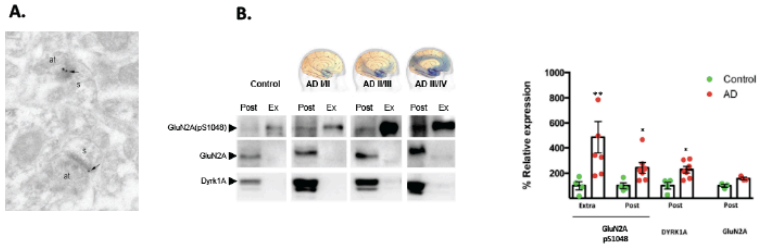

Figure 2. GluN2A(pS1048) phosphorylation is localised at the glutmatergic synapses and levels increase along AD progression and are localised at the extrasynaptic compartment.

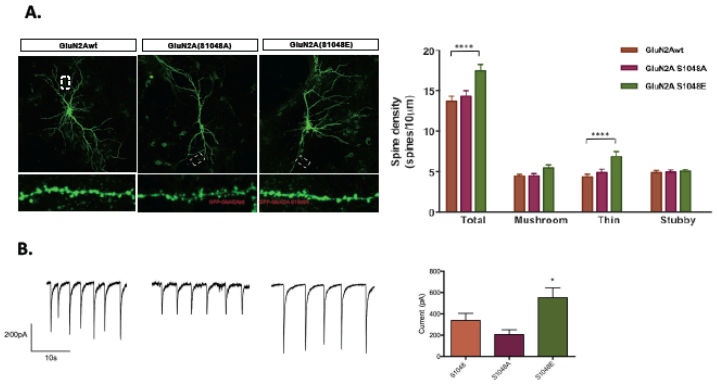

Figure 3. AD-associated GluN2A(pS1048) phosphorylation alteres neuronal morphology (A) and affects NMDAR-mediated neurotransmission (B), potentially leading to neuronal dysfunction.