SYNAPTIC MARKERS IN PRECLINICAL ALZHEIMER'S DISEASE

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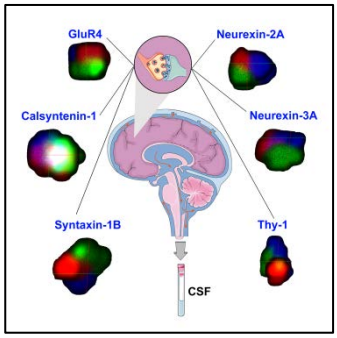
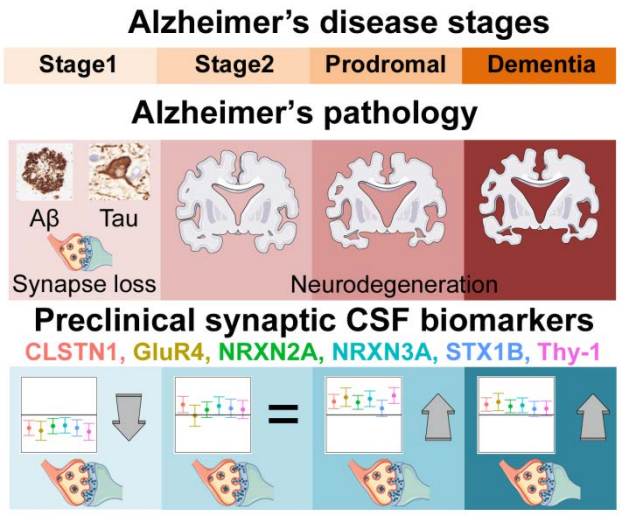


1. Project summary

The aim of this project is to widen our understanding of synaptic damage in Alzheimer's disease (AD). The specific objectives were divided into two main sections: 1) Characterize the synaptic proteome in cerebrospinal fluid (CSF) in AD with the aim of discovering synaptic proteins as potential early biomarkers of the disease. 2) Characterize the molecular composition of human entorhinal cortex synapses and identify the earliest changes induced by AD. To achieve these objectives, the following experimental design was proposed: Objective 1) The best candidates for synaptic CSF biomarkers in AD were selected based on data generated from the literature and a pilot study. Of the 251 synaptic proteins identified that are detectable in the CSF, 22 were selected for further study by mass spectrometry (MS). Targeted mass spectrometry assays were developed for the 22 proteins and synaptic expression was evaluated in human brain tissue. From this study, 9 proteins were selected (Calsyntenin -1, GluR2, GluR4, Neurexin-2A, Neurexin-3A, Neuroligin-2, Syntaxin-1B, Thy-1, and Vamp-2) that were measured in 3 cohorts of CSF from control subjects and preclinical and clinical stages of the Alzheimer continuum. The study showed an overall decrease of these proteins in the preclinical phase of AD (people with biological signs of AD but without symptoms), changes that precede neurodegeneration markers. We have filed a patent to protect the exploitation of these proteins as biomarkers of synaptic degeneration and we have licensed the development of immunoassays to facilitate their detection in the clinical setting. Objective 2) For this purpose, the molecular composition of isolated synaptosome fractions was compared from 18 samples from non-pathological control subjects, and two pathologies that present with tauopathy (brain deposits of tau



Graphical Summary: We have identified and validated a panel of 6 synaptic proteins (Calsyntenin-1, GluR4, Neurexin-2A, Neurexin-3A, Syntaxin-1B and Thy-1) that are decreased in cerebrospinal fluid in the individuals at preclinical stage 1 of AD who have signs of Aβ pathology but have yet to show symptoms or widespread neurodegeneration. The same proteins are increased in individuals with Prodromal AD or dementia, reflecting a more generalized neurodegeneration in these symptomatic stages. These results show for the first time that the decrease in synaptic proteins in CSF is a very early phenomenon that precedes neurodegeneration.

protein), namely primary age-related tauopathy (PART) and AD. We observed an increase in mitochondrial proteins in the synapses of PART and AD cases compared with controls. However, we did not observe differences in the composition of the synapses between PART and AD cases. We conclude that tau pathology associated with age and AD is associated with a recruitment of mitochondrial proteins to the synapse. In summary, the study provides important information on the initial changes of a critical region for AD and has led to the identification of biomarkers for the detection of synaptic degeneration, an early event in AD pathogenesis.

2. Results obtained

Synapses are essential structures for neuronal function. Synaptic damage plays a central role in AD although it is a phenomenon that has yet to be fully studied in humans. In this project, 2 major objectives were proposed to widen our understanding of this early event in AD pathogenesis and its potential application in the early diagnosis of AD.

Objective 1) Characterize the synaptic proteome in cerebrospinal fluid (CSF) with the aim of identifying synaptic proteins as potential early biomarkers of the disease.

In order to achieve this objective, we first selected the best candidate synaptic CSF biomarkers based on data generated from the literature and a pilot study obtained from 60 CSF samples. This is the first systematic study of the CSF proteome and also includes the complete characterization of its synaptic component. Specifically, the study revealed that synaptic proteins make up 6% of the CSF proteome. Of the 251 proteins detectable in CSF, 22 were selected for further study. We developed targeted mass spectrometry tests (SRM: selective reaction monitoring) using 54 peptides labeled with isotopes corresponding to the 22 proteins of interest. We removed 12 proteins from the study due to insufficient detection in 5 CSF samples. The synaptic expression of the 10 remaining proteins was evaluated in the human brain using the array tomography microscopy available in our group and detailed in our 2 previous publications (Colom-Cadena et al., Brain 2017, Pickett et al., J Alzheimer Dis 2016). AT is particularly suitable for the study of human synapses, since it provides an improved

spatial resolution in the axial plane compared to other techniques of light microscopy (such as confocal microscopy). By obtaining ultrathin tissue sections (70 nm), individual synapses can be identified. The 3D reconstructions of individual synapses clearly show the expression of 9 of the panel proteins directly at the synapse, marked by pre- (synaptophysin) and post-synaptic (PSD-95) markers (**Fig. 1A**). However, Tenascin-R was found surrounding the synapse without making direct contact, consistent with data from the literature suggesting that Tenascin-R resides in extracellular perineuronal networks. The specificity of synaptic expression was further evaluated by quantifying the enrichment of the panel proteins in synaptic fractions from 6 human cortical tissue samples. The 2A isoform of Neurexin-2 could not be analyzed due to the lack of a commercially available antibody specific suitable for Western blotting. **Fig. 1B** shows that the 9 proteins analyzed were enriched in synaptic fractions compared to the homogenate ($p < 0.03$).

The 9 proteins with high specificity for the synapse (Calsyntenin-1, GluR2, GluR4, Neurexin-2A, Neurexin-3A, Neuroligin-2, Syntaxin-1B, Thy-1, and Vamp-2) were chosen for evaluation as markers of synapse loss in 3 cohorts of control subjects and across preclinical and clinical stages of the Alzheimer continuum from Hospital de Sant Pau in Barcelona, Fundación CITA Alzheimer and Hospital Clínic de Barcelona. The overall study (**Fig 2**) showed a global decrease of these proteins in the preclinical Stage 1 (without symptoms) and a subsequent increase in the symptomatic phases (prodromal and dementia). Of the 9 proteins, 6 (Calsyntenin-1, GluR4, Neurexin-2A, Neurexin-3A, Syntaxin-1B and Thy-1) showed a significant decrease at Stage 1 ($p < 0.05$). These results show for the first time that the decrease in synaptic proteins in CSF is a very early phenomenon that can be detected in the preclinical phase of AD that precedes neurodegeneration. We propose that reduced levels of these proteins at preclinical stage 1 may reflect reduced synaptic density in these individuals who already show signs of Aβ pathology but are yet to show symptoms, an effect that is masked by widespread neurodegeneration in later stages of the disease. The results were published in the most important international journal in the field of proteomics (Lleó et al., Mol Cell Prot 2019).

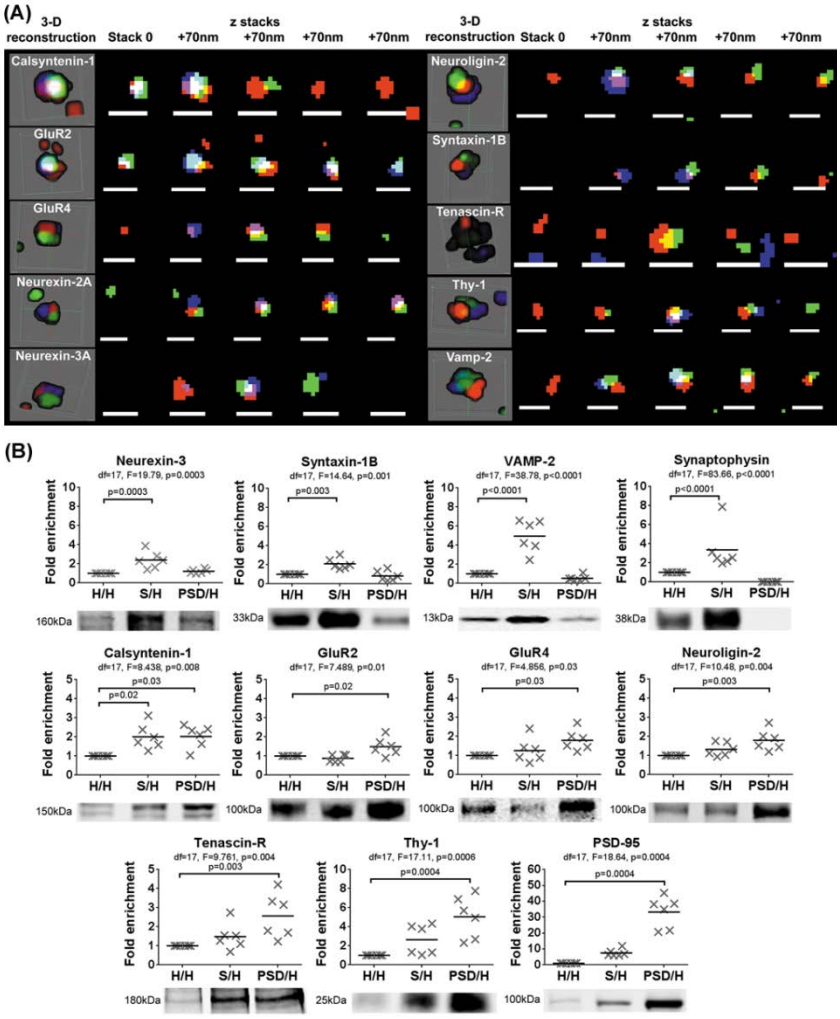


Fig 1. Expression of the panel proteins at the human cortical synapse. (**A**) Using AT microscopy, ultrathin tissue slices (70nm) from human post-mortem cortical tissue from one brain donor were immunostained for pre- (synaptophysin, red) and post- (PSD95, green) synaptic markers and the synaptic panel proteins (blue). A representative 3-D reconstruction of a representative synapse is shown for each panel protein. The segmented immunofluorescence of the 3 proteins in each individual stack (at 70nm increments) is shown to the right of the reconstruction. Scale bars representing 1μm are shown at the bottom of each z stack. (**B**) Mean fold-enrichment plotted for each panel protein in homogenate (H), synaptosome-enriched (S) and PSD-enriched (PSD) fractions taken from post-mortem human cortex (n=6). S/H and PSD/H; intensity in S or PSD fractions relative to H for the same sample. H/H; intensity in the H fraction for each sample relative to the mean intensity in the H fraction across all samples. Enrichment of the pre- (synaptophysin) and post- (PSD-95) markers is also shown. Degrees of freedom (df), F statistic and p-values for the ANOVA are shown at the top of each plot and Dunnett's p values are shown for significant pairwise comparisons (α=0.05).

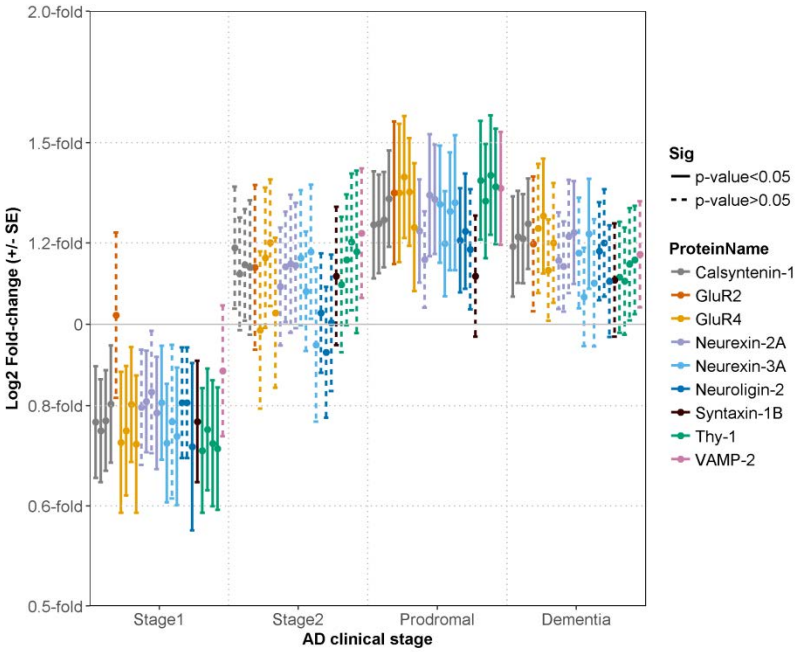


Fig 2. Synaptic panel peptide levels in the CSF across the AD continuum. The log2 fold-change (+/- standard error; SE) in CSF levels of the synaptic panel peptides and summarized protein levels are plotted for each preclinical and clinical AD stage versus cognitively normal controls. For ease of interpretation, the natural values are labelled on the y-axis on a log2 scale. The linestyle of the error bars was determined by p-value cut-offs for pairwise group comparisons using a mixed effect linear regression model (see legend). Stage1; preclinical AD stage 1, Stage2; preclinical stage 2,Prodromal; prodromal AD, Dementia; AD dementia.

Objective 2) Characterize the molecular composition of human entorhinal cortex synapses and identify the earliest changes induced by AD.

To achieve this objective, we adapted a protocol for enrichment of synaptosomes from 18 brain samples from the entorhinal cortex of controls and two pathologies that present with tauopathy (abnormal deposits of tau protein in the brain), namely primary age-related tauopathy (PART) and AD. We first verified by Western Blot that the fractions obtained were enriched for the presynaptic fraction and the fractions were then analyzed by mass spectrometry at the Genomic Regulation Center. The proteins found in the 3 groups were compared. Using PANTHER, an online tool for the analysis of biological pathways (www.pantherdb.org), we annotated the proteins to a biological process and compared the average fold-change related to each process in AD cases compared to controls. We observed lower levels of proteins associated with vesicle secretion and synaptic signaling, which could reflect the synapse degeneration associated with AD (Fig 3). On the other hand, we saw that proteins with a mitochondrial function were elevated in the AD synaptic fractions (**Fig 3**). The PART cases demonstrated the same changes compared to controls. We conclude that tau pathology characteristic of aging and AD is associated with a recruitment of mitochondrial proteins to the synapse. This study provides important information about the initial changes in a critical region for AD and we propose that the proteins associated with mitochondrial pathways could be new markers of the initial processes

in diseases presenting with tauopathy. A manuscript detailing these results is currently in preparation.

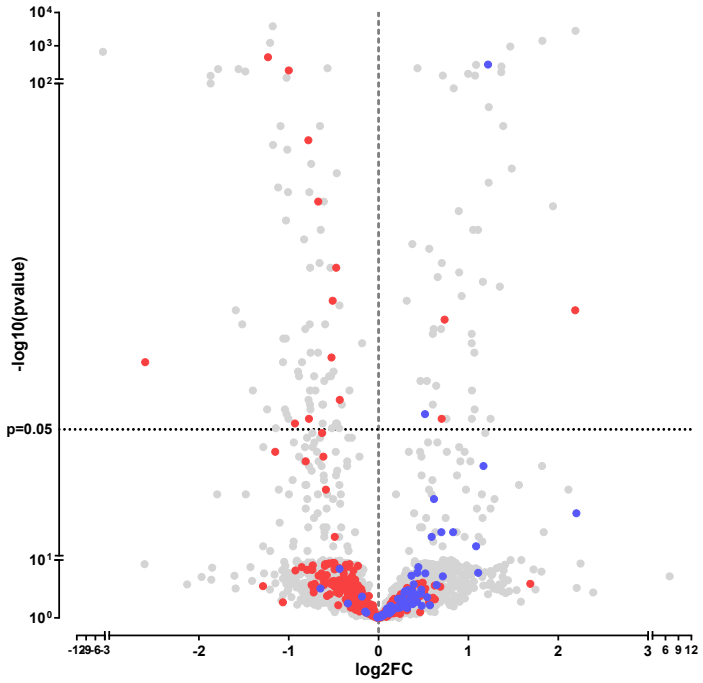


Fig 3. Compositional changes of entorrhinal cortex synapses associated with AD. Relative expression (log2FC) of the 3130 proteins quantified in synaptosome fractions in AD cases versus controls. Proteins associated with the secretion of synaptic vesicles or the synaptic signalling are marked in red. Proteins associated with mitochondrial functions are marked in blue.

In summary, this project has provided novel information about the initial synaptic changes in a critical region for AD and has allowed the identification of new biomarkers that could be used to detect synapse degeneration, an early event in AD pathogenesis, in living subjects. These new biomarkers have the potential to be useful in a variety of neurological and psychiatric diseases, and open up many avenues for further investigation (see our publication, Lleó et al., Clinica Chimica Acta 2019).

3. Relevance and possible future implications

This project has led to very relevant results for the scientific community and with possible practical applications for patients with Alzheimer's and other neurodegenerative diseases. Specifically, the synaptic proteins discovered in the CSF in this project (Objective 1) may represent markers of progression in Alzheimer's disease and other related disorders or early diagnosis markers in at-risk subjects (for example, in cases with a family history).

In addition, as a direct consequence of this project, a patent for 10 synaptic proteins has been submitted to protect their exploitation as biomarkers of synapse degeneration and a Belgian company, ADx Neurosciences, has been licensed for the development of 4 protein immunoassays that will facilitate application of the biomarkers on a large scale in patients with neurological diseases. The development of further studies that demonstrate a wider clinical use is a key step for their incorporation into clinical practice. We hope that in the near future, some of these biomarkers may be incorporated into clinical routine or in clinical trials of disease-modifying drugs.

4. Generated literature

Scientific articles

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Colom-Cadena M; Pegueroles J, Herrmann AG, Henstridge CM; Muñoz L, Querol-Vilaseca M, San Martín-Paniello C, Luque-Cabecerans J, Clarimón J, Belbin O, Nuñez R, Blesa R, Smith C, McKenzie AC, Frosch MP, Roe A, Fortea J, Andilla J, Loza-Álvarez P, Gelpi E, Hyman BT, Spires-Jones T, Lleó A. Synaptic phosphorylated a-synuclein in dementia with Lewy bodies. Brain 2017; 140: 3204-14.

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Lleó A, et al. Proteomic alterations at the entorhinal synapse in brains with Tau pathology. In preparation.

Patents

European Patent: Inventors: Olivia Belbin, Alberto Lleó, Alejandro Bayés, Juan Fortea, Daniel Alcolea.

Priority date: 16 March 2018.

Application number: EP18382175.0

Title: Markers of synaptopathy in neurodegenerative disease.

Licenses

Entities: IIB-SantPau, Barcelona; ADx Neurosciences, Belgium

Reason for the license: Development of antibodies and immunoassays suitable for the in vitro diagnostic market for 4 synaptic biomarkers identified by the team at the IIB-Sant Pau.

Duration: 2018-2025