Role of ANXA2, HSP90AA1 and PTK2B in synaptic homeostasis through microglial cells in the human entorhinal cortex in Alzheimer’s disease

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Research Article

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Abstract

Background

Alzheimer’s disease (AD), the most prevalent neurodegenerative disorder worldwide, is clinically characterized by cognitive deficits. Neuropathologically, AD brains accumulate deposits of amyloid-β (Aβ) and tau proteins. Furthermore, these misfolded proteins can propagate cell to cell in a prion-like manner and induce native proteins to become pathological. The entorhinal cortex (EC) is the earliest area affected by tau accumulation along with volume reduction and neurodegeneration. Neuron–glia interactions are now a focus; however, the role of microglia and astroglia in the pathogenesis of AD remains unclear. Proteomic approaches allow the determination of changes in the proteome to better understand the pathology underlying AD and therefore the identification of possible biomarkers.

Methods

Bioinformatic analysis of proteomic data was performed to compare the EC from AD vs. non-AD human brain tissue. To validate proteomic results, western blot, immunofluorescence and confocal studies were carried out.

Results

The findings revealed that the most disturbed signaling pathway was synaptogenesis, with the impairment of the growth and branching function of the dendrites and axons. Due to their involvement in synapse function, relationship with Aβ and tau proteins and interactions in a pathway analysis, three proteins were selected for further study: HSP90AA1, PTK2B and ANXA2. These proteins showed colocalization with neurons and/or astroglia and microglia and with Aβ and tau proteins.

Conclusions

Taken together, HSP90AA1, PTK2B and ANXA2 may play a significant role in synaptic homeostasis through microglial cells in AD.

Background

Alzheimer’s disease (AD) is the most prevalent neurodegenerative disorder worldwide, and its prevalence is rapidly growing due to the aging population (1). It is clinically characterized by cognitive deficits and memory dysfunction. Neuropathologically, deposits of amyloid-β (Aβ) and tau proteins are found in AD brains (2). These misfolded proteins can act in a prion-like manner, propagating in a cell-to-cell manner through neurons and/or glial cells and inducing native proteins to become pathological (3, 4). Accumulating evidence points to a prominent role of astroglia and microglia in AD pathogenesis (5). However, whether both glial cell populations facilitate the clearance (6, 7) and/or the spread (8–10) of Aβ and tau pathological proteins remains unclear.

Since tau accumulation occurs in a predictable manner, six neuropathological stages have been established for AD (11). In addition to the locus coeruleus, the entorhinal cortex (EC) is one of the earliest areas involved in tauopathy (Braak stage I). From the EC, tau aggregates spread out from within the medial temporal lobe and beyond to the rest of the cortex. Importantly, the EC is the principal entrance of cortical information into the hippocampus through the perforant pathway (12). Because of its unique location in the cortical-hippocampal circuit, the EC constitutes an
essential connectomic hub (13) for memory encoding and retrieval (14, 15). In fact, medial temporal lobe atrophy, especially in the hippocampus and EC, is one of the hallmarks of AD and is used as a diagnostic criterion (16).

Neuronal loss and volume reduction in the EC have been largely reported (17, 18). There is a clinical need to identify biomarkers for diagnosis and therapeutic strategies to protect against EC degeneration. Determining changes in the proteome is a potential tool to better understand the pathology underlying AD (19–22). Proteomic approaches in the human EC have been scarce but have identifying alterations related to protein phosphorylation (23) and ion transport function (24) in patients with AD. A recent proteomic study in our laboratory revealed interesting up- (S100A6, PPP1R1B, BAG3 and PRDX6) and downregulated (GSK3B, SYN1, DLG4 and RAB3A) proteins related to neurodegeneration and astrogliosis in the human EC in AD (18).

In the present study, a bioinformatic analysis of proteomic data was performed in the EC in AD. Specifically, synaptogenesis was the most affected signaling pathway, with a marked decrease in the growth and branching function of dendrites and axons. Heat shock protein HSP 90-alpha (HSP90AA1), protein-tyrosine kinase 2-beta (PTK2B) and annexin-2 (ANXA2) were selected because of their involvement in synapse function, their relationship with Aβ and tau pathological proteins, and their connections in an interactome identified in a pathway analysis. We analyzed the specific expression patterns of these proteins in particular cell types, neurons, astroglia and microglia, to disentangle cell-type-specific contributions to the disease pathology. Findings point to microglia as major players in synaptic function. Additionally, HSP90AA1, PTK2B and ANXA2 could have an important role in synaptic homeostasis, inducing the polarization and migration of microglia to inflammation sites in neurons, or the promotion of Aβ clearance through microglial activation. This information will help to elucidate AD pathogenesis, thus improving diagnostics and therapeutics for AD.

**Methods**

**Proteomic-bioinformatic analysis**

On the basis of findings from a previous proteomic study through sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS) of the human EC in our laboratory (Dataset S1a) (18), a complementary bioinformatic analysis was performed. The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD029359 (Username: reviewer_pxd029359@ebi.ac.uk; Password: 9ZBBqh6c). Differentially expressed proteins (DEPs) were established with a P value < 0.01 and thresholds set to ≥ 1.8 for upregulation and ≤ 0.55 for downregulation.

Interactome and pathway analyses were performed using machine learning-based bioinformatic QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity). SYNGO (Synaptic Gene Ontologies) https://syngoportal.org/ was applied to analyze synapse function. BioGRID 4.4 (Database of Protein, Genetic and Chemical Interactions) https://thebiogrid.org/ was used as a repository of protein interactions with pathological markers (APP and MAPT). Venn diagrams of the data were constructed using Venny https://bioinfogp.cnb.csic.es/tools/venny/. Finally, the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins database) website (https://string-db.org/) was used to establish the protein–protein interaction networks of some differentially expressed proteins of interest and to create four cluster networks with the k-means algorithm.
Postmortem human brain samples were provided by Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Biobanco en Red de la Región de Murcia (BIOBANC-MUR), Biobanco de Tejidos de la Fundación CIEN (BTCIEN) and Biobanco del Principado de Asturias (BPA); integrated in the Spanish National Biobanks Network; and then processed following standard operating procedures with the approval of the Clinical Research Ethics Committee of Ciudad Real University Hospital (PID2019-108659RBI00). Two experimental groups were used: $n = 8$ AD cases (mean age $\pm$ SEM: 80.63 $\pm$ 3.267) and $n = 8$ age-matched non-AD cases (mean age $\pm$ SEM: 74.50 $\pm$ 2.732). Information about the cases used is detailed in Table 1.

### Table 1

Demographic and clinic-pathological features of the individuals used in the present study.

<table>
<thead>
<tr>
<th>Case</th>
<th>DxAP</th>
<th>Assay</th>
<th>Braak Stage</th>
<th>Sex</th>
<th>Age (years)</th>
<th>PMD (hh:mm)</th>
<th>Brain weight (g)</th>
<th>Original fixation</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AD</td>
<td>IF</td>
<td>VI</td>
<td>F</td>
<td>87</td>
<td>15:30</td>
<td>990</td>
<td>Formaldehyde</td>
<td>Sepsis</td>
</tr>
<tr>
<td>2</td>
<td>AD</td>
<td>IF</td>
<td>V</td>
<td>F</td>
<td>80</td>
<td>4:00</td>
<td>910</td>
<td>Formaldehyde</td>
<td>Respiratory infection</td>
</tr>
<tr>
<td>3</td>
<td>AD</td>
<td>IF</td>
<td>VI</td>
<td>F</td>
<td>85</td>
<td>2:00</td>
<td>1150</td>
<td>Formaldehyde</td>
<td>Cardiorespiratory arrest</td>
</tr>
<tr>
<td>4</td>
<td>AD</td>
<td>WB</td>
<td>VI</td>
<td>M</td>
<td>90</td>
<td>4:30</td>
<td>1070</td>
<td>Frozen no-fixed</td>
<td>Cardiorespiratory arrest</td>
</tr>
<tr>
<td>5</td>
<td>AD</td>
<td>WB</td>
<td>V-VI</td>
<td>F</td>
<td>91</td>
<td>5:00</td>
<td>n.a.</td>
<td>Frozen no-fixed</td>
<td>n.a.</td>
</tr>
<tr>
<td>6</td>
<td>AD</td>
<td>WB</td>
<td>VI</td>
<td>F</td>
<td>76</td>
<td>11:10</td>
<td>900</td>
<td>Frozen no-fixed</td>
<td>Cardiorespiratory arrest</td>
</tr>
<tr>
<td>7</td>
<td>AD</td>
<td>WB</td>
<td>VI</td>
<td>M</td>
<td>69</td>
<td>2:25</td>
<td>n.a.</td>
<td>Frozen no-fixed</td>
<td>Multi-organic failure</td>
</tr>
<tr>
<td>8</td>
<td>AD</td>
<td>WB</td>
<td>VI</td>
<td>F</td>
<td>67</td>
<td>4:15</td>
<td>n.a.</td>
<td>Frozen no-fixed</td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>9</td>
<td>NAD</td>
<td>IF</td>
<td>-</td>
<td>F</td>
<td>75</td>
<td>10:30</td>
<td>1050</td>
<td>Formaldehyde</td>
<td>Cardiogenic shock</td>
</tr>
<tr>
<td>10</td>
<td>NAD</td>
<td>IF</td>
<td>-</td>
<td>F</td>
<td>81</td>
<td>5:00</td>
<td>1100</td>
<td>Formaldehyde</td>
<td>Multi-organic failure</td>
</tr>
<tr>
<td>11</td>
<td>NAD</td>
<td>IF</td>
<td>-</td>
<td>F</td>
<td>62</td>
<td>2:00</td>
<td>1050</td>
<td>Formaldehyde</td>
<td>Cardiorespiratory arrest</td>
</tr>
<tr>
<td>12</td>
<td>NAD</td>
<td>WB</td>
<td>-</td>
<td>F</td>
<td>83</td>
<td>7:20</td>
<td>1320</td>
<td>Frozen no-fixed</td>
<td>Intestinal ischemia</td>
</tr>
<tr>
<td>14</td>
<td>NAD</td>
<td>WB</td>
<td>-</td>
<td>M</td>
<td>68</td>
<td>4:10</td>
<td>1350</td>
<td>Frozen no-fixed</td>
<td>Sepsis</td>
</tr>
<tr>
<td>15</td>
<td>NAD</td>
<td>WB</td>
<td>-</td>
<td>F</td>
<td>82</td>
<td>4:00</td>
<td>800</td>
<td>Frozen no-fixed</td>
<td>Respiratory failure</td>
</tr>
<tr>
<td>16</td>
<td>NAD</td>
<td>WB</td>
<td>-</td>
<td>M</td>
<td>68</td>
<td>4:00</td>
<td>1220</td>
<td>Frozen no-fixed</td>
<td>Cardiorespiratory arrest</td>
</tr>
</tbody>
</table>
To standardize sample conditions from different brain banks, all formalin-fixed blocks were fixed by immersion in 4% paraformaldehyde. For cryoprotection, blocks were submerged in a phosphate-buffered (PB) solution of 2% dimethyl sulfoxide (DMSO) and 10% glycerol for 48 h and finally in a PB solution of 2% DMSO and 20% glycerol for 48 h as well. Later, tissue was cut with a freezing sliding microtome into a series of 50 µm thick coronal sections. Series were stored in cryoprotective solution and kept at −20 ºC until further processing.

**Immunofluorescence**

For immunofluorescence, epitopes from human formaldehyde-fixed samples (Table 1) were unmasked by boiling tissue sections under pressure for 2 min in citrate buffer. After unmasking, sections were immersed in formic acid for 3 min and rinsed in phosphate-buffered saline (PBS) or Tris-buffered saline (TBS; 0.05 M NaCl, 0.05 M Tris, HCl pH 7.6). Endogenous peroxidase activity was inhibited using 1% H$_2$O$_2$ for 20 min. Tissue was then immersed in blocking buffer for 30 min at room temperature and incubated overnight at 4°C with primary antibodies (for details, see Table 2). Then, sections were incubated for 2 h at room temperature with Alexa Fluor 488, 568, or 647 anti-multiple species antibodies (1:200 in TBS with 0.3% Triton X-100; Invitrogen), counterstained with DAPI (0.01% in TBS, Sigma–Aldrich) for 5 min in the dark and coverslipped with PVA-DABCO (Sigma–Aldrich). Images were captured with a Zeiss LSM 800 confocal microscope coupled to Zen 2.3 software.
Table 2
Details of antibodies used in this study.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Manufacturer</th>
<th>Catalog nº</th>
<th>Species</th>
<th>Assay</th>
<th>Dilution</th>
<th>Blocking Buffer</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iba-1</td>
<td>Abcam</td>
<td>ab5076</td>
<td>Goat polyclonal</td>
<td>IF</td>
<td>1:1000</td>
<td>TBS + 0.3%TX-100 + 10%NDS</td>
<td>Alexa Fluor® 647 donkey</td>
</tr>
<tr>
<td>GFAP</td>
<td>Abcam</td>
<td>ab53554</td>
<td>Goat polyclonal</td>
<td>IF</td>
<td>1:500</td>
<td>TBS + 0.3%TX-100 + 10%NDS</td>
<td>Alexa Fluor® 647 donkey</td>
</tr>
<tr>
<td>β-Amyloid</td>
<td>Cell signaling</td>
<td>2454</td>
<td>Rabbit polyclonal</td>
<td>IF</td>
<td>1:250</td>
<td>PBS + 0.3%TX-100 + 2%NDS</td>
<td>Alexa Fluor® 488 donkey</td>
</tr>
<tr>
<td>β-Amyloid</td>
<td>Cell signaling</td>
<td>2450</td>
<td>Mouse monoclonal</td>
<td>IF</td>
<td>1:1000</td>
<td>TBS + 0.3%TX-100 + 10%NDS</td>
<td>Alexa Fluor® 568 donkey</td>
</tr>
<tr>
<td>Tau</td>
<td>Cell signaling</td>
<td>46687</td>
<td>Rabbit monoclonal</td>
<td>IF</td>
<td>1:100</td>
<td>PBS + 0.3%TX-100</td>
<td>Alexa Fluor® 488 donkey</td>
</tr>
<tr>
<td>Neuro-Chrom™ Pan Neuronal</td>
<td>Sigma–Aldrich</td>
<td>MAB2300</td>
<td>Mouse monoclonal</td>
<td>IF</td>
<td>1:100</td>
<td>TBS + 0.3%TX-100 + 10%NDS</td>
<td>Alexa Fluor® 568 donkey</td>
</tr>
<tr>
<td>ANXA2</td>
<td>Abcam</td>
<td>ab41803</td>
<td>Rabbit polyclonal</td>
<td>IF</td>
<td>1:100</td>
<td>TBS + 0.3%TX-100 + 10%NDS</td>
<td>Alexa Fluor® 488 donkey</td>
</tr>
<tr>
<td>HSP90α</td>
<td>Invitrogen</td>
<td>MA3-010</td>
<td>Mouse monoclonal</td>
<td>IF</td>
<td>1:20</td>
<td>TBS + 0.3%TX-100 + 10%NDS</td>
<td>Alexa Fluor® 568 donkey</td>
</tr>
<tr>
<td>PYK2</td>
<td>Abcam</td>
<td>ab32571</td>
<td>Rabbit monoclonal</td>
<td>IF</td>
<td>1:100</td>
<td>TBS + 0.3%TX-100 + 10%NDS</td>
<td>Alexa Fluor® 488 donkey</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Cell signaling</td>
<td>2118</td>
<td>Rabbit monoclonal</td>
<td>WB</td>
<td>1:2000</td>
<td>Milk 5%</td>
<td>Polyclonal Goat Immunoglobulins/HRP</td>
</tr>
</tbody>
</table>

IF: Immunofluorescence, WB: Western blot.

Protein extraction and western blotting
Frozen-tissue samples (Table 1) were disrupted with a pellet pestle (Sigma–Aldrich) and homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 0.5% Na-deoxycholate) with a protease inhibitor cocktail (Sigma–Aldrich). Homogenates were then shaken for 2 hours at 4°C, followed by centrifugation for 5 min at 12,000 g at 4°C. Subsequently, supernatants were collected. Protein concentration was determined using a Bicinchoninic Acid Kit for Protein Determination (Sigma–Aldrich) and a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific).

Equal amounts of lysate proteins (30 µg) from human samples (Table 1) were loaded onto 10% polyacrylamide gels for SDS–PAGE and electrophoretically transferred to nitrocellulose (HSP90AA1) or PVDF (ANXA2 and PTK2B) membranes. GAPDH was used as a housekeeping protein. Membranes were blocked with nonfat dry milk in TTBS (0.1% Tween-20, 0.06 M NaCl, and 0.2M Tris-hydroxymethyl-aminomethane pH 8.8) for 60 min and incubated with primary antibodies (Table 2) overnight at 4º C. Membranes were then washed with TTBS and incubated with the appropriate peroxidase-conjugated secondary antibodies (1:5000). Band intensity was imaged with Syngene G:Box (GeneSys software) after incubation with Enhanced Chemiluminescence reagents (Thermo Fisher Scientific) and analyzed with ImageJ.

**Statistics**

All statistical analyses were conducted using GraphPad Prism 6 Software (GraphPad Inc., San Diego, CA, USA, v.6). Data are expressed as the mean ± SEM, and statistical comparisons were made using t tests or Mann–Whitney U tests for normally or nonnormally distributed data, respectively. The statistical significance level was established at α = 0.05.

**Results**

**Proteomic-bioinformatic analysis**

The total number of DEPs in the human EC was 139, with 52 upregulated and 87 downregulated proteins (Dataset S1b). Ingenuity Pathway Analysis was used to investigate the potential implication of the observed changes in metabolic pathways and protein interaction networks. The analysis of the top ten canonical pathway revealed the Synaptogenesis Signaling Pathway in first place with a negative z-score (Fig. 1A). Inhibited functions in this relevant pathway are the branching of neurites or dendritic growth/branching through the upstream regulator PPARA (peroxisome proliferator-activated receptor alpha), leading to organismal death (Fig. 1B). More specifically, increased and decreased function annotations in relation to synapses are shown in Fig. 1C, where a substantial reduction in the growth and branching of neurites is highlighted (Dataset S1c).

To further analyze synaptic homeostasis, we performed a SYNGO analysis, for which cellular component and biological process enrichment analysis in synapses revealed that 37/139 DEPs were involved (Dataset S1d). The synapse Gene Ontology (GO) term (GO:0045202) revealed 9 specific proteins (AP2M1, ARHGDIA, CORO1A, CYFIP2, EEF1A2, FGA, RPLP2, RPS25, TBC1D24; p value: 8.97e-12). The alteration was also relevant in both the presynapse (GO:0098793), with 7 altered proteins (AP1G1, CADPS, PFN2, PRKACA, PTK2B, SCRNN1, SYNJ1; p value = 4.12e-6), and the postsynapse (GO:0098794), with 6 altered proteins (ACTR2, CYFIP1, PFN2, PPP1R1B, PRKACA and PTK2B; p value = 1e-4). PTK2B, affected in the presynapse and postsynapse, was also involved in postsynaptic density (GO:0099092), postsynaptic modulation of chemical synaptic transmission (GO:0099170) and the regulation of postsynaptic density assembly (GO:0099151) (Dataset S1e-f).
To determine how many proteins from DEPs were related to the A\(\beta\) (APP) and tau (MAPT) pathological proteins, we generated a Venn diagram (Fig. 1D). Sixteen proteins matched A\(\beta\) (APP), and 20 matched tau (MAPT), of which only 3 were common (GSK3B, HSP90AA1 and PPIA).

To characterize in detail the human EC in AD, proteomic data were functionally analyzed (Fig. 1E). Several direct and indirect interactions among A\(\beta\) (APP) and DEP proteins were noted when APP was incorporated into the interactome map. Specifically, 8 increased proteins (AHNAK, AIF1, ANXA2, H2AZ2, H4C1, LMNA, MYL12A and PRDX6) and 14 decreased proteins (ACOT7, ARMT1, COPS2, DLG4, EPB41L3, GSK3B, HSP90AA1, PHYH1PL, PPIA, PRKACA, PTK2B, RAN, RASAL1 and TPP2) were involved. Remarkably, the presence of AIF1 (synonyms of IBA1), related to microglial cells, is shown in the interactome map. In addition, STRING analysis of 34 DEPs revealed four different clusters related to axonal formation in yellow, vesicular transport in blue, chaperone response in green, and synapses and inflammation in red (Figure S1), which include the characterized proteins of our previous proteomic study: S100A6, PPP1R1B, BAG3, PRDX6, GSK3B, SYN1, DLG4 and RAB3A.

After bioinformatic analyses of proteomic data, three proteins were selected (two downregulated, HSP90AA1 and PTK2B, and one upregulated, ANXA2) for further experiments based on different criteria. The first criterion was statistical significance based on the \(p\) value (< 0.01) and fold change (≥ 1.8 for upregulated and ≤ 0.55 for downregulated expression) established to select DEPs. Second, the three proteins were selected due to their interactions with A\(\beta\) and/or tau protein extracted from the BIOGRID analysis. More specifically, ANXA2 was related to A\(\beta\), PTK2B was related to tau, and HSP90AA1 was related to both. Third, all three proteins showed direct or indirect functional interactions with A\(\beta\) protein and microglia in an interactome map. Fourth, HSP90AA1, PTK2B and ANXA2 showed interactions in the STRING analysis with the characterized proteins from our previous study, such as PRDX6, S100A6, BAG3, GSK3B or DLG4. Moreover, PTK2B was also selected due to its known involvement in altered synapses in AD.

**Immunofluorescence and western blot analysis**

To validate our findings in a spatial context, we performed immunofluorescence analysis to qualitatively check the down- or upregulation of the HSP90AA1, PTK2B and ANXA2 proteins identified in our proteomic analysis. The expression of HSP90AA1, PTK2B and ANXA2 was investigated in non-AD and AD cases (Fig. 2A and B, D and E, G and H, respectively). HSP90AA1 (corresponding to the HSP90\(\alpha\) antibody) expression was lower in AD cases than in non-AD cases (Fig. 2A, B). PTK2B (corresponding to the PYK2 antibody) expression was also lower in AD cases than in non-AD cases (Fig. 2D, E). Finally, ANXA2 expression was higher in AD cases, emphasizing a morphological change from rounded cells in non-AD cases to ramified cells in AD cases (Fig. 2H, G).

Selected proteins were also quantitatively validated using western blotting. No statistically significant differences were found for HSP90AA1 (\(p\) value = 0.5317), PTK2B (\(p\) value = 0.0952) or ANXA2 (\(p\) value = 0.4127) (Fig. 2C, F, and I, respectively). All proteins showed a trend according to proteomic data analysis without reaching statistical significance, probably due to the high intrahuman variability among samples.

**Colocalization**

Immunofluorescence analysis of selected proteins with microglial (IBA1), astroglial (GFAP) or neuronal (PAN) markers was performed to assess spatial relationships. In addition, the pathological markers A\(\beta\) and tau were investigated together with HSP90AA1 and ANXA2.
HSP90AA1 colocalized with microglial (Fig. 3A-C) and astroglial (Fig. 3D-F) cells. On the other hand, HSP90AA1 was found in the vicinity of Aβ plaques and colocalized with Aβ (Fig. 3G-I) and tau (Fig. 3J-L) deposits in AD cases. PTK2B colocalized with neuronal (Fig. 4A-C) and microglial cells (Fig. 4D-F). ANXA2 overlapped with microglial (Fig. 5A-C) and astroglial (Fig. 5D-F) cells. Moreover, ANXA2 showed an intense relationship with Aβ plaques (Fig. 5G-I, Movie S1). In addition, we observed astrocytes specifically around Aβ plaques that coexpressed ANXA2 (Fig. 6). Interestingly, triple colocalization of ANXA2, HSP90AA1 and IBA1 was observed in microglial cells with a rounded morphology but not in those with a ramified morphology (Fig. 7).

**Discussion**

To better characterize the relevance of DEPs and their implication in the pathology of AD, a complementary bioinformatic analysis of human EC proteomic data was performed. A total of 139 significant DEPs (52 upregulated and 87 downregulated) were identified. The most altered signaling pathway in the EC of AD cases was synaptogenesis (Fig. 1A, B and C). Later, SYNGO analysis was performed to elucidate the DEPs involved in synapse function (Dataset S1d-f). We also evaluated the existence of an association between altered DEPs and Aβ (APP) and tau (MAPT), characteristic pathological proteins (Fig. 1D). Furthermore, to characterize in detail direct and indirect interactions based on pathway analysis, DEPs were represented in an interactome map (Fig. 1E). Finally, based on bioinformatic and proteomic analysis, two downregulated proteins, HSP90AA1 and PTK2B, and one upregulated protein, ANXA2, were selected as the DEPs of interest for western blot, immunofluorescence and confocal studies.

The results revealed that synaptic homeostasis was disrupted, specifically at the synaptogenesis level, with a marked decrease in the growth and branching functions of dendrites and axons (Fig. 1A, B and C). This would lead to neurodegeneration (Fig. 8), widely described in AD (17, 18). Synaptic pathology occurs early in AD and has been correlated with cognitive impairment (25). However, the molecular mechanisms that lead to synaptic dysfunction remain unclear. Most research focuses on the harmful consequences of soluble toxic forms of Aβ and tau at synapses (26, 27). For this reason, we constructed a Venn diagram of DEPs related to Aβ (APP) and tau (MAPT), where PTK2B matched tau, ANXA2 was related to Aβ, and HSP90AA1 matched both (Fig. 1D). In a representative interactome map of functional relationships, the central node, APP, was linked to different proteins, including HSP90AA1, PTK2B and ANXA2 (Fig. 1E). Notably, the network revealed indirect relationships with AIF1, which is related to microglial cells. Glial-mediated neuroinflammation is also involved in synaptic dysfunction (5, 27). In particular, recent research findings suggest that microglia and astroglia might contribute to synapse loss by engulfing synaptic structures (27). Microglia and astroglia have recently been considered crucial players in the pathogenesis of AD, but their protective or harmful roles remain unclear (5). Evidence suggests that both populations could be involved in either the clearance or, conversely, the spread of Aβ and tau pathological proteins (6–9). One of the main goals of this study was to characterize the contribution of HSP90AA1, PTK2B and ANXA2 to neurons, microglia and/or astroglia in AD to establish neuron-glia cell interactions (Figs. 3, 4 and 5, respectively). Moreover, the spatial relationships with Aβ and tau were investigated for HSP90AA1 and ANXA2.

HSP90 is an essential chaperone that regulates proper protein folding in the cell (28). Two main subtypes are expressed in mammals: HSP90β (HSP90AB1), which is constitutively expressed, and HSP90α (HSP90AA1), which is enriched in the brain and an inducible molecular chaperone that participates in the stress response (28, 29). In cooperation with its cochaperones, HSP90 is capable of regulating tau phosphorylation and dephosphorylation (29). In fact, HSP90AA1 (a downregulated protein in the present study) colocalized with tau protein (Fig. 3L). Several lines of investigation support the idea that the inhibition of HSP90 is a promising way to reduce tau pathology (30–32). Hsp90 was found to promote Aβ clearance through the activation of microglial phagocytosis in AD (Fig. 8) (28, 29, 32, 33). HSP90AA1 is also considered a microglial activation marker in Parkinson’s disease (34). In our study, HSP90AA1
colocalized with microglia (Fig. 3C) and was distributed around Aβ plaques (Fig. 3I). Moreover, we demonstrated for the first time the colocalization of HSP90AA1 with astrocytes (Fig. 3F). This interaction should be further studied to elucidate its role in AD.

PTK2B is a nonreceptor cytoplasmic tyrosine kinase predominantly expressed in neurons (35), and its colocalization was verified in the present study (Fig. 4C). It plays an important role in synaptic function and is involved in NMDA receptor regulation, hippocampal-related memory, spine structure modulation, postsynaptic regions organization, and synaptic plasticity (36). This is in accordance with our SYNGO results where PTK2B was involved in synaptic function, being affected in both pre- and postsynapses (Dataset S1f). There is currently controversy regarding its role in synaptic function: PTK2B mediates Aβ-induced synaptic loss (37); however the hippocampal overexpression of PTK2B improves a transgenic mouse model of AD, rescuing the loss of synaptic markers such as PSD95 (38). PTK2B accumulation represents an early pathological marker corresponding to progressive pathological stages of tau in AD patients and in transgenic mice (39). In fact, hyperphosphorylated tau was found to colocalize with PTK2B in the human AD brain (39). GSk3β, the main kinase phosphorylating tau protein, is activated by PTK2B (40, 41). Additional investigations revealed that PTK2B could act as a direct tyrosine kinase of tau (42). PTK2B activity displays differing effects on Aβ and tau signaling. PTK2B activity mediates toxic Aβ signaling but suppresses tau phosphorylation, protecting against tauopathy in AD (41). This protein seems to be required for normal macrophage polarization and migration toward sites of inflammation (Fig. 8) (43). We have shown for the first time that PTK2B is also expressed in microglial cells in the EC in AD (Fig. 4F).

ANXA2, a calcium-regulated membrane-binding protein, is present in growth cones and axonal branches of neurons (44). Knowledge of the involvement of ANXA2 in the pathology of AD is limited. It has been identified as a tau-interacting protein (45). The tau-ANXA2 interaction could retain tau protein in the axonal compartment of neurons (46). ANXA2 was found to be upregulated in the EC in AD, which could control the mislocalization of tau. This could point to a compensatory mechanism of the affected neurons in patients. There are few studies on Aβ to date. One study implicated ANXA2 as a regulator of Aβ metabolism facilitating autophagosome-lysosome fusion to decrease Aβ (47). In the present study, ANXA2 showed an intense spatial relationship with Aβ plaques in the EC in AD (Fig. 5I). Remarkably, astrocytes were located around Aβ plaques that coexpressed ANXA2 (Fig. 6). Therefore, the presence of ANXA2 could be involved in astroglia containing Aβ plaques. Regarding glial cells, our results showed a colocalization of ANXA2 with both astroglia and microglia in the EC in AD (Fig. 5C and F). A single study showed that ANXA2 was expressed by reactive astrocytes (identified by prominent cytoplasm and processes with strong GFAP immunoreactivity) in the human hippocampus, whereas quiescent astrocytes were minimally immunoreactive (48). On the other hand, ANXA2 has been linked to Aβ-mediated microglial activation and proinflammatory responses through tissue plasminogen activator (tPA) signaling pathways, which produce plasmin to degrade Aβ peptides (Fig. 8) (49). Interestingly, triple colocalization of ANXA2, HSP90AA1 and IBA1 was observed specifically in amoeboid microglia (Fig. 7), which is indicative of activated microglia, in contrast to the resting ramified morphology (50). Therefore, the joint interaction of ANXA2 and HSP90AA1 could be necessary to activate microglia toward their phagocytic form to degrade Aβ deposits.

All three proteins investigated in the present study showed a relationship with microglial cells. It has been described that microglia show different states during the AD course. Microglia have a beneficial role at the beginning of the disease, limiting the toxic accumulation of Aβ and tau through clearance or phagocytosis (5, 7, 51). Additionally, microglial cells are located around different Aβ species, compacting and corralling Aβ plaques (52). However, there is also considerable evidence supporting that activated microglia can be harmful in AD progression (5, 51). Thus, microglia become unable to protect against amyloid burden and exacerbate tau pathology (10, 53). Additionally, microglia can directly cause synaptic loss through the engulfment and removal of synapses and can secrete
proinflammatory factors that can damage neurons either directly or indirectly by activating neurotoxic astrocytes (27, 51, 54–56). In particular, it has been shown that reactive astrocyte subtype A1 is induced by activated microglia via the secretion of IL-1α, TNF and C1q cytokines (54). This type of neurotoxic astrocyte has been found in brain tissue in several neurodegenerative diseases, including AD. As a result, A1 astrocytes lose many astrocytic functions, such as support for neurons, synapse formation and function, and pruning synapses and myelin debris by phagocytosis (54, 57). More specifically, A1 astrocytes induced the formation of fewer and weaker synapses and lost their ability to promote synaptogenesis. Furthermore, A1 astrocytes secrete a neurotoxin that leads to the death of neurons and oligodendrocytes (54). This suggests that A1 astrocytes, induced by activated microglia, are toxic to the synapse and that their presence could contribute to neurodegeneration and disease progression (Fig. 8).

This study elucidates important features of synaptic dysfunction in the EC in AD. However, the main limitations are the low sample size and the advanced stages (V-VI) of the cases used. Therefore, future studies with larger cohorts at different disease stages could shed light on the progression of synaptic pathology across AD.

**Conclusions**

In conclusion, this report implicates the synaptogenesis as the most disrupted signaling pathway in the human EC in AD. HSP90AA1, PTK2B and ANXA2 could modulate synaptic homeostasis via microglial cells as follows: HSP90AA1 could induce the clearance of Aβ at synapses through an incremental increase in microglial phagocytosis; PTK2B, mainly expressed in neurons, could promote the polarization and migration of microglia to inflamed areas of synaptic structures; and ANXA2, also enriched in neurons, could mediate Aβ-mediated microglial activation and the inflammatory response and, subsequently, promote Aβ degradation at synapses. In addition, activated microglia can induce neurotoxic astrocytes, leading to neurodegeneration (Fig. 8). These statements further implicate microglial cells in the propagation of neurodegenerative and neuroinflammatory processes in AD. Therefore, a better understanding of microglia–synapse signaling events is needed to prevent synaptic dysfunction, neurodegeneration, and cognitive decline.

**Abbreviations**

Aβ  
Amyloid-β  
AD  
Alzheimer’s Disease  
AIF1  
Allograft Inflammatory Factor 1  
ANXA2  
Annexin-2  
DEPs  
Differentially Expressed Proteins  
DMSO  
Dimethyl Sulfoxide  
EC  
Entorhinal Cortex  
GFAP  
Glial Fibrillary Acidic Protein  
GO
Gene Ontology
HSP90AA1
Heat Shock Protein HSP 90-Alpha
IBA1
Ionized Calcium-Binding Adaptor Molecule 1
PB
Phosphate-Buffered
PBS
Phosphate-Buffered Saline
PTK2B/PYK2
Protein-Tyrosine Kinase 2-Beta
STRING
Search Tool for the Retrieval of Interacting Genes/Proteins database
SWATH-MS
Sequential Window Acquisition of All Theoretical Fragment Ion Mass Spectra
SYNGO
Synaptic Gene Ontologies
TBS
Tris-Buffered Saline.

Declarations

Ethics approval and consent to participate

Postmortem human brain samples were collected and processed following standard operating procedures with the approval of the Clinical Research Ethics Committee of Ciudad Real University Hospital (PID2019-108659RBI00). These processes included obtaining the donors' written consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analyzed during the current study are available in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD029359 (Username: reviewer_pxd029359@ebi.ac.uk; Password: 9ZBBqh6c). The data supporting the findings of this study are available in Supplementary Information. Raw data are available from the corresponding author, upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

VAL, IUB and AFC contributed to experimental design and data collection; VAL, SVC and MGR performed the experiments and analyzed data; VAL and DSS contributed to interpretation of the proteomic data and statistical analysis; VAL and AMM wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY INFORMATION

Additional file 1: Dataset S1.
Proteomic-bioinformatic data supporting the findings of this study.

Additional file 2: Figure S1.
Protein-protein interactions of the human EC in AD. STRING analysis of 34 DEP distributed in four network clusters; gray ellipses indicate the three markers characterized in this study (modified of Astillero-Lopez V. et al; 2022).

Additional file 3: Movie S1.
ANXA2 showing an intense relationship with Aβ plaques.

References


Proteomic analysis of the human EC in AD. (A) The top ten canonical pathways classified according to the z score. (B) Graphical summary of the synaptogenesis signaling pathway, the top canonical pathway, with the associated functions and the affected upstream regulators. (C) Increased and decreased functions in relation to synapses. (D) Common and unique differential DEPs related to AD pathology. Venn diagram showing the overlap between DEPs and Aβ (APP) and/or tau (MAPT) pathological proteins. Gene names are indicated for the corresponding overlapping areas of interest. (E) Representative functional protein interactome map for DEPs and APP.
Figure 2

Validation of proteomic results through immunofluorescence and western blot analyses: HSP90AA1, PTK2B and ANXA2. Coronal sections of the human EC immunofluorescently stained for HSP90AA1 (HSP90α antibody) in non-AD (A) and AD (B) cases, PTK2B (PYK2 antibody) in non-AD (D) and AD (E) cases and ANXA2 in non-AD (G) and AD (H) cases. Western blot quantification and band images of HSP90AA1 (C), PTK2B (F) and ANXA2 (I). The graphs show the mean ± SEM. Scale bar = 40 µm for A-B and G-H and 25 µm for D-E.
Figure 3

Glial and pathological colocalization of HSP90AA1. Images showing the colocalization among HSP90AA1, glial markers, and pathological proteins. Coronal sections of the human EC immunofluorescently stained for HSP90AA1 and IBA1 for microglia (A-C), GFAP for astrocytes (D-F), Aβ (G-I) and tau (J-L) in AD cases. Arrows point to colocalizations. Scale bar = 20 µm.
Figure 4

Neuronal and microglial colocalization of PTK2B. Images showing the colocalization among PTK2B and neuronal and glial markers. Coronal sections of the human EC immunofluorescently stained for PTK2B and PAN for neurons in non-AD cases (A-C) and for IBA1 for microglia in AD cases (D-F). Scale bar = 10 µm.
Figure 5

Glial and pathological colocalization of ANXA2. Images showing the colocalization among ANXA2, glial markers, and Aβ pathological protein. Coronal sections of the human EC immunofluorescently stained for ANXA2 and IBA1 for microglia (A-C), GFAP for astrocytes (D-F), and Aβ (G-I) in AD cases. Scale bar = 20 µm.
Figure 6

Triple colocalization of ANXA2, Aβ plaques and astrocytes in AD. (A) Coronal section of the human EC immunofluorescently stained for ANXA2, Aβ and GFAP for astrocytes and detail (B). Arrows and arrowheads indicate Aβ plaques with and without ANXA2, respectively. Orthogonal view of the z-stack (C) and each of its channels in green ANXA2 (D), red Aβ (E) and purple astrocytes (F). Scale bars for A = 70 µm, B = 35 µm, C = 20 µm and D, E, F = 55 µm.
Figure 7

Triple colocalization of ANXA2, HSP90AA1 and microglia in AD. Coronal sections of the human EC immunofluorescently stained for ANXA2 (A, E, I), HSP90AA1 (B, F, J) and IBA1 (C, G, K) for microglia. Orthogonal views of corresponding z-stacks (D, H, L). Each z-stack corresponds to a specific number of slices. Scale bar = 20 µm.
Figure 8

Role of HSP90AA1, PTK2B and ANXA2 in synaptic decline through microglial cells in AD. HSP90AA1 promotes Aβ clearance through the activation of microglial phagocytosis in AD. Its downregulation decreases microglial activation, phagocytosis and consequently Aβ clearance at synapses. PTK2B, expressed in neurons, promotes normal macrophage polarization and migration to inflamed areas in synaptic structures. However, PTK2B is downregulated, and consequently, microglial cell activation and migration to Aβ deposits are reduced and contribute to neurodegeneration and synapse disruption. ANXA2, present in neurons as well, could contain Aβ pathology through autophagosome-lysosome fusion and the activation of microglial cells. Therefore, its upregulation could increase autophagosome-lysosome fusion and Aβ-mediated microglial activation and subsequently promote Aβ degradation. On the other hand, active microglia secrete proinflammatory cytokines that can damage neurons either directly or indirectly by activating neurotoxic astrocytes. Additionally, ANXA2 is expressed in reactive astrocytes, which lose
many normal astrocytic functions, including neuron support, synapse formation and function. As a result, it leads to fewer and weaker synapse formation and a decline in synaptogenesis. Therefore, synaptic homeostasis is disrupted with a marked decrease in the growth and branching of dendrites and axons and neurodegeneration in the EC in AD.

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**Supplementary Files**

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- DatasetS1.xlsx
- Supplementaryfigure1600dpi.tif
- MovieS1.avi