Amyloid-beta peptides trigger premature functional and gene expression alterations in human-induced neurons

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Abstract

Background

Alzheimer's disease (AD) is the most prevalent cause of dementia in the elderly, characterized by the presence of amyloid beta (Aβ) plaques, neurofibrillary tangles, neuroinflammation, synapse loss and neurodegeneration in the brain. The amyloid cascade hypothesis postulates that deposition of Aβ peptides is the causative agent of AD pathology, but we still lack comprehensive understanding about the molecular mechanisms connecting Aβ peptides to neuronal dysfunctions in AD. In this work, we investigated the early effects of Aβ peptides accumulation on the functional properties and gene expression profiles of human-induced neurons (hiNs).

Methods

We exposed 6-weeks-old hiNs to low concentrations of cell-secreted Aβ oligomers or synthetic Aβ and performed time-lapse time microscopy to detect fast calcium transients as an indirect readout of neuronal electrical function. Next, we used single-nucleus RNA sequencing (snRNA-seq) to probe early Aβ-mediated gene expression alterations in hiNs and human-induced astrocytes (hiAs). Lastly, we leveraged snRNA-seq data to identify patterns of intercellular communication modulated by Aβ oligomers.

Results

We show that hiNs acutely exposed to low concentrations of both cell-secreted Aβ peptides or synthetic Aβ₁₋₄₂ exhibit alterations in the frequency of calcium transients suggestive of increased neuronal excitability. We also show that cell-secreted Aβ up-regulates the expression of several synaptic-related genes and down-regulates the expression of genes associated with metabolic stress mainly in glutamatergic neurons and to a lesser degree in GABAergic neurons and astrocytes. These neuronal alterations correlate with activation of SEMA5, EPHA and NECTIN signaling pathways, which are important regulators of synaptic plasticity.

Conclusions

Our findings indicate that slight elevations in Aβ concentrations are sufficient to elicit transcriptional changes in human neurons with long lasting consequences to neural network activity and suggest that at least part of the effects of Aβ on synapses might be mediated by semaphorin, ephrin and nectin signaling pathways.

Background
Alzheimer's disease (AD) is a neurodegenerative disorder associated with severe cognitive impairment in which memory loss is one of the most predominant features. Worldwide, AD accounts for 60 to 80 percent of dementia cases and represents an increasing burden for ageing populations [1]. Analysis of post-mortem human brains reveal two key features of AD, namely the presence of amyloid plaques - extracellular accumulations of amyloid-β (Aβ) peptides, that are derived from the proteolytic processing of the amyloid precursor protein (APP); and neurofibrillary tangles - intracellular accumulations of the microtubule-associated protein tau. The identification of familial, AD-linked mutations in the genes for amyloid-β precursor protein (APP) and presenilin (PS1 and PS2) associated with deregulation of Aβ peptide production suggests that APP metabolism is at the heart of the disease process through the statement of the amyloid cascade hypothesis [2]. Remarkably, recent treatments developed from this hypothesis and based on immunotherapies against Aβ peptides have shown significant but limited clinical effects in slowing down disease progression [3]. However, even if encouraging, we are still far from benefiting from an effective treatment and we greatly need a better understanding of the pathophysiological processes of AD potentially related to Aβ peptides to develop complementary and original therapeutics approaches.

Alterations in neuronal electrical activity and network oscillations are among the first signals in the brain of AD patients and are intimately associated with Aβ deposits even prior to the onset of clinical symptoms [4–8]. In rodents, treatment with soluble Aβ peptides can lead to neuronal hyperexcitation [9, 10] and modulation of excitatory synaptic activity, in turn, regulates the proteolysis of APP and release of Aβ peptides [11, 12], creating positive feedback between Aβ accumulation and neuronal electrical activity/synapse dysfunction. Early impairment of neuronal circuits is also observed in the brain of transgenic AD animal models, including the APP23xPS45 double-transgenic mice [13] and the 5XFAD mice that over-expresses human amyloid precursor protein (APP) and presenilin 1 (PS1) harboring five familial AD mutations [14, 15]. Moreover, human-induced pluripotent stem cells (hiPSC)-derived neurons carrying mutations in APP or PSEN1 show altered electrical properties [16], suggesting that Aβ peptides modulate the functional properties of human neurons. However, the precise molecular mechanisms associated with Aβ-mediated neuronal hyperexcitability and the exact contribution of Aβ1-42 peptides for this process remains elusive.

In this work, we investigated the early functional and gene expression alterations in human-induced neurons (hiNs) and astrocytes (hiAs) exposed to low concentrations of cell-secreted Aβ peptides. We show that a single exposure to low concentrations Aβ peptides is sufficient to promote an increase in the frequency of calcium transients in hiNs associated with specific synaptic-related gene expression alterations in glutamatergic neurons. These results suggest that slight (and repetitive) changes in Aβ concentrations in the brain may have long lasting consequences for neural networks by impacting gene expression in glutamatergic neurons.

**Methods**

1. **Maintenance of hiPSCs and neural induction**
For this project, we took advantage of a commercially available hiPSC cell line (ASE-9109, Applied StemCell Inc. CA, USA). The maintenance of the iPSC cultures was done in adherence with manufacturer's protocols which can be found on the webpage of Stemcell Technologies. Briefly, hiPSCs were maintained in mTeSR1 + 5X supplemented medium in non-treated cell culture dishes/plates pre-coated with 10 µg/ml of vitronectin fresh-diluted in Cell Adhere Dilution Buffer (StemCell Technologies) during 1 h at room temperature. Full medium change was performed daily until the confluence reached about 80%, when the cells were passed. Cell number and viability were recorded using a LUNA™ Automated Cell Counter. To induce a neural lineage from iPSCs, we used either a monolayer or an embryoid body methods. Both consisting of a serum-free system validated by StemCell Technologies and based on the dual SMAD-inhibition (SMADi) method [17]. Briefly, on the monolayer system, hiPSCs are cultured at high density and passaged for 3 times in STEMdiff™ Neural Induction Medium + SMADi (StemCell Technologies) for approximately 3 weeks. Alternativly, on the embryoid body procedure, the iPSCs are seeded on AggreWell plates (StemCell Technologies) to produce the embryoid bodies. Which are then replated in classic plates followed by a Neural Rosette selection to retain the neural cells. After this period, the hiNPCS generated are maintained for up to 9 passages in treated cell culture dishes pre-coated with poly-L-ornithine (PLO) and laminin (10 µg/mL), using supplemented STEMdiff™ Neural Progenitor Medium (NPM; Stemcell Technologies) fully changed daily. PLO solution was made in water (0.001%) while laminin was diluted in PBS 0.01M with Ca^{2+} and Mg^{2+}.

2. Differentiation of hiNPCs and mixed cultures of hiNs and hiAs

To obtain mixed 2D cultures comprising hiNs and hiAs from hiNPCs, we followed the spontaneous differentiation protocol detailed by StemCell Technologies. Following this spontaneous protocol, 100,000 hiNPCs/well were plated in 24-well cell imaging plates from Eppendorf (Cat # 0030741005) pre-coated with PLO (0.001%) and laminin (10µg/mL). Cells were kept in 0.5 mL of NPC medium per well for 24 hours. Following this, equal volume of complete BrainPhy medium (supplemented with BDNF, GDNF, laminin, dibutyryl-cAMP, ascorbic acid, N2, and SM1) was added to each well to begin the process of differentiation [18]. Subsequently, media was changed in the plates bi-weekly. The media change consisted of removing half of the existing medium in each well and replacing it with an equal volume of fresh complete BrainPhys medium. Mixed cultures of hiNs and hiAs were exposed to cell-secreted or synthetic Aβ peptides (see below) after 5–6 weeks of differentiation and then processed for calcium imaging, western blotting, immunocytochemistry or snRNA-sequencing.

3. Cell-secreted Aβ peptides treatment

Chinese hamster ovary (CHO) cell lines overexpressing the human APP695 (CHO-APP^{WT}) or the London mutated APP695 (CHO-APP^{V717L}) were previously described [19]. CHO cells were grown in DMEM/F-12 1:1 medium, supplemented with 10% heat-inactivated foetal bovine serum, 0.2% Pen/Strep, 2% HT supplement and 300 µM Proline (Sigma). One day before CHO supernatant media collection, a full-medium replacement was performed with Brainphys. CHO cell conditioned Brainphys medium was
collected 24h later and Aβ concentrations were measured using Alpha-LISA kits specific for human Aβ1–X (AL288C, PerkinElmer) and Aβ1–42 (AL276C, PerkinElmer). Briefly, the human Aβ analyte standard was diluted in the BrainPhys medium. For the assay, 2 µL of cell culture medium or standard solution was added to an Optiplate-384 microplate (PerkinElmer). 2 µL of 10X mixture including acceptor beads and biotinylated antibody was then added to the wells with culture media or standard solution. Following incubation at room temperature for an hour, 16 µL of 1.25X donor beads was added to respective wells and incubated at room temperature for 1 hour. Luminescence was measured using an EnVision-Alpha Reader (PerkinElmer) at 680 nm excitation and 615 nm emission wavelengths. Control treatment was performed using unconditioned/blank Brainphys medium.

4. Synthetic Aβ₁₋₄₂ treatment

Commercially available lyophilized Aβ₁₋₄₂ (Anaspec) was reconstituted in 1% DMSO as the solvent, followed by PBS 10mM at the concentration of 1 uM and re-diluted in 500 ul/well of supplemented BrainPhys medium to reach a final concentration of 100 pM. The inverted control peptide (Aβ₄₂₋₁, Anaspec) at the same final concentration and vehicle only (1% DMSO in PBS 10 mM) were used as controls. After 5 weeks of spontaneous differentiation, half of the medium was removed from each well and kept aside for later replacement. Then, either the Aβ₁₋₄₂, the inverted peptide or the vehicle were added at proper concentration and the cells were kept in the incubator for 24 hours. After that period, the medium in each well was replaced followed by the calcium imaging procedures and protein extraction.

5. Calcium Imaging Experiments

Spontaneously differentiated 5–6 weeks cultures containing hiNs and hiAs were treated with cell conditioned medium or synthetic Aβ₁₋₄₂ (and respective controls) for 48 hours and 24 hours, respectively, prior to real-time calcium imaging recordings. Cells were incubated with Oregon Green™ 488 BAPTA-1 (OGB-1) acetoxymethyl (AM) (ThermoFisher Scientific) for 1 h immediately before the recordings. A 2.5 mM stock solution of the calcium-indicator dye was prepared in Pluronic™ F-127 (20% solution in DMSO) (ThermoFisher Scientific). 1 µL of the dye solution was added to 500 µL of existing BrainPhys medium in each well of a 24-well cell imaging plate. The other half of existing BrainPhys media from the wells of the plate was removed and kept aside at 37°C while the calcium-indicator dye was incubated in fresh BrainPhys medium. After 1 h of incubation, the medium which was kept aside was replaced to each well. The 2D cultures were then ready to be imaged using a Spinning Disk Microscope (Nikon) housed at the Institut Pasteur de Lille, Lille, France using the MetaMorph imaging software. We took 1000 images using a 20x (synthetic peptide treatment) or 40x objective (conditioned medium treatment) with 10 ms exposure time and 200 ms intervals, totalizing up to 3 minutes of recording per field. For each well, up to 5 random fields were chosen, and at least two wells from 3 (for cell conditioned medium) or 4 (for synthetic Aβ) independent culture batches were imaged for each condition.

6. Analyses of Calcium Transients
Time-series data from calcium imaging recordings were at first converted to .avi format after background subtraction by use of ImageJ software. The videos were subsequently opened on CALciumImagingAnalyzer (CALIMA) software [20]. Each video recording of a field of cells was first downscaled to 2X in terms of size with a 10X zoom and was checked for the frame average mode. Moreover, in this first detection stage, pre-set filter parameters were adjusted and applied to enable the detection of the maximum number of fluorescent neurons (regions of interest - ROIs) in each field. During this step, the detected cells were checked several times to ensure that only neurons were selected, based both on cell morphologies and the presence of fast calcium transients. Fluorescence signal noise was filtered using the median of 3 each consecutive images. Fluorescence changes in all detected ROIs was then recorded. Fast (< 400 ms) increases of fluorescence signal above 2 or more standard deviations of the average background was considered a calcium transient (or spike). Detected spikes and cross-correlation matrices of all cells from each field of imaging were extracted and exported as .csv files.

7. Single-nucleus RNA-sequencing

6-week-old mixed cultures of hiNs and hiAs were washed in the 24-well plate wells with 1 mL of Deionized Phosphate Buffer Saline 1X (DPBS, GIBCO™, Fisher Scientific 11590476). Cells were lysed with wide bore tips in 1 mL Lysis Buffer (Tris-HCL 10mM, NaCl 10mM, MgCl2 3mM, Tween-20 0,1%, Nonidet P40 Substitute 0,1%, Digitonin 0,01%, Invitrogen™ RNAseout™ recombinant ribonuclease inhibitor 0,04 U/µL). Multiple mechanical resuspensions in this buffer were performed for a total lysis time of 15 min. 500 µL of washing buffer was added (Tris-HCL 10mM, NaCl 10 mM, MgCl2 3 mM, Tween-20 0.1%, BSA 1%, Invitrogen™ RNAseout™ recombinant ribonuclease inhibitor 0,04 U/µL) and the lysis suspension was centrifuged 8 mins at 500 g at 4°C (used for all following centrifugation steps) Nuclei pellets were washed 2 times using washing buffer with one filtration step by MACS pre-separation filter 20µm (Miltenyi Biotec). Nuclei pellets were resuspended in 100 µL of staining buffer (DPBS BSA 2%, Tween-20 0,01%, 0,04 U/µl Invitrogen™ RNAseout™ recombinant ribonuclease inhibitor), 10 µL of Fc blocking reagent HumanTruStainFc™ (422302, Biolegend) and incubated 5 min at 4°C. 1µl of anti-Vertebrate Nuclear Hashtag Antibody (Total-Seq™-A, Biolegend) was added and incubated 15 mins at 4°C. Nuclei pellets were washed three times in staining buffer with one filtration step by MACS pre-separation filter 20 µm (Miltenyi Biotec) to a final resuspension in 300 µL of staining buffer for Malassez cell counting with Trypan blue counterstaining (Trypan Blue solution, 11538886, Fisherscientific). Isolated nuclei were loaded on a Chromium 10X genomics controller following the manufacturer protocol using the chromium single-cell v3 chemistry and single indexing and the adapted protocol by Biolegend for the HTO library preparation. The resulting libraries were pooled at equimolar proportions with a 9 for 1 ratio for Gene expression library and HTO library respectively. Finally, the pool was sequenced using 100pb paired-end reads on NOVAseq 6000 system following the manufacturer recommendations (Illumina).

Unique Molecular Index (UMI) Count Matrices for gene expression and for Hash Tag Oligonucleotide (HTO) libraries were generated using the CellRanger count (Feature Barcode) pipeline. Reads were aligned on the GRCh38-3.0.0 transcriptome reference (10x Genomics). Filtering for low quality cells according to the number of RNA, genes detected, and percentage of mitochondrial RNA was performed. For HTO
sample, the HTO matrix was normalized using centered log-ratio (CLR) transformation and cells were assigned back to their sample of origin using HTODemux function of the Seurat R Package. Then, normalizations of the gene expression matrix for cellular sequencing depth, mitochondrial percentage and cell cycle phases using the variance stabilizing transformation (vst) based Seurat::SCTransform function were performed.

To integrate the experimental replicates of the single cell experiments, the harmony R package (https://github.com/immunogenomics/harmony) was used. In order to integrate the datasets, the SCTransform normalized matrices was merged and PCA was performed using Seurat::RunPCA default parameter. The 50 principal components (dimensions) of the PCA were corrected for batch effect using harmony::RunHarmony function. Then, the 30 first batch corrected dimensions were used as input for graph-based cell clustering and visualization tool. Seurat::FindNeighbors using default parameters and Seurat::FindClusters function using the Louvain algorithm were used to cluster cells according to their batch corrected transcriptomes similarities. To visualize the cells similarities in a 2-dimension space, the Seurat::RunUMAP function using default parameter was used. Cell clusters were then annotated based on cell type specific gene expression markers.

To study effect of conditioned media from CHO cells expressing APP WT or APP V717L on hiNPCs-derived cells, cell type specific differential expression analysis was performed on 7578 cells (2570 “APPV717L” cells, 2262 “APPWT” cells and 2746 control cells) using wilcoxon rank-sum test after normalization and regularized variance stabilization of the raw count using SCTransform (Hafemeister and Satija, 2019). We used adjusted p-value < 0.05 and |log2FC|>0.25 as cut-offs to define differently expressed genes. Functional enrichment analysis was performed using the R package FGSEA (Fast Pre-ranked Gene Set Enrichment Analysis, version 1.26.0). To perform fgsea, we utilized a gene set list at adjusted p-value < 0.05 sorted based on log2FC from three comparisons: APPV717L x Control, APPV717L x APPWT, and APPWT x Control. For the fgseaMultilevel function, the minimum gene set size was set to 15, while the maximum was limited to 400. The significance threshold for p-values was defined as p-value < 0.05. To determine the up- or down-regulated terms within the considered ontologies between conditions, we employed the normalized enrichment score (NES). Terms with NES values greater than 0 were classified as up-regulated, while those with NES values less than 0 were classified as down-regulated. The fgsea Multilevel analysis was performed across all cell types identified within the conditions.

8. Statistical Analysis

All statistical analyses and preparation of graphs were performed using Graph-Pad Prism 8 (San Diego, CA, USA) or RStudio (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/). For all statistical comparisons, we first tested for the normality of data distribution using the Kolmogorov-Smirnov test and then compared group variances using the appropriate parametric or non-parametric tests, as indicated in figure legends. Data in plots are represented as mean + standard deviation (SD). An α-error level of p < 0.05 was considered significant.
Results

Exposure to low concentrations of Aβ peptides increases electrical activity and reduces synchronicity of hiNs

Increased concentrations of Aβ can lead to neuronal hyperexcitation [9, 10, 13] and reduced synaptic connectivity in AD animal models. To evaluate whether increased concentrations of Aβ could also affect human neurons, we treated 6-weeks-old mixed hiNs/hiAs cultures with cell secreted Aβ peptides (Fig. 1). Firstly, we measured the concentrations of soluble Aβ peptides in the conditioned medium derived from CHO cells expressing the APP<sup>V717L</sup> (CHO-APP<sup>V717L</sup>) or APP<sup>WT</sup> (CHO-APP<sup>WT</sup>) using alphaLISA and observed averaged concentrations of 700 pM Aβ<sub>1-42</sub> and and 350pM Aβ<sub>1-42</sub> in CHO-APP<sup>WT</sup> conditioned medium (Fig. 1B). As previously described [19, 21], the concentrations of Aβ<sub>1-42</sub> was 8-folds higher, whereas that of Aβ<sub>1-42</sub> was 2.5-folds higher in CHO-APP<sup>V717L</sup> compared to CHO-APP<sup>WT</sup> conditioned medium (Fig. 1B).

Forty-eight hours after treatment with conditioned medium, we labeled cells with the calcium sensitive dye Oregon green BAPTA and performed time-lapse video microscopy (Fig. 2; Supplementary Movies 1–3). Time-series fluorescence images were then analyzed using CALIMA [20] and calcium spikes were detected when fluorescence change was higher than 2 standard deviations of the average baseline (Methods). We observed a significant higher frequency of calcium spikes in hiNs cultures treated with conditioned medium derived from CHO-APP<sup>V717L</sup> compared to that from CHO-APP<sup>WT</sup> (Fig. 2C). To evaluate whether this change in the frequency of calcium spikes could be explained in part by a higher concentration of Aβ<sub>1-42</sub> in the conditioned medium from CHO-APP<sup>V717L</sup> (Fig. 1B), we performed similar calcium imaging experiments in hiNs/HiAs cultures 24 hours after treatment with 100pM of synthetic Aβ<sub>1-42</sub> or its inverted control peptide (ICP). We observed a significantly higher frequency of calcium spikes in hiNs treated with Aβ<sub>1-42</sub> compared to both vehicle and ICP (Fig. 2D). These observations suggest that the changes in calcium spike frequency observed in hiNs exposed to cell-secreted Aβ peptides are at least partly due to the higher concentrations of Aβ<sub>1-42</sub> in the CHO-APP<sup>V717L</sup> conditioned medium.

Next, we measured the synchronicity of calcium transients among hiNs by calculating the eigenvalues of cross-correlation matrices generated by crossing the activity of each individual neuron with all other neurons in the same image field [22]. Despite the increase in the frequency of calcium spikes elicited by Aβ peptides, we observed a lower cross-correlation among inter-neuron calcium spikes in cultures exposed to cell-secreted Aβ or synthetic Aβ<sub>1-42</sub> (Fig. 2F-G), suggesting a reduction in neuronal synchronization. The proportion of active cells (defined as cells showing at least one calcium spike detected during the time of observation) was similar among conditions (Cell-secreted Aβ - Control: 44.36 ± 22.27%; APP WT: 39.68 ± 15.5%; APP V717L: 42.21 ± 20.71%; Synthetic Aβ – Control: 31.31 ± 9.59%; ICP: 39.61 ± 6.85%; Aβ<sub>1-42</sub>: 36.2 ± 11.78%). Altogether, these observations indicate that exposure to low
concentrations of cell-secreted Aβ or synthetic Aβ₁₋₄₂ peptides is sufficient to alter the electrical activity of hiNs circuits.

Transcriptional changes in hiNs exposed to elevated concentrations of Aβ peptides

We next sought to identify possible changes in gene expression in human-induced astrocytes and neurons elicited by treatment with Aβ. To that, we performed snRNA-seq of mixed hiNs/hiAs cultures treated with conditioned media from CHO-APP<sup>V717L</sup> or CHO-APP<sup>WT</sup> and blank Brainphys for 48h (Fig. 1). After quality control (Methods), we recovered 7578 nuclei and identified 17 different cell types/subtypes using unsupervised clustering based on gene expression (Fig. 3A). We observed that treatment with conditioned media significantly affected the proportion of cells in clusters 1 and 3, but there was no difference between CHO-APP<sup>V717L</sup> and CHO-APP<sup>WT</sup> (Fig. 3B). To annotate main cell types in our dataset, we identified the top markers of each cluster (Supplementary Table 1) and evaluated the expression of the astrocyte markers SLC1A3 (GLAST), GFAP and TNC; pan-neuronal markers SNAP25, DCX, MAPT; glutamatergic neurons marker SLC17A6 (VGLUT2); GABAergic neurons marker GAD1 and GAD2; and neural progenitor cells (NPCs) markers HES6, CCND2 and CDK6 in different clusters (Fig. 3C). We were able to identify 2 NPCs (14 and 17), 4 astrocytes (2, 3, 4 and 5), 7 glutamatergic neurons (12, 6, 7, 9, 10, 11 and 12) and 3 GABAergic neurons clusters (8, 12 and 15). We were unable to unambiguously assign cluster 16 to any specific cell type based on its gene expression profile and therefore annotated it as “unclassified” (Fig. 3D). Although we observed significant statistical differences in the proportions of cell types within a same condition, no differences were observed when we compared the proportion of an individual cell type among conditions (Fig. 3E). We also evaluated the expression of putative Aβ ligands [23] in our cultures and found that hiNs mainly expressed acetylcholine receptor subunit alpha-7 (CHRNA7), Glutamate Ionotropic Receptor NMDA Type Subunit 1 (GRIN1), Glutamate Metabotropic Receptor 5 (GRM5) and LDL Receptor Related Protein 1 (LRP1), whereas hiAs mainly expressed LRP1 and Prion Protein (PRNP) (Fig. 3F).

To characterize early molecular alterations elicited by conditioned media treatments, we used the Wilcoxon rank-sum test to identify differently expressed genes (DEGs) in NPCs, astrocytes, glutamatergic and GABAergic neurons. We found that treatment with conditioned medium from CHO-APP<sup>WT</sup> significantly (log₂FC > 0.25 and FDR < 0.05) affected the expression of 252 genes in astrocytes, 32 in GABAergic neurons and 89 in glutamatergic neurons (Fig. 4; Supplementary Table 2). Nevertheless, a more significant effect was observed in cultures treated with conditioned medium from CHO-APP<sup>V717L</sup>, where we identified 235 DEGs in astrocytes, but 228 in GABAergic neurons and 596 in glutamatergic neurons (Fig. 4; Supplementary Table 2). Interestingly, a direct comparison of the transcriptional profile of cultures treated with conditioned medium from CHO-APP<sup>V717L</sup> or CHO-APP<sup>WT</sup> revealed a selective alteration in gene expression of glutamatergic neurons (174 DEGs in APP V717L compared to APP WT), but only 2 DEGS in GABAergic neurons and 10 DEGs in astrocytes (Fig. 4; Supplementary Table 2). Together, these data indicate that elevated levels of APP and its metabolites modulate gene expression in
astrocyte and different neuronal subtypes, and that further increase in Aβ concentrations has a more prominent effect in gene expression of glutamatergic neurons.

**Increased concentrations of Aβ affect pathways associated with oxidative stress and synapse transmission**

The above analysis is based on fold-change thresholds and could, therefore, mask small variations in gene expression between CHO-APP<sup>V717L</sup> and CHO-APP<sup>WT</sup> treated GABAergic neurons and astrocytes. To overcome this limitation, we performed a gene set enrichment analysis (GSEA), which allowed us to identify functional and pathway enrichments modulated by conditioned media treatments in each cell type based on the ranked gene expression values (Subramanian 2005). We observed significant functional enrichments for the comparisons between CHO-APP<sup>V717L</sup> vs Control but not CHO-APP<sup>WT</sup> vs Control conditions (Fig. 5). In astrocytes, treatment with conditioned medium from CHO-APP<sup>V717L</sup> up-regulated genes associated with regulation of cell-differentiation, and down-regulated genes associated with catabolic process and endoplasmic reticulum lumen (Supplementary Table 3). In neurons, up regulated genes were mainly associated with synapse-related terms, whereas down-regulated genes were associated with metabolic processes (Figure A-B; Supplementary Table 3). We also found functional enrichment for gene sets associated with neuronal intrinsic electrical properties, such as ion channels and calcium ion binding, in glutamatergic but not GABAergic neurons treated with CHO-APP<sup>V717L</sup> conditioned medium compared to controls (Fig. 5A; Supplementary Table 3). Furthermore, only glutamatergic neurons displayed functional enrichments for gene sets identified in cultures treated with conditioned medium from CHO-APP<sup>V717L</sup> compared to CHO-APP<sup>WT</sup> (Fig. 5C; Supplementary Table 3), further suggesting a higher sensibility of this neuronal subtype to Aβ. Up-regulated genes were enriched for cell adhesion, regulation of cellular component biogenesis and post-synapse membrane, whereas down-regulated genes were enriched for transported activity, mitochondrion and catalytic complex (Fig. 5C; Supplementary Table 3). Altogether, these findings indicate that at least part of the molecular alterations observed in hiNs are related to the increased Aβ concentration in CHO-APP<sup>V717L</sup> compared to CHO-APP<sup>WT</sup> conditioned medium.

**Selective effect of Aβ on synaptic processes of glutamatergic neurons**

Synapses are fundamental information processing units of the brain and synaptic dysregulation is central to AD pathology. To further characterize our transcriptomic data in a synapse context, we used SynGo, a systematic annotation of synaptic genes and ontology of synaptic processes [24]. Confirming our previous observations using GSEA, we identified the enrichment of several gene ontologies (GOs) related to synaptic biological processes in both glutamatergic and GABAergic neurons (Fig. 6; Supplementary Table 4). This enrichment was mainly evident for DEGs identified in neurons exposed to conditioned medium from CHO-APP<sup>V717L</sup> compared to controls (Fig. 6A-B), affecting both the pre- and
post-synaptic compartments, and associated with the regulation of presynaptic cytosolic calcium levels, synaptic vesicle cycle, regulation of postsynaptic membrane neurotransmitter receptor levels and synapse assembly, among other synaptic processes (Supplementary Table 4). Also, like our GSEA analysis, we only observed significant enrichments for DEGs identified in glutamatergic neurons when comparing exposed to conditioned medium from CHO-APPV717L compared to CHO-APPWT (Fig. 6D; Supplementary Table 4).

**Exposure to Aβ peptides alters neuroglial communication**

Lastly, we leveraged our snRNAseq data to infer the intercellular communication patterns in our hiNs/hiAs cultures and to evaluate how these patterns could be impacted by the treatment with CHO-APPV717L or CHO-APPWT conditioned medium. To that end, we employed CellChat to predict major signaling inputs and outputs for cells and delineates conserved and context-specific pathways across different conditions [25]. We observed consistent signaling patterns in astrocytes, glutamatergic neurons and GABAergic neurons in all 3 experimental conditions, but some conspicuous differences could also be observed (Fig. 7; Supplementary Table 5). Compared to controls, both CHO-APPV717L and CHO-APPWT conditioned media inhibited the neuregulin (NRG), epidermal growth factor (EGF) and myelin protein zero (MPZ) signaling in astrocytes, stimulated cadmium (CADM) signaling in glutamatergic neurons and CD46 signaling in astrocytes, glutamatergic and GABAergic neurons (Fig. 7A-B). We also observed specific changes in signaling patterns of cells treated with CHO-APPV717L compared to CHO-APPWT conditioned media, including a higher the relative strength of ephrin-A (EPHA), semaphorin (SEMA5) and NECTIN signaling, and a lower relative strength in midkine (MK; also known as neurite growth-promoting factor 2 - NEGF2) and fibronectin (FN1) signaling (Fig. 7C). In accordance with our previous observations in gene expression, we found that treatment with CHO-APPV717L or CHO-APPWT conditioned media increased both the outgoing and incoming interaction strength on the aggregated cell-cell communication network from all signaling pathways in glutamatergic neurons and to a lesser extent in GABAergic neurons (Fig. 7D). In contrast, the conditioned media treatment reduced the outgoing and incoming interaction strength in astrocytes (Fig. 7D). Collectively, these analyses indicate that small increases in Aβ concentrations may have important consequences for neuroglia communication.

**Discussion**

AD patients show aberrant neural network activity in the form of both hyper- and hypo-excitability, and several lines of evidence indicate that these network alterations result from changes in neuronal excitability and synaptic transmission tied to elevated Aβ levels [13, 26–28]. Yet, the molecular mechanisms underlying Aβ-induced hyper-excitability remain unclear. In this work, we show that human-induced neurons acutely exposed to low concentrations of both cell-secreted Aβ peptides (including Aβ1-42) or synthetic Aβ1-42 present alterations in the frequency of calcium transients suggestive of increased neuronal excitability. We also show that cell-secreted Aβ up-regulates the expression of several synaptic-related genes and down-regulates the expression of genes associated with metabolic processes
in mitochondria mainly in glutamatergic neurons and to a lesser degree in GABAergic neurons and astrocytes. Collectively, these findings suggest that slight elevations in Aβ concentrations are sufficient to elicit transcriptional changes in human neurons that can contribute to early alterations on neural network activity.

Aβ peptides play a central role in the pathogenesis of AD. When they accumulate abnormally in the brain, they can have detrimental effects on neurons, such as the induction of neuritic plaques, disruption of calcium homeostasis, synaptic loss and oxidative stress [29, 30], thus directly contributing to neuronal dysfunction and death. Aβ peptides also affect astrocytes [14, 31–33], which can indirectly contribute to neuronal dysfunctions in AD. Moreover, the presence of Aβ in the brain can activate microglia, the immune cells of the central nervous system, leading to neuroinflammation [34, 35]. Chronic neuroinflammation can further exacerbate neuronal damage and contribute to the progression of AD. Therefore, Aβ-mediated signaling mediates both cell-autonomous and non-cell-autonomous processes impacting neuronal function and neurodegeneration. In our work, we only model the possible effects of Aβ peptides in neurons and astrocytes. Future work should evaluate the effects of those peptides in more complex hiPSC-derived cellular system harboring neurons, astrocytes, oligodendrocytes, microglia and endothelial cells, thus better representing the complex cellular landscape of the brain.

Despite this limitation, our results reveal some important features of the acute neuronal response to low concentrations of APP and Aβ peptides, which can partly mirror early stages of AD pathology. Here, we show that exposure to CHO-APP\textsuperscript{V717L} conditioned medium, which mainly differ from CHO-APP\textsuperscript{WT} conditioned medium by a higher concentration of Aβ oligomers enriched for Aβ\textsubscript{1–42} [19, 21], leads to a higher frequency and lower synchronicity of calcium spikes in hiNs. We also describe similar alterations in cultures treated with purified Aβ\textsubscript{1–42} peptide, suggesting that acute exposition to a low concentration of Aβ\textsubscript{1–42} is sufficient and necessary to induce human neuron hyperexcitability and reduced coordination of network activity. These finding agree with previous work showing that hiPSC-derived neurons bearing AD-related mutations in presenilin-1 or amyloid precursor protein show a higher frequency of spontaneous action potential compared to isogenic gene corrected controls that is reversed via either γ-Secretase or BACE1 inhibition [16]. It also coincides with work in mice showing that low concentrations (200pM) of Aβ\textsubscript{1–42} induces an increase of frequency of miniature EPSCs, a decrease of paired pulse facilitation, an increased length of postsynaptic density and an increased expression of plasticity-related proteins [9, 26]. These effects were present upon extracellular but not intracellular application of the peptide and involved the activation of α7 nicotinic acetylcholine receptors [26], which are also expressed in a fraction of hiNs (mainly glutamatergic neurons) in our cultures.

The alterations in calcium dynamics together with the gene expression pattern of known Aβ\textsubscript{1–42} ligands in our cultures may suggest a direct effect of this peptide in the regulation of neuronal functional properties. However, our experimental design does not allow us to unambiguously disentangle the direct effects of Aβ peptides in neurons from potential indirect effects mediated by astrocytes. In fact, inclusions of aggregated Aβ in astrocytes critically affect their ability to support neuronal function [33]
and recovery of astrocytic calcium activity normalizes neuronal hyperactivity in a mouse model of amloidopathy [32], suggesting that at least part of the neuronal alterations observed in response to Aβ peptides are mediated by astrocytes. Yet, our transcriptomic data indicate that exposure to Aβ peptides directly modulate gene expression in glutamergic neurons, since only this cell type show significant alterations in gene expression when exposed to CHO-APPV717L compared to CHO-APPWT conditioned medium. Moreover, the higher expression of putative Aβ receptors in hiNs compared to hiAs, together with the predominant number of genes differentially expressed in glutamergic neurons exposed to CHO-APPV717L conditioned medium compared to GABAergic neurons or astrocytes, suggest a primarily cell-autonomous effect of Aβ-mediated signaling in glutamergic neurons.

Our transcriptional data also reveal critical effects of Aβ-mediated signaling on the regulation of metabolism, electrical activity and synaptic plasticity in hiNs, especially glutamergic ones. These finding are in accordance with previous studies in AD animal models at early stages of amloidopathy [36–38] and in the brains of patients with AD at early Braak stages [36, 39–42]. Thus, we believe that our results in hiNs may provide insightful information on the molecular mechanisms mediating Aβ-mediated neuronal hyperexcitability in AD. Moreover, our innovative approach to identify intercellular signaling pathways impacted by Aβ peptides via the identification of interactions among ligands, receptors and their cofactors expressed by hiNs and hiAs may contribute to unravel new targetable molecules to prevent Aβ-mediated toxicity.

We show that treatment with CHO-APPV717L conditioned medium activates EPHA, SEMA5 and NECTIN signaling mainly in glutamatergic neurons and, to a lesser extent, in GABAergic neurons and astrocytes. Ephrin ligands and their associated Eph receptors guide axons during neural development and regulate synapse formation and neuronal plasticity in the adult [43]. Similarly, members of the nectin and semaphorin signaling pathways regulate several aspects of synapse formation and maintenance during development and adulthood [44, 45]. Among the pathways activated in hiNs treated CHO-APPV717L compared to CHO-APPWT conditioned medium, SEMA5B regulates the elimination of synaptic connections in cultured hippocampal neurons [46], EPHA1 is a risk-modifying locus for AD [47, 48]. Therefore, it is plausible to speculate that at least part of the functional and gene expression alterations observed in hiNs exposed to Aβ peptides are mediated by those signaling pathways.

Although we cannot formerly rule out the contribution of soluble APP products rather than Aβ peptides to the alterations observed on the activity and gene expression of hiNs, the fact that they are mainly observed in cells treated with CHO-APPV717L conditioned medium strongly suggest that soluble Aβ peptides are key regulators of neuronal plasticity. On the other hand, the milder alterations observed in the expression of synaptic-related genes of hiNs exposed to the CHO-APPWT conditioned medium could suggest that even lower amounts of Aβ peptides, APP itself or other APP metabolites also affect these cells, as previously suggested in AD mouse models [9, 49]. Future studies could employ our system to evaluate the impact of Aβ and other APP metabolites at different concentrations and aggregation states to the regulation of the functional properties and gene expression in human neurons, thus further
advancing our understanding about the physiological and pathological roles of these complex signaling molecules.

**Conclusions**

Our results indicate that slight elevations in Aβ concentrations are sufficient to elicit transcriptional changes in human neurons, contributing to early alterations on neural network activity, and suggest targetable candidate intercellular signaling pathways mediating Aβ-induced neuronal hyperexcitability.

**Abbreviations**

Aβ - amyloid beta

hiNs – human-induced neurons

hiAs - human-induced astrocytes

snRNA-seq – single nucleus RNA-sequencing

APP – amyloid precursor protein

CHO - Chinese hamster ovary

CHO-APP$^{WT}$ – CHO cell line overexpressing the human APP695

CHO-APP$^{V717L}$ - CHO cell line overexpressing the human London mutated APP695

DEG – differentially expressed gene

GSEA – gene-set enrichment analysis

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**

Count table and metadata from snRNA-seq experiments are available at Mendeley Data, V1, doi: 10.17632/mjcsph7mbs.1
Time-lapse video-microscopy images are available upon reasonable request.

**Competing interests**

The authors declare no competing interests.

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**Authors' contributions**


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**References**


Figures
Figure 1

Different concentrations of $A\beta$ peptides in $\text{CHO-APP}^{\text{WT}}$ and $\text{CHO-APP}^{\text{V717L}}$ conditioned media. (A) Experimental design showing the generation of hiNs/hiAs cultures, medium conditioning, and treatment. (B) Quantification of $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides in the conditioned medium from $\text{CHO-APP}^{\text{WT}}$ or $\text{CHO-}$
APPV717L (*p<0.05; ** p< 0.01; Unpaired t-test). (C) Immunocytochemistry in 6-week-old hiNs/hiAs culture using antibodies against MAP2 (red), GFAP (green) and NESTIN (blue). Calibration bar: 20mm.

**Figure 2**

**Altered frequency of calcium transients in hiNs exposed to Aβ peptides.** (A) Snapshot of 6-week-old hiNs/hiAs culture labeled with Oregon green BAPTA (green) and treated 24h before with 100nM of HiLyte™ Fluor 555-labeled Aβ1-42 (red). (B-B’) Representative plot of fluorescence change over time in 1000 frames. Green vertical lines indicate detected calcium spikes (ΔF > 2 SDs). Dashed box is magnified in B’. (C) Quantification of calcium spikes in cultures treated with conditioned medium 48h before imaging. (D) Quantification of calcium spikes in cultures treated with 100pM synthetic Aβ1-42 24h before imaging. (E) Representative cross-correlation matrices showing the synchronicity rate of hiNs calcium transients in different conditions. (F) Graphic showing the eigenvalues of cross-correlation matrices in control and cell-secreted treated hiNs/hiAs cultures. (G) Same for cultures treated with synthetic Aβ1-42.

Statistics in C-D, F-G: *Padj< 0.05; ****Padj<0.0001; Kruskal-Wallis followed by Dunn's multiple comparison test; n= 3 independent cultures for each condition. Number of analyzed cells per condition: Blank Brainphys= 1098; APPWT= 580; APPV717L= 670; Vehicle= 2543; ICP= 3459; Aβ1-42= 2472.
Figure 3

Similar cellular composition of hiNs/hiAs cultures treated with control or CHO-cells conditioned media. 
(A) UMAP representation of the different cell clusters in hiNs/hiAs cultures. (B) Relative proportion of cell clusters. (C) Dot plot showing the expression of key cell markers used to annotate cell populations. (D) UMAP representation of the annotated cell types/subtypes in hiNs/hiAs cultures in different treatment conditions. (E) Proportion of cell types/subtypes in different conditions (statistics). (F) Dot Plot showing the expression of putative Aβ ligands in different cell types.
Figure 4

Exposure to Aβ peptides modify gene expression in hiNs and hiAs. (A) Volcano plots representing DEGs (red dots, adjusted p-value <0.05 and |log2FC| >0.25) identified in astrocytes, glutamatergic and GABAergic neurons when comparing CHO-APP<sup>WT</sup> vs control, or CHO-APP<sup>V717L</sup> vs control, and CHO-APP<sup>V717L</sup> vs CHO-APP<sup>WT</sup>.
Figure 5

**Enrichment for synaptic and metabolic pathways in hiNs treated with Aβ peptides.** (A-C) Dot Plots showing the normalized enrichment scores (NES) for pathways enriched for genes differentially expressed in glutamatergic neurons (A, C) and GABAergic neurons (B) when comparing CHO-APP^{V717L} vs control (A-B) or CHO-APP^{V717L} vs CHO-APP^{WT} (C).
Figure 6

Genes modulated by CHO-APP\textsuperscript{V717L} conditioned medium are involved in synapse organization and signaling. (A-C) Synapse-related gene ontologies for biological processes enriched for DEGs identified in glutamatergic od GABAergic neurons when comparing CHO-APP\textsuperscript{WT} vs control (A), or CHO-APP\textsuperscript{V717L} vs control (B), and CHO-APP\textsuperscript{V717L} vs CHO-APP\textsuperscript{WT}(C).
Figure 7

**Aβ peptides modulate specific signaling pathways in hiNs/hiAs.** (A-C) Heatmaps showing the relative strength of signaling pathways in astrocytes, glutamatergic and GABAergic neurons exposed to conditioned media or control treatments. Signaling pathways specific to one condition are highlighted in gray and red. (D) Dot plots showing the averaged interaction strength for incoming and outgoing interactions.
signaling in astrocytes, glutamatergic and GABAergic neurons exposed to conditioned media or control treatments. Dot sizes indicate the number of ligand-receptor pairs (“Counts”) identified in each cell type.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupTable1clusterMarkers.csv
- SupTable2DEGs.xlsx
- SupTable3GSEA.xlsx
- SupTable4syngoontologies.xlsx
- SupTable5CellChatInteractions.xlsx
- SupplementaryMovie1Control.avi
- SupplementaryMovie2CHOAPPWT.avi
- SupplementaryMovie3CHOAPPV717L.avi