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Research Article

Propranolol Reduces Phosphorylated Tau Accumulation in Patient Stem-Cell Derived Brain Organoid Models of Alzheimer's Disease

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Abstract

Previous studies using a transgenic mouse model of Alzheimer's disease demonstrated that propranolol reduces the increase in hippocampal levels of amyloid-beta-42, tau protein, and hyperphosphorylated tau protein independent of blood pressure lowering effects. However, transgenic mouse models do not adequately reflect Alzheimer's disease pathophysiology. Testing candidate molecules in models that more closely approximate human brain tissue represents an important step in validation of potential treatment approaches. We established patients brain organoids from induced pluripotent stem-cell (iPSC) of sporadic and familial (Presenilin-1 mutation) Alzheimer's disease and tested if propranolol could decrease TAU and phosphorylated TAU. At three months of differentiation, organoids were evaluated for expression of Alzheimer's disease markers, for global protein dysregulation using mass spectrometry, and treated with propranolol. Here we showed that Sporadic Alzheimer's organoids had an increased level of phosphorylated tau compared to control organoids. Global proteomic analysis showed that several TAU phosphorylating kinases were upregulated, supporting the increased level of TAU phosphorylation. Alpha-synuclein, often preceding amyloid beta accumulation in Alzheimer's disease and associated with amyloid-beta plaques, was increased. Interestingly, protein homeostasis was dysregulated: the proteasome and lysosomal degradation systems were downregulated, along with new protein syntheses such as the spliceosome and mRNA formation. Treatment of Sporadic Alzheimer's organoids with propranolol resulted in a decrease of Tau phosphorylation recapitulating the in vivo findings. The results suggest further development of propranolol and novel structural analogs may be an effective method of developing potentially disease modifying therapies for sporadic Alzheimer's disease.

Keywords: *Alzheimer's disease, brain organoid, propranolol, tau protein*

Introduction

Alzheimer's disease (AD) is one of the most significant health problems affecting older individuals, causing cognitive decline and dementia, as well as being the sixth leading cause of death in the United States. The pathophysiology underlying development of AD is complex and not entirely understood, with oxidative stress, synaptic integrity, synaptic density, neuronal death, and brain inflammation all contributing to different and varying degrees [1,2]. Alzheimer's disease is also characterized by extracellular accumulation of amyloid-beta-42 protein aggregates in the form of plaques, and intracellular accumulation of hyperphosphorylated tau as neurofibrillary tangles [1,2]. While there has been considerable progress in understanding and identifying contributing pathophysiologic mechanisms and potential etiologies, there is not yet an effective treatment or disease modifying therapy available [2]. For many years, the only two classes of medications approved for treatment of AD i.e., cholinesterase inhibitors and an N- methyl-D-aspartate receptor antagonist were purely symptomatic [2]. Recently a new medication, aducanumab, was approved by the U.S. Food and Drug Administration (FDA), but the approval was controversial, based on a retrospective analysis of data [3]. Consequently, new therapeutics with potentially disease modifying effects are still desperately needed.

Propranolol has previously been suggested to produce potentially disease modifying effects independent of its blood pressure lowering properties. In this study, they compared female Tg2576 mice to non-transgenic littermates, both groups treated with propranolol. Upon propranolol treatment Tg2576 mice showed improvement on memory behavioural tests. Additionally, Propranolol treated Tg2576 mice showed significantly reduced hippocampal levels of amyloid-beta 40 and 42 protein levels compared to saline treated Tg2576 controls [4]. Tg2576 mice do accumulate increased levels of hyperphosphorylated tau in neurons and treatment with propranolol caused the levels of hyperphosporylated tau in the hippocampus of Tg2576 mice to revert back to normal levels [4]. However, while these results were encouraging, the Tg2576 mouse model does not exactly replicate the disease state in humans in significant ways, such as not forming neurofibrillary tangles, and further study in a model more closely resembling human Alzheimer's disease pathology is needed to confirm the ability of propranolol to reduce accumulation of amyloid-beta-42 and phosphorylated tau proteins.

Human brain organoid models are three-dimensional structures that highly resemble human brain regions such as the cortex. One benefit of these models is they can be derived from stem-cells obtained from disease specific populations, such as individuals suffering from specific genetic mutations or sporadic forms of diseases of interest, as well as from normal controls [5]. In this manner it is possible to develop brain organoid models that are highly specific to different genetic forms of common diseases, as well as more common sporadic forms of the disorder. Brain organoid models of Alzheimer's disease generated using patient derived stem cells have been shown to accurately recapitulate some of the abnormalities observed to naturally occur in the brains of individuals suffering from Alzheimer's disease [5]. One drawback to brain organoids is they do not contain the same proportions of cell types found in normal brain tissue, they have an embryonic phenotype and lack of vascularization, which can limit the size and survival of the organoids and interfere with modeling disease aspects related to vasculature such as the blood-brain barrier [5].

We examined the effects of propranolol treatment on patient stem-cell derived brain organoid models of Alzheimer's disease to determine if we could reproduce the published effects of propranolol in reducing accumulation of amyloid-beta-42 and phosphorylated tau as reported in the Tg2576 transgenic mouse model. We decided to focus primarily on accumulation of phosphorylated tau for this reason since it represented the most likely measurement to significantly differ in responsiveness to propranolol between the two models. Additionally, because the brain organoid models do not have vasculature [6], this reduces the potential for any confounding issues related to the blood pressure lowering effects of propranolol.

We included brain organoid models using patient derived stem-cells from both sporadic Alzheimer's disease and familial with a specific genetic form of the disorder, the presenilin-1 mutation. This allowed us to determine if propranolol would demonstrate differential effectiveness between sporadic and genetic forms of the disease which may have implications for testing in clinical trials and eventually treatment as well.

Methods and Methods

Cerebral organoids differentiation

Cerebral organoids were generated from iPSC obtained from the Coriell Institute. iPSC was culture in mTeSR™1 until they reached about 80% confluency and were passaged at least one time before proceeding to differentiation. Organoids were generated using Stem Cells Technologies media and protocol (STEMdiff™ Cerebral). Organoids were cultured for 150 and 210 days of age.

Immunohistochemistry

Organoids were harvested and washed with PBS 3 times. Organoids were then fixed overnight with 4% PFA at 4°C. Organoids were then dehydrated with 30% sucrose overnight at 4°C and embedded in OCT embedding media. Organoids were cryosectioned to 12µm and mounted onto gelatin chromium alum double coated slides. For immune staining, antigen retrieval was performed by heating the slides in sodium citrate buffer with Tween-20 at 90°C for 10 minutes. Slides were subsequently permeabilized with 10% FBS in PBS with 0.3%triton 100x. Slides were then blocked with 10%FBS in PBS for 1hr. Primary antibodies were left overnight at 4°C and washed off the next day. Antibody used: S10013 Beta (Invitrogen/Thermo Fisher Cat# MA5-12969), GFAP (Invitrogen/Thermo Fisher Cat # 13-0300), MAP2 (Invitrogen/Thermo Fisher Cat# Pa1-10005), Ctip2 (abcam Cat # ab18465), SATB2 (abcam Cat# ab34735), FOXG1 (abcam Cat # ab18259), SOX2 (R&D Systems Cat # AF2018), Phospho-Tau (Ser296) (Invitrogen/Thermo Fisher Cat#44-752G), beta-amyloid (Cat #d54d2). Secondary antibody was added and left on for 45 minutes at room temperature. Slides were coversliped with vector vibrance mounting media.

Propranolol treatments

At 3- and 5-months post-differentiation, organoids were treated with propranolol using the following concentrations: 0.03µM, 0.1µM, 0.3µM, 1µM and 10µM. Treatments were performed for 10 days. After treatment, organoids were harvested, lysed using MSD protocol and kit (K15121-D) to quantify pTau and beta amyloid (K15199E) presence. Percentage of Tau phosphorylation as calculated using the ratio over the total tau as reported by the kit.

Mass spectrometry

Six organoids for each group were lysed by using urea lysis buffer (40 mM HEPES pH 7.5, 200 mM NaCl), containing Protease & Phosphatase Inhibitor Cocktail (cat# 78440), 10 mM MG132 and 10 M of urea. Proteins were digested in MS buffer (0.1 M Tris-HCl, cat# P-920, pH 8.5) containing 0.5 M TCEP (cat# 20491, prepared in MS buffer), 0.5 M 2-chloroacetamide (cat# 154955), 0.25 µg/µL Lys-C (cat# 125-05061, prepared in MS grade water), incubated at 37°C for 4 hours at 750 rpm; 100 mM $CaCl_2$, and 0.5 µg/µL Trypsin (cat# 90058, prepared in MS grade water) were added to the samples and incubated at 37°C for 20 hours with shaking at 750 rpm. The digested samples were then desalted using C18 columns (cat# 89870) and dried using a vacuum centrifuge. Before running mass spectrometry samples, the samples were dissolved in 0.2% FA solution, and peptide concentration was tested through Pierce Quantitative Fluorometric Peptide Assay (cat# 23290).

The LC-MS/MS experiments were performed using an EASY-nLC 1000 (Thermo Fisher Scientific) connected to a Q Exactive Orbitrap Mass Spectrometers (Thermo Fisher Scientific).

Sample (0.25 ug) was loaded onto an Easy Spray Column (25 cm × 75 µm, 2 µm C18, ES802, Thermo) and separated over 195 min at a flow rate of 0.5 µL/min with the following gradient: 2- 35% B (180 min), 35-85% B (5 min), and 85% B (10 min). Solvent A consisted of 99.9% H2O and 0.1% formic acid, and solvent B consisted of 19.9% H_2O, 80% ACN, and 0.1% formic acid.

Full MS scan was acquired at 70,000 resolutions with a scan range of 350-2000 m/z, the AGC target was 1×10^6, and the maximum injection time was 100 ms. MS2 scan was acquired at 17,500 resolutions with a scan range of 200-2000 m/z, the AGC target was 5×10^4, the maximum injection time was 64 ms, and the isolation window is 2.0 m/z. System control and data collection were performed by Xcalibur software.

The proteomic data processing was performed through Proteome Discoverer 1.4 (Thermo Scientific) using Uniprot human database and the Sequest HT Search Engine. The search allowed for a precursor mass tolerance of 10 ppm, a minimum peptide length of 6, and a minimum peptide sequences number of 1. Upon identification of dysregulated protein from control sample and correction for false discovery rate (*t*-test <0.05), we analyzed interaction protein using STRING program.

Results

Characterization of AD patient derived organoids

Human brain organoids are 3D structures useful in understanding human brain development and human brain-related disorders [6,7]. Cerebral brain organoids model human cortical development and can be useful in drug discovery research [6]. Previous studies have shown that brain organoids originated from patient-derived induced pluripotent stem cells obtained from AD patients recapitulate some features of AD, including amyloid beta accumulation and increase in tau phosphorylation. We, therefore, differentiated iPSC lines derived from a patient with sporadic (sAD), familiar AD (fAD) and a control patient (CON) into cerebral brain organoids.

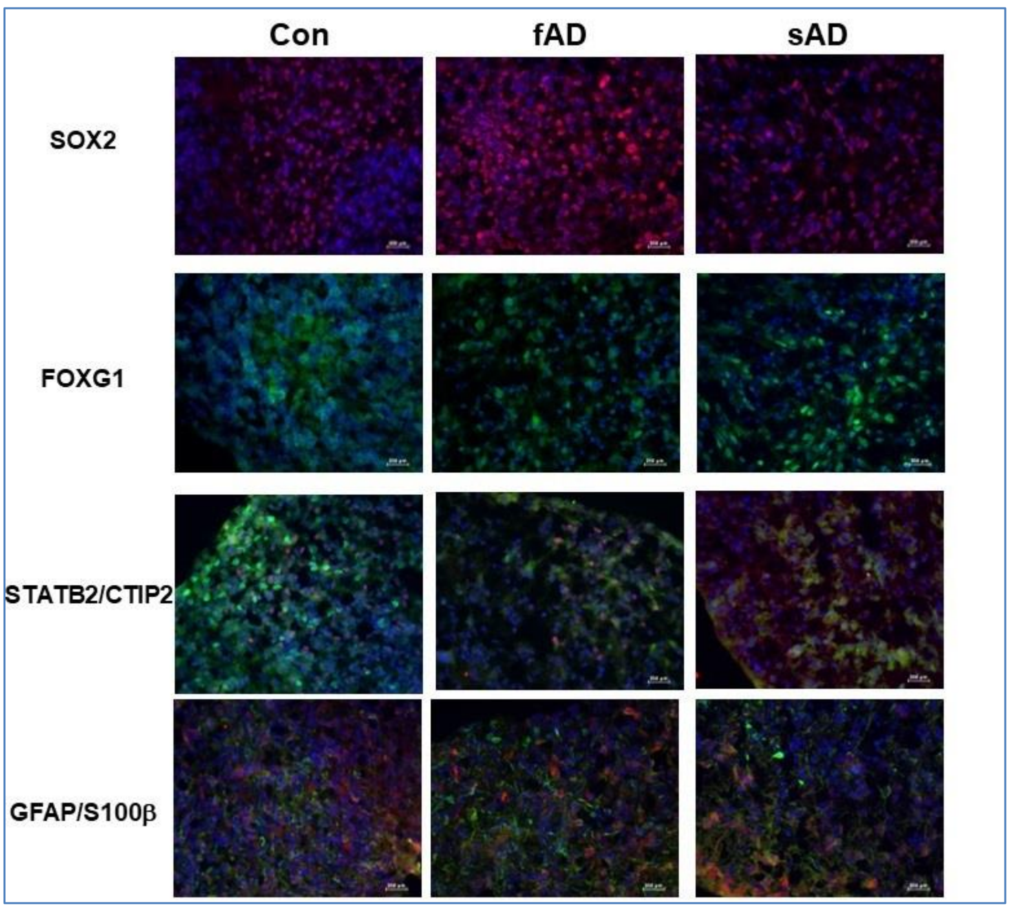

Figure 1. Developmental characterization of AD cerebral organoids at day 120. 120-day old organoids were stained for **A)** neuronal progenitor (SOX2) **B)** Fore brain (FOXG1), C) cortical layer STATB2 and (CTIP2) and D) astrocytes (GFAP, s100β) at day 120 of age.

In Figure 1 organoids showed expression of markers of proper cortical differentiation. All organoids expressed neural progenitor marker SOX2 (Figure 1A) and forebrain progenitor FOXG1 (Figure 1B). Cortical neuronal differentiation and patterning was further evaluated by the expression of CTIP2 (Figure 1C). At 3 months, we detected expression of astrocytes markers GFAP and to a certain extent S100β.

Next, we evaluated the expression of AD markers in brain organoids, including phosphorylated TAU and amyloids beta. Figure 2A shows expression of phosphorylated TAU in MAP2 expressing neurons in all brain organoids including the control both at 2 and 3 months (Figure 2A and B, respectively). We detected a small amount of amyloid-beta expression in sAD sample, but only at 2 months of differentiation (Figure 2B) and this was not detected at 3 months of differentiation (not shown). Since all organoids expressed large amounts of phosphorylated TAU, we decided to measure pTAU expression using a more quantitative and sensitive approach, using MSD pTAU quantification kit. As shown on Figure 2C sAD organoids expressed double the amount of pTAU compared to controls and fAD at pTAU upregulation was consistent also at 5 months of organoids differentiation (not shown). Next, we performed the same quantitative approach for Amyloid beta, using MSD quantification Kit, but we could not detect intracellular amyloid-beta. Overall, the data suggest that AD patient-derived brain organoids were properly differentiated and disease organoids, sAD expressed higher levels of pTAU.

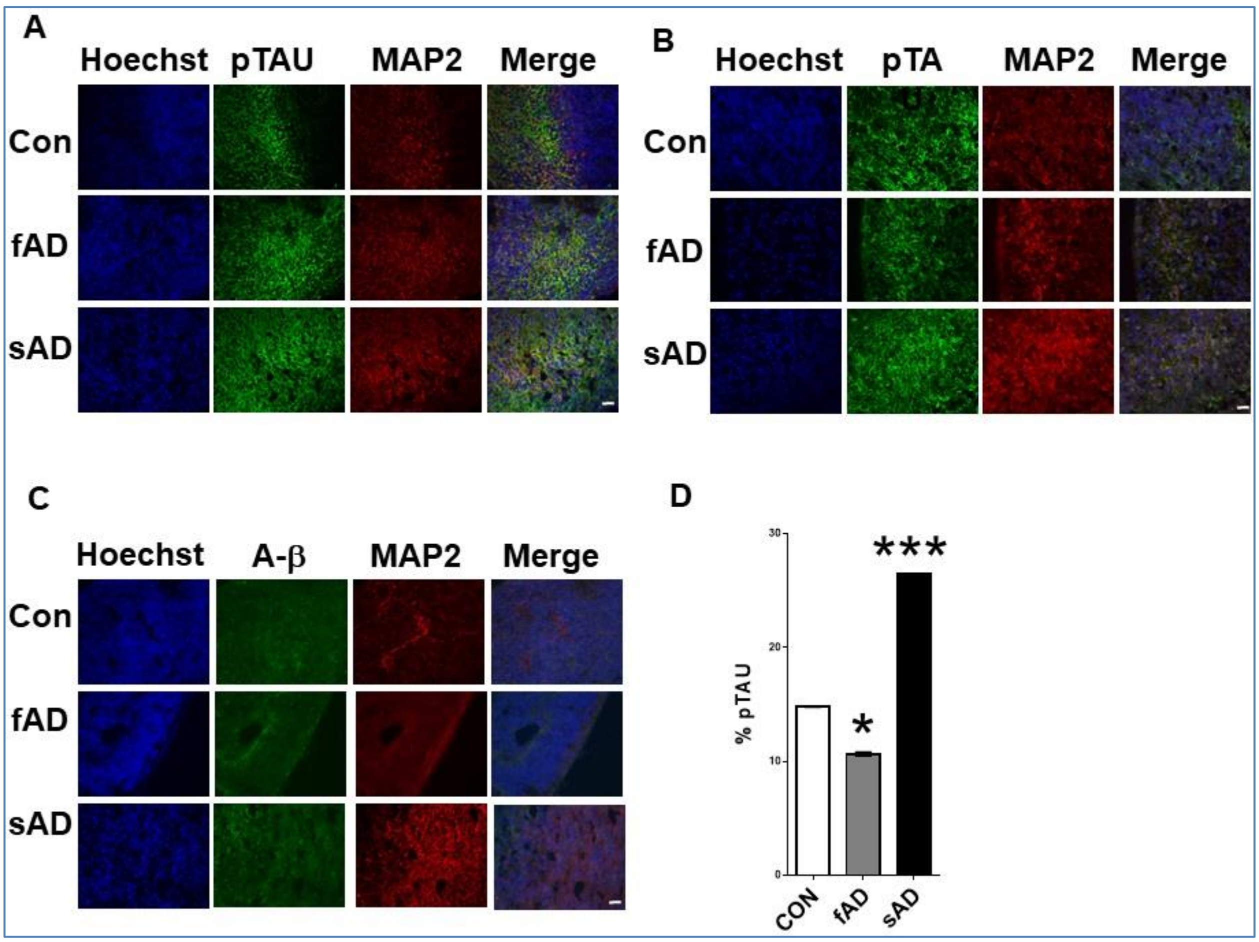

Figure 2. Characterization of AD hallmark phenotypes in cerebral organoids. A-B) Hyperphosphorylated TAU and MAP2 at 90 and 120 days, respectively **C)** Amyloid-b (A-b) and MAP2 at 90 days **D)** percentage of TAU phosphorylation (pTAU) (*t*-Test *** $p<0.001$, *$p<0.05$). White bar=200μ.

s-AD organoids have a distinctive protein expression pattern that differs from control organoids

To further understand whether cerebral brain organoids were an appropriate system to model the AD phenotype, we performed a proteomics analysis of sAD (which showed higher level of expression of pTAU) compared to control organoids at 3 months of differentiation. As shown in Figure 3, sporadic AD had a significant pool of protein dysregulated compared to control organoids (volcano plot Figure 3A) when selecting only statistical relevant protein and accounting for false discovery rate.

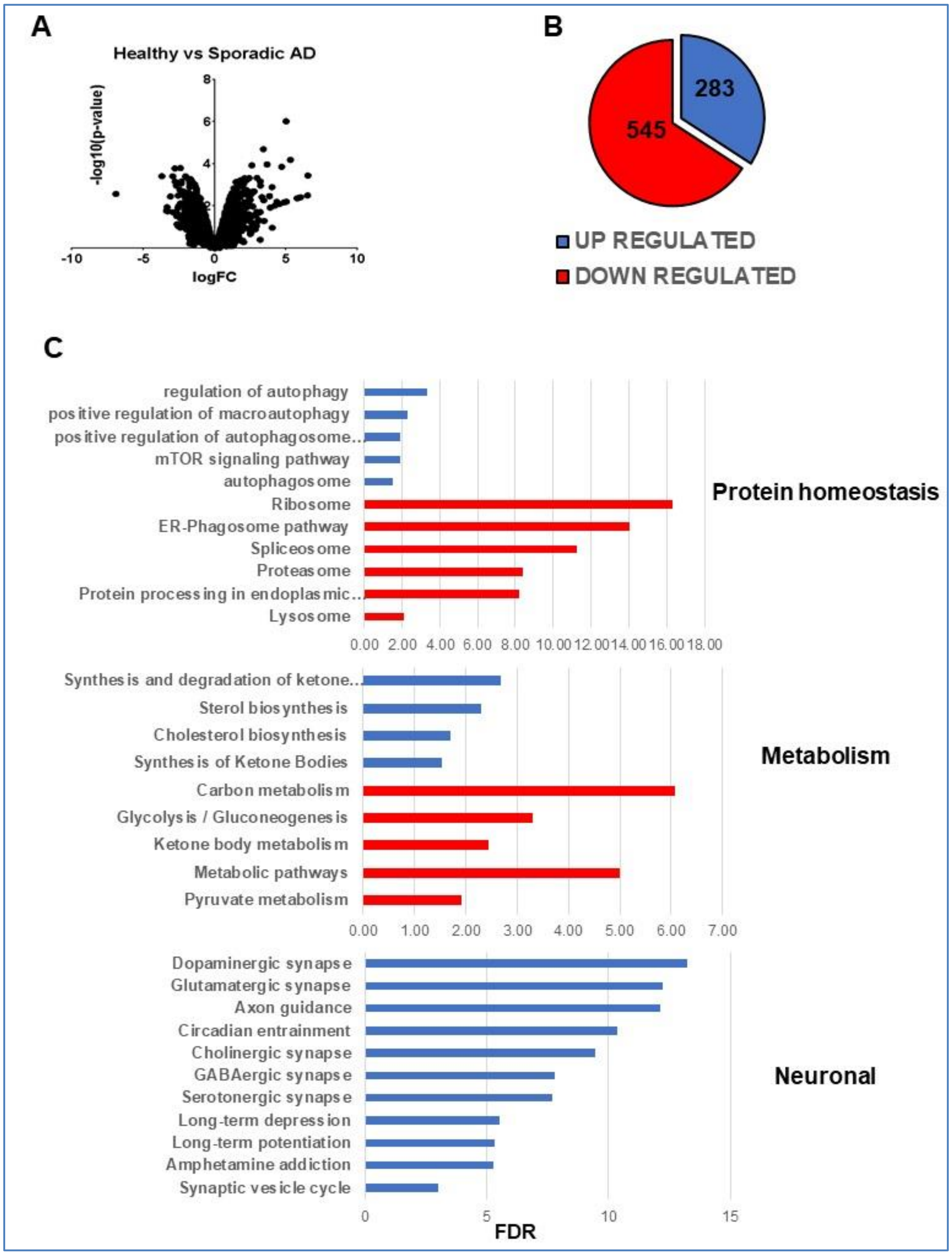

Figure 3. Proteomic Analysis of sAD organoids. Day 120 sAD organoids proteomic dysregulated proteins. **A)** volcano plot of differentially expressed protein compared to Con organoids **B)** pie plot of dysregulated protein (red down regulated, blue up regulated **C)** Reciprocal of False Discovery Rate (FDR) of pathway analysis measured using STRING to show cellular process dysregulated in sAD organoids (red bars are down regulated protein, blue bars are up regulated proteins).

Overall, 545 proteins were upregulated, and 283 proteins were downregulated in diseases organoids compared to control organoids (Figure 3B, pie plot). When we performed pathway analysis to understand which processes and signals were significantly dysregulated in sAD samples, we discovered essential and critical cellular processes were dysregulated, including pathways involved in the protein homeostasis, metabolism, and cellular pathway important for neuronal differentiation and function (Figure 3C).

Next, we analyzed more in detail proteins that were dysregulated. We used STRING program to identify dysregulated protein in AD disease sample, with the goal to identify whether dysregulated proteins interacted among each other in a specific system. Dysregulated interacting proteins were further corrected to account for the false discovery rate ($p < 0.001$). First, we report that several proteins involved in the development of AD were dysregulated in sAD samples, providing further evidence that c-proteins brain organoids have already features and markers of the disease.

Adam10, important hydrolases that cleave Amyloid protein into benign peptides [8], was significantly downregulated, suggesting a propensity of the diseased organoids to generate aggregating Amyloid-β peptides. Many proteins that have been associated with AD were significantly upregulated in diseased organoids, including TAU and α-synunclein [9]. Most importantly, many kinases reported to have TAU as a phosphorylation substrate [10] were clearly upregulated in disease organoids (Figure 4A), supporting the propensity of sAD organoids toward an AD phenotype.

Next, we noticed that the protein degradation system of the disease brain organoids was mostly dysregulated. Specifically, many proteins involved in the proteosome degradation system were significantly downregulated compared to controls.

Following the same trend, many proteins that interact in the lysosomal degradation were significantly downregulated. On the contrary, many proteins involved in autophagy were upregulated, probably as a compensatory effect (Figure 4B). The data suggest that the diseased organoids have already an impaired ability to degrade accumulated proteins, which fits with the accumulation of abnormal protein aggregates associated with AD [11-13]. Next, we identified that several interacting proteins involved in carbon metabolism were downregulated, while the ketone and cholesterol metabolism proteins were upregulated compared to the organoids controls (Figure 4C). Finally, many proteins involve with spliceosome function, and therefore gene expression were downregulated, while proteins involved with mitochondrial function and generation were upregulated in AD organoids compared to controls (Figure 4D). Our data showed that the AD organoids already have protein upregulation that promotes TAU phosphorylation and protein aggregation. Protein degradation is significantly affected, as well gene expression due to dysregulation of the spliceosome, suggesting impairment of protein homeostasis from the initial stage of AD.

Propanolol treatment decrease TAU phosphorylation

Since brain organoids show hallmarks for AD, we tested whether they can be used as a human system for drug discovery. Propranolol was previously identified to be able to decrease Tau phosphorylation and Amyloidβ accumulation in vivo using AD mouse model [4]. It was also previously shown that propranolol is an agonist of the acetylcholine receptor (alpha7 nAChR) [14] similar to the FDA approved drug galantamine, which has been one of the few drugs approved to treat AD patients. Therefore, we treated sAD and control organoids with propranolol at different concentrations to see whether it could decrease TAU Phosphorylation. As shown in Figure 5A in control organoids propranolol only slightly decreased TAU phosphorylation, while 3 and 10 μM of propranolol decreased to half the percentage of TAU Phosphorylation. Our data suggest that propranolol may be useful as a therapeutic agent for AD given its ability to decrease TAU phosphorylation in an AD human model system.

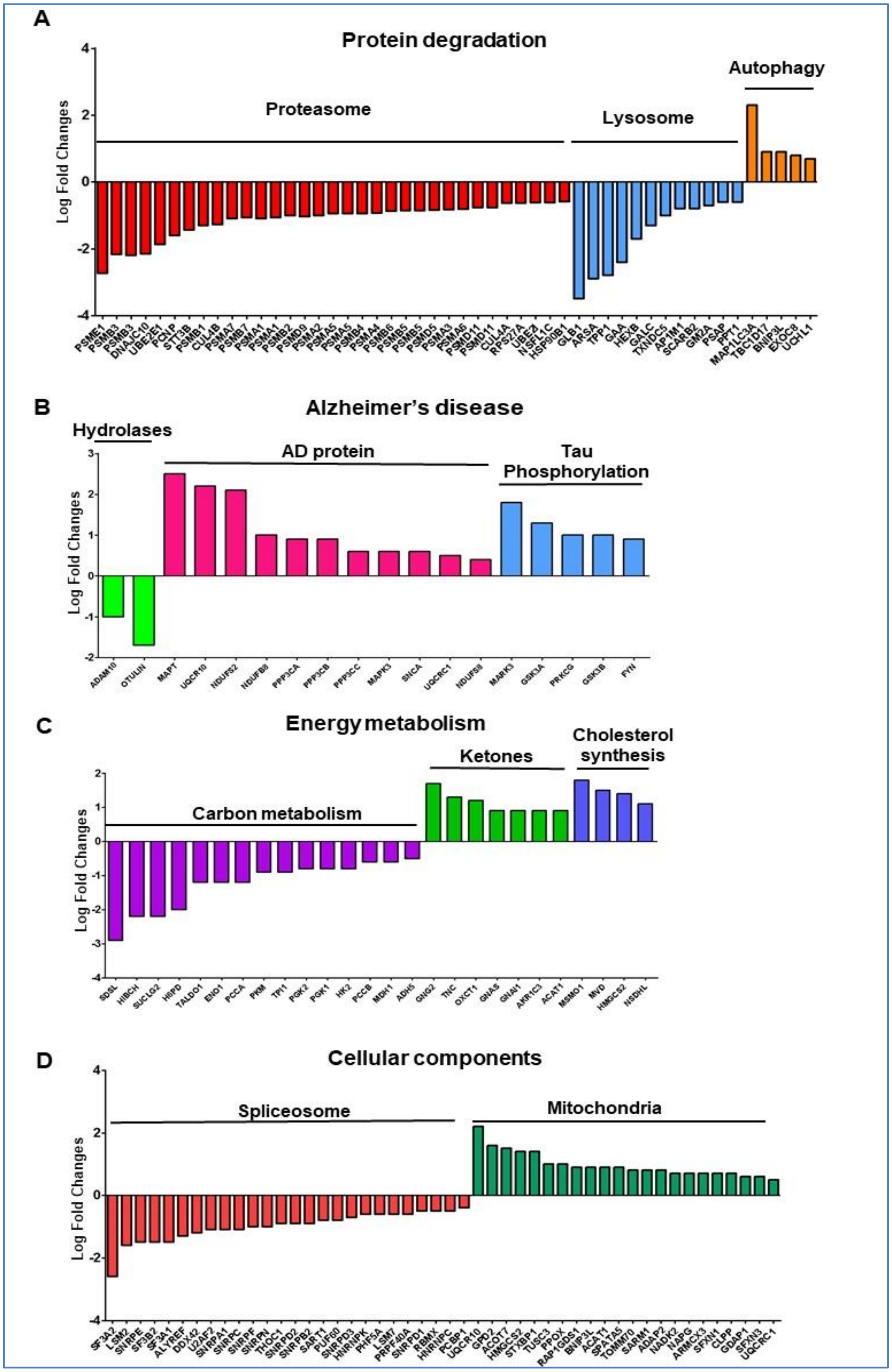

Figure 4. Dysregulated protein in sAD organoids. Graphs of significantly dysregulated proteins (*p*<0.05) Log of Fold Change LogFC in sAD organoids at day150 **A)** Alzheimer's disease related proteins **B)** protein degradation related proteins **C)** Energy and metabolism **D)** cellular components.

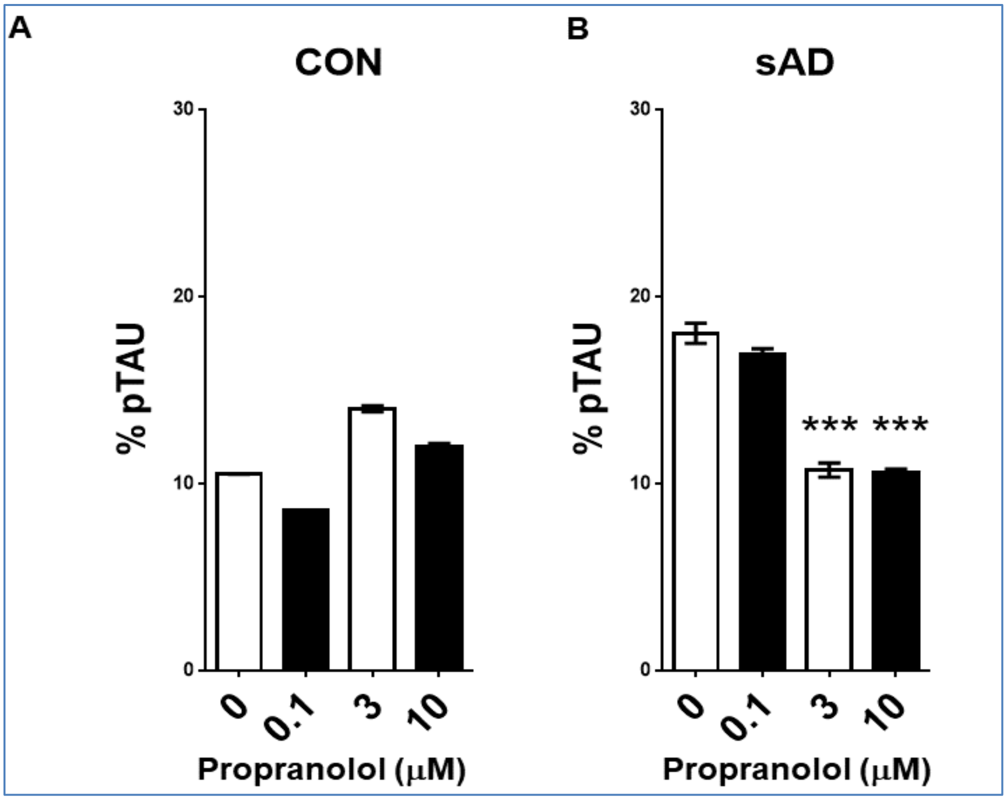

Figure 5. Propranolol treatment in sAD organoids. Percentage of Tau phosphorylation upon 10 days of propranolol treatment (0, 0.1, 3, 10 mM) in Con (A) and sAD (B) ordanoids (Day 150). (One-way Anova p<0.001).

Discussion

AD is a neurodegenerative disorder characterized mainly by abnormal protein aggregation of Amyloid-β and TAU. Here we report that cerebral cortex brain organoids differentiated from AD patients recapitulate AD hallmarks, specifically TAU phosphorylation and, therefore, can be used as a model system for drug discovery. We have tested two iPSC lines obtained from AD patients, and one of them showed an increase of TAU phosphorylation and abnormal protein expression associated with AD iPSC has an inherent variation among clones and between patients [15]. Clonal and patient variation could be the reason why we did not detect a relevant AD phenotype in all lines evaluated. Additional studies will be required to overcome this limitation and evaluate additional iPSC lines derived from AD patients. Nevertheless, the iPSC line that shows an AD phenotype can be used for disease modeling and as a drug development tool.

sAD organoids had several upregulated proteins associated with AD, including α- synuclein. α-Synuclein was initially discovered as the protein aggregation in dementia with Lewy bodies disease (DLB) [16]. It has recently been found upregulated in 50% of AD post-mortem patients with sporadic and pre-senillin associated mutation, suggesting a role in the pathophysiology of AD protein aggregation [17,18]. Most important, elevated α-synuclein levels are found in cerebrospinal fluid (CSF) of patients with mild cognitive impairment (MCI) that will develop AD at a later stage [9,19]. Finally, α-synuclein upregulation in CSF correlates also with APOE e4 genotype, the stronger association marker that correlates with an increased risk of developing AD [20].

Our proteomic analysis suggests that AD cerebral organoids are prone to accumulate protein aggregates (Amyloid-β and TAU fibrils) at an early stage. Adam 10, a metalloproteinase with α-secretase activity, is strongly downregulated in sAD. Adam10 is the major α-secretase for APP; it cleaves APP into non-aggregating or benign forms of Amyloids

[21]. Downregulation of the α-secretase will provide more substrates to the γ-secretase to produce aggregating amyloidβ products. In addition, relevant protein kinases whose substrate is TAU are upregulated, including MARK3, GSK3A, and GSK3B [10]. Our findings suggest that the cerebral brain organoids present with Alzheimer's disease markers can be a valuable model for discovering early markers of AD.

The propensity of sAD organoids to accumulate abnormal protein is also supported by dysregulation of protein degradation. Our finding showed that several subunit components of the proteasome and the lysosome system are downregulated in the sAD organoids. The proteasome is an integral system to degrade unfolded proteins and protein aggregates, and its dysfunction has been measured in post-mortem AD patient brains and AD mouse model. Several reports summarized in [13] showed that the proteasome is dysfunctional when Amyloid-β accumulates or in the presence of neurofibrillary tangles. Surprisingly, our system measured a dysfunctional protein degradation before neurofibrillary tangle formation or amyloid-β accumulation, suggesting that an impairment of the protein degradation system might precede and, therefore, contribute to protein accumulation.

In our proteomic analysis, we identified that the spliceosome system is downregulated. Aberrant mRNA splicing and aggregation of spliceosome components in neurofibrillary tangles have been reported in AD post-mortem brains. In drosophila was shown that the spliceosome components co-precipitate with phosphorylated TAU. More importantly, loss of function in spliceosome subunits results in neuronal neurodegeneration and aberrant mRNA splicing as reported in post-mortem patient brains [22]. Our data suggest that aberrant TAU phosphorylation might induce spliceosome dysfunction and further contribute to neuronal dysfunction. Overall, our proteomic data overlap published data using adult brain tissue obtained from AD patients [23]. Finally, using an in vitro system using human brain organoid we report that propranolol treatment decreases TAU phosphorylation in sAD organoids to normal levels, supporting previously published data using AD mouse model on the therapeutic potential to treat AD.

Conflicts of Interest

There are no conflicts of interests to be declared.

Acknowledgements

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