Apolipoprotein-based regulation of ganglioside metabolism upon secretase activity inhibition in iPSC-derived cerebral organoids

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Abstract

Beta and gamma-secretase inhibitors have been of pharmacological interest to reduce abeta (Aβ) formation and aggregation, a defining characteristic of Alzheimer's disease (AD). Recent research indicates that Apolipoprotein E (ApoE), another genetic risk factor for AD, can regulate secretase activity. Secretase inhibitor-induced elevation of neuronal membrane lipids has been documented in 2D models. Due to their enhanced ability to reproduce AD-like pathology and ease of performing the experimental intervention, we utilized a 3D model comprised of pluripotent stem cells generated from AD patients. We treated patient-derived ApoE3 and ApoE4 cerebral organoids with beta and gamma-secretase inhibitors to determine if organoids could reproduce the differences observed in the 2D model and if the alteration in secretase activity could affect the regulation of neuronal lipids synthesis in an ApoE-dependent manner. Ganglioside profiling was accomplished using liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) via selective reaction monitoring (SRM). The inhibitor administration elevated the levels of ganglioside and ceramide in ApoE4-derived organoids. Since gangliosides are known to enhance Aβ fibrillogenesis, our study implies that reduction in secretase activity affects neuronal membrane architecture that could eventually aggravate AD, particularly in patients with the ApoE4 isoform. In addition, the ability of organoids to replicate results from other experimental models demonstrates their potential to improve translatability significantly.

Keywords: Alzheimer's disease, Ganglioside, Cerebral Organoids, Secretase, Liquid chromatography/electrospray ionization tandem mass spectrometry.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder marked by a progressive loss of the structure and function of neurons, leading to cell death. This could result in the deterioration of cognitive and motor skills. It is the most frequent cause of dementia and typically affects those over 65[1]. Although the precise etiology of AD is unknown, a mix of malfunctions in complex regulatory processes, such as endocytosis, lipid metabolism, and vesicle trafficking, have been identified[2–4].

One of the hallmarks of AD is the formation of amyloid beta (Aβ) plaques in the brain. Beta and gamma secretases cleave amyloid precursor protein (APP) and contribute to forming Aβ plaques. This makes secretase inhibitors a fascinating pharmacological target for lowering Aβ synthesis and slowing the course of AD[5]. It is well recognized that secretases, such as β-site APP cleaving enzyme 1 (BACE1), considerably impact neuronal lipid metabolism and function[6,7]. Lipids are abundant in the brain and essential for sustaining normal brain processes such as supporting the structural integrity of the brain, enabling signal transduction, and myelination[4]. Disruption in lipid metabolism is linked to various neurological diseases, including AD[8]. This could posit that disturbances in secretase activity may lead to changed lipids levels, thus, increasing the risk for AD[9,10]. Sialylated glycosphingolipids called gangliosides are widely distributed throughout the brain, particularly in the neurons. Studies have reported that BACE1 could regulate ganglioside metabolism by affecting the activity of other enzymes involved in their synthesis and degradation, potentially modulating their levels[6]. BACE1 has also been shown to cleave gangliosides and generate new ganglioside species that may have

different biological activities than the original [11]. The precise mechanisms by which secretases affect gangliosides are not fully understood and require further research.

The pathogenesis of AD is complicated since it involves a myriad of cell types, tissue architectures, and metabolic pathways. Another challenge is the lack of a disease model that sufficiently replicates the disease's complexity and pathological characteristics and the hardship of obtaining human brain samples[12]. However, the recent development of cerebral organoids, 3D models of the human brain, has provided new insight into the disease[13]. The organoids have demonstrated relevant features of AD, such as the production of amyloidogenic variants of the Aβ protein and increased tau phosphorylation, as well as the ability to form a variety of brain-like regions with complex tissues[14,15]. Also, the current animal transgenic models are insufficient for sporadic AD (sAD), the most prevalent form of AD, with the ApoE4 allele being a significant risk factor for sAD[16]. Organoids can aid in their study by incorporating specific genetic mutations[17].

In this study, we show that induced pluripotent stem cells (iPSC) derived cerebral organoids from sAD patients recapitulated the pathological features of the disease, along with the expected effect upon treatment with secretase inhibitors. We also assessed the effect of these inhibitors on neuronal lipids. Due to increased sensitivity, specificity, and precision, a mass spectrometry (MS) assay via selected reaction monitoring (SRM) was employed to analyze gangliosides. In addition to quantifying major gangliosides, the assay was also utilized to identify the molecular composition of lipids, including the fatty acid composition and the head group structure. In sum, by employing our MS-based ganglioside profiling assay in the brain organoids, we identified a hitherto unreported link

in which ApoE allele-dependent ganglioside metabolism dysregulation was observed upon secretase inhibition in cerebral organoids.

Materials and Methods

Materials

The BACE1 inhibitor was purchased as BACE inhibitor C3 (β-Secretase Inhibitor IV), and the gamma secretases inhibitor was purchased as Compound E (γ-Secretase Inhibitor XXI) from EMD Millipore (City?, MA, USA). Ammonium acetate (Cat# A11450) and ammonium formate (Cat# A11550) were from Fisher Chemical (Hampton, NH, USA). LC-MS grade acetonitrile (can, Cat# 34967), isopropanol (IPA, Cat# 34965), and formic acid (FA, for MS ~98% purity) were from Honeywell (Charlotte, NC, USA). LC-MS grade methanol (Cat# 0013687802BS) was from Biosolve Chimie (Dieuze, France). Acetic acid (≥99.8%) was from Penta Chemicals (Chrudim, Czech Republic). Isotopically-labeled ganglioside internal standards (GM1 and GM3) were synthesized by Lukas Opalka (Faculty of Pharmacy, Charles University, City, Country). The ultrapure water was prepared in the purification system (arium® Comfort System, Sartorius, City, Country).

Cerebral organoid culture and treatment with β and γ-secretase inhibitors

3D cultures, or cerebral organoids, were generated following published protocols[13,15]. To treat the organoids with β and γ-Secretase inhibitors, a previously published protocol was followed with minor modifications[12]. The organoids were treated for 50 days, starting from day 50 to day 100. BACE Inhibitor C3 (5uM) and Compound E (6nM) were continuously added to the differentiation medium. Organoids were harvested from two

independent batches of CO differentiation (N=5 each). Control samples were either non-treated (NTR) or treated with dimethyl sulfoxide (DMSO), the solvent used to dilute the inhibitors.

Sample preparation and Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) for the analysis of gangliosides

Lipid extraction followed by sequential protein extraction was performed as previously reported by our group[18]. Briefly, the harvested organoids were freeze-dried and homogenized. Lipids were extracted upon addition of 80% IPA to the dry homogenate, followed by vortex and sonication. The sample was further centrifuged, and the lipid extract was removed from the residual protein pellet. The lipid extracts were stored at -20°C until analysis.

The lipid extract was mixed with isotopically labeled GM1 (0.3uM) and GM3 (0.15uM) internal standards for relative ganglioside quantitation. For the ganglioside assay, ApoE3/3 (N=10) and ApoE4/4 (N=10) control organoids, along with ApoE3/3 (N=10) and ApoE4/4 (N=10) sAD organoids were utilized. The analysis of gangliosides through LC/ESI-MS/MS was followed based on the same protocol (Ref). Using a Agilent 1200 uHPLC system coupled to a ESI 6495 Triple Q MS., we analyzed eight subclasses of gangliosides – GD3, GM3, GM2, GD2, GM1, GD1a, GD1b, and GT1b. We also analyzed molecular species with different ceramide chains in each subclass. They were identified based on product ions reflecting the fragmentation behavior based on the fatty acid chain based on accurate m/z values and retention times per the predicted retention behavior. The detailed list of the SRM transitions is available in Tab S1. Data were

processed and analyzed in Mass hunter Quantitative Analysis software (Agilent Technologies, Santa Clara, USA).

Statistical analysis

The graphs were plotted using GraphPad Prism 9.0.0 (GraphPad Software, Inc., La Jolla, CA, USA). The data were normally distributed, as assessed by the Shapiro-Wilk test ($p > 0.05$). Groups were compared using Student's two-tailed t-test (two groups). To identify any separation in the widespread data, principal component analysis was performed using open access tool on Metaboanalyst (Metaboanalyst 5.0[19]). The data were scaled using the z-score method before PCA. The figures were prepared using BioRender scientific illustration software (BioRender.com). Details are provided in individual figure legends.

Results

1. The effect of suppression of secretase activity on Aβ production in cerebral organoids

We treated sAD-E3 (N=4) and sAD-E4 cerebral organoids (N=4) with β and γ-secretase inhibitors and measured the levels of Aβ40 and Aβ42 production through ELISA. We utilized NTR and DMSO-treated organoids (N=4) as controls. There was a significant decrease in Aβ40 and Aβ42 levels in both sAD-E3 and sAD-E4 organoids. No differential change was observed between E3 and E4 organoids upon inhibitor treatment. We also did not observe any significant difference in the Aβ40 and Aβ42 levels between NTR and DMSO conditions, irrespective of the ApoE genotype (Fig 1).

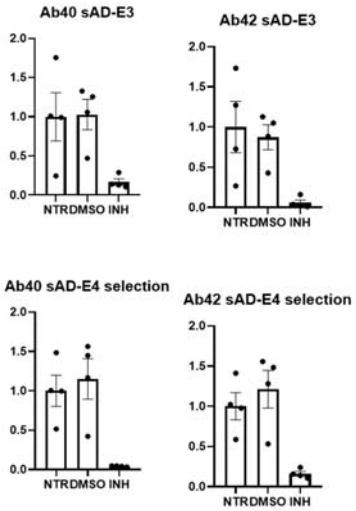

Figure 1. Effect of beta and gamma-secretase inhibitor activity on Aβ production. sAD-E3 and sAD-E4 organoids were treated with BACE inhibitor C3 and Compound E for 50 days, and levels of Aβ40 and Aβ42 in the organoid were determined by ELISA. Each point refers to the organoid (N=4). The levels of DMSO and INH are relative to the NTR levels.

2. Characterization of gangliosides in the cerebral organoids

Separation and classification of gangliosides according to their ceramide type was done in the RPLC column in both positive and negative ionization mode. Predictably, gangliosides were separated from the most hydrophilic to the hydrophobic molecules. Precursor ions with GM1, GM2, and GM3 were detected in the state of a single charge, with GD1 and GT1 detected in the state of a double charge. A common fragment ion at m/z 290 composed of sialic acid ions (SA2) was used as the product ion in the negative mode. The ceramide moiety of gangliosides influenced the elution of different molecular species. The retention behavior of gangliosides followed the patterns related to the number of carbon atoms in fatty acyl chains and the number of double bonds (Fig 2). Gangliosides were eluted from d16:1-18:0 (d18:1-16:0) to d18:1-22:0. We could also distinguish between regioisomeric gangliosides such as GD1a and GD1b using product ions such as SA-SA2 (/m/z 581). In total, we profiled eight subclasses of gangliosides,

identifying 70 molecular species in a single cerebral organoid. Interestingly, the most prevalent species were C16 and C18 fatty acyl chains, in contrast to the prevalent C18 and C20 fatty acyl chains in adult human brains[20].

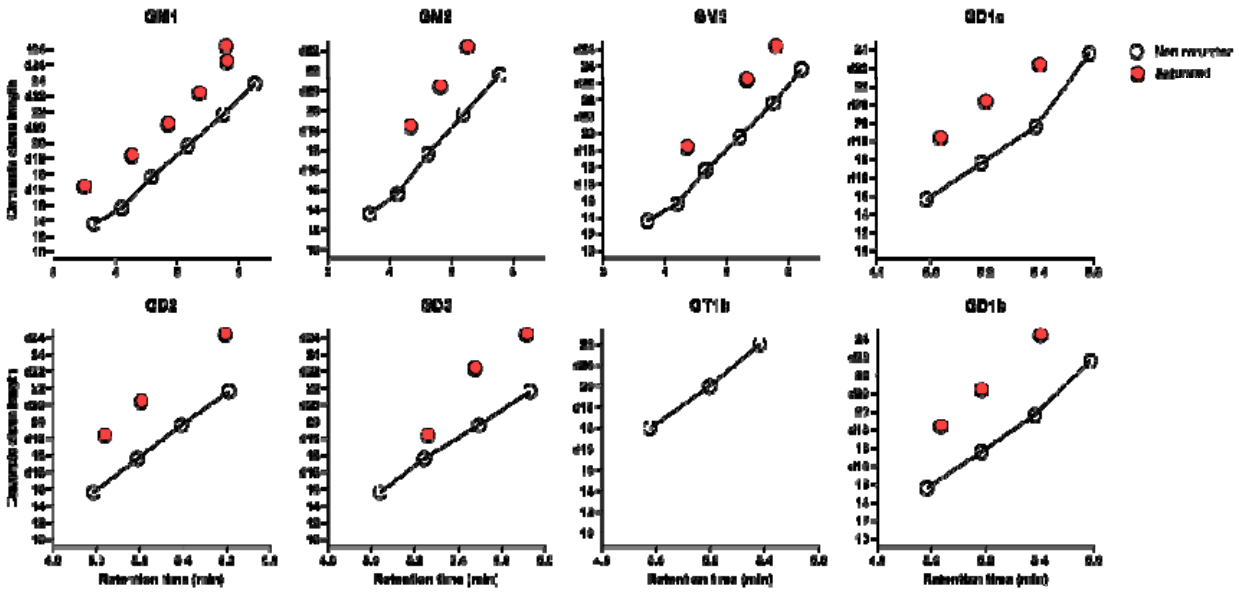

Fig 2. The retention time of gangliosides profiled in cerebral organoids for fatty acid chain lengths ranging from C14:0 to C24:0. The red dots (o) indicate the non-saturated fatty acid chains, whereas the white dots (o) indicate saturated fatty acid chains. A total of 60 molecular species were characterized in a single cerebral organoid.

3. Suppression of secretase activity increased the levels of gangliosides in ApoE dependent manner in cerebral organoids

Based on our method to characterize gangliosides, we performed relative quantitation of the species mentioned above in two batches of organoids (N=10).

PCA plot of the organoids showed no separation in the ApoE3/3 isoform upon treatment with β and γ-Secretase inhibitors; however, we observed slight separation in the ApoE4/4 line (Fig 3a). Upon closer look at the total ganglioside concentrations, we observed a

significant increase in their levels in sAD-E4 organoids treated with inhibitors. These results were limited to the ApoE4/4 line alone, with no upregulation observed in the ApoE3/3 line (Fig 3b, S1).

We were also interested in the effect of inhibitors on the precursors involved in the formation of gangliosides. Strikingly, we also found a dramatic increase in dihydroceramide and ceramide species in the sAD-E4 organoids, which contrasted with the sAD-E3 organoids, where their levels had significantly declined upon inhibitor treatment (Fig S2).

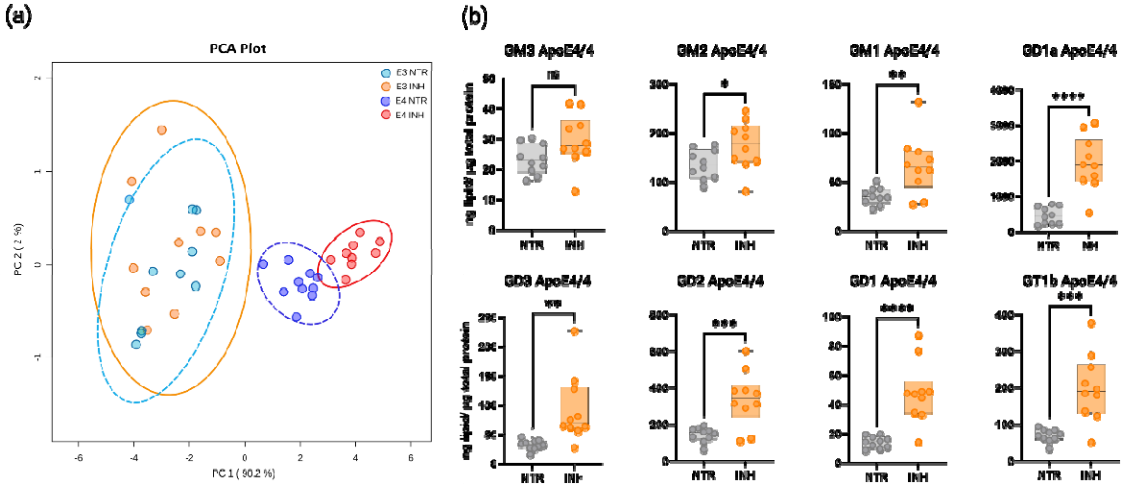

Figure 3. Effect of reduction of secretase activity on levels of gangliosides. sAD-E3 and sAD-E4 organoids were treated with reagent names for 50 days at a single concentration of 100uM. The level of gangliosides in the NTR and inhibitor-treated organoids were measured by LC-ESI/MS in combination with SRM. (a) Principal component analysis (PCA) of N=10 organoids, each from NTR sAD-E3, NTR sAD-E4, INH sAD-E3, and INH sAD-E4 conditions. (b) Total ganglioside levels in NTR and INH cerebral organoids. Each box plot indicates the minimum and maximum with a median of 10 values. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 (Groups were compared using two-tailed t-test).

4. Exogenous addition of GM1 to ApoE3 and ApoE4 genotype cerebral organoids

It has been reported that gangliosides, especially glycosyltransferases, increase the life of beta secretases and their activity, leading to heightened cleavage of APP[6]. Thus, we were also interested in whether adding exogenous GM1 impacted Aβ40 and Aβ42 levels and whether any ApoE-dependent effect could be observed.

Through immunohistochemistry performed with labeled exogenous GM1, we could visualize the incorporation of additionally provided GM1 in the organoids (Figure 4a). However, we could not identify any change in Aβ40 and Aβ42 levels in either E3 or E4 organoids upon adding GM1 (Fig 4b).

Measurement of cerebral organoid samples by LC/ESI-MS/MS for gangliosides revealed an increase in levels of GM2 and GD1a species which are direct degradation products and precursors of GM1, respectively, upon treatment with GM1 (Fig 4c). Interestingly we also observed a decline in b-series gangliosides (Fig S3). This result was most prominent in the ApoE3/3 line.

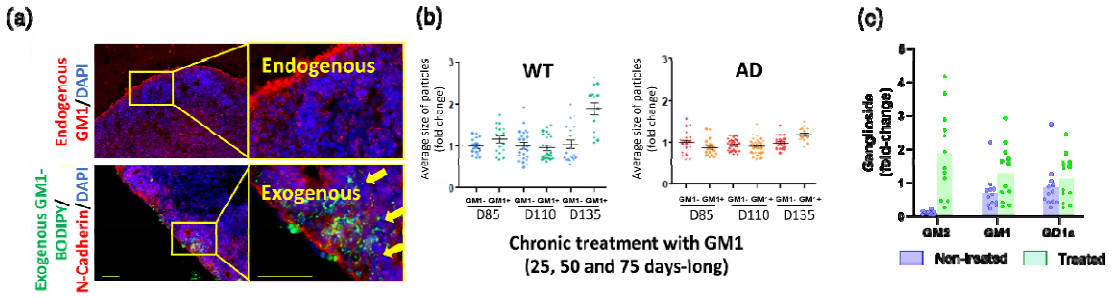

Fig4. Effect of application of exogenous GM1 on Aβ and ganglioside production. Wild-type (WT) and AD ApoE3/3 and ApoE3/4 organoids were treated with GM1 for over 100 days at a single concentration of 100uM, and the size of Aβ particles in the organoid was determined by ELISA, whereas gangliosides were relatively quantified using LC-ESI/MS in combination with SRM. (a) Indirect immunofluorescent staining images depict the endogenous GM1 and the labeled exogenous GM1 in the cerebral organoids. (b) Size of the Aβ particles in WT and AD organoids at three-time points - D85, D110, and D135. The dots depict the number of Aβ plaques taken for the analysis. The result of GM1 treatment is presented as relative to the non-treated organoids. (c) Total ganglioside levels of GM1, GM2, and GD1a in NTR and GM1 treated organoids in WT ApoE3/3 organoids. The columns represent the average±standard deviation. The dots represent individual organoids.

Discussion

As anticipated, treatment of cerebral organoids with secretase inhibitors led to a considerable decrease in Aβ40 and Aβ42 relative to control organoids in the E3 and E4 lines. Interestingly, we also detected a minor rise in Aβ40 levels relative to Aβ42 in the ApoE3 line, in sharp contrast to the ApoE4/4 line, which had greater Aβ42 levels than Aβ40 levels (Fig 1). A plausible explanation could be ApoE4's ability to bind to the secretase complex and suppress the synthesis of Aβ40, as previously reported[21].

There is evidence that the accumulation of gangliosides, GM3 and GM1, can facilitate the development of the GAβ complex, resulting in a conformational shift serving as a seed for fibrillogenesis[22–25]. An age-dependent rise in GM1 has also been found in synaptosomes containing the ApoE4 isoform, but the increase was not as significant in synaptosomes containing the ApoE3 isoform[26]. We utilized a targeted approach established for the study of gangliosides to profile molecular species relative to one another. Thus, the accumulation of gangliosides observed in the sAD-E4 cerebral organoids indicates the potential to aggravate AD etiology further.

Secretases such as BACE1 are known to be elevated in the brains of AD patients, making them an attractive therapeutic target[16,27]. Elevated expression of GM1, GM2, or GD2 increases β- and γ-site cleavages of APP[28]. β-1,4 N-acetylgalactosaminyltransferase (B4GALNT1), one of the glycosyltransferases responsible for producing these complex gangliosides, is known to affect BACE1 stability by inhibiting its lysosomal degradation and boosting its protein levels[6]. This ultimately results in a surge of the beta-cleavage of APP. The ApOE4 isoform, a known hereditary risk factor for AD, can not only exacerbate neurodegeneration[16,29] but can also influence the activity of γ -secretase by

raising the amount of γ-secretase complex attached to it, thus escalating the synthesis of Aβ42[30]. This demonstrates an unsolved mechanism through which ApoE isoform-dependent neuronal lipid dysregulation mediated by decreased secretase activity may contribute to AD pathogenesis. Upregulation of ganglioside metabolism previously observed in neuritic cell lines treated with secretase inhibitors was replicated precisely in organoids[9] (Fig 3b).

Our attempts to detect dysregulation in Aβ levels upon organoid treatment with exogenous GM1 were unsuccessful (Fig 4b). It is conceivable that the mere incorporation of this lipid into the cell surface probably does not affect APP processing. As previously documented, surface-adherent exogenous gangliosides are incapable of altering the stability of BACE1, resulting in no change in APP cleavage[6].

Conclusion

In conclusion, we demonstrated that a decrease in secretase activity modifies ganglioside levels in ApoE4 genotype cerebral organoids, which could contribute to AD pathology's genesis. In the future, it will be intriguing to see if ApoE4's propensity to sequester secretases directly impacts glycosyltransferases and ganglioside levels. Modulation of ganglioside metabolism could present an opportunity to develop a way to prevent and treat AD. Our study further proved the efficacy of cerebral organoids in exhibiting AD-like pathophysiology. This model has the potential to dramatically expedite research and drug discovery in Alzheimer's disease, as well as provide valuable insights into its underlying causes.

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Funding

This work was supported by the Project Internal Grant Agency of Masaryk University (No. CZ.02.2.69 / 0.0 / 0.0 /19_073 / 0016943), Grant Agency of Masaryk University (GAMU project No. MUNI/G/1131/2017), the Czech Health Research Council (AZV project No. NV19-08-00472), the RECETOX research infrastructure (the Czech Ministry of Education, Youth, and Sports–MEYS, LM2018121), CETOCOEN EXCELLENCE Teaming (Horizon2020, 857560 and MEYS, 02.1.01/0.0/0.0/18_046/0015975), Czech Science Foundation (GACR; no. 18-25429Y, GA20-15728S, and 21-21510S), by the European Regional Development Fund - Project INBIO (No. CZ.02.1.01/0.0/0.0/16_026/0008451). DB was supported by funds from NF Neuron, Alzheimer NF, and by Career Restart Grant from Masaryk University (MUNI/R/1697/2020).

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