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Amyloid- β Production Via Cleavage of Amyloid- β Protein Precursor is Modulated by Cell Density

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Abstract

Mounting evidence suggests that Alzheimer disease (AD) is caused by the accumulation of the small peptide, A β , a proteolytic cleavage product of amyloid- β protein precursor (A β PP; or APP). A β is generated through a serial cleavage of APP by β - and γ -secretase. A β 40 and A β 42 are the two main components of amyloid plaques in AD brains, with A β 42 being more prone to aggregation. APP can also be processed by α -secretase, which cleaves APP within the A β sequence, thereby preventing the generation of A β . Little is currently known regarding the effects of cell density on APP processing and A β generation. Here we assessed the effects of cell density on APP processing in neuronal and non-neuronal cell lines, as well as mouse primary cortical neurons. We found that decreased cell density significantly increases levels of A β 40, A β 42, total A β , and the ratio of A β 42:A β 40. These results also indicate that cell density is a significant modulator of APP processing. Overall, these findings carry profound implications for both previous and forthcoming studies aiming to assess the effects of various conditions and genetic/chemical factors, e.g. novel drugs on APP processing and A β generation in cell-based systems. Moreover, it is interesting to speculate whether cell density changes *in vivo* may also affect APP processing and A β levels in the AD brain.

Keywords

Alzheimer's disease; amyloid- β ; amyloid- β protein precursor; cell density

INTRODUCTION

AD is an insidious and progressive neurodegenerative disease and the primary cause of dementia in the elderly. It is a genetically complex and heterogenous disorder and based on the age of onset, it has two primary forms: early or late-onset of AD. More than 200 fully penetrant mutations in the amyloid- β protein precursor (*APP*), presenilin 1 (*or PSEN1*), and presenilin 2 (*PSEN2*) have been linked to early-onset familial AD (FAD) (<60 years old; 5–10% cases) [1–3], whereas 90–95% cases are late-onset AD (>60 years old) and a variant (ϵ 4) of the gene encoding apolipoprotein E (*APOE*) has been associated with this disease type. Pathologically, AD is characterized by two hallmarks: amyloid plaques primarily composed of A β , and neurofibrillary tangles made up of the hyperphosphorylated tau. Multiple lines of evidence implicate excessive A β load in the brain as the primary cause of

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AD [1, 4, 5]. A β is a ~4 kDa peptide produced by a serial proteolytic cleavage of APP (Fig. 1) [6]. The initial cleavage of APP can be carried out by either α -, or β -secretase (or BACE1). α -Secretase cleavage produces sAPP α and the α -C-terminal fragment (α -CTF or APP-C83) while β -secretase cleavage produces sAPP β and β -C-terminal fragment (β -CTF or APP-C99). C83 and C99 can be further cleaved by γ -secretase, which produces the P3 peptide and A β , respectively [1, 2, 5, 7]. A β can be 37-43 amino acids long depending on the γ -secretase cleavage site. While A β 42 and A β 40 are the two primary A β species, A β 42 is more prevalent than A β 40 in amyloid plaques. Most early-onset FAD mutations in *APP*, *PSEN1*, and *PSEN2* increase the ratio of A β 42:A β 40, which drives the aggregation of A β into neurotoxic oligomeric assemblies [1, 2]. All three secretases are transmembrane proteins. α -Secretase activity can be carried out by at least 3 transmembrane proteins, ADAM9, 10 and 17 belonging to the adamalysin (or a disintegrin and metalloproteinase, ADAM) protein family [6]. Generally, these proteases play roles in cell adhesion, proteolytic processing, and ectodomain shedding of cell surface proteins. BACE1 (also named as Asp2, or memapsin 2), a type 1 transmembrane protein, has been identified as the β -secretase protein, which belongs to the pepsin family of aspartyl proteases [8]. γ -Secretase is a heterogeneous protein complex, which has been shown to contain at least four transmembrane proteins: presenilin (PS1), presenilin enhancer 2 (PEN2), nicastrin, and anterior pharynx-defective 1 (APH-1) [1, 9, 10]. APP processing by α -, β - and γ -secretases is highly regulated, and the misregulation of APP processing is believed to underlie the pathogenesis of AD, particularly by increasing total A β levels or the A β 42:A β 40 ratio [1, 5]. AD brain is characterized by tremendous neuronal death and significantly decreased cell density, particularly in late stage of AD. However, currently, little is known regarding how A β levels and APP metabolism are altered by cell density. Thus, in this report, we set out to determine whether A β generation and APP metabolism are altered by cell density. We utilized both neuronal and non-neuronal cell models, as well as mouse primary cortical neurons in this study. We demonstrated that A β 40, A β 42, A β (40+42) levels, and the ratio of A β 42:A β 40 are all significantly increased with decreases in cell density. Thus, these findings carry profound implications for previous and forthcoming studies assessing the effects of various conditions and genetic/chemical factors on AD pathogenesis, e.g. novel drugs on APP processing and A β generation in cell-based systems.

MATERIALS AND METHODS

Cell culture and mouse primary cortical neuron culture

Human neuroglioma H4 cells that stably over-express human APP751 (H4-APP751) cells have been reported previously [11–14]. Chinese hamster ovary (CHO) cells that stably over-express human APP751 (CHO-APP751) have been reported elsewhere [14, 15]. These cell lines were cultured on regular tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 200 μ g/ml G418. Mouse primary cortical neurons were obtained from Brainbits (E18) and were cultured on poly-D-lysine/laminin-coated plates in B27/Neurobasal medium supplemented with 1X GlutaMAX (Invitrogen) [13].

Chemicals and antibodies

The APP C-terminal antibody (targeting the last 19 amino acids of APP751, APP750 or APP695; 1:1000) was a polyclonal antibody, was purchased from Sigma (catalog #: A8717), and can detect full-length APP, and APP-CTFs (APP-C83 and APP-C99). β -Actin antibody (1:10,000) was purchased from Sigma. The HRP-conjugated secondary antibodies (anti-mouse and anti-rabbit) (1:100,000) were purchased from ThermoFisher.

Cell lysis and protein amount quantification

Cells were lysed in M-PER (Mammalian Protein Extraction Reagent) (Thermoscientific) with 1X Halt protease and phosphatase inhibitor cocktail (Thermoscientific). The lysates were collected and centrifuged at 13,000 rpm (Eppendorf centrifuge 5417) for 10 minutes. Pellets were discarded and supernatants were transferred into a new microcentrifuge tube [16]. Total protein was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce) [13].

Western blotting analysis

Western blotting analysis was carried out by the method described previously [13, 16–18]. Briefly, after centrifugation and protein concentration measurement, an equal amount of each protein sample was applied to electrophoresis followed by membrane transfer, antibody incubation, and signal development. The VersaDoc imaging system (Bio-Rad) was used to develop the blots and the Quantity One software (Bio-Rad) was used to quantify the proteins of interest by subtracting the background, following the protocols described previously [13, 16, 17]. Signals from full-length APP (APP-FL), APP-C83, APP-C99, and β -actin were assessed and quantified. β -Actin was used as an internal loading control.

A β measurement

A β measurement was performed following the manufacturer's suggested protocols and was described previously [13, 17]. In brief, A β 40 and A β 42 levels were quantified using a sandwich enzyme-linked immunosorbent assay (ELISA), and were normalized to the protein concentration from the same cell lysates. The media from H4-APP751 cells were analyzed using the Wako Human kit to detect the levels of A β 40 (catalog #:292-62301) and A β 42 (catalog #:298-62401). The A β 40 kit from Signet was also utilized to validate the A β 40 results from Wako. The media from CHO cells and mouse primary cortical neurons were analyzed using the Wako Human/Rat kit to detect the levels of A β 40 (catalog #:294-62501) and A β 42 (catalog #: 290-62601). Briefly, the Wako ELISA kit used an assay based on colorimetric changes. First, 100 μ l standard and treatment samples were incubated at 4°C with plate seal overnight, and then the solution was gently washed off and 100 μ l of the HRP-conjugated antibody solution was added into each well and incubated for 2 hours. The solution was then washed off and the TMB solution was added with 30 minutes incubation. Lastly, 100 μ l Stop solution was added into each well and the absorbance was immediately read at 450nm with a microplate reader.

α - and β -Secretase activity assay

We used the fluorometric reaction-based kits that detect the APP cleavage activity level of α - and β -secretase. The assays were performed according to the manufacturer's protocols (R&D Systems) and were described elsewhere [19].

Data analysis

A β 40 and A β 42 levels from each sample, as well as fluorometric readouts from the secretase activity experiment were normalized to the protein concentrations from the same cell lysates [11, 13, 18]. To account for any differences in loading, the levels of full-length APP and its CTFs (C83 and C99) were normalized to the β -actin levels from the same lane. The ratios of C83 and C99 to full-length APP were calculated by dividing C83 or C99 by APP-FL levels from the same lane. The ratios of total A β to C99 were calculated by dividing total A β levels by C99 levels from the same samples. The observations from higher cell densities were normalized to lowest cell densities, unless otherwise specified, and were demonstrated as mean \pm S.E. from at least three independent experiments [11, 13, 18]. We used a two-tailed *t* test to compare the differences between two experimental groups. The Bonferroni correction

analysis was used to correct for multiple comparisons within a single experiment. P values 0.05 were considered statistically significant.

RESULTS

A β 40 and A β 42 levels vary depending on cell density

We initially employed a neuronal cell line to assess the effects of varying cell densities on A β levels and APP processing. The stable human neuroglioma H4-APP751 cell line were plated in 6-well cell culture plates with different numbers (50K or 50 thousand, 100K, 150K, 200K, 250K, 300K and 400K) [13, 17, 18]. The surface area of one well in a 6-well plate is 9.6cm² and the seeding densities were approximately 5, 10, 15, 20, 25, 30, and 40 X 10³ cells/cm², respectively. Cells were harvested after 48 hours. There was no observed cell death, but differences in morphology were observed at different densities. As expected, cells plated at lower cell densities exhibited less cell-cell contact and occupied less area based on our subjective observation. In addition, individual cells plated at lower densities possessed a larger cell size than individual cells at higher densities. Concordantly, cells at higher densities exhibited more cell-cell contact and occupied greater area, and individual cell revealed more circular shape and smaller size at higher densities.

Conditioned media were harvested and subjected to ELISA to measure A β levels. For each sample, both A β 40 and A β 42 levels were measured and normalized to cell lysate protein concentration. At the plating of 100K, the levels of A β 40 and A β 42 exhibited a trend of, but not significant increase versus 50K (Fig. 2A-2B). At plating numbers from 200K to 400K, both A β 40 and A β 42 levels were significantly lower versus 100K ($p < 0.05$; Fig. 2A-2B). For example, plating at 400K, A β 40 and A β 42 levels decreased 61.0%, and 68.0%, respectively, compared to plating at 100K ($p < 0.01$; Fig. 2A-2B). The regression expression of A β 40 is represented as a polynomial function: $y = -5E-06x^2 + 2E-05x + 1.1469$ ($R^2 = 0.8057$) (a fitting curve with highest R^2 value was chosen) (Fig. 2A). The regression expression of A β 42 is represented as a polynomial function: $y = 2E-07x^2 - 0.0024x + 1.2664$ ($R^2 = 0.8615$) (Fig. 2B). Next, we assessed A β (40+42) levels and the A β 42:A β 40 ratios at varying cell densities. Here we used the levels of A β (40+42) to represent total A β since these two A β species constitute the primary A β components. Plating at 100K revealed a trend to, but not significant increase in the level of total A β versus 50K (Fig. 2C). At plating of 200K to 400K, total A β levels markedly declined with increases in cell density versus 100K ($p < 0.05$; Fig. 2C). Levels of A β (40+42) could be represented using a polynomial function: $y = -3E-06x^2 - 0.0006x + 1.178$ ($R^2 = 0.8275$). Similarly, the ratio of A β 42:A β 40 was decreased with increasing seeding densities, and presented as a polynomial function: $y = 4E-06x^2 - 0.0027x + 1.1257$ ($R^2 = 0.7922$) (Fig. 2D). For example, the A β 42:A β 40 ratio was 30.9% lower at 250K versus 100K ($p < 0.01$).

Cell type-specific differential effects in APP processing have been previously observed [16, 20, 21]. Therefore, we next validated the effect of cell density on A β levels utilizing different cell models. First, we utilized a non-neuronal cell model, chinese hamster ovary cells stably over-expressing human APP751 (CHO-APP751). CHO-APP751 cells of different numbers (50K, 100K, 150K, 200K, 250K, 300K, 350K and 400K) were initially seeded and incubated on 6-well cell culture plates and allowed to grow for 48 hours. Conditioned media were collected and applied to ELISA to measure A β levels. As a result, similar to what was observed in H4-APP751 cells, there was no cell death at each density, but there were differences in morphology at different densities. We showed that both A β 40 and A β 42 levels significantly decreased with increases in cell density ($p < 0.05$; versus 50K) (Fig. 3A-3B). For example, at plating of 400K, A β 40 and A β 42 levels decreased by 80.2% and 83.1%, respectively, versus 50K ($p < 0.01$; Fig. 3A-3B). In addition, total A β levels significantly declined with increases in cell density compared to those at 50K ($p < 0.05$; Fig.

3C). For example, plating at 400K, total A β level decreased by 80.4% versus 50K ($p < 0.01$; Fig. 3C). Similar to what was observed in H4-APP751 cells, the A β 42:A β 40 ratio was reduced with increasing cell densities (Fig. 3C). For example, the A β 42:A β 40 ratio decreased by 27.5% at plating of 150K versus 50K ($p < 0.01$; Fig. 3C).

A β 40 and A β 42 levels vary depending on cell density in mouse primary cortical neurons

Next, we studied the effects of cell density on A β levels using mouse primary cortical neurons. The average size of a mouse primary neuron is approximately 25% of the size of an H4 or CHO cell. Therefore, in order to arrive at final cell densities for the mouse primary cortical neurons that would be similar to those of the H4-APP751 and CHO-APP751 cell lines, we used 24-well poly-D-lysine coated plates. Specifically, mouse primary cortical neurons were initially seeded at different densities (200K, 250K, 300K, 400K and 600K), and allowed to grow for 48 hours. Conditioned media were collected and subjected to ELISA to measure A β levels. (We had to start with 200K as the lowest seeding density in order to achieve reliable measurements of A β and control protein concentrations in the mouse primary neuronal cultures.) Our data showed that the levels of A β 40, A β 42, A β (40+42), and the ratio of A β 42:A β 40 markedly decreased with increases in cell density when compared to the lowest starting density of 200K ($p < 0.05$; Fig. 4A-4D). For example, comparing the plating of 600K to 200K, the A β 40 level decreased by 45.3% ($p < 0.01$; Fig. 4A), the A β 42 level declined by 74.9% ($p < 0.01$; Fig. 4B), the total A β level dropped by 51.2% ($p < 0.01$; Fig. 4C), and the ratio of A β 42:A β 40 decreased by 53.2% ($p < 0.01$; Fig. 4D). Thus, studies of H4-APP751 cells, CHO-APP751 cells, and mouse primary cortical neurons consistently revealed that the levels of A β 40, A β 42, total A β , and A β 42:A β 40 ratios were inversely correlated with cell density.

APP proteolytic processing varies depending on cell density

In the next set of experiments, we asked whether APP processing was altered with changes in cell density. H4-APP751 cells of different numbers were seeded on 6-well cell culture plates and allowed to grow for 48 hours. Cell lysates were collected and applied to Western blotting analysis. Samples of 50K and 100K densities were not applied due to their low protein concentrations for the Western blotting analysis. Equal amounts of protein were loaded onto the electrophoresis gel. We observed that full-length APP (or APP-FL) were represented with immature (*N*-glycosylated) and mature (*N*, *O*-glycosylated) forms of APP, which has been observed in previous reports [16, 17, 20, 22]. The levels of full-length APP and its CTFs (C83 and C99) were quantified and normalized to β -actin from the same samples [11, 16, 17] and compared to 150K versus the higher densities (200K to 400K). With increased cell density, full-length APP exhibited a trend toward decreased levels (Fig. 5A-5B). Next we measured the levels of APP proteolytic products APP-C83 and APP-C99, as well as the ratios of C83/APP and C99/APP. All of these measures varied inversely with cell density. For example, comparing the values at 400K to 150K, the APP-C83 level decreased by 65.6% ($p < 0.05$), the C83/APP ratio declined by 39.3%, the APP-C99 level decreased by 80.0% ($p < 0.01$), and the ratio of C99/APP decreased by 68.5% ($p < 0.01$) (Fig. 5C-5D). Thus, these data suggested that the APP processing activities of α - and β -secretase were attenuated with increases in cell density.

We also used the ratio of A β (40+42):C99 to assess γ -secretase processing of APP-C99. The levels of A β (40+42) (normalized to cell lysate protein concentrations) were divided by the C99 levels from the same samples. The ratios of A β (40+42):C99 at higher seeding densities were compared to those at 150K. We found that the level of A β (40+42):C99 initially increased at higher plating densities and reached a plateau at 300K (Fig. 5E). For example, at initial seeding of 400K, the ratio of A β (40+42):C99 increased by 276.3% versus 150K ($p < 0.01$; Fig. 5E). Thus, these data suggested that γ -secretase processing of APP is

potentiated with increases in cell density, which is different from the changes in α - and β -secretase processing of APP.

Next we utilized CHO-APP751 cells to validate our observation of cell density-dependent APP processing in H4 cells. CHO-APP751 cells of different numbers were seeded on 6-well cell culture plates and allowed to grow for 48 hours. Cell lysates were collected and applied to Western blotting analysis, excluding the samples from the densities of 50K and 100K due to low protein concentrations. Equal amount of protein from each sample was loaded onto the electrophoresis gel. Full-length APP, APP-C83 and APP-C99 levels were quantified and normalized to β -actin from the same samples. Full-length APP levels at higher densities were normalized to the values at 150K. Our data showed that at higher cell densities, full-length APP levels were markedly decreased versus those at 150K (Fig. 6A-6B). As an example, at the plating of 400K, full-length APP levels decreased by 54.2% compared to those at 150K ($p < 0.05$; Fig. 6A-6B).

We next measured levels of APP proteolytic fragments (C83 and C99), their ratios to full-length APP, as well as the ratio of $A\beta(40+42)$ to C99 in response to increasing cell density. Our data showed that both C99 and C83 levels and their ratios to APP markedly decreased with the increasing cell density (Fig. 6C-6D). For example, at the plating of 400K, the ratio of C83:APP decreased by ~80%, and the ratio of C99:APP decreased by ~60%, respectively, when compared to those at 150K ($p < 0.05$; Fig. 6C-6D). In contrast, at higher plating densities, the ratio of $A\beta(40+42)$:C99 had a trend to initially increase from 150K to 250K, then start to decrease > 250K (Fig. 6E). Collectively, our data show that APP processing and $A\beta$ generation are profoundly influenced by changes in cell density.

Validation of altered APP processing with changes in cell density

We next employed a fluorometric-based *in-vitro* assay for measuring α - and β -secretase activity levels to validate the effects of cell density on APP processing in H4-APP751 cells. We used 10cm-plates to obtain sufficient protein amounts for the assay, as recommended by the manufacturer. Based on the cell surface area, seeding densities of 0.5M (million), 1M, 1.5M, 2M and 3M in 10cm-plates are equivalent to those at 100K, 200K, 250K, 300K and 400K in 6-well plates, respectively. Cells were plated and harvested after 48 hours. Cell lysates were collected using the extraction buffer provided by the manufacturer and applied to the fluorometric assay. The fluorometric readouts were normalized to the cell lysate protein concentration of each sample. The normalized fluorometric levels at higher densities were compared to those at 100K. Our data showed that at higher seeding densities, both α - and β -secretase activities were attenuated when compared to those at 100K (Fig. 7A-7B). For example, at plating of 400K, β -secretase activity decreased by 45.8% versus 100K ($p < 0.01$; Fig. 7A), while α -secretase activity decreased by 34.5% versus 100K ($p < 0.01$; Fig. 7B). These data suggest that the effects of cell density on APP processing and $A\beta$ levels are modulated, at least in part by changes in the activity levels of α - and β -secretase.

DISCUSSION

AD pathogenesis is believed to be primarily triggered by $A\beta$ accumulation in the brain [1–5]. Thus, identifying factors that influence APP processing and $A\beta$ generation may help elucidate the etiology and pathogenesis of AD. Here, we tested cell density as a putative effector of APP processing using multiple cell models. We found that $A\beta$ levels and APP processing are strongly influenced by cell density. These findings have profound implications for thousands of previously published studies assessing APP processing and $A\beta$ generation *in vitro*. In addition, the data described here may be relevant to changes in APP processing and $A\beta$ generation in the AD brains as cell densities are altered with disease progression. First, we showed that both $A\beta_{40}$ and $A\beta_{42}$ levels vary inversely with cell

density. Second, we found that the ratios of A β 42:A β 40 are also reduced with increasing cell density. Importantly, the increase of A β 42:A β 40 ratio has been identified in more than 200 FAD mutations in APP, *PSEN1*, and *PSEN2*, which drive the aggregation of A β into neurotoxic oligomeric assemblies [1, 2]. Thus, in previous countless studies assessing the effects of various conditions and factors on A β levels and the A β 42:A β 40 ratio, study-to-study variations in cell density may have had profound and heretofore-unnoticed effects on the reported experimental conclusions.

Along with these findings, we show that A β levels and APP processing by proteolytic secretases are altered at different cell densities. Particularly, both α - and β -secretase processing of APP are potentiated, whereas γ -secretase processing appear to be attenuated with decreasing cell density overall (based on the ratio of A β /C99). In the neuronal cell model (H4-APP751), γ -secretase processing of APP reaches a maximum level at high densities (300K-400K), and becomes attenuated at lower densities (150K-250K). In the non-neuronal model (CHO-APP751), γ -secretase processing of APP achieves a maximum level at medium cell densities (250K), and is attenuated at both lower densities (150–250K) and higher densities (250K-400K).

PS1/ γ -secretase can process several other substrates apart from APP, e.g. molecules regulating cell-cell contact and signal transduction [23–26]. The cadherin molecules, primarily E(epithelial)- and N(neuronal)-cadherin, form cadherin-based adherens junctions (CAJs) at sites of cell-cell interaction and modulate fundamental intercellular functions, e.g. cell-cell interaction and development [26, 27]. When cell densities are high, PS1 is concentrated at the intercellular sites on the cell surface in CAJs and promotes CAJ assembly and cell-cell interaction [23]. However, when cells are exposed to stimuli, e.g. apoptosis and calcium influx, PS1/ γ -secretase cleaves full-length E-cadherin and a transmembrane E-cadherin CTF, derived from a metalloproteinase cleavage [26]. This cleavage leads to the disassembly of the CAJs, and increases soluble β - and α -catenin. The cytosolic pool of β -catenin has been indicated to be a key regulator of the Wnt signaling pathway [28, 29], which is associated with not only APP processing and A β metabolism [30], but also the pathogenesis of AD and other neurological disorders [29]. Moreover, over-expressing PS1 leads to elevated cell–cell interaction and aggregation, while this function is lost with the artificial loss-of-function *PSEN1* mutation, D257A [23]. Collectively, these multiple lines of evidence indicate that γ -secretase activity may be regulated by cell density and vice-versa, and support our current finding that γ -secretase processing of APP may also be dependent on cell density.

In summary, we show that A β 40 and A β 42 levels, total A β levels, and the ratios of A β 42:A β 40 vary inversely with cell density. Moreover, the activities of both α - and β -secretase were potentiated with decreases in cell density. Finally, based on the ratio of A β /C99, γ -secretase activity appears to be attenuated with decreases in cell density, although, additional studies will be necessary to confirm this. Overall, these findings carry profound implications for both previous and forthcoming studies investigating the effects of various conditions and genetic/chemical factors on APP processing and A β generation in cell-based systems. Moreover, it is interesting to speculate whether cell density changes *in vivo* may also affect APP processing and A β levels in the AD brain.

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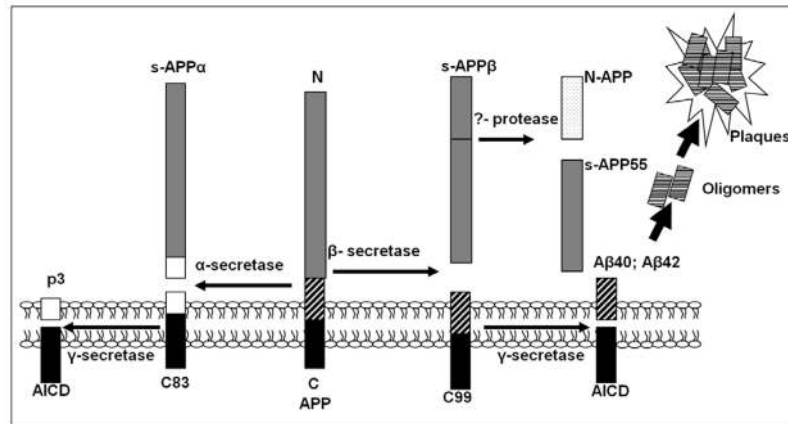


Figure 1. Summary of the proteolytic processing of APP

The early-onset familial AD gene *APP* encodes amyloid β -protein precursor, which generates $A\beta$ through the serial proteolytic cleavage by β - and γ -secretase. β -secretase cleavage produces the secreted, ~90kDa protein, sAPP β , and the β -carboxy-terminal fragment, β -CTF (or C99). sAPP β is the substrate of an unidentified secretase, which produces N-APP (containing the N-terminal 286 amino acids of APP; ~35kDa) and s-APP55 (~55kDa). C99 can be cleaved by γ -secretase and gives rise to $A\beta$ and AICD (APP intracellular domain). In contrast to this amyloidogenic process by β - and γ -secretase, APP undergoes an alternative cleavage pathway which precludes $A\beta$ generation. This pathway is initiated by α -secretase and produces sAPP α and the carboxy-terminal fragment, α -CTF (or C83). C83 can be further cleaved by γ -secretase to produce P3 and AICD.

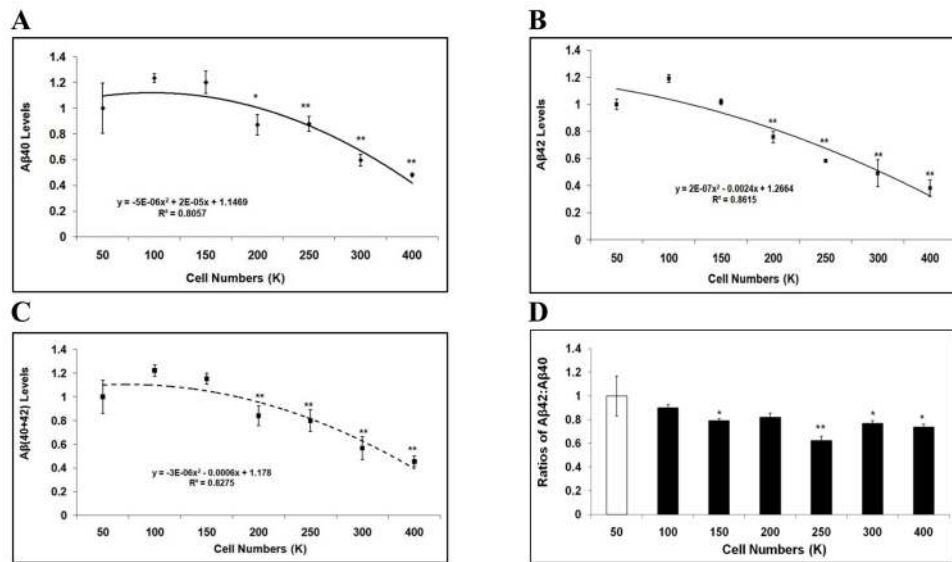


Figure 2. Aβ40 and Aβ42 levels vary depending on cell density in H4-APP751 cells
 Cells of different numbers were seeded on 6-well plates. Conditioned media and cell lysates were collected after 48h. Cell lysates were applied to BCA assay to calculate protein concentration. Conditioned media were applied to ELISA to calculate Aβ40 and Aβ42 levels, which were then normalized to protein concentration. The Aβ40 and Aβ42 values represented in the figure were normalized values. The values of Aβ40, Aβ42, Aβ(40+42), and the Aβ42:Aβ40 ratio from higher cell densities were compared to those from 100K. **A-C** At higher seeding densities (200K-400K), the levels of Aβ40 (**A**), Aβ42 (**B**), and Aβ(40+42) (**C**) significantly decreased versus 100K. **D**. The ratio of Aβ42:Aβ40 was also reduced with increasing seeding densities (n=3 for each treatment group; mean ± S.E.; *p<0.05; **p<0.01).

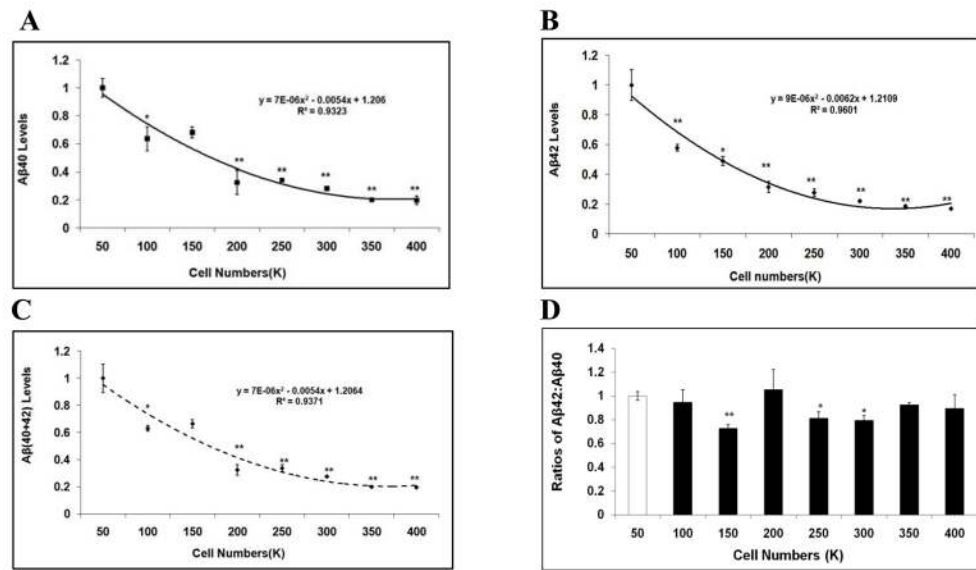


Figure 3. Aβ40 and Aβ42 levels vary depending on cell density in CHO-APP751 cells

Cells of different numbers were seeded on 6-well plates and harvested after 48h. Cell lysates were applied to BCA assay to calculate protein concentration. Conditioned media were applied to ELISA to calculate Aβ40 and Aβ42 levels, which were then normalized to protein concentration. The values of Aβ40, Aβ42, Aβ(40+42), and the Aβ42:Aβ40 ratios from higher cell densities were compared to the values at 50K. **AC** At higher seeding densities, the levels of Aβ40 (**A**), Aβ42 (**B**), and Aβ(40+42) (**C**) significantly decreased versus 50K. **D**. The ratio of Aβ42:Aβ40 was also reduced with increasing seeding densities (n=3 for each treatment group; mean ± S.E.; *p<0.05; **p<0.01).

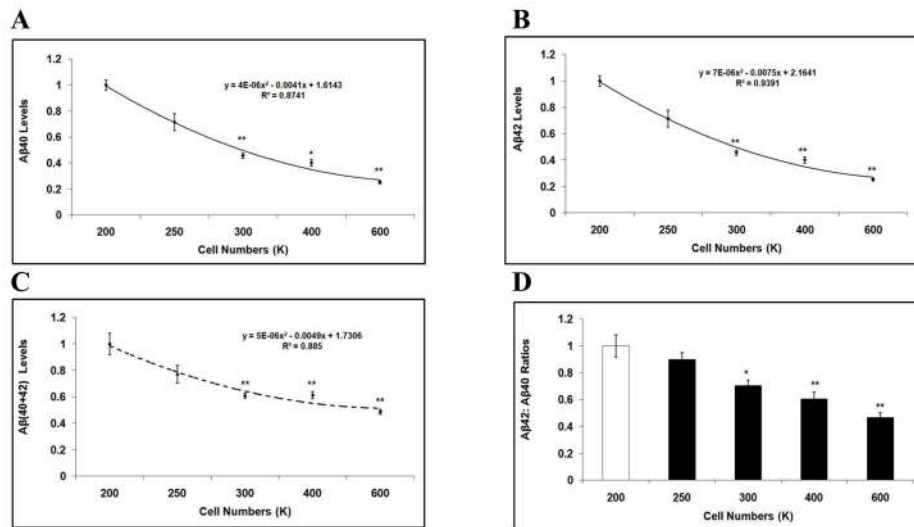


Figure 4. Aβ40 and Aβ42 levels vary depending on cell density in mouse primary cortical neurons

Mouse primary cortical neurons were seeded on poly-D-lysine/laminin-coated 24-well plates and harvested after 48h. Cell lysates were applied to BCA assay to calculate protein concentration. Conditioned media were applied to ELISA to calculate Aβ40 and Aβ42 levels, which were then normalized to protein concentration. The values of Aβ40, Aβ42, total Aβ, and the Aβ42:Aβ40 ratios from high seeding densities were compared to those from 200K. **A-D** At higher seeding densities (300K-600K), the levels of Aβ40 (**A**), Aβ42 (**B**), Aβ(40+42) (**C**), and the ratios of Aβ42:Aβ40 (**D**) significantly decreased compared to those at 200K (n=3 for each treatment group; mean ± S.E.; *p<0.05; **p<0.01).

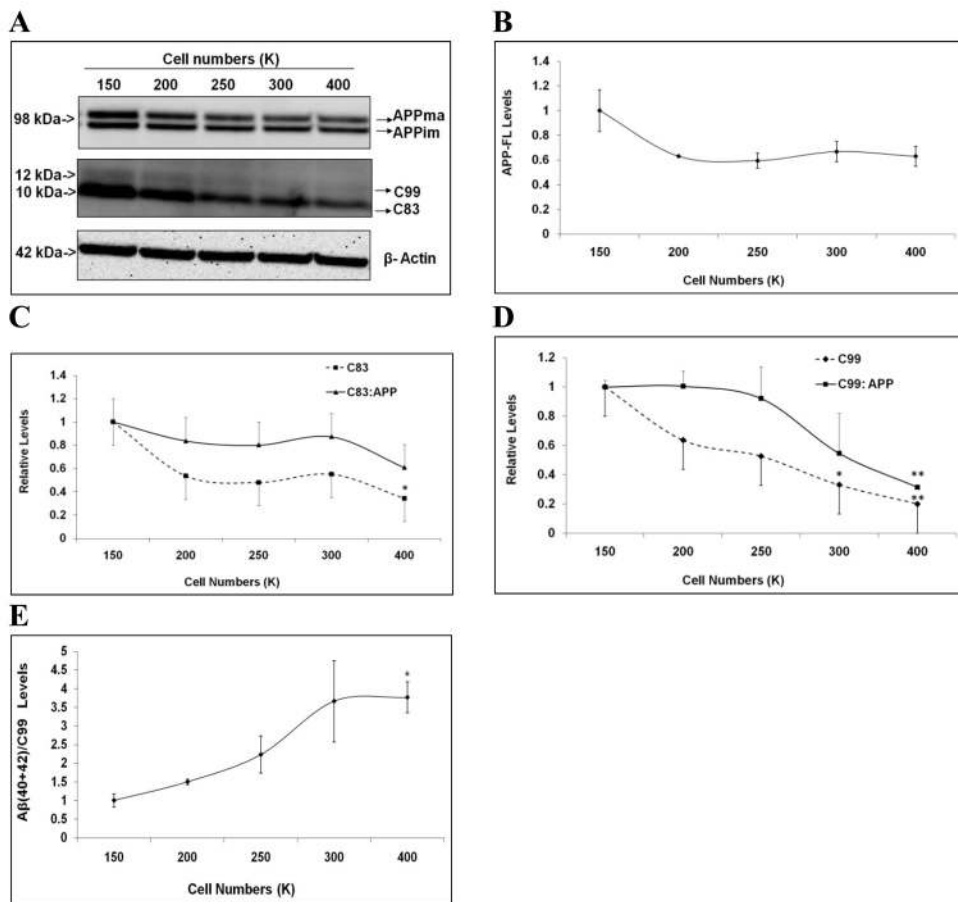


Figure 5. APP proteolytic processing varies depending on cell density in H4-APP751 cells
A-D. H4-APP751 cells were seeded on 6-well plates with different densities. Cell lysates were collected after 48h and applied to Western blotting analysis. The levels of full-length APP, APP-CTFs (C83 and C99), and their ratios to APP from higher seeding densities were compared to those at 150K. At higher cell densities, full-length APP levels had a trend to decrease versus 150K (**A-B**). The levels of C83 and C99, as well as their ratios to full-length APP decreased at higher seeding densities (**C-D**). **E.** The ratios of A β (40+42):APP-C99 were obtained by normalizing the levels of A β (40+42) to APP-C99 levels from the same samples, and the ratios from higher seeding densities were compared to those at 150K. The ratios of A β (40+42):APP-C99 increased with higher seeding densities (n=3 for each treatment group; mean \pm S.E.; *p<0.05; **p<0.01).

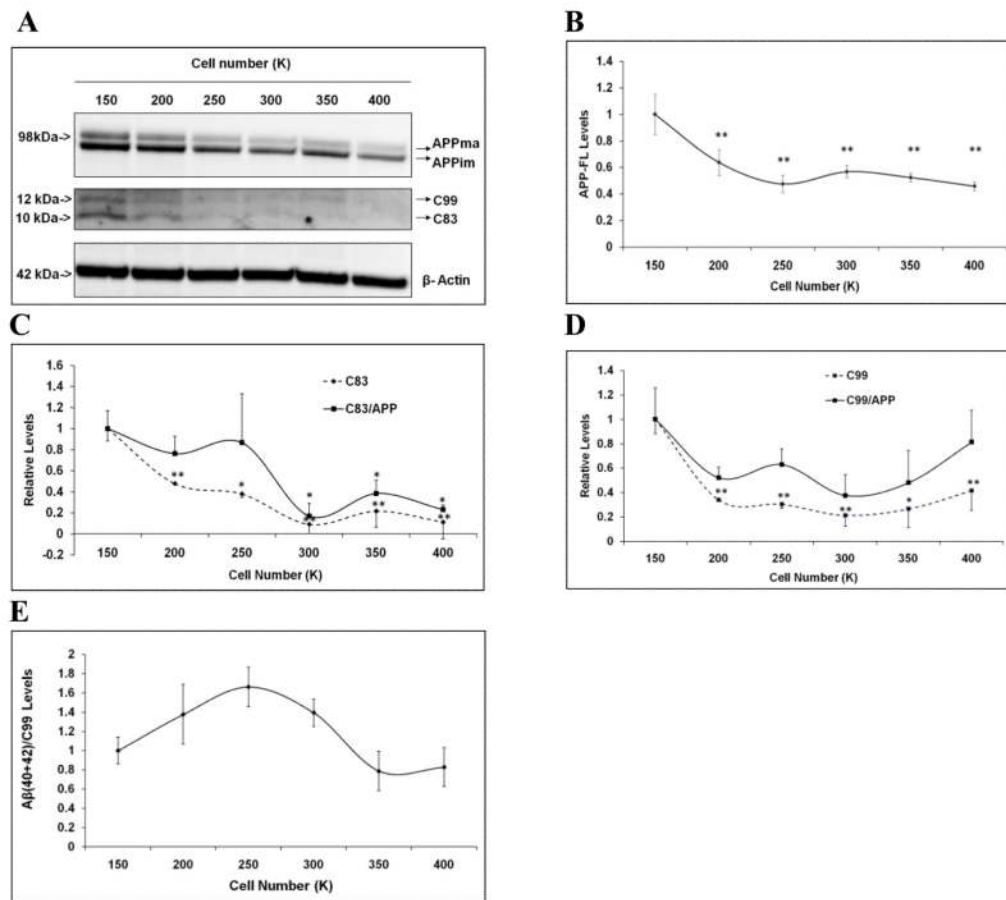


Figure 6. APP proteolytic processing varies depending on cell density in CHO-APP751 cells

A-D. CHO-APP751 cells were seeded on 6-well plates with different densities. Cell lysates were collected after 48h and applied to Western blotting analysis. The levels of full-length APP, APP-CTFs (C83 and C99), and their ratios to APP from higher seeding densities were compared to those at 150K. At higher cell densities (200K-400K), full-length APP levels significantly decreased versus those at 150K (**A-B**). At higher seeding densities, the levels of C83 and C99, as well as their ratios to full-length APP decreased versus 150K (**C-D**). **E.** The ratios of $A\beta(40+42)$:APP-C99 were obtained by normalizing the levels of $A\beta(40+42)$ to APP-C99 levels from the same samples. The ratios of $A\beta(40+42)$: APP-C99 initially increased with higher seeding densities (versus 150K), but then began to decrease with seeding densities > 250K (n=3 for each treatment group; mean \pm S.E.; *p<0.05; **p<0.01).

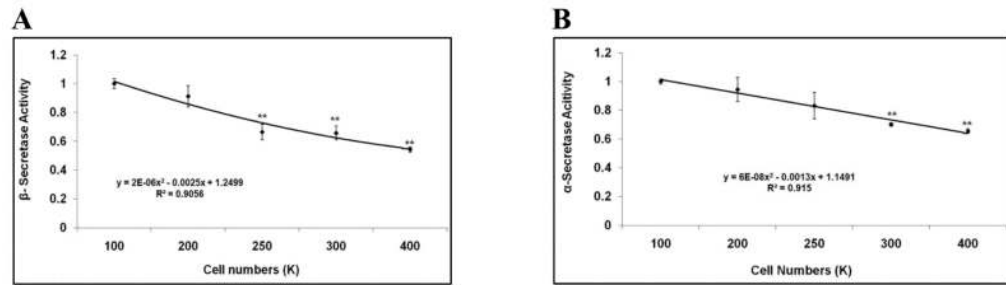


Figure 7. Validation of altered APP processing with changes in cell density

A-B, H4-APP751 cells were seeded on 10cm-plates with different densities (0.5M, 1M, 1.5M, 2M, and 3M; equivalent to the densities of 100K, 200K, 250K, 300K, and 400K in 6-well plates, respectively). Cell lysates were collected after 48h and were applied to the BCA assay to determine protein concentration and the fluorometric assay to measure α - or β -secretase activity. The fluorometric readout was normalized to the protein concentration for each sample. Normalized fluorometric values at higher cell densities were compared to those at 100K. At higher seeding densities, the APP processing activity of α - and β -secretase decreased when compared to those at 100K (n=3 for each treatment group; mean \pm S.E.; *p<0.05; **p<0.01).