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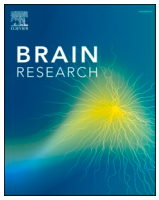

Research paper

Amyloidogenic and non-amyloidogenic pathways of amyloid precursor protein processing in oligodendrocytes

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ABSTRACT

Excessive accumulation of toxic amyloid-β (Aβ) species in the brain is a major pathological process triggering neurodegeneration in Alzheimer's disease (AD). Recent studies indicate that both neurons and glial cells, including oligodendrocyte lineages (OLs), contribute to brain homeostasis and affect AD pathology; however, the roles of oligodendrocyte precursor cells (OPCs) and oligodendrocytes (OLGs) in AD remain to be fully elucidated. This study examined Aβ production and related protein expression in primary cultured OLs. Primary cultured OLs produced Aβ40 and Aβ42 and expressed amyloid precursor protein (APP), β-secretase (BACE1) and γ-secretase (PS1) as well as α-secretase (ADAM10). OLGs express APP770 in addition to APP695. Treatment with a γ-secretase inhibitor reduced Aβ40 and Aβ42 production levels derived from OPCs/OLGs and suppressed OPC differentiation. Additionally, conditioned media from OLGs improved neuronal cell viability under oxidative stress and contained higher levels of sAPPα compared to OPCs. The neuroprotective effect of OLG was diminished by a sAPPα inhibitor, suggesting that OLG-derived sAPPα protects neurons under oxidative stress. These findings revealed that OLs produce pathogenic Aβ40/42 via the amyloidogenic pathway and secrete neuroprotective sAPPα via the non-amyloidogenic pathway. Elucidating the pathological shift from beneficial non-amyloidogenic to harmful amyloidogenic processes in OLs during AD onset and progression would provide crucial insights into novel therapeutic approaches.

1. Introduction

Increasing life expectancy has dramatically increased the prevalence of dementia, with an estimated 7 million people in Japan and 60 million globally projected to develop dementia by 2025. Alzheimer's disease (AD), the most common cause of dementia in later life, currently lacks disease-modifying treatments except anti-Aβ antibody therapy. The progressive accumulation of toxic Aβ species in the brain parenchyma and vasculature is known to be a major pathological process that triggers neuronal degeneration and synaptic loss in AD. Cerebral amyloid angiopathy (CAA), characterized by Aβ deposits in cerebral blood vessels, often coexists with AD, worsening its progression by impairing Aβ clearance and neurovascular function (Kisler et al., 2017).

Aβ is produced by the sequential cleavage of the amyloid precursor protein (APP), mediated through proteolytic processing by β- and γ-secretases. In the amyloidogenic pathway, β-secretase (BACE1) cleaves APP, releasing the extracellular soluble APPβ fragment (sAPPβ) and the membrane-anchored C-terminal fragment (CTFβ). γ-secretase, a multiprotein complex consisting of presenilin (PS), nicastrin, Aph1, and Pen2, cleaves CTFβ and generates Aβ peptides such as Aβ40 and Aβ42. In contrast, in the non-amyloidogenic pathway, α-secretase, including ADAM10, cleaves within the Aβ peptide domain and generates sAPPα and CTFα without producing Aβ peptides (Wang et al., 2017). Aβ40 and Aβ42 can aggregate into amyloid fibrils. Aβ42 is the major component of parenchymal plaques in the brain of patients with AD, while Aβ40 is the major component of cerebrovascular plaques in CAA (Haass and Selkoe, 2007). Therefore, these secretases have been identified as therapeutic targets for the treatment of AD/CAA.

In addition to neurons, glial cells, such as microglia, astrocytes, and oligodendrocytes (OLGs), contribute to brain homeostasis, and the dysregulation of these cell types affects the pathophysiology of AD. Although the roles of microglia and astrocytes in AD pathogenesis have

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been increasingly reported, the influence of oligodendrocyte lineages (OLs) on AD onset and progression remains unclear. OLGs play a critical role in maintaining the neural system through myelination-dependent and −independent pathways. Oligodendrocyte precursor cells (OPCs), which can differentiate into OLGs (Rivers et al., 2008), are widely distributed in the brain and, beyond serving as a reservoir for OLGs, have multiple aspects in regulating neurovascular function (Maki et al., 2013). Aβ induces OLG death through mitochondrial disruption and oxidative stress (Xu et al., 2001). Conversely, OLGs produce Aβ, and suppression of OLG-derived Aβ reduces the accumulation of Aβ in the brain and improves neurological dysfunction (Rajani et al., 2024; Sasmita et al., 2024). In addition, oligodendrocyte and myelin dysfunction drive Aβ accumulation in mouse models of AD (Depp et al., 2023). Taken together, a detailed investigation of the amyloidogenic and non-amyloidogenic pathways of amyloid precursor protein processing in OLs would be crucial. Hence, in this study, we investigated the expression levels of Aβ and its related proteins in primary cultured OLs and their significance for pathological and neuroprotective functions.

2. Results

2.1. OLGs produce more Aβ40 and Aβ42 than OPCs

In addition to neurons, microglia and astrocytes have been reported to generate Aβ40 and Aβ42 (Oberstein et al., 2015). Although a previous report showed that OLGs generate both Aβ40 and Aβ42 *in vitro* (Skaper et al., 2009), no reports have compared Aβ production between OPCs and OLGs using quantitative analysis. Therefore, we first quantified Aβ production in OPCs and OLGs in a primary culture system. Cultured OLs were obtained from the cerebral cortices of neonatal rats, as previously described (Yasuda et al., 2020). Using the same primary culture system, we have already confirmed the high purity of OLs through IHC, flow cytometry, and Western blot (Yasuda et al., 2020). We found that conditioned media from each cell type, collected after 24 h incubation in DMEM, contained Aβ40 and Aβ42 as measured by ELISA, and OLGs produced the higher amount of Aβ than OPCs (Fig. 1A–B, N = 4). After the treatment with DAPT, a γ-secretase inhibitor, the production levels of Aβ40 and Aβ42 in OLGs were significantly reduced without apparent cell death (Fig. 1C–E, N = 4). DAPT treatment did not consistently result in significant suppression of Aβ production in OPCs (data not shown). The underlying mechanism remains uncertain, but it is possible that OPCs and OLGs exhibit differences in the relative activity or regulation of β-secretase and γ-secretase.

2.2. OPCs and OLGs express APP, BACE1, PS1 and ADAM10

Aβ peptides are generated by the proteolytic cleavage of APP by β-secretase (BACE1) and γ-secretase (PS1). OLGs have been reported to express APP and BACE1 *in vitro* (Sasmita et al., 2024; Skaper et al., 2009), but detailed investigations of the expression levels of APP, BACE1 and PS1 in primary cultured OPCs/OLGs remain to be performed. Therefore, we evaluated these proteins using western blotting and semi-quantitative PCR. This confirmed that both OPCs and OLGs expressed APP695, BACE1, and PS1 at both protein and gene levels (Fig. 2A–B). These results suggest that OPCs and OLGs generate Aβ40 and Aβ42 from APP in a cell-autonomous manner. Furthermore, as OPCs differentiated into OLGs, mature OLGs began to express APP770, the expression level of which was negligible in OPCs and low in neurons (Fig. 2C, N = 3). Immunofluorescence analysis also showed that OPCs and OLGs expressed APP, BACE1 and PS1 (Fig. 3A–C, Supplementary Fig. S1A–C). Additionally, sAPPα is generated by the proteolytic cleavage of APP by α-secretase (ADAM10) via the non-amyloidogenic pathway. We found that OLGs also express higher levels of ADAM10 compared with OPCs and neurons as shown by western blot (Fig. 2A) and immunofluorescence analysis (Fig. 3D, Supplementary Fig. S1D). ADAM10 is also shown to be expressed in OLs at gene level (Tabula Muris et al., 2018).

2.3. DAPT suppresses OPC differentiation in a dose dependent manner

Since DAPT, a γ-secretase inhibitor, suppresses the production of Aβ in OPCs/OLGs as reported in neurons (Voytyuk et al., 2018), γ-secretase inhibitors may have promising therapeutic potential for patients with AD (Panza et al., 2010). Therefore, we investigated the effect of DAPT on

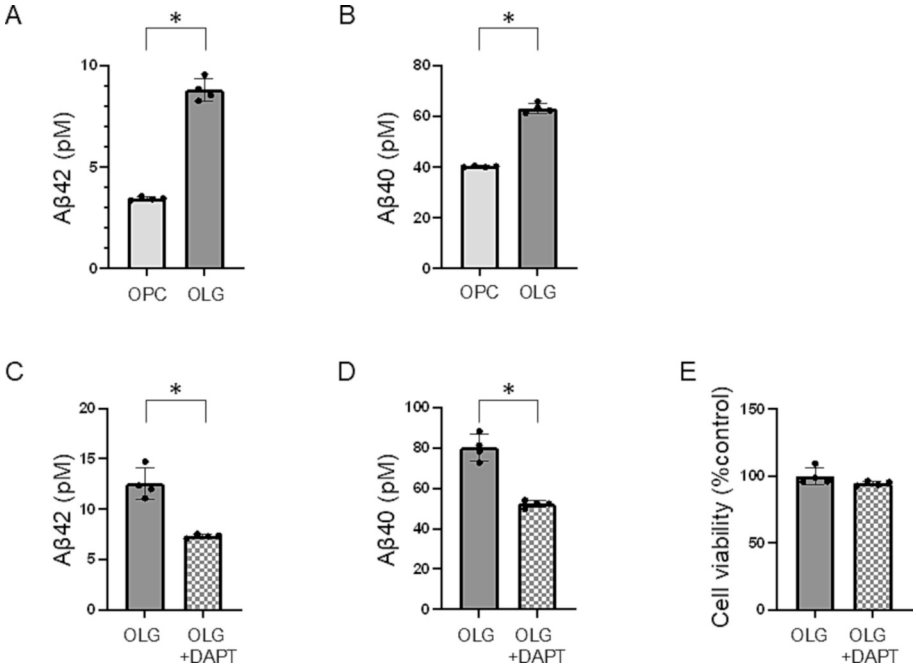

Fig. 1. OPCs and OLGs generate Aβ40 and Aβ42. Both OPC- and OLG- conditioned media, which were collected after 24 h incubation in DMEM, contain Aβ40 and Aβ42 measured by ELISA, and OLGs generate higher amounts of Aβ40 and Aβ42 compared with OPCs (A–B, N = 4). After the treatment with γ-secretase inhibitors (DAPT), the production levels of Aβ40 and Aβ42 were decreased significantly (C–D, N = 4). Results are expressed as mean ± SD per group. *p < 0.05.

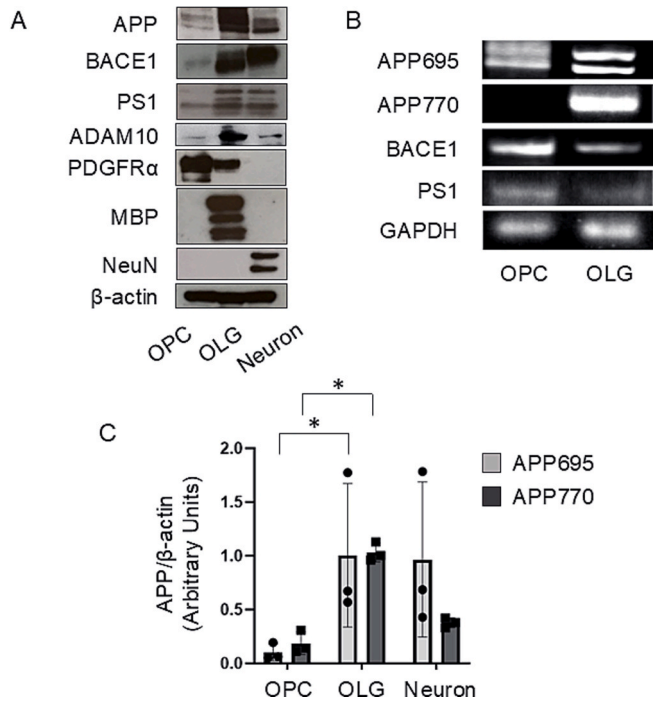

Fig. 2. OPCs and OLGs express APP, BACE1, PS1. Both OPCs and OLGs expressed APP695, BACE1, PS1, and ADAM10, as shown by western blot (A) and semi-quantitative PCR (B), respectively. Moreover, OLGs expressed higher levels of APP770 than OPCs and neurons. The histogram shows the quantitative analysis of APP expression levels normalized by internal controls (β-actin) in western blotting (C, N = 3). Results are expressed as mean ± SD per group. *p < 0.05.

OPC differentiation, which is a pivotal factor in AD pathogenesis (Desai et al., 2010). OPCs were cultured in differentiation media containing vehicle or DAPT for 7 days. DAPT-treated OPCs showed a lower expression ratio of MBP (a marker for mature OLGs) per PDGFR-α (a marker for immature OPCs) (Fig. 4A–B, N = 3), compared with vehicle-treated OPCs by western blotting. DAPT-treated OPCs also showed shorter and fewer processes in microscopic images (Fig. 4C). These results suggest that γ-secretase inhibitor (DAPT) suppresses OPC maturation, although it reduces both Aβ40 and Aβ42 levels derived from OLGs.

2.4. OLGs-derived sAPPα protects neurons under oxidative stress

As OLGs produce higher levels of Aβ40 and Aβ42 than OPCs, OLGs may have a negative impact on AD pathology, such as neuronal degeneration and synaptic damage, through excessive Aβ production. On the other hand, OLs may produce neuro-supportive sAPPα as OLGs express higher levels of ADAM10 than OPCs and neurons as shown above. As expected, we found that OLG-CM contained higher levels of sAPPα than OPC-CM and neuron-CM (Fig. 5A, N = 3). To investigate the effects of OPC/OLG-derived factors on neurons, we performed media transfer experiments. SH-SY5Y neuronal cells were cultured under oxidative stress (50 μM H_2O_2) with OPC/OLG-derived conditioned media (OPC/OLG-CM) and basal media (DMEM) for 24hr, and then the viability of SH-SY5Y neuronal cells was assessed by WST assay. Notably, OLG-CM, but not OPC-CM (data not shown), alleviated the decrease in viability of basal media-treated SH-SY5Y neuronal cells under oxidative stress (Fig. 5B, N = 5–6). These results suggest that OLGs have more neuroprotective effects under oxidative stress than OPCs. Furthermore, the neuroprotective effect of OLG was diminished by an sAPPα inhibitor, GI254023X (5 μM), suggesting that OLG-derived sAPPα protects neurons under oxidative stress (Fig. 5C–D, N = 5).

2.5. OLG-induced neuroprotection under oxidative stress is mediated by the MEK/ERK pathway

To determine the underlying mechanisms of OLG-mediated neuroprotective effects, we investigated the MEK/ERK signaling pathway. OLG-CM increased the level of phosphorylated extracellular signal-regulated kinase (ERK) 1/2 under H_2O_2-induced oxidative stress, which was blocked by co-treatment with U0126, a MEK/ERK inhibitor (Fig. 6A–B, N = 6). Correspondingly, U0126 canceled the protective effects of OLG-CM in neurons under oxidative stress (Fig. 6C, N = 4). These results suggested that OLG-induced neuroprotection under oxidative stress was mediated by the MEK/ERK pathway.

3. Discussion

In this study, we used primary culture to demonstrate the following findings: 1) Both OPCs and OLGs generate Aβ40, Aβ42, and sAPPα to a higher extent in OLGs by a cell-autonomous manner as they express APP, BACE1, PS1, and ADAM10 at gene and protein levels. 2) OLGs, not OPCs, express APP770 in addition to APP695. 3) The γ-secretase inhibitor reduces the Aβ production from OPCs/OLGs while suppressing OPC differentiation. 4) OLGs, but not OPCs, may support neurons partially via sAPPα secretion (Fig. 7).

OLs constitute a substantial population of glial cells and are the largest type of non-neuronal cells in the brain (von Bartheld et al., 2016). As OLGs produce higher levels of Aβ peptides and sAPPα than OPCs, these OLG-derived factors may have pivotal effects on AD pathology. Considering that excessive Aβ oligomers are harmful (Butterfield et al., 2001; Ganguly et al., 2017), while sAPPα is generally beneficial for neuronal and synaptic function (Mockett et al., 2017), OLGs could have dual and opposing roles in AD-related pathological conditions, depending on the context. We found that normal OLGs protect damaged neurons under oxidative stress partially through the secretion of sAPPα. However, injured OLGs in patients with AD might transform into a different phenotype, which generates more toxic Aβ oligomers and a lower amount of sAPPα, leading to the acceleration of neuronal degeneration in AD (Depp et al., 2023). The dual role of OLs in AD suggests they can both support neurons and contribute to neurodegeneration. Early in the disease, OLG-derived sAPPα may promote neuronal survival, but as OL function declines, increased Aβ production may drive plaque formation and toxicity. This shift from non-amyloidogenic to amyloidogenic processing may be a key transition in AD, highlighting the need for strategies to enhance OL-mediated neuroprotection while reducing Aβ production.

Although recent studies have revealed that diverse phenotypes of microglia and astrocytes contribute to AD progression in both detrimental and restorative ways (Dzamba et al., 2016), detailed information regarding phenotypic changes in OLs in AD is scarce. Elucidating these phenotypic changes in OLs concerning APP processing will provide crucial insights into novel therapeutic approaches for AD. Moreover, understanding the alterations in OPC/OLG-derived Aβ strains (e.g., monomer vs. oligomer, Aβ40 vs. Aβ42) and other APP metabolites (such as AICD, sAPPβ, NFTα, and NFTβ) under pathological conditions (e.g., glutamate stress, abnormal neuronal activity, hypoxia) would be a promising target for future research. In particular, Aβ oligomers (AβOs) are highly toxic species that induce synaptic dysfunction, neuroinflammation, and tau abnormalities, via various receptors including NMDA receptors, leading to oxidative stress, Ca^{2+} dyshomeostasis, and mitochondrial dysfunction (Araki, 2023; Viola and Klein, 2015). Given that AβOs may exert greater toxicity than Aβ fibrils, investigating the role of oligodendrocyte-derived AβOs in AD could provide new insights into disease mechanisms and therapeutic strategies.

The APP gene can be alternatively spliced to produce different isoforms, including APP695, APP751, and APP770 (Selkoe, 2001). APP770 and APP751 contain a serine protease inhibitory domain known as the Kunitz protease inhibitor (KPI) domain, whereas APP770 contains a KPI

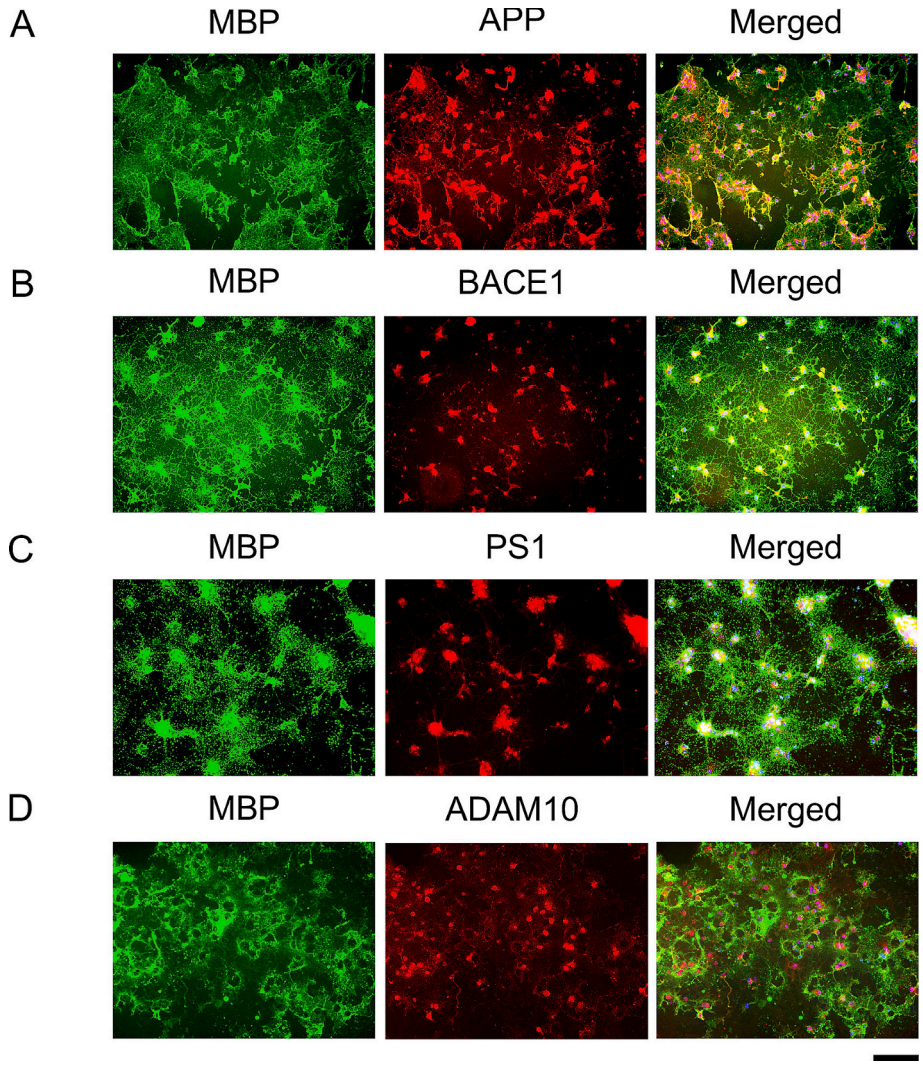

Fig. 3. Immunofluorescence of cultured OLGs. The OLGs were stained for MBP (an OLG marker) with APP (A), BACE1 (B), PS1 (C), and ADAM10 (D). Scale bar indicates 100 μm. These data demonstrate that cultured OLGs express APP, BACE1, PS1, and ADAM10.

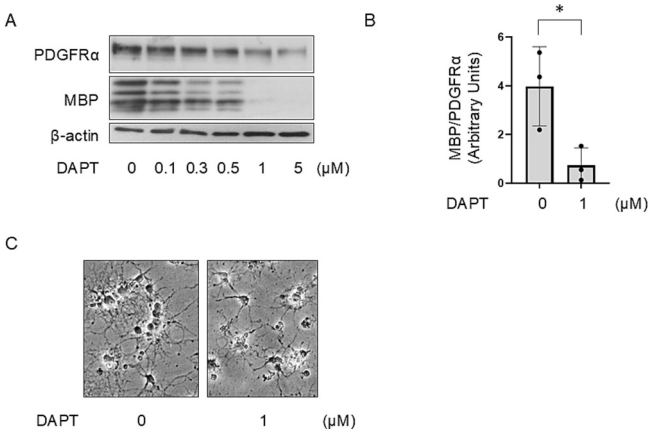

Fig. 4. DAPT suppresses OPC differentiation in a dose-dependent manner. OPCs were treated with DAPT in the differentiation medium for 7 days. OPC differentiation was inhibited in a dose-dependent manner, as shown by the decreased expression ratio of MBP (mature OLG) per PDGFRα (immature OPC) in western blotting (A–B, N = 3) and microscopic observations (C). The results are expressed as mean ± SD per group. *$p < 0.05$.

domain plus an OX2 domain (Mahdi et al., 1995). OLGs produce more Aβ40 and Aβ42 than OPCs, likely due to increased expression of APP770, which enhances amyloidogenic processing. Additionally, variations in β- and γ-secretase activity, intracellular trafficking, and proteolytic processing may contribute to the observed differences in Aβ levels. In the brain, neurons generally express APP695 (Wertkin et al., 1993), whereas endothelial cells express APP770 (Kitazume et al., 2010). The KPI-APP derived from endothelial cells regulates platelet aggregation and hemostasis, although the roles of OX2 remain poorly understood. Additionally, neuronal expression of KPI-APP exhibits a substantial amyloidogenic potential (Ho et al., 1996), and the protein and mRNA levels of APP-KPI are elevated in the AD brain, potentially contributing to Aβ accumulation (Bordji et al., 2010). According to our findings, as OPCs differentiate into OLGs, mature OLGs express APP770, while its expression levels are negligible in OPCs and low in neurons. APP770, which contains a KPI domain, may promote amyloidogenic processing and Aβ production, potentially contributing to Aβ accumulation. Additionally, its involvement in coagulation and inflammation may impact cerebrovascular integrity and neuroinflammation in AD. The detailed roles of OLG-derived APP-KPI and OX2 under normal and pathological conditions should be clarified in future studies.

Since reducing Aβ burden is fundamental for AD treatment, γ-secretase inhibitors are considered a therapeutic option to decrease Aβ accumulation. However, γ-secretase inhibitors block not only the

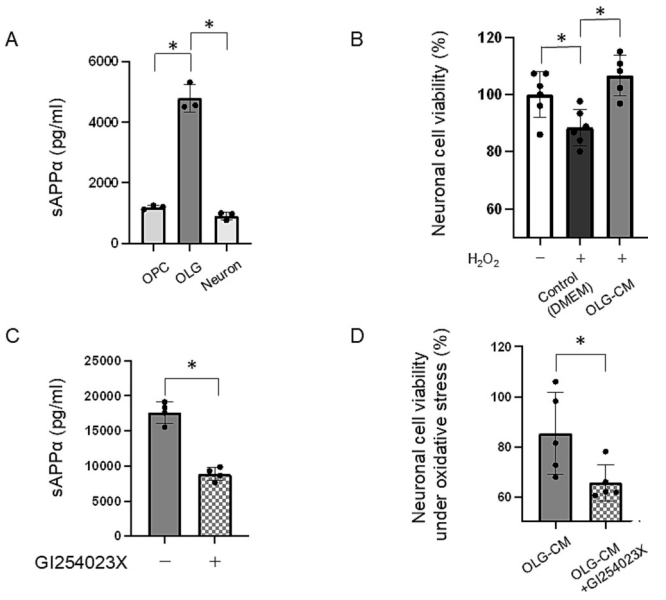

Fig. 5. OLGs may protect neurons under oxidative stress via sAPPα secretion. OLG-derived conditioned media (OLG-CM) protects damaged SH-SY5Y neuronal cells under oxidative stress with 24 h exposure of 50 μM H_2O_2 (A, N = 6). OLGs produce a higher amount of sAPPα compared with OPCs (B, N = 3). GI254023X, an sAPPa inhibitor, suppresses the production of sAPPa from OLGs (C, N = 4) and reduces neuronal cell viability under oxidative stress (D, N = 5). Results are expressed as mean ± SD per group. *p < 0.05.

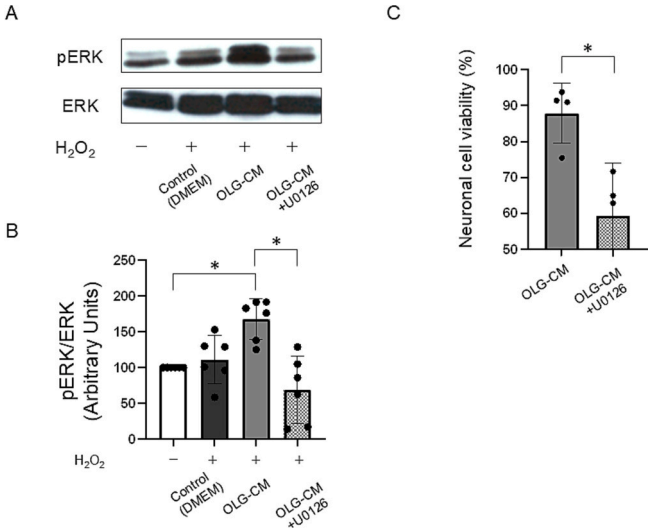

Fig. 6. OLG-induced neuroprotection under oxidative stress is mediated by the MER/ERK pathway. SH-SY5Y neuronal cells were incubated with the control or OLG-derived conditioned media. Western blotting was performed to evaluate the levels of phosphorylated extracellular signal-regulated kinase (ERK) using anti-phosphorylated ERK (p-ERK) 1/2 or anti-ERK 1/2 antibodies. OLG-CM increased the level of phosphorylated extracellular signal-regulated kinase (ERK) 1/2 under H2O2-induced oxidative stress, which was blocked by co-treatment with the MEK/ERK inhibitor U0126 (A–B, N = 6). Correspondingly, U0126 canceled the protective effects of OLG-CM in neurons under oxidative stress (C, N = 4).

amyloidogenic pathway but also other pathways, such as Notch, ErbB, and N-cadherin, which are essential for maintaining the pool of various stem cells and regulating cell fate appropriately (Maarouf et al., 2008). For example, Notch, a type I transmembrane receptor, releases its intracellular domain (NICD) after cleavage by the γ-secretase complex. Subsequently, NICD translocates to the nucleus and activates the transcription of Notch target genes, promoting oligodendrogenesis in progenitor cells and OPC maturation via Deltex1 (Cui et al., 2004). ErbB signaling has also been reported to contribute to the generation and regulation of oligodendrocyte lineage cells (Brinkmann et al., 2008). In our study, we showed that the γ-secretase inhibitor DAPT decreased both Aβ40 and Aβ42 levels derived from OPCs and OLGs, while suppressing OPC maturation in a dose-dependent manner. The underlying mechanisms of γ-secretase inhibitor-mediated suppression of OPC maturation may be attributable to the inhibition of oligo-supportive signaling pathways, including Notch and ErbB. Inhibiting γ-secretase may disrupt these pathways, impairing OPC maturation. Previous studies have demonstrated that γ-secretase inhibitors exhibit limited efficacy in clinical trials due to severe side effects, including intestinal toxicity, immune dysregulation, and Notch signaling disruption (Panza et al., 2010). Our findings suggest that, in addition to these well-established limitations, γ-secretase inhibition disrupts OPC maturation, which could impair remyelination and neurovascular function in AD pathology.

In conclusion, we demonstrated that OLs produce Aβ40/42, pathogenic proteins in AD, via the amyloidogenic pathway, and secrete sAPPα, which has neuroprotective and neurogenic functions, via the non-amyloidogenic pathway. Given that oligodendrocyte lineage cells play more diverse and critical roles in regulating the neurovascular system in the brain than previously understood, these findings may shed light on previously unrecognized aspects of AD pathophysiology. Further studies are needed to clarify how oligodendrocyte-derived Aβ peptides and related factors influence AD development and to establish novel therapeutic strategies against the disease.

4. Materials and methods

To investigate the roles of OLs in APP processing and their contributions to AD pathology, we conducted a series of *in vitro* experiments using primary OLs. Below, we describe the detailed methodologies employed in these investigations.

4.1. Primary glial cell cultures

Primary OPCs and OLGs were prepared from cerebral cortices of 1–2 day old Sprague-Dawley rats. Isolated cerebral cortices were incubated for 15 min at 37°C in Lebovitz, 0.25 % trypsin, and deoxyribonuclease. Dulbecco's Modified Eagle's medium (DMEM) containing 20 % fetal bovine serum (FBS) was added to the cortical cells and mixed until the cells broke into single cells. After putting the suspended cells through a 40 μm cell strainer, they were centrifuged at 1000 rpm for 5 min. The supernatants were then removed, and the pellets were suspended in DMEM containing 20 % FBS. Subsequently, the dissociated cortical cells were plated in poly-d-lysine-coated flasks and cultured in DMEM supplemented with 20 % FBS and 1 % penicillin/streptomycin. The medium was changed every 2 days and mixed glial cells, including OPCs, astrocytes and microglia, were grown. After the cells were confluent, the flasks were shaken for 1 h on an orbital shaker (220 rpm) at 37°C to remove the microglia. The medium was replaced with fresh medium and shaken overnight. The medium was then collected and plated on non-coated tissue culture dishes for 1 h at 37 °C to eliminate possible contamination by astrocytes and microglia. After 1 h, the non-adherent cells were collected, passed through a 40 μm cell strainer, spun at 1000 rpm for 5 min, and seeded at 2.0×10^4 cells/mL in Neurobasal media containing glutamine, 1 % penicillin/streptomycin (PS), 10 ng/mL platelet derived growth factor (PDGF)-AA, 10 ng/mL fibroblast growth factor (FGF)-2, and 2 % B27 supplement onto poly-l-ornithine-coated plates. To obtain OLGs, OPCs were cultured in differentiation media (DMEM containing 1 % PS, 10 ng/mL ciliary neurotrophic factor and 15 nM Triiodothyronine) for 7 days.

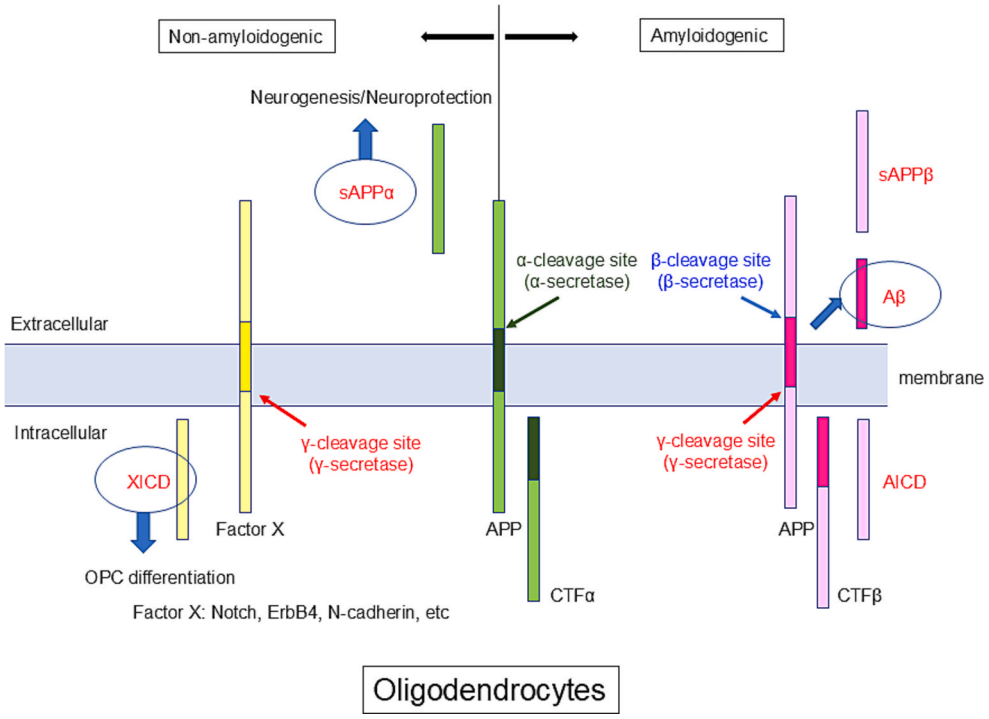

Fig. 7. Amyloidogenic and non-amyloidogenic pathways of amyloid precursor protein processing in oligodendrocytes. Oligodendrocytes produce pathogenic Aβ via an amyloidogenic pathway and secrete neuroprotective sAPPα via a non-amyloidogenic pathway through an autocrine manner. γ-secretase cleaves not only APP but other transmembrane factors, such as Notch, ErbB, and N-cadherin, which are related to OPC differentiation.

4.2. Neuronal cell culture

Cortical neuronal cultures were prepared from 17th-day of pregnant Sprague Dawley rat embryos (Shimizu Laboratory Supplies), as described previously (Kinoshita et al., 2019). Briefly, the cortices were dissected and dissociated. Cells were plated on dishes coated with poly-D-lysine in DMEM, containing 5 % heat-inactivated fetal bovine serum, and 1 % penicillin/streptomycin, at a density of 200,000–250,000 cells/cm^2. After seeding for 24 h, the medium was changed to NB medium containing 0.5 mM glutamine, 1 % penicillin/streptomycin, and 2 % B27 supplement. Cultured neurons were used for experiments 14 days after seeding.

4.3. ELISA

The concentrations of Aβ40, Aβ42 and sAPPα in the condition media were measured by the Human/Rat β-Amyloid 40 ELISA high sensitive kit (Wako), Human/Rat β-Amyloid 42 ELISA high sensitive kit (Wako), and sAPPα ELISA high sensitive kit (IBL) according to the manufacturer's instructions. To minimize the influence of culture conditions, conditioned media (CM) was prepared following a standardized protocol (Maki et al., 2018). Cells were washed with PBS, incubated in basal medium without FBS or growth supplements for 24 h, and centrifuged at 10,000 × g for 5 min at 4 °C to remove debris. While differences in cell derivation may contribute to variations in Aβ production and sAPPα levels, this approach ensures comparable conditions for CM analysis.

4.4. Western blot analysis

Samples were collected from the cell lysates. Each sample was separated by SDS-Page on a 5–20 % acrylamide gel (ATTO). The proteins were transferred to a Polyvinylidene Difluoride (PVDF) membrane (Millipore) and blocked with 5 % fat-free milk for 1 h. Transferred membranes were washed three times for 15 min by TBS-T buffer (200 mM Tris, 1,370 mM NaCl, and 1 % tween), followed by the blotting with the designated first antibodies at 4 °C overnight. The membranes were washed three times with TBS-T buffer for 15 min each, and blotted with secondary antibodies dissolved in TBS-T at room temperature for 1 h. After three 15-min washes with TBS-T buffer, the membranes were visualized using ECL western blotting detection reagents (GE Healthcare) or Chemi-Lumi One Super (Nacalai tesque).

4.5. Immunofluorescence

Cells were washed with ice-cold phosphate-buffered saline (PBS), followed by incubation with 4 % paraformaldehyde for 15 min. After three washes with PBS containing 0.1 % Triton X-100, the cells were incubated with 3 % bovine serum albumin in PBS for 1 h. The cells were then incubated with primary antibodies against MBP (Thermo Scientific, 1:200) with APP (IBL), BACE1 (Cell Signaling Technology, 1:200), PS1 (Millipore, 1:200), and ADAM10 (Sigma, 1:200) respectively at 4 °C overnight. After removing the primary antibodies with PBS, the cells were incubated with secondary antibodies for 1 h at 22 ± 2°C. Finally, the nuclei were counterstained using VECTASHIELD with DAPI (Vector Laboratories). Images were analyzed using a fluorescence microscope (BZ-X 710; KEYENCE) interfaced with a digital charge-coupled device camera and an image analysis system.

4.6. Antibodies

The following antibodies were used for western blot analysis: APP(C) (IBL, 2 μg/mL), ADAM10 (Sigma, 1:1000), BACE1 (Cell Signaling Technology, 1:1000), PS1 (Millipore, 1:500), PDGFR-α (R&D systems, 1:1000), MBP (Thermo, 1:500), NeuN (Millipore, 1:1000), Erk (Cell Signaling Technology, 1:1000), pErk (Cell Signaling Technology, 1:1000), β-actin (Sigma, 1:5000).

4.7. Semi-quantitative PCR

Total DNA was extracted from both OPCs and OLGs using the RNeasy

Mini Kit (QIAGEN), and cDNA was synthesized from total DNA using the First-Strand cDNA Synthesis Kit (GE Healthcare). The cDNA was used as a template for semi-quantitative PCR with gene-specific primers, Taq polymerase (E × Taq (Takara), or PrimeSTAR MAX (Takara). The primers used for the semi-quantitative PCR were as follows:

App695; GCGGATGGACGTTTGTGAGA (forward), CAGGTACTTGTCGACTGCGT (reverse). App770; ACTGAGTCTGTGGAGGAGGT (forward), CAGGTACTTGTCGACTGCGT (reverse). Bace1, CAGTCCTTCCGCATCACCAT (forward), AACATGAAGAGGCGCAGAT (reverse). Presenilin1; TGGTGTGGTCGGGATGATTG (forward), GCTTCTGGGTCTCCTTCAGC (reverse). GAPDH; ATTTGGCCGTATCGGACGCC (forward), CCAGCCTTCTCCATGGTGGT (reverse).

4.8. γ-secretase inhibitor treatment

DAPT, γ-secretase inhibitor, was added to OPCs and OLGs in different concentrations (0, 0.1, 0.3, 0.5, 1, 5 μM). After incubating at 37 °C for 24 h, we measured the expression levels of Aβ40 and Aβ42 in the media derived from both OPCs and OLGs. Moreover, DAPT was added to OPCs in differentiation media for 7 days to evaluate its effect on OPC differentiation capacity.

4.9. Media transfer experiment under oxidative stress

The conditioned media (CM) was prepared as previously described (Maki et al., 2018). Cell cultures were washed with PBS and maintained in basal medium without FBS or growth supplements for 24 h. The CM was then collected and centrifuged at 10,000 × *g* for 5 min at 4 °C to remove the cells and debris. The CM was stored at −80 °C until use. SH-SY5Y neuronal cells were treated with 50 μM H_2O_2 for 24 h in DMEM, OPC-, or OLG-conditioned media. After incubation, the Premix WST-1 Cell Proliferation Assay System (Takara) was used to evaluate cell viability.

4.10. Assessment of cell proliferation using the WST assay

Cell proliferation and survival were assessed using the WST reduction assay kit (Cell Counting Kit-8; Dojindo). The WST assay is a sensitive colorimetric method used to determine cell viability. Cells were incubated with 10% WST solution for 1 h at 37 °C, and then the absorbance of each medium (DMEM, OPC CM, and hypoxic OPC CM) was measured using a microplate reader with a test wavelength of 450 nm and a reference wavelength of 630 nm.

4.11. Statistical analysis

Experiments were performed 3– 4 times independently. All quantitative data were analyzed using GraphPad Prism 6.0. (Graph Pad Software Inc., La Jolla, CA, USA). Quantitative data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test. The Mann–Whitney *U* test was used to explore differences in non-normally distributed variables between the two groups. All values are expressed as mean ± standard deviation (SD). Statistical significance was set at $P < 0.05$.

Ethical Approval

All procedures were performed in accordance with the guidelines for animal experimentation from the ethical committee of Kyoto University. The institutional Animal Care Committee of the Kyoto University Graduate School of Medicine approved all protocols.

CRediT authorship contribution statement

Misaki Hida: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ken Yasuda:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Masaru Toyokawa:** Visualization, Methodology, Investigation, Formal analysis, Data curation. **Megumi Asada-Utsugi:** Writing – review & editing, Supervision, Formal analysis, Data curation, Conceptualization. **Shintaro Toda:** Supervision, Conceptualization. **Narufumi Yanagida:** Supervision, Conceptualization. **Ryosuke Takahashi:** Supervision, Funding acquisition. **Ayae Kinoshita:** Supervision, Funding acquisition, Conceptualization. **Takakuni Maki:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions Statement

M.H. conducted experiments, analyzed the data, designed the study and drafted the manuscript; K.Y. and M.T. conducted experiments, analyzed the data. K.Y., M.A-U., S.T, N.Y. conceived the experiments and made critical revision of the manuscript for important intellectual content; A.K. and R.T. supervised and made critical revision of the manuscript for important intellectual content. T.M. designed the study, handled funding, supervised overall experiments and drafted the manuscript. All authors reviewed the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brainres.2025.149601.

Data availability

Data will be made available on request.

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