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Studies On The Novel Function Of Amyloid Precursor Protein In Glial Differentiation Of Neural Stem Cells

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STUDIES ON THE NOVEL FUNCTION OF AMYLOID PRECURSOR PROTEIN IN GLIAL DIFFERENTIATION OF NEURAL STEM CELLS

by

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ABSTRACT

Although amyloid β (Aβ) deposition has been a hallmark of Alzheimer's disease (AD), the physiological function of amyloid precursor protein (APP) is not clear. Our results suggested that high concentration of APP induces glial differentiation while physiological level of APP promotes migration and differentiation of neural stem cell (HNSC). HNSCs were mainly differentiated into astrocytes when they are transplanted into APP transgenic mouse brain or treated with a high concentration of secreted-type APP (sAPP) in culture.

Staurosporine (STS) induced a distinctive astrocytic morphology in NT-2/D1 neural progenitor cells with expressions of APP and astrocyte-specific markers, glial fibrillary acidic protein (GFAP), aspartate transporter, and glutamate transporter-1. Expression of APP is correlated with GFAP expression in both mRNA and protein level in this experiment. Inhibition of APP expression by RNA interference (RNAi) or treatment with MEK1 inhibitor (PD098059), which reduces APP expression by suppressing ERK phosphorylation, abolished GFAP expression. These results indicate that STS induces glial differentiation of neuronal progenitor cells by increasing APP levels through activation of ERK pathway.

We also found that APP-induced glial differentiation of neural progenitor NT-2/D1 cells is mediated by activation of IL-6/gp130 and notch signaling pathway. Treatment of APP activated IL-6/gp130 signal pathway via protein-protein interaction between APP and

gp130 and it increased the gene expressions of CNTF, gp130 and JAK1, and phosphorylation of STAT3 while gene silencing of CNTF, JAK1 or STAT3 by RNAi, or treatment the cells with antibodies recognizing gp130 suppressed GFAP expression, indicating these molecules are crucial for APP-induced glial differentiation. Thus treatment of sAPP may promote glial differentiation of neural progenitor cells by activation of IL-6/gp130 signaling cascade.

Treatment of sAPP increased the generation of notch intracellular domain as well as gene expression of Hes1 but did not change expression levels of notch or its ligands. We also found protein-protein interaction of APP and notch using immunoprecipitation suggesting that glial differentiation of NT-2/D1 cells is mediated by the physical interaction between APP and notch. N-terminal domain of APP (1-205 a.a.) alone can bind to notch and activate these signaling cascade in NT-2/D1 cells. Thus, APP may induce glial differentiation through activation of IL-6/gp130 and notch signal cascade by binding with its N-terminal domain. Taken together, our results suggest that APP regulates neural stem cell (NSC) differentiation through IL-6/gp130 and notch signaling pathway. Furthermore, the activation of both glial differentiation mechanisms may be necessary to potentiate APP-induced glial differentiation of NSC.

Altered APP metabolism in Down syndrome and Alzheimer's disease may accelerate premature glial differentiation of NSCs, resulting in gliosis found in these diseases. Although it is not clear that how adult neurogenesis contributes to maintain normal brain function, destruction of neuroreplacement mechanism by NSCs may pose a problem.

We may also have to consider effect of APP on the stem cell therapy for these diseases, since HNSCs may not properly differentiate into neurons under these pathological conditions.

ACKNOWLEDGMENTS

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Finally, I give the special and most important thanks to God who is my savior and shepherd.

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LIST OF ACRONYMS/ABBREVIATIONS

AD	Alzheimer's disease
ADAM10	A Disintegrin and Metalloproteinase 10
AICD	APP Intracellular Domain
APOE	Apolipoprotein E
APL	Amyloid beta (β4) Precursor Like
APLP	Amyloid beta (β4) Precursor Like Protein
APP	Amyloid Precursor Protein
BACE	Beta Site APP Cleaving Enzyme1
BMP	Bone Morphogenic Protein
bHLH	Basic Helix-Loop-Helix
CAPPD	Central APP Domain
CSL	CBF1/Su(H)/Lag-1
CNTF	Ciliary Neurotrophic Factor
DS	Down's syndrome
ERK	Extracellular Receptor Kinase
FAD	Familiar Alzheimer's Diseases
GFAP	Glial Fibrillary Acidic Protein
Hes1	Hairy and Enhance of the Split1
HNSC	Human Neural Stem Cell
JAK	Janus Kinase
JIP	Jun NH_2 Terminal Kinase Interacting Protein

LIF	Leukemia Inhibitory Factor
LRP receptor	LDL Receptor related Protein Receptor
NICD	Notch Intracellular Domain
mDab1	Mouse Disabled1
OSM	Oncostatin M
PDGF	Platelet Derived Growth Factor
RA	all-*trans* retinoic acid
sAPP	Secreted Amyloid Precursor Protein
siRNA	Small Interference RNA
SR-A	Class A Scavenger Receptor
STAT3	Signal Transducer and Activator of Transcription 3
STS	Staurosporine
SVZ	Subventricular Zone
TUNEL assay	Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling assay
VEGF	Vascular Endothelial Growth Factor

GENERAL INTRODUCTION

General background of Alzheimer's diseases

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases, resulting in a progressive, irreversible brain disorder such as dementia. It attacks and slowly destroys the mind of the afflicted person. More than 4.5 million Americans are believed to have AD and by 2050, the number could increase to 14 million. Approximately 59,000 victims die and 350,000 new cases of AD are diagnosed each year [1]. In general, pathology of AD is caused by the deposition of amyloid plaque and the appearance of the neurofibrillary tangles. The major symptoms of the disease include memory loss, confusion, impaired judgment, personality changes, disorientation, and loss of language skills [2].

Genetic studies have showed that missense mutations in the APP and presenilins 1/2 , encoded on chromosome 21, 14, and 1, respectively, cause early-onset familiar AD (FAD) [3-5]. Mutations of those genes enhance AD by upregulating the proteolytic process of APP, resulting in increase of Aβ peptide generation as well as extracellular senile plaque deposition. In the case of the late-onset of AD, variants of apolipoprotein E (APOE) gene are a highly suspected factor[6]. While APOE ε4 allele is closely associated with APP processing by modulating cholesterol level in the brain [7], APOE ε3 allele modulates tau metabolism by interacting with tau [8].

Biology of APP and Aβ

APP is a type 1 single transmembrane protein, consisting of 695-770 amino acids. The APP gene is approximately 240 Kb, located on chromosome 21, and harbors 19 exons [9]. Depends on alternative splicing of exon 7, 8, and 15, several different type of splice variants (APP695, APP751, and APP770) are generated in the cells with tissue specifically and each splice variants have distinctive characteristics. For example, APP770, containing exon 7, has a function in blood clotting since it contains Kunitz-type proteinase inhibitor (KPI) domain [10]. However, APP695, lacking KPI domain, is specifically expressed in neuron [11, 12].

The APP family consists of APP, APLP1, APLP2 [13-15], APL-1 in *C.elegans* [16], and APPL in *Drosophila* [17]. These APP molecules harbor several consensus motifs such as E1 and E2 and show higher sequence homology as well. Therefore, APP families share functional similarity and show functional redundancy in in-vivo Alzheimer's studies.

Due to the cytotoxicity of Aβ peptides in the brain, mechanism of the proteolytic processing of APP has been intensively studied to understand the pathophysiology of AD. Generation of Aβ peptides result from various combination of the cleavage by β-, and γ-secretases. The major proteolytic cleavage of APP is performed by α-secretase (ex, ADAM10) [18], cleaves between residues Lys612 and Leu613 of APP, whereas β-secretase (ex, BACE1) cleaves APP after Met596 and Tyr606 [19-21]. Cleavage of α- and β-secretase produces soluble APPs (sAPPα and sAPPβ) as well as α- and β- carboxyl terminal of APP fragments (CTFs) of APP. Then, γ-secretase cleaves C-terminal of APP

to liberate either amyloidogenic Aβ peptides or p3 in combination with BACE1 or ADAM10, respectively [22]. Furthermore, APP intracellular domain, functioning phosphoinositide-medidated calcium signaling [23], apoptosis [24], and transcription regulation [25], is generated by γ-secretase activity. Therefore, β- and γ-secretase has a critical role in Aβ generation and senile plaque deposition in AD (Figure 1.).

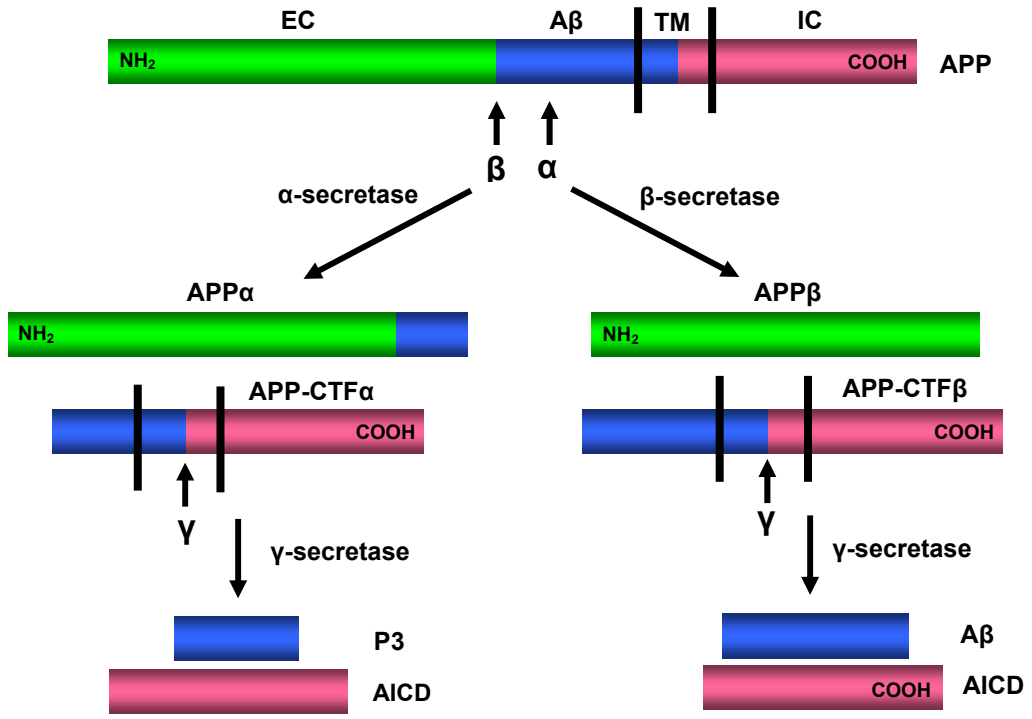

Figure 1: Schematic diagram of sequential processing of APP by α-, β-, γ-secretases. EC: extracellular, IC: intracellular, TM: transmembrane. Aβ domain is highlighted in blue.

Structural and functional properties of APP

Despite the wealth of studies regarding the physiological function(s) of APP, there is little consensus between its function in vitro and in vivo. The main reason that physiological function(s) of APP is (are) still under debates is caused by the molecular complexity of APP. Due to its molecular complexity, various functions have been attributed in the same domain of APP in the CNS. Therefore, to investigate the physiological function of APP, the structural property of APP should be well understood. In general, functional domain of APP can be classified with E1 domain, central APP domain (CAPPD), E2 domain, and APP intracellular domain (AICD) [26].

E1 domain harbors growth-factor like domain (GFLD) (23-128 a.a) which has been know to have cysteine rich region and heparin binding site[27]. Since it contains highly positively charged surface, GFLD functions like a growth factor through protein-protein interaction with other counterpart molecules. Current studies have showed that GFLD is associated with cell adhesion, which contributes to neurite outgrowth and synaptogenesis[28]. Then, E1 domain is followed by the well-studied E2 domain. E2 domain contains RERMS sequence[29] and heparin-sulfate proteoglycan binding site[30], function as a growth promoting factor. AICDs are generated by subsequent cleavage of APP by γ-secretase/nicastrin complex. Since this domain has several important structural properties, AICD has been extensively studied and found that it is associated with multiple signaling pathways[23]. The structural properties of the cytoplasmic region of APP suggest that APP might have a role as a G-protein coupled receptor, via G_o binding domain in AICD[31]. Above all, AICDs modulate signaling pathway by interacting

with various partners, including Fe65, JIP, X11/Mint, and mDab1, via YENPTY motif[32] (Figure 2.).

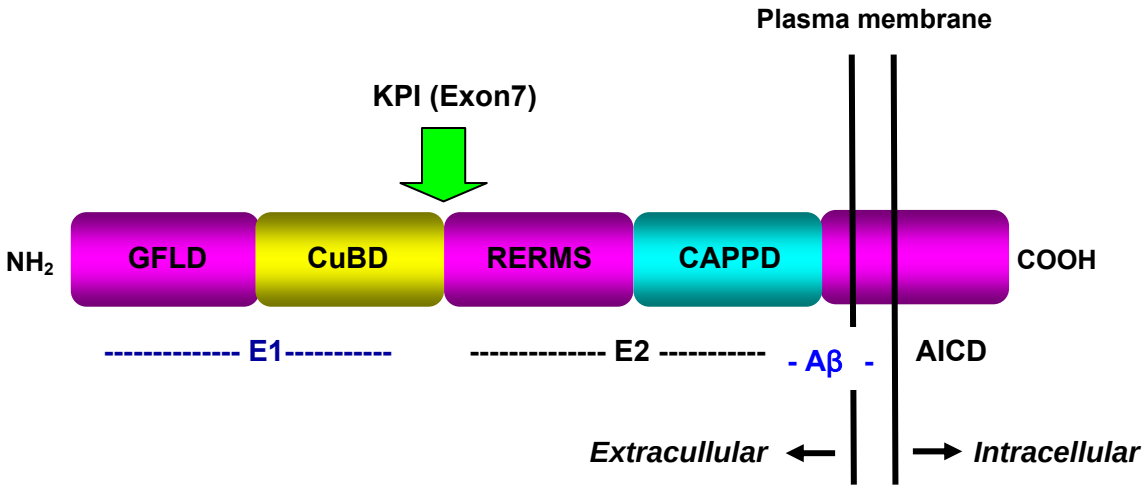

Figure 2: Schematic diagram of domain organization of APP. E1 domain is composed with N-terminal growth factor like domain (GFLD) and copper-binding domain (CuBD). E1 is followed by E2/central APP domain (CAPPD) which is, so called, carbohydrate domain. Aβ indicates that Aβ domain. In APP751 and APP770, KPI domain is included by alternative splicing.

Glial differentiation of neural stem cells

During vertebrate central nervous system (CNS) development, progenitor cells are, specifically subdivided into certain location. After being settled down to specific region, those progenitors start to proliferate to increase the number of the cells during post-gastrulation stage (expansion phase) and then are differentiated into neuron (neurogenic phase) and glia (gliogenic phase) during mid-gestation and postnatal stage, respectively[33]. Accumulated evidences suggest that neurons are typically differentiated before astrocytes and oligodendrocytes with the exception of a few sites where postnatal and adult neurogenesis occur during later development, such as the subventricular zone (SVZ) of the fore brain. Recent studies show that these timing of the sequential differentiation events are finely modulated by both intrinsic and extrinsic cues.

During neurogenic phase (around E12-13), several morphogens induce neuronal differentiation with selective or instructive manner. Platelet-derived growth factor (PDGF) [34] and vascular endothelial growth factor (VEGF) [35] is involved in the promotion of neuronal differentiation by selectively enhancing neuronal population. Inversely, Wnt signaling pathway is also implicated with instructive neuronal differentiation by upregulating Neurogenin 1, 2 (Ngn1, 2), proneural basic helix-loop-helix transcription factors. Additionally, Wnt signaling pathway has a potent suppressive role on FGF2, one of the gliogenic factors [36-38].

In the later stage of the neural differentiation (around postnatal period), glial differentiation is induced by various factors. To date, IL-6 cytokine families, bone morphogenic factors (BMPs), basic fibroblast growth factor (bFGF), and Notch signaling has been known for an important mechanism which is involved in the glial differentiation process [39].

IL-6-induced JAK/STAT signaling pathway is a central part of the glial differentiation process [40] (Figure 3). This gliogenic signaling pathway is stimulated by several IL-6 family cytokines such as leukemia inhibitory factor (LIF) [41], ciliary neurotrophic factor (CNTF) [42], IL-6, and oncostatin M (OSM) [43]. Once these IL-6 cytokine families interact with their cognate receptors (ex, IL-6 receptor α), the resultant complexes are then associated with gp130, crucial signal transducing component of IL-6 receptor[44]. Formation of these receptor heterodimers stimulates a downstream transcription factor, signal transducer and activator of transcription (STAT) proteins by activation of JAKs, especially JAK1 and JAK2. Predominantly, STAT3 molecules get phosphorylation, and then the phosphorylated STAT3s form dimerization and undergo translocation into the nucleus. Finally, STAT3 dimers bind to the putative STAT3 binding element of GFAP promoter, typical astrocytic marker, for glial differentiation[45].

Notch signaling has been known to control cell fate through local cell-cell interaction. During development process, Notch suppresses neuronal differentiation in many organisms in vivo and in vitro (Figure 3). When ligands (*ex.*, Delta and Jagged) bind to Notch, it results in proteolytic cleavage of Notch receptors by γ-secretase/nicastrin

complex to release the signal-transducing Notch intracellular domain (NICD). Generated NICDs translocate into nucleus and interact with the nuclear protein which is referred to as CBF1/Su(H)/Lag-1 (CSL) [46]. Complex of CSL and NICD activates expression of primary target genes of Notch such as Hairy and enhancer of split (Hes) gene families. Then, Hes, a basic helix-loop-helix (bHLH) type transcriptional repressor, suppresses expression of the proneural transcription factors such as Mash1 and NeuroD[47, 48].

Interesting feature of glial differentiation is that signaling mechanisms, associated with glial differentiation, are strengthened by cross-talking. Glial differentiation is enhanced by cross-talking between IL-6 and Notch signaling pathways by interacting with Hes1 and JAK2. When Notch target gene, Hes1, expression is upregulated, it physically interact with JAK2. Then, these complexes interact with STAT3 and enhance accessibility of STAT3 molecule to STAT3 binding element of GFAP promoter [49].

Although BMPs have multiple functions in the neural differentiation, it is also associated with glial differentiation during late gestation or postnatal stage[50]. During differentiation process, Smad1, stimulated by BMPs, makes a complex with transcription coactivator, CBP/P300, and STAT3 molecules. Then, those complexes have higher accessibility to STAT3 binding element of GFAP promoter[51]. Therefore, overall glial differentiation process is promoted by cross-talking between these gliogenic signaling pathways.

Though some of these gliogenic factors are expressed throughout the neural differentiation process, they show different effect to the neural progenitor cells along

with the developmental stage. This stage specificity is modulated by the intrinsic factor such as methylation status. Depends on the methylation status of CpG island within the STAT3 binding element of STAT3 promoter and histone H3, it determines the stage specificity of gliogenic factors. If STAT3 promoter is methylated and histone H3 is demethylated, it may not be able to induce glial differentiation of neural stem cells since STAT3 complexes are not be able to access to GFAP promoter[52, 53].

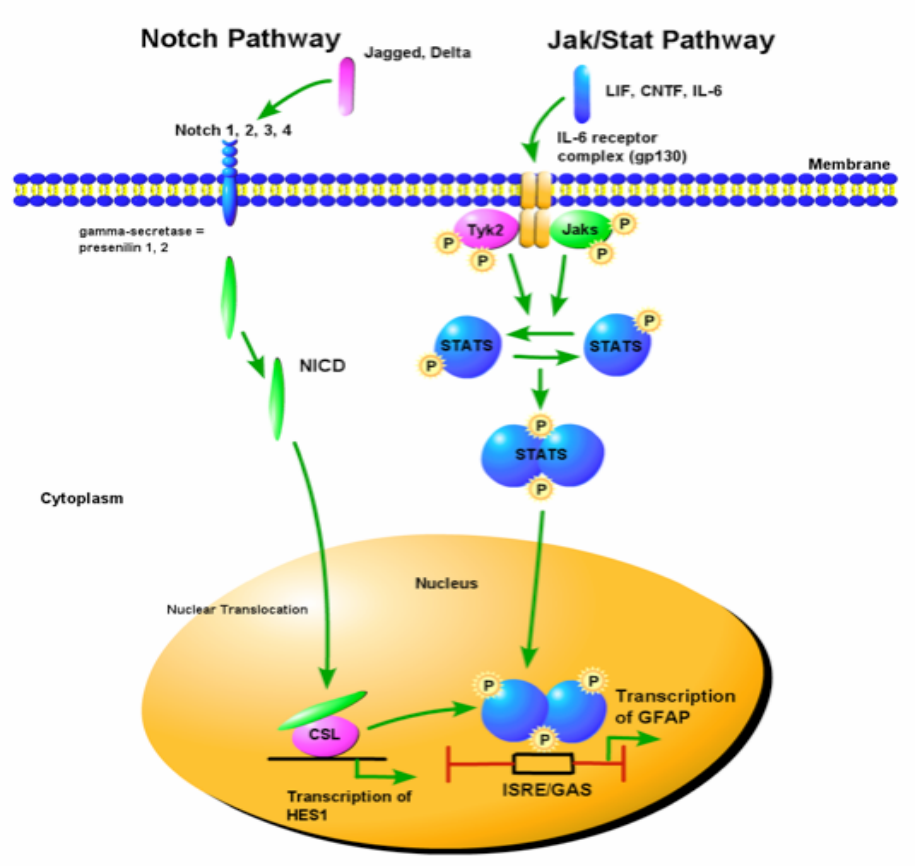

Figure 3: Schematic diagram of two representative glial differentiation mechanisms.

Rationale and aims

Although APP is one of the most intensively studied molecules because of it importance in pathology of AD, the physiological function of APP is still controversial and needs to be elucidated. However, several structural properties of APP have been revealed as a growth factor or a ligand. Crystal structure of the cystein-rich, heparin-binding N-terminal domain of APP reveals a growth-factor like domain configuration [27]. Moreover, several lines of reports have introduced the novel function of APP as a ligand which interacts with various receptors, such as the class A scavenger receptor (SR-A) [54] and LDL receptor related protein (LRP) receptor [55]. These studies indicate that secreted type of soluble APP may modulate cellular function such as differentiation via certain receptors, similar with extrinsic factors in glial differentiation. On the other hand, since DS is a trisomy of chromosome 21, contains the APP gene, DS patients have extra copies of the APP gene in their genetic makeup. Therefore, DS patients usually show high level expression of APP and similar symptoms on their later onset. Bhan *et al*. reported that most of Down's syndrome (DS) patient's neural stem cells were mainly differentiated into astrocytes [56]. Due to the functional property of N-terminal domain of APP, overexpressed APP affects cell fate specification of neural stem cells in DS patients.

As mentioned above, although several gliogenic factors have been reported up to now, there remains the possibility of other factors that might induce glial differentiation by unknown mechanism. Thus, here, we proposed that sAPP may be a novel extrinsic factor which is necessary for the glial differentiation of neural progenitor cells. The main

purpose of the studies is to elucidate the novel function of APP as a gliogenic factor and specific glial differentiation mechanism, associated with sAPP-induced glial differentiation of neural stem cells. The proposed study is innovative, since our research may elucidate novel function and specific molecular mechanism of APP in stem cell biology and may also provide perspective into understanding the mechanism of pathophysiology of neurodegenerative diseases such as AD. Thus, our study is significant because it will provide a vast knowledge of the novel function and mechanisms of APP during HNSC differentiation and this information will be crucial for developing innovative strategies for the prevention and treatment of AD, including regulation of APP level, interference of APP catabolism by manipulating specific signal cascade and/or suppression of IL-6 and/or Notch signaling by blocking the effect of APP.

THE AMYLOID PRECURSOR PROTEIN INDUCES GLIAL DIFFERENTIATION OF HUMAN NEURAL STEM CELL

Introduction

The discovery of multipotent neural stem cells (NSCs) in the adult brain, suggesting that the regeneration of neurons can occur throughout life, has brought revolutionary changes in the theory of neurogenesis [57]. Neural stem cells have been isolated from embryonic and adult mammalian [58, 59] tissue including the human central nervous system (CNS) [60]. These cells can be propagated *in vitro* using a serum-free medium containing epidermal growth factors (EGF) and basic fibroblast growth factors (FGF-2) [61]. Many studies have shown that NSCs can be transplanted and incorporated into the developing mammalian brain and can differentiate into neuronal and glial cells [62-64]. Even in the adult brain, at least some environmental cues seem to remain, as was shown for the rostral migratory system, where stem cells extensively migrated from the subventricular zone (SVZ) to the olfactory bulb [65] and to cognitively relevant brain regions [66]. *In vitro* cultivated and transplanted NSCs migrated using routes similar to endogenous NSCs and then were differentiated in the adult brain. These cells also showed a pattern of broad distribution in the cortex and hippocampus [67]. Moreover, transplanted animals showed improved cognitive functions and functional synapse formation, implying that these cells are functionally capable of being incorporated into the existing neural network [67, 68]. While essential factors for the migration of neuroblasts during embryonic development have been studied extensively in the past, it is still unclear if these factors are also responsible for NSC migration in the adult brain. So far,

it has been suggested that polysialylated NCAM[69], Eph/ephrin signaling [70], and b1 integrins [71] are the involved in neuronal chain migration. Additionally, our recent study indicates that reelin, an extracellular matrix protein required for cortical patterning during development, also affects NSC migration in the adult [72].

Many functions have been attributed to the amyloid precursor protein (APP), including a role as a kinesin I cargo receptor [73]. Nevertheless, APP-knockout mice are viable and display only a minor phenotype [74, 75], suggesting that APP function both during development and later in the maturing process can be at least partially compensated by its family members [e.g., amyloid precursor-like protein 1, 2 (APLP1, APLP2)] [76]. Several studies have shown that APP has growth factor-like properties and can increase proliferation [77], or together with the cytosolic adaptor protein Fe65 can enhance cell motility in cell culture models [78]. Moreover, crystal structure of the heparin-binding N-terminal domain of APP shows a growth factor-like domain structure, supporting evidence of growth factor as a function of APP [27].

Here we report that APP is necessary for the migration and differentiation of HNSCs using well-defined *in-vitro* culture differentiation model by eliminating exogenous factors [79]. Our data, i.e., HNSCs transplanted into APP-knockout mice showed significantly less migration compared to transplanted wild-type controls, suggesting that APP may function as a crucial cue *in vivo* during adult neurogenesis. Moreover, since excessive environmental APP induced gliogenesis in HNSCs, these results implicate a deficiency of stem cell biology by altered APP metabolism in Alzheimer's disease (AD) pathology.

Materials and methods

TUNEL assay

The Fluorescein Apoptosis Detection System (Promega, Madison, WI) was used to assay the DNA fragmentation of HNSCs at 3 DIV. The protocol was followed according to the manufacturer's suggestions with only minor modifications. Briefly, HNSCs were fixed in methanol at -20°C for 20 min. HNSCs were permeabilized in 0.2% Triton X-100 solution in PBS for 5 min, and equilibrated for 10 min with equilibration buffer. The TdT enzyme reaction with fluorescein-12-dUTP was performed at 37°C in a dark humid chamber for 60 min. All the nuclei of HNSCs were counter-stained by propidium iodide (red) and the DNA fragmentation-positive cells were stained in green.

This study was performed under IRB protocol #2001-0316, which meets the criteria for exemptions defined in the U.S. Department of Health and Human Services Regulations for the Protection of Human Subjects (45 CFR 46). Optimized conditions for propagation and maintenance of HNSCs have previously been described in detail [61, 67, 79]. Briefly, HNSCs were cultured in serum-free supplemented growth medium consisting of HAMS-F12 (Gibco, BRL, Burlington, ON), antibiotic-antimycotic mixture (Gibco), B27 supplement (Gibco), human recombinant FGF-2, EGF (R&D Systems, Minneapolis, MN), and heparin (Sigma, St. Louis, MO) and incubated at 37°C in a 5% CO2 humidified incubation chamber (Fisher, Pittsburgh, PA). Human NSCs were differentiated in serum-free basal medium Eagle (BME, Gibco), which contains Earle's

salt and L-glutamine in the absence of FGF-2 and EGF without the addition of other extrinsic differentiation factors.

sAPP preparation

APP cDNA comprising nucleotides -2 to 2358 in expression vector pORFex13 was a gift from Dennis Selkoe. DNA encoding the sequence from leu18 to lys613 human APP695 was amplified by PCR using following primers : 5'-CTG GAG CTA CCC ACT GAT-3'and 5'-CTT CAA GTA GTA GTT TTT ACT-3'. The DNA fragment was gel-purified using a GeneClean II (Bio 101) kit, then cloned into the Pml 1 site of the pPICZ expression vector (Invitrogen) downstream of the *pichia pastoris* alcohol oxidase (AOX1) promoter. To transform *pichia pastoris*, DNA was linearized with Pme I, mixed with *pichia pastoris* strain SMD1168 (Invitrogen), and electroporated. Expression was induced by exposure to methanol for 29 hr as described in the Invitrogen instruction manual. After induction, the culture supernatant was filtered to remove any residual particles, and then loaded on a 5 ml heparin-sepharose column (Amersham Pharmacia Biotech). The column was washed with 0.01 M sodium phosphate buffer, pH 7 until no protein was detected in the outflow. Then a 100 ml, 0-2 M linear NaCl gradient in 0.01 M sodium phosphate pH 7 buffer was applied to the column. The purified truncated form of sAPP695 was sequenced and C-terminal carboxypeptidase analysis revealed it was truncated at amino acid 505.

Stereotactic injection of HNSCs into mice

Mature (3-4 month old) male mice (8 in each group) were deeply anesthetized with sodium pentobarbital (50mg/kg, i.p.). Using the bregma as a reference point, about 10^5 of HNSCs preincubated with BrdU (3μM for 3 days) were slowly injected into the right lateral ventricle (AP -1.4; ML 1.8; DV 3.8mm) of the brain using a stereotaxic apparatus (David Kopff). Immunosuppressant was not given to the animals. All animal experiments were conducted under Animal Protocol #00-184 approved by the Animal Care Committee of the University of Illinois at Chicago. Four weeks post-transplantation, animals were sacrificed and the brains were removed for analysis with immunohistochemistry.

Immunohistochemistry

Detailed methods for immunohistochemistry were previously described[11,24]. Briefly, after fixation, samples were washed in phosphate-buffered saline with 0.2 % Triton X (PBST), then incubated in PBST containing appropriate normal sera. Next, the samples were incubated overnight (up to 12 hr) with primary antibodies, and then incubated with secondary antibodies for 1.5 hr in a dark humidified chamber after washing with PBS. Next, the samples were washed thoroughly in PBS and coverslipped with VECTASHIELD® DAPI Mounting Media (Vector). Primary antibodies: 4G8 monoclonal antibody, mouse IgG2b (Senetek), 1:50; 6E10 monoclonal antibody, mouse IgG1 (Senetek), 1:50; 22C11 anti-Alzheimer Precursor Protein A4, mouse IgG (Chemicon), 1:100; anti-βIII-tubulin monoclonal, mouse IgG2b, clone SDL.3D10 (Sigma), 1:1000; anti-GFAP goat anti-glial filament protein, N-terminal human affinity purified, goat IgG

(Research Diagnostics Inc., Flanders, NJ), 1:400. Secondary antibodies coupled to FITC or TRITC were purchased from Jackson Immuno Research.

RT-PCR analysis

Total RNA was extracted from the cells with Trizol reagent (Invitrogen) according to the manufacturer's protocol. One μg of the total RNA was reverse-transcribed (RT) and amplified by the SuperScript™ ONE-STEP™ RT-PCR system (Invitrogen) with the following primers: GFAP (+) 5'-AAGCAGTCTACCCACCTCAG-3', (-) 5'-ATCCCTCCCAGCACCTCATC-3'; APP (+) 5'-CTTGAGTAAACTTTGGGACATGGCGCTGC-3', (-) 5'-GAACCCTACGAAGAAGCC-3'; LIF (+) 5'-CTGTTGGTTCTGCACTGGA-3', (-) 5'-GGGTTGAGGATCTTCTGGT-3'; Delta (+) 5'-TGCTGGGCGTCGACTCCTTCAGT-3', (-) 5'-GCCTGGCTCGCGGATACACTCGTCACA-3'; Jagged-1 (+) 5'-ACACACCTGAAGGGGTGCGGTATA-3', (-) 5'-AGGGCTGCAGTCATTGGTATTCTGA-3'; Hairy/Enhancer of split 1 (Hes1) (+) 5'-CGGACATTCTGGAAATGACA-3', (-) 5'-CATTGATCTGGGTCATGCAG-3'; β-actin (+) 5'-GACAGGATGCAGAAGGAGAT-3', (-) 5'-TTGCTGATCCACATCTGCTG-3'. RT-PCR condition was a reverse transcription at 55˚C for 30 min; a pre-denaturation at 94˚C for 2min; 25-32 cycles of 94˚C for 15 sec; 55-56˚C for 30 sec and 72˚C for 30 sec; and a post-extension at 72˚C for 5 min. Cycle number was adjusted in order to keep the PCR amplification in a log-phase for semi-quantization. Ten μl of the reaction was separated in a 2% E-gel (Invitrogen).

Results

HNSC differentiation is preceded by apoptosis

We have previously shown that under serum-free unsupplemented media conditions, HNSCs grown as neurospheres migrate and differentiate into βIII-tubulin, glial fibrillary acidic protein (GFAP), and O4 immunopositive cells, markers for neurons, astrocytes, and oligodendrocytes, respectively [79]. These results suggest that HNSCs are capable of producing endogenous factors necessary for their own differentiation and survival. To assess the migration and differentiation process in more detail, we employed a time-lapse video microscopic study. During the early stages (1-3 days in vitro, DIV) of serum-free differentiation, differentiating HNSCs appear to reach out to some HNSCs, which exhibit a shrunken morphology similar to cells undergoing apoptotic cell death (Figure 4). To further assess cell death type, we used the TUNEL assay to detect *in situ* DNA fragmentation, an early marker of apoptosis, in HNSCs differentiated with/without serum. Under serum-free differentiation conditions, the cells that displayed small shrinkage followed by detachment from the neurosphere were TUNEL positive. In contrast, fewer than 5% of TUNEL-positive cells that were detected had differentiated in media supplemented with 10% fetal calf serum (Figure 5 *a,b*). The increase in apoptosis without serum is probably due to growth factor deprivation, similar to that described for neurons [80]. Nevertheless, at later stages of serum-free differentiation, HNSCs migrate and differentiate without further apoptosis, and are able to survive in unsupplemented media for more than 3 weeks [79], indicating the existence of a self-supporting system.

Thus, it seems likely that a factor(s) released from apoptotic cells may induce differentiation and migration of HNSCs.

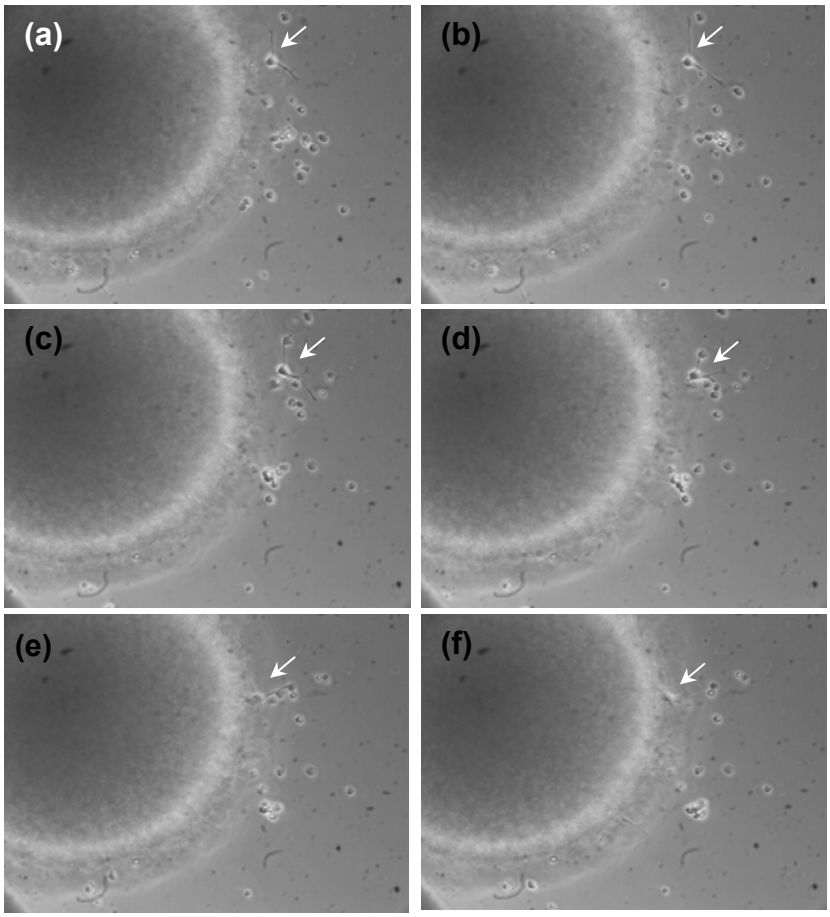

Figure 4: Time-lapse video microscopy shows differentiating HNSCs migrate toward apoptotic cells at 2 DIV. Differentiating HNSCs (arrow) in a serum-free unsupplemented medium migrate away from the neurosphere cluster (***a***). Interestingly, these cells extend their processes onto nearby morphologically shrunken cell(s), which are apparently apoptotic cells (***b***), followed by a retraction of the processes (***c***), which are now attached to the apoptotic cells (***d***), back into the neurosphere cluster (***e***). This response of differentiating HNSCs to the apoptotic cells strengthened the possibility that some component(s) of apoptotic cells serve as a "factor(s)" capable of influencing the physiological activity of differentiating HNSCs.

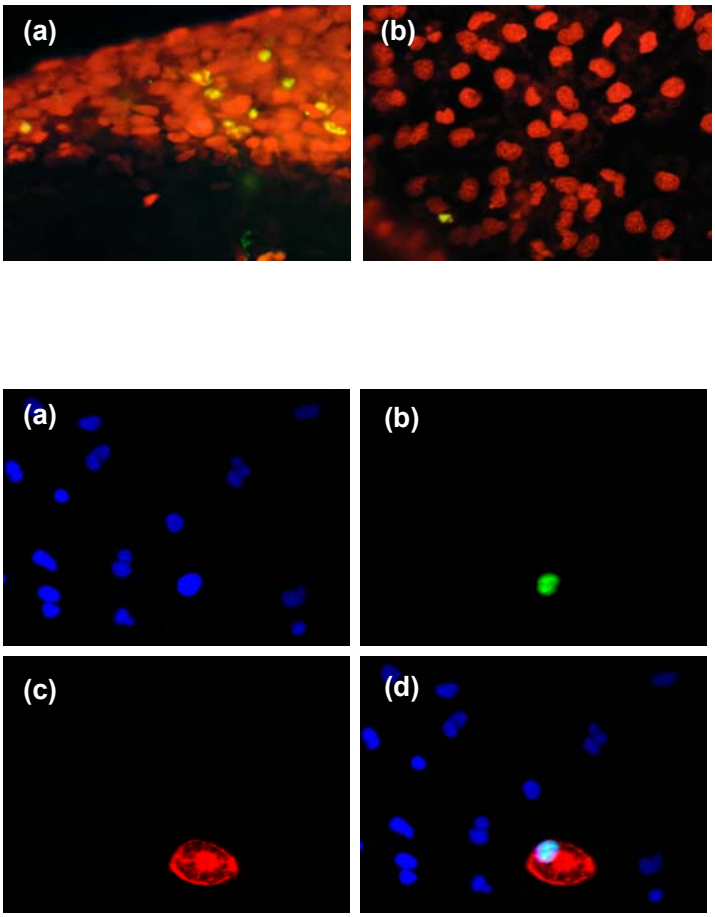

Figure 5: APP expression is increased in apoptotic cells under the serum-free differentiation condition. The TUNEL signal for DNA fragmentation increased at 3 DIV, indicating HNSCs underwent much greater degree of apoptotic cell death (yellow) during differentiation in a serum-free unsupplemented medium (*a*) compared with serum differentiation conditions (*b*). All these nuclei of HNSCs were counter-stained by propidium iodide (red) and the DNA fragmentation positive cells were visualized with fluorescein (green). All these nuclei of HNSCs were counter-stained by DAPI (*c*, blue) The TUNEL signal (*d*, green) and immunoreactivity for APP recognized by 22C11 monoclonal antibody (*e*, red) are co-localized in the shrunken morphology cell in the serum-free differentiation condition (*f*).

APP is increased in apoptotic HNSCs

Since it is known that APP levels are elevated in apoptotic cells [81], we tested whether this is also true for HNSCs under serum-deprived conditions. We combined the TUNEL assay and immunocytochemistry with a well-characterized monoclonal antibody recognizing the N-terminal growth factor-like domain of APP (22C11). Under serum-free conditions, TUNEL-positive cells showed strong expression whereas non-apoptotic cells exhibited only background levels of APP (Figure 5c-e), indicating that APP levels are also elevated in apoptotic HNSCs.

APP involved in migration or differentiation of HNSCs

We further hypothesized that under serum-free conditions, APP derived from apoptotic HNSCs might serve as a migration and differentiation factor for neighboring HNSCs. To investigate if APP is involved in the migration and differentiation process, we tested whether the 22C11 antibody inhibits APP function on HNSC migration. We treated HNSCs with a variety of concentrations of 22C11 under serum or serum-free differentiation conditions for 3 days. The addition of 22C11 (500 ng/ml) completely inhibited migration and differentiation of HNSCs under serum-free unsupplemented conditions (Figure 6). In contrast, 22C11 treatment did not affect the migration of HNSCs when fetal bovine serum was added to the culture (data not shown). This result suggest that APP may be involved in the induction of the differentiation and migration of HNSCs, whereas fetal calf serum contains (possibly independent of APP) many other factors promoting migration and differentiation.

To investigate whether the N-terminal secreted form of APP might be sufficient to induce migration of HNSCs, we treated HNSCs with an exogenous recombinant human secreted APP (sAPP) produced in yeast [82], which contained 95% sAPP695T (ending at amino acid 505 of 695) and 5% sAPP695 (ending at the alpha cut). The addition of recombinant sAPP to cell culture media dose-dependently (25, 50 and 100 ng/ml) increased migration and differentiation of HNSCs (Figure 7 a-d) under serum-free conditions. At the highest dose (100ng/ml), neurospheres were completely absent.

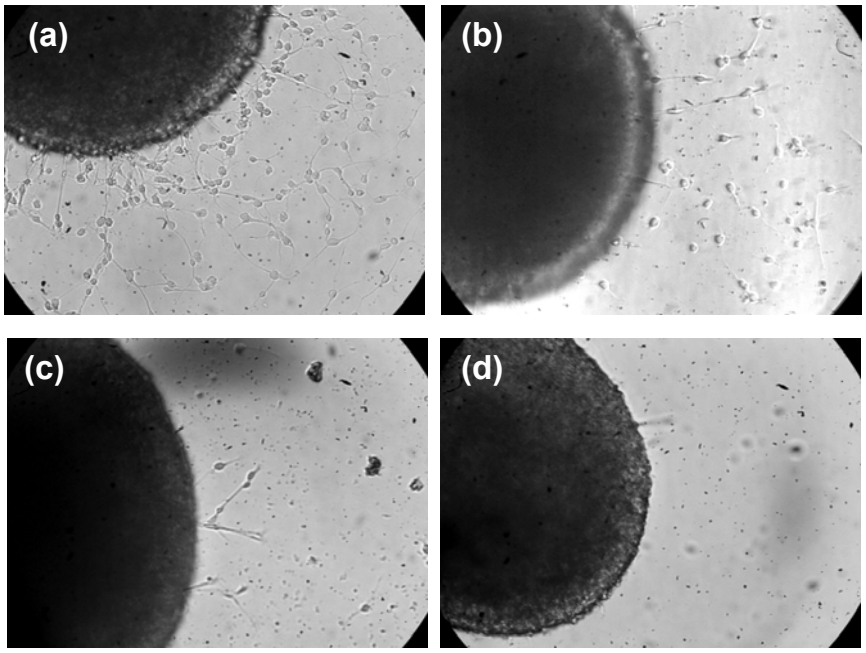

Figure 6: Treatment with 22C11 antibody recognizing the N-terminal of APP dose-dependently inhibited differentiation of HNSCs in non-serum unsupplemented media. The panels show the typical differentiation pattern of HNSCs under treatment with 22C11 (a; control, b; 125 μg/ml, c; 250 μg/ml, d; 500 μg/ml) at 3DIV.

sAPP increases gliogenesis of HNSCs

We also characterized the cell population of sAPP-treated HNSCs at 5 DIV under serum-free conditions by double-immunofluorescent labeling of GFAP and βIII-tubulin (Figure 7 *e-h*). Treatment with sAPP dose dependently (25, 50, and 100 ng/ml) increased the population of GFAP-positive cells from an average of 45% in controls (no sAPP) to an average of 83% with the highest concentration of sAPP treatment (100 ng/ml at 5 DIV).

Interestingly, the lowest dose of sAPP treatment (25 ng/ml) increased both glial and neuronal differentiation. However, higher doses of sAPP (50 and 100ng/ml) dose-dependently decreased βIII-tubulin-positive cells (neurons) in the total population of differentiated HNSCs, from an average of 51% in controls to an average of 13% with the highest concentration of sAPP treatment (Figure 7*i*). Expressions of genes related to JAK/STAT signaling (LIF, CNTF, JAK1 and STAT3, Figure 8A) and Notch signaling (Delta, Jagged1 and Hes1, Figure 8B) were increased in NT-2 cells treated with sAPP (100 ng/ml) for overnight. This result indicates activation of astrocytic differentiation mechanisms. Although the mechanism for the glial promoting effect of sAPP on HNSCs is not yet clear, APP may increase glial differentiation by influencing the cell fate decision of HNSCs. Since sAPP treatment did not increase the apoptosis related gene expression in HNSCs, selective death of proneural progenitors in the neurospheres can be ruled out (Fig. 8C).

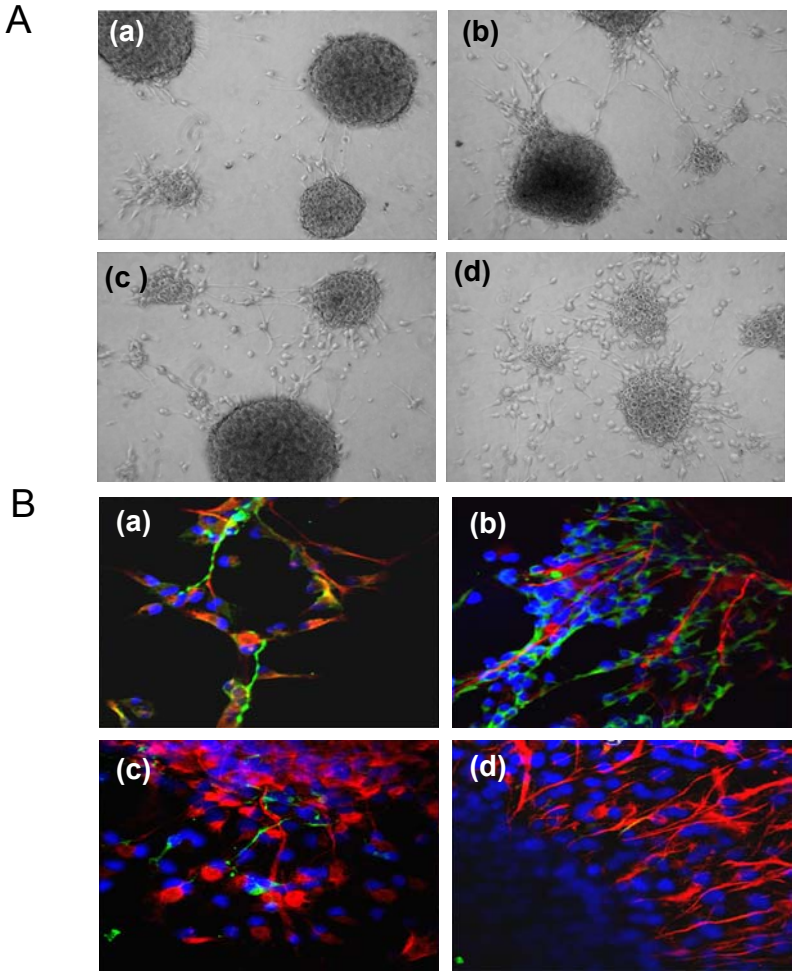

Figure 7: Secreted amyloid precursor protein (sAPP) alters migration and differentiation of HNSCs. Treatment of HNSCs culture with recombinant sAPP for 5 days dose-dependently (a; control, b; 25 ng/ml, c; 50 ng/ml and d; 100 ng/ml) increased the migration and differentiation of HNSCs under the serum-free unsupplemented condition. The cell population of sAPP-treated HNSCs at 5 DIV in the serum-free differentiation condition was further characterized by double immunofluorescence staining with GFAP (red) and βIII-tubulin (green) markers for astrocytes and neurons, respectively. All nuclei were counterstained with DAPI (blue). The panels show typical morphology and differentiation patterns of HNSCs in e; control culture and cultures treated with f; 25 ng/ml, g; 50 ng/ml and h; 100 ng/ml of sAPP. At low dose of sAPP (25 ng/ml), increased glial (red) and neuronal (green) differentiations were observed compared with the control (f). At a higher dose of sAPP treatment, many glially differentiated HNSCs (red) were observed (g and h).

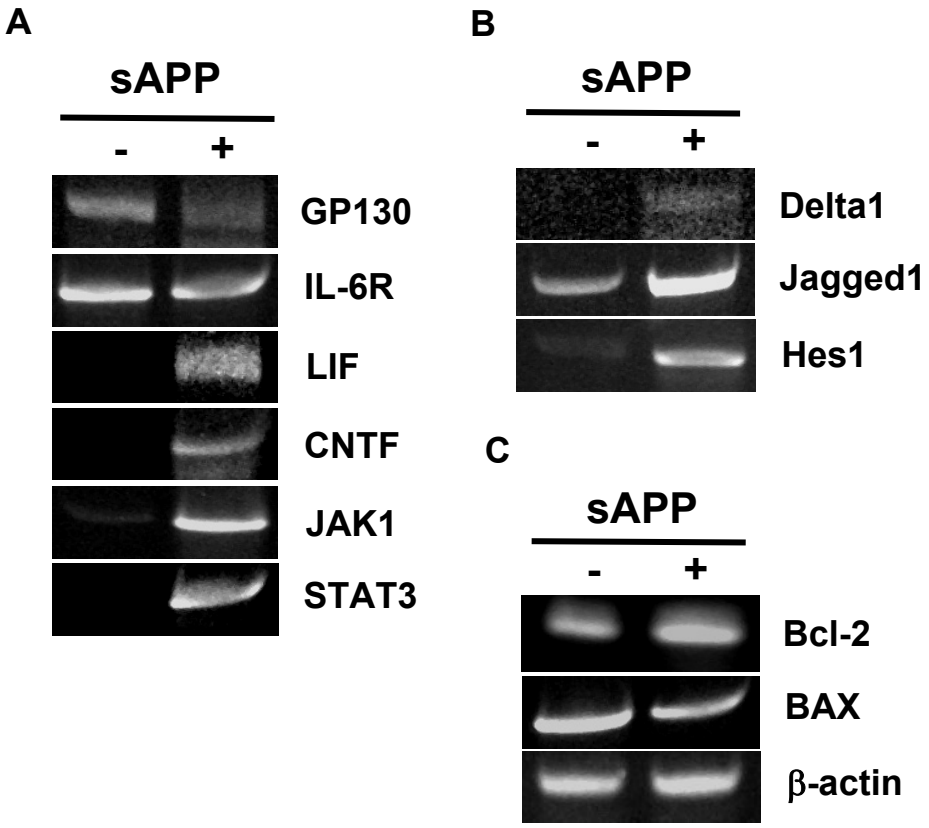

Figure 8: Gene expression pattern after sAPP treatment in neuronal progenitors. Expressions of genes related to JAK/STAT signaling (LIF, CNTF, JAK1 and STAT3) and Notch signaling (Delta, Jagged1 and Hes1) were increased in NT-2 cells treated with sAPP (100ng/ml) for overnight. This result indicates activation of astrocytic differentiation mechanisms. However, genes associated with apoptosis (Bcl-2 and BAX) did not change.

HNSCs migration and differentiation is reduced in APP-knockout mice

Based on our *in vitro* data, we further investigated whether APP might have an impact on the migration or differentiation of NSCs in adults *in vivo.* To address this question, we transplanted HNSCs into the brains of APP knockout [74] and control mice at 3 months of age. To differentiate between host and transplanted cells, HNSCs were labeled *in vitro* by the incorporation of bromodeoxyuridine (BrdU) into the nucleus DNA before transplantation. These labeled cells (about 10^5) were subsequently injected unilaterally into the cerebral lateral ventricle of c57/black wild-type (WT) and APP-knockout mice. Immunohistochemical examination of wild-type brain sections 4 weeks after transplantation revealed migration and differentiation patterns similar to our previous study with HNSCs transplanted into aged memory-impaired rats [67]. Cells distributed bilaterally in the singular and parietal cortexes (layer II, IV and V) (Fig. 9*a*) and hippocampus (CA1, CA2 and dentate gyrus) (Fig.9*c*) were intensely and extensively immunopositive for BrdU and human βIII-tubulin. The transplanted HNSCs also differentiated into GFAP-immunopositive cells that co-localized with the neuronal fibers of layer III in the cortex (Fig.9*e*). These donor-derived BrdU-immunopositive cells were much larger than the host brain cells. We did not detect the above-mentioned morphologies and distributions of βIII-tubulin- or GFAP-positive cells in wild-type control mice that had not received HNSC transplantation.

Although transplanted HNSCs in APP-knockout mice also differentiated into βIII-tubulin- and GFAP- positive cells, the distribution and migration patterns were not symmetric

and differentiated cells were far fewer compared to injected wild-type mice. Despite the rather uniform βIII-tubulin-positive cell distribution and structure in the hippocampus of APP-knockout mice (Fig. 9*d*), βIII-tubulin-positive HNSCs were only detected near the injection site of the cortex and lacked apical dendrites (Fig. 9*b*). These results indicate that APP plays an important role in neurogenesis in the adult brain.

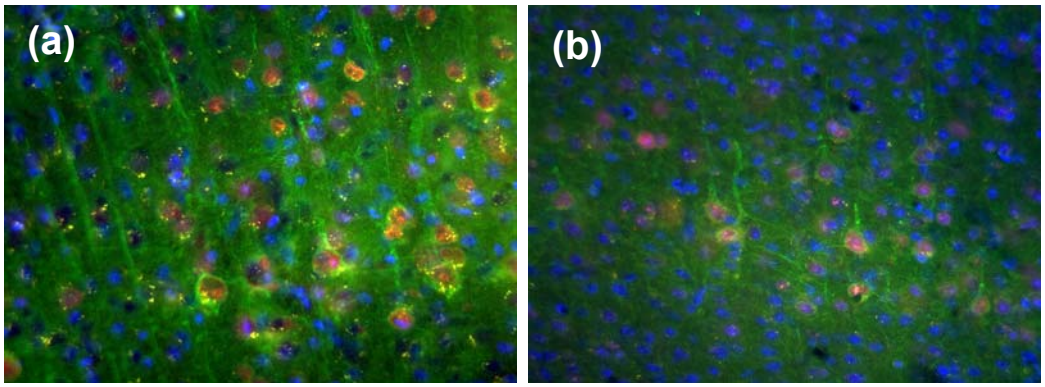

Figure 9: HNSCs transplanted into the APP knockout mice brain show fewer migrations and differentiations compare with cells transplanted into wild-type control mice. To distinguish HNSCs from the host cells HNSCs were prelabeled by BrdU *in vitro* before unilateral ventricle injection. We immunostained the brain with βIII-tubulin, GFAP, and BrdU, markers for neurons, astrocytes, and transplanted HNSCs at 4 weeks after injection, respectively. All nuclei were counterstained with DAPI (blue). In wild-type mice, BrdU (red) and βIII-tubulin (green) immunopositive cells were distributed bilaterally in the parietal cortexes (*a*) and pyramidal layer of the hippocampus (*c*) indicating the neuronal differentiation of transplanted HNSCs. HNSCs also differentiated into GFAP-immunopositive cells (red) that associated with the βIII-tubulin (green) stained neuronal fibers of layer III in the cortex (*e*). The differentiation and distribution patterns of the transplanted HNSCs are similar to those we found in our previous transplantation study with rats[11].In APP knockout mice, βIII-tubulin-positive cells (green) with BrdU (red) positive nuclei, neurally differentiated HNSC-transplants were only detected near the injection site in the cortex and these cells showed abnormal morphologies (*b*). In the hippocampus, although the number of HNSC-derived cells was reduced, βIII-tubulin and BrdU-immunopositive cells had quite similar positioning and structure to these cells found in wild-type mice (*d*).

Discussion

Our study clearly showed that APP function is relevant for HNSC migration and differentiation *in vitro* and *in vivo*. We found that under non-serum differentiation of HNSCs, APP levels are elevated in apoptotic cells. This result is consistent with other studies showing that damaged neurons and neurons committed to apoptosis are strongly immunopositive for APP [83, 84]. Time-lapse observation of HNSC cultures under non-serum differentiation conditions showed migration of differentiating HNSCs toward the apoptotic cells *in vitro.* We demonstrated that treatment with recombinant sAPP promoted the migration and differentiation of HNSCs, and 22C11 antibody-mediated neutralization of APP inhibited these effects in a dose-dependent manner. Since amyloidogenic fragments produced from APP are reported to be released into the extracellular space from neuronal cells under serum-deprived conditions [80], we hypothesize that under serum-free differentiation conditions, APP released from apoptotic cells serves as a differentiation and/or migration factor for neighboring HNSCs. Although the mechanism of 22C11-mediated inhibition of the migration and differentiation of HNSCs is not clear, since the epitope of 22C11 lies within the growth factor-like domain of APP, 22C11 may prevent receptor or ligand interactions by binding to biologically active sites of APP. It is known that sAPP activates MAPK (ERK) in PC12 cells *via* the *ras* pathway [85]. *Ras* in turn, can activate *rac* [86], a *rho*-family GTPase controlling lamellipodia formation through a focal complex assembly, and thereby also activating cell motility. Focal complex assembly is an integrin-mediated process requiring extracellular matrix and rho/rac activity[87]. Sabo and colleagues [78] have shown that APP can enhance cell motility in MDCK cells in association with Fe65, which

colocalizes with APP at focal contacts. Since Fe65 over expression is known to increase secretion of APP [88], one could argue that APP secreted from neighboring cells act as a migrational cue through focal adhesion assembly. Since b1-integrins have been shown to be necessary for the chain migration of neuroblasts [71], the connection between integrin-mediated migration and APP could be a clue for understanding the mechanism of the regulation of stem cell motility by APP. In contrast to another study showing that sAPP significantly enhanced proliferation of neural stem cells [89], we did not observe this effect in our studies, probably because our assay was done under non-serum conditions without any proliferation factors.

Besides increasing differentiation and migration, a high concentration of sAPP increased the glial cell population of differentiated HNSCs *in vitro*, indicating that the over-production of APP fragments may influence the cell fate decision of HNSCs and reduce neural differentiation. Another possibility is that higher concentrations of sAPP may eliminate HNSC populations differentiating into neurons, since high APP expression in neuronal cell lines is reported to cause apoptotic cell death by caspase-3 activation [90], although the latter possibility can probably be excluded because sAPP treatment did not increase expression of apoptosis related genes in HNSCs.

Gene expression data after sAPP treatment of the cells also show increased expression of genes related to Notch and JAK/STAT signaling indicating activation of these cascades. Since these signaling cascades are known to involve in glial differentiation of

stem cells, sAPP might affecting on these cascades to induce glial differentiation of HNSCs. However, detailed studies are needed to confirm the mechanism of action.

Recently, Bahn et al., reported that NSCs from Down's syndrome patients differentiated into astrocytes rather than neurons [56]. They have also reported a morphological abnormality of neurally-differentiated NSCs, which we have also seen in HNSCs transfected with wtAPP. Since Downs' syndrome patients have inherited three copies of APP that resides on chromosome 21, this abnormal differentiation may result from an overdose of the APP gene. Furthermore, since Down's patients develop AD by age 40, these findings might also have implications in AD pathology. Although it is not clear whether adult neurogenesis is essential for normal cognitive function in aging, it is tempting to speculate that the altered APP metabolism that impairs proper NSC migration and differentiation could be a part of pathological process of AD, since aged transgenic APP mice exhibit neocortical neuronal loss [91]. Furthermore, although the rate of neurodegeneration in the adult brain may be minimal, it may be that in the long run, such a deficit might significantly reduce normal brain function. In addition to these drawbacks, transplantation therapy of AD with HNSCs may not be effective in an environment where APP metabolism is altered and might lead to excessive gliogenesis. Thus, we may have to consider the regulation of APP processing to develop effective HNSC transplantation therapy in AD patients.

Regulation of stem cell biology by APP raises a question regarding Ab immunization, which may also reduce APP fragments. Could Ab immunization be helpful for

maintaining stem cell function in AD? Our current study has shown for the first time that APP is one of the factors required for HNSC migration in the adult brain. Migration of transplanted HNSCs to the cerebral cortex was markedly reduced in APP-knockout mice compared to wild-type controls. Since migration from the subventricular zone to the neocortex could be found in adult macaques [66], lack of APP expression in APP-knockout mice might be a major factor in preventing the migration of HNSCs into this region, indicating that APP regulates NSC migration in the adult brain. Although it is conceivable that APP expression in transplanted HNSCs may partially compensate for the APP deficit in the host brain, this effect seems to be minimal, since the absence of environmental APP clearly alters the migration pattern of transplanted HNSCs. Thus, APP might function as an environmental cue rather than as a cell-cell contact mechanism. In mammals, APP is part of a gene family comprising three paralogues: APP, APLP1, and APLP2. Evolutionary studies have revealed a remarkable homology between these proteins, specifically in the N-terminal and C-terminal domains [14, 92]. Additionally, mouse double-knockouts of both APP and APLP2 result in 80% mortality during the first week postnatally [93], indicating that APLPs may compensate for the lack of APP in the APP-knockout mouse. The successful migration of transplanted HNSCs into the hippocampus of APP knockout mice, as we have observed in the current study, may thus be explained by the redundant function of APP, APLP1, and APLP2. This redundancy is also supported by the finding that APLP1 and APLP2 mRNAs are highly abundant in the granule cells of the dentate gyrus in humans [94]. It would be interesting to extend our studies to double-knockout mice to confirm this hypothesis. These results indicate that environmental or sAPP factors may be important in regulating the migration

and differentiation of HNSCs in the adult brain. Since HNSCs may play important roles in neurodegeneration, if APP is indeed involved in the regulation of HNSCs as we propose, destruction of the APP system may jeopardize the maintenance of normal adult brain function.

AMYLOID PRECUROSR PROTEIN IS INVOLVED IN STAUROSPORINE INDUCED GLIAL DIFFERENTIATION OF NERUAL STEM CELLS

Introduction

Human embryonic teratocarcinoma cells (NT-2/D1) derived from a testicular germ cell tumor have been intensively used as an experimental model for investigating neural differentiation [95-97]. Unlike post-mitotic CNS neurons or neuroblastoma, NT-2/D1 cells still possess some multipotency and distinctive developmental characteristics, which resemble the nature of neural stem cells [98]. By treating with all-*trans* retinoic acid (RA), NT-2/D1 cells are known to progressively differentiate into post mitotic neurons in 3-5 weeks, expressing full neuronal characteristics and capable of forming functional synapses [99, 100]. Recently, RA treatment has also been reported to differentiate NT-2/D1 into astrocytes (NT2/A) [99], which express astrocyte specific markers such as glial fibrillary acidic protein (GFAP), astrocyte-specific glutamate transporter-1 (GLT-1)/ excitatory amino acid transporters (EEAT)-2 and aspartate transporter (GLAST)/EAAT-1.

Staurosporine (STS), indolo (2, 3-alpha) carbazole, has been extensively used as a protein kinase C inhibitor [101] or apoptosis inducer in neuronal precursors [102], neurons [103], and other tumor cell lines. However recent studies indicate STS also inhibits cell proliferation and induces neuronal and glial differentiation of murine embryonic stem cells [104], PC12 pheochromocytoma [105], and C6 glioblastoma [106]. Although the target and mechanism of the tropic effects of STS remains to be determined, STS may have properties not only as an apoptotic inducer but also as a differentiation inducer. In the

current study, we try to elucidate mechanisms of STS-induced astrocytic differentiation using NT-2/D1 cells.

The amyloid precursor protein (APP) is a 695- to 770-amino acid, membrane spanning glycoprotein. To date, the cytotoxicity of Aβ peptides, generated from APP by sequential cuts with γ- and β-secretases, has been extensively studied since Aβ deposition is a major pathophysiology of Alzheimer's Disease (AD) [107]. While physiological function of APP has not been well documented, structure of APP suggests that APP might function as a receptor *via* G_o binding domain [108], or as a ligand *via* soluble N-terminal domain [28, 109]. Secreted type of APP (sAPP) promoted neurite outgrowth of primary neuronal cultured cells [28] as well as proliferation of neural stem cells [109]. Over expression of APP reduced the number of apoptotic neurons deprived of NGF in dorsal root ganglion [110], which may occur through p38 MAPK-dependent phosphorylation and activation of myocyte enhancer factor-2 [111]. These results indicate that APP has properties of growth and anti-apoptotic factors. Here, we demonstrate that APP is playing an important role in STS-induced astrocytic differentiation of NT-2/D1 cells.

Materials and methods

Reagents and antibodies

STS (Sigma), PD098059 (Sigma) and SB239060 (Calbiochem) was dissolved in dimethyl sulfoxide and stored at –80˚C until use. Primary antibodies: rabbit anti-GFAP antibody (Promega); mouse anti-APP antibody (22C11) (Chemicon); mouse anti-ERK

antibody (BD Transduction Laboratory); and mouse anti-phospho-ERK1/2 (pT202/pY204) antibody (BD Transduction Laboratory. Secondary antibodies: anti-mouse IgG and anti-rabbit IgG horseradish peroxidase-conjugated antibodies (Jackson Immunoresearch Laboratory) were used.

Cell culture

The NT2/D1 cells were seeded ($5x10^6$ cells per 150 mm petri dish) in Dulbecco's modified Eagle's medium with F-12 (DMEM/F-12; Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (Novacell), 1% antibiotic-antimycotic (Invitrogen), 4 mM glutamine (Invitrogen) and maintained in a humidified atmosphere of 5% CO^2/95% air at 37˚C. The cells were passed twice a week by short exposure to trypsin/EDTA (Invitrogen). For the experiments, $1x10^6$ NT-2/D1 cells were plated in a 6 well tissue culture plate and subsequently treated with 10 or 40 nM STS. Cells were rinsed twice in ice-cold phosphate-buffered saline (PBS, pH7.4) then mRNA and protein samples were extracted for further analysis. Enhanced green fluorescent protein (EGFP) expression in the cells were detected as green fluorescence signals under a microscope (Leica, model DMRB) after fixing with 4% Para formaldehyde.

siRNA preparation

Human APP695 (Genbank access number: A33292) mRNA target sequences were designed using Ambion target finder software. The mRNA target sequences of human APP695 was 5'- AATCTTTGGAACAGGAAGCAG-3' (1094-1114). siRNA PCR products were synthesized using a Silencer™ Express kit (Ambion). Then, silencing efficacy was

determined using RT-PCR. As a control, we prepared EGFP siRNA PCR products (accession number: U55763, target sequence: 106-127) as follows. In the first round PCR, hU6 promoter (GenBank accession #M14486, gene sequence 64-355) and 27 nt of U6 5'-coding sequence was amplified with a primer set (U6-F: 5'-TCTTTGGAATTCAAGGTCGGGCAGGAAGAGGGCCTA-3', U6-R: 5'-CGCGGATCCTAGTATATGTGCTGCCGAAGC-3') using plasmid pUC-hU6 [112] as a template. Simultaneously hairpin-siRNA for EGFP gene consists of 21 nt sense strand of siRNA, 9 nt spacer and 21 nt antisense strand of siRNA was generated by PCR using another primer set (siEGFP-F: 5'-CGC GGA TCC GGC GAT GCC ACC TAC GGC AAG CTC GAG ATC-3', siEGFP-R: 5'-GCT CTA GAG GCG ATG CCA CCT ACG GCA AGG ATC TCG AGC T-3'). After 100-times dilution, first round PCR products were mixed and used as template for the second round PCR reaction. Since 5' end of siEGFP-F primer contains complementary sequence to 3' end of hU6 promoter region, the second round PCR using U6-F primer and siEGFP-R primer produced hU6-siEGFP. The first and second round PCR reaction condition were both pre-incubation at 95˚C for 5 min; 35 cycles of 95˚C for 40 s, 55˚C for 30 s and 72˚C for 30 s; and post-extension at 72˚C for 5 min. To produce PCR fragment containing hU6 promoter with a random sequence for using as a nonspecific siRNA in the cells, we used a primer set U6-F and U6-R1 (5'-AAA AAT TCT AGA TGT AAA AAT AGT GTT GTG TGC CTA GGA TAT GTG CTG CCG AAG CGA GCA C-3') using plasmid pUC-hU6 as a template.

Transfection

Transient transfection of pEGFAP-C1 (BD Clontech), pCEP-APP-GFP, pCEP-APP695 (kindly provided by Dr. Beth Ostaszewski, Harvard Medical School), pGFAP-GFP-S65T (kindly provided by Dr. Albee Messing, University of Wisconsin Madison) and siRNA PCR fragments (U6-random, siGFP and siAPP) was performed with Lipofectamine^{TM} 2000 (Invitrogen) on sub confluent NT-2/D1 cells in a 6-well culture plate, according to the manufacturer's protocol. Transfection efficiency in NT-2/D1 cell was determined by transfection of 0.5 μg of pEGFP-C1, and it was generally around 80%.

RT-PCR analysis

Total RNA was extracted from the cells with Trizol reagent (Invitrogen) according to the manufacturer's protocol. One μg of the total RNA was reverse-transcribed (RT) and amplified by the SuperScript^{TM} ONE-STEP^{TM} RT-PCR system (Invitrogen) with the following primers: GFAP (+) 5'-AAGCAGTCTACCCACCTCAG-3', (-) 5'-ATCCCTCCCAGCACCTCATC-3'; APP (+) 5'-CTTGAGTAAACTTTGGGACATGGCGCTGC-3', (-) 5'-GAACCCTACGAAGAAGCC-3'; GLT-1 (+) 5'-GACAGTCATCTTGGCTCAGA-3', (-) 5'-AATCCACCATCAGCTTGGCC-3'; GLAST (+) 5'-CTGCTCACAGTCACCGCTGT-3', (-) 5'-AGCACGAATCTGGTGACGCG-3'; EGFP (+) 5'-CAAGGACGACGGCAACTACAAGAC-3', (-) 5'-GCGGACTGGGTGCTCAGGTAGTGGT-3'; β-actin (+) 5'-GACAGGATGCAGAAGGAGAT-3', (-) 5'-TTGCTGATCCACATCTGCTG-3'. RT-PCR condition was a reverse transcription at 55˚C for 30 min; a pre-denaturation at 94˚C for 2min; 25-32 cycles of 94˚C for 15 sec; 55-56˚C for 30 sec and 72˚C for 30 sec; and a

post-extension at 72˚C for 5 min. Cycle number was adjusted in order to keep the PCR amplification in a log-phase for semi-quantization. Ten μl of the reaction was separated in a 2% E-gel (Invitrogen).

Immunoprecipitation and western blot analysis

Protein samples were prepared by lysing the cells using an ice-cold lysis buffer consist of 1% NP40, 150 mM NaCl, 50 mM Tris pH8.0 and Complete Protease Inhibitor Tablets (Boehringer). The protein concentration of each sample was measured by Bio-Rad protein assay (Bio-Rad). Lysates were immunoprecipitated with an antibody against APP, GFAP, and ERK molecules using protein A-Sepharose (Amersham Bioscience). Then, precipitates and, in some cases, cell lysates were heated at 70ºC for 10 min in a sample loading buffer and separated on NuPAGE^{TM} 4-12 % Bis-Tis Gel (Invitrogen) for 45 min at 200 V and transferred to a PVDF membrane (30V, 60 min). Membranes were blocked with 5% skim milk in PBS for 1h at room temperature and probed at 4˚C overnight with primary antibody in 5% skim milk. The membranes were washed 3 times for 5min each with PBS containing 0.05% Tween 20 (pH7.4) and then incubated with horseradish peroxidase-conjugated secondary antibodies in 5% skim milk for 2h at RT. After 3 times washing with PBS containing 0.05% Tween 20, immunoreactive bands were visualized by using ECL plus (Amersham Bioscience) chemiluminescence reagent.

Cloning and expression of the recombinant human APP695

We amplified human APP695 gene from pCEP-APP695 by PCR using EPPENDORF® HotMasterMix (2.5×, Eppendorf). The commercially synthesized primers were R-hAPP (+): 5'-CACCCTGGAGGTACCCACTGAT-3' and R-hAPP (-): 5'-TCATTTTTGATGATGAACTTG-3' for protein expressed with a His tag at the N terminus. The PCR product was separated by gel electrophoresis on a 1.2% E-gel (Invitrogen), and the appropriate band was extracted using a gel extraction kit (Quiagen). Then, blunt-end directional cloning was performed by a topoisomerase reaction into a pET100 vector (Champion pET Directional TOPO Expression and Cloning Kit, Invitrogen). The gene insertion and its directionality as well as the integrity of the pET100 vector were verified by the sequencing facility at the University of Central Florida. BL21 Star DE3 (Invitrogen) competent cells were transformed for protein expression and grown in Luria-Bertani broth at 37˚C to an absorbance ~0.8 at 600nm. Then, protein expression was induced under ampicillin (50μg/ml) and 1 mM isopropyl-β-thiogalactopyranoside at 37˚C for 4h. Cells were harvested by centrifugation at 10,000×g for 25 min.

Purification of human APP695

Cells were resuspended in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0] and disrupted by sonication. Cell lysates were collected by centrifugation (2000g at 4˚C for 20 min). The supernatant was directly applied to Ni-NTA column (invitrogen), which is washed with a buffer solution containing 50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole pH 8.0, to remove nonspecifically bound protein. The His-tagged protein was eluted with buffer containing 50 mM NaH_2PO_4, 300 mM

NaCl, and 150 mM imidazole pH 8.0. The purified protein was then stored at –20˚C. The sample was more than 95% pure, as assessed by SDS-PAGE.

Image analysis

Gel or Western blot images were captured by KODAK Image Station 2000MM. Optical density of the target bands were analyzed using ImageJ (Ver 4.1, NIH) and expressed in mean±SD from experiments performed in triplicates.

Results

Induction of glial cell like morphological change of NT-2/D1 cells by STS

As a further test for function of STS as a differentiation inducer, we analyzed morphological changes after STS treatment. NT-2/D1 cells grew as a monolayer culture of neuroepithelial cells with a high nuclear to cytoplasmic ratio and prominent nucleoli resembling embryonic carcinoma cells (Figure 10A-a). Morphological changes started right after day 1 of treatment with 40 nM STS (Figure 10A-b). Dramatic change of morphology occurred after 7 days treatment of 40 nM STS (Figure 10A-c) and these changes continued during 14 days treatment of STS (Figure 10A-d). Based on morphological features, protoplasmic and polygonal irregular star-like cells, STS treated cells showed distinctive astrocytic cell morphology (Figure 10A-c, d), indicating that STS treatment induced astrocytic differentiation of NT-2/D1 cells.

GFAP expression in STS-treated NT-2/D1 cells

Although physiological function of GFAP during astrocytic differentiation is still unclear, GFAP has been used as a typical maker for astrocytic differentiation [113, 114]. To characterize the morphological changes in NT-2/D1 after STS treatment, we analyzed GFAP expression in these cells using RT-PCR (Figure 10B). Levels of GFAP mRNA were increased by STS-treatment compared to the basal level of GFAP mRNA in control cells with time dependent manner up to 24 hours. These cells also expressed astrocyte-specific glutamate transporter-1 (GLT-1)/ excitatory amino acid transporters (EEAT)-2 and aspartate transporter (GLAST)/EAAT-1 (Figure 10C).

We also confirmed GFAP promoter activation by STS treatment using NT-2/D1 transfected with GFAP promoter driven GFP plasmid vector (pGFAP-GFP-S65T) as a reporter system. As shown in Figure 10D and E, 10 nM STS treatment induced both mRNA and protein expression (green fluorescence) of GFP. These results indicate that STS induces astrocytic differentiation of NT-2/D1 cells.

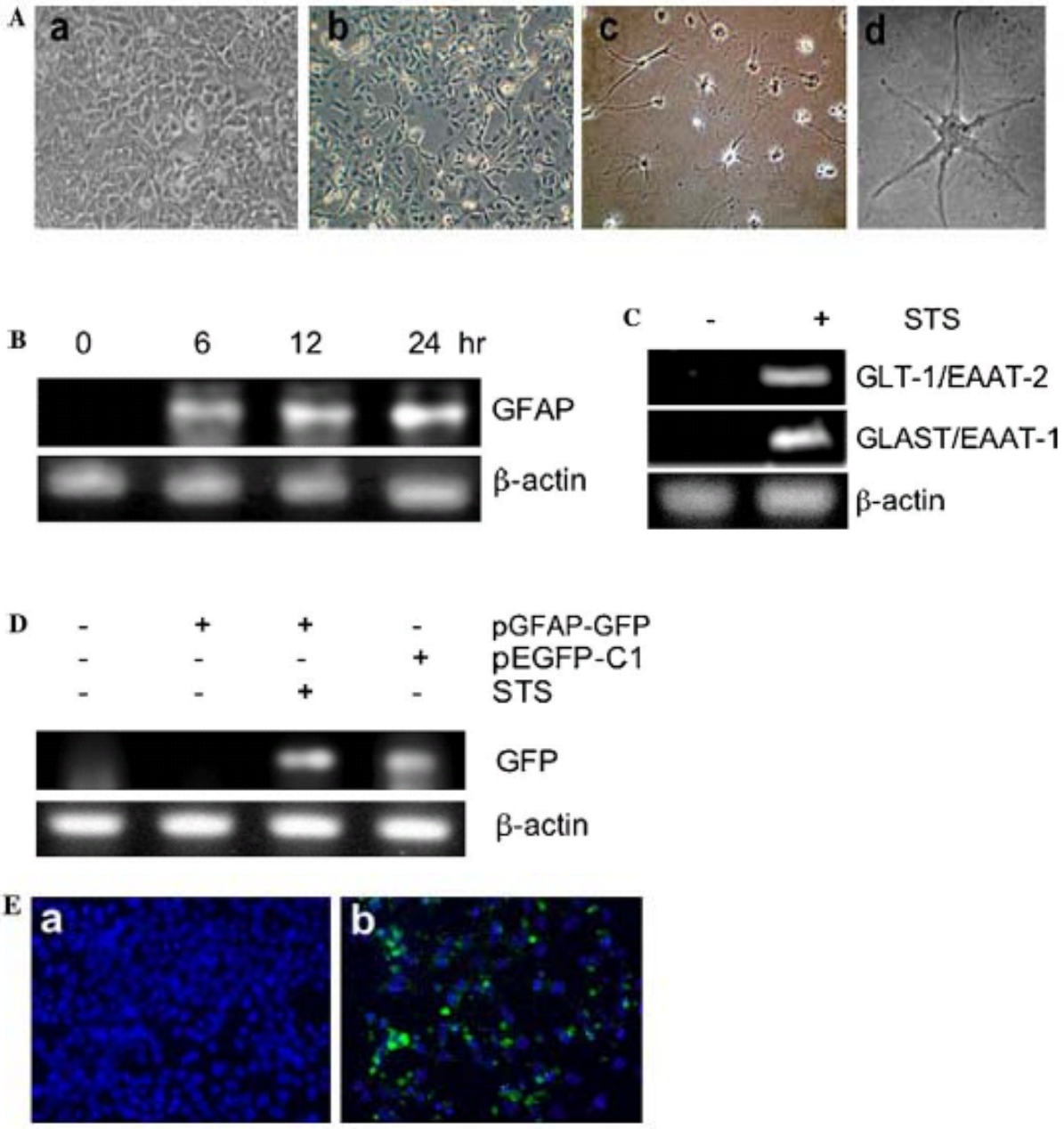

Figure 10: Morphological changes of NT-2/D1 cells and induction of astrocyte specific gene expression by STS. (A) NT-2/D1 cells grew as a monolayer culture resembling embryonic carcinoma cells (a, x100). Morphological changes started right after day 1 of treatment with 40 nM STS (b, x100). Astrocyte like morphology was observed after 7 days (c, x100) and 14 days (d, x400) treatment with STS. (B) RT-PCR analysis of GFAP gene expression in NT-2/D1 cells treated with 10 nM STS for 0, 6, 12 and 24 hrs shows

time-dependent increase of GFAP gene expression. Expression of β-actin was examined as a loading control. (C) RT-PCR analysis shows treatment with 10 nM STS for 24 h induced astrocyte specific glutamate transporter expressions (GLT-1/EAAT-2 and GLAST/EAAT-1) in NT-2/D1 cells. (D) RT-PCR analysis of mRNA of GFP transcribed by GFAP promoter in NT-2/D1 cells transfected with pGFAP-GFP-S65T (1μg each) as a reporter system. After 2days, the cells treated with 10 nM STS for 24 h expressed GFP gene. (E) Fluorescence image of GFP in NT-2/D1 cells transfected with pGFAP-GFP-S65T with STS (a) and without 10 nM STS (b) treatment for 24h. Then cells were fixed with 4% para formaldehyde and analyzed under the fluorescent microscope.

Increased gene expression level of APP during STS-induced astrocytic differentiation

Both mRNA and protein expression of APP in NT-2/D1 time-dependently increased after 10 nM STS treatment (Figure 11A and B). In addition, concentration of sAPP also time-dependently increased in the media of NT-2/D1 cell culture (Figure 11B). Then, the increased catabolism of APP showed the correlation with the GFAP expression in the NT-2/D1 cells (Figure 11B). This result suggests that STS-induced APP expression may have an important role in astrocytic differentiation of NT-2/D1 cells.

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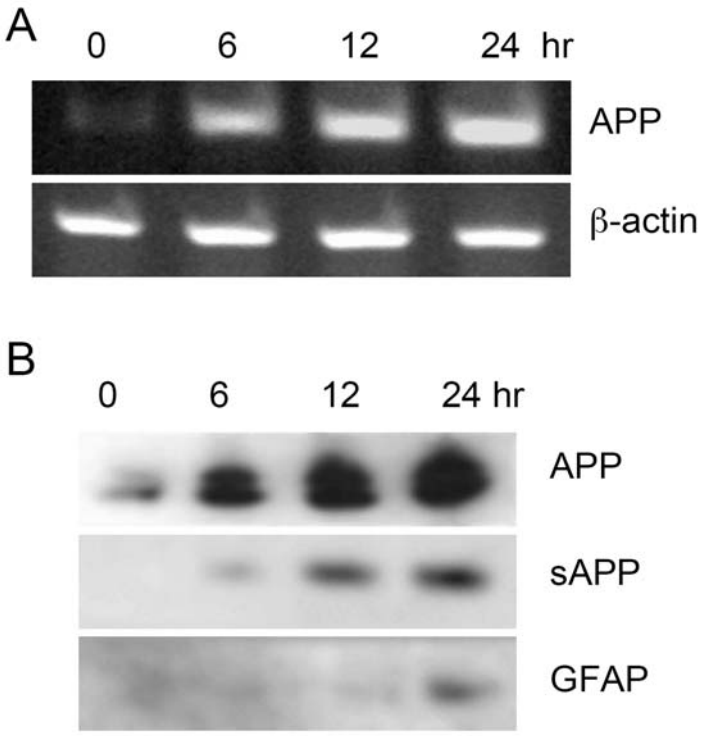


Figure 11: Increased gene expression of APP during STS-induced gliogenesis. (A) RT-PCR analysis of APP gene expression in NT-2/D1 cells treated with 10 nM STS. (B) Western blot analysis of APP, sAPP and GFAP protein expression in NT-2/D1 cells treated with 10 nM STS. Samples for APP and GFAP were isolated from the cell lysates; sample for sAPP was isolated from the NT-2/D1 cell culture media. The samples were subjected to immunoprecipitation with mouse-anti-APP Ab (22C11) or rabbit-anti-GFAP Ab. Then the immunoprecipitants were analyzed by Immunoblot analysis using a mouse-anti-APP Ab (22C11) or rabbit-anti-GFAP Ab. Western data show time dependent increase of these proteins

sAPP induced GFAP expression in NT-2/D1 cells

To determine whether expression of APP can affect stem cell differentiation, we applied various amounts (0, 1, 10, and 100 ng) of recombinant APP695 protein in NT-2/D1 cells, neural progenitor cell lines, overnight and examined GFAP expression. A distinctive increase of GFAP expression was observed with a dose-dependent manner except 500ng APP treated cells compared to the control (Figure 12A). Although expression of GFAP was somehow decreased in higher dose treated cells, still GFAP expression level was higher than the control. We already reported STS enhances gene expression of GFP by stimulating GFAP promoter activity (Figure. 10D and E). However, it wasn't clear whether STS has a direct affect on the GFAP promoter or GFAP promoter activity is regulated by other factors, such as APP, which is induced by STS treatment. Thus, we examined GFAP promoter activity by assessing GFP expression using NT-2/D1 cells transfected with GFAP promoter driven GFP expression vector (pGFAP-GFP-S65T) (Figure 12B). When APP695 expression vectors (pCEP-APP695) were overexpressed in reporter vector transfected cells, gene expression of GFP was detected. Therefore, these results suggest that APP is involved in astrogliogenesis of NT-2/D1 cells in STS treated condition.

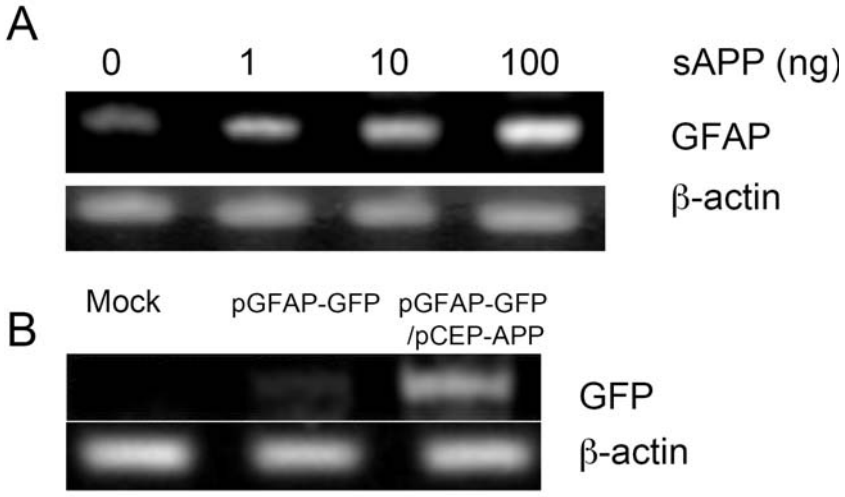


Figure 12: Increased GFAP expression in either treatment of APP and overexpression of APP. (A) RT-PCR analysis of GFAP gene expression in NT-2/D1 cells treated with various amount (0, 1, 10, and 100 ng) of recombinant APP shows dose-dependent increase of GFAP gene expression. Expression of β-actin was examined as a loading control. (B) RT-PCR analysis of mRNA of GFP transcribed by GFAP promoter in NT-2/D1 cells transfected with pGFAP-GFP-S65T (1μg each) as a reporter system. Only APP expression vector (pCEP-APP695) was co-transfected, expression of GFP mRNA can be examined

Gene silencing of APP expression suppresses GFAP expression

Recently small interference RNA (siRNA) has been extensively used for elucidating specific functions of the gene of interest [115]. To confirm the involvement of APP in STS-induced astrocytic differentiation, we investigated whether STS function in NT-2/D1 is abolished by knocking-down the gene expression of APP using siRNA. We constructed specific siRNA PCR products (siAPP-PCR), which specifically recognize APP. Small interference APP-PCR significantly decreased protein expression of APP–GFP fusion protein in NT-2/D1 cells transfected with pCMV-APP-GFP (Figure 13 A-d). Control PCR products (U6-random sequence PCR) did not inhibit APP-GFP fusion protein expressions.

Small interference APP PCR also showed significantly suppressed APP and GFAP gene expressions in NT-2/D1 cells treated with STS, while control siRNA PCR products (siGFP-PCR), which recognize GFP, didn't suppress APP and GFAP expression (Figure 13 B and C). These findings suggest that APP is a crucial molecule for astrocytic differentiation of NT-2/D1 cells induced by STS treatment.

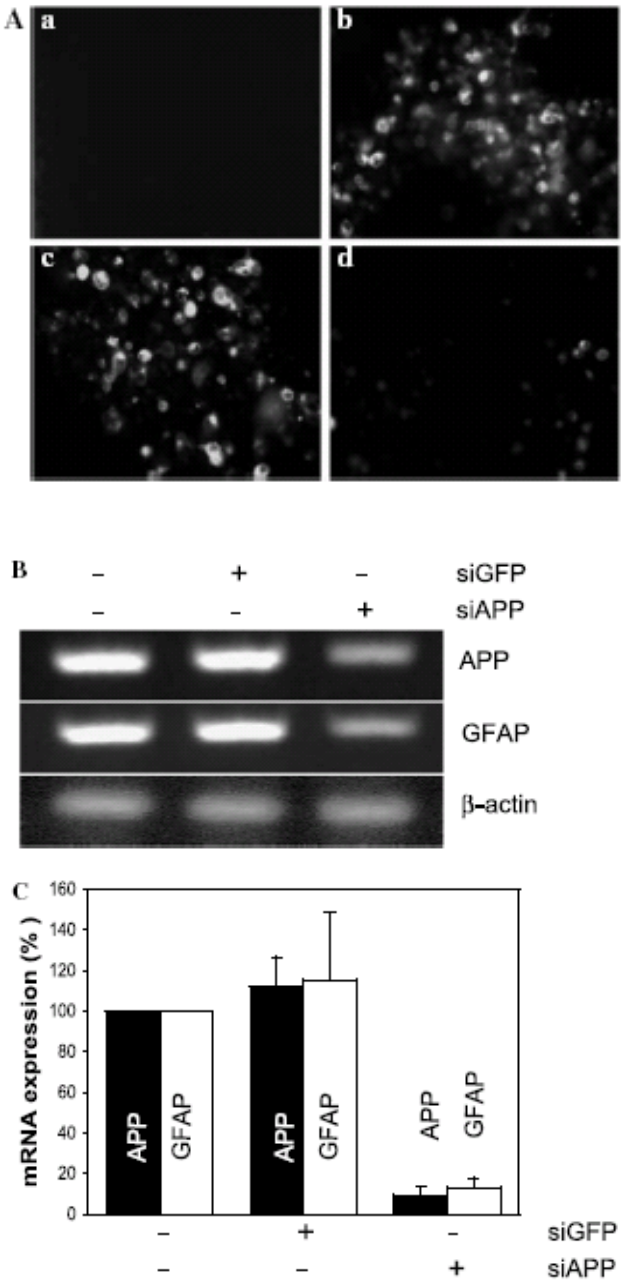
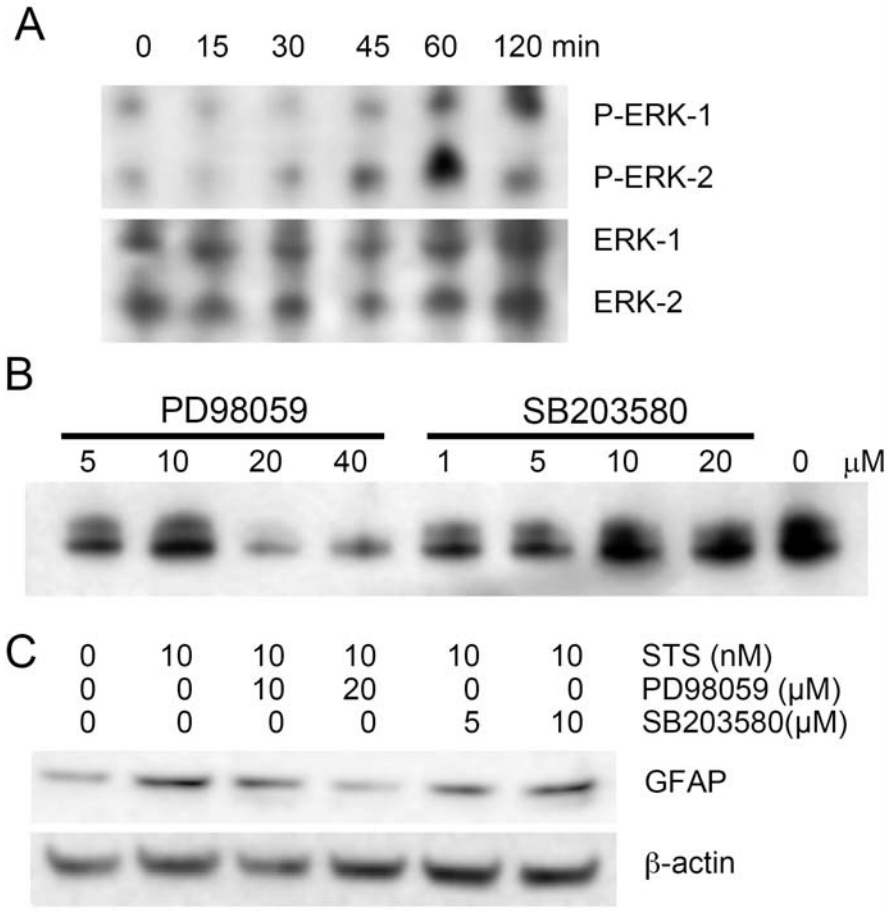


Figure 13: Assessment of APP functions by RNAi for APP in NT-2/D1 cells. (A) A mock-transfected NT-2/D1 cells, as negative controls, show no auto fluorescent signal (a). NT-2/D1 cells transfected with 1µg of pEGFP-C1, as positive controls, show significant level of green fluorescent signals (b). NT-2/D1 cells in both c and d received transfection of 1 µg of pCEP-APP-GFP to express APP-GFP fusion protein. Co-

transfection of 1 μg of U6-random PCR products expressing random sequence does not suppress expression of the APP-GFP fusion protein (c). Co-transfection of 1 μg of siRNA PCR products targeting APP significantly suppresses expression of the APP-GFP fusion protein (d). Experiments were repeated at least three times with similar results. (B) Silencing of APP reduced GFAP expression stimulated by 10 nM STS in NT-2/D1 cells. Gene expression levels of APP and GFAP were assessed by RT-PCR in NT-2/D1 cells treated with 10 nM STS were transfected with each 1 μg of siRNA of PCR products targeting GFP (siGFP) or APP (siAPP) for 2 days. Transfection of siAPP but not siGFP significantly reduced the expression of APP as well as GFAP mRNA levels in the NT-2/D1 cells. (C) The data (means ± SD, n=3) presented are % changes of mRNA expression of APP and GFAP compare to the control, NT-2/D1 cells only treated with 10 nM STS, as 100%.

STS stimulate APP expression via ERK pathway

Although STS is known to be a PKC inhibitor [101], it is also reported to activate mitogen-activated protein kinase (MAPK) signal transduction [116], which is a major pathway responsible for APP catabolism [117]. Treatment of NT-2/D1 cells with 10 nM STS time-dependently increased phosphorylation of ERK1/2 (p44/42 MAP kinases) after 45 min of incubation (Figure 14 A). To further test the role of ERK signaling in STS-induced APP expression, we pretreated NT-2/D1 cells with PD098059 [118], a selective inhibitor of MEK1 or SB203580 [119], a selective inhibitor of p38 MAPK for 30 min prior to a 10nM STS treatment with PD098059 or SB203580 for 24h. PD098059 inhibited STS-induced APP (Figure 14 B) and GFAP (Figure 14 C) production at a concentration of 20μM or greater, whereas SB203580 didn't significantly alter APP and GFAP production. Similarly, mRNA expression of APP also was inhibited by PD098059 at a concentration of 20μM or greater (Figure 14 D and E). In addition, mRNA expression levels of GFAP decreased according to the decrease of APP expression (Figure 14 D and E). These results indicate that STS-induced APP and GFAP expressions are mediated by ERK1/2 signaling pathway not by p38 MAPK pathway and that STS-induced astrocytic differentiation is mediated via ERK pathway by stimulating APP production.


A
0 15 30 45 60 120 min
P-ERK-1
P-ERK-2
ERK-1
ERK-2
B
PD98059
SB203580
5 10 20 40 1 5 10 20 0 μM
C
0 10 10 10 10 10 STS (nM)
0 0 10 20 0 0 PD98059 (μM)
0 0 0 0 5 10 SB203580(μM)
GFAP
β-actin

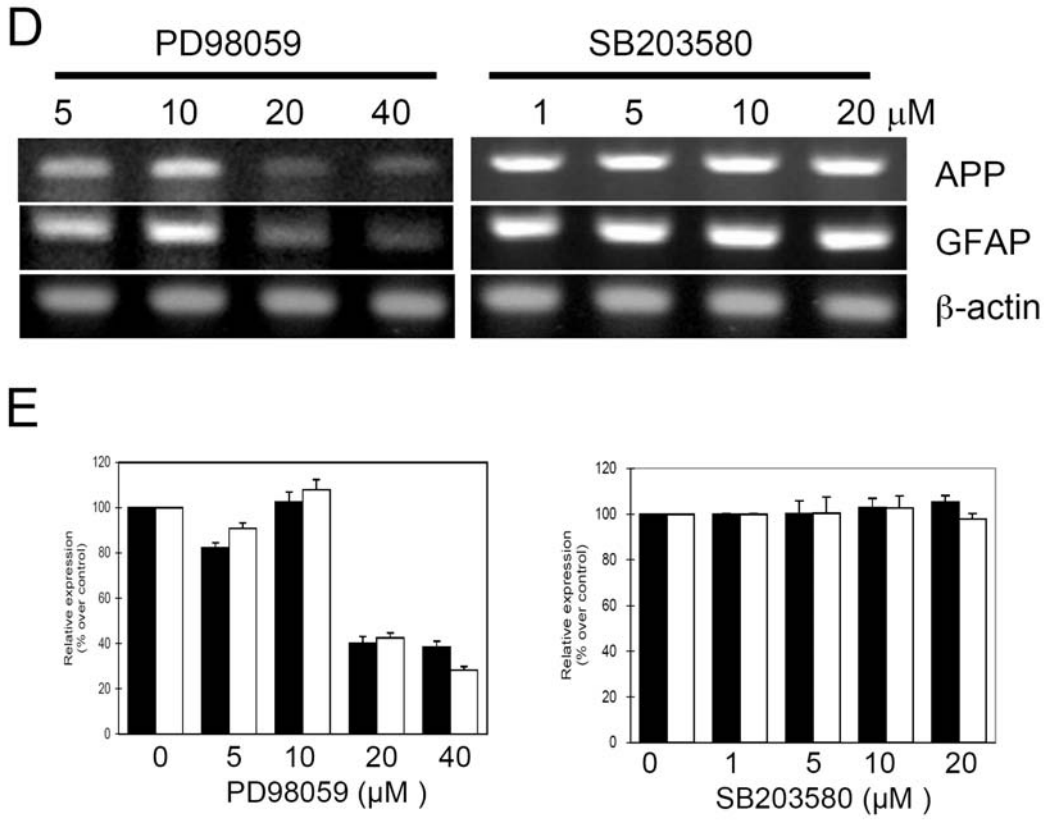


Figure 14: The involvement of ERK1/2 pathway in STS-induced astrocytic differentiation of NT-2/D1. (A) NT-2/D1 cells were plated at a density of $1x10^6$ cells and then treated with 10 nM STS for indicated time period to induce astrocytic differentiation. Cell lysates were subjected to immunoprecipitation with anti-human-pan-ERK Ab. Parallel blots were probed with anti-phospho-p44/42 ERK Ab (P-ERK-1/2) and anti-pan-ERK Ab (ERK-1/2). Phosphorylation of ERK1/2 increased progressively after 45 min. (B) PD098059, a selective MEK1 inhibitor, decreased APP expression activated by 10 nM STS in NT-2/D1 cells. While SB203580, a selective p38 MAPK inhibitor, did not affect APP expression. (C) PD98059, decreased GFAP expression activated by 10 nM STS in NT-2/D1 cells, while SB203580 did not affect GFAP expression. (D) PD98059, decreased GFAP and APP gene expression activated by 10 nM STS in NT-2/D1 cells, while SB203580 did not affect the gene expression. Gene expressions of APP and GFAP were examined by RT-PCR in NT-2/D1 cells pretreated with various concentrations of PD098059 or SB203580 for 30 min and exposed to 10 nM STS for 24 h. (E) Semi quantitative image analysis of gene expression data from (D). The data (means ± SD, n=3) presented are % increase of mRNA expression of APP and GFAP compare to the control as 100%.

Discussion

NT-2/D1 cells have been thought to exclusively differentiate into pure neurons as a neuronal progenitor. However, recent studies show astrocytic differentiation of NT-2/D1 cells by RA treatment [97, 99], indicating that it is a good *in vitro* model system for analyzing regulation of neural differentiation. In the current study, we report that STS induces astrocytic differentiation of NT-2/D1 by increasing APP expression via activation of ERK1/2 signaling cascade.

Typical astrocytic stellate morphology appeared in the culture after 7 days of treatment with STS and more than 90 % of cells showed this feature by week 2. These cells expressed high levels of not only GFAP, a typical astrocyte marker but also astrocyte-specific glutamate transporters such as GLAST/EAAT1 and GLT1-EAAT2, which are used for removing excessive glutamate to maintain a physiological level of extracellular glutamate concentration [120], indicating that STS differentiated NT-2/D1 cells into astrocytes. Furthermore, STS treatment increased green fluorescent in NT-2/D1 transfected with reporter vector (pGFAP-GFP-S65T), indicating activation of GFAP promoter by STS.

Apoptosis is closely associated with differentiation during development of the neuronal system [121]. Recent reports also show STS, a potent apoptosis inducer, causes early neural stem cell like differentiation of embryonic stem cells [104]. Although the mechanism is not clear, STS is also reported to induce astrocytic phenotypes in C6 glial cells [106].

Thus apoptotic cascade triggered by STS may be involved in the astrocytic differentiation.

We found that APP expression, which is reported to be increased during apoptosis [122], increased in NT-2/D1 cell culture after STS treatment. Although to date, most of APP studies have been focused on neurotoxicity of Aβ, several lines of evidence suggest that APP affects mechanisms of anti-apoptosis and neurite outgrowth [28, 111]. Thus, the increased expression levels of APP may be a compensation mechanism of the apoptotic condition created by STS treatment. Down's syndrome (DS) is associated with a high incidence of AD with massive gliosis [123, 124]. Recently Bahn *et al.* [56] demonstrated that neurospheres derived from DS patients almost exclusively differentiated into GFAP positive cells. Since DS patients have trisomy of chromosome 21, where the APP gene is localized, we associate these incidents to an over expression of APP and speculate that STS-induced APP expression might play a crucial role in glial differentiation of NT-2/D1 cells.

In this study, knocking down APP expression of NT-2/D1 cells using RNA interference (RNAi) against APP reduced GFAP expression induced by STS treatment. Although STS may induce astrocytic differentiation of NT-2/D1 through various signaling mechanisms, this result suggests that at least APP is involved in the induction of GFAP expression during STS-induced glial differentiation.

MEK1 inhibitor (PD098059), which reduces phosphorylation of ERK1/2, dose-dependently reduced expression of APP and GFAP. Since APP expression is regulated by phosphorylation of ERK1/2 [125] and STS increases phosphorylation of ERK1/2 [116], STS may increase APP expression level by ERK1/2 phosphorylation. On the other hand p38 MAP kinase inhibitor (SB203580) did not show any significant effect on APP and GFAP expression. These results indicate that STS may induce GFAP expression through the up regulation of APP by increasing ERK1/2 phosphorylation.

It is not clear how adult neurogenesis is essential for normal cognitive function in aging. Although the rate of endogenous neuroregeneration in the adult brain may be minimal, in the long run, pathologically-altered APP metabolism in AD or DS causes a defect in neurogenesis and significantly harms normal brain function. This fact could also prevent successful neuroreplacement therapy for AD using NSC by shifting the differentiation pattern of the transplanted cells into glial cells rather than into neurons. Thus, in order to use stem cell transplantation as a potential strategic intervention therapy for AD or DC. Regulation of APP levels and/or modifications of the APP signal pathways within the cells may need to be developed along with a better understanding of how the mechanisms of APP function in neural stem cell biology.

THE NOVEL FUNCTION OF AMYLOID PRECURSOR PROTEIN IN GLIAL DIFFERENTIATION IS MEDIATED THROUGH IL-6/GP130 SIGNALING PATHWAY

Introduction

During neural development, neurons are typically differentiated before astrocytes and oligodendrocytes with the exception of a few sites of postnatal and adult neurogenesis such as the subventricular zone (SVZ) of the fore brain [126, 127]. Current studies showed that different kind of population of brain cells can be sequentially generated from individual progenitors by cell intrinsic factors as well as extrinsic cues from surrounding environment [128]. Generally, glial differentiation is occurred during relatively late embryonic stage (mouse E16-17 day) and the postnatal period by various factors [129]. To date, interleukin (IL-6) cytokine families [130], bone morphogenic factors (BMPs) [131], basic fibroblast growth factor (bFGF) [132], and Notch signaling [133] has been known as an important gliogenic factors, functioning only in E15 or more order cortical progenitors. Although these gliogenic factors also exist in the earlier embryonic stage (around E11-12), they promote proliferation of neural stem cells instead of glial differentiation by various factors such as DNA methylation [53] and bHLH transcription factor (Neurogenin 1 or Neurogenin 2) [134].

IL-6-induced JAK/STAT signaling pathway is a central part of the glial differentiation process [40]. There are reported several IL-6 family cytokines such as leukemia inhibitory

factor (LIF), CNTF, IL-6, and oncostatin M (OSM) [130]. Once these IL-6 cytokine families interact with their cognate receptors (ex, IL-6 receptor α), the resultant complexes are then associated with gp130 [44], crucial signal transducing component of IL-6 receptor. Subsequently, formation of receptor heterodimers stimulates a downstream transcription factor, signal transducer and activator of transcription (STAT) proteins by activation of JAKs, especially JAK1 and JAK2. Predominantly, STAT3 molecules get phosphorylation, and then the phosphorylated STAT3s form dimerization and undergo translocation into the nucleus. Finally, STAT3 dimers bind to the putative STAT3 binding element of GFAP promoter, typical astrocytic marker, for glial differentiation

Despite above-mentioned reports have intensively showed the mechanisms of glial differentiation, there still remain other factors that induce differentiation by unknown mechanisms. The APP is a membrane spanning glycoprotein consisting of a 695- to 770-amino acid [9]. To date, the cytotoxicity of Aβ peptides, generated by subsequent cleavage of β- and γ-secretase, has been focused due to its cytotoxicity in the brain, leading to the pathophysiology of AD [135]. Although several physiological function of APP has been reported, it is still controversial and needs to be clarified [26]. However, crystallography studies showed that the structure of N-terminal domain of APP (23-128 a.a residues) is harboring growth factor-like structure which possesses three disulfide bridges and cystein rich regions [27]. Moreover, this domain include heparin binding site which is usually positively charged basic region. Furthermore, several studies provided evidence for the view that released sAPP may operate as a factor that is able to evoke coordinated cellular response in specific target cells [85, 136, 137]. For example, sAPP

interacts with the class A scavenger receptor (SR-A), showing the physiological function of sAPP as a ligand [54]. In this receptor-ligand interaction, negatively charged N-terminal domain of APP (191-264 a.a) was identified as an essential domain for physical interaction with SR-A. Thus, these reports suggest secreted type of APP may regulate cellular function via certain receptors.

On the other hand, several lines of reports showed the potential role of APP in the stem cell biology. DS patients have 3 copies of chromosome 21[138]. Since APP coding gene is also located in chromosome 21, DS patients express high level of APP and shows similar symptoms of AD on their later stage [138, 139]. Interestingly, while normal subjects' neural stem cells were differentiated into neuron and glia with a half ratio, most of DS patients' neural stem cells were differentiated into astrocytes [56]. Therefore, this indicates that high concentration of soluble APP possibly affects cell fate specification of neural stem cells of DS patient. Previously, we reported that treatment of high concentration of sAPPα or overexpression of APP enhanced the glial differentiation of HNSCs [140]. Moreover, we also found that an important role of APP in staurosporine (STS)-induced glial differentiation of neural progenitor, NT-2/D1 cells [141].

In the present study, we examined specific glial differentiation mechanism stimulated by sAPP in NT-2/D1 cells. Then, we found that IL-6 cytokine signaling pathway is crucial for the sAPP–induced glial differentiation. Therefore, these results implicate a deficiency of stem cell biology by altered APP metabolism in AD pathology.

Materials and methods

Reagents and antibodies

Jak2 inhibitor, AG490 (tyrphostin B42; Sigma) were dissolved in dimethyl sulfoxide and stored at -80℃ until use. Recombinant sAPPα protein (Sigma) was solubilized with dH_2O and stored at -80°C until use. The following antibodies (Abs) were used: mouse β-III tubulin Ab (Sigma); rabbit anti-GFAP Ab (Promega); mouse anti-APP Ab (22C11) (Chemicon); rabbit anti-human STAT3 Ab (Chemicon); rabbit anti-phospho-STAT3 (Tyr705) Ab (Cell Signaling); mouse anti-CD130 (gp130) Ab (Chemicon); anti-phospho-tyr Ab (4G10) (Upstate) and rabbit anti-β-actin Ab (Cell Signaling). For the secondary Abs, anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary Abs (Jackson Immunoresearch Laboratory) were used.

Cell culture and transfection

The NT-2/D1 cells were seeded ($5x10^6$ cells per 150 mm petri dish) in Dulbecco's modified Eagle's medium (DMEM/F-12; Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS; Novacell), 1% antibiotic-antimycotic (Invitrogen), 4 mM glutamine (Invitrogen) and maintained in a humidified atmosphere of 5% CO^2/95% air at 37ºC [97, 142]. The cells were passed twice a week by short exposure to 0.25% trypsin/0.01% EDTA (Invitrogen). For the experiments, 1×10^6 NT-2/D1 cells were plated in a 6 well tissue culture plate and subsequently, APP-induced differentiation of NT-2/D1 cells was evaluated for expression of astrocytic and neuronal markers by RT-PCR

and Western blot analysis under the treatment of recombinant sAPP. Transient transfection of various siRNA PCR fragments (siJAK1, siSTAT3 and siCNTF), which suppress the gene expression of JAK1, STAT3 and CNTF, respectively, were performed with Lipofectamine™ 2000 (Invitrogen) on subconfluent NT-2/D1 cells in a 6-well culture plate, according to the manufacturer's protocol. Transfection efficiency was determined by transfection of 0.5 μg of pEGFP-C1 (Clontech). Cells were rinsed twice in ice-cold phosphate-buffered saline (PBS, pH7.4) after 48h of the incubation and mRNA samples were extracted for further analysis.

RT-PCR analysis

Total RNA was extracted from the cells with Trizol reagent (Invitrogen) according to the manufacturer's protocol. One μg of the total RNA was reverse-transcribed and amplified by the SuperScript™ ONE-STEP™ RT-PCR system (Invitrogen) with the following primers: IL-6R (+) 5′-CATTGCCATTGTTCTGAGGTTC-3′, (-) 5′-AGTAGTCTGTATTGCTGATGTC-3′; gp130 (+) 5′-AAGGATGGTCCAGAATTCAC-3′, (-) 5′-CCTTCACTGAGGCATGTCGC-3′, (-); GFAP (+) 5'-AAGCAGTCTACCCACCTCAG-3', (-) 5'-ATCCCTCCCAGCACCTCATC-3'; CNTF (+) 5'-CAGGGCCTGAACAAGAACAT-3', (-) 5'-GCCAACAAAACATGGAAGGT-3'; LIF (+) 5'-CTGTTGGTTCTGCACTGGA-3', (-) 5'-GGGTTGAGGATCTTCTGGT-3'; JAK1 (+) 5'-TGCTCCTGAGTGTGTTGAGG-3', JAK1 (-) 5'-AGGTCAGCCAGCTCCTTACA-3'; STAT3 (+) 5'-TTTCACTTGGGTGGAGAAGG-3', STAT3 (-) 5'-GCTACCTGGGTCAGCTTCAG-3'; β-actin (+) 5'-GACAGGATGCAGAAGGAGAT-3', (-)

5’-TTGCTGATCCACATCTGCTG-3’. Ten µl of the reaction were then separated on a 2% E-gel (Invitrogen).

Immunoprecipitation and western blot analysis

Protein samples were prepared by lysing the cells using an ice-cold lysis buffer consist of 1% NP40, 150 mM NaCl, 50 mM Tris pH8.0 and Complete Protease Inhibitor Tablets (Boehringer). The protein concentration of each sample was measured by Bio-Rad protein assay (Bio-Rad). Lysates were immunoprecipitated with an antibody against STAT3 and gp130 molecules using protein A-Sepharose (Amersham Bioscience). Then, precipitates were heated at 70ºC for 10 min in LDS (Lithium Dodecyl Sulfate) sample loading buffer (1×) and separated on NuPAGE^{TM} 4-12 % Bis-Tis Gel (Invitrogen) for 45 min at 200 V and transferred to PVDF membrane (30V, 60 min). Membranes were blocked with 5% skim milk in PBS for 1h at RT and probed at 4ºC overnight with primary antibody in 5% skim milk. The membranes were washed 3 times for 5min each with PBS containing 0.05% Tween 20 (pH7.4) and then incubated with horseradish peroxidase-conjugated secondary antibodies in 5% skim milk for 2h at RT. After 3 times washing with PBS containing 0.05% Tween 20, immunoreactive bands were visualized by using ECL plus (Amersham Bioscience) chemiluminescence reagent.

siRNA preparation

Human STAT3 (Genbank access number: NM_003150), Jak1 (Genbank access number: AB219242) and CNTF (Genbank access number: NM 000614) mRNA target sequences were designed using ambion target finder software. The mRNA target

sequences of human STAT3, Jak1 and CNTF were 5'-TATCATTGACCTTGTGAAA-3' (1919-1938), 5'-CCACATAGCTGATCTGAAA-3' (2710-2729) and 5'-TACAAGATCCCCCGCAATG-3' (475-494), respectively. siRNA PCR products were synthesized using a Silencer™ Express kit (Ambion). Then, cells were transfected with siRNAs and incubated for 48 hr. The silencing efficacy was confirmed by RT-PCR.

Immunocytochemistry

Detailed methods for immunocytochemistry were previously described [140]. Briefly, for HNSC staining, after fixation, samples were washed in phosphate-buffered saline with 0.2 % Triton X (PBST), then incubated in PBST containing appropriate normal sera. Next, the samples were incubated overnight (up to 16 hr) with primary antibodies, and then incubated with secondary antibodies for 1.5 hr in a dark humidified chamber after washing with PBS. Next, the samples were washed thoroughly in PBS and coverslipped with VECTASHIELD® DAPI Mounting Media (Vector).

Image analysis

Gel and Western blot images were captured by KODAK Image Station 2000MM (KODAK). Optical density of the target bands were analyzed by ImageJ software (NIH-IMAGE Version 4.1) and expressed in Mean±SD form experiments performed in triplicates.

Results

sAPP induces glial differentiation via IL-6 cytokine related signaling pathway

To investigate the physiological role of sAPP as a gliogenic factor, HNSCs were treated with recombinant sAPPα (100ng/ml) and were differentiated under serum-free unsupplemented conditions. HNSCs treated with sAPPα revealed a significantly higher level of GFAP-positive cells compared to control HNSCs at 5DIV (Figure 15A). While the HNSC controls were differentiated into neurons and glial with a relatively even ratio, treatment of sAPPα induced massive glial differentiation of treated HNSCs. These results indicate that an overdose of the wtAPP causes gliogenesis rather than neurogenesis *in vitro*.

To investigate how sAPP induces glial differentiation of neural stem cells, first, we examined the potential involvement of IL-6 cytokine related signaling pathway. As we mentioned above, Santiago-Garcia *et al.* reported the novel function of APP as a ligand for the SR-A of macrophages [54]. Concurrently, the structural property of N-terminal domain of sAPP also indicated that secreted soluble APP may have a growth factor like function which interacts with cell surface receptor[27]. Thus, we hypothesized that released sAPP may stimulate IL-6 cytokine related signaling pathway, similar to other IL-6 ligand such as LIF, CNTF and *etc.* To verify whether sAPP activates IL-6/gp130 signaling, we examined the gene expression of various molecules related with IL-6 cytokine families, such as IL-6R, gp130, CNTF, LIF, JAK1, and STAT3, in the presence

of sAPP (Figure 15B). Consistently with immunohistochemistry data, the treatment of sAPP promoted expression of GFAP, a typical astrocyte marker [113]. While IL-6 R and LIF gene expression level was not changed, expression level of gp130 and CNTF was substantially increased by treatment of sAPP. Among downstream signaling molecules, although expression of STAT3 does not show any significant changes in mRNA level, expression level of JAK1 was increased by treatment of sAPP. In general, various IL-6 cytokine families have induced different cellular response by stimulating certain 'preferred' molecules from IL-6 receptors, JAKs, and STAT molecules [143]. In this context, our results suggest that sAPP induces glial differentiation of NT-2/D1 cells through IL-6 signaling cascade by specific upregulation of expressions of gp130, CNTF and JAK1.

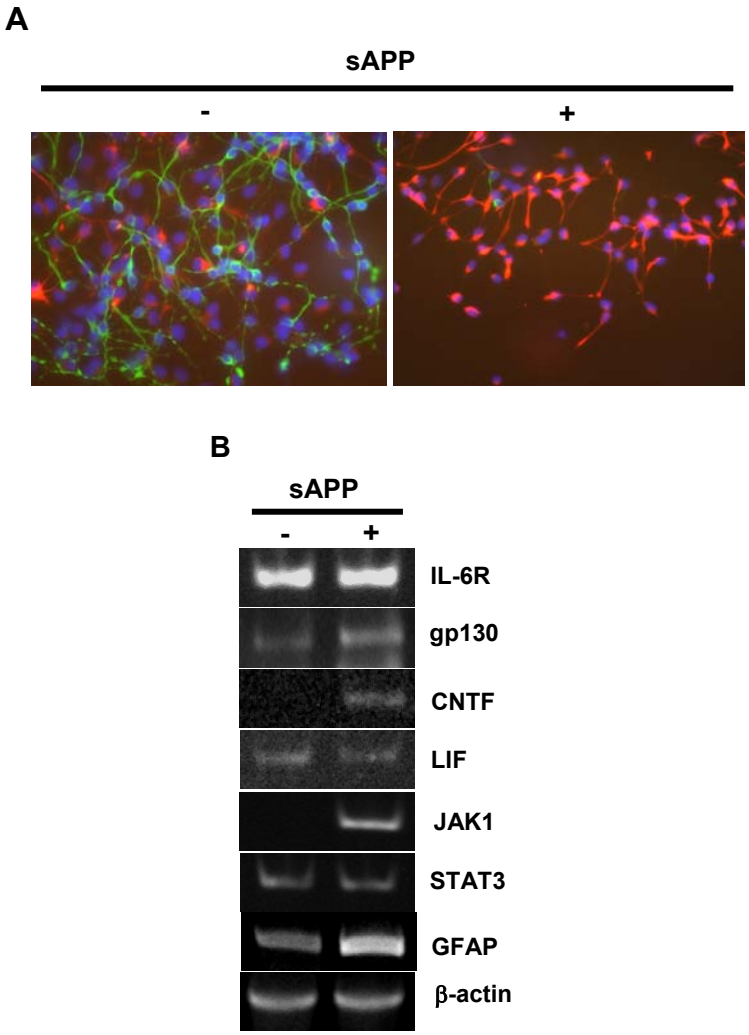


Figure 15: Differentiation of HNSCs and IL-6/gp130 related gene expressions were altered by high concentration of sAPP. (A) APP transfection increases the glial population of HNSCs *in vitro*. Double immunofluorescence staining with GFAP (red) and βIII-tubulin (green) markers for astrocytes and neurons, respectively. All nuclei are counterstained with DAPI (blue). HNSCs transfected with mammalian expression vectors containing genes for wild-type APP (wtAPP) differentiated under serum-free unsupplemented conditions displayed a significantly reduced level of neural differentiation (*a*) compared with HNSCs transfected with the vector alone at 5DIV (*b*). The abnormal morphology of the processes is shown in neuronally differentiated HNSCs (green) with wtAPP gene transfection (*a*). (B) sAPP–induced gene expression changes, which are related with IL-6 signaling cascade, were assessed by RT-PCR. Recombinant protein sAPP (100ng/ml) was applied to NT-2/D1 cells for 2 hr, then, mRNAs were extracted for RT-PCR analysis.

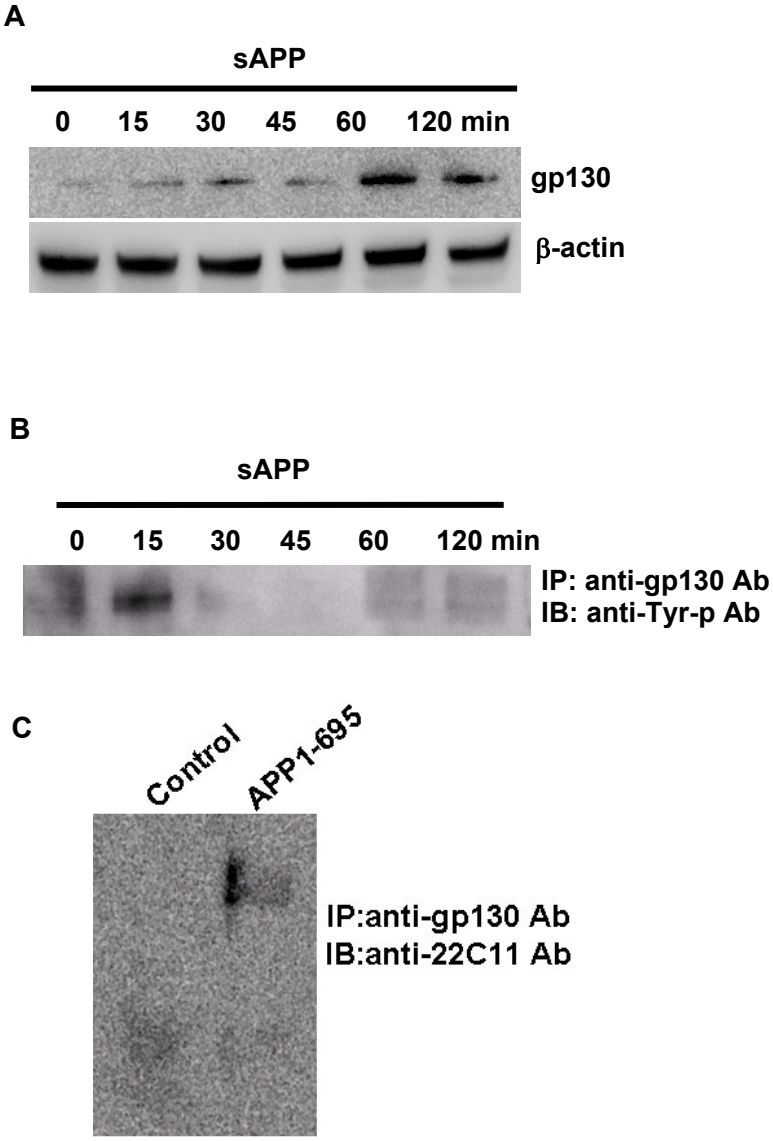
sAPP affects expression and phosphorylation of gp130 during glial differentiation

For further investigation, we examined critical changes of the IL-6/gp130 signaling cascade related molecules from upstream signaling pathway. Since expression of gp130 was significantly promoted by sAPP, we examined the functional importance of gp130, which is known as a signaling subunit of IL-6 receptor, in the presence of sAPP by measuring expression and phosphorylation of gp130 (Figure 16A) [144].

When IL-6 cytokine families interact with non-signal transducing receptors (ex, IL-6Rα and LIF-Rα), those receptor/ligand complexes came to interact with gp130. Then, those complexes are phosphorylated by JAKs and subsequent phosphorylation events are occurred. Treatment of sAPP induced slight increase of gp130 expression from 15 min. sAPP-induced gp130 expression reached its highest level at 60 min after treatment, then slightly decreased at 120 min. As mentioned above, interaction of gp130 with IL-6 cytokines induces conformational changes and recruit various tyrosine kinases, for instance, JAKs (JAK1, JAK2, JAK3, and TYK2) and tyrosine phosphatase such as an SH2 domain containing protein tyrosine phosphatase (SHP-2) (Figure 16B). We thus examined phosphorylation level of gp130 after treatment of sAPP. As shown in Fig. 16B, treatment of sAPP induced early on-set of tyrosine phosphorylation of gp130. Treatment of sAPP induced phosphorylation of gp130 in early on-set (15 min) and later on it was undetectable until 45min. However, phosphorylation of gp130 was come back to basal level from 60 min again [145]. Subsequently, phosphorylated gp130 may recruit downstream signaling molecules such as JAK/STAT to send signals to downstream.

Since expression and phosphorylation of gp130 was immediately changed by treatment of sAPP, we postulated that sAPP may directly interact with gp130 receptor subunit, similar with other ligands. To examine interaction between APP and gp130, we did an immunoprecipitation with anti-gp130 antibody and blotted with anti-22C11 antibody. Then, our result indicates that sAPP can stimulate IL-6/gp130 signaling cascade by direct interaction with gp130 receptor (Figure 16C).

In Figure 16D and E, we applied anti-gp130 (CD130) Ab to block the effect of sAPP on IL-6/gp130 signaling pathway since anti-gp130 Ab interferes correct formation of ligand/receptor complexes [146]. Then, GFAP expression and phosphorylation level of STAT3 was assessed. Treatment of anti-gp130 Ab successfully downregulated GFAP expression as well as STAT3 phosphorylation in the presence of sAPP (Fig. 16D and E). These results suggest that signals, stimulated by sAPP, are transduced via gp130 for glial differentiation of neural progenitor NT-2/D1 cells.


A
sAPP
0 15 30 45 60 120 min
gp130
β-actin
B
sAPP
0 15 30 45 60 120 min
IP: anti-gp130 Ab
IB: anti-Tyr-p Ab
C
Control
APP1-695
IP:anti-gp130 Ab
IB:anti-22C11 Ab

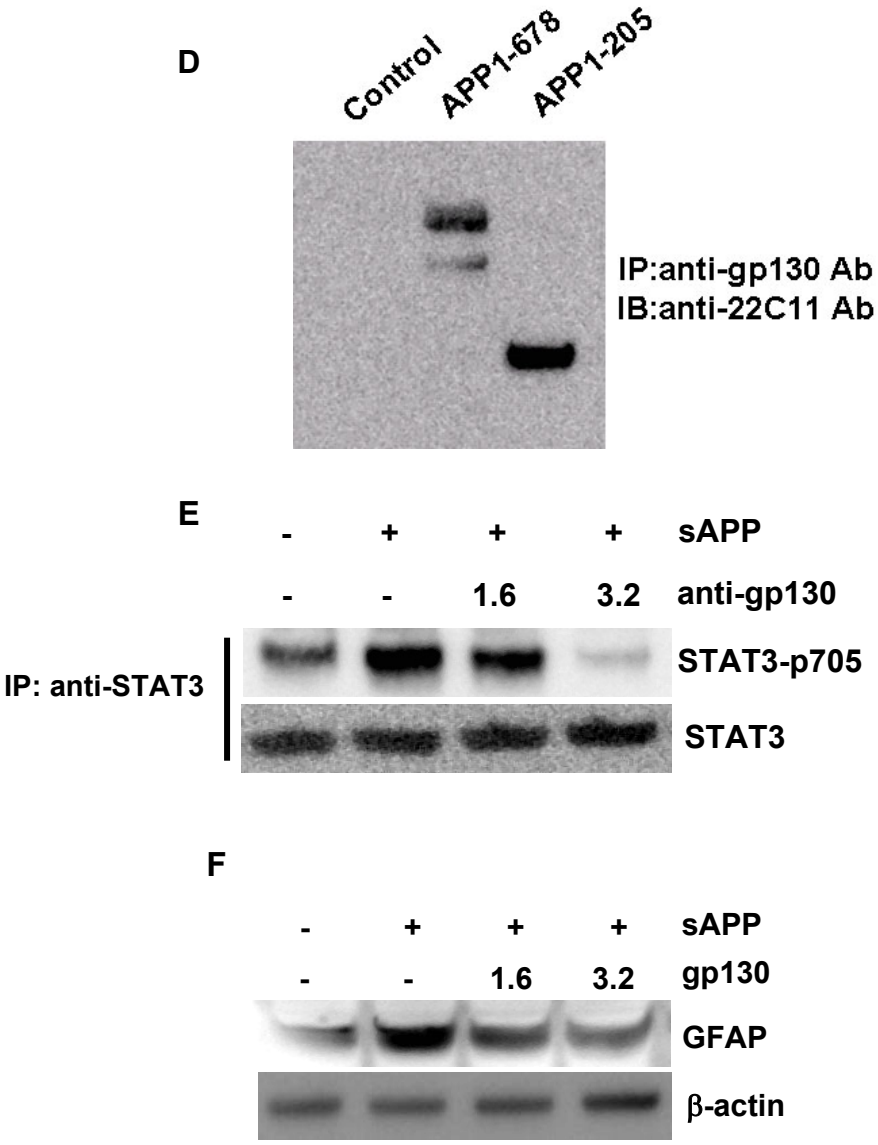


Figure 16: sAPP induces glial differentiation by upregulating expression and phosphorylation of gp130 in neural progenitor, NT-2/D1 cells. sAPP induced gp130 expression was measured by western blot using anti-gp130 Ab in a various time frame (0-15-30-45-60-120 min). (B) sAPP induced phosphorylation of gp130 was assessed by co-immunoprecipitation. Protein extracts from sAPP treated NT-2/D1 cells were immunoprecipitated with anti-gp130 Ab. Then, membranes were probed with anti-p-Tyr (4G10) Ab. NT-2/D1 cells were treated with sAPP for 16 hr in the presence/absence of the anti-gp130 blocking Ab and the expression changes of GFAP (C) and phosphorylation of STAT3 were examined (D) by western blot analysis.

sAPP induced glial differentiation is mediated via intracellular mediators of IL-6 signaling pathway

It has been previously documented that phosphorylation of downstream signaling molecules of gp130, such as JAKs and STAT3, are observed after stimulation of the IL-6 family of cytokines [130]. Although gene expression level of STAT3 wasn't changed, the importance of STAT3 shouldn't be underestimated since phosphorylation status is also crucial for the function of STAT3 in glial differentiation (Figure 15B) [147]. We thus examined whether treatment of sAPP induces phosphorylation events in STAT3. When 100ng/ml of sAPP was applied into NT-2/D1 cells, it promoted phosphorylation of STAT3-p-Tyr705 residues from 30 min after sAPP treatment with time-dependent manner (Figure 17A) [145].

Recently small interference RNA (siRNA) has been intensively used as a tool for studying the function of gene of the interest [148]. Here, we constructed siRNA PCR products against JAK1 and STAT3 to verify the role of those molecules in sAPP-induced gliogenesis. These siRNA PCR products showed successful silencing efficacy on expression of JAK1 and STAT3 (Figure 17B). When these siRNA PCR products were transfected into NT-2/D1 cells, the application of siRNA downregulated GFAP expression by knocking down JAK1 and STAT3 expression even under the treatment of sAPP (Figure 17C). Therefore, those results suggest that sAPP-induced glial differentiation is mediated by JAK/STAT in IL-6/gp130 signaling pathway.

On the other hand, we also examined the function of JAKs in sAPP-induced glial differentiation mechanism by inhibiting JAKs using selective inhibitor (AG490; Tyrphostin B42) as well as siRNA. Since phosphorylation of STAT3 at Tyr-705 residues are catalyzed by the JAKs (JAK1, JAK2, JAK3, and TYK2), we hypothesized that the application of JAK inhibitor might suppress phosphorylation of STAT3 molecules as well as GFAP expression. As demonstrated in Figure 17C, the transfection of siRNA of JAK1 efficiently knocked-down gene expression of GFAP under the presence of sAPP. Furthermore, as shown in Figure 18A and B, treatment of AG490 suppressed GFAP expression as well as phosphorylation of STAT3-p-Tyr705 [149]. Taken together, these results indicate that sAPP–induced gliogenesis is mediated by IL-6/gp130 signaling pathway through gp130 and downstream signaling molecules, JAKs and STAT3.

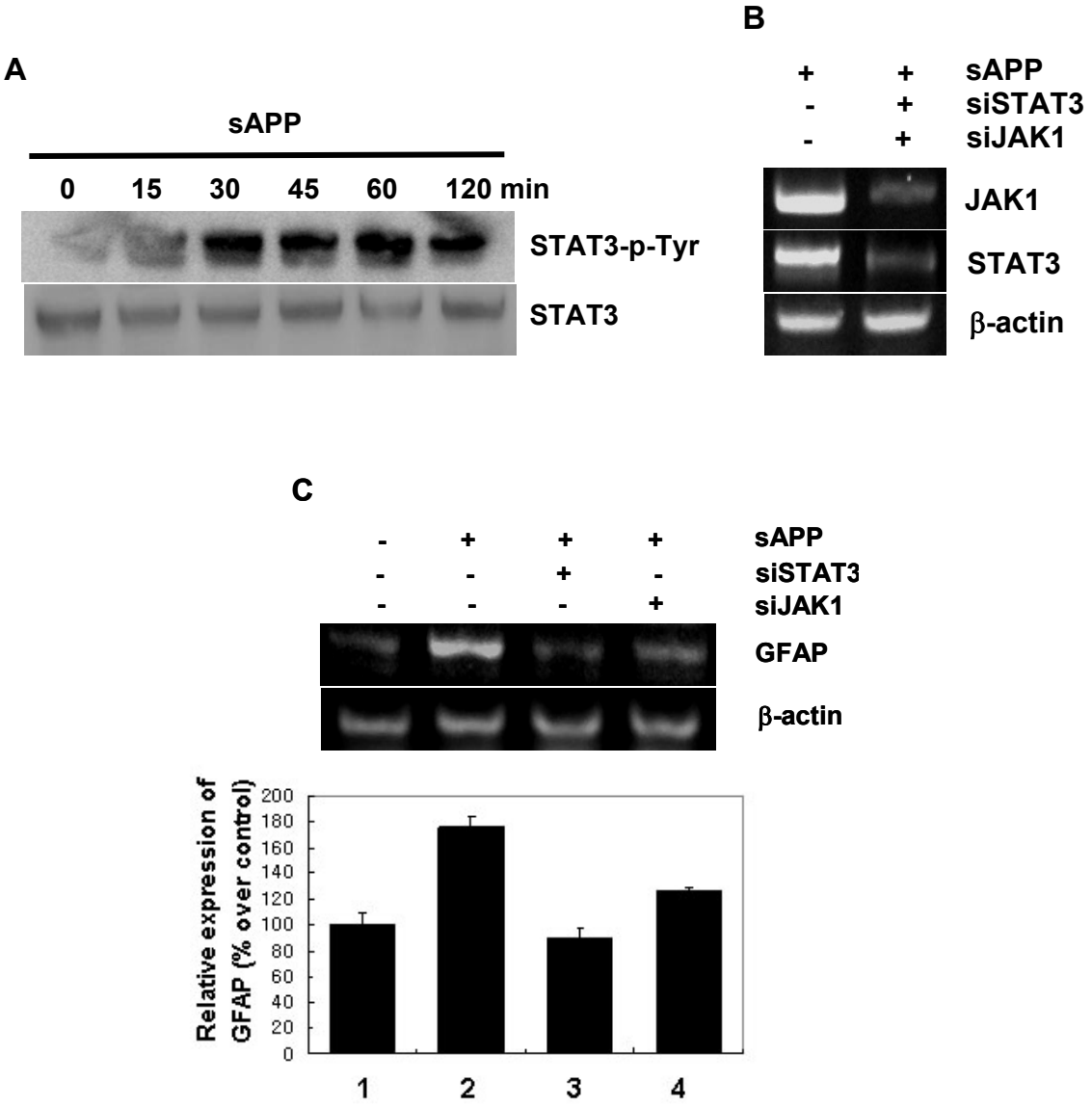


Figure 17: sAPP-induced glial differentiation is mediated by STAT3 in IL-6/gp130 signaling pathway. (A) Phosphorylation of STAT3 was analyzed by western blot. For immunoprecipitating STAT3 molecules, protein extracts from sAPP treated NT-2/D1 cells were applied with anti-STAT3 Ab. Then, phosphorylation of STAT3 molecules were assessed by probing with anti-STAT3-P705. Phosphorylation of STAT3 was also assessed in a various time frame (0-15-30-45-60-120 min). (B) Efficacy of siRNA targeting on JAK1 and STAT3 were assessed. NT-2/D1 cells were transfected with siRNAs of JAK1 and STAT3 and grown for 2 days. Then, sAPP were treated for 16 hr. To measure the efficacy of siRNA, RT-PCR was performed using JAK1 and STAT3 recognizing primers. (C) NT-2/D1 cells were transiently transfected with siRNA targeted to JAK1 and STAT3 for 2days and then incubated the cells for 16 hr with sAPP. Total

RNAs were analyzed for expression of GFAP. Expression of ββ-actin was examined as a loading control.

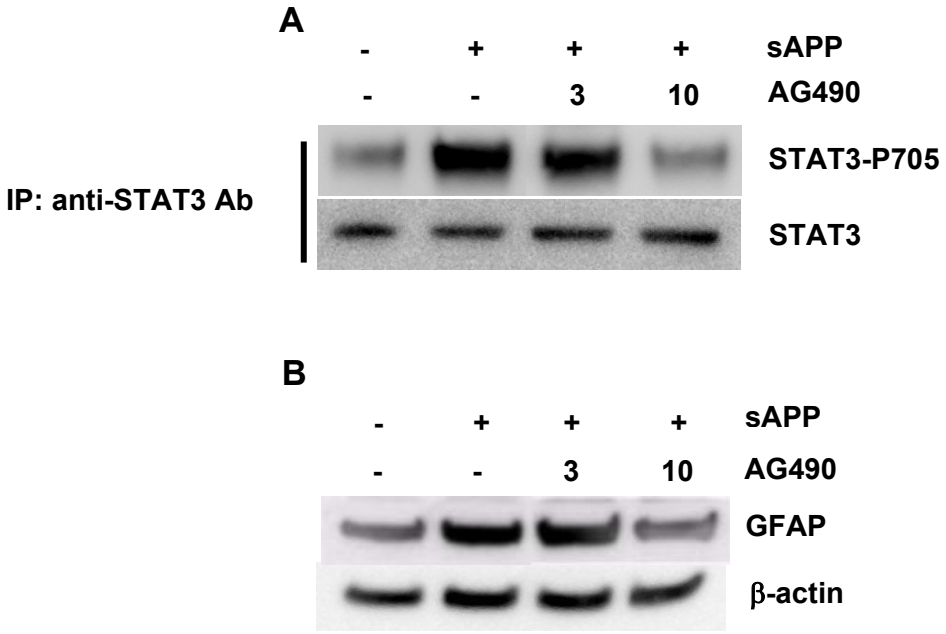


Figure 18: sAPP-induced glial differentiation is mediated by JAKs in IL-6/gp130 signaling pathway. JAK2 was also involved in sAPP induced astrocytic differentiation via IL-6 signaling pathway. To inhibit the function of JAK2, NT-2/D1 cells were preincubated with JAK2 inhibitor, AG490, for 1 hr. Subsequently, 100ng/ml of sAPP was treated into NT-2/D1 cells for 16 hr. To investigate the function of JAK2 in sAPP-induced glial differentiation, western blot analysis was performed to detect GFAP expression and STAT3 phosphorylation. Then, Treatment of AG490 suppressed STAT3 phosphorylation (A) as well as expression of GFAP with dose-dependent manner (B).

sAPP-induced CNTF expression is important for glial differentiation

CNTF is one of the IL-6 cytokine family extrinsic factors which are associated with glial differentiation of neural stem cells. Upregulation of CNTF expression was observed in a short-term (2 hr, Fig 15B) as well as long-term treatment (1-2-3-4-5 day, Figure 19A) of sAPP. This result suggests that sAPP-induced glial differentiation is potentiated by the increase of CNTF expression in NT-2/D1 cells. Although it is still not known how sAPP enhances CNTF expression, based on recent reports, upregulation of CNTF is triggered by the positive autoregulatory mechanism. To investigate function of CNTF in the context of s APP-induced glial differentiation, we also constructed and transfected siRNA, specifically silencing CNTF expression, into NT-2/D1 cells (Figure 19B). Then, versatile siRNA of CNTF suppressed GFAP expression level with dose-dependent manner in the presence of sAPP. Although still further studies are needed, this result indicates that s APP might stimulate IL-6/gp130 signaling pathway by promoting CNTF expression.

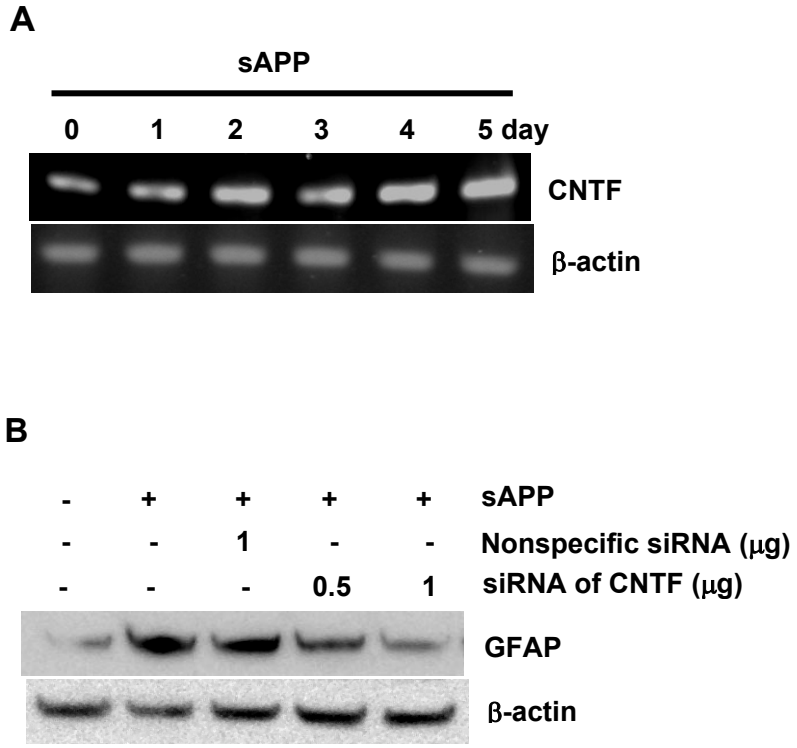


Figure 19: sAPP induces glial differentiation via upregulation of CNTF expression. (A) sAPP–induced CNTF gene expression changes were assessed by RT-PCR. 100ng/ml of sAPP was applied to NT-2/D1 cells for 0-1-2-3-4-5 days, then, mRNAs were extracted for RT-PCR. Expression of β-actin was examined as a loading control. (B) To elucidate the function of CNTF, siRNA of CNTF and non-specific siRNA was generated and transiently transfected into NT-2/D1 cells for 2 days and then cells were incubated for 16 hr with sAPP. Total RNAs were analyzed for expression of GFAP. Expression of β-actin was examined as a loading control.

Discussion

Glial differentiation is occurred at relatively later stage (E16, 17 or perinatal stage) of the development of the mammalian CNS. During this stage, glial differentiation of the neural stem cell is tightly modulated by various extrinsic (ex, LIF, CNTF, Notch, and BMPs) and intrinsic factors (ex, Ngn1, Ngn2, and DNA methylation). Extensive current studies have revealed that IL-6/gp130 signaling pathway is the central part of the glial differentiation of neural stem cells. When ligands, such as LIF and CNTF, bind to receptor, subsequent phosphorylation of gp130, JAK and STAT3 are occurred. Then, phosphorylated STAT3 complexes translocate into the nucleus and stimulate target gene expression by interacting with STAT3 binding element of the target gene such as GFAP. Interestingly, IL-6/gp130 signaling pathway is efficiently modulated by autoregulatory loop to coordinate with other part of the glial differentiation machinery such as Notch, and BMPs.

Although APP has been intensively studied due to the cytotoxicity of the Aβ peptides in the brain, we have reported novel function of sAPP as a gliogenic factor in stem cell differentiation. In our previous studies, higher concentration of sAPP induced massive glial differentiation of HNSCs under the condition of basal media differentiation. Since basal media doesn't contain serum, we can rule out the effect of any other growth factors and trophic factors [79]. Thus, this result suggests that the unique function of sAPP as a gliogenic factor in the HNSCs. Furthermore, in our previous studies, we investigated the function of sAPP in the glial differentiation, induced by STS [104]. Despite STS has been used as a PKC inhibitor or apoptosis inducing reagent, recently, it also

showed the novel function as a terminal differentiation reagent. Under the treatment of STS, neural progenitor NT-2/D1 cells were differentiated into glia, showing GFAP expression as well as astrocyte-specific morphological changes. Concurrently, expression level of APP was also upregulated by the treatment of STS by stimulating ERK signaling cascade whereas the application of siRNA of APP efficiently silenced expression of GFAP even in the presence of STS. Although our finding suggests that APP has a crucial correlation with GFAP expression during the glial differentiation, further studies are still needed to clarify the specific mechanisms, implicated in the promotion of glial differentiation. To answer that question, we investigated whether treatment of sAPP activates major glial differentiation mechanism such as IL-6/gp130 signaling pathway.

In the present study, we found that the novel function of sAPP as a gliogenic factor in neural progenitor, NT-2/D1, cells. Treatment of sAPP (100ng/ml) induced massive glial differentiation of HNSCs while untreated HNSCs were differentiated into neuron and glia evenly, showing consistent with our previous reports (Figure 21A). To elucidate whether IL-6/gp130 signaling pathway is crucial for sAPP-induced glial differentiation, we examined gene expression pattern of IL-6 relevant genes such as IL-6R, gp130, CNTF, LIF, JAK1, STAT3, and GFAP under the treatment of sAPP (Figure 15B). Since different kind of the IL-6 cytokine subfamilies induce distinct patterns of expression and phosphorylation status of signaling molecules involved in IL-6/gp130 signaling pathway, probably, IL-6/gp130 signaling may respond distinctively to treatment of sAPP [143]. In our experimental condition, treatment of sAPP enhanced expression of gp130, CNTF,

JAK1 and GFAP. Therefore, there results suggest that IL-6/gp130 signaling pathway is stimulated by treatment of sAPP.

Next, we examined the expression and phosphorylation status of gp130, signaling subunit of IL-6 receptor, in the presence of sAPP (Figure 16A and B). The expression of gp130 was gradually increased by sAPP up to 60 min and slightly downregulated at 120 min (Figure 16A). Treatment of sAPP also caused massive phosphorylation of gp130 in the relatively early stage (15 min). The phosphorylation of gp130 was disappeared quickly and then, came back to basal level at the later stage (60, 120 min) [145]. The gp130, in general, is phosphorylated by JAKs in the early stage of signal transduction. The ligand/gp130 complex is rapidly dephosphorylated by the various phosphatases (ex, SOCSs and SHPs) and internalized into the cytoplasm[145]. Therefore, our finding suggests that upregulation of gp130 expression may be a compensatory mechanism to maintain homeostasis of gp130 expression level.

Although sAPP could activate IL-6/gp130 signaling via upregulation of gp130, CNTF, JAK1, increase of those gene expressions does not explain immediate phosphorylation (< 15min) of gp130. Thus, we hypothesized that sAPP may directly activate IL-6/gp130 signaling cascade by physical interaction with gp130, similar to other IL-6 ligands. Our immunoprecipitation result indicates that sAPP could directly interact with gp130. Inversely, the application anti-gp130 Ab, which recognizes a ligand binding domain of gp130 receptor [150], potently suppressed downstream signal transduction, such as STAT3 phosphorylation and GFAP expression, by blocking the effect of sAPP on gp130

receptors. Therefore, gp130 is a critical component in the sAPP-induced glial differentiation for the transduction of outside signals, generated by sAPP.

JAK/STAT pathway has been extensively studied due to its regulatory role in various biological systems such as an immune system and developmental biology. Especially, JAK/STAT signaling pathway is a crucial system, implicated in astrocytic marker GFAP expression, in glial differentiation process. In the present studies, treatment of sAPP enhanced phosphorylation of STAT3-p-Tyr705 with time-dependent manner (Figure 17A). For suppression of the function of STAT3, siRNA of STAT3 PCR products was transfected into NT-2/D1 cells (Figure 17B, C). GFAP expression, triggered by sAPP, was drastically suppressed by the siRNA of STAT3.

On the other hand, we examined the involvement of JAKs in sAPP-induced glial differentiation. The conformational change of gp130 recruits non-receptor kinases such as JAKs. Subsequently, activated JAKs phosphorylate STAT3 molecules to form STAT3 homodimer complexes. Then, these STAT3 complexes are translocated into nucleus and transactivate GFAP expression. When we transfected siRNA of JAK1 (Figure 17C) and treated chemical JAK2 inhibitor, AG490 (Figure 18A, B), it efficiently suppressed STAT3 phosphorylation as well as GFAP expression. These results indicate that JAK/STAT is a crucial intracellular mediator of the s APP-induced glial differentiation of NT-2/D1 cells.

Next, we investigated how sAPP stimulates IL-6/gp130 signaling cascade for induction of glial differentiation. Treatment of sAPP induced expression of CNTF, one of the important extrinsic gliogenic factor, in an early on-set of gliogenesis (< 120 min) (Figure 15B) as well as in the longer period (up to 5 days) (Figure 19A). To elucidate the function of CNTF in sAPP-induced glial differentiation, we generated and transfected siRNA of CNTF (Figure 19B). Silencing CNTF expression potently suppressed GFAP expression levels in the presence of sAPP. Though still further studies are necessary, we thus postulated that treatment of sAPP may also promote glial differentiation by upregulating CNTF expression in NT2-D1 cells.

Taken together, treatment of sAPP may activate IL-6/gp130 signaling pathway by direct protein-protein interaction with gp130 and/or increase of CNTF expression. Then, serial phosphorylation events are occurred in gp130, JAKs, and STAT3 molecules for the signal transduction, triggered by sAPP. Moreover, expression of gp130 and JAK1 may be promoted by the positive autoregulatory loop of the IL-6/gp130 signaling pathway to strength glial differentiation process.

Our present results address us to novel paradigm in understanding of the pathophysiological mechanism associated with DS or AD. While Aβ has been a hallmark due to its clinical significance in AD, we showed the gliogenic function of sAPP may be also a crucial factor, inducing glial differentiation of neural stem cell in AD patient brain. Since the collections of neural progenitor population is still resided and compensate the damaged cells in the brain, in a younger age, function of brain might be

maintained normally by replacing defected cells with neurally differentiated endogenous stem cells even under the high concentration of APP. However, in an aged brain, decreased population of neural stem cells is not sufficient for replacing defected cells under high-dose sAPP because sAPP-induced gliogenesis of neural stem cells overwhelm neural differentiation. In the present studies, we found that massive glial differentiation, caused by sAPP, may degrade the function of brain in AD or DS. Although further studies are necessary, regulating environmental APP level would provide better strategies for AD or DS by improving neurogenesis of endogenous neural stem cells.

THE NOVEL FUNCTION OF AMYLOID PRECURSOR PROTEIN IN GLIAL DIFFERENTIATION IS ASSOCIATED WITH NOTCH SIGNALING PATHWAY

Introduction

The amyloid precursor protein (APP) is a membrane spanning glycoprotein consisting of 695- to 770-amino acids [9]. To date, the cytotoxicity of Aβ peptides, which are generated by subsequent β- and γ-secretases cleavage of APP, has been extensively studied to understand the pathophysiology of Alzheimer's disease (AD) [9, 135, 151, 152]. Despite the wealth of studies regarding the physiological function(s) of APP, there is no consensus on its physiological function [26]. Thus, the physiological function(s) of APP remains to be elucidated. A function of APP has been suggested as a ligand for receptors, such as the class A scavenger receptor [54]. Since crystal structure of the heparin-binding N-terminal domain of APP resembles a growth-factor like domain [27, 30, 153], N-terminal soluble APP (sAPP) may act as a growth factor. In our previous studies, we found that treatment with α cleaved sAPP or over-expression of APP induced glial differentiation in a human neural stem cells (HNSCs) culture [140]. We also found that APP plays a critical role in staurosporine (STS)-induced glial differentiation of NT-2/D1 cells [141]. Interestingly, Bhan *et al.* showed that NSCs isolated from Down's Syndrome (DS) patients, who display AD like pathology later in their life, differentiated into mainly astrocytes while NSCs from healthy subjects produced both neurons and astrocytes [56]. Since DS patients have trisomy of chromosome 21, which contains gene coding APP, high levels of APP expression in DS patients may responsible for the abnormal differentiation pattern of

NSCs as well as the AD pathology found in DS [139, 154-156]. Taken together, these findings suggest that APP may involved in glial differentiation of NSCs.

Glial differentiation of NSCs is induced by various factors during the relatively late embryonic stage (mouse E16-17) and postnatal period [39, 157, 158]. These factors include IL-6 cytokine families [130, 159], bone morphogenic factors (BMPs) [160], basic fibroblast growth factor (bFGF) [52], and Notch ligands [161, 162]. Notch signaling has been shown to control cell fate through local cell to cell interactions. During development, Notch suppresses neuronal differentiation *in vivo* and *in vitro* [47, 48]. When ligands bind to Notch, proteolytic cleavage of Notch receptors is occurred by γ-secretase/nicastrin complex to release the signal-transducing Notch intracellular domain (NICD) [163]. Proteolytically cleaved NICDs translocate into the nucleus and interact with the nuclear protein, referred to as CBF1/Su(H)/Lag-1 (CSL) [46]. The CSL and NICD complex activates the expression of primary target genes of Notch, such as Hairy and enhancer of split (Hes) gene families [164]. Following activation, Hes suppresses the expression of transcription factors which are involved in neuronal differentiation such as Mash1 and NeuroD. On the other hand, glial differentiation is strengthened by cross-talking between IL-6 and Notch signaling pathways. IL-6 signaling pathway has been known as a central part of gliogenesis. Subsequent phosphorylation of gp130, Janus kinases (JAKs), and signal transducer and activator of transcription 3 (STAT3) induces glial differentiation of neural stem cells. Interestingly, current study reported that glial differentiation is potentiated by interacting with Hes1 and JAK2 because these protein complexes facilitate accessibility of STAT3 to DNA binding element of GFAP promoter.

In the present study, we demonstrate that sAPP may be a novel extrinsic factor that induces glial differentiation of neural progenitor cells through the Notch signaling pathway.

Materials and methods

Reagents and antibodies

The γ-secretase inhibitor, L-685,458 (Sigma), was dissolved in dimethyl sulfoxide and stored at -80°C until use [165]. Recombinant sAPPα protein (Sigma) was dissolved in purified water and stored at -80°C until use. The following antibodies (Abs) were used: rabbit anti-GFAP Ab (Promega); goat anti human-glial filament protein, GFAP (N-terminal human affinity purified, 1:400, Research Diagnostics Inc., Flander, NJ); mouse anti human - amyloid beta antibody, 4G8 (1:50, Senetek); mouse IgG1 6E10, (1:50, Senetek); mouse IgG anti-Alzheimer Precursor Protein A4, 22C11 (1:100, Chemicon); mouse IgG2b anti-human βIII-Tubulin, clone SDL3D10 (1:1000, Sigma); Sheep anti Bromodeoxyuridine (BrdU) (1:300, Sigma for detection of cells derived from transplanted HNSCs); rabbit anti-human STAT3 Ab (Chemicon); mouse anti-phospho-STAT3 (Ser727) Ab (Chemicon); rabbit anti-phospho-STAT3 (Tyr705) Ab (Cell Signaling); rabbit anti-activated Notch Ab (Abchem); mouse anti-GFP Ab (Zymed); and rabbit anti-β-actin Ab (Cell Signaling); anti-mouse IgG and anti-rabbit IgG horseradish peroxidase-conjugated Abs (Jackson Immunoresearch Laboratory); anti-mouse, anti goat, anti-sheep (1:500) conjugated with fluorescein (FITC) or rhodamine (TRITC) (Jackson IR Laboratories, Inc.).

Cell culture and transfection

NT2/D1 cells [142] were seeded at a density of $5x10^6$ cells per 150 mm petri dish in Dulbecco's modified Eagle's medium (DMEM/F-12; Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS; Novacell), 1% antibiotic-antimycotic mixture (Invitrogen), 4 mM glutamine (Invitrogen) and maintained in a humidified atmosphere of 5% CO^2/95% air at 37ºC[97]. The cells were passed twice a week by short exposure to 0.25% trypsin/0.1% EDTA (Invitrogen). For all experiments, 1×10^6 NT-2/D1 cells were plated in a 6-well cell culture plate and subsequently, APP-induced differentiation of NT-2/D1 cells was evaluated for the expression of astrocytic and neuronal markers by RT-PCR and Western blot analysis under the treatment of condition media or various concentrations of recombinant APP.

Transient transfections were performed with vector constructs for pCDNA3.1; pCEP-APP695 (residues 1-695); pCMVIg-APP.1 (residues 1-678), pCMVIg-APP.2 (residues 1-205), [pCMVIg-APP.1 and pCMVIg-APP.2 (generously provided by Dr. Thomas Suhduf (University of Texas Southwestern, USA)[166]]; pBOS-ZEDN1 (a kind gift from Dr. Gerry Weinmaster, UCLA, USA)). All transfections were performed with Lipofectamine™ 2000 (Invitrogen) on sub confluent NT-2/D1 cells in a 6-well culture plate according to the manufacturer's protocol. Cells were rinsed twice in ice-cold phosphate-buffered saline (PBS, pH7.4) and 48h after the transfections, mRNA samples were extracted for further analysis.

Construction of pGFAP-GFP-S65T stable transfectant

NT-2/D1 cells were transfected with pGFAP-GFP-S65T (provided by Dr. Albee Messing, University of Wisconsin-Madison) using Lipofectamine™ 2000 (Invitrogen) [167]. After selection with 400μg/ml geneticin, G418 (Invitrogen) for 15-20 days, single colonies were picked and tested for reporter assay. The pGFAP-GFP-S65T stably transfected cells were cultured in DMEM/F-12 supplemented with 10% heat inactivated FBS, 1% antibiotic-antimycotic, 4 mM glutamine and 200μg/ml G418.

RT-PCR analysis

Total RNA was extracted from the cells with Trizol reagent (Invitrogen) according to the manufacturer's protocol. One μg of the total RNA was reverse-transcribed and amplified by the SuperScript™ ONE-STEP™ RT-PCR system (Invitrogen) with the following primers: GFAP (+) 5'-AAGCAGTCTACCCACCTCAG-3', (-) 5'-ATCCCTCCCAGCACCTCATC-3'; Delta1 (+) 5'-TGCTGGGCGTCGACTCCTTCAGT-3', (-) 5'-GCCTGGCTCGCGGATACACTCGTCACA-3'; Jagged-1 (+) 5'-ACACACCTGAAGGGGTGCGGTATA-3', (-) 5'-AGGGCTGCAGTCATTGGTATTCTGA-3'; Hes1 (+) 5'-CGGACATTCTGGAAATGACA-3', (-) 5'-CATTGATCTGGGTCATGCAG-3'; hNotch1 (+) 5'-GATGCCAACATCCAGGACAACATGGG-3', hNotch1 (-) 5'-GGCAGGCGGTCCATATGATCCGTGAT-3'; hNotch2 (+) 5'-ACATCATCACAGACTTGGTC-3', hNotch2 (-) 5'-CATTATTGACAGCAGCTGCC-3'; EGFP (+) 5'-CAAGGACGACGGCAACTACAAGAC-3', (-) 5'-GCGGACTGGGTGCTCAGGTAGTGGT-3'; β-actin (+) 5'-

GACAGGATGCAGAAGGAGAT-3', (-) 5'-TTGCTGATCCACATCTGCTG-3'. Ten µl of the reaction mixtures were then analyzed on a 2% E-gel (Invitrogen). Gel images were captured using a KODAK Image Station 2000MM (KODAK).

Immunoprecipitation and western blot analysis

Protein samples were prepared by lysing the cells with ice-cold lysis buffer consisting of 1% NP40, 150 mM NaCl, 50 mM Tris pH8.0 and Complete Protease Inhibitor Tablets (Boehringer). The protein concentration of each sample was measured by Bio-Rad protein assay (Bio-Rad). Lysates were immunoprecipitated with antibodies against GFAP, STAT3 and NICD molecules using protein A-Sepharose (Amersham Bioscience). Following immunoprecipitation, the samples Then immunoprecipitants or cell lysates were heated at 70ºC for 10 min in LDS (Lithium Dodecyl Sulfate) sample loading buffer (1×) and separated on NuPAGE^{TM} 4-12 % Bis-Tis Gel (Invitrogen) for 45 min at 200 V and transferred to a PVDF membrane (30V, 60 min). Membranes were blocked with 5% skim milk in PBS for 1h at RT and probed at 4ºC overnight with primary antibody in 5% skim milk. The membranes were washed 3 times for 5min each with PBS containing 0.05% Tween 20 (PBS-T, pH7.4) and incubated with horseradish peroxidase-conjugated secondary antibodies in 5% skim milk for 2h at RT. After 3 times washing with PBS-T, immunoreactive bands were visualized by using ECL plus (Amersham Bioscience) chemiluminescence reagent. Western blot images were captured with KODAK Image Station 2000 MM (KODAK).

Stereotactic injection of HNSCs into mice

8 months old male APP23 transgenic [168], and wild-type mice were deeply anesthetized with a 1:1 mixture of Ketamine (100 mg/kg) and Xylazine (20 mg/kg) and mounted onto a stereotaxic apparatus (ASI Instrument, USA). Using bregma as a reference point, approximately 10^5 cells HNSCs were suspended in 10 μl PBS, and slowly injected into the right lateral ventricle (coordinates: anterior posterior (A/P)–0.6 mm; medial lateral (M/L) +1.0 mm; dorsal/ventral (D/V) +2.4 mm) of each mouse using a 25 μl Hamilton gastight syringe (Hamilton, Reno, NV, USA) attached with a 22-gauge beveled needle.

Immunohistochemistry

Detailed methods for immunohistochemistry have previously been described [67, 169]. Briefly, 6 weeks post-transplantation, animals were sacrificed by an overdose of a 1:1 mixture of Ketamine (100 mg/kg) and Xylazine (20 mg/kg), and a subgroup of animals (n=6-8) were transcardially perfused with phosphate buffer (PBS). Brains were removed, dissected into the hippocampus and cortex, and tissue samples were maintained on dry ice and stored at -80° C until Western blotting experiments were performed. The remaining group of mice with HNSC implants (n=6) were transcardially perfused with PBS and 4% paraformaldehyde (pH 7.4). Brains were removed and post-fixed for 8-12 h in 4% paraformaldehyde, and cryoprotected in 20% sucrose-PBS overnight. The brains were subsequently cut on a cryostat (20 μm coronal free floating sections) and kept in PBS at 4° C. For fluorescent immunohistochemical analysis, sections were washed three times and blocked with 3% donkey serum in PBS

containing 0.25 %Triton X-100 for 1 hr at RT. After serum blocking, sections were incubated with a combination of primary antibodies, diluted in PBS containing 0.25% Triton X-100 and with 3% normal donkey serum overnight at 4°C. After 3 washes in PBS-T, sections were incubated with corresponding secondary antibodies for 2 hr at RT. After a final wash in PBS-T, sections were mounted and cover slipped with Vectashield with DAPI (Vector Laboratories, Inc., Burlingame, CA) and observed using a Leica DMRB fluorescent microscope. Microscopic images were taken with an Axiocam digital camera (Carl Zeiss) mounted on the DMRB and processed using the QIMAGING with Q Capture software (Qimaging Corporation).

Immunocytochemistry

For fluorescent immunocytochemistry of the HNSCs, Treatment of recombinant sAPPα and transfection of APP expression vector was performed. Cells were fixed in 4% paraformaldehyde for 20 min at RT, and then washed in PBS-T, and thereafter incubated in PBS-T containing 3 % normal donkey serum. Next, the samples were incubated overnight at 4(up to 12 hr) with the following primary antibodies mouse anti human - amyloid beta antibody, 4G8 (1:50, Senetek); mouse IgG1 6E10, (1:50,Senetek); mouse IgG anti-Alzheimer Precursor Protein A4, 22C11 (1:100, Chemicon); mouse IgG2b anti-human βIII-Tubulin, clone SDL3D10 (1:1000, Sigma) and goat anti human-glial filament protein, GFAP (N-terminal human affinity purified, 1:400, Research Diagnostics Inc., Flander, NJ). After washing with PBS, samples were incubated with corresponding secondary antibodies coupled to FITC or TRITC for 1.5 hr in a dark humidified chamber. Next, the samples were washed thoroughly in PBS and

cover slipped with Vectashield mounting media with DAPI (Vector) for fluorescent microscopic observation.

Results

Increased glial differentiation of HNSCs in APP23 transgenic mice

To examine whether APP has an effect on the differentiation of HNSCs in adult mice *in vivo,* we transplanted HNSCs into the cerebro lateral ventricle of APP23 transgenic and wild type (WT) mice at 8 and 12 months of age. To identify the transplanted HNSCs in the host brain, anti-human specific GFAP antibody was used for the fluorescent immunohistochemistry. Majority of the transplanted HNSCs differentiated into GFAP-positive astrocytes in the cortex of APP23 transgenic mice 4weeks after transplantation while less GFAP-positive cells were found in the WT mice (Figure 20A-a and b). In 12 month old APP23 mice with progressed Aβ pathology, more activated astrocytes were found surrounding the area of Aβ deposits (Figure 20A-c). These results indicate that high level of APP in the transgenic mouse induces glial differentiation of HNSCs and Aβ deposits may attract and/or activate astrocytes. We compared the protein expression levels of APP, GFAP and NICD in the cerebral cortex of 8 month WT and APP23 mice using Western blot analysis (Figure 20B). Both APP and GFAP expressed at higher level in APP23 mice compared to WT mice. APP23 transgenic mice also express higher level of NICD, indicating activations of Notch signaling.

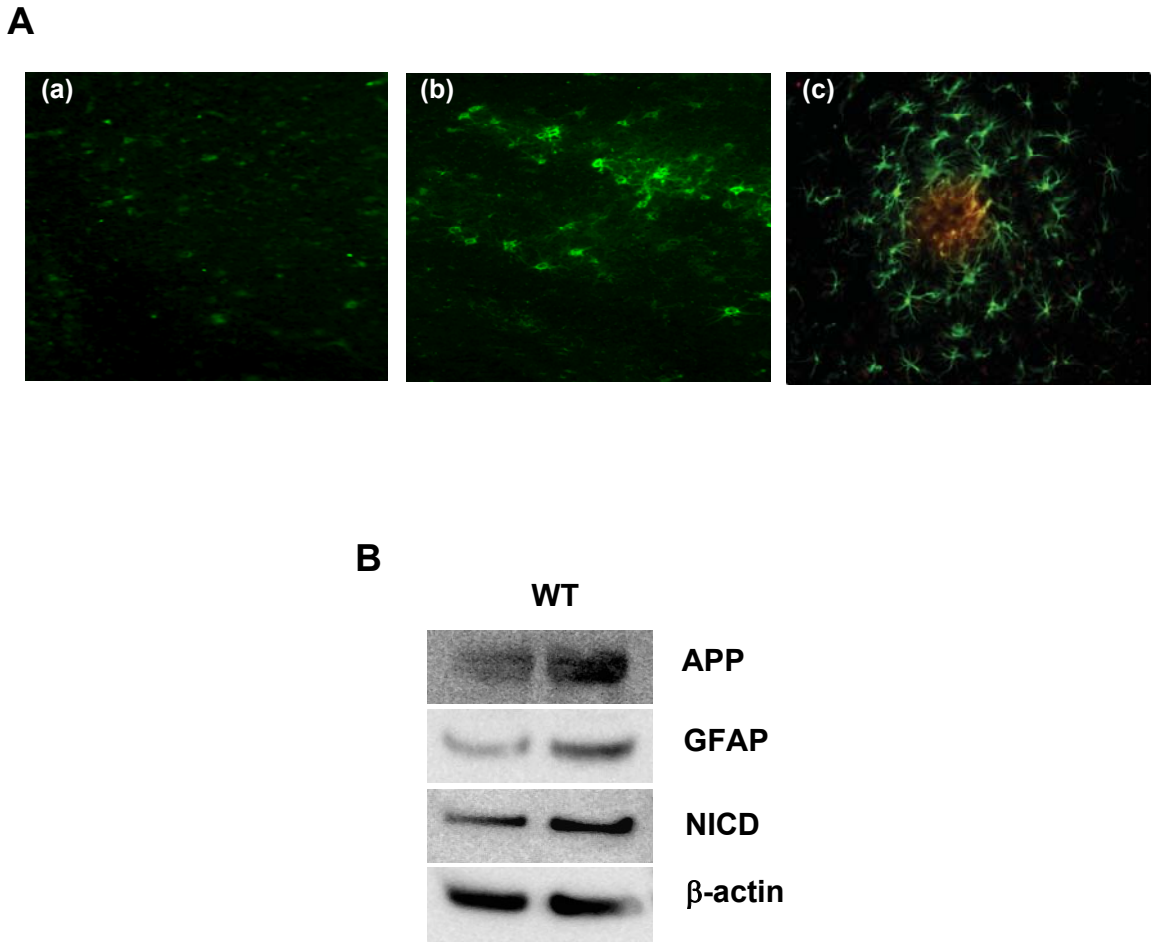


Figure 20: Differentiation of HNSCs into astroglial cells *in vivo*. (A) Representative fluorescent immunohistochemical images in the hippocampus of WT and APP23 mice 4-6 weeks after HNSCs transplantation. a: WT (8 months old), b: APP23 (8 months old), and c: Active gliogenesis was detected around plaque-like formations in the hippocampus of 12 months old APP23 transgenic mice after HNSC-transplantation. GFAP (green), Abeta stained with 4G8 (red). (B) Western blot analysis of protein expression of APP, GFAP, and NICD in 8 months old WT and APP23 transgenic mice

sAPP increases glial differentiation of HNSCs

To investigate effect of APP on HNSC differentiation, we analyzed the cell population of differentiating HNSCs treated with sAPP for 5 days under serum-free conditions by double-immunofluorescent staining for GFAP and βIII-tubulin, markers for astrocytes and neurons respectively (Figure 21A). Treatment with recombinant APP (sAPP) dose dependently (25, 50, 100 ng/ml) increased the population of GFAP-positive cells from 45% to 83%. Interestingly, a lower dose of sAPP treatment (25 ng/ml) increased both GFAP and βIII-tubulin-positive cells. However, higher doses of sAPP (50 and 100 ng/ml) dose-dependently decreased βIII-tubulin-positive cells from 51% to 13% of the total population of differentiated HNSCs (Figure 21A). These results suggest that sAPP increases both glial and neuronal differentiations at the lower dose, but in the higher dose sAPP suppresses neuronal and promotes glial differentiations. The sAPP may be influencing the cell fate decision of HNSCs because sAPP treatment did not increase Terminal transferase dUTP nick end labeling (TUNEL) signals, marker for apoptosis, in the HNSCs culture thus selective death of proneural progenitors in the neurospheres may be ruled out (data not shown).

Overexpression of APP *in vitro* reduces neurogenesis of HNSCs

HNSCs were transfected with mammalian expression vectors (pCEP-APP) containing wild-type APP695 (wtAPP) gene and were differentiated under serum-free unsupplemented conditions. After 5 days differentiation, HNSCs transfected with wtAPP differentiated into a significantly higher number of GFAP-positive cells (*: $p<0.01$, $n=3$)

compared to a control, HNSCs transfected with the vector alone (Figure 21B). Neurons differentiated from HNSCs transfected with wtAPP comprised of less than 7%+SE of the total number of cells (Figure 21B), whereas the controls showed a 54%+SE neuronal population (Figure 21A). Furthermore, neurally differentiated cells derived from HNSCs transfected with wtAPP showed abnormal morphology, reduced length and grossly misshaped neurite, similar to the neurons derived from NSCs isolated from Down syndrome patients[56]. These results indicate that an overdose of the wtAPP gene, which may produce an increased amount of sAPP fragments, suppress normal neuronal differentiation and causes gliogenesis rather than neurogenesis of NSCs *in vitro*.

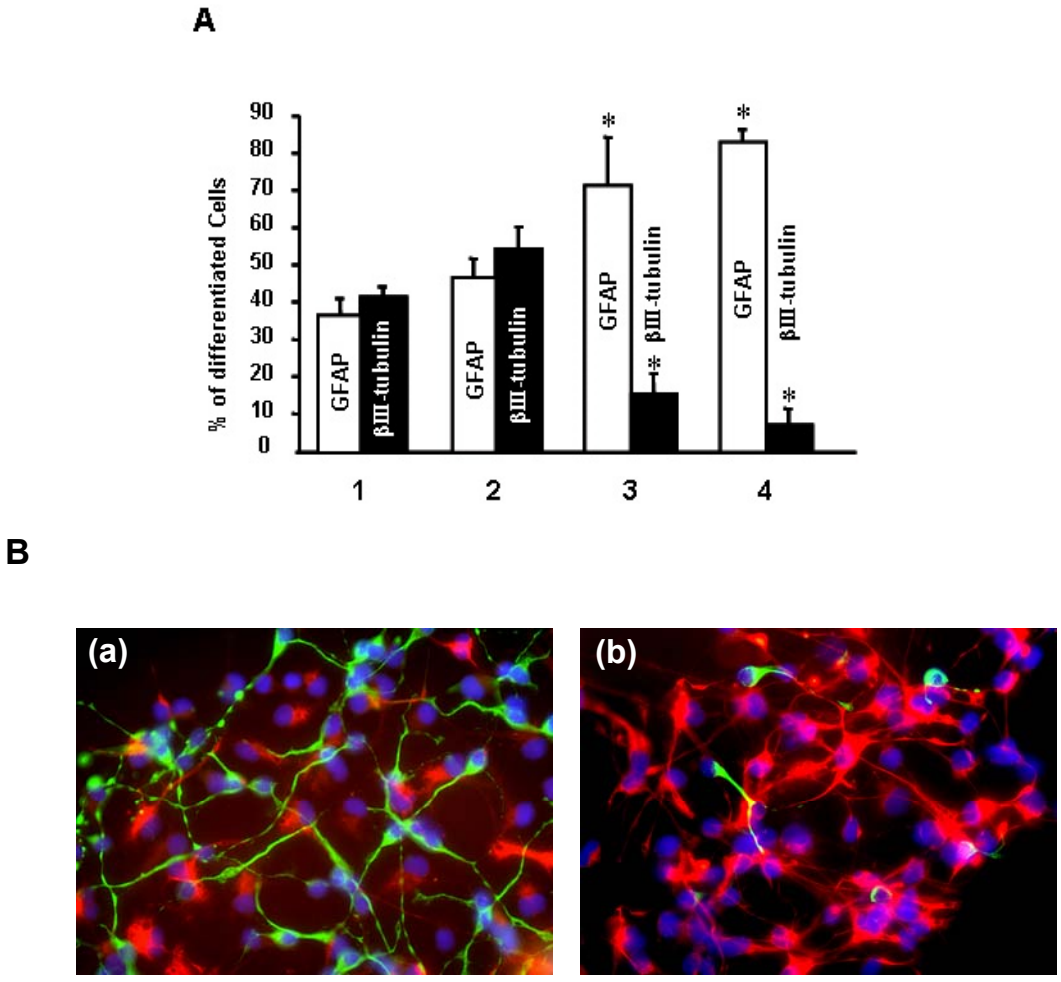


Figure 21: Migration and differentiation of HNSCs were altered by treatment of sAPP and overexpression of APP. (A) Treatment of HNSCs culture with recombinant sAPP for 5 days dose-dependently increased the migration and differentiation of HNSCs under the serum-free unsupplemented condition (1; control, 2; 25 ng/ml, 3; 50 ng/ml and 4; 100 ng/ml). The cell population of sAPP-treated HNSCs at 5 DIV in the serum-free differentiation condition was further characterized by double immunofluorescence staining with GFAP and βIII-tubulin, markers for astrocytes and neurons, respectively. All nuclei were counterstained with DAPI. At a higher dose of sAPP treatment, many HNSCs differentiated into glial cells (red). For quantitative population analysis, the number of GFAP or βIII-tubulin immunopositive cells was counted versus total cell numbers of DAPI-labeled nuclei. All data values are expressed as mean percentages (±S.E.M.). One-factor ANOVA followed by post hoc analysis (Student-Newman-Keuls) was used to demonstrate statistically significant differences between experimental groups and control groups (*: $p<0.01$). (B) APP transfection increases the glial

population of HNSCs *in vitro*. Double immunofluorescence staining with GFAP (red) and βIII-tubulin (green). All nuclei are counterstained with DAPI (blue). HNSCs transfected with mammalian expression vectors containing genes for wild-type APP (wtAPP) differentiated under serum-free unsupplemented conditions displayed a significantly reduced level of neural differentiation (***a***) compared with HNSCs transfected with the vector alone at 5DIV (***b***). The abnormal morphology of the processes is shown in neuronally differentiated HNSCs (green) with wtAPP gene transfection (***a***).

sAPP induced GFAP expression in NT-2/D1 cells

To analyze mechanism of the sAPP function to regulate differentiation process of NSCs, we examined effects of sAPP on differentiation of NT-2/D1 neural progenitor cells. Expression of GFAP was increased in NT-2/D1 by treatment with recombinant sAPP with a time- (Figure 22A) and dose-dependent manner (Figure 22B). We also examined effect of sAPP on GFAP promoter activity using NT-2/D1 cells stably transfected with a GFAP promoter driven GFP expression vector (pGFAP-GFP-S65T) as a reporter system. Treatment of NT-2/D1 cells transfected with pGFAP-GFP-S65T with sAPP, showed a dose-dependent increase of GFP protein expression, indicating that sAPP induces glial differentiation by activation of GFAP promoter (Figure 22C). For further investigation, we applied 22C11 antibodies, which can recognize and capture the N-terminal domain of APP, to neutralize the effect of sAPP in glial differentiation of NT-2/D1 cells. We observed that treatment with 22C11 effectively antagonized the effect of sAPP in glial differentiation of NT-2/D1 cells by suppressing GFAP expression levels (Figure 22D and E). Therefore, these results indicate that sAPP is involved in glial differentiation of NSCs.

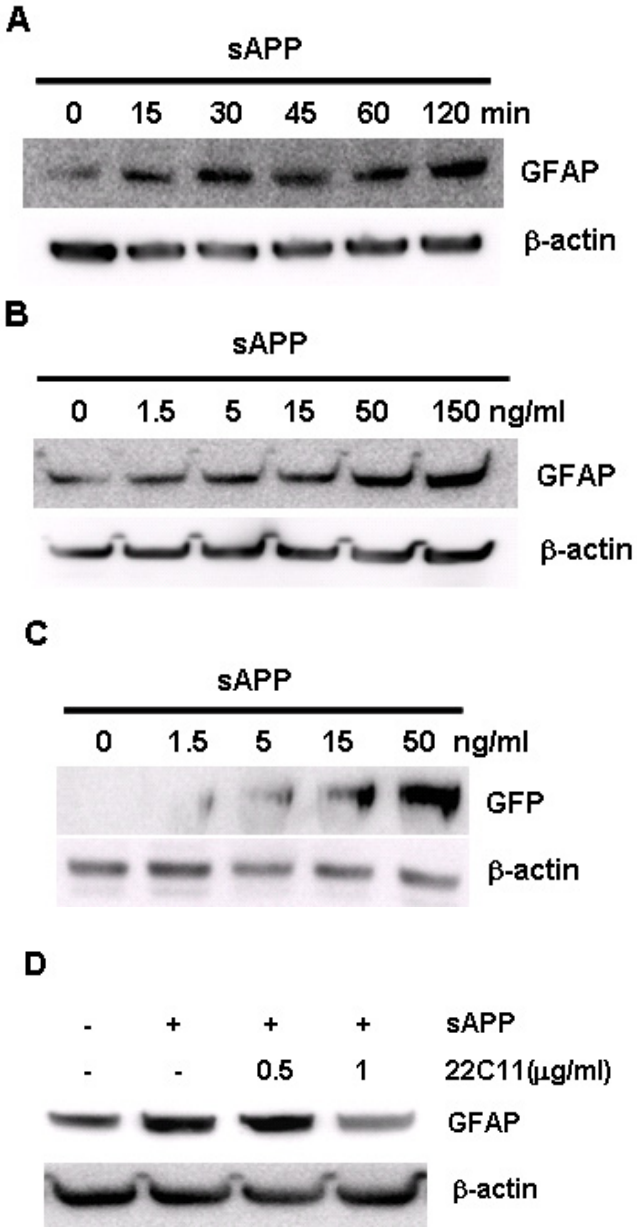


Figure 22: Induction of GFAP expression in sAPP treated NT-2/D1 cells. (A) Western blot analysis for measuring GFAP expression. Cells were grown in 100ng/ml of recombinant sAPP containing media for 0, 15, 30, 45, 60 and 120 min. Expression of β-actin was examined as a loading control. (B) Western blot analysis of GFAP expression which is induced by various concentration (0, 1.5, 5, 15, 50, and 150 ng/ml) of

recombinant sAPP. Expression of β-actin was examined as a loading control. (C) Western blot analysis of GFP expression which is transcribed by GFAP promoter. HEK293 Cells were transfected with pGFAP-GFP-S65T vectors as a reporter system. To investigate the function of sAPP on GFAP promoter, cells were treated with a variety of concentration (0, 1.5, 5, 15, and 50 ng/ml) of sAPP. (D) NT-2/D1 cells were treated with sAPP for 18 hr in the presence of the anti-APP neutralizing Ab (22C11) and observed the expression changes of GFAP by western blot analysis. Experiments were repeated at least three times with similar results.

sAPP induces glial differentiation via Notch signaling pathway

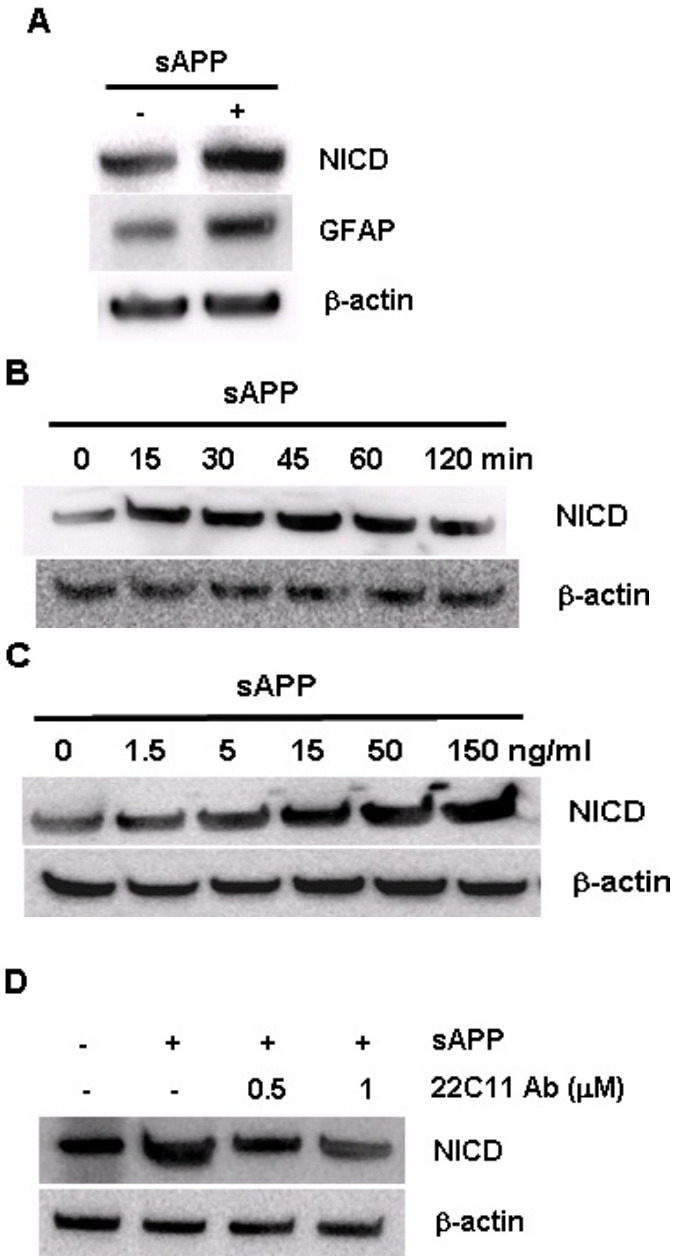
Based on our in *vivo* findings where higher NICD generation was observed in APP23 mice brain compared to WT, we hypothesized that over expression of sAPP may stimulate Notch proteolysis and nuclear translocation of NICD resulting in glial differentiation. To test whether sAPP stimulates the Notch signaling pathway, we assessed NICD generation i*n vitro* using NT-2/D1 cells treated with sAPP. sAPP promoted NICD generation as well as increased GFAP protein expression in NT-2/D1 cells (Figure 23A). These results are, consistent with our *in vivo* results showing that high dose of sAPP upregulate GFAP expression as well as stimulate an increased NICD generation (Figure 23B). Next, we investigated both the time- and dose-dependent effects of sAPP on the generation NICD (Figure 23B and C). NICD generation was observed 15 min after treatment with sAPP (100ng/ml). Since NICDs were generated in an early stage, it suggests that sAPP may stimulate Notch signaling cascade in a similar manner as the Notch ligands, Jagged and Delta. Upon examination of the dose dependent effects of sAPP, a gradual increase in NICD generation was observed in NT-2/D1 cells following treatment with different doses of sAPP (Figure 23B and C). In contrast, application of 22C11 potently suppressed the generation of NICDs in a dose dependent manner (Figure 23D). These results provide further evidence that the notch signaling pathway is stimulated by treatment with sAPP.

sAPP stimulates Notch signaling pathway via γ-secretase/nicastrin complex

NICDs are generated by γ-secretase/nicastrin complex under the stimulation of Notch ligands such as Delta and Jagged1, thus, if sAPP-induced gliogenesis is truly mediated via Notch signaling pathway, we would expect that the expression of GFAP, Hes1 and NICD generation would be suppressed by treatment with γ-secretase inhibitors such as, L-685,458. To further validate whether sAPP stimulates the Notch signaling cascade, we pretreated various doses (1, 3, and 10 μM) of L-685,458 to NT-2/D1 cells to suppress γ-secretase activity. Treatment with L-685,458 inhibited both NICD generation and GFAP expression even in the presence of sAPP (Figure 23E). In addition, application of L-685,458 potently suppressed the expression of Hes1, a target gene of Notch (Figure 23F). Taken together, these results strongly suggest that sAPP-induced glial differentiation is mediated through the Notch signaling pathway.

Recently novel regulatory mechanism in glial differentiation was documented that Notch and JAK/STAT pathways cross-talk through physical interaction between Hes1 and JAK2 [49]. These protein complexes facilitate phosphorylation of STAT3 and maximize the accessibility of STAT3 to STAT3 binding elements of target promoters including GFAP. Although sAPP can directly promote phosphorylation of STAT3 through gp130/IL-6 signaling pathway, somehow, APP-induced Notch signaling activation is also involved in the phosphorylation status of STAT3 by regulating Hes1 expression. We thus aimed to examine whether sAPP maximizes glial differentiation by cross-talk between the Notch and gp130/STAT3 signaling pathways. As shown in Fig. 23E and F,

Hes1 and GFAP expression were upregulated in NT-2/D1 cells treated with sAPP. Conversely, treatment of L-685,458 efficiently inhibited Hes1 and GFAP expression by antagonizing NICD generation. These results suggest that APP-induced Notch signaling activation also can increase GFAP expression level through gp130/IL-6 related signaling pathways through cross talk. Thus, to investigate whether APP also enhances gliogenesis via cross talk with the gp130/IL-6 signaling pathway, phosphorylation of STAT3-Tyr705 was examined after treatment with APP [45]. Treatment of L-685,458 also suppressed phosphorylation of STAT3-Tyr 705 even in the presence of sAPP (Figure 23G). This is probably caused by downregulation of Hes1 gene expression as reported previously [49]. Although further mechanistic studies are needed, these results suggest that sAPP potentiates gliogenesis by cross-talk between Notch and gp130/IL-6 signaling pathways.


A
sAPP
-
+
NICD
GFAP
β-actin
B
sAPP
0 15 30 45 60 120 min
NICD
β-actin
C
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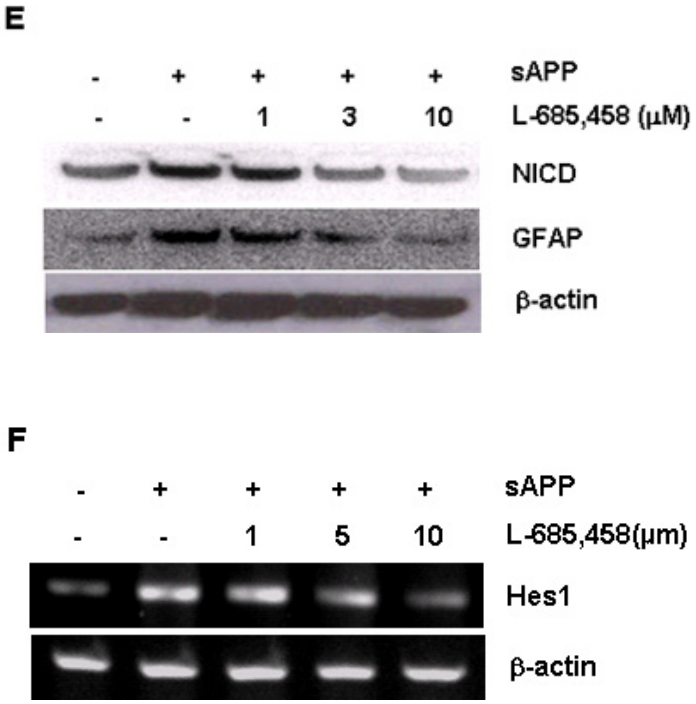

Figure 23: sAPP stimulates Notch signaling pathway during gliogenesis. (A) Treatment of sAPP (100ng/ml) promoted GFAP expression as well as NICD generation. (B) and (C) sAPP induced generation of NICD was time- and dose- dependent manner as indicated above. Generation of NICD was measured by western blot analysis using anti-active Notch Ab (B and C). (D) sAPP induced NICD generation was suppressed by neutralizing antibody (22C11). (E) and (F) sAPP induced NICD generation was mediated by stimulation of γ-secretases. L-685,458 (γ-secretase inhibitor) suppressed expression of GFAP and generation of NICD with dose dependently. Expression of GFAP and generation of NICD was assessed by western blot analysis using anti-GFAP Ab and anti-activated Notch Ab, respectively. Furthermore, Hes1 gene expression level was examined after treatment of sAPP. Then, dose-dependent effect of L-685,458 was observed by RT-PCR. (G) sAPP enhances cross-talk between Notch and gp130/STAT3 signaling pathway. For detection of phosphorylation of STAT3-Tyr-705, immunoprecipitation was performed with anti-STAT3 Ab. Then, western blot analysis was executed using anti-STAT3-Tyr-705 Ab. Expression of β-actin was examined as a loading control. Experiments were repeated at least three times with similar results.

Interaction between sAPP and Notch is crucial for glial differentiation

There are two possible mechanisms regarding APP-induced Notch signaling activation. One is that sAPP may stimulate the Notch signaling cascade by upregulating expression of Notch receptors or Notch ligands. The other mechanism may be a direct protein-protein interaction between APP and Notch because several studies reported that APP can physically interact with Notch. To investigate how sAPP stimulates the Notch signaling pathway, we first assessed the gene expression levels of Notch related molecules, such as Delta1, Jagged1, Hes1, hNotch1 and 2, in NT-2/D1 cells after sAPP treatment (Figure 24A). We observed that the expression of Hes1was drastically increased following treatment with sAPP, whereas no change in the expression of Notch ligands (Delta1, Jagged1) and Notch receptors (hNotch1 and 2) were observed. Our findings indicate that APP-induced Notch signaling activation may not occur by upregulation of the expression of Notch ligands or Notch receptors. Therefore, to examine the possibility of the latter mechanism, the physical interaction of APP and Notch was tested by using immunoprecipitation (Figure 24C). First to identify possible crucial domain(s) of APP for the physical interaction, we constructed truncated mutants, APP1-678 and APP1-205, which encode sAPPα (1-678) and N-terminal domain of APP (1-205), and transfected these mutants, along with an APP expression vector (APP 1-695) into NT-2/D1 cells. Interestingly, we found that the N-terminal domain of APP (1-205) was sufficient for the physical interaction with Notch. This result is in agreement with recent reports which have showed physical interactions of APP with Notch. Hence we confirm here that APP interacts with Notch directly and for the first time we

demonstrate that a protein-protein interaction is important for sAPP-induced glial differentiation, mediated by the Notch signaling cascade.

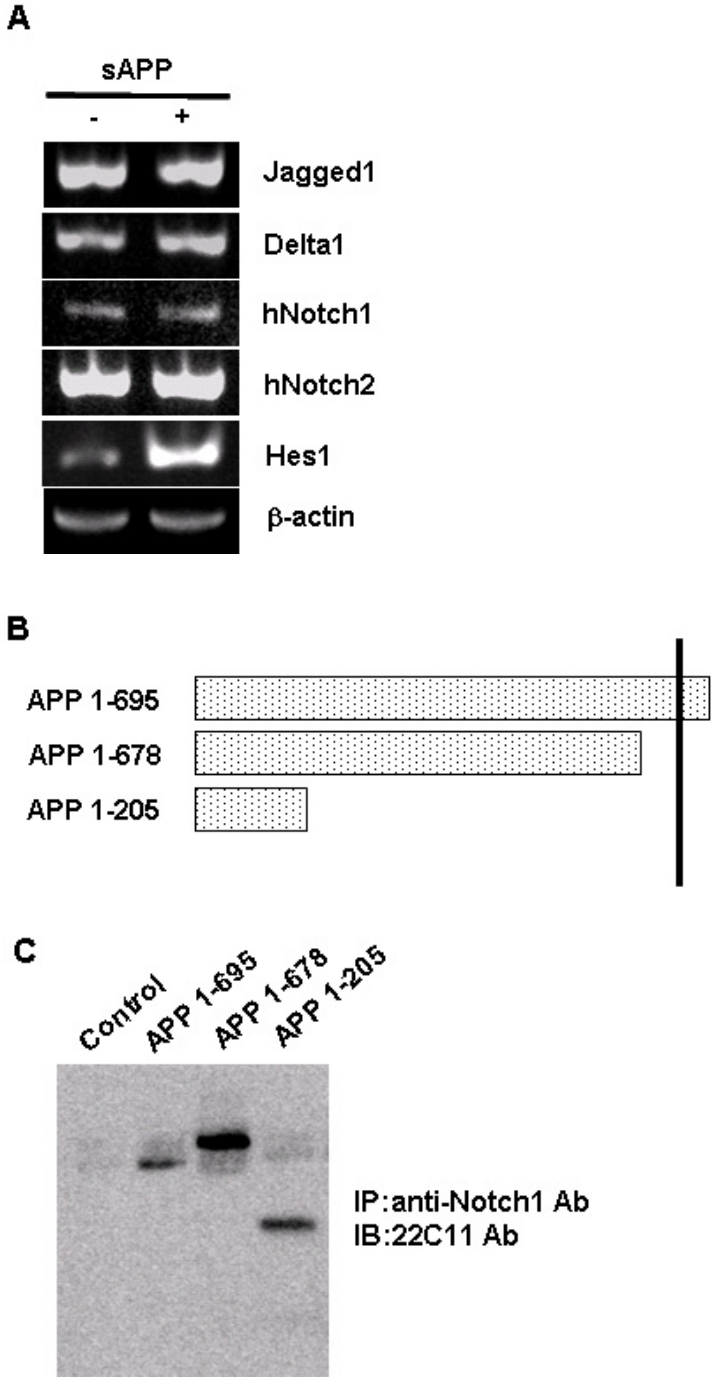

Figure 24: Physical interaction of APP and Notch is crucial for stimulation of gliogenesis. (A) sAPP–induced gene expression changes, which are related with Notch signaling cascade, were assessed by RT-PCR. 100ng/ml of recombinant sAPP protein was applied to NT-2/D1 cells for 2 hr, then, mRNAs were extracted using Trizol for RT-PCR. Then, most of Notch related genes were not affected by treatment of sAPP except Hes

1. Expression of β-actin was examined as a loading control. (B) Schematic diagram of truncated mutant of APP clones. Several truncated N-terminal domain of APP (1-695, 1-678, and 1-205 a.a.) was used for functional analysis. (C) Protein-protein interaction of APP and Notch was tested by using immunoprecipitation. Proteins were extracted from NT-2/D1 cells using NP40 lysis buffer. Then, protein complexes were precipitated by anti-Notch Abs and western blot analysis were performed by using anti-22C11 Abs, Experiments were repeated at least three times with similar results.

N-terminal domain of APP is sufficient for induction of glial differentiation

Since the structure of APP harbors a variety of functional domain, sAPP has shown various physiological functions in vivo as well as in vitro. However, several reports demonstrate that N-terminal domain of APP has a growth factor like domain because it contains cystein rich domain and heparin binding site which are involved in protein-protein interaction. We thus aimed to identify whether N-terminal domain of APP is sufficient for gliogenesis. For that purpose, we transfected NT-2/D1 cells with three different APP truncated mutant expression vectors of APP [pCMV-APP695 (1-695), pCMVIg-APP.1 (1-678), and pCMVIg-APP.2 (1-205)] in. Next, GFAP, Hes1 expression and NICD generation were examined to investigate the potential function of these various N-terminal domains of APP in gliogenesis. As shown in Figure 25, N-terminal domain of APP showed sufficient capability to induce gliogenesis by enhancing GFAP and Hes1 expression and NICD generation. Several earlier studies have reported that N-terminal domain of APP promotes proliferation of stem cells because of its growth factor like structure. Especially, Callie et al. showed that soluble form of APP enhances proliferation of progenitor cells in adult subventricular zone by acting as a cofactor of EGF [109]. However, we didn't find any enhanced proliferation in our experimental conditions, instead we found that treatment of sAPP slightly reduced cell proliferation of NT-2/D1 cells (data not shown) Therefore, our present study suggests that the N-terminal domain of soluble APP is a potent gliogenic factor to neural precursor cells.

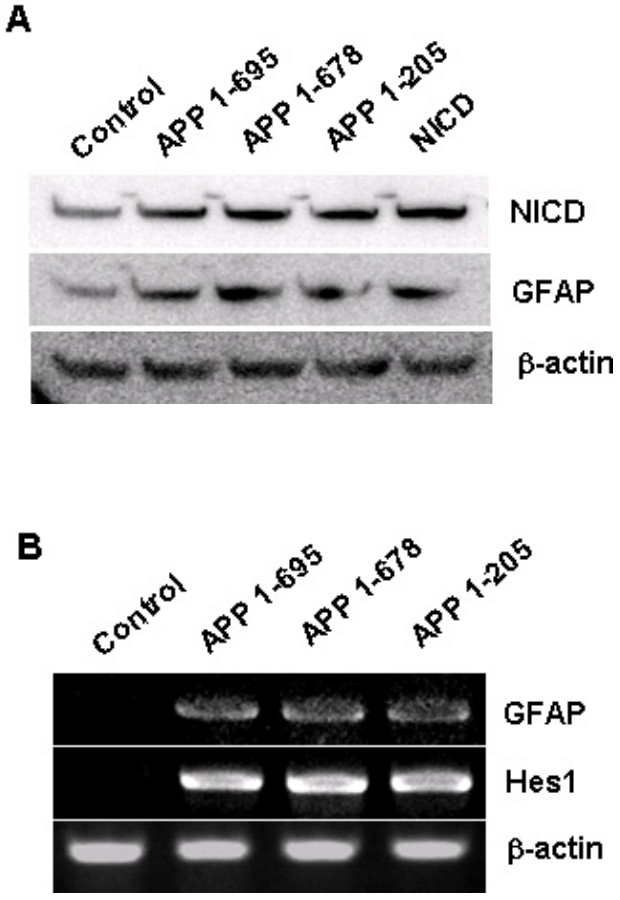

Figure 25: N-terminal of sAPP is sufficient for glial differentiation. (A) N-terminal domain of APP induced expression of GFAP as well as generation of NICD. Condition media, collected from sAPP and their mutants transfected HEK293 cells, were added into NT-2/D1 cells. Then, protein extracts were imunnoprecipitated and probed with anti-active Notch Ab and anti-GFAP Ab, respectively. (B) N-terminal domain of APP enhanced mRNA expression level of both GFAP and HES1, target gene of NICD. Condition media were treated into NT-2/D1 cells and total mRNAs were collected using Trizol. Then, RT-PCR was performed to amplify target genes using GFAP and HES1 recognizing primer. Expression of β-actin was examined as a loading control. Experiments were repeated at least three times with similar results.

Discussion

APP has been extensively studied in relation to Aβ deposition, which is one of the hallmarks of the pathophysiology in AD. In the present study, we report a novel physiological function of sAPP and the mechanisms involving sAPP regulation of cell fate specification of neural progenitor cells. In general, glial differentiation process is judged by the expression of GFAP as well as S100β. Although there still remains unclear whether it is sufficient for identification of gliogenesis, here, we used GFAP as a marker for the glial differentiation. In our previous studies, we have clarified that sAPP has a crucial role in altering expression of astrocytic specific markers such as GFAP, astrocyte-specific glutamate transporter-1(GLT-1)/ excitatory amino acid transporter-2 (EEAT-2), and aspartate transporter-2 (GLAST)/ EEAT-1 expression as well as morphological changes of neural precursor NT-2/D1 cells [141]. Although it has been reported earlier that sAPP promotes proliferation of NSC both *in vitro* and *in vivo* [89, 109], we observed opposite findings in which treatment of sAPP did not increase the proliferation of NT-2/D1 cells in our experimental condition (data not shown). Instead we detected that treatment of sAPP slightly suppressed cell proliferation of NT-2/D1 cells. Furthermore, sAPP induced GFAP expression in a time- and dose-dependent manner by stimulating GFAP promoter activity in neural precursor cells. In addition, high levels of APP induced glial differentiation of HNSCs in vitro as well as in vivo. Therefore, our data indicates that sAPP is closely associated with the glial differentiation process of neural precursor cells. To elucidate the molecular mechanisms involved in sAPP-induced gliogenesis, we investigated the specific mechanisms of APP-induced glial differentiation. In general, bFGF, BMPs, IL-6 cytokine families, and Notch have been the

most known representatives for the signaling pathway associated with gliogenesis of the neural stem cells. Recently, He *et al*. proposed that the Jak/STAT pathway is central to the gliogenic machinery and postulated a framework for understanding the control of gliogenesis during development [40]. On the other hand, Notch canonical pathway regulates gliogenesis by either suppressing transactivation of neurogenic bHLH transcription factors (Mash1 and NeuroD) or cooperating with STAT3 of IL-6 signaling pathway.

In previous reports, several lines of reports addressed that possible association of Notch signaling pathway in glial differentiation. In adult DS cortex, the upregulation of Notch1 and Hes1 expression was observed [170] as well as massive gliogenesis of HNSCs [56]. As discussed before, since AD and DS shows similar characteristics in terms of the expression level of APP [171, 172] and symptom of the late on-set [123], these results maybe caused by high concentration of soluble APP. Interestingly, our in vivo study also suggests that the involvement of Notch signaling pathway in APP-induced glial differentiation (Figure 20B). When we examined the protein level of APP, GFAP and NICD in the cortex of both wild type control and APP23 mice using Western blotting, NICD generation showed higher correlation with GFAP expression level in the cerebral cortex of APP23 mice compared to WT mice. In accordance with increased APP protein expression, increases in GFAP protein expression as well as an increase of NICD generation were observed in the APP23 transgenic mice. Therefore, we examined Notch signaling pathway as a possible candidate signaling pathway in sAPP-induced glial differentiation.

When sAPP was applied to neural precursor cells, it potently generated NICDs with time- and dose-dependent manner and eventually turned on gene transcription of Hes1, neuronal repressor. Since these sequential events were usually executed by recruiting γ-secretase/nicastrin complex under the stimulation of Notch ligands, we examined whether sAPP can also stimulate Notch signaling pathway similarly with other Notch ligands such as Deltas or Jaggeds. To determine the specific mechanisms, a γ-secretase inhibitor, L-685,458, was used to suppress the function of γ-the secretase/nicastrin complexL-685,458, inhibited the generation of NICD and induction of Hes1 gene expression efficiently. Furthermore, the expression of GFAP protein and the phosphorylation of STAT3 were also diminished by L-685,458. In agreement with our findings, a recent study reported that the Notch signaling pathway can cross-talk with the JAK/STAT3 signaling pathway through interaction of Hes1 and JAK2 [49]. These complexes enhance the accessibility of STAT3 homodimers to promoter sites and potentiate gene expression of target genes. These findings suggested that treatment of sAPP may induce gliogenesis by enhancing GFAP expression via Notch signaling pathway. On the other hand, sAPP may also suppress glial differentiation by inhibiting the expression of the neurogenic transcription factors such as NeuroD and Mash1 [173]. However, in our experimental conditions, we could detect gene expression of NeuroD as well as Mash1 in both regular and sAPP treated NT-2/D1 cells. (data not shown) Thus, this indicates that treatment of sAPP may selectively force cell fate specification of neural precursors into glia cells by stimulating the Notch signaling cascade.

Structure of APP 695 largely consists of several characteristic elements. The N-terminal APP 695 is composed of a signal peptide for the trafficking, a cystein-rich domain (CRD), a Zinc-binding motif, and acidic sequences [26]. The Central APP domain (CAPPD) consists of a large domain which doesn't contain cystein residues and a short linker sequence that harbors α- and β-secretase cleavage sites [26]. The C-terminal of APP harbors a transmembrane region and a cytoplasmic tail. Several lines of evidence suggested that APP has a structural property similar to a growth factor and ligand. Thus, we tested here whether the N-terminal domain of APP is enough to induce glial differentiation of neural stem cells. To elucidate the functional properties of the N-terminal domain of sAPP in glial differentiation, we examined the expression of GFAP and Hes1, as well as generation of NICDs after treatment with conditioned media which contained full length APP695, APP1-678, and APP1-205. Treatment of APP and truncated mutants of APP promoted gene expression of GFAP and Hes1 as well as NICD generation. These findings suggest that sAPP may function as a ligand for Notch in neural stem cells treated with APP.

Up to date, it has been in veil how sAPP stimulates these signaling cascades. We thus hypothesized that there may be two possible ways to stimulate gliogenesis by treatment with sAPP. First, treatment with sAPP may promote the expression of various ligands or receptors, associated with Notch receptors, such as Delta and Jagged. The other possibility is that sAPP may directly interact with Notch molecules to induce signals for gliogenesis, similar with Notch ligands. If our first hypothesis is correct, treatment of sAPP would thus induce an increased expression of ligands associated with the Notch

signaling pathway and sequentially stimulate downstream signaling cascade. However, our findings indicate that this is not the case since there were no significant changes in Notch related gene expression except for the Hes1 gene, target of NICD (Fig. 24A). In the latter case, physical interaction of sAPP with Notch may be the most feasible way to induce glial differentiation of neural stem cells. To examine our hypothesis, we performed immunoprecipitation and found that sAPP interacts with Notch. Then, we found that sAPP physically interacts with Notch (Figure 24C) In support of our findings, several recent studies have demonstrated protein-protein interaction between APP and Notch [170, 174, 175].

In conclusion, our present findings address a novel paradigm in the understanding of glial differentiation mechanisms, which may aid in the development of novel therapeutic strategies for AD. Current limitation for the clinical use of stem cell therapy in AD is the low efficacy of neuronal differentiation of transplanted stem cells. Even if stem cells were transplanted into the brains of AD patient's brain, these implanted cells would mainly differentiate into glia due to the higher concentrations of sAPP *in vivo*. However, if glial differentiation related signaling pathways are tightly regulated, we may improve the success rate of stem cell therapy for AD. Therefore, knowledge acquired from this study may help to understand molecular mechanism of neurodegenerative disease such as AD and find advanced treatment or prevention methodology for AD.

GENERAL DISCUSSION

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases leading to dementia. Since almost 4.5 million patients suffer from AD in America and the number of patients will be increased to 14 million by year 2050, effective method for treatment or prevention is urgently necessary [1]. Although cytotoxicity of Aβ has been intensively studied due to its clinical importance, recent studies have shown the limitation of Aβ theory, such as the irrelevance with phenotypic characteristics of the amyloid precursor protein (APP) knock-out mice [176]. Thus, it tends to lead our concerns on physiological function of APP in the brain. Despite various physiological functions of APP have been reported such as neurite outgrowth, cell proliferation, and neural migration, physiological function of APP is still controversial and remained in veil because there are little consensus between *in vitro* and *in vivo* [26]. In the present studies, we investigated the novel function of APP in stem cell biology, especially, regarding the differentiation of neural stem cells.

Since serum-free unsupplemented condition does not contain any factors related with differentiation and growth, NSCs may not be differentiated and survived properly in this harsh condition. However, previously, we have shown that HNSCs can be differentiated into neurons, astrocytes, and oligodendrocytes and those cells can be survived up to 3 weeks under the serum free media condition. Thus, our previous results indicate that some unknown factors, produced endogenously, may affect their own differentiation as well as survival in the serum-deprived condition. In the present study, we observed the

elevation of APP expression in apoptotic cells, which has a shrunken morphology, under the condition of serum-free differentiation of HNSCs. The migration and differentiation of HNSCs were promoted by the treatment of recombinant sAPP with a dose-dependent manner. Inversely, when the function of APP was neutralized by N-terminal domain recognizing 22C11 antibody, the effect of APP on HNSCs was drastically antagonized. Thus, under serum-free differentiation conditions, APP released from apoptotic cells may serve as a differentiation and/or migration factor for neighboring HNSCs.

However, a high concentration of sAPP increased the glial cell population of differentiated HNSCs *in vitro*. Our results suggest that physiological concentration of APP enhances the migration and overall differentiation of HNSCs, whereas, higher concentration of APP, mainly differentiated into glia. Although exact mechanism is still unclear, several studies showed that elevation of neurogenesis of NSCs was observed in an early on-set AD patient brain while a massive gliosis is occurred in a late on-set AD patient brain [177, 178]. Based on our results, we might postulate that differentiation of early on-set AD patient's NSCs is promoted by the slight elevation of APP expression level. Since APP level does not reach to the gliogenic threshold, NSC of early stage AD patient may not be differentiated into glia. Inversely, most of the stem cell population of AD patient may be differentiated into glia because, as on the disease progressed, the amount of APP may be increased and will be beyond the gliogenic threshold in the brain.

Our *in vivo* study also may support the fact that high dose of APP might cause glial differentiation since higher expressions of APP and GFAP, typical astrocyte marker, were observed in APP23 tg mice compared to wild type mice. Although our results do not directly represent the effect of APP on differentiation of NSCs, at least, it indicates APP level correlates with GFAP expression in the brain. Consistently, Bahn *et al.* [56] demonstrated that neurospheres derived from DS patients almost exclusively differentiated into GFAP positive cells. This may result from an overdose of the APP gene, since Downs' syndrome patients have inherited three copies of APP that resides on chromosome 21.

We examined the function of APP in STS-induced glial differentiation of NT-2/D1 cells. STS has been used as a PKC inhibitor [101]as well as an apoptosis inducing reagent [102]. Additionally, several studies showed novel function of STS as a terminal differentiation inducer in murine embryonic stem cells [104], PC12 pheochromocytoma [105], and C6 glioblastoma [106]. Although a molecular mechanism of STS in differentiation remains unknown, STS-induced differentiation may be associated with an apoptosis mechanism. Since the elevation of APP is observed in apoptotic cells [80], we postulated increased APP may influence glial differentiation of NT-2/D1 cells under the apoptotic condition triggered by STS.

We found that treatment of STS induces apoptosis in NT-2/D1 cells by assessing the cell viability as well as the DNA fragmentation, a hall mark of apoptosis. Typical astrocytic morphological changes and typical astrocytic marker such as GFAP,

GLAST/EAAT1 and GLT1-EAAT2 were appeared in the presence of STS [120], suggesting that STS induces glial differentiation of NT-2/D1 cells.

We found that treatment of STS gradually increased APP expression as well as secretion during glial differentiation. Recent studies demonstrated APP has an anti-apoptotic function via phosphorylation of myocyte enhancer factor 2 [28, 111]. Thus, the increased expression and secretion levels of APP might be a compensatory mechanism against STS-induced apoptotic condition.

For further studies, gene silencing of APP was performed to examine involvement of APP in STS-induced glial differentiation. We found that knocking-down expression of APP significantly reduced GFAP expression in the presence of STS. Although STS may induce glial differentiation of NT-2/D1 through various signaling mechanisms, this result suggests that at least APP is associated with the induction of GFAP expression during STS-induced glial differentiation.

To investigate how STS regulate APP level, we examined APP catabolism/metabolism. Since APP expression is regulated by phosphorylation of ERK1/2 [125] and STS increases phosphorylation of ERK1/2 [116], we postulated that STS may increase APP expression by promoting ERK1/2 phosphorylation during glial differentiation. We found that MEK1 inhibitor (PD098059), which inhibits phosphorylation of ERK1/2, significantly reduced expression of APP and GFAP with dose-dependent manner. These results indicate that

STS may induce GFAP expression through the upregulation of APP by increasing ERK1/2 phosphorylation.

To elucidate mechanism relevant to APP-induced glial differentiation, we examined the potential involvement of IL-6/gp130 and notch signaling pathway [179]. Since different members of the IL-6 cytokine have shown induction of distinct patterns of expression and phosphorylation status of signaling molecules involved in IL-6/gp130 signaling pathway [143], treatment of APP may induce distinctive cellular responses. In our experimental condition, treatment of sAPP activates IL-6/gp130 signaling pathway via a physical interaction with gp130. Beside the result of immunoprecipitation, treatment of anti-gp130 antibodies, blocking a ligand binding site of gp130 [150], also showed that the interaction between APP and gp130 is crucial for activation of IL-6/gp130 signaling since the effect of sAPP was suppressed by anti-gp130 antibodies.

While a rapid phosphorylation of gp130 was induced in an early stage, phosphorylation of gp130 was quickly reduced back to basal level, presumably, by the internalization of ligand/IL-6R/gp130 complexes. In previous reports, when gp130 interacts with its ligands, it is targeted for degradation and gp130 should be synthesized before it appears in the membrane for preparing next cellular event [145]. Thus, treatment of sAPP significantly may promote the expression of gp130 from 60 min to maintain a certain amount of gp130 expression in the cell surface as a compensatory mechanism [40].

Our results have shown that treatment of sAPP promoted the expression of gp130, CNTF, and JAK1 in early son-set (< 2hr) for glial differentiation. Thus, sAPP may also stimulate CNTF expression to activate the IL-6/gp130 signaling cascade. The expression of CNTF, a potent gliogenic factor [179], was increased in an early on-set of gliogenesis (< 120 min) as well as up to 5 days in the presence of sAPP. However, since the application of siRNA of CNTF decreased GFAP expression, it reveals that CNTF may have the crucial function in APP-induced glial differentiation. Though further studies may be needed, treatment of sAPP may also induce glial differentiation by upregulating CNTF expression in NT2-D1 cells.

JAK/STAT signaling pathway is an important regulatory system, implicated in GFAP expression, in glial differentiation [45]. Conformational changes of gp130 recruits non-receptor kinases such as JAKs and activated JAKs phosphorylate STAT3 molecules to form STAT3 homodimer complexes. Then, STAT3 complexes are translocated into nucleus to turn on GFAP expression. To examine whether signaling transducing molecules such as JAKs and STAT3 in APP-induced glial differentiation, we used siRNA and pharmacological inhibitor for suppressing the function of JAK1/2 or STAT3 molecules. Although treatment of sAPP enhanced phosphorylation of STAT3-p-Tyr705, siRNA of STAT3, JAK1 and JAK2 inhibitor (AG490) inhibited signal transduction, caused by APP, and diminished GFAP expression. Therefore, present results suggest that JAK/STAT molecules are crucial intracellular mediators of the sAPP-induced glial differentiation of NT-2/D1 cells.

The notch signaling pathway also has been known as an important glial differentiation mechanism of NSCs [162]. The treatment of sAPP promoted the generation of NICD and Hes1 gene expression, suggesting the activation of notch signaling in NT-2/D1 cells. However, since treatment of γ-secretase inhibitors (L-685,458) suppressed NICD generation, Hes1 expression, and GFAP expression in the presence of sAPP, our results indicate that sAPP can induce glial differentiation of neural progenitor cells via notch signaling pathway.

We examined mechanisms associated with APP-induced notch signaling activation to elucidate how sAPP stimulate notch signaling pathway. One possibility is that sAPP may stimulate notch signaling cascade by increasing the expression of notch receptors and/or ligands such as Delta and Jagged. However, this possibility should be ruled out because mRNA expression level of Notch1, 2, Jagged, and Delta was not changed by treatment of sAPP. Instead, we found that APP can stimulate notch signaling cascade by physical interaction with notch receptors using immunoprecipitation. Several recent studies also have demonstrated a protein-protein interaction between APP and Notch in support of our findings [170, 174, 175]. Interestingly, N-terminal domain of APP (1-205) was enough to interact with Notch and promotes NICD generation as well as Hes1 expression. Also, APP (1-205) domain was sufficient to induce GFAP expression level in both protein and mRNA as well. It may be the reason that treatment of N-terminal recognizing antibodies (22C11) suppresses NICD generation as well as GFAP expression since a ligand/receptor binding is blocked by 22C11. Therefore, protein-

protein interaction of APP with gp130 and notch receptor may be the crucial for the induction of glial differentiation.

Previously, Kamakura *et al.* demonstrated IL-6 signaling and notch can cross-talk and have a synergistic effect through interaction of Hes1 and JAK2 [49]. These complexes enhance the accessibility of STAT3 homodimers to promoter sites and potentiate the expression of target genes (ex, GFAP). Our results also indicate that treatment of γ-secretase inhibitor suppressed GFAP expression as well as STAT3 phosphorylation via cross-talking between IL-6/gp130 and notch signaling pathway. However, APP-induced glial differentiation may be not modulated by a synergistic way but induced by activation of both IL-6 and notch signaling for the glial differentiation. If APP-induced glial differentiation is occurred synergistically, although one signaling cascade (IL-6/gp130 or notch) is blocked by antibodies, siRNAs, or chemical inhibitors, we could observe certain level of GFAP expression, higher than control, because APP can still activate the other signaling pathway. However, when one signaling cascade is suppressed, overall GFAP expression level was decreased almost back to basal level. Therefore, our findings indicate that the activation of both two signaling cascade may be necessary for APP-induced glial differentiation.

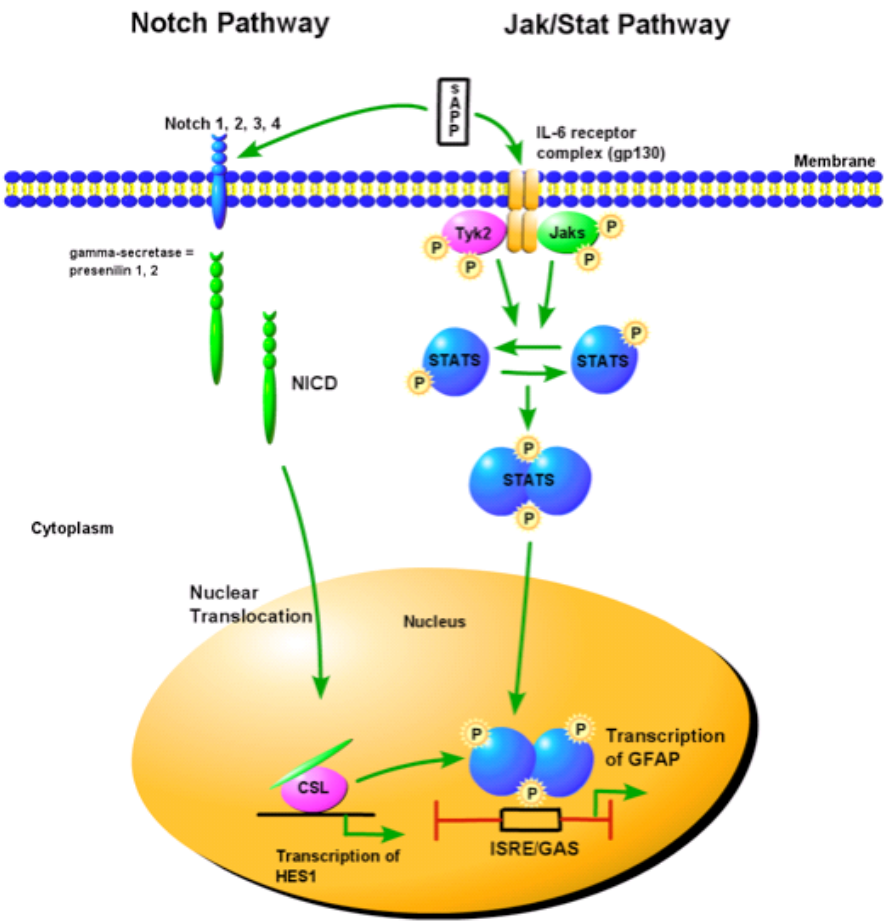

Figure 26: Schematic diagram of sAPP-induced glial differentiation mechanism.

Although the rate of neurogenesis of endogenous NSCs in the AD patient brain is slightly promoted in their early on-set [177, 178], in the long run, pathologically-altered APP metabolism in AD or DS may cause a defect in neurogenesis and significantly destroy normal brain functions due to massive glial differentiation of endogenous NSCs. A gliogenic APP function could also prevent successful stem cell therapy for AD using NSC by influencing the differentiation of the transplanted cells into glial cells rather than into neurons. Thus, in order to use stem cell transplantation as a potential strategic intervention therapy for AD or DC, regulation of environmental APP levels and/or modifications of the APP signal pathways within the cells may need to be developed. Therefore, regulation of APP level could be a promising strategy to increase neurogenesis in AD brain.

APPENDIX: COPYRIGHT PERMISSION

Some parts (Chapter 2 and 3) of this dissertation are based upon the following publications:

1. Y.-D. Kwak, E. Choumkina, K. Sugaya ‘Amyloid precursor protein is involved in staurosporine induced glial differentiation of neural progenitor cells’ Biochemical and Biophysical Research Communications 344 (2006) 431–437.

2. Y.-D. Kwak, C.L. Brannen, T. Qu, H.M. Kim, X. Dong, P. Soba, A. Majumdar, A. Kaplan, K. Beyreuther, and K. Suagaya ‘Amyloid Precursor Protein Regulates Differentiation of Human Neural Stem Cells’ Stem Cells and Development 15 (2006) 381–389.

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