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A TAG-1/APP signalling pathway through FE65 negatively modulates neurogenesis in *Mus musculus* (Linnaeus, 1758)

Dissertation

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Contents

1. Introduction

1.1 Amyloid Precursor Protein

Alzheimer's disease (AD) is the most common form of dementia in the elderly. It is a progressive brain disorder that gradually destroys a person's memory, orientation, judgment and reasoning. As the disease progresses, individuals may also experience changes in personality and behaviour, such as anxiety, suspiciousness or agitation, as well as delusions or hallucinations (Mattson, 2000). Affected individuals develop a gradual and progressive decline in cognitive and functional abilities as well as behavioural and psychiatric symptoms leading to a vegetative state and ultimately death (Turner, 2003). The duration of the illness may vary from 3 to 20 years (Bertram and Tanzi, 2005).

Amyloid beta peptide (Aβ) deposition, one of the hallmarks of AD, is thought to be the primary driver of neurodegeneration and cognitive decline leading to dementia (Glenner and Wong, 1984; Selkoe, 1991). The central role of Aβ deposition has been strongly supported by a wealth of evidences, including data from genetic studies of AD (Tanzi and Bertram, 2005). Amyloid precursor protein (APP) is expressed from early embryonic stages, suggesting it may have physiological functions in development, in addition to its pathological role in AD. Full-length APP and other fragments of APP after proteolytic cleavage have been found to be involved in various physiological functions, including proliferation of neural stem cells (Caillé et al., 2004), axonal transport (Buxbaum et al., 1998b; Gunawardena and Goldstein, 2001), cell adhesion and neurite outgrowth (Breen et al., 1991; Fossgreen et al., 1998; Soba et al., 2004).

1.1.1 The structure of APP

APP is a type I transmembrane glycoprotein consisting of a large extracellular domain, a single transmembrane domain and a short cytoplasmic tail. The

extracellular domain of APP contains a protease inhibitor domain, a heparin binding domain (Mok et al., 1997) and a Cu2+ and Zn2+ binding domain (Hesse et al., 1994; Bush et al., 1993; Gralle, 2007). The extracellular domain of APP also contains an N-glycosylation site (Pahlsson and Spitalnik, 1996; Gralle, 2007). These characteristics of the extracellular domain of APP suggest that the extracellular domain of APP may play a very important role in interaction with other proteins. Consistent with this idea, LDL-receptor-related protein (LRP) (Ulery et al., 2000ab; Cam et al., 2005), fibulin (Ohsawa et al., 2001) and F-spondin (Ho and Sudhof, 2004) interact with the extracellular domain of APP. The transmembrane of APP interacts with γ-secretase complex, which cleaves APP within the membrane, releasing the large extracellular domain of APP (sAPP), Aβ and the intracellular domain of APP (AICD) from full-length APP. sAPP, Aβ and AICD are all functional fragments involved in physiological or/and pathological processing. Compared to the large extracellular domain, the intracellular domain of APP is very small. However, a variety of signalling adaptor proteins bind to the small cytoplasmic tail of APP, including Go (Brouillet et al., 1999), Fe65 (Borg et al., 1996), X11 (Borg et al., 1996; 1998), JIP-1 (Matsuda et al., 2001), and APP-BP1 (Chow et al., 1996). These adaptor proteins are implicated in mediation of the proteolytic processing and the signalling transduction of APP.

The APP gene, located on the long arm of chromosome 21 (Goldgaber et al., 1987), contains 18 exons spanning more than 170kb (Yoshikai et al., 1990). Alternative splicing generates APP mRNAs encoding three major isoforms APP695, APP751, and APP770 (containing 695, 751 and 770 amino acids, respectively) (Zheng et al., 1995). All these three isoforms can generate Aβ following cleavage by secretases. APP751 and APP770 contain a domain homologous to the Kunitz-type serine protease inhibitors (KPIs; Kitaguchi et al., 1988; Ponte et al., 1988) that is absent in APP695 (Zheng et al., 1995). APP751

and APP770 are expressed in both the brain and other tissues, whereas APP695 is expressed predominantly in neurons (Neve et al., 1988; Zheng et al., 1995).

APP is conserved among mammalian species. Two APP homologs, known as APP like protein 1 (APLP1) and APP like protein 2 (APLP2), have been identified in both human and mouse. The APP homologs lack the Aβ region, and so cannot generate Aβ. However, they are proteolytically processed in the same manner as APP (Eggert et al., 2004), to release an Aβ-like peptide or P3-like peptide following cleavage by β- and γ-secretase or α- and γ-secretase, respectively. Concomitantly, they liberate the corresponding intracellular domains (ICDs; Eggert et al., 2004; Walsh et al., 2003), which are proposed to be involved in cellular signalling pathways (Walsh et al., 2003; Cao and Südhof, 2001).

1.1.2 Secretases and proteolytic cleavage of APP

The best-studied post-translational modification of APP is its proteolytic processing (De Strooper and Annaert, 2000; Selkoe, 1999). APP is cleaved at the level of the plasma membrane, or within the lysosomal and the endoplasmic reticulum/Golgi compartments of the cell, to generate sAPP, the C-terminal fragment CTF99, Aβ and the membrane-associated carboxyl-terminal fragment of APP (AICD). Proteolytic cleavage of APP first involves α-secretase (ADAM10) or β-secretase (BACE) generating (i) extracellular fragments, named sAPP-α and sAPP-β, respectively, and (ii) transmembrane fragments or CTFs (C-terminal fragments), CTF83 and CTF99, respectively. Later CTF83 and CTF99 are cleaved by the γ-secretase complex (presenilin, nicastrin, APH-1, PEN 2), which cleavage sites within the transmembrane helix, to release heterogeneous Aβ fragments or P3 peptides and AICD. Proteolytic cleavage by α- and γ-secretase precludes the generation of Aβ and so is known as the non-amyloidogenic processing pathway. When β- and γ-secretase cleaves APP, Aβ will be released. Hence this is called the amyloidogenic processing pathway (**Fig. 1**). APLP1 and APLP2 are also proteolytically processed and generate AICD, but do not yield Aβ

peptide because the primary amino acid sequence in that region is particularly divergent from APP (Eggert et al., 2004).

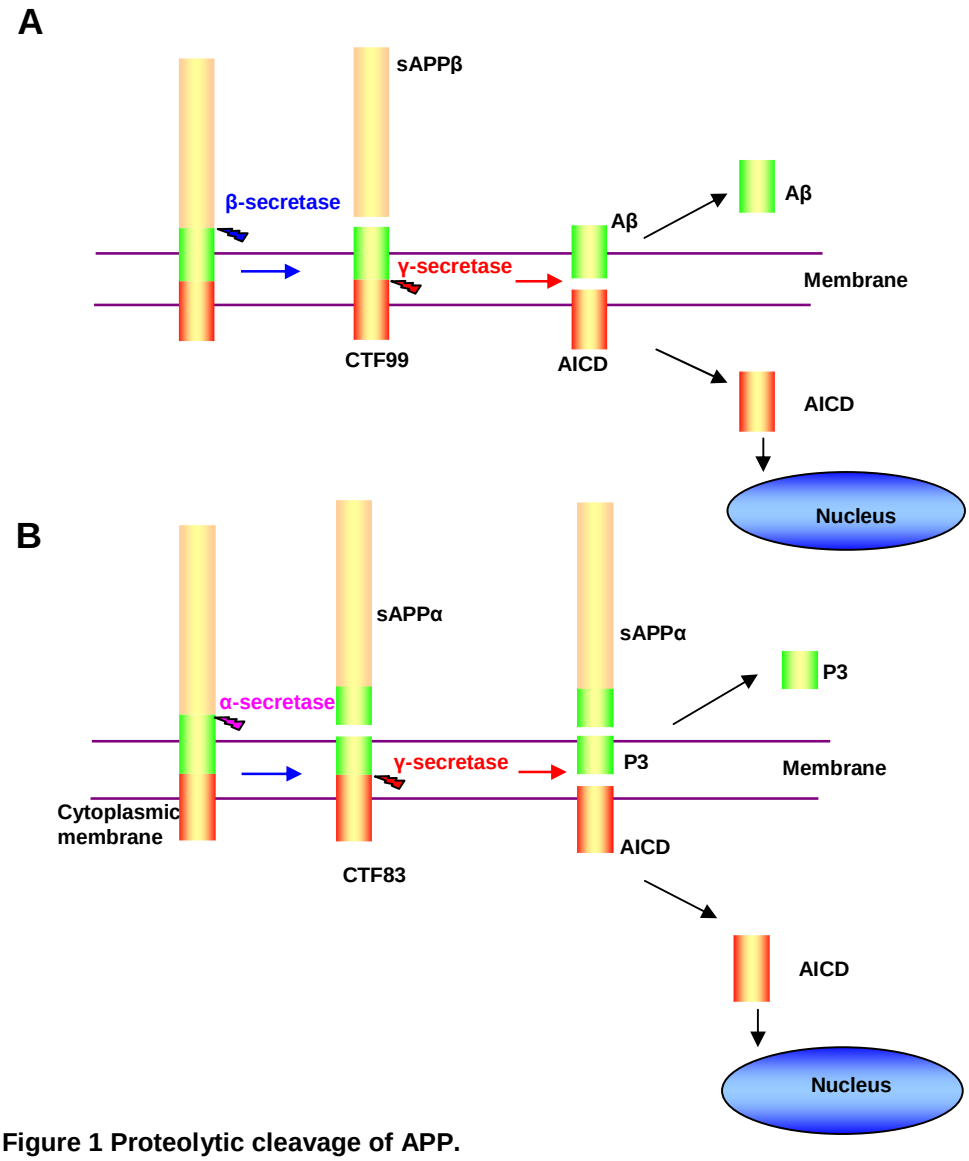

Figure 1 Proteolytic cleavage of APP.
A: Amyloidogenic processing pathway. APP is cleaved by β-secretase to generate sAPPβ and CTF99. CTF99 is further cleaved by γ-secretase to generate and Aβ and AICD. Aβ and sAPPβ are secreted to the extracellular of the cells. AICD is supposed to release from the membrane to the cytoplasm. **B**: Nonamyloidogenic processing pathway. APP is cleaved by α-secretase to generate sAPPα and CTF83. CTF99 is further cleaved by γ-secretase to generate and P3 and AICD. P3 and sAPPα are secreted to the extracellular of the cells. AICD is supposed to release from the membrane to the cytoplasm.

1.1.2.1 α-secretase

The majority of APP is cleaved by α-secretase within the Aβ sequence thus precluding Aβ generation (Ikezu et al., 1998). This theoretically prevents further amyloidogenesis in AD. The α-secretase processing of APP results in the shedding of the APP ectodomain (APPs). Several reports implicate members of a disintegrin and metalloprotease family (ADAM) (Schlondorff and Blobel, 1999) in α-secretase processing of APP. The ADAM family, a large protein family, is involved in cell-cell interactions and also in the processing of several other membrane-anchored proteins in addition to APP, such as TNFα, Notch, Delta, and others (Black et al., 1997). ADAM10 is a major α-secretase candidate (Lammich et al., 1999). Overexpression of ADAM10 increases α-secretase activity (Lammich et al., 1999). A dominant negative form of ADAM10 with a point mutation in the zinc-binding site was been found to inhibit α-secretase activity, whereas sAPPα production was not totally abolished (Lammich et al., 1999), suggesting that other proteins may contribute in α-secretase cleavage of APP as well.

Another member of the ADAM family, tumor necrosis factor-α (TNF- α) – converting enzyme (TACE or ADAM17), is another candidate for α–secretase cleavage of APP. The inhibition or knockout of TACE decreases the release of the α-cleaved product sAPPα (Buxbaum et al., 1998a). However, cells lacking TACE still have a residual α-secretase activity that cannot be increased by phorbol esters (Buxbaum et al., 1998a) and TACE may play a role in PKC-dependent α-secretase activity (Suh and Checler, 2002). In addition to ADAM10 and TACE, MDC9 (also known as meltrin γ), a membrane-anchored metalloprotease, was suggested to have α-secretase-like activity and to mediate the proteolytic cleavage of APP (Koike et al., 1999).

1.1.2.2 β-secretase

Alternatively, APP may be cleaved by β-secretase to generate secreted sAPPβ and a CTF99 transmembrane fragment. β-secretase has various names including BACE (β-site APP cleaving enzyme), Asp-2 (aspartyl protease 2), and memapsin-2 (membrane anchored protease of the pepsin family) (Hussain et al., 1999; Lin et al., 2000; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). Most researchers now use the name BACE-1 because it reflects the activity of the protein. BACE-2 (Asp-1, memapsin-1), which share 64% homology with BACE-1 in structure, is a second β-secretase (Yan et al., 1999).

BACE-1 and BACE-2 belong to a new family of aspartyl proteases closely related to the pepsin family (Hong et al., 2000). BACE-1 is highly expressed by neurons and has been demonstrated to be the major β-secretase responsible for Aβ generation in the CNS (Cai et al., 2001; Roberds et al., 2001; Luo et al., 2001). In contrast, BACE-2 is mainly expressed in peripheral tissues, but also to some extent in brain (Hussain et al., 2001; Farzan et al., 2000; Bennett et al., 2000). Both BACE-1 and BACE-2 can cleave APP not only at the Asp1 site of Aβ, but also at Glu11 sites of the Aβ sequence, generating an N-terminally truncated peptide that is considered more amyloidogenic and more neurotoxic than full-length Aβ (Pike et al., 1995). BACE-2, can also cleave the Aβ sequence between Phe19 and Phe20 close to the α-secretase site, resulting in less Aβ generation (Yan et al., 2001; Bennett et al., 2000). Thus, BACE-2 can be considered an alternative α-secretase (Farzan et al., 2000). In BACE-1 knockout mice, the secretion of Aβ1-40/42 and Aβ11-40/42 is abolished and the mice seem healthy and show no gross histological abnormalities (Cai et al., 2001; Luo et al., 2001), thus BACE-1 seems a good target for drug development. Use of siRNA targeting BACE-1 indeed reduces amyloid production and the neurodegenerative and behavioural deficits in APP transgenic mice (Singer et al., 2005). Memory deficits and cholinergic dysfunction in a mouse model of AD are rescued by BACE-1 deficiency (Ohno et al., 2004).

BACE-1, as a transmembrane protein, however, has been found involved in physiological processes in CNS development as well. Spatial memory and emotional deficits have been reported in BACE-1 knockout mice, which are possibly due to presynaptic deficits caused by the absence of BACE-1, suggesting that BACE-1 is involved in learning and memory (Laird et al., 2005). BACE-1 also plays a role in myelination. BACE-1 deficiency can cause hypomyelination in both PNS and CNS, by regulation of neuregulin cleavage (Willem et al., 2006; Hu et al., 2006). Moreover, BACE-1 has been found to cleave the β1 and β2 subunits of sodium channels (Wong et al., 2005; Kim et al., 2007), by which it can modulate the activity and expression of sodium channels (Ong et al., 2005; Kim et al., 2005; 2007). In BACE-1 knockout mice, the sodium channel density seemed decreased although not to a statistical significant extent and the inactivation of sodium channel currents shifted toward more depolarized potentials (Dominguez et al., 2005).

1.1.2.3 γ-secretase

Cleavage of CTF99 or CTF83 by γ-secretase generates AICD and Aβ peptides or P3 peptides (Selkoe, 2001). The γ-secretase complex consists of presenilin-1 or -2–nicastrin–aph1–pen2 (Esler et al., 2002; Lee et al., 2002; Li et al., 2000ab; Steiner et al., 2002；Wolfe et al., 1999b). The presenilins (PSs) are the core proteins in the γ-secretase complex. The nicastrin, anterior pharynx defective 1 (APH1) and PS enhancer 2 (PEN2) modulate the catalytic function of γ-secretase via interacting with presenilins (Spasic and Annaert, 2008). The presenilins are transmembrane proteins consisting of 7 to 9 highly hydrophobic domains. The C-terminal domain is proposed to be involved in the binding of the transmembrane domain of candidate substrate proteins (Annaert et al., 2001). Consistent with the fact that the cleavage of the substrates by γ-secretase happens within the membrane, the C-terminal domain of PS is unusually hydrophobic and therefore closely associated with the membrane (Annaert and Strooper, 2002). The transmembrane domain 6 (TMD6) and the C-terminal part of PS starting from

transmembrane domain 7 (TMD7) are much conserved in evolution, suggesting that this area is very important for the function of γ-secretase (Annaert and Strooper, 2002). Aspartates (Asp257 or Asp385) in TMD6 and TMD7 areas are important for the catalytic function of γ-secretase, because mutation of either Asp257 or Asp385 in PS abolishes γ-secretase activity (Wolfe et al. 1999a; Nyabi et al., 2003). Thus, PS is taken as a novel type of aspartyl protease. Notably, these aspartatic catalytic sites are separated from the binding sites of PS. As reported by Annaert et al., PS forms a ring structure with its transmembrane domains. The binding site and the catalytic sites are separately located at different parts of the “ring” (Annaert et al., 2001).

The transmembrane domain of PS is important for its cleavage activity. However, the particular sequence of the transmembrane domain of the substrate is of little important for cleavage by PS. As reviewed by Annaert and Stroope, the regulation of PS-mediated cleavage appears to depend on the shedding of the ectodomain triggered by other extracellular secretases such as α-secretase or β-secretase. The type I integral transmembrane protein can become a substrate for PS once the length of its ectodomain drops below 200-300 amino acids (Annaert and Strooper, 2002). Apart from the length of the ectodomain, oligomerization of the substrate proteins is also important for cleavage by PS. Disrupting the oligomerization of the substrate proteins by mutation abolishes cleavage by PS (Annaert and Strooper, 2002). The fact that PS is quite relaxed in its cleavage sequence requirement is consistent with the fact that the substrates of PS are very diverse: there are more than 30 type I transmembrane proteins found as the substrate for PS (Spasic and Annaert, 2008). APP interacts with the PS1-binding domains via the 11-amino acid-long sequence located on the C terminal site from the γ40-cleavage site (Annaert et al., 2001). This region contains the mutation site that causes familial AD. However, whether the mutation in this region alters the binding of APP to PS remains to be further investigated (Annaert and Strooper, 2002)

PS is implicated to have an essential role in physiological processes, such as apoptotic events (Wolozin et al., 1996); Wnt/β-catenin signalling (Xia et al., 2001); Ca2+ signalling (Leissring et al., 2000; Tu et al., 2006), and normal neurogenesis, and neuronal survival (Shen et al., 1997). Various lines of evidence suggest that PS1 is required for normal neurogenesis. The ventricular zone of $PS1^{-/-}$ brains is markedly thinner than that of wild-type brains by embryonic day 14.5 (E14.5), indicating impairment in neurogenesis. Bilateral cerebral cavitation caused by massive neuronal loss in specific subregions of the mutant brain is prominent after E16.5. PS1 expression was specifically inactivated in the CNS of adult mice, causing a significant decrease in Aβ production, but with remarkably few problems for the general well-being of the animals (Feng et al., 2001; Yu et al., 2001). Nevertheless, the mice showed discrete deficiencies in assays measuring long-term spatial memory (Yu et al., 2001). Feng et al. suggests that the absence of PS1 causes a reduction in neurogenesis in the dentate gyrus of the hippocampus and propose that this neurogenesis is important for the periodic clearance of outdated hippocampal memory traces (Feng et al., 2001). Although quite intriguing, this suggestion was made based on the results of one type of experiment, and it is also not clear whether the PS was indeed inactivated in the neuronal stem cells in question. Another group has reported that lack of PS1 leads to premature differentiation of neural progenitor cells, indicating a role for PS1 in a cell fate decision between postmitotic neurons and neural progenitor cells (Handler et al., 2001). Neural proliferation and apoptotic cell death during neurogenesis are unaltered in $PS1^{-/-}$ mice, suggesting that the reduction in the neural progenitor cells observed in the $PS1^{-/-}$ brain is due to premature differentiation of progenitor cells, rather than to increased apoptotic cell death or decreased cell proliferation. In addition, the premature neuronal differentiation in the $PS1^{-/-}$ brain is associated with aberrant neuronal migration and disorganization of the laminar architecture of the developing cerebral hemisphere. In the ventricular zone of $PS1^{-/-}$ mice,

expression of the Notch1 downstream effector gene, Hes5, is reduced and expression of the Notch1 ligand, Delta1 (Dll1), is elevated, whereas expression of Notch1 is unchanged (Handler et al., 2001). PS also plays a role in regulating the stability of cytosolic beta-catenin, a protein involved in Wnt signalling correlating with enhanced cell proliferation. The PS1 A246E mutation stimulated the proliferation of progenitor cells in the dentate gyrus of PS1-deficient adult mice, but did not influence their survival or differentiation, suggesting that the PS1 A246E mutation influences cell growth putatively via abnormal beta-catenin signalling in vivo (Chevallier et al., 2005). These results provide direct evidence that PS1 controls neuronal differentiation in association with the downregulation of multiple signalling pathways during neurogenesis.

1.1.3 Regulated intramembrane proteolysis (RIP)

Transmembrane proteins can be cleaved within the plane of the membrane to liberate cytosolic fragments that enter the nucleus to regulate gene transcription. This mechanism is called **R**egulated **I**ntramembrane **P**roteolysis (RIP). RIP is conserved from bacteria up to the higher vertebrates and is involved in various biological functions such as in embryonic development, in cholesterol homeostasis, and in the unfolded protein response (Brown et al., 2000). There are several common features of RIP: **i**) The proteins undergoing RIP span the membrane bilayer at least once; Ire1, ErbB, Notch and APP, for example, are type I transmembrane proteins oriented with their NH2 termini in the lumen and their COOH termini in the cytosol. ATF6, SREBP are type II transmembrane proteins with their NH2 termini in the cytosol. **ii**) The transmembrane cleavage does not occur until the bulk of the protein on the extracytosolic (luminal or extracellular) face has been removed by a primary cleavage. The primary cleavage is a prerequisite for the secondary intramembrane cleavage by shortening the extracytosolic segment to less than 300 amino acids. **iii**) The released proteolytic fragments can activate receptors (e.g., the *Drosophila* epidermal growth factor receptor by Spitz) or can regulate gene transcription

(e.g., the cytoplasmic fragments of SREBP or Notch) (Brown et al., 2000). For example, the intracellular domain of Notch (NICD), after being processed by RIP, binds directly to the downstream transcription factors of evolutionarily conversed C promoter binding factor/suppressor of Hairless/lag-1 (CSL) family or Deltex, and translocates into the nucleus to activate gene expression.

1.1.4 Release of AICD

APP is thought to be processed by RIP as well. APP is cleaved by intramembrane-cleaving proteases, the PS/γ-secretase complex, only after removal of the ectodomain by α- or β-secretase. The regulation of the RIP of APP, in contrast to that of Notch, is not very well understood. One way of regulating the first substrate cleavage in RIP is by ligand binding. Notch, for example, is inserted into the cell surface after being synthesized and glycosylated to become a mature receptor protein (Munro et al., 2000; Blaumueller et al., 1997). At the cell surface, it is only after its binding to the Delta or Jagged ligand that the Notch receptor is cleaved in its ectodomain by the metalloprotease TNFα converting enzyme (TACE) and becomes a substrate for PS/γ-secretase (Brou et al., 2000; Mumm et al., 2000). This shedding of the Notch extracellular portion is believed to be indispensable for the third enzymatic cleavage of Notch in the intramembrane region by PS1-dependent γ-secretase (Kopan and Goate, 2000). Several binding partners of APP have been identified. For example, low density lipoprotein (LDL) receptor-related protein (LRP) (Cam et al., 2005), the low density lipoprotein (LDL) receptor-related protein 1B (LRP1B) (Cam et al., 2004), F-spondin (Ho et al., 2004), PDGF (Gianni et al., 2003), laminin (Kibbey et al., 1993) and furin (Hwang et al., 2006) interact with APP, modulating the proteolytic cleavage of APP.

LRP interacts with APP via its ligand binding domain II and IV (Cam et al., 2005). Expression of LRP facilitates APP processing via the amyloidogenic pathway (Cam et al., 2005). Cells overexpressing APP that are transiently transfected with

LRP produce higher levels of Aβ and lower levels of sAPPα compared with mock-transfected cells (Ulery et al., 2000ab; Cam et al., 2005). The ligand binding domain II and IV of LRP contains the NPxY motifs, which are required for receptor endocytosis (Chen et al., 1990), suggesting its possible role in APP endocytosis. Consistently, further studies indicate that the fast rate of endocytosis of LRP facilitates the trafficking of APP within the endocytic compartments. CHO cells stably expressing wild-type LRP have less cell surface APP than control cells transfected with empty vector, whereas those stably expressing endocytosis-defective LRP accumulate APP at cell surface (Cam et al., 2005). The retention of APP on the cell surface decreases Aβ generation but increases sAPPα (Cescato et al., 2000). Therefore, LRP leads to preferential APP processing by the amyloidogenic pathway via accelerating the endocytic accumulation of APP. The low density lipoprotein (LDL) receptor-related protein 1B (LRP1B), another member of the low density lipoprotein receptor family, which shares high homology with the LRP, binds to APP as well. However, distinct from LRP, which accelerates internalization of APP, LRP1B accumulates APP at the cell surface, which is likely related to the slow endocytosis rate of LRP1B. Consistently, the level of Aβ generation is decreased by LRP1B (Cam et al., 2004). Together, both LRP and LRPBP modulate APP processing via regulation of endocytosis of APP. Moreover, the cytoplasmic tails of the LRP and LDL receptors also bind to Fe65 and mammalian Disabled, adaptor proteins involved in the intracellular signal transduction of APP, providing a possibility that LRP and LDL receptors play a role in APP signalling (Trommsdorff et al., 1998). However, whether LRP and LRPBP modulate proteolytic process of APP by RIP remains to be further investigated.

F-spondin, a protein associated with extracellular matrix, has been found to interact with APP and regulate the proteolysis of APP. However, the role of F-spondin in cleavage of APP seems contradictory. Some reports suggest that F-spondin reduces generation of C-terminal fragments of APP (CTFs) via inhibiting

the cleavage of APP by BACE (Ho and Sudhof, 2004). Another study suggests that F-spondin has an opposite function on APP proteolytic cleavage to generate CTFs in an ApoE receptor-dependent manner (Hoe et al., 2005). F-spondin has been observed to play a role in inhibition of transcriptional activity in an artificial GAL4-fused luciferase system. However, more direct evidence is required to clarify whether F-spondin can trigger the RIP of APP (Ho and Sudhof, 2004). Additionally, PDGF is reported to induce cleavage of APP through a src-Rac-dependent pathway (Gianni et al., 2003). Furin is reported to enhance the α-cleavage of APP (Hwang et al., 2006). Laminin is also suggested as a binding partner of APP (Kibbey et al., 1993). However, no clear evidence has been provided indicating that any of these molecules modulates the proteolytic cleavage of APP by RIP.

An important aspect of RIP is that the released proteolytic fragments can activate receptors (e.g. activation of the *Drosophila* epidermal growth factor receptor by Spitz) or can regulate gene transcription (e.g. the cytoplasmic fragments of SREBP or Notch) (Brown et al., 2000), the question is whether AICD generated by RIP is involved in such regulatory processes. Transcriptional activity of AICD has been detected in an artifical luciferase system, where a Gal4 binding motif on the luciferase reporter gene attracts the GAL4 fused AICD (Cao and Südhof, 2001; 2004). A series of candidate AICD target genes have been identified, including teraspanin KA11/CD82, glycogen synthase kinase-3β (GSK-3β), Neprilysin, APP (Baek et al, 2002; Kim et al, 2003; Pardossi-Piquard et al, 2005; Von Rotz et al, 2004). However, it has been recently argued that activation of these genes is not directly modulated by AICD (Hébert et al., 2006; Waldron et al., 2008). A recent study revealed that expression of EGFR is under the control of AICD. A direct binding of endogenous AICD to the EGFR promoter was provided as evidence of that AICD has transcriptional activity (Zhang et al., 2007).

NICD, after release from the Notch receptor, interacts with either Deltex1 or CSL and is further translocated into the nucleus to modulate the expression of the target gene (Hu et al., 2003; Cui et al., 2004; Schroeter et al., 1998). Various intracellular molecules are indicated to bind to the intracellular domain of APP, such as Fe65, X11, Go protein, PAK3 APP-BP1, JIP and Dab1 (Russo et al., 2005).

1.1.5 Intracellular Binding Partners of APP

1.1.5.1 Fe65 family

The Fe65 family, which consists of Fe65, Fe65-like (Fe65L or Fe65L1), and Fe65L2, has been identified to interact with the C-terminus of APP in yeast two-hybrid screens (Borg et al., 1996; Bressler et al., 1996; Duilio et al., 1998; Fiore et al., 1995; Guénette et al., 1996; Tanahashi and Tabira, 1999; Zambrano et al., 1997). They are also "polyvalent" in possessing three protein interaction domains: a WW domain and two PTB domains (PTB1 and PTB2) with distinct binding specificities. The PTB_2 domain of Fe65 binds either the intracellular domain of full-length APP or AICD. This binding is critical for activation of the APP signalling pathway although the exact relationship between Fe65 and AICD remains to be further investigated. In an artificial luciferase system in which APP was fused with Gal4, AICD itself was found to have no transcriptional activity, whereas on binding with Fe65, transcriptional activity was strongly enhanced (Cao and Südhof, 2001; 2004). NICD, after liberating from Notch, binds to CSL and translocates into the nucleus, where it regulates the transcription of downstream genes (Schroeter et al., 1998). AICD and Fe65 have been suggested to have an analogous relationship: AICD is highly labile and binds Fe65, which like CSL, is present in the cytoplasm and nucleus. Fe65 stabilizes and promotes nuclear translocation of AICD in a manner similar to CSL and NICD (Kimberly et al., 2001). However, some studies claim that AICD does not translocate into the nucleus. It appears that after release from full-length APP, membrane anchored-AICD activates FE65 by causing a conformational change,

and the latter enters the nucleus to form a molecular complex with CP2–LSF–LBP-1c or Tip60, which modulates gene expression (Cao and Südhof, 2001;2004; Cupers et al. 2001b; Kimberly et al. 2001). The AICD degrades soon. On co-expression of APP and Fe65 in Hela cells, both APP and FE65 localize at the nucleus. However, when co-expressed with APP*, a mutant form of APP that abolishes the binding between APP and Fe65, Fe65 alone has been observed in nucleus. These results indicate that full-length APP locks Fe65 in the cytoplasm, preventing it entering the nucleus (Cao and Südhof, 2004; Minopoli et al., 2001). The activated nuclear Fe65 binds Tip60, which is believed to have transcriptional activity, and another coactivator, to regulate the transcription of downstream genes. However, it has also been suggested that Fe65 itself has transcriptional activity, while AICD and Tip60 serve merely as positive/negative modulators (Yang et al., 2006). Therefore, how these molecules contribute to the APP signalling pathway and regulation of downstream genes needs to be further investigated.

The PTB_2 domain of Fe65 also binds the C-terminus of full length APP via a KPI domain, providing a potential scaffold between APP and LRP (Kinoshita et al., 2001). Interaction of the Fe65 PTB domain with APP requires the -GYENPTY- motif as well as threonine-668, 14 residues to the N-terminal side of the internalization sequence. Phosphorylation of threonine-668 of APP impairs Fe65 interaction suggesting that adaptor protein interactions with APP may be differentially regulated by its phosphorylation–dephosphorylation (Ando et al., 2001). A WW domain interacts with proline-rich ligands, such as Mena (mammalian enabled) that binds actin and thus links Fe65 and APP to cytoskeletal dynamics and cellular motility and morphology (Ermekova et al., 1997), and c-Abl tyrosine kinase. The PTB1 domain of Fe65 binds the transcription factor complex CP2–LSF–LBP-1c or Tip60, and this complex is found in nuclear fractions (Zambrano et al., 1998 Cao and Südhof, 2001). The PTB1 domain of Fe65 also binds the low-density lipoprotein receptor-related

protein (LRP) that serves as a receptor for ApoE and α-2 macroglobulin that scavenge secreted Aβ (Trommsdorff et al., 1998). With Fe65L coexpression, LRP degradation is accelerated in vitro (Guénette et al., 2002).

Fe65 itself can be phosphorylated by c-Abl tyrosine kinase at Tyr547, and the phosphorylation of $Fe65^{Tyr547}$ enhances the transcription activity of APP but does not increase the interaction between APP and Fe65 (Perkinton et al., 2004). In addition, Fe65 and Fe65L accelerate secretory processing and maturation of APP and promote APPsα and Aβ secretion in H4 cells and Madin Darby canine kidney (MDCK) cells (Guénette et al., 1999; Sabo et al., 1999). These results may be cell-type dependent, however, because Fe65 stabilizes immature APP and inhibits APPs and Aβ secretion in HEK 293 cells (Ando et al., 2001). Fe65L1 potentiates γ-cleavage of C99 to promote AICD generation and Aβ secretion (Chang et al., 2003). Fe65L2 also promotes Aβ secretion but does not otherwise affect cellular APP metabolism (Tanahashi and Tabira, 2002). But it is to be noted that both Fe65L1 and Fe65L2 do not modulate transcriptional activity of AICD (Tanahashi and Tabira, 2002; Chang et al., 2003). Therefore, Fe65 has multiple roles in cellular processes via binding to various molecules through its different domains (**Fig. 2**).

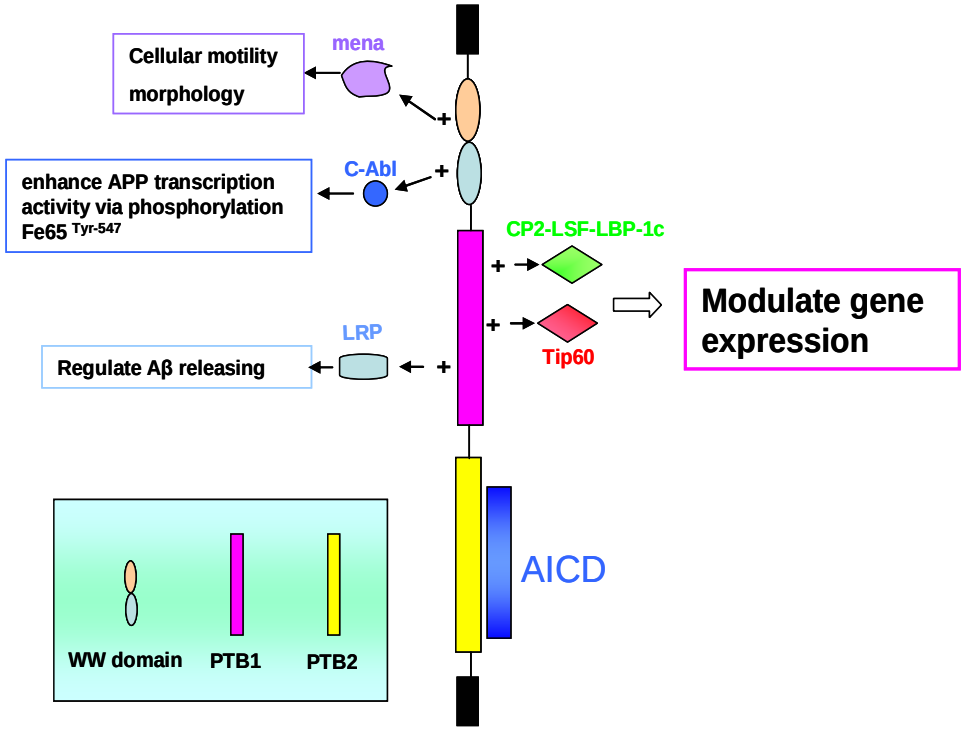

Figure 2 Structure and binding partners of Fe65.
FE65 interacts with mena and C-Abl through the WW domain. The PTB1 domain of FE65 interacts with CP2-LSF-LBP-1c, Tip60 or LRP. Fe65 interacts with AICD via its PTB2 domain.

Fe65 is expressed at high levels in neurons (Bressler et al., 1996). In adult mouse brain, Fe65 is highly expressed in neurons in the hippocampus, cerebellum, thalamus, and midbrain, with some expression in a subset of astrocytes in the hippocampus. Fe65 expression is also developmentally regulated, with levels declining after embryonic day 15 and increasing again progressively from postnatal day 10 to adulthood (Kesavapany et al., 2002). The dynamic expression of Fe65 suggests that Fe65 may play a role during central nerve system development. APP co-localizes with Fe65 at synaptic sites and in distal domains of neuronal growth cones, particularly actin-rich lamellopodia (Sabo et al., 2003). Increase of cellular motility has been observed with APP and Fe65 co-expression (Sabo et al., 2001). In Fe65 and Fe65L1 double knockout

mice, cortical dysplasia has been observed, and a similar phonotype is found in APP, APLP1 and APLP2 triple knockout mice, suggesting that APP-Fe65 signalling or Fe65 family proteins are involved in cytoskeleton motility (Guénette et al., 2006). Fe65 also blocks cell cycle progression via down-regulating thymidylate synthase expression (Bruni et al., 2002).

1.1.5.2 X11 family

X11 family members, including X11α, β, and γ, contain divergent N-termini but highly conserved C-termini consisting of a PTB domain and two PDZ domains. While X11γ expression is ubiquitous, X11α and β are expressed only in the brain (Borg et al., 1999; Hase et al., 2002; McLoughlin et al., 1999; Okamoto and Sudhof, 1997;1998). During murine embryonic development, X11β and X11γ are expressed before embryonic day 10.5 (E10.5), whereas X11α expression starts later between E12.5 and E14.5 (Ho et al., 2003). In the adult mouse, both X11α and β are widely expressed, but levels vary among brain regions. X11α is highly expressed in the olfactory system, piriform and entorhinal cortex, the substantia nigra, throughout the cortex, and other brain regions. X11α expression is restricted to neurons and enriched in axons (Okamoto et al., 2000). In contrast, X11β is often found in somatodendritic compartments (Nakajima et al., 2001).

The PTB domain of X11α interacts with APP, APLP1, and APLP2 specifically (Miller et al, 2006). Eight residues of the APP peptide -QNGYENPTYKFFEQ- make specific contacts with the PTB of X11α according to crystal structural analysis (Zhang et al., 1997). Further mutational analysis reveals that the -YENP- tetrapeptide is essential for X11α interaction. Although mutation of the second tyrosine residue in the -YENPTY- sequence to alanine does not impair X11α or Fe65 interaction, this mutation significantly alters APP cellular trafficking and processing, perhaps due to a lysosomal targeting defect (Borg et al., 1996; King et al., 2003). Both secretory and endocytic pathways of APP trafficking are impaired by X11α (King et al., 2004b). Consistently, cellular APP levels are

increased, while the generation of Aβ is reduced by X11α (Borg et al., 1998; Sastre et al., 1998; Hill et al., 2003). However, the reduction of Aβ generation may be due to the impairment in γ- but not β-cleavage of APP (King et al., 2004ab). Because there is no any inhibitory effect of X11α on γ-cleavage activity in a cell-free system, the inhibition of Aβ secretion by X11α may be via impaired trafficking of APP to subcellular compartments containing active γ-secretase complex (King et al., 2004ab). Consistently, an interaction between X11s and presenilin has been found (Biederer et al., 2002). The inhibition of Aβ secretion by X11α is more significant in Swedish mutated APP (APPswe) (King et al., 2004ab). Human X11α and human APPswe double transgenic mice reveal a significant decline in Aβ40 levels in brain homogenates and rescue of age-dependent amyloid plaque deposition in brain compared to age-matched human APPswe transgenic control mice (Lee et al., 2003). Consistent with the fact that X11α binds to APP via its PTB domain, coexpression of APP with the isolated PTB domain of X11α mimics most of the modulatory effects of intact X11α on APP metabolism (Mueller et al., 2000). Like X11α, X11β stabilizes cellular APP and decreases APPsα and Aβ secretion (McLoughlin et al., 1999), while the effect of X11γ on APP metabolism is unknown.

1.1.5.3 Go Protein

The first direct evidence in support of this notion that APP acts as a receptor protein was the finding that the APP cytodomain interacted with the heterotrimeric Go protein via the His^{657}-Lys^{676} domain of APP695 (Nishimoto et al., 1993; Brouillet et al., 1999). Stimulation of G_o by FAD APP is required to cause aberrant neuronal cell cycle entry and apoptosis (Lang et al., 1995; McPhie et al., 2003). Pertussis toxin, which inactivates the heterotrimeric G proteins G_o and G_i, inhibits the apoptosis and DNA synthesis caused by familial Alzheimer's disease (FAD) APP mutants; the apoptosis and DNA synthesis are rescued by co-expression of a pertussis toxin-insensitive G_o (McPhie et al., 2003). These data are consistent with the reports that the His^{657}-Lys^{676} domain of

APP-695 activates the heterotrimeric GTP-binding protein G_o in a $GTP_\gamma S$-inhibitable manner (Nishimoto et al., 1993; Lang et al., 1995). Moreover, an antibody, 22C11, to the extracellular domain of APP can act as a ligand mimetic (Okamoto et al., 1995) to activate G_o protein, demonstrating that APP may be a G protein-coupled receptor.

1.1.5.4 PAK3

The p21-activated kinase 3 (PAK3), a serine/threonine kinase, is another binding protein of the C-terminus of APP (McPhie et al., 2003). PAK3 is involved in the control of cytoskeleton dynamics, possibly affecting cognition by regulating neuronal structures. Mice lacking expression of PAK3 are impaired in both synaptic plasticity and cognition (Meng et al., 2005). PAK3 also has been implicated in the DNA synthesis and neuronal apoptosis caused by FAD mutants of APP (McPhie et al., 2003). A kinase inactive mutant of PAK3 inhibits FAD APP-mediated neuronal apoptosis and DNA synthesis; this effect is abolished by deletion of the PAK3 APP-binding domain or by co-expression of a peptide representing this binding domain. A 26-amino acid peptide representing the APP binding domain in PAK3 has been shown to inhibit FAD APP-mediated cell cycle entry (McPhie et al., 2003). These data suggest that both the kinase activity of PAK3 and also its interaction with APP are important for the FAD APP signalling pathway.

A model has been proposed, in which APP is part of a G_o protein-centred complex including PAK3 that transduces extracellular signals to the cytoplasm. One of the downstream molecules in the PAK3-mediated pathway is the c-Jun N-terminal kinase 3 (JNK3). JNK3 is highly expressed and activated in postmortem brains of individuals with AD (Zhu et al., 2001). JNK3 is associated with neurofibrillary tangles, and JNK up-regulation co-localizes with phosphorylated tau (Zhu et al., 2001), a microtubule associated protein which is hyperphosphorylated in AD. Aberrant phosphorylation of tau by JNK3 causes the

formation of oligomeric tau fibrils that may be viewed as “pretangles” (Sato et al., 2002). In a transgenic mouse model of AD, JNK activation is associated with amyloid deposits and phosphorylated tau. Age-dependent increased JNK activity is correlated not only with increased amyloid deposition in this mouse model but also loss of functional synapses similar to that observed in AD brain (Savage et al., 2002). All these evidence suggests that APP-Go-PAK3-JNK3, as a potential pathway, contributing the pathological process of AD. Moreover, JNK3 also phosphorylates APP at threonine668 site (numbering from APP695). This phosphorylation prevents the binding of APP to Fe65 (Kimberly et al., 2005).

1.1.5.5 APP-BP1

APP-binding protein 1 (APP-BP1) binds the cytoplasmic domain of APP (Chow et al., 1996). The interaction of APP with APP-BP1 activates a pathway leading to the conjugation of NEDD8, a ubiquitin-like protein, to its target. APP-BP1 forms a heterdimer with Uba3, and together to activate NEDD8 (Cope and Deshaies, 2003). Activated NEDD8 neddylates members in the cullin family. Cullins are scaffold proteins for the E3 ubiquitin ligase complex, and neddylation of cullin enhances its ability to promote ubiquitination (Read et al., 2000; Wu et al., 2000). NEDD8 has been found in ubiquitinated neurofibrillary tangles in AD brain (Mori et al., 2005). Overexpression of FAD mutants of APP in neurons results in an increase in expression of APP-BP1 in lipid rafts. Consistent with this result, Overexpression of APP-BP1 is found in lipid rafts in at-risk regions of human AD brain relative to cognitively intact controls (Chen et al., 2003). Moreover, inhibition of the neddylation pathway in neurons by expression of a dominant-negative mutant of hUbc12 prevents FAD APP-mediated cell cycle entry and apoptosis (Chen et al., 2000; Chen et al., 2003). Recently, APP-BP1 has been further shown to play a role in inhibition of Aβ generation (Chen et al., 2007). All this evidence has suggested a role of APP-BP1 in Alzheimer’s disease.

1.1.5.6 JIP family

C-Jun N-terminal kinase (JNK) interacting protein-1a (JIP-1a), JIP-1b, and JIP-2 are members of the that possess PTB domains and also interact with the -YENPTY- motif of APP (Inomata et al., 2003; Matsuda et al., 2001; Scheinfeld et al., 2002). JIP-1a is a splice variant which lacks a complete PTB domain that is not found in humans. JIP-1a and JIP-2 bind weakly to APP and do not affect its processing. JIP-1b interaction enhances JNK-mediated threonine-668 phosphorylation indicating that JIP-1b may function as a scaffold between APP and JNK (Inomata et al., 2003; Taru et al., 2002a). JIP-1b may also link APP and kinesin light chain-1 (Inomata et al., 2003; Matsuda et al., 2003). Similar to X11α and β, JIP-1b interaction with APP stabilizes immature APP and inhibits APPs, Aβ40, and Aβ42 secretion in vitro (Taru et al., 2002b). In addition to JIP-1b serving to scaffold APP to the kinesin light chain, kinesin-1 may directly interact with the APP C-terminus via the tetratrico peptide repeat (TPR) domain of the kinesin light chain (Kamal et al., 2000). The kinesin–APP or kinesin–JIP-1b–APP complex may mediate fast axonal transport of vesicles containing APP, presenilin, and BACE (Kamal et al., 2001).

1.1.5.7 Other intracellular proteins

Disabled-1 (Dab1) and autosomal recessive hypercholesterolemia (ARH) protein interact with APP via their PTB domains (Homayouni et al., 1999; Howell et al., 1999; Noviello et al., 2003). Their effects on APP processing still remain unknown. Recent study has revealed that down-regulation of ARH expression increases cellular APP levels (Noviello et al., 2003). The PTB domain of Shc and the Src homology 2 (SH2) domain of Abl may interact with APP in a phosphorylation-dependent manner, suggesting a possible role of APP in tyrosine kinase-mediated signal transduction (Russo et al., 2002).

Other proteins without PTB domains may interact with the APP C-terminus, but little is known of their potential modulatory effects on its processing or the *in vivo* significance of their interaction. These putative APP binding partners include UV-

damaged DNA binding protein (UV-DDB) that recognizes the -YENPTY- motif (Watanabe et al., 1999). Protein interacting with the APP tail 1 (PAT1), a microtubule-binding protein, mediates intracellular transport of APP through the secretory pathway. Mutation of tyrosine to alanine in the -YTSI- sequence of APP precludes PAT1 interaction (Zheng et al., 1998). Finally, caveolin-3 may bind to APP and promote its β-cleavage (Nishiyama et al., 1999).

In summary, various proteins bind to the intracellular domain of APP. These proteins bind to the intracellular domain of APP in a competitive-way. For example, contemporary binding of Fe65 or X11 to APP seems to be impossible, and that of Fe65 and Go is not probable (Russo et al., 1998). More evidences indicate Fe65 is a good candidate to be involved in RIP of APP, providing that APP, like notch, may be a signalling receptor which is modulated by ligand binding and RIP.

1.1.6 Physiological roles of APP and other fragments of APP

We have introduced the procession of APP, the ligands and the adaptor proteins of APP. All these information has implicated that APP, as a signalling receptor, may involved in various physiological function, such as in cell–cell interaction, cell adhesion, protease inhibition (for the longer APP isoforms), and neurite outgrowth (Breen et al., 1991; Ghiso et al., 1992; Salinero et al., 2000; Schubert and Behl, 1993; Yamazaki et al., 1997). The catabolites of APP, sAPPα/β and A β, play physiological function as well.

1.1.6.1 Role of APP in cell adhesion and neurite outgrowth

Increasing evidence suggests a role of APP in cell adhesion and neurite outgrowth (Coulson et al., 2000). APP was stained as the irregular punctuate pattern, which is similar to the staining patterns of other CAMs, on the neuronal cell surface and also on its synaptic localization (Breen et al. 1991; Schubert et al. 1991). The Fab′ fragments of anti-APP antisera were found to inhibit Neuro2a

cells, which endogenously express APP, binding to a collagen substrate. Same APP antibodies have been found to inhibit both neuron-neuron and neuron-glial binding, but not glial-glial adhesion. In addition, a repression of neurite outgrowth cells has been observed in presence of the antibodies (Breen et al. 1991). These data suggest that APP may play a role in the mediation of both cell-cell and cell-substrate adhesion (Breen et al. 1991). Consistently, the down-regulation of cellular APP expression using an antisense strategy also reduces the strength of cell-substrate adhesion (Coulson et al., 1997). Furthermore, an overexpression of the protein in *Drosophila* results in a blistered wing phenotype, which is suggestive of an alteration in cell-cell adhesion in the dorsal and ventral epithelial cell layers during development (Fossgreen et al., 1998).

APP interacts with a number of elements of the extracellular matrix such as collagens I and IV (Breen et al., 1991; Beher et al., 1996), laminin (Multhaup et al., 1992; Kibbey et al., 1993), fibronectin (Narindrasorasak et al., 1995), and heparan sulfate proteoglycans (HSPGs) (Narindrasorasak et al., 1991), and glycosaminoglycans (Multhaup, 1994), supporting its role in cell-substrate adhesion. Three members of the APP family, APP, APLP1 and APLP2 interact with each other in a homo- or hetero-dimeric way, and trans-dimerizations have been shown to promote cell-cell adhesion (Soba et al., 2005).

Post-translational modifications of proteins play a key role in determining these adhesive properties (Breen et al., 1998). APP can form the core protein of a chondroitin sulfate proteoglycan (Shioi et al., 1992), which is termed appican (Shioi et al., 1993), and it may also exist as an HSPG (Schubert et al., 1989). The attachment of chondroitin sulfate chains is restricted to the Ser619 amino acid residue of the L-form of the protein (that lacks exon 15) (Pangalos et al., 1995a). The expression of appican, which can exist in either a membrane-bound or a secreted protein (Shioi et al., 1993), is restricted to neural cells (Pangalos et al., 1995b; Shioi et al., 1993), although the secreted appican may contain the

complete amyloidogenic sequence, suggesting an altered processing pathway of the proteoglycan form of the APP (Salinero et al., 1998). The presence of the chondroitin sulfate side chain is important for the adhesive function of APP, as appican demonstrates an increased adhesive potential when compared with the non-chondroitin sulfate proteoglycan form of APP (Pangalos et al., 1996), and the secreted form of appican can also interact with elements of the extracellular matrix in order to promote the adhesion of neural cells (Wu et al., 1997).

1.1.6.2 Role of APP in axonal transport

Deficient axonal transport has been implicated in several neurodegenerative diseases, including AD (Praprotnik et al., 1996; Dhaenens et al., 2004). Axonal transport is essential for maintaining the structure and function of nerve cells. APP is transported as a cargo anterogradely to the axonal terminus (Sisodia et al., 1993) and serves as a vesicular receptor for the anterograde motor protein kinesin-1 (Kamal et al., 2001). The cytoplasmic domain of APP apparently binds to the kinesin light chain thereby acting as the link between the transport vesicle and the kinesin motor complex. Overexpression of human APP or loss-of-function of APPL in Drosophila caused same axonal transport phenotypes as in kinesin and dynein mutants (Gunawardena and Goldstein, 2001). These observations were further confirmed and extended in mice, demonstrating that APP is needed for the transport of an axonal compartment containing APP, the neurotrophin receptor TrkA, BACE, PS1, GAP43, and synapsin I, but not synaptophysin or synaptotagmin. Thus apparently all proteases needed to generate Aβ are present in this compartment and, accordingly, Aβ production in this fraction could be demonstrated. Interestingly, a mutation in PS1 involved in early-onset AD leads to kinesin-1-based axonal transport deficits, presumably through deregulation of glycogen synthase kinase 3β (GSK3β) (Pigino et al., 2003).

Stokin and colleagues examined the temporal relationship between pathological changes and axonal abnormalities in AD patients and two different animal models of AD (Stokin et al., 2005). They showed axonal swellings containing vesicular components in the cholinergic system, a transmitter closely linked to cognition, early in the course of AD. Of note, similar swellings were also seen in mice overexpressing either of two forms of mutant APP months before Aβ deposition. APP processing to Aβ, is linked to transport because reduced expression of kinesin-1, the anterograde transport motor, increases Aβ42 and leads to increased Aβ deposition as plaques. Even more intriguing, with kinesin-1 reduction, Aβ deposits are confined to proximal axons, as opposed to distal terminals (Buxbaum et al., 1998a), which is consistent with reduced transport, suggesting that an axonal transport deficit might be a proximal and causative factor in the pathogenesis of AD. APP is transported along with various Jun N-terminal kinase (JNK) pathway molecules, raising the possibility that axonal transport blockage stimulates the generation and release of Aβ and activation of JNK pathway molecules (Zhu et al., 2002), which will then be retrograde transported back to the cell body to transduce repair signals.

As such, these studies demonstrate that axonal blockage interferes with axonal transport early in the course of AD, well preceding amyloid pathology in mouse models of AD (Stokin et al., 2005), suggesting axonal transport deficits at a central preeminent point in the disease pathogenesis. However, this work was seriously challenged by another laboratory recently (Lazarov et al., 2005), and more research will be needed before any conclusions can be made. It is crucial to know the relationship between axonal transport abnormalities and intracellular Aβ levels and other known early pathological alterations; whether axonal transport deficits occur in mild cognitive impairment (MCI) or whether they are only present in frank AD and what causes the axonal transport deficits. As APP is not significantly elevated even in AD patients bearing APP mutations, it also

remains to be determined whether various mutant APPs at normal levels interfere with axonal transport and its potential mechanisms.

1.1.6.3 Role of APP in neural stem cells

sAPP is normally present in brain tissues and circulates in the cerebrospinal fluid (CSF) (Palmert et al., 1989). Several *in vitro* studies have suggested sAPP enhances synaptogenesis, neurite outgrowth, cell survival and adhesion (Mattson, 1994). sAPP was then identified to stimulate proliferation of neural stem cells isolated from the embryonic brain (Hayashi et al., 1994; Ohsawa et al., 1999). Intracerebral infusion of sAPP increases synaptic density and improves memory performance (Roch et al., 1994). Evidence that sAPP plays a role in neural stem cell division comes from initial studies showing that sAPP stimulates the proliferation of *bona fide* neural stem cells *in vitro* (Hayashi et al., 1994; Ohsawa et al., 1999), as do epidermal growth factor (EGF) or fibroblast growth factor (FGF) in the same cultures. It is known that EGF intracerebral infusion induces type-C cells to divide and invade adjacent zones, and that this EGF-expandable population of type-C cells exhibits properties of multipotential stem cells *in vitro* (Doetsch et al., 2002). Consistently, sAPP-binding sites are also present on both type-C cells and type-A cells that correspond to the migrating neuroblasts (Caillé et al., 2004). Intracerebral sAPP infusion results in a similar increase in the number of EGF-responsive type-C cells through direct regulation of their proliferation. Moreover, they further proved that there are binding sites for secreted N-terminal nonamyloidogenic APP (sAPP) on epidermal growth factor (EGF)-responsive neural stem cells in the subventricular zone of the adult brain, where sAPP acts as an EGF cofactor to stimulate proliferation of these cells, as sAPP is necessary for, but cannot replace, full EGF activity (Caillé et al., 2004). Another important issue raised by Caille and colleagues concerns the lack of sAPP binding in the dentate gyrus, the other well-characterized neurogenic region of the adult brain, implicating that regulation of neurogenesis in the dentate gyrus is substantially different from that in the SVZ. This result opens the

hypothesis that changes in the levels of sAPP could influence activity of the neurogenic regions of the adult brain in normal and pathological conditions, although very little is known about the intracellular signalling mechanisms by which sAPP triggers neural stem cell division (Conti and Cattaneo, 2005).

Aβ, another fragment of APP, also has a role in regulation of neural stem cells. Aβ has been found to trigger a Fas- and caspase independent apoptotic pathway in cultured neural precursor cells and to promoter the adherence and neuronal differentiation of neural precursor cells (Millet et al., 2005). Moreover, enhanced neurogenesis has been observed in Alzheimer's disease transgenic (PDGF-APPsw, ind) mutant mice, which overexpress the Swedish and Indiana APP mutations. In this mouse, BrdU incorporating cells and cells expressing immature neuronal marker increase in both hippocampus and subventricular zone (SVZ), two neuroproliferative regions in the adult CNS (Jin et al., 2004ab). This result is consistent with the observation that neurogenesis increases in the hippocampus of AD patients.

This evidence suggests that APP and APP related-fragments have various functions in regulation of neural precursor cells. Investigation of how they regulate neural precursor cells is important for understanding both the physiological role of APP and the pathological processes of Alzheimer's disease, since, abnormal cell cycle procession and neurogenesis and gliogenesis have been observed in AD patients (Kuhn et al., 2007).

In summary, APP plays multiple roles in the development and maintenance of the physiological function of the CNS, such as in neural progenitor cells, cell adhesion, neurite outgrowth, and axonal transport. The generation of Aβ and sAPP is always accompanied by AICD generation. There is no evidence yet as to whether or how the RIP of APP takes part in any of these functions via AICD. Thus, identification of the function and mechanism of RIP of APP is important for

understanding not only the physiological role of APP but also the pathological development and mechanism of Alzheimer's disease.

1.2 Neural stem cells

Neural stem cells are a heterogeneous population of mitotically-active and self-renewing cells of both the developing and the adult CNS, which are capable of multipotent differentiation into neurons, astrocytes and oligodendrocytes (Temple, 2001; Gage, 2000).

1.2.1 Developmental lineage of neural stem cells

Since neurogenesis and gliogenesis occur during different stages of development, it was assumed, for many years that neurons and glia in the CNS were produced from distinct precursor pools that diverged early during embryonic development. A novel hypothesis of neural stem cell lineage states that the embryonic neuroepithelial stem cell gives rise to the radial glial cells in the embryo, which act as stem cells in the fetal brain, from which the postulated astrocytic stem cell in the adult brain is derived (Alvarez-Buylla et al., 2001; Doetsch, 2003; Merkle et al., 2004).

1.2.1.1 Radial glia cells as the neural stem cells in early developmental stage

VZ Wilhelm His examined the structure of early neuroepithelium with the histological technology at the nineteenth century and found that two morphologically different cell groups existing in this region: the rounded mitotic cells near the neural tube lumen and the elongated cells. He interpreted the rounded mitotic cells as neural precursors and proposed that the elongated ones generated glial cells (His, 1889; Alvarez-Buylla et al., 2001). From this work, neuroepithelial cells in the early neural tube were believed to generate two distinct precursor pools: neural precursors and glial precursors. One century later Schaper and Sauer showed that the elongated cells and the rounded mitotic cells of the early neuroepithelium were actually the same cells at different stages of the cell cycle (Alvarez-Buylla et al., 2001). This work indicates that neuroepithelial cells may undergo a developmental lineage. More recently, the

elongated cells were believed to be radial glia, which might be neural stem cells in early developmental stage (Alvarez-Buylla et al., 2001).

Radial glial cells share several structural features with neuroepithelial cells. Both cell types have their soma in the VZ, and possess longer processes that extend towards the pial surface (Alvarez-Buylla et al., 2001). These processes have been thought to guide the neural blast cells to migrate to their final position (Rakic, 1978). In addition, both cell types express the intermediate filament nestin (Hockfield and McKay, 1985), which has also been described in cultured neural stem cells. Moreover, radial glial cells undergo mitosis and interkinetic nuclear migration in a fashion very similar to neuroepithelial stem cells (McKay, 1989). The difference between radial glial cells and neuroepithelial cells is the "glial" adjective. Radial glial cells have cellular and molecular characteristics of astroglia. Similar to astroglia, radial glial cells contain glycogen granules and other ultra structural characteristics of astrocytes, however, neuroepithelial cells do not have such characteristics. Furthermore, radial glial cells express the astrocytes-specific glutamate transporter GLAST, S100β, glutamine synthase (GS), vimentin, and tenascin-C (TN-C) and GFAP. These molecules are all absent from neuroepithelial cells (Gotz and Barde, 2005).

Radial glial cells isolated from mouse forebrain and rat neocortex can generate both neurons and glia *in vitro* (Malatesta et al., 2000). With the retrovirus labeling method, radial glial cells have been found that could proliferate and generate neurons *in vivo* (Noctor et al., 2001). Moreover, the progeny of radial glial cells have temporal and spatial specific characteristics that are the same as those of neural stem cells (Temple, 2001). Radial glial cells isolated from the early embryonic stage, such as E14~E16, have been found to generate primarily neurons and few glia. However, when radial glial cells were isolated from E18, they produced mostly glia and only a few neural colonies were observed. At the peak of neurogenesis (about E14), radial glial cells located at the dorsal

telencephalon are more neurogenic than that of ventral telencephalon (Gotz and Barde, 2005). Therefore, all of these lines of evidence indicate that radial glia might be the neural stem cells during early development.

1.2.1.2 Astrocytes as neural stem cells in the adult central nerve system

Most radial glial cells in mammalian brain disappear within days to weeks after birth. Studies of adult mouse brain suggest that neural stem cells indeed exist in the adult CNS. Cells isolated from adult brain can be self-renewing in response of epidermal growth factor (EGF) or/and basic fibroblast growth factor (bFGF) *in vitro* (Reynolds and Weiss, 1992; Morshead et al., 1994; Gage et al., 1995; Weiss et al., 1996). On withdrawal of these growth factors, the cells can differentiate into neurons, astrocytes and oligodendrocytes, suggesting that neural stem cells exist in the adult nervous system (McKay, 1997). Subventricular zone (SVZ) and hippocampus have been further identified as two major neurogenic regions in the adult CNS (Gould, 2007). However, what kind of cells are the neural stem cells in the adult brain? At the beginning, ependymal cells were considered to be the neural stem cells in adult brain, since ependymal cells can function as stem cells under specific culture conditions (Johansson et al., 1999ab). However, further studies using other culture conditions suggested that ependymal cells are not neural stem cells (Chiasson et al., 1999; Doetsch et al., 1999; Laywell et al., 2000). In addition, there is lack of convincing evidence that ependymal cells can divide *in vivo* (Doetsch et al., 1999). Recent studies identified that the neural stem cells in the adult subventricular zone (SVZ) have characteristics of fully differentiated glia. SVZ astrocytes can self-renew in presence of growth factors and differentiate into neurons *in vitro*. Ablation of GFAP-expressing astrocytes prevents neurosphere formation from SVZ tissue, suggesting that SVZ astrocytes are the primary adult stem cells (Imura et al., 2003; Morshead et al., 2003). With a virus lineage labelling method, astrocytes in SVZ were found to self-renew and differentiate into neurons in olfactory bulb (Doetsch et al., 1999). Distinct types of cells have been observed to exist in the

SVZ. The A type cells are neuroblast, which differentiate into neurons and migrate into the ofactory bulbs. The C type cells are highly proliferative cells, which express EGFR receptor on the cell surface. The B type cells are astrocytes which are believed to be the neural stem cells (Doetsch et al., 1999). After eliminating type A and type C cells in SVZ with cytosine-β-D-arabinofuranoside (Ara-C), the spared astrocytes can divide and produce new type A and type C cells, generating the new entire SVZ (Doetsch et al., 1999), further confirming that astrocytes are the neural stem cells in SVZ. Similarly, astrocytes in the dentate gyrus of the hippocampus behave as neural stem cells, which can give rise to granule neurons (Seri et al., 2001). In addition to SVZ and hippocampus, the neocortex, striatum, and amygdala but not the substantia nigra, may also have the neurogenic capacity (Gould, 2007), although it remains controversial as to whether this capacity is expressed under normal physiological conditions.

In summary, like other types of cells, neural stem cells have a developmental lineage. Neural epithelial cells are the earliest neural stem cells, which generate radial glial cells. At E12 (embryonic day), they begin to have the characteristics of radial glia. In most brain regions in the mouse, radial glial cells constitute the majority of progenitors by E13/E14, and then they remain present until the end of neurogenesis and neural migration, when their remainder transform into astrocytes (Gotz and Barde, 2005). In the adult brain, astrocytes behave like neural stem cells in adult brain, which can proliferate and generate diverse cell types (**Fig. 3**).

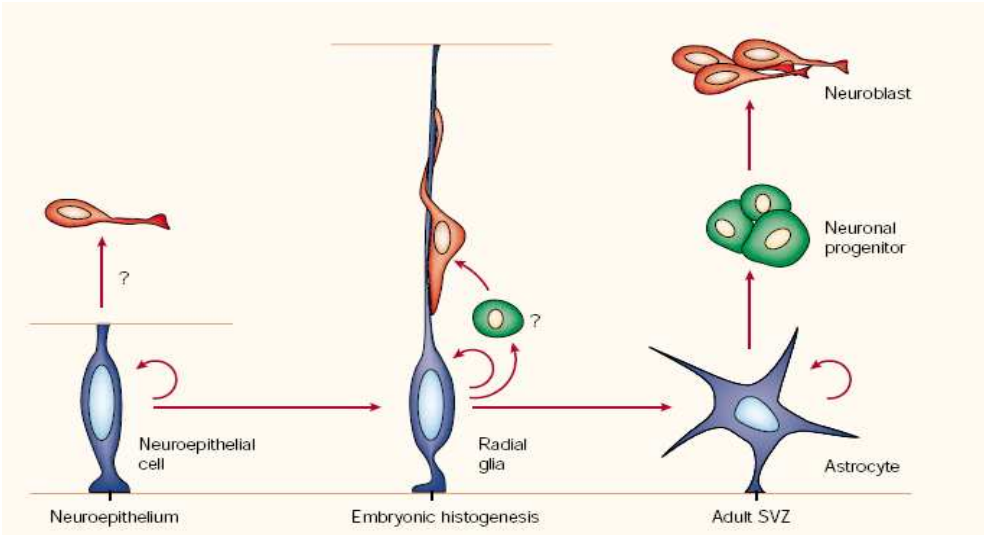

Figure 3 Unified hypothesis for neural stem-cell development (Alvarez-Buylla et al., 2001).
Left panel: Neuroepithelial cells (purple) are neural stem cells in the early stage of embryonic development. Middle panel: Radial glia might be neural stem cells. Radial glia might produce neurons directly or indirectly through transit amplifying cell types (green). The generated neurons migrate into the cortex along the fiber of their progenitor. Right panel: Astrocytes are the neural stem cells in the adult brain. They self-renew and produce neurons possibly through intermediate cell types.

1.2.2 Cell fate determination in neural stem cells

1.2.2.1 Neural stem cells acquire both temporal information and spatial information

In the CNS, different cell types arise in a precise temporal order and migrate and locate in particular regions. During development, neurons arise first, and are then followed by astrocytes and oligodendrocytes. In mouse, neurogenesis commences around embryonic 12 days (E12), peaks at E14, and finishes around E18. Astrocytes arise around only after E16 (Bayer and Altman, 1991). Most oligodendrocytes are produced after birth when the neurogenesis is already finished. The number of axons and oligodendrocytes during development also

matches, with a ratio such that one oligodendrocyte myelinates as many as 40 axonal segments (Salzer, 2003). In this way, the amount and the position of the glial cells are matched to the neuronal circuitry that is initially established. This “neuron first, glia later” cell-genesis pattern has been observed only in the vertebrates. In the lower organisms such as flies, however, both neurons and glia appear coincidentally (Bayer and Altman, 1991).

The characteristics of neural stem cells may explain such timing-controlled cell genesis in the vertebrates. Neural stem cells have temporal-restricted and spatial-restricted characteristics. The progeny generated by neural stem cells isolated at various developmental stages are different. In clonal culture systems, most of cortical cells isolated from E10 mice generate clones containing neurons only, while only 10~20% generate mixed clones containing both neurons and glial cells. There are only about 1% of clones containing only astrocytes. However, the percentage of astrocyte containing clones increases to about 20% in cells isolated from E15 mouse brain (Qian et al., 2000). Additionally, Qian et al. found that isolated neural stem cells from the cerebral cortex change their capacity to make neurons versus glia over time in a serum free culture system, suggesting that multipotent stem cells change their properties over time (Qian et al., 2000). This point has been further confirmed by *in vivo* data. Late-embryonic ferret progenitor cells cannot make cells appropriate for younger stages when transplanted into early cerebral cortex (Desai and McConnell, 2000). Neural stem cells from different regions also have different progenies. Mid/hindbrain progenitor cells are unable to generate telecephalic phenotypes after E13.5 in mouse (Olsson et al., 1997). Progenitor cells in the ventral neural tube in vertebrate embryos initially produce motor neurons and later produce oligodendrocytes, whereas dorsal progenitor cells produce interneuron and astrocytes (Bertrand et al., 2002). Neural crest stem cells from E14 embryos undergo primarily neurogenesis in gut but gliogenesis in sciatic nerves. Even

when transplanted into the same *in vivo* environment, gut and sciatic nerve neural crest stem cells adopt different cell fates (Bixby et al., 2002).

However, how are neural stem cells modulated by temporal and spatial information? All of these data suggest that intrinsic signals play an important role in modulating the temporal sequential generation of neurons and glia from neural stem cells. However, early stem cells co-cultured with early cortical slices adopt neural phenotype, whereas the same cells co-cultured with postnatal cortical sliced generate predominantly glia cell types (Morrow et al., 2001), suggesting that extrinsic environment factors regulate the cell fate of neural stem cells as well. Thus, these intrinsic and extrinsic signals co-operate during development to control cell fate determination. The same signals may promote different cell fates depending on the cellular context, and particularly the activity of other signalling pathways. Exploring how these signals co-operate and control cell fates is essential for not only understanding the developmental properties of cortex but the characteristics of neural stem cells, thereby providing theoretical support for clinical application of neural stem cells.

There are no established makers to unequivocally identify neural stem cells so far, so it is difficult to distinguish neural stem cells from neural precursor cells and neural progenitor cells, which share most characteristics of neural stem cells. In the following text, I refer to all the cells with the properties of neural stem cells as neural progenitor cells (NPCs).

1.2.2.2 Signalling pathways and factors promoting neuronal fate

The NPCs commit to their neural fate via two waves of expression of basic Helix-loop-Helix (bHLH). Neurogenesis is initiated by proneural genes, which express earlier in the ectoderm, and commit the multipotent cells to neural lineage. Neural

differentiation is further ensured by the expression of neural differentiation genes, which promote the maturation and differentiation of NPCs.

1.2.2.2.1 Basic Helix-loop-Helix

Basic helix-lop-helix (bHLH) factors are transcriptional transactivators, which have a bHLH domain, a structural motif that is responsible for their DNA binding and dimerization properties. The bHLH domains bind to DNA sequences that contain a core hexanucleotide motif, CANNTG, known as an E-box, to form bHLH heterodimers. The transcriptional activity of bHLH factors is mediated by the interaction of bHLH heterodimers with coactivators, such as P300/CBP and PCAF. Neurogenesis is mediated by two broad categories of bHLH factors at different stages: **proneural bHLH factors**, which are involved in initiating neurogenesis, and **neuronal differentiation bHLH factors**, which are involved in mediating terminal neuronal differentiation (Bertrand et al., 2002).

1.2.2.2.1.1 Proneural genes

Proneural genes initiate neurogenesis

Proneural genes, which encode the transcription factors of the bHLH class, are both necessary and sufficient, in the cortex of the ectoderm, to initiate the development of neural lineage and to promote the generation of progenitors that are committed to differentiation. Proneural proteins share several features that define them as “proneural”. **i**) All known proneural genes share similar biochemical properties with other bHLH factors. **ii**) They are expressed in the ectoderm, before any sign of neural differentiation become apparent. **iii**) They are both required and sufficient to promote the generation of neural progenitor cells from the ectoderm (Bertrand et al., 2002).

Mash1, Neurogenin1 (Ngn1) and Neurogenin1 (Ngn2), and possibly Math1 and Math5 have been established to have clear proneural activity. Three proneural bHLH transcription factors Ngn1, Ngn2 and Mash1 are expressed in the

telencephalon. During development, these proneural factors are expressed at very low levels while neural progenitors are being specified and a transient increase in their expression causes the initiation of neurogenesis (Lo et al., 1991; Guillemot and Joyner, 1993; Sommer et al., 1996). Consistently, neurogenesis defects have been observed in Mash1, Ngn1 or Ngn2 single knockout mice (Sun et al., 2001; Fode et al., 2000). In the cortical progenitor cells derived from Ngn1 null mutant mice, neurogenesis has been disrupted whereas gliogenesis has been enhanced (Sun et al., 2001; Fode et al., 2000). Ngn1 also is required for promoting generation of vestibular sensory hair cells of the inner ear (Raft et al., 2007). In addition to promoting neurogenesis of the cortex, Ngn2 also determines the neuronal fate in distal cranial ganglia (Fode et al., 1998). Ngn2 is expressed in the ventricular zone of the ventral midbrain as well, where dopaminergic neurons are generated. Analysis from Ngn null mutant mice suggests that Ngn2 is required for the differentiation of Sox2(+) ventricular zone progenitors into Nurr1(+) postmitotic dopaminergic neuron precursors in the intermediate zone, and that it is also likely to be required for their subsequent differentiation into tyrosine hydroxylase-positive dopaminergic neurons in the marginal zone (Kele et al., 2006; Andersson et al., 2006). In mash1 knockout mice, decreases in GABAergic interneurons in the ventral telecephalon, a loss of olfactory sensory neurons in olfactory epithelium, and association with a loss of progenitor population (Casarosa et al., 1999; Cau et al., 2002) has been observed. When both mash1 and Ngn2 are absent, the failure of progenitor cell specification results in dramatic reduction in the number of cortical neurons (Fode et al., 2000).

The role of proneural genes in inducing neural differentiation has further been confirmed by gain of function (GOF) analysis. Ectopic expression of Ngn genes in the surface ectoderm of *Xenopus* and zebrafish embryos, or in the neural tube of chick embryos, causes generation of supernumerary neural progenitors, driving progenitors away from division, and promoting neural differentiation

(Mizuguchi et al., 2001; Blader et al., 1997; Ma et al., 1996). Ectopic expression of mash1 in both cortical progenitors and *Xenopus* also resulted in the same phenotype, promoting neural differentiation (Farah et al., 2000; Ferreiro et al., 1994; Turner et al., 1994).

Math1 and Math5 also contain a bHLH domain. Overexpression of Xath1 induces ectopic neuronal differentiation in the non-neural ectoderm (Kim et al., 1997). Although without inducing early neural makers, and ectopic expression of Math1 in chick neural tube leads to precocious differentiation of epithelial cells (Gowan et al., 2001). Moreover, math1 can induce ectopic chrodotonal organs in *Drosophila*, and can partially rescue the proneural defects in *ato*-null files (Ben-Arie et al., 2000). Overexpression of Xath5 in *Xenopus* or Cath5 in chicken promotes RGC differentiation (Matter-Sadzinski et al., 2001). The mice lacking Math1 fail to form hair cells in the inner ear (Bermingham et al., 1999), cerebellar granule cells (Ben-Arie et al., 1997), and dorsalmost population of spinal interneurons (D1 interneurons) (Bermingham et al., 2001). Math5 is required for the differentiation of Retinal ganglion cells (RGCs). In mice or Zarafish lacking Math5, the number of RGCs decreases, while the number of cone photoreceptors increase, implying that Math5 is involved in specifying a switch in cell fate between RGCs to cones (Brown et al., 2001; Kay et al., 2001; Wang et al., 2001). Conversely, Overexpression of Xath5 in *Xenopus* or Cath5 in chicken, promotes RGC differentiation (Matter-Sadzinski et al., 2001).

Proneural genes inhibit gliogenesis

The expression of proneural genes peaks at the height of neurogenesis and subsides during astrocyte differentiation (Ma et al., 1999; Sommer et al., 1996), suggesting that proneural genes may act to suppress astrocyte formation. Agreeing with this idea, overexpression of Ngn1 blocks the formation of astrocytes (Sun et al., 2001). LOF studies further confirm the inhibition of proneural genes on astrocyte formation. In Ngn1 null mutant mice, the cortical

progenitor cells differentiate to form more astrocytes while neurogenesis has been disrupted (Sun et al., 2001; Fode et al., 2000). In Mash1/Math3 double knock-out or Mash1/Ngn2 double knock-out mice, the cells that should normally become neurons adopt the glial fate instead (Tomita et al., 2000; Nieto et al., 2001). Thus, proneural genes promote neurogenesis not only by driving progenitor cells from the cell cycle, but also by inhibiting the alternative glial cell fate. Suppression of gliogenesis by proneural genes might be independent of DNA binding. The Jak-Stat pathway is known to promote gliogenesis triggered by cytokines via a transcriptional programme induced by the Stat3/smad/CBP transcriptional coactivator complex. Ngn1 has been found to disrupt this Stat3/smad/CBP transcriptional coactivator complex. Moreover, the phosphorylation of Stat3 in response to cytokine treatment, which is required for signal transduction of Jak-Stat pathway, is blocked by Ngn1 (Sun et al., 2001). Such a repressive function of proneural genes on gliosis is consistent with the fact that no gliogenesis occurs in the neurogenic period.

1.2.2.2.1.2 bHLH differentiation genes

bHLH differentiation genes are members of the NeuroD/Nex family, including NeuroD, NeuroD (also called NeuroD-related factor, NDRF), and Nex (also called Math2) (Ross et al., 2003). Like proneural bHLH genes, bHLH differentiation genes also are E-box binding transcription activators that, when overexpressed in cultured progenitor cells, are also sufficient to induce cell cycle arrest and neuronal differentiation (Farah et al., 2000). However, there are several features that differentiate bHLH differentiation genes from proneural genes: **i**) Distinct from those proneural genes localized in the ventricular zone, where the cell population is enriched with multipotent progenitors, differentiation bHLH genes are expressed in the cortical plate (Schwab et al., 1998), by immature neurons. **ii**) Proneural genes participate in both neural and glial fate determination of neural progenitors (Nieto et al., 2001), whereas bHLH differentiation genes drive progenitors to differentiate only into neurons (Lee et al., 2000). **iii**) In mice lacking

differentiation genes, such as Nex or NeuroD2, defects in the differentiation and survival of cerebellar and hippocampal neurons have been observed, which are clearly distinct from the loss of progenitors observed in mice that lack the proneural genes Mash1 or/and Ngn (Olson et al., 2001; Schwab et al., 2000). These distinct characteristics of bHLH differentiation and proneural genes suggest that they modulate neural development sequentially in a stepwise manner over time, with the bHLH proneural genes being expressed earlier to select progenitor cells to neural specific cell fate, while bHLH differentiation genes promote neural progenitors to become mature neurons. Consistently, proneural genes, in fact, are required for the expression of the differentiation genes. When Ngnr (a *Xenopus* Ngn gene), Xath3 and NeuroD are expressed sequentially in *Xenopus,* the ectopic expression of Ngnr induces the expression of both Xath3 and NeuroD, whereas Xath3 and NeuroD can cross-active each other, but do not induce Ngnr expression, suggesting that Ngnr acts as upstream activator for the expression of NeuroD (Ma et al., 1996; Perron et al., 1999).The analysis of mouse models further confirms this idea. Ngn1 or Ngn2 is required for the expression of Math3 and neuroD in cranial sensory neurons (Fode et al., 1998; Ma et al., 1999), and mash1 acts upstream of Ngn1 and NeuroD in the olfactory epithelium (Cau et al., 2002). Thus, proneural genes initiate neurogenesis with transient expression, and then, by inducing the expression of the NeuroD family of differentiation genes, promote neurons to terminal differentiation.

1.2.2.2.1.3 Other bHLH factors modulate neurogenesis via regulating the activity of proneural bHLH proteins

Proneural bHLH factors and neuronal differentiation bHLH factors cooperate to finish the two developmental waves of neurogenesis in a sequential, stepwise manner. However, there are other bHLH factors, which are neither defined as “proneural” or “differentiational” bHLH proteins, involved in cell fate determination by regulation of the activity of proneural genes, such as Hes and Id. These

factors are categorized as repressor-type bHLH genes and activator-type bHLH genes according to their modulatory function on proneural genes.

Hes factors inhibit neurogenesis via antagonizing proneural genes

Three conserved domains are required for the function of Hes factors: the bHLH, orange and WRPW domains. Hes factors form dimmers and bind to the DNA through its bHLH domain (Sasai et al., 1992). Unlike other bHLH factors, which bind E-box (CANNTG) with a higher affinity, Hes factors have a higher binding affinity for the N-box (CACNAG) than for the E-box. The WRPW domain containing the carboxy-terminal Trp-Arg-Pro-Trp sequence is involved in the recruitment of the TLE/Groucho corepressor and required for the transcriptional repression activity (Paroush et al., 1994; Grbavec and Stifani, 1996). The orange domain of Hes factors is less conserved, and confers specificity for protein-protein interaction (Dawson et al., 1995).

There are seven members in the Hes family. Among these, Hes1, Hes3 and Hes5 are highly expressed on neural stem cells (Sasai et al., 1992; Akazawa et al., 1992; Allen and Lobe, 1999). They are expressed in the ventricular zone throughout the telencephalon, where they sustain progenitors in an undifferentiated, proliferative state and inhibit their differentiation into neurons (Nakamura et al., 2000; Hirata et al., 2000; Ishibashi et al., 1995; Ohtsuka et al., 2001). Hes1 mutant mice show premature neuronal differentiation in vivo, with a 2-fold excess of neurons in the forebrain at E13.5 (Ishibashi et al., 1995; Nakamura et al., 2000). While Overexpression of Hes1 in the developing cortex causes a perturbation of neuronal differentiation (Ishibashi et al., 1994). Accordingly, suppression of Hes1 expression in ES-derived neural stem cells with antisense oligonucleotides causes neuronal differentiation (Kabos et al., 2002). Hes5 has a similar function to Hes1 on neurogenesis. Consistently, in Hes1; Hes5 double knock-out mice, many progenitor cells are not maintained and prematurely differentiate into neurons (Ohtsuka et al., 1999; Ishibashi et al.,

1995; Cau et al., 2000; Hatakeyama et al., 2004). Neuroshpheres derived from Hes1; Hes5 double knock-out mice do not expand properly whereas the wild-type neuroshpheres do so extensively (Ohtsuka et al., 1999). This evidence indicates that both Hes1 and Hes5 are essential for maintenance and proliferation of neural stem cells and, when in absence of them, multipotent progenitor cells prematurely differentiate into neurons.

Neuroepithelial cells are the initial stem cells, which will develop into radial glial cells later. Hes1 and Hes3 expression occurs at the neuroepithelial stage. After E8.5 onward, Hes5 expression starts and Hes3 expression is down-regulated, while Hes1 expression is maintained. Despite the defects in neuronal differentiation and neurosphere maintenance observed in Hes1/Hes5 double knock-out mice, not all of the neuroepethelilal cells and radial glial cells are disrupted, suggesting that other factors, such as Hes3, may compensate for Hes1 and Hes5 deficiency. Agreeing with this notion, in Hes1;Hes3;Hes5 triple knock-out mice, many neuroepithelial cells prematurely differentiate into neurons (Hatakeyama et al., 2004), and virtually all radial glial cells prematurely differentiate into neurons by E10 at the expense of the later born cell types: later born neurons, oligodendrocytes, astrocytes and ependymal cells (Hatakeyama et al., 2004). Thus, Hes1, Hes3 and Hes5 are essential to generate cells in correct numbers and with full diversity by maintaining neural stem cells until later stages.

What are the mechanisms underlying the inhibition of neuronal differentiation by Hes factors? Up-regulation of the activator-type bHLH genes such as Mash1 and Math3 (Hatakeyama et al., 2004) has been observed in Hes-mutant mice, suggesting that the inhibition of neuronal differentiation by Hes factors may occur via depression of the proneural activity of proneural genes. Hes factors repress the activity of proneuronal genes via two distinct pathways. One is that Hes factors repress transcriptional expression of proneural genes via binding to DNA. Hes factors form homodimers and heterodimers with bHLH family members and

bind to DNA elements called N boxes (CACNAG) to repress the expression of target genes, such as mash1 (Chen et al., 1997; Davis and Turner, 2001). Transcriptional repression is mediated by the interaction of Hes proteins with transcription corepressors of the Groucho/transducin-like enhancer of split (Gro/TLE) family, via a conserved tetrapeptide motif (WRPW) (Paroush et al., 1994). The histone deacetylase activity associated with these repressor complexes modifies chromatin structure, making DNA inaccessible to transcriptional activation (Näär et al., 2001). The other way is that Hes factors antagonize the activity of proneural proteins is through interacting physically with proneural bHLH proteins. *Espl* repressors (*Drosophila* homologs of Hes factors) interact with proneural proteins and with the corepressor Groucho. This allows the recruitment of Groucho to the promotors at which proneural bHLH/E protein dimers are bound, resulting in the repression of proneural bHLH target genes (Giagtzoglou et al., 2003). Importantly, Hes factors do not require a DNA binding domain to inhibit proneural protein function, but are tethered to DNA through their ability to interact with proneural bHLH proteins.

Notably, although both Hes1 and Hes5 repress the activity of proneural genes to inhibit neurogenesis, Hes1 and Hes5 inhibit the activity of proneural genes via different mechanisms. Studies from olfactory placodes of Hes1 or Hes5, or Hes1/Hes5 double mutant mice indicate that Hes1 regulates neurogenesis by interactions at the level of Mash1, while Hes5 regulates neurogenesis by interactions at the level of Ngn1 (Cau et al., 2000).

Hes factors promote gliogenesis

Proneural genes promote neurogenesis while inhibiting gliogenesis. Hes factors seem to have the same function. In the retina, forced expression of hes1 or hes5 promotes the conversion of retinal progenitors into Müller glia (Furukawa et al., 2000; Hojo et al., 2000), whereas disruption of the Hes1 or Hes5 gene causes a decrease in the number of Müller glial cells that form (Furukawa et al., 2000;

Hojo et al., 2000). Similarly, in progenitors derived from the spinal cord, overexpression of Hes1 causes differentiation into astrocytes (Wu et al., 2003). When cortical progenitors are cultured in vitro, treatment with BMP to induce astrocyte differentiation causes a rapid increase in the expression of Hes5, suggesting that induction of Hes5 is part of the astrocyte differentiation program (Nakashima et al., 2001). However, when hes1 or hes5 was ectopically expressed early in the developing telencephalon, it failed to promote astrocytic differentiation (Ohtsuka et al., 2001). Instead, this mis-expression of Hes factors maintained cells in a precursor-like state. Thus, an increase in Hes activity prior to the neurogenic phase may maintain cortical precursors in an undifferentiated state, whereas an increase in Hes expression subsequent to the neurogenic phase may support astrocytic differentiation. The distinct functions of Hes on gliogenesis dependent on the stage of developmental may be explained by the fact that Hes factors alone are not sufficient for the regulation of astrocyte formation, but rather require to work in combination with other factors such as Stats and Samds.

Expression of Hes factors is regulated by Notch

Expression of Hes1 and Hes5 is regulated by Notch signalling (Jarriault et al., 1995; Ohtsuka et al., 1999). Notch, a transmembrane protein, is activated by the ligands Delta and Jagged. Upon activation, Notch is cleaved to release the intracellular domain (NICD), which is transferred into nucleus and forms a complex with the DNA-binding protein RBP-Jκ (Honjo, 1996; Selkoe and Kopan, 2003). RBP-Jκ itself is a transcriptional repressor and represses Hes1 and Hes5 expression by binding to their promoters. However, when RBP-j forms a complex with NICD, this complex becomes a transcriptional activator and induces Hes1 and Hes5 expression. Thus, Notch activation leads to upregulation of Hes1 and Hes5 expression. Notch inhibits neuronal differentiation and maintains neural stem cells via Hes1 and Hes5. In the absence of Hes1 and Hes5, Notch fails to inhibit neuronal differentiation (Gaiano and Fishell, 2002; Ohtsuka et al., 1999).

Consistent with the notion that Hes1 and Hes5 also promote gliogenesis in the late developmental stage, Notch is involved in astrocyte formation as well. In cultured adult hippocampal progenitors, Notch promotes astrocytic differentiation (Tanigaki et al., 2001). The intracellular domain of Notch has been shown to directly activate the GFAP promoter (Ge et al., 2002).

Thus, Notch signalling inhibits neuronal differentiation via His factors, which can repress the activity of proneural genes. However, in Ngn1, Ngn2 and mash1 single knock-out mice, failure to express Delta and Serrate/Jagged has been observed to be accompanied by deficits in neurogenesis. Delta and Serrate/Jagged are the ligands of Notch, which are transmembrane proteins expressed in the neighbouring cells. Overexpression of *Xenopus* Ngn1 can activate expression of Delta1 in *Xenopus* embryos (Ma et al., 1996), whereas lack of Dll1 expression has been found in Ngn2 mutant mice (Fode et al., 1998). The Notch ligands Dll1 and Dll3, and the target of Notch signalling Hes5, fail to be expressed in the Mash1 mutant ventral telencephalon (Casarosa et al., 1999). Thus, proneural genes can depress proneural activity in the neighbouring cells by upregulating the expression of Notch ligands and so activating Notch signalling. Activated Notch up-regulates Hes1 and Hes5 expression, which in turn, represses the activity of proneural genes. This phenomenon is called "**lateral inhibition**", by which, only parts of progenitor cell population are allowed to differentiate, while the others have to maintain in a proliferative state (Beatus and Lendahl, 1998). Lateral inhibition modulated by Notch-Hes1/Hes5 signals provides a negative-feedback loop to allow neurogenesis to persist from embryonic development to adulthood and to ensure that appropriate numbers of cortical cells are generated during development (Beatus and Lendahl, 1998).

However, Hes6, another member of the Hes family, provides a positive-feedback loop in proneural genes to promote neuronal differentiation. Hes6 expression follows that of Ngn but precedes that of neuronal differentiation genes. Several

lines of evidence have suggested that Hes6 expression is induced by proneural genes but not by Notch signalling. Ectopic expression of Hes6 in *Xenopus* embryos promotes neurogenesis (Koyano-Nakagawa et al., 2000). Recent studies have revealed that Hes6 represses transcription of the Hes5 genes and Hes1, thus functioning as a negative regulator of Notch signalling (Fior and Henrique, 2005; Bae et al., 2000; Gao et al., 2001). Conversely, Hes6 may be repressed by Hes5 activity. In cells committing to differentiation, Hes6 is up-regulated by proneural genes and then, represses Notch activity to promote neuronal differentiation in these cells. In neural progenitors, Notch signalling produces an initial burst of Hes5 activity, which in turn represses Hes6, to prevent these progenitors from differentiating. Thus, Hes5/Hes6 forms a negative feedback circuit in the Notch signalling pathway to regulate the neuronal differentiation of neural precursor cells (Fior and Henrique, 2005).

In summary, proneural genes commit neural stem cells to neural cell fate, while these differentiated neural progenitors up-regulate expression of Notch ligands, to activate Notch signalling. Active Notch signalling then in turn inhibits neural differentiation via Hes1 and Hes5, which represses the activity of proneural genes. This lateral inhibition modulated by Notch signalling can be neutralized by Hes6, which represses the activity of Hes1 and Hes5.

Ids inhibit neurogenesis by antagonizing proneural genes

Ids (inhibitors of differentiation) are members of bHLH family, although they lack the basic DNA binding region. Ids are also expressed in the ventricular zone of the telencephalon (Jen et al., 1997), where they have a similar function to the Hes factors, serving to inhibit precocious differentiation of cortical progenitors. In mice lacking both Id1 and Id3, cortical progenitors exit from the cell cycle prematurely and undergo accelerated neuronal differentiation (Lyden et al., 1999). Interestingly, forced expression of Ids in cultured cells blocks the differentiation of neurons and oligodendrocytes (Toma et al., 2000; Kondo et al., 2000; Wang et

al., 2001b). In contrast, the formation of astrocytes is not inhibited by Ids (Nakashima et al., 2001).

Id inhibits neurogenesis via repressing the activities of proneural genes. Proneural genes such as Mash, Ngn, and members of the oligo and neuroD families are required to form heterodimers with E proteins, for their DNA binding and transcription activity. Id genes have a bHLH domain, through which the Id proteins have high affinity for E proteins (Norton, 2000). Thus, Id can compete with these genes, forming heterodimers with E protein that cannot bind to DNA. Therefore, in contrast to Hes factors, which inhibit the activity of proneural genes by recruitment of corepressors, Ids inhibit the activity of proneural genes by sequestration.

1.2.2.2.2 Other transcriptional factors modulate neurognesis

In addition to bHLH, other transcriptional factors also modulate neurogenesis as an intrinsic mechanism, such as Sox family, Pax6 and REST.

Sox1-3 inhibits neural differentiation via hindering the capacity of proneural genes to induce downstream events of neuronal differentiation (Neuro family), while the expression level of proneural proteins is unaffected by Sox1-3 (Bylund et al., 2003). Proneural proteins, however, can down-regulate the expression of Sox1-3 (Bylund et al., 2003). Thus, Sox1-3 provides one control mechanism that ensures neuronal differentiation only when is sufficient expression of proneural genes. Sox21, a member of the SoxB2 group, promotes neurogenesis (Sandberg et al., 2005). Although the ability of Sox21 to promote neurogenesis is independent of the intrinsic status of proneural protein activity, the ability of proneural proteins to commit cells to neuronal differentiation seems to be based on their capability to up-regulate the level of Sox21 expression. Therefore, the balance between Sox1-3 and Sox21 determines whether neural precursors exit from cell cycle to differentiate (Sandberg et al., 2005). Whereas SoxB proteins

modulate neurogenesis, other members of Sox family, such as SoxE and SoxD family, are reported to be involved in gliogenesis (Wegner and Stolt, 2005; Stolt et al., 2006)

Pax6 is a direct activator of Ngn2 transcription in cortical progenitors (Scardigli et al., 2003). Consistently, reduction in the number of cortical neurons has been observed in the absence of Pax6 (Hack et al., 2005; Heins et al., 2002). Pax6 can also induce neurogenesis in postnatal astrocytes in a proneural gene-independent way (Heins et al., 2002).

The transcription factor REST (RE1-silencing transcription factor, also known as neuron-restrictive silencer factor NRSF), contains a DNA binding domain localized within the cluster of eight zinc fingers, as well as repressor domains at the N and C termini. REST/NRSF represses transcription of its target gene by binding to its binding site/neuron-restrictive silencer element (RE1/NRSE) that is present in the target genes' regulatory regions (Ballas et al., 2005).

1.2.2.2.3 Signalling pathways modulate neurogenesis

As mentioned, transcriptional factors play an important role in initiation and determination of neurogenesis. These transcriptional factors, such as proneural genes, inhibit gliogenesis while they promote neurogenesis. In this way, they ensure enough time for neurogenesis. The wave of expression of these transcription factors is essential for modulating neurogenesis. For example, earlier expression of proneural genes initiates neurogenesis, and further induces expression of differentiation bHLH factors which promote neural differentiation, to ensure neurogenesis is complete by production of mature neurons (Ross et al., 2003). Therefore, neurogenesis has been thought of as a default direction of cell fate determination mostly via these intrinsic factors. However, we should also notice that these intrinsic factors are modulated by extrinsic cues. Hes factors, for instance, are modulated by Notch pathway. These extrinsic cues modulate

neurogenesis, via transducing their signals through their cell surface receptor and down-stream cellular signalling elements. Modulation of cell fate by such signalling pathway provides a very good mechanism for neural progenitor cells to adopt the environment. Several signalling pathways have been identified as playing a role in modulating neurogenesis, for example, Notch signalling, Wnt and the MEK-ERK-RSK-C/EBP pathway. Among them, the Notch and Wnt signalling pathways modulate neurogenesis via regulation of the activity of proneural bHLH proteins, whereas the activity of proneural bHLH proteins is not affected by activation of the MEK-ERK-RSK-C/EBP pathway, but their activity is required for the modulation of neurogenesis by MEK-ERK-RSK-C/EBP pathway.

1.2.2.2.3.1 The Notch signalling pathway and cell fate decision

Notch is a type I transmembrane protein and can be processed by regulated intramembrane proteolysis (RIP) after ligand binding, releasing its activated intracellular domain (NICD) to interact with other downstream effectors and then regulating diverse biological functions. Constitutive expression of the intracellular domain of the Notch receptor (NICD) in the retina of *Xenopus lavis* or the developing eyes of *D. melanogaster* inhibits neuronal fates (Coffman et al., 1993; Dorsky et al., 1995; Fortini et al., 1993), suggesting that the NICD is the key effector in the Notch signalling pathway to inhibit neuronal differentiation. This idea is further confirmed in the developing mammalian cortex, where expression of a retroviral vector carrying a constitutively activated NICD at early embryonic stages (E9.5) also inhibited neuronal differentiation (Gaiano et al., 2000; Mizutani and Saito, 2005). When expressed at later embryonic stages (E14.5) or at postnatal stages, however, both dispersed and periventricular astrocytes are detected, suggesting that an initial inhibition of neural differentiation is followed by a promotion of gliogenesis (Gaiano et al., 2000; Mizutani and Saito, 2005). This idea has been further confirmed by serials of additional studies. For example, activation of Notch signalling in rat retinal progenitors inhibits the differentiation of neuronal types but promotes the expression of Müller glia

makers (Bao and Cepko, 1997; Furukawa et al., 2000). The same phenotype has been observed in zebrafish. Notch1 activation blocks neuronal differentiation, whereas it leads to excessive and premature gliogenesis (Scheer et al., 2001). Consistently, Delta1 (Dll1)-dependent notch signalling in the chick retina inhibits neuronal differentiation (Henrique et al., 1997), whereas the reduction of Notch signals promotes neuronal differentiation, while a dose-dependent increase in the number of ganglion cells generated has been observed (Austin et al., 1995). These results are consistent with the notion that Hes factors are the major downeffectors of Notch signalling pathway. Hes factors inhibit neurogenesis by repressing the activity of proneural genes while promotes gliogenesis.

However, Hes factors are not the sole effectors of Notch signals. Myelin-associated glycoprotein (MAG), one of the components of myelin, has been found to be another effector of Notch signals. After activation by F3/contactin or NB3, two novel ligands of Notch receptor, activated NICD binds to Deltex1, instead of RBP-Jκ, forming a transcriptional coactivator complex to activate the transcriptional activity of MAG. Notch promotes the formation of oligodendrocytes by this pathway (Hu et al., 2003; Cui et al., 2004). Brain Lipid Binding protein (BLBP) and the neuregulin receptor ErbB2 (Anthony et al., 2005; Gaiano et al., 2000), another two target genes of Notch signalling have been found (Anthony et al., 2005; Gaiano et al., 2000). These genes are activated by Notch through distinct pathway through CSL/CBF-1 and Deltex, respectively (Patten et al., 2006). The neuregululiln1-erB2 signalling pathway also plays an important role downstream of Notch signalling in inducing and maintaining radial glial cells, since down-regulation of neuregulin1 or erb2 results in premature transformation of radial glial cells into astrocytes (Schmid et al., 2003). Moreover, Notch has been proposed to induce gliogenesis directly through activation of the GFAP promoter by a transcriptional complex involving the Notch intracellular domain (Notch-ICD) and the DNA binding protein CSL/CBF-1 (Ge et al., 2002). CSL represses GFAP transcription in neural progenitors when bound with the co-

repressor N-CoR (Hermanson et al., 2002), whereas when N-CoR is replaced with NICD, CSL activates GFAP transcription at the onset of gliogenesis, suggesting that CSL switches from a transcriptional repressor function to that of an activator after deterring its binding from N-CoR to NICD. RBP-Jκ can bind directly to the GFAP promoter as well and promote transcription when the Jak-Stat pathway is coincidentally activated (Ge et al., 2002). When the Jak-Stat pathway is not activated, RBP-Jκ instead binds to a repressive cofactor protein, NCoR, which functions to repress gliogenesis (Hermanson et al., 2002).

In summary, Notch signals have diverse functions on the cell-fate decision during development via distinct effectors. During early stages of development, Notch inhibits neurogenesis, while in the later developmental stages it promotes the formation of astrocytes and oligodendrocytes via different down-stream signals. How do the Notch signals switch their function from neurogenesis to gliogenesis, or oligodendrogenesis? Although Notch signalling promotes gliogenesis but inhibits neurogenesis, Notch signals do not act as the switch that initiates gliogenesis while terminating neurogenesis. In post-migratory NCSCs, ligand-induced Notch activation promotes gliogenesis accompanied by an irreversible loss of the potential for neuronal differentiation (Morrison et al., 2000). In neurospheres derived from adult spinal cord, Notch activation inhibits both neuronal and glial differentiation, whereas the inactivation of the endogenous Notch signals through the expression of a dominant-negative Delta construct, which is an antagonist of the pathway, promotes neuronal fate at the expense of astrocytes (Yamamoto et al., 2001). In adult hippocampal stem cells, Notch signals repress differentiation into both neurons and oligodendrocytes, while committing the cells to an astroglial fate (Tanigaki et al., 2001). Activated Notch seems to enhance astroglial differentiation in long-term neurosphere cultures that are derived from embryonic telencephalic precursors, but seem to suppress both neuronal and astroglial differentiation in neurospheres derived from cultured neonatal forebrain cells (Hitoshi et al., 2002). These experiments have shown

that Notch controls cell fate in these cells in a stepwise manner, first by promoting glia at the expense of neurons, and then by promoting astrocytes, while repressing oligodendocyte differentiation (Grandbarbe et al., 2003). Therefore, Notch signals could be regulated by different controlling mechanisms depending on the developmental cortex. Notch signals can be modulated either by the quantity of the receptors or the ligands on the cell surface or via the down-stream signals at the transcriptional level (Mukherjee et al., 2005). Consistently, neurospheres derived from Dll1-knockout mice are biased towards neuronal differentiation at the expense of both astrocytes and oligodendrocytes (Grandbarbe et al., 2003). The generation of neurospheres derived from the brains of RBPjk-knockout mice is compromised due to the near absence of neural stem cells, as has likewise been observed in neurospheres from Notch1 knock-out mice (Hitoshi et al., 2002).

1.2.2.2.3.2 The Wnt signalling pathway

NPCs determine their differentiational fate upon the cellular cortex through Wnt signalling pathway as well. Wnt proteins signals through a receptor complex composed of members of the Frizzled (Fz) and low-denstity lipoprotein receptor-related protein (Lrp) families, and activated a number of intracellular signalling pathways including the β-catenin/TCF pathway (Brantjes et al., 2002; Wodarz and Nusse, 1998). Recent studies reveal that the Wnt/β-catenin signalling pathway inhibits neuronal differentiation. Overexpression of Wnt7A or of a stabilized form of β-catenin then leads to cell cycle arrest and neuronal differentiation of cortical progenitors, while expression of Wnt signalling inhibitors, such as Axin and Dkk1, results conversely in an inhibition of neuronal differentiation (Hirabayashi et al., 2004). Induced expression of the proneural bHLH genes, Ngn1 and Ngn2, seems to be the mechanism underlying the enhanced neurogenesis by Wnt signalling. The promoters of the proneural proteins Ngn1 and Ngn2 can be directly activated by a β-catenin/TCf complex in transfected cells (Hirabayashi et al., 2004; Israsena et al., 2004), and,

consistently, Ngn2 expression is reduced in mice with a cortical-specific deletion of β-catenin (Machon et al., 2003). Wnt signalling is also required for neurogenesis in the adult hippocampus (Lie et al., 2005; Zhou et al., 2004). Notably, the role of Wnt signalling in promoting neurogenesis is restricted in the later cortical progenitors, and expression of stabilized β-catenin at early stages of development (E10.5) actually inhibits neuronal differentiation (Hirabayahshi and Gotoh, 2005).

1.2.2.2.3.3 The MEK-ERK-RSK-C/EBP pathway

In addition to bHLH, C/EBPs also play an important role in neurogenesis. C/EBPs are one family of transcription factors, composed of basic leucine zipper DNA binding proteins (C/EBPα, β, γ, δ, ε, ζ), that recognize a common DNA binding sequence (Williams et al., 1991). Phosphorylated C/EBP, can bind to the promotor of α-tubulin or math2, activating expression of these proneural genes (Ménard et al., 2002; Uittenbogaard et al., 2007). The phosphorylation of C/EBP is activated by a serial signalling cascade. Activated MEK/ERK causes activation of Rsk, and then Rsk phosphorylates C/EBP at the Thr 217 site. The C/EBP also can be directly phosphorylated by active ERK (Williams et al., 1995). Some growth factors such as FGF and PDGF enhance neurogenesis via activation of the MEK-C/EBP pathway. However, C/EBPs act only as differentiation factors responsible for initiating the transcription of early neural genes, rather than as key “determinination” factors in the same sense as bHLHs to determine the cell fate of cortical progenitors. Expression of appropriate bHLHs is a requisite for the neurogenic action of C/EBPs. C/EBPs have to collaborate with bHLHs to function as growth factors-regulating differentiation signals in cortical progenitor cells (Ménard et al., 2002). The collaboration between C/EBPs and bHLHs possibly occurs via P300/CBP, which are transcriptional coactivators involved in multiple signalling pathways. P300/CBP bind to neurogenic bHLHs such as neurogenin as well as to C/EBPs, thereby potentially provided a link between bHLHs and activated C/EBPs during neurogenesis (Ménard et al., 2002).

1.2.2.3 Signalling pathways and factors modulating gliogenesis

As mentioned, proneural genes and their downstream bHLH downstream genes determine the neural fate of multipotent NPCs. Neurogenesis can be modulated by signalling pathways such as Notch, Wnt and MEK-ERK-RSK-C/EBP signalling pathways. These signalling pathways provided a context-dependent and temporal-dependent mechanism to ensure the precise timing of neurogenesis occurs and the number of generated neurons during developmental cortex. Gliogenesis starts from E16, when neurogenesis has almost finishes (Bayer and Altman, 1991). The question is why there is no gliogenesis in the neurogenic period and how the gliogenesis is triggered when neurogenesis almost finishes. Neurogenic progenitor cells become gliogenic if placed in a postnatal cortical environment (Morrow et al., 2001), suggesting that gliogenesis is triggered mostly by extrinsic cues. Recent studies reveal that some cytokines such as Cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF), and leukemia inhibitor factor (LIF), secreted from neurons, can trigger the gliogenesis (Bonni et al., 1997; Johe et al., 1996; Nakashima et al., 1999b; Barnabé-Heider et al., 2005). The Jak-Stat pathway, which can be activated by cytokines, plays a pivotal role in triggering gliogenesis. Cytokines induce heterodimerization of β-subunits of the co-receptors LIFRβ and gp130, by which Jaks are activated. Activated Jaks further phosphorylate Stats. Upon phosphorylation, Stats form dimers and translocate into the nucleus, where they act as transcriptional factors, via interacting with P300/CBP, activating of the transcription of GFAP and S100β, two astrocyte genes (Bonni et al., 1997; Nakashima et al., 1999ab; Stahl and Yancopoulos, 1994). Consistently, a profound deficit in astrocyte formation has been observed in mice lacking either LIFRβ or gp130. Neural precursors derived from gp130 knock-out or LIFRβ knock-out mice are deficient in astrocyte formation (Koblar et al., 1998; Nakashima et al., 1999a). An acute knockdown of gp130 in cortical precursors caused a decrease in the number of precursors that generated early astrocytes *in vitro*, and a decrease in astrocyte formation *in vivo*

(Barnabé-Heider et al., 2005). Similarly, inhibition of Stat signalling abolishes the ability of cytokines to induce astrocyte formation both *in vitro* (Bonni et al., 1997; Rajan and McKay, 1998) and *in vivo* (Barnabé-Heider et al., 2005).

Notch signalling pathway, as mentioned, induces gliogenesis. However, the induction of gliogenesis by the notch signalling pathway only occurs at the later stage of embryonic development (Ohtsuka et al., 2001). And RBP-Jκrepresses gliogenesis when Jak-Stat pathway is activated (Hermanson et al., 2002). Thus, it seems that Jak-Stat pathway plays a key role in gliogenesis. Another example is nuclear factor-1 (NF1) family, also known as CTF or CAAT box, transcription factors, which are composed of four members in vertebrates (NF1-A, -B, -C, and X) (Gronostajski, 2000). A NF1-binding site has been detected in the GFAP promoter (Krohn et al., 1999; Gopalan et al., 2006; Cebolla and Vallejo, 2006). Consistently, a promotive function of NF1A/B on gliogenesis has been observed (Shu et al., 2003; Steele-Perkins et al., 2005; Deneen et al., 2006). However, NF1 is expressed by neural precursor cells immediately prior to gliogenesis, suggesting that it may induce gliogenesis via collaboration with cytokine-induced pathways. EGF and FGF2, two growth factors, are shown to promote gliogenesis as well. Similarly, both of them promote gliogenesis in a cytokine-dependent manner (Song and Ghosh, 2004).

1.2.2.3.1 Mechanism of competition between neurogenisis and gliogenesis

Extrinsic cues are very important for inducing the gliogenic switch. However, expression of some cytokines, such as neuropoietin and cardiotrophin-like cytokine, occurs during the neurogenic period but still fails to induce gliogeneis (Derouet et al., 2004; Uemura et al., 2002). This indicates that the responsiveness of cortical precursors is different at distinct developmental stages. During the neurogenic period, the gliogenic competence of the neural precursors is silent (Miller and Gauthier, 2007). There are several mechanisms underlying the silencing of gliogeneic competence during the neurogenic period. As

mentioned above, the JAK-STAT pathway plays an essential role in cytokine-induced gliogenesis. Binding to p300/CBP is required for the transcriptional activity of STATs on gliogenic genes (Bonni et al., 1997). P300/CBP acts as a coactivator in multiple signalling pathways. During the neurogenic period, Ngn binds and sequesters p300/CBP from interaction with Stats, thereby inhibiting gliogenic transcription (Sun et al., 2001). Such a repressive mechanism on gliogenesis during the neurogenic period provides a good explanation as to why no gliogenesis happens during the neurogenic period even when there are also some cytokines expressed. This mechanism has been further confirmed in BMP-induced cell genesis. In contrast to the cytokines that induce gliogenesis, BMPs have dual effects on neural cell genesis. For example, BMP2 instructively promotes gliogenesis during the gliogenic period, but enhances neurogenesis of cortical precursors during the neurogenic period (Li et al., 1998; Mabie et al., 1999; Gross et al., 1996; Gomes et al., 2003). BMPs are reported to bind to their heterotrimeric serine/theronine kinase receptors to signal largely via activation of the downstream transcription factors, Smads 1,5 and 8 (Chen et al., 2004). Activated Smads can form a complex with either p300/CBP:Stat or p300/CBP:Ngn (Nakashima et al., 1999b). As mentioned, Ngn and Stats bind to P300/CBP in a compatible manner, by which neurogenesis or gliogenesis are promoted individually (Sun et al., 2001). However, binding of Smad1 to p300/CBP is independent of interaction between p300/CBP and the Stats and ngn1, providing a potential molecular explanation for the dual actions of BMP2. In this model, during the gliogenic period, when ngn1 levels are very low, exposure to BMP2 and gliogenic cytokines causes formation of a Smad:p300/CBP:Stat complex that transactivates gliogenic genes. Under these conditions, BMPs also cause expression of inhibitory HLHs such as Id1, which can antagonize any neurogenic genes in the same precursor, thereby ensuring the precursors make glia and not neurons (Nakashima et al., 2001). In contrast, during the neurogenic period, precursors expressed high levels of bHLH genes like Ngn1, and BMP2 exposure causes formation of a Smad: p300/CBP:Ngn1 complex that inhibits

gliogenesis by sequestering p300/CBP from interaction with the Stats, and that can participate in transactivation of neuronal genes (Sun et al., 2001). Therefore, p300/CBP can bridge multiple pathways to control the switch between neurogenesis and gliogenesis during development.

Acitivation of a MEK-C/EBP pathway enhances neurogenesis in the presence of FGF and PDGF, and inhibits gliogenesis induced by CNTF, thereby provides a mechanism whereby growth factors can selectively bias progenitors to become neurons during development (Ménard et al., 2002). SHP2, a growth factor regulated phosphatase (Neel BG et al., 2003), is reported to play an important role in such processions of cell fate decision in neural precursors. SHP2 instructs neural precursors to become neurons but not astrocytes. Knockdown of SHP2 leads to a delay of neurogenesis and an enhancement of gliogenesis (Gauthier et al., 2007). SHP2 is an integral upstream component of the RTK-MEK-ERK-C/EBP pathway, which is recruited to many RTKs upon activation and is essential for sustained MEK-ERK activation (Neel et al., 2003). SHP2 is also recruited to the activated gp130 receptor and negatively modulates the gp130-Jak-Stat pathway (Lehmann et al., 2003; Ernst and Jenkins, 2004). Consistently, SHP2 instructs precursors to adopt neurogenic fate rather than gliogenic fate via activating the neurogenic RTK-MEK-ERK-C/EBP pathway, while inhibiting the gliogenic gp130-Jak-Stat pathway, thereby ensuring that recursors that are biased to a neural fate do not attempt to become a glia even in the presence of cytokines (Gauthier et al., 2007).

1.2.2.3.2 Other mechanisms underlying repression of gliogenesis

DNA methylation

In addition to the competive mechanism between the gliogenic pathway and the neurogenic pathway, DNA methylation and/or chromatin modifications have been revealed to be involved in repression of gliogenesis. The STAT3 binding site in the GFAP promoter is preferentially methylated in neurogenic versus gliogenic

cortical precursors and, this methylation inhibits STAT3 association and gfap transcription (Takizawa et al., 2001). S100β genes are also methylated in early cortical precursors (Namihira et al., 2004). When DNA methyltransferase 1 (dnmt1) is conditionally knocked-out in neural precursors, decreased numbers of neurons, precocious astrogenesis, and an aberrant up-regulation of gfap and s100β genes have been observed (Fan et al., 2005). Interestingly, genes in the Jak-stat pathway are also depressed in dnmt1 knock-out precursors, and STAT3 inhibition abolishes the observed increase in gliogenesis (Fan et al., 2005). A subsequent study provided further support for the idea that methylation of genes in the Jak-Stat pathway regulates the gliogenic potential of cortical precursors and demonstrates that cytokines themselves depressed the pathway, thereby defining a positive feedforwad loop (He et al., 2005). Thus, in early neural precursors, DNA methylation represses genes encoding astrocyte-specific genes and the gp130-Jak-Stat pathway, and this repression is lifted as precursors develop.

Transcriptional repression

N-coR, a corepressor for multiple transcription factors, acts by forming a complex with histone deacetylases and is involved in the repression of gliogenesis during the neurogenic period. The cortical precursors derived from N-CoR knock-out mice have been observed to fail to self-renew, but instead to differentiate into astrocytes. Consistently, premature of astrocyte formation and gliosis has been shown in the N-CoR knock-out forebrain (Hermanson et al., 2002). These observations indicate a repressive role of N-CoR on gliogenesis. Moreover, the repressive function of N-CoR on gliogenesis is dependent on Notch signalling. Binding of N-coR to CSL or RBP-Jκ is required for its repressive activity on promoters of gliogenic genes (Hermanson et al., 2002; Ge et al., 2002). Recent studies reveal that this repressive action of N-CoR on gliogenesis is regulated by environmental signals. A complex of EICD:TAB2:N-CoR:RBP-Jκ forms with the activation of Neuregulin-ErbB signalling. Binding of neuregulin to its single

transmembrane receptor ErB4 leads to cleavage and release of the receptor intracellular domain, EICD, which forms a complex with TAB2 and N-CoR. This complex then translocates into nucleus, where it represses the transcriptional activity of gliogeneic genes together with RBP-Jκ (Sardi et al., 2006). However, the nuclear location of N-CoR can be disrupted by cytokines such as CNTF, which translocate N-CoR to the cytoplasm (Hermanson et al., 2002). Therefore, this evidence suggests another model where, during neurogenic period a compelx of EICD:TAB2:N-CoR: RBP-Jκ represses gliogenesis by directly binding to the GFAP promoter in the nuclei. However, when sufficient numbers of neurons generate sufficient amount of cytokines, this pathway is inhibited by translocating N-CoR to the cytoplasm. At same time, cytokines activate the Jak-Stat pathway to promote gliogenesis.

TLX, an orphan nuclear receptor, is found to bind to the promoter of GFAP gene, and repress the transcription activation of GFAP induced by LIF. The number of proliferative cells decreases, whereas the astrocytes increase in the DG and SVZ from TLX null mice (Shi et al., 2004).

Therefore, both intrinsic facors and extrinsic cues control the property of NPCs. Neurogenesis is initited by expression of proneural genes, and further promoted by differentiation to ensure the terminal differentiation. Gliogensis is triggered mostly by extrinsic cues, while it has a silenced repressive mechanism. Such repressive mechanism ensures the neurogenic property of NPCs in the competition between neurogenesis and gliogenesis during the neurogenic period. Signalling pathways can be regulated through their ligands and intracellular downstream signalling. Thus, signalling pathways provide a good mechanism to ensure the precise time, position and extent of cell genesis occurs. As mentioned in this chapter, various signalling pathways are co-ordinated together to control the property of NPCs. Neural stem cell therapy has become a new technique in clinics. So understanding the mechanism underlying control and regulation of

NPCs is not only important for developmental study, but for the clinical application.

2. The aim of study

Amyloid Precursor Protein (APP) is the precursor protein of β-amyloid (Aβ), which forms deposition that is considered to be a marker of the pathology of Alzheimer's disease (AD) (Suh and Checler, 1997). Regulated intramembrane proteolysis (RIP) is involved in signalling transduction of various transmembrane proteins (Brown et al., 2000). Notch signalling pathway, for example, is of one of those underlying regulation by RIP. On binding of the classic ligands, such as Delta, Serrate, and Lag-2 (collectively called DSL), to its EGF repeats (Rebay et al., 1991), Notch's core signalling mechanism involves release of its intracellular domain (NICD) through RIP. NICD then translocates to the nucleus and interacts with CSL (CBF-1, Suppressor of Hairless, and Lag-1) transcription factors to activate target genes, such as HES (Weinmaster, 2000). Similar to the Notch receptor, APP is a type I membrane protein with a large extracellular domain, a single transmembrane region, and a short cytoplasmic tail (Selkoe and Kopan, 2003). APP is suggested for a long time as a receptor molecule, which is supposed to be processed by RIP. Indeed, APP can be cleaved by α- and β-secretases, and releases its intracellular domain (AICD), a 47 amino-acid polypeptide by a γ-secretase cleavage mechanism within the lipid bilayer in a presenilin-dependent manner (Selkoe and Kopan, 2003). The regulation of AICD release is thought to be triggered by extracellular or intracellular cues (Mönning et al., 1995; Kopan and Ilagan, 2004). However, despite years of studies, these cues had not yet been identified.

The F3/contactin family consists of F3/contactin (Gennarini et al., 1989), NB-2 (Ogawa et al., 1996), NB-3 (Ogawa et al., 1996), TAG-1 (Furley et al., 1990), Big-1 (Yoshihara et al., 1994) and Big-2 (Yoshihara et al., 1995). Each molecule has six Ig domains and four fibronectin type III repeats in the extracellular portion and is anchored to the cell membrane via a glycosylphosphatidylinositol (GPI) linker at the COOH terminal (Karagogeos, 2003). F3/contactin and NB3 are the ligands of

Notch receptor, which trigger the RIP of Notch, releasing NICD from the memberane. NICD is translocated into the nucleus and transcriptional activates expression of myelin associated glyprorein (MAG) via Deltex1 to promote maturation and differentiation of oligodendrocytes (Hu et al., 2003; Cui et al., 2004). In this study, we have identified TAG-1, another member of the F3/Contactin family, as a ligand of APP that can trigger RIP and release AICD.

APP is best known for its involvement in the pathogenesis of Alzheimer's disease (Selkoe, 2004). APP generates AICD after proteotic cleavage by serials secretases in addition to Aβ. Deposition of Aβ is associated with other pathological markers of AD such as cell death in the central nervous system (CNS), accumulation of amyloid plaques, and the appearance of neurofibrillary tangles (Suh and Checler, 2002). Reduction or prevention of Aβ deposition is widely believed to be desirable for slowing the progress of, or preventing the development of AD. However, recent studies reveal that APP also plays important physiological functions during development. For example, APP promotes neurite outgrowth of neurons and axonal transportation in the sciatic nerve (Buxbaum et al., 1998a; Gunawardena and Goldstein, 2001). sAPP regulates proliferation of neural progenitors in the adult subventricular zone (SVZ) (Caillé et al., 2004). This study raises the possibility that the APP may regulate the differentiation of neural progenitor cell (NPCs). The fact that expression of APP starts as early as embryonic day (E) 8 (Fisher et al., 1991), further suggests the possible role of APP in embryonic NPCs. Thus we have further investigated the function of APP and its RIP in NPCs.

We have identified a novel signalling pathway, TAG-1/APP/AICD, which inhibits neurogenesis in a Fe65-depdendent manner during development.

3 Materials and Methods

3.1 Materials

3.1.1 Antibodies

3.1.1.1 Primary antibodies

Antibody	Abbreviation	Clone	Producer
Anti-APP	22C11	Mouse, monoclonal; clone:22C11	Millipore (MAB348)
	C7	Rabbit, polyclonal; Affinity purified	Prof. Selkoe
	171610	Rabbit, polyclonal; Affinity purified	Calbiochem (171610)
	A8717	Rabbit, polyclonal; Affinity purified	Sigma-Aldrich (A8717)
	MAB343	Mouse, monoclonal	Millipore (MAB343)
Anti-phoAPP(Thr668)	phoAPP	Rabbit, polyclonal	Cell signaling
Anti-TAG-1	TG1	Rabbit, polyclonal	Prof. Kazutaka Watanabe
	TG3	Rabbit, polyclonal	Prof. Domna Karagogeos
	1C12	Mouse, monoclonal	Prof. Andrew J. Furley
	4D7	Mouse, monoclonal	Prof. Andrew J. Furley
	Goat TAG-1	Goat, polyclonal	Santa Cruz Biotechnology (sc-13684)
Anti-F3	F3	Rabbit, polyclonal	Prof. Melitta Schachner
Anti-APLP1	APLP1	Rabbit, polyclonal	Calbiochem (171615)

Anti-APLP2	APLP2	Rabbit, polyclonal	Calbiochem (171616)
Anti-Fe65	Fe65 (3H6)	Mouse, monoclonal; Clone: 3H6	Millipore (05-758)
	Fe65	Goat, polyclonal	Santa Cruz Biotechnology (sc-19751)
Anti-Nestin	Nestin	Mouse, monoclonal; Clone: Rat 401	Millipore (05-758)
Anti-Sox2	Sox2	Rabbit, polyclonal	Millipore (AB5603)
Anti-MAP(2a+2b)	MAP2	Mouse, monoclonal; Clone: AP-20	Sigma-Aldrich (M1406)
Anti- βIII tubulin	TUJ1	Mouse, monoclonal; Clone: AP-20	Sigma-Aldrich (T5076)
Anti-Gal4	Gal4(630403)	Mouse, monoclonal	Zymed (630403)
Anti-c-myc (9E10)	myc	Mouse, monoclonal	Santa Cruz Biotechnology (sc-40)
anti-V5	V5	Mouse, monoclonal	Invetrogen (R960-25)
Anti-activated Notch1	Notch1	Rabbit, polyclonal	Abcam (ab8925)
Anti- Notch2	Notch2	Rabbit, polyclonal	Abcam (ab8926)
Anti-Hes1	Hes1	Rabbit, polyclonal	Millipore (AB5702)
Anti-Hes5	Hes5	Rabbit, polyclonal	Abcam (ab25374)
anti-γ-tubulin	Tub	Mouse, monoclonal; clone: GTU-88	Sigma-Aldrich (T6557)

3.1.1.2 Secondary Antibodies

All HRP-coupled secondary antibodies were purchased from Amersham and were used a dilution of 1:5000 for western blotting. All Fluro488 or 555 coupled secondary antibodies were purchased from Invetrogen and were used at a dilution of 1:200.

3.1.2 Plasmids

1. pCMV-LacZ : Transfection control plasmid encoding bacterial β-galactosidase under control of the CMV promoter (Cao and T. Südhof, 2001;2004). Provided by Prof. T. Südhof.

2. pG5E1B-luc: Gal4 reporter reporter plasmid (Cao and T. Südhof, 2001;2004). Provided by Prof. T. Südhof.

3. pMst: Gal4 expression vector driven by the SV40 promoter derived from pM (Clontech) by mutating the stop codon before the Gal4 DNA binding domain (Cao and T. Südhof, 2001;2004). Provided by Prof. T. Südhof.

4. pMst-APP: encodes APP-Gal4 (Cao and T. Südhof, 2001;2004). Provided by Prof. T. Südhof.

5. pMst-APP*: encodes APP*-Gal4 (Cao and T. Südhof, 2001;2004). Provided by Prof. T. Südhof.

6. pCMV-Fe65: encodes full-length rat Fe65 (711 residues) (Cao and T. Südhof, 2001;2004). Provided by Prof. T. Südhof.

7. pCMV-BACE1: encode human BACE1 (Ho and T. Südhof, 2004). Provided by Prof. T. Südhof.

8. pCMV-PS1: encode human PS1. Provided by Prof. T. Südhof.

9. pRC-TAG-1: encodes full-length TAG-1. Cloned by Dr. Zhi-cheng Xiao.

10. pCDF1-AICD59: encoding mouse AICD59, Subcloned by Dr.Wu-lin Yang

11. pCDF1-C99-Gal4: encoding mouse C99-Gal4, Subcloned by Dr.Wu-lin Yang
12. pCDF1-APPV5: encodes APP-V5, V5 is fused at the C-termial of APP695. Subcloned by Dr.Wu-lin Yang

3.1.3 Cell lines

Cell Lines	Provider	Producer
CHOTAG-1	CHO cells stably express mouse TAG-1	Prof. Karagogeos
CHOAPP	CHO cells stably express human APP695	Prof. Schachner
CHOL1	CHO cells stably express human L1	Prof. Schachner
CHOAPP-Fc	CHO cells stably express the extracellular domain of mouse APP695	Prof. Schachner
CHOTAG-1-Fc	CHO cells stably express the extracellular domain of mouse TAG-1	Dr. Zhi-cheng Xiao

3.1.4 Proteins

Proteins	Producer
Recombinant human TAG-1 protein	R&D system
Recombinant human F3 protein	R&D system
TAG-1-Fc	Purified from CHOTAG-1-Fc cells
F3-Fc	Purified from CHOF3-Fc cells
L1-Fc	From Prof. Schachner
APP-Fc	From Prof. Schachner
TAXIg-GST	From Prof. Karagogeos
TAXFNIII-GST	From Prof. Karagogeos

3.1.5 Solution

Common Solutions and Reagents	
PBS	Merck
TBS	Merck
TAE	Merck

RIPA buffer	Santa Cruz Biotechnology
DNA ladder	iDNA
Protein ladder	Bio-Rad
Laminin Sample Buffer	Bio-Rad
Trincine Smaple Buffer	Bio-Rad
SDS-running buffer	Merck
Transfer Buffer	25 mM Tris base; 0.2 M Glycine; 20% Methanol (Merck)
Protease inhibitor cocktail	Roche

3.1.6 Culture Medium

Culture Medium	**Reagents**	
NPC Culture Medium	DMEM (Gibco)	1 X
	N2	1 X
	bFGF	20ng/ml
	EGF	20ng/ml
NPC Differentiation Medium	DMEM (Gibco)	1 X
	N2	1 X
	FCS	0.5%
Cell Line Culture Medium	DMEM (high sucrose)	1 X
	FBS	10%
	Penicillin	100U/ml

3.2 Methods

3.2.1 Molecular Biology

3.2.1.1 Construct APP-Fc fusion protein

Mouse APP 695 cDNA encoding for the neuronal isoform of APP (from Dr S. Sisodia) was subcloned into the pblue Bac vector using the BamHI and SacI restriction sites. To generate the fusion protein containing the extracellular domain of APP with the Fc part of human immunoglobulin G at its COOH-terminal end (APP-Fc), primers for the SacI restriction site at the 5´end (CTGACGGAACCAAGACCACCG) and for the COOH terminal end of the APP extracellular domain (terminating at amino acid position 624; SWISS-Prot accession number P12023) at the 3'end (GCTGAAGATGTGGGTTCGAACAAA) were used, introducing a new BclI restriction site at the 3´end.

3.2.1.2 Construct TAG-1-Fc fusion protein

A soluble form of TAG1-Fc recombinant protein was produced in 293T cells. The signal sequence of the GPI-anchor of mouse TAG1 was substituted with human IgG-Fc followed by a termination codon. The recombinant cDNA was inserted at the Hind III-Not I sites of pDX, a modified pcDNA3 vector with an amplification-promoting sequence (APS) (Hemann et al., 1994) upstream of the CMV site. The vector was introduced into 293T cells.

3.2.1.3 Construct F3-Fc fusion protein

A soluble form of F3-Fc fusion protein was produced in Ltk-/- cells. The region encoding the signal sequence mouse F3 GPI-anchor was substituted with human IgG Fc followed by a termination codon. The recombinant F3-Fc cDNA was inserted at the Hind III-Not I sites of pDX, a modified pcDNA3 vector with an amplification-promoting sequence (APS) (Hemann et al., 1994) upstream of the CMV site.

3.2.1.4 Production of the TAX-GST fusion protein

The sequences for the Ig and FNIII domains of TAG1 were inserted into the pGEX-KG vector to produce the GST-tagged fusion proteins. The recombinant vectors were introduced into the TOP10 strain of *E. coli*, which was subsequently

induced by IPTG (Bio-Rad Laboratories, Hercules, CA, USA). The recombinant GST fusion proteins were purified by using GST beads (Sigma-Aldrich) as instructed by the manufacturer.

3.2.1.5 Cloning of AICD59 and C99

The fragment of AICDC59 was obtained by PCR amplification of the indicated coding sequence of human APP cDNA using the primers: 5′-GGCGTCTAGAGCCACC ATGATAGCGACAGTGATCGTCATCACC-3′ and 5′-GGCGGCGGCCGCCTA GTTCTGCATCTGCTCAAAGA-3. The initial methionine (underlined) was artificially introduced. The product was digested with XbaI and NotI and subcloned into the XbaI-NotI site of pCDF1-MCS1-EF1-copGFP (System Biosciences, Mountain View, CA, USA). The construct was confirmed by sequencing and Western blotting. The fragment of Gal4-C99 was obtained by PCR amplification from plasmid pMstAPP (Gal4) using the primers: 5'-GCGC TCTAGA GCCACC ATGGATGCAGAATTCCGACATG -3' and 5′-GGCGGCGGCCGCCTA GTTCTGCATCTGCTCAAAGA-3. The product was digested with XbaI and NotI and subcloned into the XbaI-NotI site of pCDF-MCS1-EF1-copGFP. The construct was confirmed by sequencing.

3.2.1.6 Cell lines Culture

CHO cells and MEF cells were maintained under the cell line culture medium. Cells were digested with 0.25% trypsin-EDTA (Gibco) after becoming confluent, and passaged.

3.2.1.7 Culture of neural progenitor cells

Telencephalic lateral ventricle walls isolated from E14 embryos were dissociated. Isolated tissues were digested with 0.05% trysin-EDTA for 20 mins. Cells were collected and resuspended in NPC culture medium. Resuspended cells were seeded into 12-well or 24-well dish and cultured in NPC culture medium. Neurosphere were formed after 5~7 days culturing.

3.2.1.8 NPC differentiation assay

Second passage neurospheres were collected and dissociated into single cells. The cell suspension was seeded into 24-well dishes with 20,000 cells per well for 24hrs recovery. Cells were changed into NPC differentiation medium next day and cultured for 7~8 days. Cells were further fixed and immunocytochemistry was performed.

3.2.1.9 Immunocytochemistry

Cells were fixed with 4% paraformadehyde (PF) for 15~20 mins at room temperature. After brief washing with PBS, cells were permeablized with PBS containining 0.01% TritonX100 for 5 mins. After another wash with PBS, cells were blocked with 10% normal goal serum (NGS) in PBS, further stained with primary antibodies, followed with secondary antibodies. After drying, coverslips were mounted with mounting medium containing DAPI (Dako).

3.2.1.10 Immunohistochemistry

Embyronic brain were dissociated and fixed with 4% PF for overnight at 4 ◦C. After premealization for 10 mins with PBS containing 0.3% TritonX100, Tissues were washed, blocked and stained with primary antibodies, followed with secondary antibodies. After drying, tissues were mounted with mounting medium.

3.2.1.11 TUNEL assay

TUNEL assay was performed according to the manufacturer's protocol (Chemicon).

3.2.1.12 Transfection

Transient transfection of cell lines was performed using the Effectene Kit (Qiagen).

Transient transfection of NPCs was performed by using the Nucleofeto II Kit (Amaxa Biosystem). Neurospheres were collected and dissociated to single cells mechanically. After centrifuge, cells were resuspended into 100ul nucleofector solution in a density of 0.5~1.0X10^6/100ul, mixed with 2ug DNA and performed electric transfection. Transfected cells were diluted in NPC culture solution and seeded into 4-well dish.

3.2.1.13 Cell adhesion assay

CHO cells were stably transfected with pcDNA 3.1(-) containing the mouse cDNA sequence of APP 695. APP-, F3-, TAX-, TAG1- and mock-transfected CHO cells were cultured in DMEM containing 10% fetal calf serum. 35mm tissue culture petri dishes (Becton Dickinson, Franklin Lakes, NJ USA) were coated with methanol-solubilized nitrocellulose and then with proteins (12 μM) for 2 hrs at 37oC in a humidified atmosphere. Subsequently, the dishes were washed and blocked overnight with 2% heat-inactivated fatty acid-free BSA (Sigma-Aldrich). After rinsing the dishes, cells (TAG1-transfected CHO or APP-transfected CHO) were plated in 2 ml of DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum at a density of 1.5 x 106cells/ml. At 0.5 hr (in the adhesion test), the cells were gently washed and fixed with 2.5% glutaraldehyde and stained with 0.5% toluidine blue (Sigma-Aldrich) in 2.5% sodium carbonate. Blockage of adhesion was carried out using polyclonal anti-TAG1 (1:100) or anti-APP (1:100) antibodies for 0.5 h pre-incubation. Cells adhering to the various spots were photographed and counted. The results were analyzed by Newman-Keuls test with $p<0.05$ being considered significant.

3.2.1.14 Luciferase assay

The APP-Gal4 assay system has been previously described (Cao and südhof, 2001; 2004). L1-, TAG1- and TAX-transfected CHO cells as well as CHO cells were co-transfected with the following plasmids (**i**) pG5E1B-luc (Gal4 reporter plasmid, 0.1 μg DNA); (**ii**) pCMV-LacZ (β-galactosidase control plasmid, 0.05 μg

DNA); (**iii**) pMstAPP (Gal4) or pMstC99 (Gal4) (0.1 μg DNA); (**iv**) pCMV5-Fe65 (Fe65) (0.1 μg DNA), in 24-well dishes using an Effectene Transfection kit (Qiagen, Valencia, CA, USA). Additionally, cells cultured in 24-well dishes were used for AICD-Gal4, C99-Gal4, APP*-Gal4 (with NPTY to NATA mutation), AICD59*-Gal4 (with NPTY to NATA mutation), and Fe65-Gal4 luciferase reporter assays.

For the transactivation assay in NPCs, wells were coated with L1-Fc, TAG1-Fc, F3-Fc or laminin protein (8 nM). Each well received 10 times the amount of DNA as used for the CHO cells and the transfection was performed using a Nucleofector System (amaxa Biosystems, Gaithersburg, MD, USA).

To examine the role of γ–secretase in this tansactivation, different concentrations of γ–secretase inhibitors (L-685, 458: 2 or 4μM; DAPT: 10, 20, 30, or 40μM; Calbiochem) were applied to tansfected cells, while DMSO was used as a control.

The Hes1 luciferase reporter assay has been previously described (Hu et al., 2003). CHO cells were transfected with 0.1ug pGVB-Hes luciferase reporter plasmid, luciferase internal plasmid and TAG-1 cDNA, F3 cDNA or NICD cDNA as well as empty vector as a control.

Twenty four to about 36 hours post-transfection, cells in each well were washed and resuspended in 150 μl 1× passive lysis buffer (Promega). The cell suspension was vigorously vortexed and kept on ice for 15 minutes to break the cells. After centrifugation at 13,200 rpm for 3 minutes, the supernatant was taken for Hes1 luciferase reporter assays by using the Steady-Glo Luciferase Assay Kit (Promega). For each sample, 70 μl supernatant was mixed thoroughly with 70 μl luciferase assay buffer and immediately measured for luciferase activity in a Lumimeter (Promega).

To monitor the transfection efficiency, the β-galactosidase expression plasmid pCMV/lacZ was co-transfected as an internal control. After cell lysis, 10 µl lysate of each sample was mixed with 10 µl 2× β-Gal assay buffer (Promega) in a 96-well dish (Iwaki) and incubated in the dark at 37 °C for 30 minutes. Then the enzymatic reaction was stopped by adding 50 µl 1M Na2CO3 to the mixture followed by vigorous vortex. The relative amount of β-Gal in each well was measured at 495 nm in a spectrometer (Bio-Rad). To normalize the luciferase assay, the reading of each sample in Lumimeter was divided by the reading of the same sample in β-Gal assays.

Raw data from at least four independent experiments were used to determine the relative reporter activity.

3.2.2 Biochemistry

3.2.2.1 Purification of TAG-1-Fc, F3-Fc protein

CHO cells stably expressing TAG-1-Fc or F3-Fc were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% ultra-low IgG fetal bovine serum (FBS) (Gibco) for one day, after which another same volume of serum-free DMEM was added to the cells. Two days later, another 2 times volume of serum-free DMEM was added to the cells. The cells were then kept under normal culture conditions for one week. After that, the conditioned medium was collected and balanced by adding 10% (v:v) 1M Tris-HCl (pH8.0). To avoid clogging columns, the medium was centrifuged at 10,000 g for 15 min and the cell pellet was discarded. The supernatant then passed through the Protein A agarose column (Roche) at 4℃ as instructed and TAG -1-Fc or F3-Fc was eluted from the column. The concentration of F3-Fc protein was determined by the BCA protein assay kit (Bio-Rad) and the purity was confirmed by SDS-PAGE-Coomassie blue and Western blotting.

The solutions for protein purification by Protein A agarose columns were as follows:

Name	Buffer
Equilibration buffer	100 mM Tris-HCl, pH8.0
Washing buffer1	100 mM Tris-HCl, pH8.0
Washing buffer2	10 mM Tris-HCl, pH8.0
Elution buffer	100 mM glycine, pH3.0
Neutralization buffer	1.0 M Tris-HCl, pH8.0

3.2.2.2 Fc pull-down assays

Freshly prepared cerebral hemispheres of adult rats were harvested and solubilized in RIPA buffer containing a protease inhibitor cocktail. The buffer homogenate was centrifuged at 13,000g for 1 hr at 4 ^{o}C and the supernatant was incubated for 45 min at room temperature with protein A-coupled agarose beads that had been incubated with APP-Fc. After washing the beads, proteins were eluted with SDS-PAGE sample buffer and immunoblotted with anti-TAG-1 antibodies.

3.2.2.3 GST pull-down assays

Freshly prepared cerebral hemispheres of adult rats were harvested and solubilized in RIPA buffer containing a protease inhibitor cocktail. The buffer homogenate was centrifuged at 13,000g for 1 hr at 4 ^{o}C and the supernatant was incubated for 45 min at room temperature with glutathione-agarose beads that had been incubated with TAXIg-GST or TAXFNIII-GST. After washing the beads, proteins were eluted with SDS-PAGE sample buffer and immunoblotted with anti-APP antibodies.

3.2.2.4 Co-immunoprecipitation

Mouse brains and NPCs were lysed with RIPA buffer containing a protease inhibitor cocktail (Roche, Basel, Switzerland). For immunoprecipitation, lysates were precleared with protein A-coupled agarose beads for 1 hr and incubated

with APP or TAG1 antibodies together with protein A-coupled agarose for overnight at 4 ^{o}C. Samples were washed with RIPA buffer before the beads were re-suspended in SDS buffer and boiled for 3-5 mins. Samples were subjected to SDS-PAGE. Western blot analysis was performed and developed with ECL reagents (Amersham Biosciences, Piscataway, NJ, USA).

3.2.2.5 Western blotting for AICD detection in CHO and MEF cells and E15 mouse brain

Protein extraction for detection of AICD in cell lines was as previously described (Hébert et al., 2006). Briefly, cells were lysed in cell lysis buffer (1% Triton X-100, 50mM HEPES pH 7.6, 150mM NaCl, 1mM EDTA and complete protease inhibitors). APP-transfected CHO cells were co-transfected with cDNAs of Fe65, PS1 and TAG1 or pRC vector as control or CHO cells were co-transfected with cDNAs of APP-V5, Fe65, BACE1 and TAG1 or pRC vector as control. After transfection, cells were cultured in DMEM (Gibco) for 24 h and 20mM NH_4Cl was added into the medium for culture for another 24h.

MEF cells were transfected with 1 µg, 2.5 µg or 5 µg of pRC-TAG1 vector or 2.5 µg empty pRC vector as control and collected 48 h after transfection or MEF cells were treated for 3 hr at 37 ^{o}C with recombinant human TAG1 (R&D systems, USA) or recombinant human F3-Fc (R&D systems). Total proteins were prepared by direct extraction in Tricine sample buffer (Bio-Rad Laboratories) containing a protease inhibitor cocktail (Roche), sonication and boiling for 5 min. The proteins were loaded onto 16% SDS-Tricine polyacrylamide gel and transferred onto PVDF membranes.

Protein extraction to detect AICD in mouse brain was as previously described (Hébert et al., 2006). Briefly, E15 mice brains were homogenized and sonicated in buffer (20mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 10% glycerol, 5 mM EDTA, 20 mM NH4Cl) containing 0.5% TritonX-100, 5mg/ml chymostatin, and

5mg/ml leupeptin, and incubated on ice for 15 min, centrifuged at 16000 X g for 15 min and boiled for 5 min. Protein levels were quantified (Bio-Rad Laboratories). The protein was applied to 12% SDS-Tricine polyacrylamide gels and transferred onto PVDF membranes.

The PVDF membranes were warmed by intermittent microwave irradiation (5 x 10 s irradiation at 5 min intervals). The PVDF membranes were blocked with 5% dry fat milk in TBST plus 0.05% Tween 20 and incubated with primary antibodies against the C-terminal of APP (A8717, Sigma-Aldrich), TAG-1 (TG1 from Prof. Watanabe) and γ-tubulin (Sigma-Aldrich) in TBST containing 5% dry fat milk overnight at 4 ^{o}C and for 1h at room temperature. The membranes were washed with TBST and incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG or donkey anti-rabbit IgG (Amersham Biosciences) for 2h at room temperature. ECL Plus or ECL Advance Western Blotting Detection Reagents (Amersham Biosciences) were used to visualize the immunoreactive proteins. Loading controls between Western blot lanes were normalized according to the γ-tubulin signal.

3.2.3 Quantification and Statistic analysis

Immunocytochemistry on cultured cells and immunostaining of tissue sections were performed as previously described. For quantification of immunofluorescence, images of fields of cultured cells were captured by digital photomicrograph under a 10X objective systematically from top-to-bottom and left-to-right across the entirety of each coverslip. All labeled cells were then counted in each photomicrograph. The proportion of neurons was quantified as the numbers of Tuj1+ or MAP2+ cells divided by the total number of DAPI+ cells in the same fields. Each experiment was repeated on 3 to 7 mice.

The statistics were performed using one-way-ANOVA or Student's t test, as appropriate. In all the graphs, the error bars indicate either standard error of the mean (SEM) or Standard deviation (s.d.). *: P<0.05; **: P<0.001.

3.2.4 Deficient Mice

3.2.4.1 Generation of Deficient Mice

$APP^{-/-}$ mice: The mouse APP gene was inactivated by deleting a 3.8Kb sequence encoding its promoter and first exon, which encodes the ATG translation initiation codon and the signal peptide of the APP (Zheng et al., 1997).

$TAG\text{-}1^{-/-}$ mice: The mouse TAG-1 gene was inactivated by replacing a 5Kb sequence encoding its exon II-VI with the neomycin resistance gene (Fukamauchi et al., 2001).

$APP^{-/-}TAG\text{-}1^{-/-}$ mice: were generated by intercrossing APP homozygous and TAG1 homozygous ($APP^{-/-}$ X $TAG\text{-}1^{-/-}$) mice. Animals heterozygous for both loci were intercrossed to each other ($TAG\text{-}1^{+/-}$&$APP^{+/-}$ X $TAG\text{-}1^{+/-}$&$APP^{+/-}$) to generate APP and TAG1 doubly deficient ($TAG\text{-}1^{-/-}$&$APP^{-/-}$) offspring.

3.2.4.2 Genotyping of the deficient mice

3.2.4.2.1 Genomic DNA purification

Genomic DNA was isolated from the tails of the mice using DNeasy Blood & Tissue Kit (69506, QIAGEN).

3.2.4.2.2 PCR Reagents

All PCR reagents were from Tag PCR core Kit (201225, QIAGEN).

3.2.4.2.3 Primers

All primers were synthesized by Resarch Biolabs.

Mouse	Name	Primers
APP	APP wt	5'-CTGCTGCAGGTGGCTCTGCA-3'
	APP common	5'-CAGCTCTATACAAGCAAACAAG-3'
	APP mutant	5'-CCATTGCTCAGCGGTGCTGTCCAT-3'
TAG-1	TAG1 wt	5'-GAAGCACTCAGCCCTAGAAGA-3'
	TAG1 common	5'-CTTTGCCACATTGTGCTGTG-3'
	TAG1 mutant	5'-GAAGACAATAGGAGGCATGC-3'
Fe65	Fe65 wt	5'-CTTGATCATCAACTCCCAGG-3'
	Fe65 common	5'-GCTTGAGTCCTCATCACTG-3'
	Fe65 mutant	5'-CGCCTTCTTGACGAGTTC-3'

3.2.4.2.4 PCR conditions

Mouse	PCR condition	Pattern of Bands
APP	60◦C : 30secs 94◦C: 5mins **94◦C: 30secs** **60◦C: 30secs** **68◦C: 1min** (30 cycles) 72◦C: 10mins	APP-/- allele: 470bp APP+/+ allele: 250bp APP+/- allele: 470bp + 250bp
TAG-1	94◦C: 10mins 75◦C: 3mins 55◦C: 3mins **72◦C: 3min** **95◦C: 3min** **56◦C: 45secs** (35 cycles) 72◦C: 6.5min	TAG1-/- allele: 320bp TAG1+/+ allele: 540bp TAG1+/- allele: 320bp + 540bp

Fe65	94◦C: 5mins **94◦C: 30secs** **60◦C: 30secs** **68◦C: 1min** 72◦C: 10mins	Fe65-/- allele: 398bp Fe65+/+ allele: 258bp Fe65+/- allele: 398bp + 258bp

4 Results

4.1 Cell adhesion assay suggests that TAG-1 and APP bind to each other

F3 and its homologue NB-3 have been identified as functional ligands for the Notch receptor (Hu et al., 2003; Cui et al., 2004). Given that the RIP processing of APP is strikingly similar to that of the Notch receptor, we hypothesized that members of the F3 family could act as APP ligands. To investigate the potential interaction between APP and members of the F3 subfamily, cell adhesion assays were performed. When F3-transfected CHO cells (CHOF3) or non-transfected CHO cells were seeded onto APP-Fc (recombinant APP extracellular domain in fusion with the Fc part of immunoglobulin) spotted culture dishes, little adhesion was observed (**Fig. 4A**, **B)**. When CHO cells transfected with TAG-1 (CHOTAG-1) were seeded onto culture dishes spotted with APP-Fc, the cells readily adhered to the APP protein spots (**Fig. 4A**, **B**). This adhesion could be blocked by pre-treating the cells with anti-TAG-1 antibody or the culture dish with anti-APP antibody (22C11; **Fig. 4A**, **B**), indicating that the interaction of TAG-1 with APP contributes to adhesion. The reciprocal adhesion assays were performed by plating APP-transfected CHO cells (CHOAPP) onto culture dishes with TAG-1-Fc protein spots. We observed similar adhesion of the cells to the protein spots (**Fig. 4A**, **B**). The adhesion was blocked by neutralization of coated TAG-1 protein spots or cell membrane-bound APP with their respective antibodies (**Fig. 4A**, **B**). Likewise, control non-transfected CHO cells did not adhere to the TAG-1 protein spot (**Fig. 4A**, **B**).

TAG-1 is a GPI-anchored molecule containing six Ig domains and four fibronectin type III (FNIII) repeats (Karagogeos, 2003). To identify the APP binding domains in TAX (the human homologue of TAG-1), GST fusion proteins of the six immunoglobulin (Ig) domains (TAXIg-GST) and four fibronectin type III (FNIII) repeats (TAXFNIII-GST) of TAX were used as coated protein substrates for CHOAPP cells. The cells bound to spots of both proteins (**Fig. 4A**), indicating

that APP has at least two binding sites located in the TAG-1 Ig domains and FNIII repeats. Consistently, control non-transfected CHO cells adhered to neither of these two proteins nor GST alone (**Fig. 4A**).

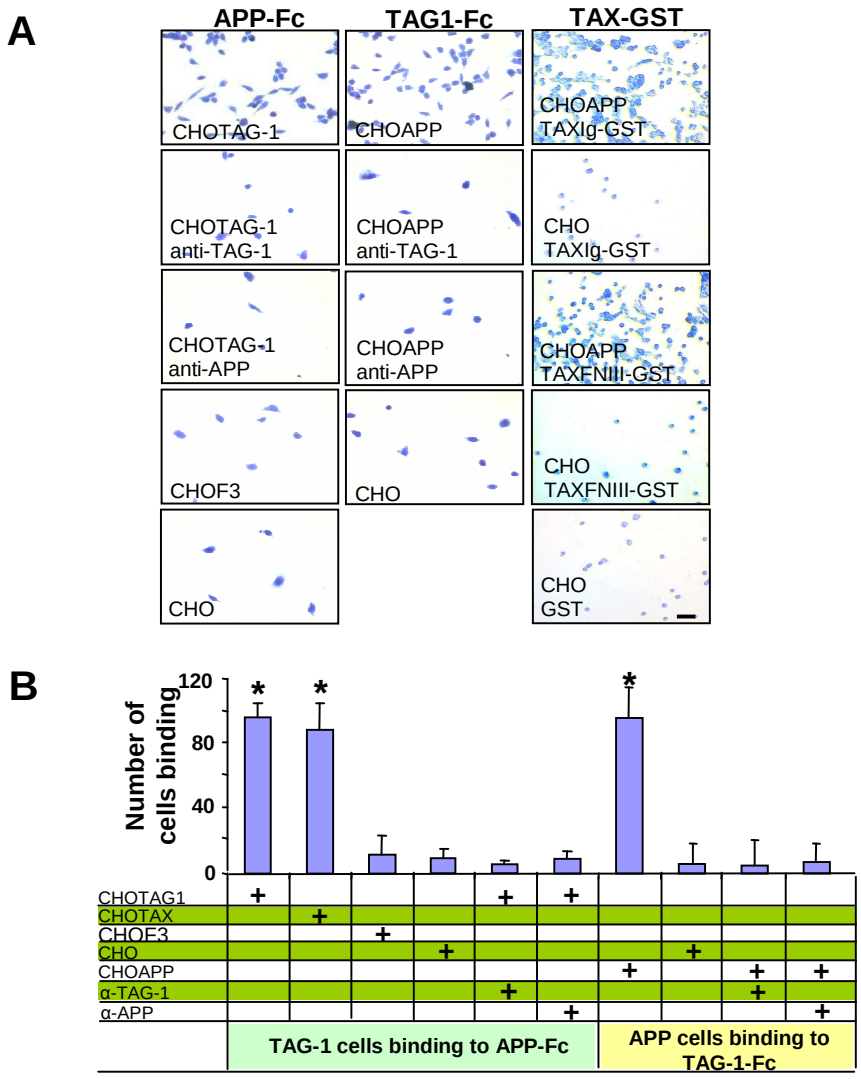

Figure 4. Cell adhesion assay indicates that TAG1 interacts with APP.
(**A**) TAG-1 transfected CHO cells (CHOTAG-1), but not F3 transfected CHO cells (CHOF3) and CHO cells (CHO), adhered to APP-Fc protein spots. Adhesion of CHOTAG-1 cells to APP-Fc was blocked by anti-TAG-1 and anti-APP antibodies. APP transfected CHO cells (CHOAPP), but not CHO cells, adhered to spots coated with TAG-1-Fc protein, TAXIg-GST, and TAXFNII-GST, but not GST. Adhesion of CHOAPP cells to TAG-1-Fc was blocked by anti-TAG-1 and anti-APP antibodies. Scale bar is 20 µm. (**B**) Quantification of CHOTAG1 cells adhering to APP-Fc substrate, CHOAPP cells adhering to TAG-1-Fc substrate, and the effects of blocking with anti-TAG-1 and anti-APP antibodies. Results shown as mean ± s.d., n=5-6, ** p<0.001. Experiments were performed by Dr. Zhi-cheng Xiao.

4.2 APP and TAG-1 associate as a protein complex

We performed co-immunoprecipitation (co-IP) in adult mouse brains to confirm the interaction between TAG-1 and APP. The results showed that several antibodies against TAG-1, including 1C12, 4D7 and TG3, but not IgG and an antibody against F3, could precipitate APP from the brain of wild-type mice (**Fig. 5A**), but could not precipatate APP from TAG-1$^{-/-}$ brain (**Fig. 5B**). Vice versa, an anti-APP antibody (171610) could precipitate TAG-1 from the brain of wild-type mice (**Fig. 5C**), but failed to precipitate TAG-1 from APP$^{-/-}$ brain (**Fig. 5D**). Inputs (**Fig. 5A**) and immunoprecipitation controls (**Fig. 5B**, **D**) from the respective wild-type and knockout mouse brains confirmed the specificity of co-immunoprecipitation and the antibodies used.

APP like protein 1 (APLP1) and APP like protein 2 (APLP2) are homologous to APP. They share similar structure to APP, except that they lack the Aβ sequence (Suh and Checler, 2002). To identify whether TAG-1 also interacts with APLP1 and APLP2, we performed co-immunoprecipitation. Antibodies against APP, PhoAPP (Thr668), APLP1 could precipitate APLP1, whereas anti-TAG-1 antibody (1C12) and non immune IgG could not do so (**Fig. 5E**), indicating that TAG-1 does not associate with APLP1. Similarly, in contrast to antibodies against APP, APLP2 could precipitate APLP2, anti-TAG-1 antibody (1C12) failed to precipitate APLP2 (**Fig. 5F**). These results suggest that TAG-1 only interacts with APP, but neither APLP1 nor APLP2.

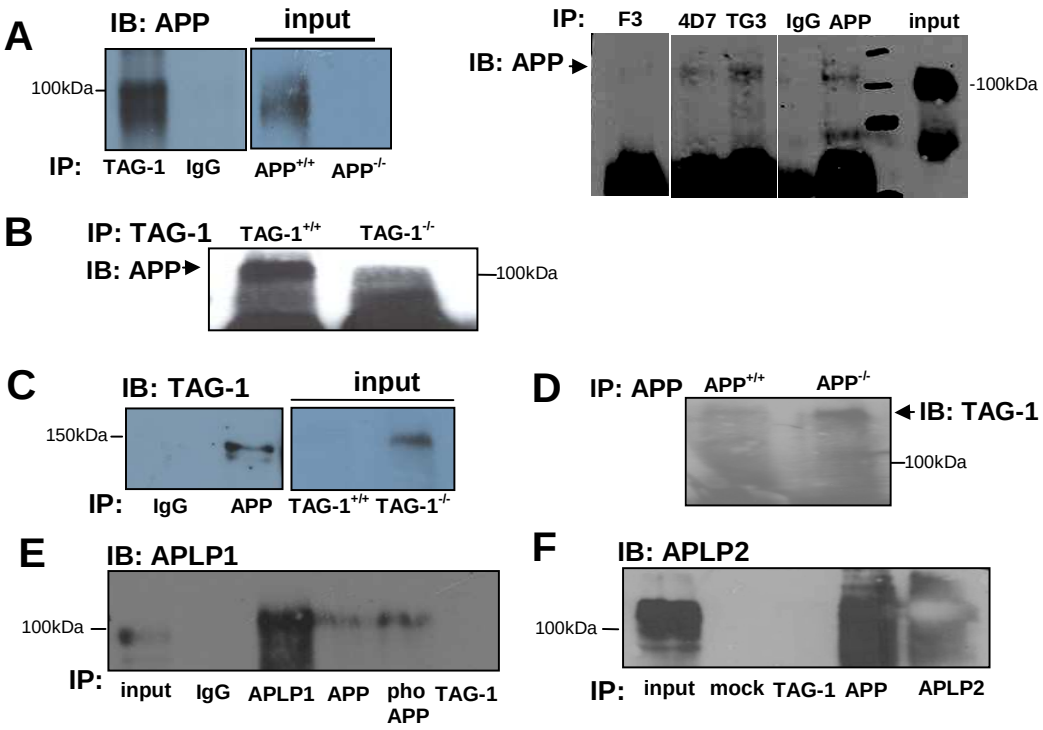

Figure 5. Co-immunoprecipitation assay (Co-IP) indicates TAG1 and APP associate as a protein complex.
(**A**) Brain lysates of wild-type mice were co-immunoprecipitated (IP) using anti-TAG-1 antibody (1C12) and non-immune IgG, and probed (IB) with anti-APP antibody. Blotting of input from wild-type ($APP^{+/+}$) and APP knockouts ($APP^{-/-}$) brain lysates confirmed the specificity of the antibody. (**B**) Using anti-TAG-1 antibodies to immunoprecipitate APP from brains lysates of TAG-$1^{+/+}$ and TAG-$1^{-/-}$. (**C**) Reciprocal assays using anti-APP antibody (171610) to capture the protein complex and anti-TAG-1 antibodies to detect the binding partner. (**D**) Using anti-APP antibodies to immunoprecipitate TAG-1 from brains lysates of $APP^{+/+}$ and $APP^{-/-}$. (**E**, **F**) Brain lysates of wild-type mice were co-immunoprecipitated using antibodies against APLP1, APLP2, APP, PhoAPP (Thr668), TAG-1, and non-immune IgG and probed with anti-APLP1 (**E**) and anti-APLP2 (**F**) antibodies.

To further confirm that TAG-1 associates with APP, we performed Fc-pull-down assays in a mixture of APP-Fc, mouse brain lysate and protein-A agarose beads. Consistent with the cell adhesion results, APP-Fc precipitated TAG-1 from the mouse brain (**Fig. 6**).

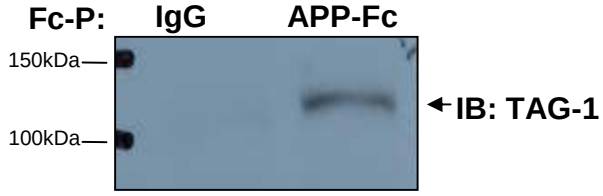

Figure 6. Fc pull-down assay indicates TAG-1 and APP associate as a protein complex.
Using APP-Fc (Fc-P) to capture the protein complex and anti-TAG-1 antibodies to detect the binding partner. IgG served as the control.

The cell adhesion assay suggested that both Ig domains and fibronectin type III (FNIII) repeats of TAG-1 bind to APP (**Fig. 4A**). To further confirm this point, GST-pull down assay was performed. In contrast to GST, both TAXIg-GST and TAXFNIII-GST precipitated APP from mouse brain as well as from CHOAPP cells (**Fig. 7**). These results demonstrate that TAG-1 and APP interact with each other (**Fig. 4**, **5**, **6**, **7**).

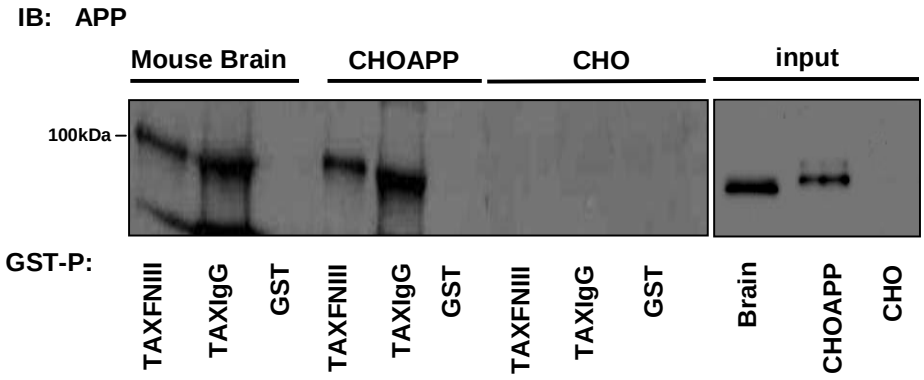

Figure 7. GST Pull-down assay indicates TAG1 and APP associate as a protein complex.
Mouse brain, APP-transfected CHO cell (CHOAPP), and CHO cell lysates were precipitated (GST-P) using TAXFNIII-GST, TAXIg-GST, and GST and probed with anti-APP antibody. Brain, CHOAPP and CHO inputs are shown in the right panel. GST-pull down assays were helped by Dr. Xiao-ying Cui.

4.3 APP localizes in the fetal neural stem cell niche

Given that TAG-1 interacts with APP, next we asked where this interaction occurs. APP is expressed by neuroepithelial cells of the cortical ventricular zone at embryonic stage (Lopez-Sanchez et al., 2005). APP immunoreactivity has also been detected in radial glia (Trapp and Hauer, 1994). Both neuroepithelial cells and radial glia are neural stem cells (Alvarez-Buylla et al., 2001). Moreover, sAPP, the secreted N-terminal nonamyloidogenic APP is found to stimulate proliferation of the cells from either *in vitro* embryonic neurospheres or C cells in adult SVZ (Caillé et al., 2004). All this evidence suggests that APP may be expressed by neural stem cells. To support this notion, we studied the localization of APP in embryonic day 14 (E14) mouse brain by immunofluoresence (IF) labelling using anti-APP antibody (C7). IF showed that APP was co-localized with nestin, a neural progenitor cell marker, in the walls of the lateral ventricles (LV) of wild-type E14 mouse (**Fig. 8A**), a neurogenic region in fetal mouse brain. The immunoreactivity of APP was not detected in $APP^{-/-}$ LV (**Fig. 8B**) by the same anti-APP antibody, indicating a specificity of the anti-APP antibody (C7) used here.

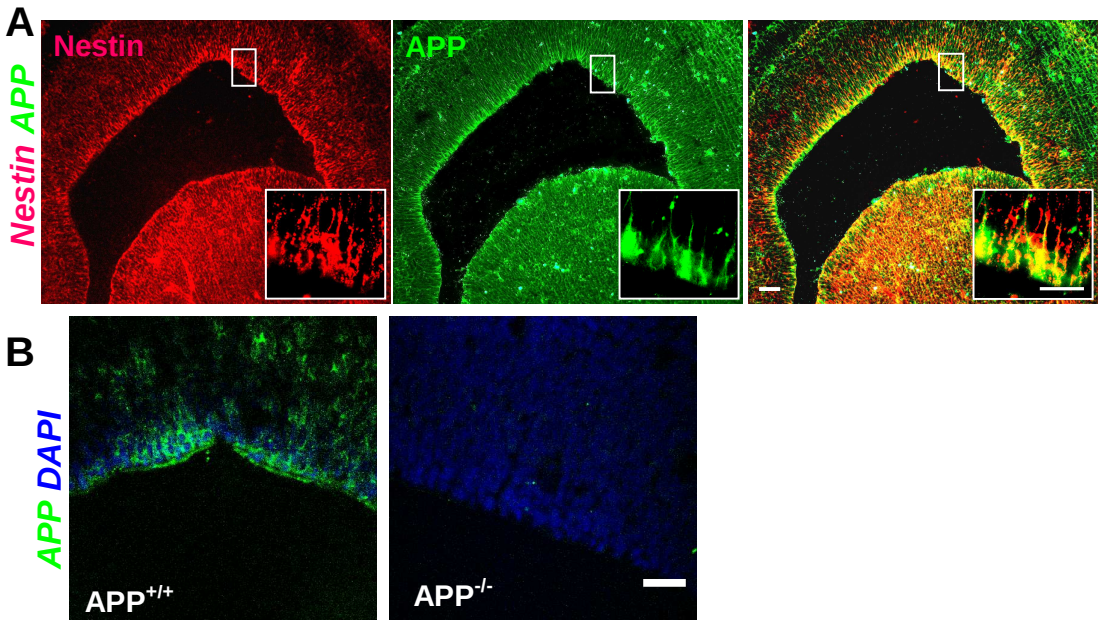

Figure 8. APP localizes in the fetal neural stem cell niche.
(**A**) Double immunostaining for nestin (red) and APP (green) in the walls of the lateral ventricles (LV) in E14 mouse brain. Bars are 50µm and 20µm in higher magnification images. (**B**) Anti-APP antibody failed to stain in the LV of E14 $APP^{-/-}$ brain. Bar is 20 µm.

4.4 TAG-1 localizes in the neurogenic ventricular zone

Next, we performed immunofluoresence (IF) labelling using antibodies against TAG-1 (TG1) to check the location of TAG-1 in E14 mouse brain. Similar to APP, TAG-1 co-localized with nestin in neurogenic lateral ventricles zone (LV) of wild-type E14 mouse (**Fig. 9A**). The specificity of TAG-1 antibody was confirmed by absence of immunoreactivity in $TAG\text{-}1^{-/-}$ LV (**Fig. 9B**).

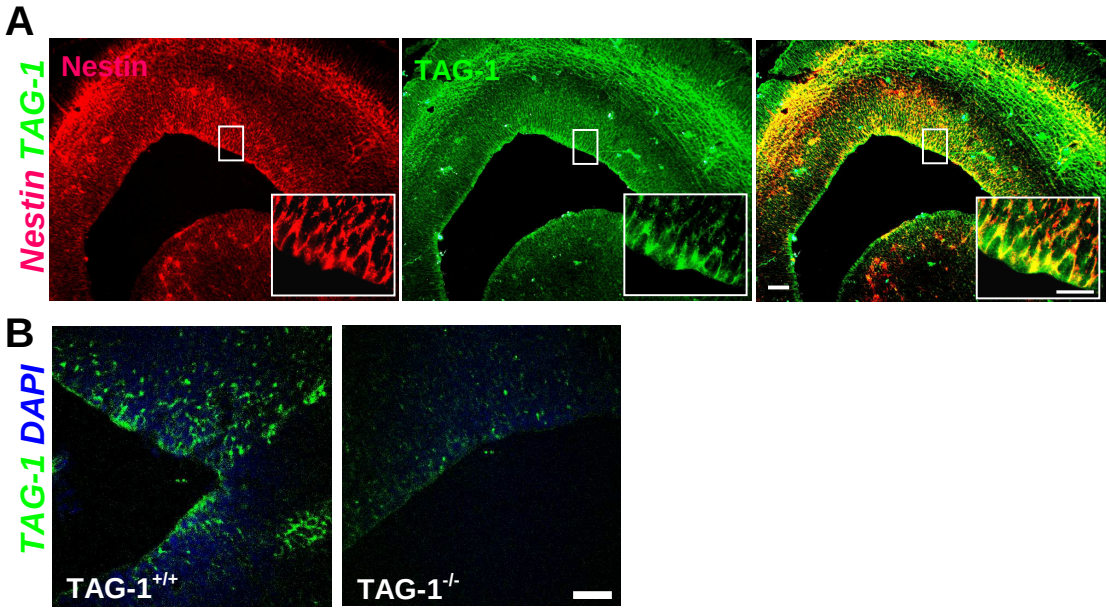

Figure 9. TAG-1 localizes in the fetal neural stem cell niche
(**A**) Double immunostaining for nestin (red) and TAG-1 (green) in the walls of the lateral ventricles (VZ) in E14 mouse brain. Bars are 50µm and 20µm in higher magnification images. (**B**) Anti-TAG-1 antibody failed to stain in the VZ of E14 TAG-$1^{-/-}$ brain. Bar is 20 µm.

4.5 TAG-1 and APP co-localize in the neurogenic ventricular zone

TAG-1 and APP interacts with each other. Both TAG-1 and APP localize in the walls of the lateral ventricles (LV) of E14 mouse. We further performed immunofluorescence (IF) labelling using antibodies against TAG-1 (TG1) and APP to check whether both molecules co-expressed in the same nestin-positive cells in E14 LV. IF indicated that TAG-1 colocalized APP in the walls of the lateral ventricles (LV) of E14 mouse (**Fig. 10**).

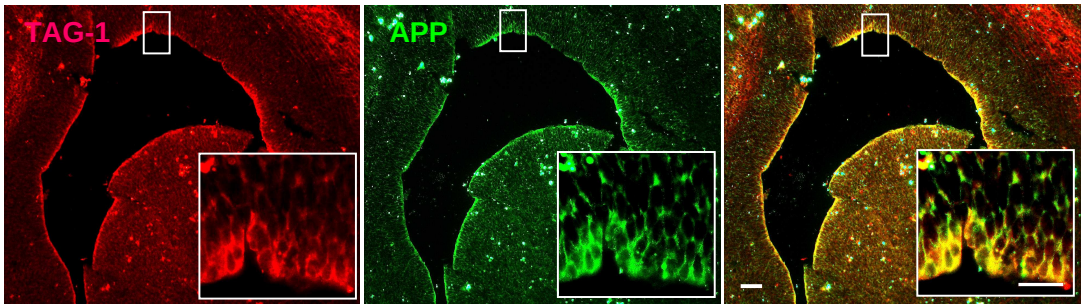

Figure 10. APP and TAG-1 co-localize in the fetal neural stem cell niche.
Double immunostaining for TAG-1 (red) and APP (green) in the walls of the lateral ventricles in E14 mouse brain. Bars are 50µm and 20µm in higher magnification images.

4.6 TAG-1 and APP co-localize in neural progenitor cells

The colocalization of TAG-1 and APP in nestin-positive cells in E14 LV suggests that both molecules are expressed by neural progenitor cells (NPCs). To further confirm this notion, we isolated NPCs from E14 mouse telencephalic ventricular walls. NPCs were cultured in presence of both bFGF and EGF, which are mitogen to maintain NPCs cells in self-renewal status. The cells were double-stained with antibodies against APP (C7 for **Fig. 11A;** MAB343 for **Fig. 11B**) or TAG-1 (TG1 for **Fig. 11C**; 4D7 for **Fig. 11D**) and the neural progenitor markers, nestin (**Fig. 11A** and **C**) or Sox2 (**Fig. 11B** and **D**). Co-localization of APP or TAG-1 with nestin or Sox2 was observed, indicating that both APP and TAG-1 are expressed by NPCs. Moreover, the cells were double-stained for APP (22C11) and TAG-1 (TG3). Consistent with the result obtained from staining on fetal brain slices, TAG-1 and APP colocalize with each other in cultured NPCs **(Fig. 11E**). The expression of TAG-1 and APP on NPCs was further confirmed by western blotting the cell lysates from E14 NPCs using anti-TAG-1 (TG1) and anti-APP (22C11) antibodies. TAG-1 and APP bands were detected respectively in both mouse brain and NPCs (**Fig. 11F**).

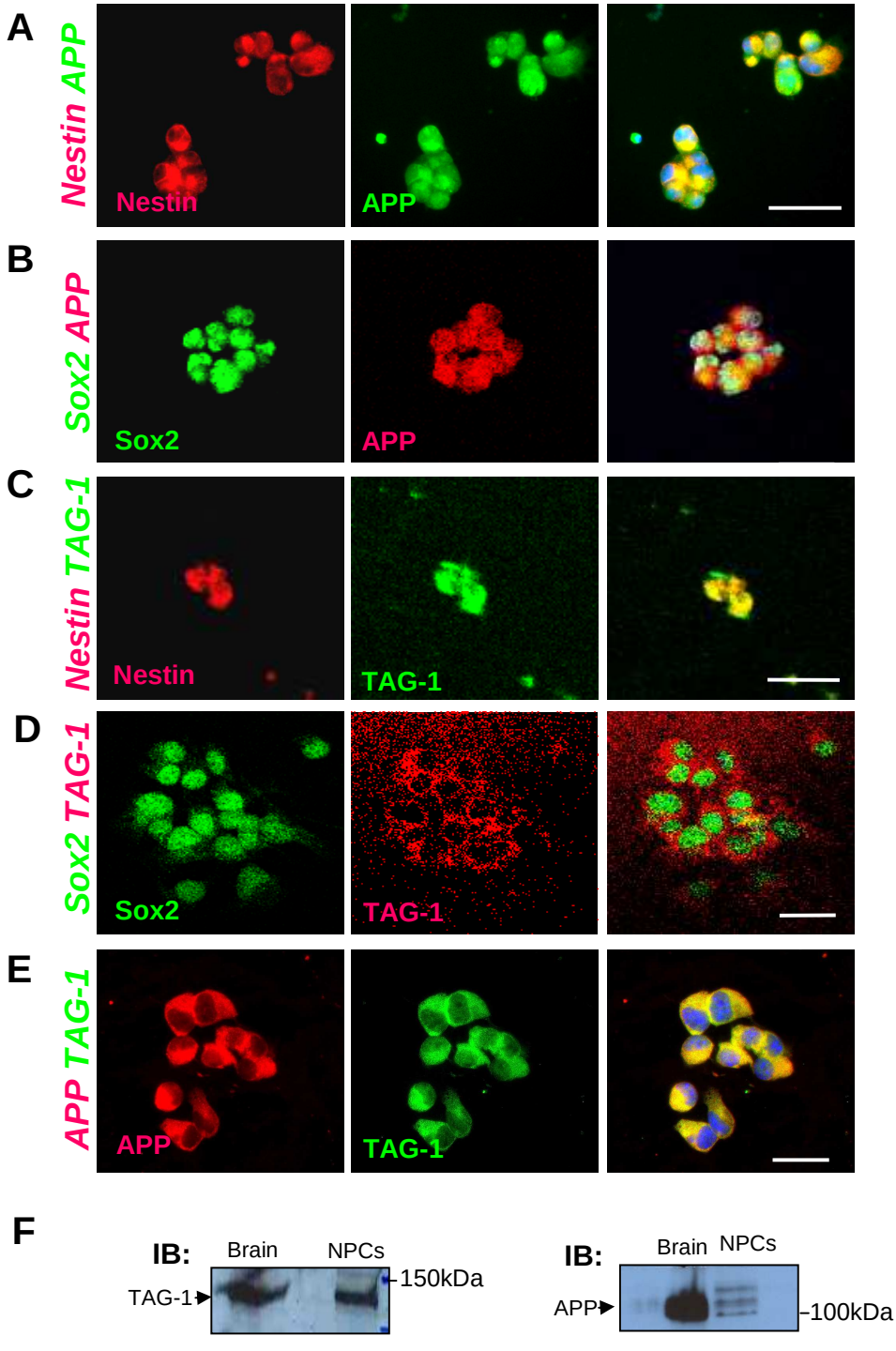

Figure 11. TAG-1 and APP are expressed and colocalize in neural progenitor cells (NPCs).
(**A**-**E**) NPCs isolated from the lateral ventricles of E14 mouse brain were double-stained for APP (**A**, **B**, **E**) and TAG-1 (**C**, **D**, **E**) and with neural precursor cell markers, nestin (**A**, **C**) or Sox2 (**B**, **D**). (**F**) Mouse brain samples and NPCs lysates were western blotted with antibodies against TAG-1 (TG1) and APP (22C11). Bars are 20 µm in **A**, **B**, **C**, **D** and **E**.

4.7 TAG-1 and APP interact with each other in neural progenitor cells

To investigate whether TAG-1 and APP interact with each other in NPCs, immunoprecipitation was performed in NPCs lysates using anti-TAG-1 (1C12), anti-F3 antibodies, and detected with anti-APP (22C11) antibodies (**Fig. 12A**). In contrast to F3, TAG-1 could precipitate APP from NPCs lysates. Vice versa, APP (171610) also could precipitate TAG-1 (TG1) from NPCs lysates (**Fig. 12B**). These results suggest that the interaction between these two molecules may also occur in NPCs.

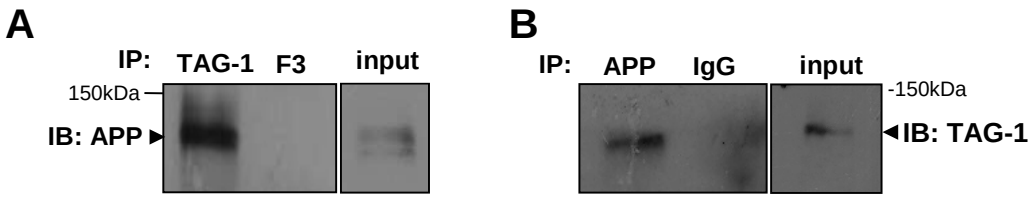

Figure 12. TAG-1 and APP associate with each other in neural progenitor cells (NPCs).
(**A**) NPCs lysates of E14 wild-type mice were co-immunoprecipitated using anti-TAG-1 (1C12) or anti-F3 antibodies and probed with anti-APP antibody (22C11). (**B**) Reciprocal assays using anti-APP antibody (171610) or non-immune IgG to capture the protein complex and anti-TAG-1 antibody (TG1) to detect the binding partner.

4.8 Developmental expression profile of APP and TAG-1 indicates they may be involved in neurogenesis

Given that TAG-1 and APP interact with each other and are co-expressed by neural progenitor cells (NPCs), we investigated the function of this interaction on NPCs. Cell genesis during development arises in a very precise temporal order. During development, neurons arise first, and are then followed by astrocytes and oligodendrocytes. In mice, neurogenesis commences around embryonic 12 days (E12), peaks at E14, finishes around E18. Astrocytes arise around only after E16 (Bayer and Altman, 1991). To get some clue of the function of the interaction between APP and TAG-1, we investigated the developmental expression profile of TAG-1 and APP. Fetal brain lysates from E10 to P0 mouse brain were

analysed by western blots with antibodies against TAG-1 (TG1), APP (A8717) and γ-tubulin (**Fig. 13**). TAG-1 was expressed from E12, while APP was detectable as early as from E10. TAG-1 was most strongly expressed from E16 and E18, while APP expression continued to increase to P0 (**Fig. 13**). Thus, both APP and TAG-1 are expressed during fetal brain development, with APP switching on as cortical neurogenesis plateaus at E14 to E15, and TAG-1 peaking as cortical neurogenesis declines fro E16 and E18 (Rodier, 1977). Both APP and TAG-1 are upregulated as neurogenesis declines during brain development, suggesting that APP and TAG-1 may play a repressive role in neurogenesis.

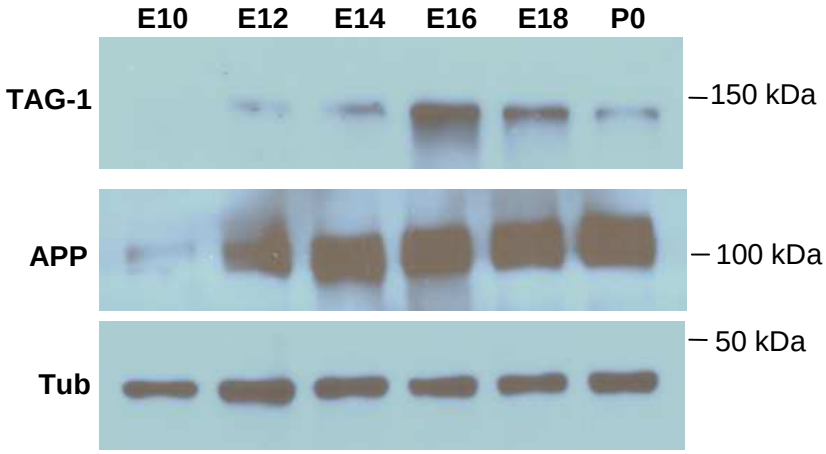

Figure 13. Developmental expression of TAG-1 and APP.
Protein lysates from E10, E12, E14, E16, E18 and P0 mouse brain were Western blotted with anti-TAG-1 (TG1) and anti-APP (A8717) antibodies. Blotting with anti-γ tubulin (Tub) antibody served as loading control.

4.9 APP inhibits neurogenesis

APP null mice show increased mortality after birth. Adult APP null mice are usually smaller than wild-type mice but at E14 there is no gross phenotypic differences observable. To assess the role of APP in modulation of neurogenesis, NPCs were isolated from the E14 telencephalic ventricular walls of

$APP^{-/-}$ mice and an *in vitro* differentiation assay was performed. Briefly, isolated NPCs were seeded into 24-well dishes at clonal density and cultured for 7-8 days in NPC differentiation medium. Neurons, astrocytes and oligodendrocytes can be detected after 7-8 days culturing in this protocol. After 7 to 8 days *in vitro* differentiation, cells were double-stained for class III β-tubulin (TUJ1, green; **Fig. 14A**) or MAP2 (red; **Fig. 14B**), two markers for differentiated neurons, and DAPI (blue; **Fig. 14A** and **B**). TUJ1-positive or MAP2-positive cells were quantified relative to the number of DAPI-positive cells. There were 36.63 ± 1.12% cells differentiating into TUJ1-positive cells and 29.44 ± 1.04% cells differentiating into MAP2-positive from $APP^{-/-}$ NPCs, which is significantly higher than from $APP^{+/+}$ NPCs, where only 26.81 ± 1.36% cells differentiated into TUJ1-positive cells and 19.66 ± 2.20% cells differentiated into MAP2-positive cells (not shown). We further normalized the percentage of TUJ1-positive cells or MAP2-positive cells from $APP^{+/+}$ mice as 100%. 37% significant increase in TUJ1-positive cells (**Fig. 14A**) and 50% significant increase in MAP2-positive cells (**Fig. 14B**) were observed in $APP^{-/-}$ mice versus wild-type littermates. These results indicate that APP inhibits neurogenesis.

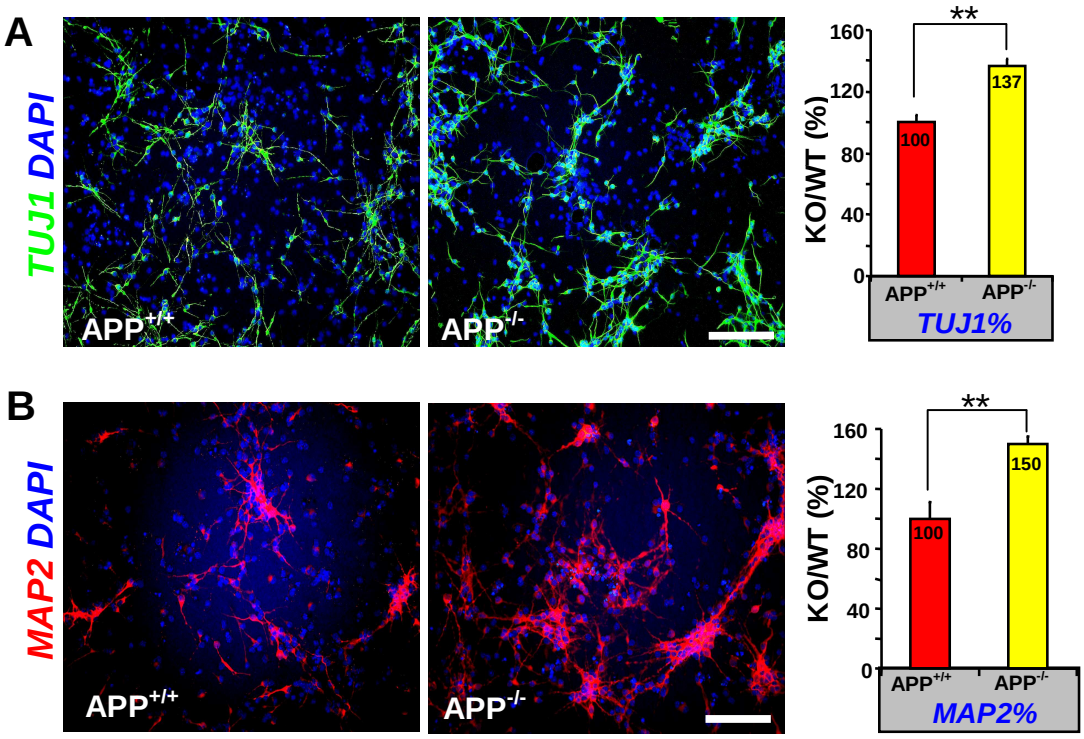

Figure 14. APP inhibits neurogenesis.
NPCs were isolated from APP$^{+/+}$ mice (WT) and APP$^{-/-}$ mice (KO). After 7-8 days *in vitro* differentiation, the cells were double-stained for TUJ1 (**A**) or MAP2 (**B**) and DAPI (**A** and **B**). The numbers of TUJ1 (**A**) and MAP2 (**B**) positive cells were counted and expressed as a percentage of the number of DAPI positive cells, and further normalized to the respective wild-type littermate controls (shown in the right panels). Bars are 100 µm. Results are means ±s.e.m., n=3-6, ** p<0.001.

4.10 TAG-1 inhibits neurogenesis

The same method was applied to analyse the modulation of neurogenesis by TAG-1. TAG-1$^{+/+}$ mice show no gross phenotypic abnormalities, although the adult mice exhibit elevated expression of adenosine A1 receptors in the hippocampus and enhanced seizure susceptibility to convulsant stimuli (Fukamauchi et al., 2001). After 7 to 8 days *in vitro* differentiation, cells were double-stained for TUJ1 (green; **Fig. 15A**) or MAP2 (**Fig. 15B**), two markers for

differentiated neurons, and DAPI (blue; **Fig. 15A** and **B**). Both TUJ1- and MAP2-positive cells were significantly increased in TAG-$1^{-/-}$ mice compared with wild-type littermates. There were 39.55 ± 1.34% cells differentiating into TUJ1-positive cells, or 34.07 ± 1.50% cells differentiating into MAP2-positive from TAG-$1^{-/-}$ NPCs. However, there were only 26.86 ± 0.90% cells were TUJ1-positive or 20.51 ± 0.82% cells differentiating into MAP2-positive in TAG-$1^{+/+}$ mice (not shown). After normalization, 48% increase in TUJ1-positive cells and 66% increase in MAP2-positive cells were observed in TAG-$1^{-/-}$ mice comparing to wild-type littermates (**Fig. 15A** and **B**). Thus, Like APP, TAG-1 plays a negative role in modulating on neurogenesis as well.

4.11 No difference is detected between TAG-$1^{+/+}$ and TAG-$1^{-/-}$ NPCs in apoptosis

To investigate whether the increment in neurogenesis in TAG-$1^{-/-}$ could be caused less cell death, we performed the TUNEL assay. NPCs were isolated from the E14 telencephalic ventricular walls of TAG-$1^{-/-}$ mice, After 7 to 8 days *in vitro* differentiation, cells were double-stained for TUNEL and DAPI. The apoptotic cells are identified by both the immunoreactivity of TUNEL and the morphology of cells. In this experiment, we did not observe any significant difference in the number of apoptotic cells between TAG-$1^{+/+}$ and TAG-$1^{-/-}$ NPCs (**Fig. 16**).

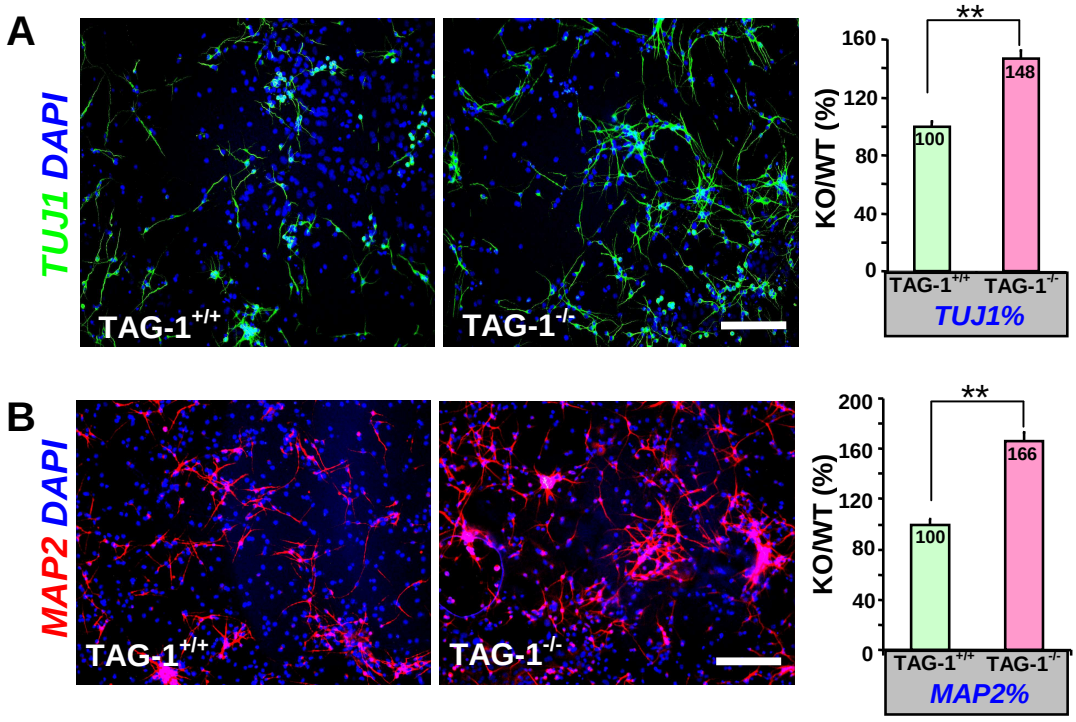

Figure 15. TAG-1 inhibits neurogenesis.
NPCs were isolated from TAG-$1^{+/+}$ mice (WT) and TAG-$1^{-/-}$ mice (KO). After 7-8 days *in vitro* differentiation, the cells were double-stained for TUJ1 (**A**) or MAP2 (**B**) and DAPI (**A** and **B**). The numbers of TUJ1 (**A**) and MAP2 (**B**) positive cells were counted and expressed as a percentage of the number of DAPI positive cells, and further normalized to the respective wild-type littermate controls (shown in the right panels). Bars are 100 µm. Results are means ±s.e.m., n=3-6, ** p<0.001.

4.12 Both TAG-1 and APP inhibit neurogenesis

To identify whether TAG-1 and APP modulate neurogenesis via a convergent pathway, we generated TAG-$1^{-/-}$APP$^{-/-}$ foetuses. The double knockout mice were usually developmentally lethal and only very few mice survived to birth but the foetuses could survive to E14. Consistent with our observations in singly deficient mice, TUJ1-positive cells were significantly increased in TAG-$1^{-/-}$APP$^{-/-}$ mice compared with wild-type littermates (**Fig. 17**). 42.47 ± 1.05% cells were TUJ1-positive in TAG-$1^{-/-}$APP$^{-/-}$ mice, while only 30.73 ± 0.69% TUJ1-positive cells were detected in TAG-$1^{+/+}$APP$^{+/+}$ littermates (not shown). These results demonstrate that the interaction between TAG-1 and APP may be involved in

modulation of neurogenesis during the early stages of CNS development (**Fig. 14**, **15** and **17**). Notably, the increase in TUJ1- positive cells (38%) in TAG-1$^{-/-}$ APP$^{-/-}$ mice is similar to that in TAG-1$^{-/-}$ (48%) or APP$^{-/-}$ (37%) mice (**Fig. 14** and **15**), suggesting that the function of APP and TAG-1 on neurogenesis is not divergent and may be via a common signalling pathway.

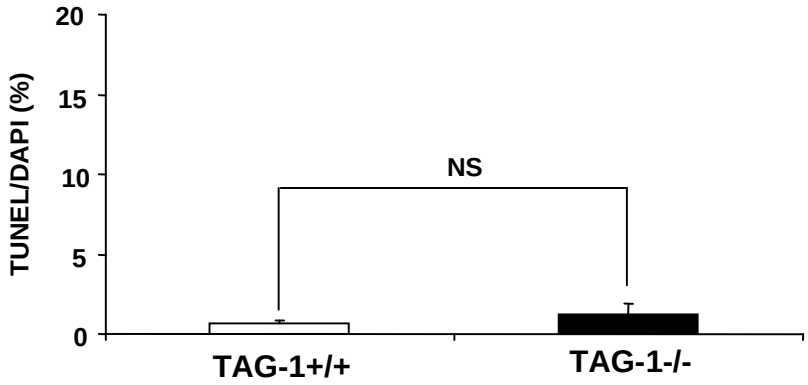

Figure 16. Apoptosis displays no difference between TAG-1$^{+/+}$ and TAG-1$^{-/-}$ NPCs.
NPCs were isolated from TAG-1$^{-/-}$ and TAG-1$^{+/+}$ mice. After 7-8 days *in vitro* differentiation, the cells were double-stained for TUNEL and DAPI. TUNEL-positive cells were counted and expressed as a percentage of the total number of DAPI positive cells. Results are means ± s.e.m., n=3, NS: non-significant.

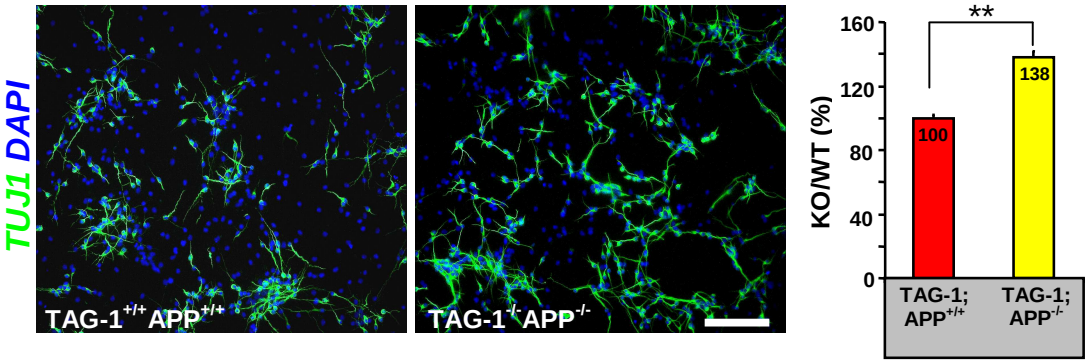

Figure 17. APP and TAG-1 inhibit neurogenesis.
NPCs isolated from TAG-1$^{+/+}$APP$^{+/+}$ mice (WT) and TAG-1$^{-/-}$APP$^{-/-}$ mice (KO) were double-stained for TUJ1 and DAPI. The number of TUJ1 positive cells were counted and expressed as a percentage of the number of DAPI positive cells, and normalized to the respective wild-type littermate control. The bar is 100 µm. Results are means ± s.e.m., n=3, ** p<0.001.

4.13 APP acts as the receptor of TAG-1 to inhibit neurogenesis

APP is suggested to be a receptor-like protein, which can transduce intracellular signals after binding to its ligand. Given that the modulation of neurogenesis by TAG-1 and APP is via a convergent signalling pathway, to further check the notion that APP acts as the receptor of TAG-1 in modulating neurogenesis, we treated TAG-$1^{-/-}$ and TAG-$1^{-/-}$APP$^{-/-}$ fetal NPCs with soluble TAG-1 protein (70nM and 140nM) during differentiation. The cells were double-stained for TUJ1 and DAPI and quantified. After the TAG-1 treatment, the number of TUJ1-positive cells was significantly decreased compared to the PBS-treated control group in the fetal NPCs from TAG-$1^{-/-}$ mice (**Fig. 18A** and **B**). However, similar treatment could not reverse the abnormally increased neurogenesis in TAG-$1^{-/-}$APP$^{-/-}$ mice (**Fig. 18C** and **D**). This result suggests that APP acts as a receptor of TAG-1 in modulating neurogenesis.

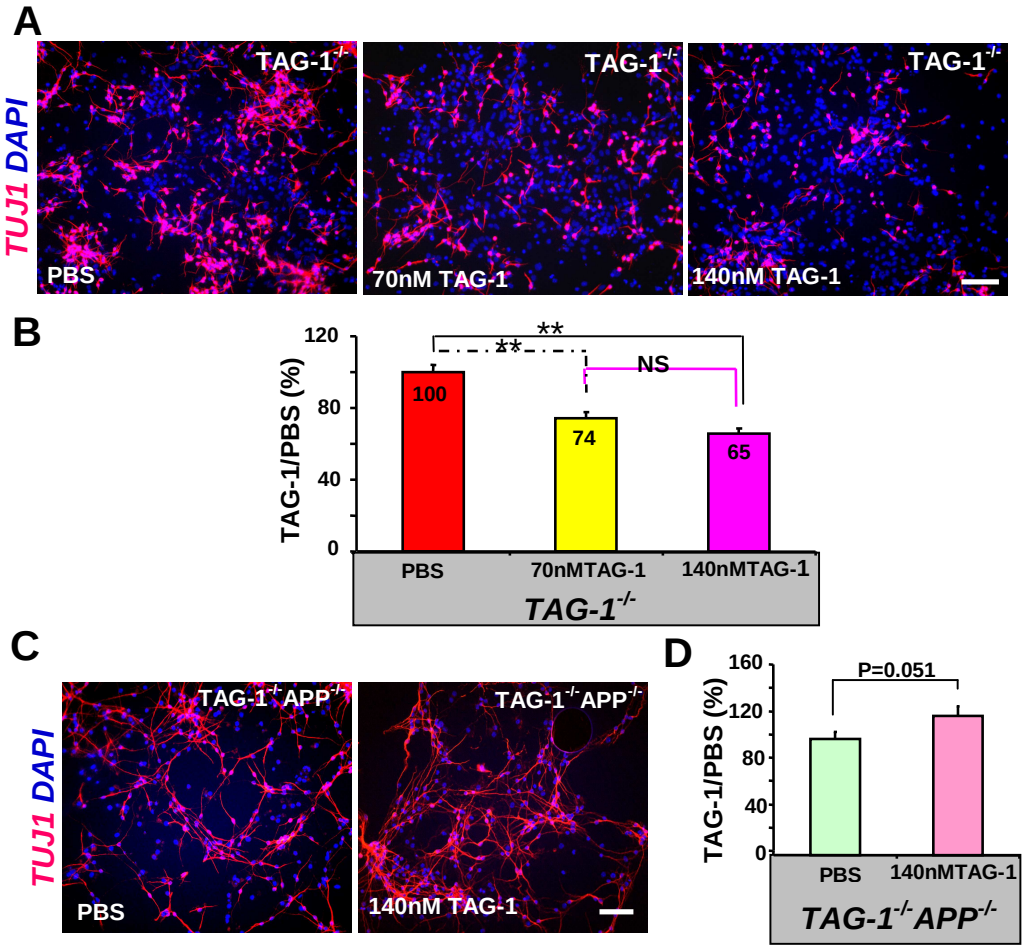

Figure 18. TAG-1 rescues the abnormal enhanced neurogenesis of TAG-1$^{-/-}$ NPCs but not TAG-1$^{-/-}$APP$^{-/-}$ NPCs.

(**A**, **B**) TAG-1 protein could rescue enhanced neurogenesis in TAG-1$^{-/-}$ NPCs. (**A**) NPCs were isolated from TAG-1$^{-/-}$ mice and treated with TAG-1 protein (70 nM and 140 nM) and PBS as a control. After differentiation, the cells were double-stained for TUJ1 and DAPI. (**B**) The numbers of TUJ1 positive cells were counted and expressed as a percentage of the number of DAPI positive cells, and further normalized to the respective PBS controls. (**C**, **D**) TAG1 protein failed to rescue enhanced neurogenesis in TAG-1$^{-/-}$APP$^{-/-}$ NPCs. NPCs were isolated from and TAG-1$^{-/-}$APP$^{-/-}$ mice and treated with TAG-1 (140 nM) and PBS as a control. After differentiation, the cells were double-stained for TUJ1 and DAPI. The numbers of TUJ1 (red) positive cells were counted and expressed as a percentage of the number of DAPI positive cells, normalized to the respective PBS controls. Bars in **A** and **C** are 100 µm. Results are means ±s.e.m., n=3-6, ** p<0.001. NS: non-significant.

4.14 TAG-1 stimulates AICD release in an artificial luciferase system

A wealth of evidence suggests that APP may be regulated by regulated-intramembrane proteolysis (RIP). Upon binding to its ligand, APP can be cleaved sequentially by secretases, releasing its intracellular fragments. We investigated whether TAG-1 could regulate AICD release in an artificial luciferase reporter system (Cao and Südhof, 2001). In this system, the yeast Gal4 DNA-binding domain was inserted into the intracellular tail of full-length APP at the cytoplasmic boundary of the transmembrane region (Cao and Südhof, 2001). Only after γ-secretase cleavage can the AICD-Gal4 element be released to drive luciferase reporter activity via the Gal4 response element. This system measures release of AICD but does not demonstrate that AICD is involved in endogenous transcriptional activation. We introduced this reporter system together with Fe65 into non-transfected CHO and CHOL1, CHOTAG-1 or CHOTAX cells. A significant upregulation of the reporter activity was observed in both CHOTAG-1 and CHOTAX cells, but neither in the control CHO cells nor CHOL1 cells (**Fig. 19A**). To exclude the possibility that different expression level of APP and FE65 caused the difference of luciferase response in non-transfected CHO and CHOL1, CHOTAG-1 or CHOTAX cells, western blot analysis was performed. No significant difference in expression levels of APP and Fe65 was observed in non-transfected CHO and CHOL1, CHOTAG-1 or CHOTAX cells (**Fig. 19B**), suggesting that upregulation of reporter activity is due to release of AICD-Gal4. Moreover, two specific γ-secretase inhibitors, L-685,458 and DAPT, reduced the TAG-1-triggered release of AICD in a dose-dependent manner (**Fig. 19C and D**). Therefore, these results suggest that TAG-1 can trigger AICD release from full-length APP in a γ-secretase-dependent manner.

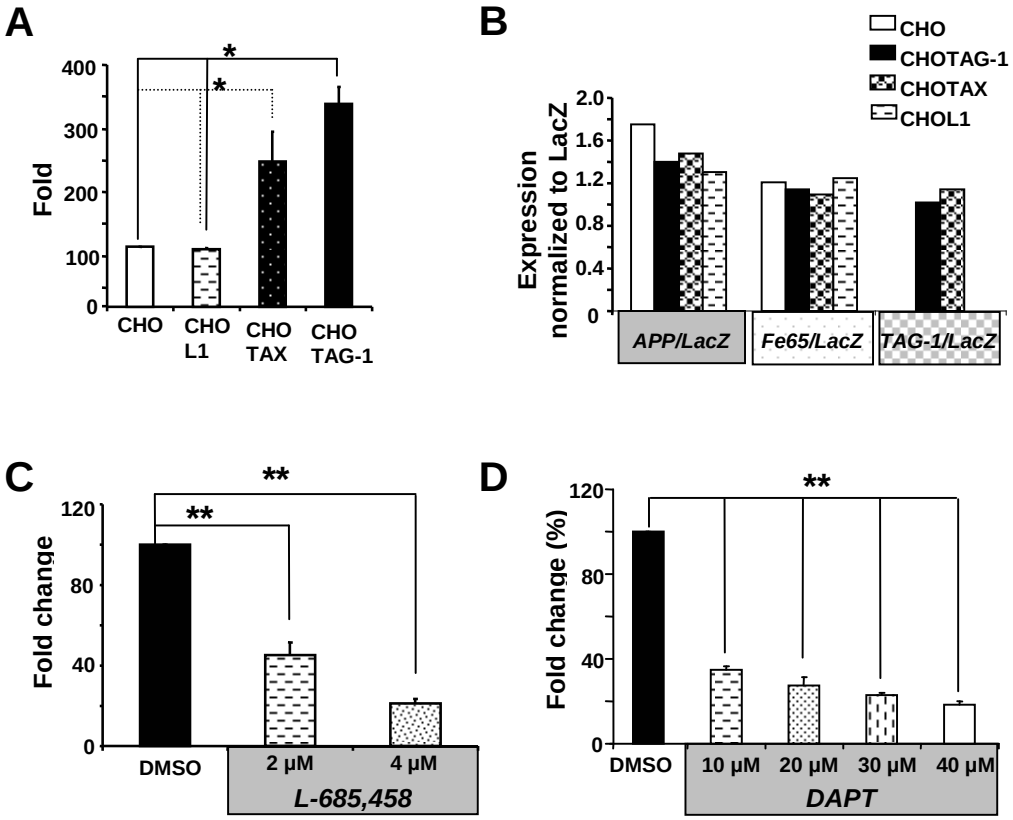

Figure 19. Luciferase assay for release of AICD-Gal4 in CHOTAG-1 and CHOTAX cells.

(**A**) CHOL1, CHOTAX, CHOTAG-1 and CHO cells were transiently co-transfected in 24-well culture dishes with pG5E1B plasmid, an APP-Gal4 construct, Fe65 plasmid, and luciferase internal control plasmid. Cleavage of APP to release AICD-Gal4 activates the luciferase reporter. Normalized luciferase activities in whole-cell lysates from CHOL1, CHOTAX and CHOTAG-1 were determined and expressed relative to activity in lysates prepared from CHO cells. (**B**) Whole-cell lysates were Western blotted using antibodies against TAG-1 (TG1), APP (22C11), Fe65 (3H6), Gal4 (630403) and myc. Expression of APP, Fe65 and TAG-1 were quantified relative to LacZ-myc, which served as internal control. (**C** and **D**) Luciferase activities in CHOTAG-1 cells were significantly reduced by two γ-secretase inhibitors (L-685,458: 2 µM and 4 µM; DAPT: 10 µM, 20 µM, 30 µM and 40 µM). Results are means ± s.e.m, n=3-6, ** p<0.001. Experiments were performed by Dr. Wu-lin Yang.

4.15 Extracellular domain of APP is essential for the functional interaction between APP and TAG-1

The extracellular cleavage is a prerequisite for the following process of RIP (Brown et al., 2000). The extracellular domain of APP contains various motifs for the interaction with other proteins, such as protease inhibitor domain, heparin binding domain (Mok et al., 1997), Cu2+ and Zn2+ binding domain (Hesse et al., 1994; Bush et al., 1993; Gralle, 2007). Moreover, an N-glycosylation site has been found located in the extracellular domain of APP (Pahlsson and Spitalnik, 1996; Gralle, 2007). To check whether the extracellular domain of APP could be essential for the release of AICD triggered by TAG-1, we transfected a modified luciferase reporter system (C99-Gal4), in which the extracellular domain of APP was deleted from the aforementioned APP-Gal4 construct, together with Fe65 into TAG-1-transfected CHO cells (**Fig. 20A**). There was no significant difference between non-transfected CHO and CHOTAG-1 cells (**Fig. 20B**), suggesting that extracellular domain of APP is required for triggering AICD luciferase activity by TAG-1. Similarly, the expression level of C99-Gal4 and Fe65 was not changed between the two groups (not shown).

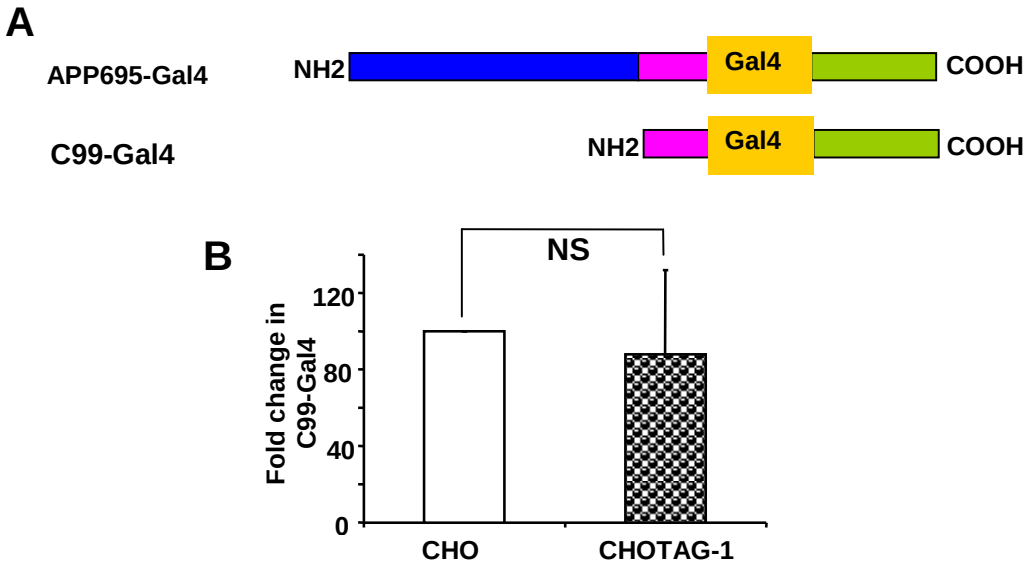

Figure 20. TAG-1 fails to trigger lufierase response in cells transfected with C99-Gal4.
(**A**) Schematic description of C99-Gal4 construct. (**B**) CHO cells and CHOTAG-1 cells were transiently co-transfected in 24-well culture dishes with pG5E1B plasmid, a C99-Gal4-construct, Fe65 plasmid, and luciferase internal control plasmid. Normalized luciferase activities in CHOTAG-1 cells were determined and expressed relative to activity in CHO cells. Results are means ± s.e.m, n=3, NS: non-significant. Experiments were helped by Dr. Wu-lin Yang.

4.16 TAG-1 triggers AICD release

To explore the notion that TAG-1 triggers AICD release after binding with full-length APP, we investigated the AICD level by western blotting. APP-transfected CHO cells were co-transfected with cDNAs of Fe65, presenilin-1 (PS1) and TAG-1 or empty vector (plasmid pRC) as control. Cell lysates were subjected to western blotting using antibodies against the C-terminal of APP (A8717), TAG-1 (TG1) and γ-tubulin. Transfection with TAG-1 cDNA, but not the empty vector, significantly increased the AICD release in the CHOAPP cells (**Fig. 21A** and **B)** and this increase could be blocked by a γ-secretase inhibitor, L-685,458 (5 μM; **Fig. 21D**). The level of APP expression was not different between these two groups (**Fig. 21A** and **C**). Moreover, CHO cells were co-transfected with cDNAs

of APP-V5, Fe65, BACE1 and TAG-1 or pRC vector (Mock) as control. Total proteins were western blotted using antibodies against TAG-1 (TG1), V5 (R960-25) and γ-tubulin (**Fig. 21E**). Western blotting with anti-V5 antibody showed that TAG-1 transfection increased AICD-V5 release in CHO cells compared with the mock transfection control (**Fig. 21E**).

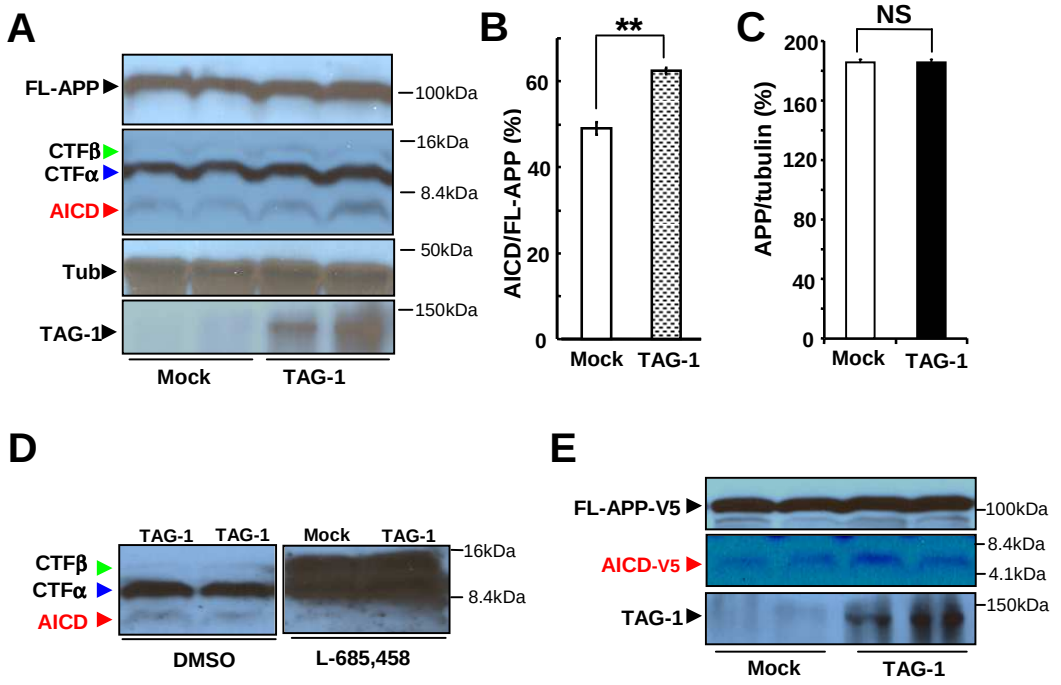

Figure 21. TAG-1 triggers AICD release from full-length APP.
(**A**) CHOAPP cells were co-transfected with cDNAs of Fe65, PS1 and TAG-1 or pRC vector (Mock) as control. Total proteins obtained from the cells was analysed by western blotting using antibodies against C-terminal APP (A8717), TAG-1 (TG1) and γ-tubulin (Tub). (**B**, **C**) AICD and full-length APP (FL-APP) bands were quantified relative to FL-APP and γ-tubulin. (**D**) The AICD release triggered by TAG-1 in CHOAPP cells was significantly reduced by a γ-secretase inhibitor (L-685,458, 5 µM). (**E**) CHO cells were co-transfected with cDNAs of APP-V5, Fe65, BACE1 and TAG-1 or pRC vector (Mock) as a control. Total proteins were analyzed by western blotting using antibodies against APP (A8717), TAG-1 (TG1), V5 (R960-25) and γ-tubulin (Tub). Results are means ± s.e.m, n=3-6, ** $p<0.001$. NS: non-significant. Western blots were helped by Mr. Toshi Futagawa

4.17 TAG-1 triggers endogenous AICD release

To ascertain whether TAG-1 could also stimulate endogenous release of AICD, we transfected mouse embryonic fibroblast (MEF) cells with empty pRC vector or various doses of TAG-1 cDNA. There was a dose-dependent increase in the production of endogenous AICD (**Fig. 22A** and **B**). Moreover, we also compared the effects of two concentrations of TAG-1 protein on endogenous AICD production in MEF cells. Again there was a dose-dependent increase in production of endogenous AICD on treatment with TAG-1 (**Fig. 22C** and **D**). The effect of TAG-1 on endogenous AICD production was further confirmed by investigation of AICD expression in E15 brains of TAG-$1^{+/+}$, TAG-$1^{+/-}$ and TAG-$1^{-/-}$ mouse embryos. There was a reduction in expression of endogenous AICD in the heterozygous and homozygous TAG-1 null mouse brains that corresponded with the reduction in expression of TAG-1 (**Fig. 22E**). In addition to AICD production, which requires γ-secretase cleavage, CTF-α and CTF-β were detected in the *in vitro* cell culture experiments. There was a marked preponderance of CTF-α over CTF-β but both CTF-α and CTF-β increased, in addition to AICD in response to TAG-1 (**Fig. 21A; Fig. 22A**, **C** and **E**) suggesting that, while α-secretase cleavage dominates, both α- and β-secretase cleavage increase in response to TAG-1.

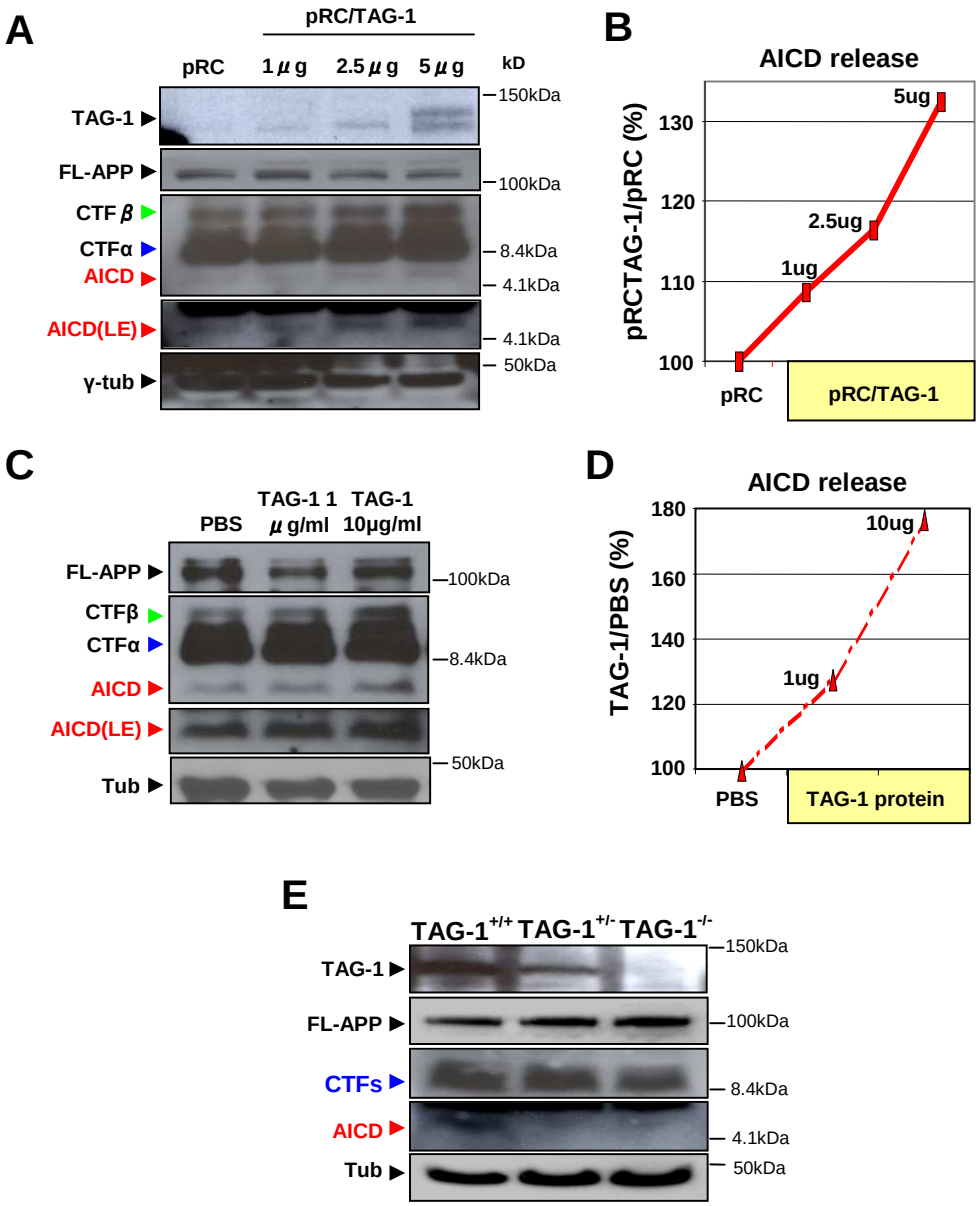

Figure 22 TAG-1 triggers endogenous AICD release.
Total proteins were western blotted using antibodies against C-terminal APP (A8717), TAG-1 (TG1) and γ-tubulin. (**A**, **B**) MEF cells were transfected with 1 µg, 2.5 µg or 5 µg of TAG-1 cDNA in pRC vector or 2.5 µg empty pRC vector as control. (**C**, **D**) MEF cells were treated for 3 hr at 37 ◦C with PBS or 1 µg/ml or 10 µg/ml TAG-1. (**E**) E15 brain from TAG-$1^{+/+}$, TAG-$1^{+/-}$ and TAG-$1^{-/-}$ mouse embryos. LE: longer exposure. FL-APP: full length APP. Western blots were helped by Mr. Toshi Futagawa.

4.18 TAG-1 triggers AICD release in NPCs

TAG-1 and APP inhibit neurogenesis in a convergent signalling pathway where APP acts as the receptor of TAG-1. TAG-1 triggers AICD to release from the full-length APP in CHOAPP, MEF and fetal mouse brains. To further examine whether TAG-1 is a function ligand of APP in NPCs, we used the luciferase reporter system in cells isolated from TAG-1$^{-/-}$APP$^{-/-}$ embryos to investigate whether TAG-1-APP interaction could modulate AICD release in fetal NPCs. After transfection with the APP luciferase reporter system, *TAG-1$^{-/-}$APP$^{-/-}$* NPCs were cultured as monolayers loaded onto culture dishes co-coated with TAG-1-Fc, L1-Fc, or F3-Fc and laminin. As expected, TAG-1-Fc, but not F3-Fc, L1-Fc or laminin, strongly triggered reporter activity (**Fig. 23A**), indicating that AICD can be released. A specific γ-secretase inhibitor (L-685,458) blocked the induction of AICD release by TAG-1 in a dose-dependent manner (**Fig. 23B**), indicating that γ-secretase is involved in the TAG-1 triggered RIP process in the TAG-1$^{-/-}$APP$^{-/-}$ fetal NPCs. Similar to the observations in both CHOTAG-1 and CHOTAX cells (**Fig. 19**), these experiments demonstrate that AICD-dependent activity in the luciferase reporter system is also regulated by the interaction of TAG-1 and APP in NPCs.

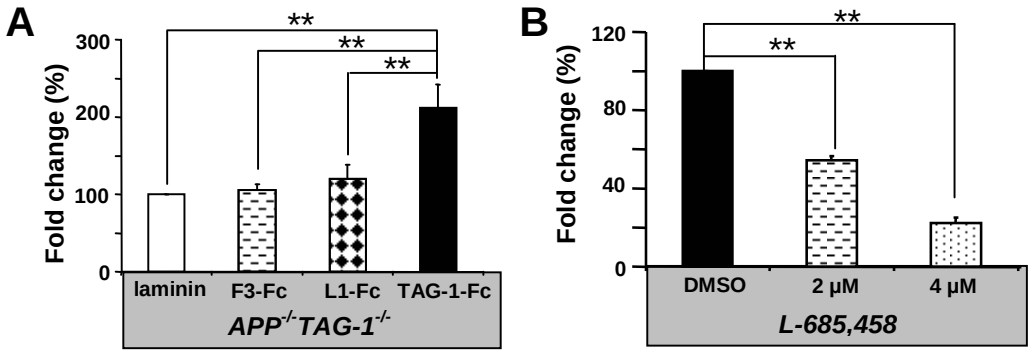

Figure 23 TAG-1 triggers AICD release in NPCs.
TAG-1 triggered release of AICD-Gal4 in NPCs of TAG-$1^{-/-}$$APP^{-/-}$ double deficient mice. NPCs isolated from TAG-$1^{-/-}$$APP^{-/-}$ mice were transiently co-transfected with pG5E1B plasmid, an APP-Gal4 construct, Fe65 plasmid, and luciferase internal control plasmid. (**A**) The transfected NPCs were cultured in 24-well culture dishes substrate-coated with laminin, F3-Fc, L1-Fc, and TAG-1-Fc as indicated. Normalized luciferase activities in whole-cell lysates were determined and expressed relative to the activity in lysates prepared from laminin treated cells. (**B**) TAG-1 triggered luciferase activity in TAG-$1^{-/-}$$APP^{-/-}$ NPCs was significantly reduced by a γ-secretase inhibitor (L-685,458, 2 µM and 4 µM, respectively). Results are means ± s.e.m, n=3-6, ** p<0.001.

The release of AICD requires γ-secretase. NPCs were treated with γ-secretase inhibitor, which could abolish the release of AICD from full-length APP, and subjected into neurogenesis analysis after 7-8 days *in vitro* differentiation. TUJ1-positive neurons increased significantly in the presence of γ-secretase inhibitor comparing to the DMSO control group in $APP^{+/+}$ mice (**Fig. 24A**), suggesting that the release of AICD may be required for the inhibition of neurogenesis by TAG-1-APP signalling pathway. However, γ-secretase inhibitor enhanced neurogenesis of NPCs from $APP^{-/-}$ mice as well (**Fig. 24B**). This may be due to inhibition of the Notch signalling pathway by γ-secretase inhibitor in the presence of APP.

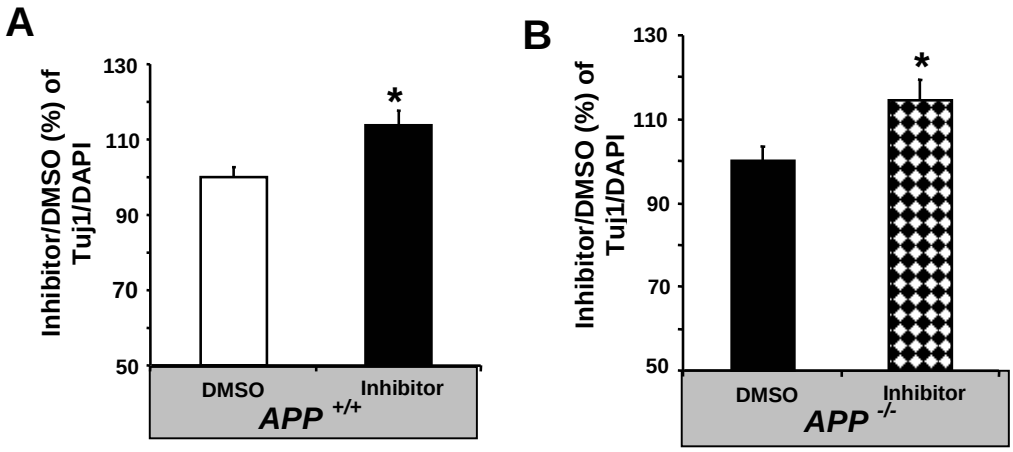

Figure 24. Effects of γ-secretase inhibitor on neurogenesis.
To investigate the effect of a γ-secretase inhibitor on neurogenesis, we treated (**A**) $APP^{+/+}$ and (**B**) $APP^{-/-}$ NPCs with Mw167 (20μM) and DMSO as a control. After 7-8 days *in vitro* differentiation, the cells were double-stained for TUJ1 and DAPI. The number of TUJ1 positive neurons were counted and expressed as a percentage of the total number of DAPI positive neurons, normalized to the DMSO control condition. Results are means ± s.e.m, n=3-6, * $p<0.05$.

4.19 AICD is necessary for the negative modulation of neurogenesis by the TAG-1-APP signalling pathway

To test whether AICD is required for the negative modulation of neurogenesis by the TAG-1-APP signalling pathway, we constructed AICD59, an intracellular active fragment of APP released after TAG-1-APP interaction, in a pCDF1-MCS1-EF1-copGFP vector, where green fluorescent protein (GFP) was expressed together with AICD59, but under control of another promoter. We transfected AICD59 to empty vector containing GFP alone into $TAG\text{-}1^{-/-}$ fetal NPCs and performed neurogenesis assay after 3 days *in vivo* differentiation. The number of TUJ1-positive cells in the AICD59 transfected group was significantly decreased compared to the control group transfected with the empty vector containing GFP alone (**Fig. 25;** $p<0.001$).

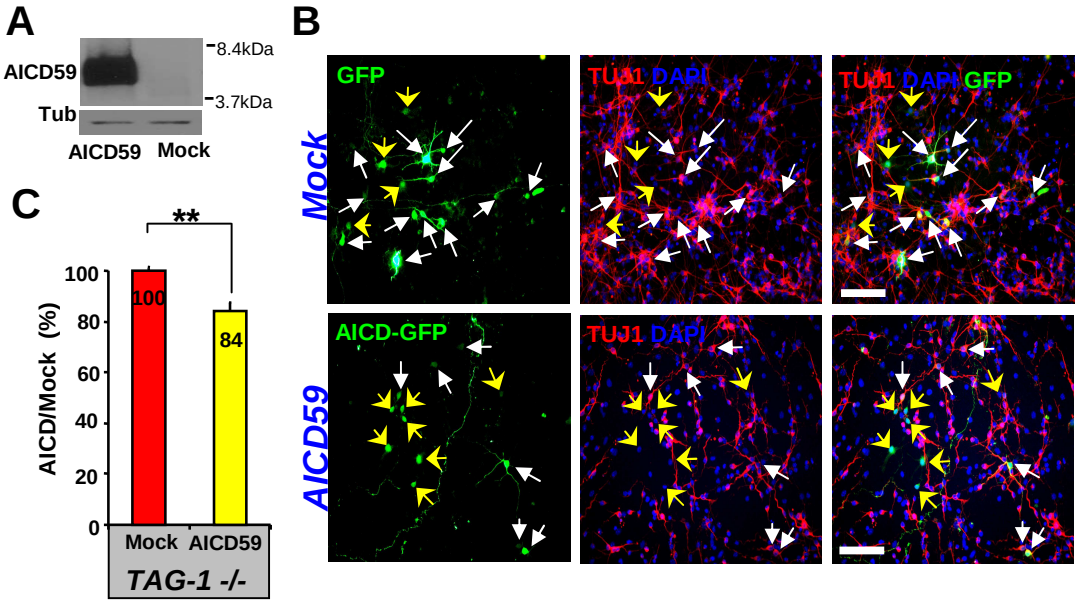

Figure 25 Enhanced neurogenesis of TAG-1$^{-/-}$ NPCs is reduced by AICD.
(**A**) The human AICD59 cDNAs were cloned into the pCDF1-MCS1-EF1-copGFP mammalian expression vector. HEK293 cells in 60-mm plates were transfected with AICD59 or empty vector (Mock) and harvested 24 hrs after transfection. The cell lysate was subjected to western blot using antibodies against c-terminal APP (AICD59) and γ-tubulin (Tub). (**B**) NPCs were isolated from TAG-1$^{-/-}$ and co-transfected with AICD59 in a pCDF1-MCS1-EF1-copGFP vector (AICD59) and empty vector containing only GFP (Mock) as a control. After differentiation, the cells were double-stained for TUJ1 and DAPI. White arrows indicate neurons infected with empty vector or AICD59, and yellow arrows indicate non-neurons infected with empty vector or AICD59. (**C**) Tuj1-positive green cells were quantified relative to the total number of green cells, and further normalized the percentage of neurons derived from AICD59-transfected NPCs (AICD59) relative to from NPCs transfected with empty vector (Mock). Bars is 100 μm. Results are means ± s.e.m, n=3-6, ** p<0.001.

AICD is suggested to be involved in apoptosis in neuronal cells (Minopoli et al., 2006). To investigate whether cell death could be responsible for the phenotype that AICD reduces neurogenesis in TAG-1$^{-/-}$ NPCs, we performed TUNEL assay in TAG-1$^{-/-}$ NPCs transfected either empty vector or AICD. No any detectable difference was observed in the number of apoptotic cells between the two groups (**Fig. 26**).

In summary, TAG-1 is a functional ligand of APP, which can trigger the RIP of APP and cause the intracellular domain of APP (AICD) to be released from full-length APP. The TAG-1-APP signalling pathway inhibits neurogenesis via AICD. However, what are the down-stream elements after AICD?

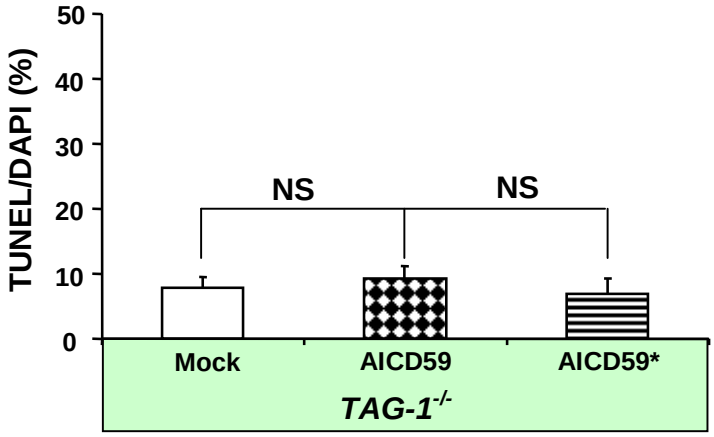

Figure 26 Both AICD59 and AICD59* do not induce apoptosis in TAG-1$^{-/-}$ NPCs.
To investigate whether overexpression of AICD induces apoptosis in TAG-1$^{-/-}$ NPCs, NPCs were isolated from TAG-1$^{-/-}$ mice and transfected with AICD59, AICD59 mutant (with the NPTY to NATA mutation; AICD59*) or empty vector containing only GFP (Mock) as a control. After 7-8 days *in vitro* differentiation, the cells were double-stained for TUNEL and DAPI. TUNEL-positive cells were counted and expressed as a percentage of the total number of green cells. Compared to the control vector group, there were no detectable differences. Results are means ± s.e.m, n=3, NS: non-significant.

4.20 Fe65 is expressed in the neurogenic ventricular zone and NPCs and negatively modulates neurogenesis

The mammalian Fe65 protein family consists of Fe65, Fe65L1 and Fe65L2. This class of scaffolding proteins has three structural domains, which include a WW and two phosphotyrosine binding domains (PID1-PTB1 and PID2-PTB2) that mediate protein–protein interactions. All Fe65 protein family members bind members of the APP protein family (APP, APLP1 and APLP2) through the C-terminal PID2-PTB2 domain (Mcloughlin and Miller, 2008). It has been suggested that Fe65 plays a role in AICD-dependent transcriptional activation, but, unlike Fe65, the interactions between Fe65L1 and Fe65L2 with APP do not activate

APP-dependent transcription (Mcloughlin and Miller, 2008). Therefore, we investigated whether Fe65 acts as a downstream element in the TAG-1-APP signalling pathway regulating neurogenesis. We studied the localization of the expression of Fe65 in E14 mouse brain and in isolated NPCs from E14 mouse telencephalic ventricular walls by double IF labelling using antibodies against Fe65 and nestin or Sox2. In the E14 mouse brain, IF showed that Fe65 was co-localized with nestin in the ventricular walls (**Fig. 27A**). NPCs were double-stained for Fe65 and Sox2 (**Fig. 27B**). Moreover, western blotting detected a Fe65 band in both NPCs and total mouse brain (**Fig. 27C**). These results demonstrate that Fe65, like TAG-1 and APP, is expressed by NPCs in the neural stem cell niche.

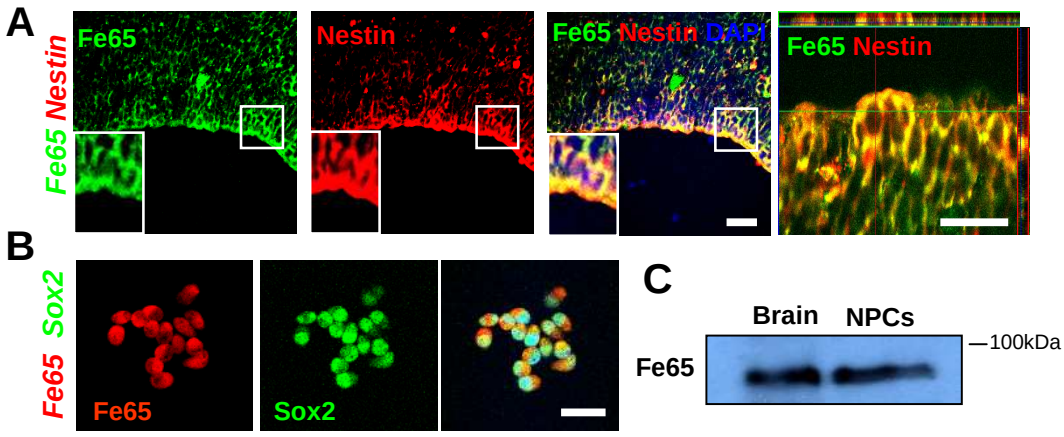

Figure 27 Fe65 is expressed in telencephalic ventricular walls of E14 mouse brain and NPCs derived from E14 mouse telencephalic ventricular walls.
(**A**) Double immunostaining for nestin (red) and Fe65 (green) in the walls of the lateral ventricles in E14 mouse brain. (**B**) NPCs isolated from the lateral ventricles of E14 mouse brain were double-stained for Sox2 (green) and Fe65 (red). (**C**) Mouse brain samples and the NPCs lysate were Western-blotted with antibodies against Fe65. Bars in **A** and **B** are 20 µm.

We further performed neurogenesis analysis in NPCs from $Fe65^{-/-}$ mice. 41.11 ± 0.96% TUJ1-positive cells differentiated from $FE65^{-/-}$ NPCs, while only 29.78 ± 0.87% TUJ1-positive cells were observed after differentiation from $FE65^{+/+}$ NPCs (not shown). After normalization, a 38% increase in TUJ1-positive cells was

detected after differentiating from Fe65$^{-/-}$ NPCs comparing to from Fe65$^{+/+}$ NPCs (**Fig. 28**). TUNEL assay showed that the number of apoptotic cells was not different between Fe65$^{-/-}$ and Fe65$^{+/+}$ NPCs (**Fig. 29**). Thus, similar to TAG-1$^{-/-}$, APP$^{-/-}$ and TAG-1$^{-/-}$APP$^{-/-}$ mice, Fe65 deletion also leads to abnormal enhancement of neurogenesis.

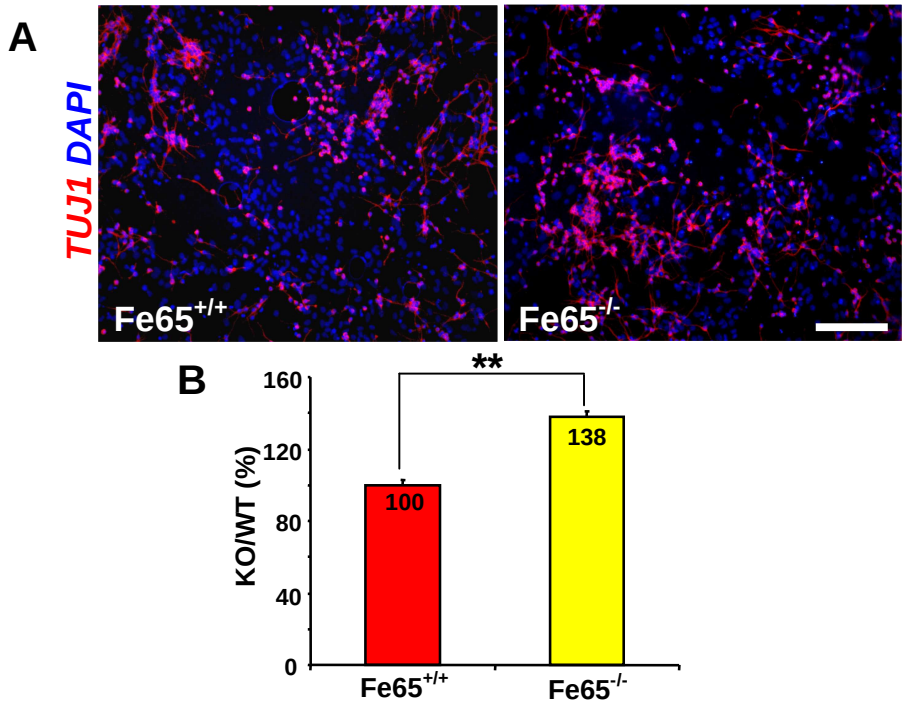

Figure 28 Fe65 inhibits neurogenesis.
(**A**) NPCs were isolated from Fe65$^{+/+}$ mice (WT) and Fe65$^{-/-}$ (KO) mice. After 7-8 days *in vitro* differentiation, the cells were double-stained for TUJ1 and DAPI. (**B**) The number of TUJ1 positive neurons was counted and expressed as a percentage of the number of DAPI positive cells and normalized to the respective wild-type littermate control. Scale bar in **A** is 20 µm. Results are means ± s.e.m, n=3, ** p<0.001.

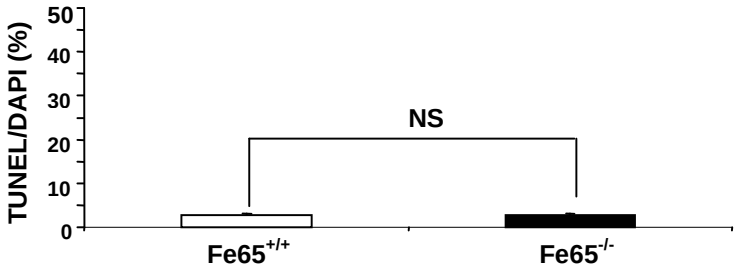

Figure 29 Fe65 does not induce apoptosis in NPCs.
NPCs were isolated from $Fe65^{+/+}$ and $Fe65^{-/-}$ mice. After 7-8 days *in vitro* differentiation, the cells were double-stained for TUNEL and DAPI. The number of TUNEL positive cells were counted and expressed as a percentage of the total number of DAPI positive cells. NS: non-significant.

4.21 Fe65 is a downstream element of TAG-1-APP signalling during modulation of neurogenesis

The aforementioned observations imply that Fe65 may act as a downstream element in the TAG-1-APP signalling pathway. To test this hypothesis, we introduced a luciferase reporter system in which Gal4 is fused to the N-terminal of Fe65 (Cao and Südhof, 2004) into CHOTAG-1 cells. When co-transfected with APP, but not when transfected with the empty vector as a control, a significant upregulation of reporter activity was observed in CHOTAG-1 cells (**Fig. 30A**). Notably, co-transfection with an APP mutant (APP*) that abolishes Fe65 binding and transactivation (NPTY to NATA mutation; Cao and Südhof, 2004) did not increase the TAG-1 triggered Fe65-dependent transcriptional activity (**Fig. 30A**). A specific γ-secretase inhibitor, DAPT, reduced the TAG-1 triggered Fe65-dependent transcriptional activity (**Fig. 30A**). We introduced this luciferase reporter system into CHO cells. When TAG-1 cDNA, but not TAG-1 alone or the empty vector control, was co-transfected with APP cDNA, but not the APP* mutant, a significant upregulation of reporter activity was observed, which could be reduced by DAPT (**Fig. 30B**). Moreover, we investigated whether both Fe65 and TAG-1 could modulate intracellular release of AICD in wild-type fetal NPCs. After transfection with the APP-Gal4 luciferase reporter system and Fe65, NPCs were cultured as monolayers loaded onto culture dishes co-coated with TAG-1-

Fc and laminin. As expected, Fe65 increased the AICD-dependent reporter activity in the NPCs compared to mock control transfection with the empty vector (**Fig. 30C**). Consistently, after TAG-1-Fc treatment, the AICD-dependent reporter activity was even more strongly increased in the Fe65-transfected NPCs compared with the non-treated group (**Fig. 30C**). Thus, these results demonstrate that TAG-1 regulates not only AICD-dependent, but also Fe65-dependent, activity in luciferase reporter systems in a γ-secretase dependent manner suggesting that TAG-1 stimulation of APP leads to intracellular release of AICD and activation of Fe65.

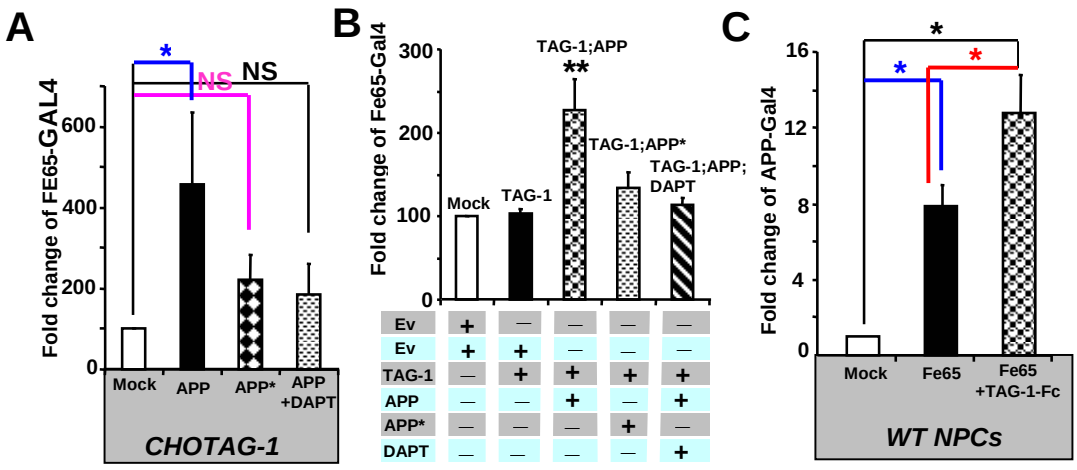

Figure 30 Fe65 is a down-stream element of TAG-1-APP signalling pathway.
(**A**) Activity of Fe65-Gal4 in CHOTAG-1 cells. CHOTAG-1 cells were transiently co-transfected in 24-well culture dishes with pG5E1B plasmid, a Fe65-Gal4 construct, luciferase internal control plasmid, and APP cDNA or an APP mutant (with the NPTY to NATA mutation; APP*) as well as empty vector (Mock). A γ-secretase inhibitor (DAPT: 40 μM) significantly reduced luciferase activity. (**B**) Activity of Fe65-Gal4 in CHO cells. CHO cells were transiently co-transfected in 24-well culture dishes with pG5E1B plasmid, a Fe65-Gal4 construct, luciferase internal control plasmid, TAG-1 cDNA and APP cDNA or an APP* mutant as well as empty vector (Ev; Mock). DAPT (40 μM) significantly reduced luciferase activity. (**C**) TAG-1 triggered AICD release in wild-type NPCs. NPCs were transiently co-transfected with pG5E1B plasmid, an APP-Gal4 consrtuct, Fe65 plasmid, and luciferase internal control plasmid. The transfected NPCs were cultured in 24-well culture dishes with or without TAG-1 protein coating. Normalized luciferase activities in whole-cell lysates were expressed relative to activity in lysates prepared from TAG-1 transfected CHO cells (**A** and **B**) or CHO cells (**B**) or NPCs (**C**) with mock control transfection. Results are means ± s.e.m or s.d. (**C**), n=3-5, ** p<0.001, * p< 0.05. NS: non-significant.

Next, we investigated whether TAG-1 could modulate neurogenesis of NPCs in the absence of Fe65. TAG-1 protein was applied to trigger the TAG-1-APP signalling pathway in fetal NPCs isolated from either $Fe65^{+/+}$ or $Fe65^{-/-}$ mice. The number of TUJ1- positive cells was significantly decreased in the $Fe65^{+/+}$ mice by TAG-1 protein, compared to the PBS-treated group (**Fig. 31**). However, TAG-1 could not reverse the abnormal increase in neurogenesis in the $Fe65^{-/-}$ mice (**Fig. 31**). Thus, Fe65 is required for signalling transduction in the TAG-1-APP signalling pathway.

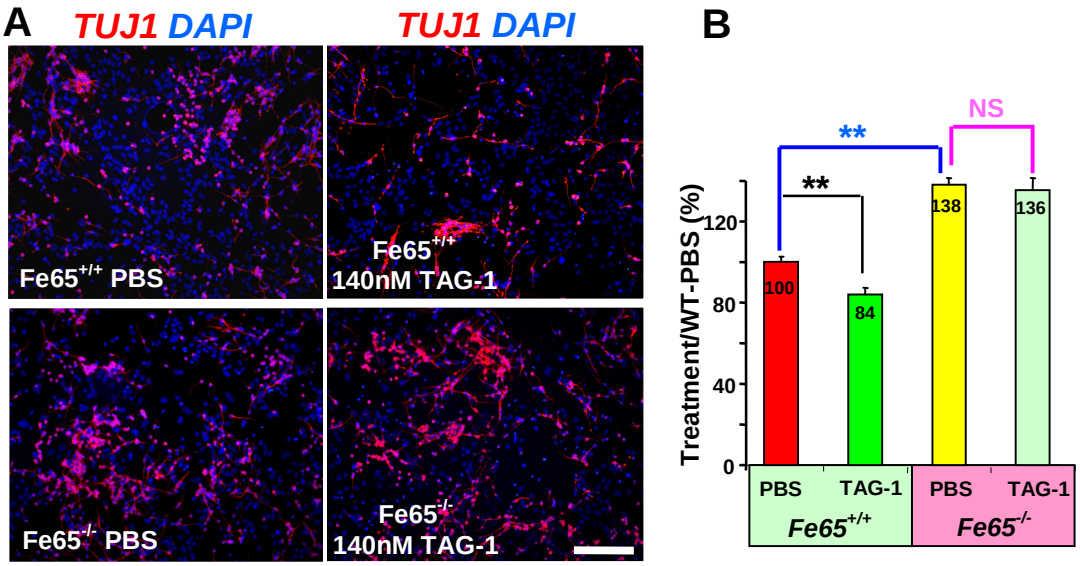

Figure 31 Fe65 is required for modulation of neurogenesis by TAG-1.
(**A**) NPCs were isolated from $Fe65^{+/+}$ and $Fe65^{-/-}$ mice and treated with TAG-1 and PBS as a control. After 7-8 days *in vitro* differentiation, the cells were double-stained for TUJ1 and DAPI. (**B**) TUJ1-positive cells were quantified relative to the number of DAPI-positive cells, and normalized to the wild-type PBS control. Results are means ± s.e.m, n=3, ** p<0.001. NS: non-significant.

The release of AICD from full-length APP is required for the negative modulation of the TAG-1-APP signalling pathway on neurogenesis. Interaction between Fe65 and AICD is important for further transduction of signalling (Cao and Südhof, 2004). To check whether interaction of Fe65 and AICD could occur in the modulation of neurogenesis by the TAG-1-APP signalling pathway, we transfected an AICD59 mutant construct (AICD59* that has a NPTY to NATA mutation), into TAG-$1^{-/-}$ NPCs. In contrast to AICD59, AICD59* could not reverse the abnormal enhancement of neurogenesis in TAG-$1^{-/-}$ NPCs (**Fig. 32**). There was no detectable difference in apoptosis between each group (**Fig. 26**). Given that the abnormal enhancement of neurogenesis in the TAG-$1^{-/-}$ NPCs can be reversed by either application of TAG-1 (**Fig. 32**) or transfection of AICD59 (**Fig. 32**), and that AICD59* abolishes Fe65 binding and transactivation (Cao and Südhof, 2004), together these observations strongly support the notion that Fe65 acts as a downstream element in the TAG-1-APP signalling pathway negatively regulating neurogenesis.

In summary, we have identified the TAG-1 is a functional ligand of APP. Upon binding with TAG-1, APP is cleaved and releases its intracellular domain (AICD), which further interacts with Fe65. We have demonstrated that this TAG-1-APP signalling pathway inhibits neurogenesis (**Fig. 33**).

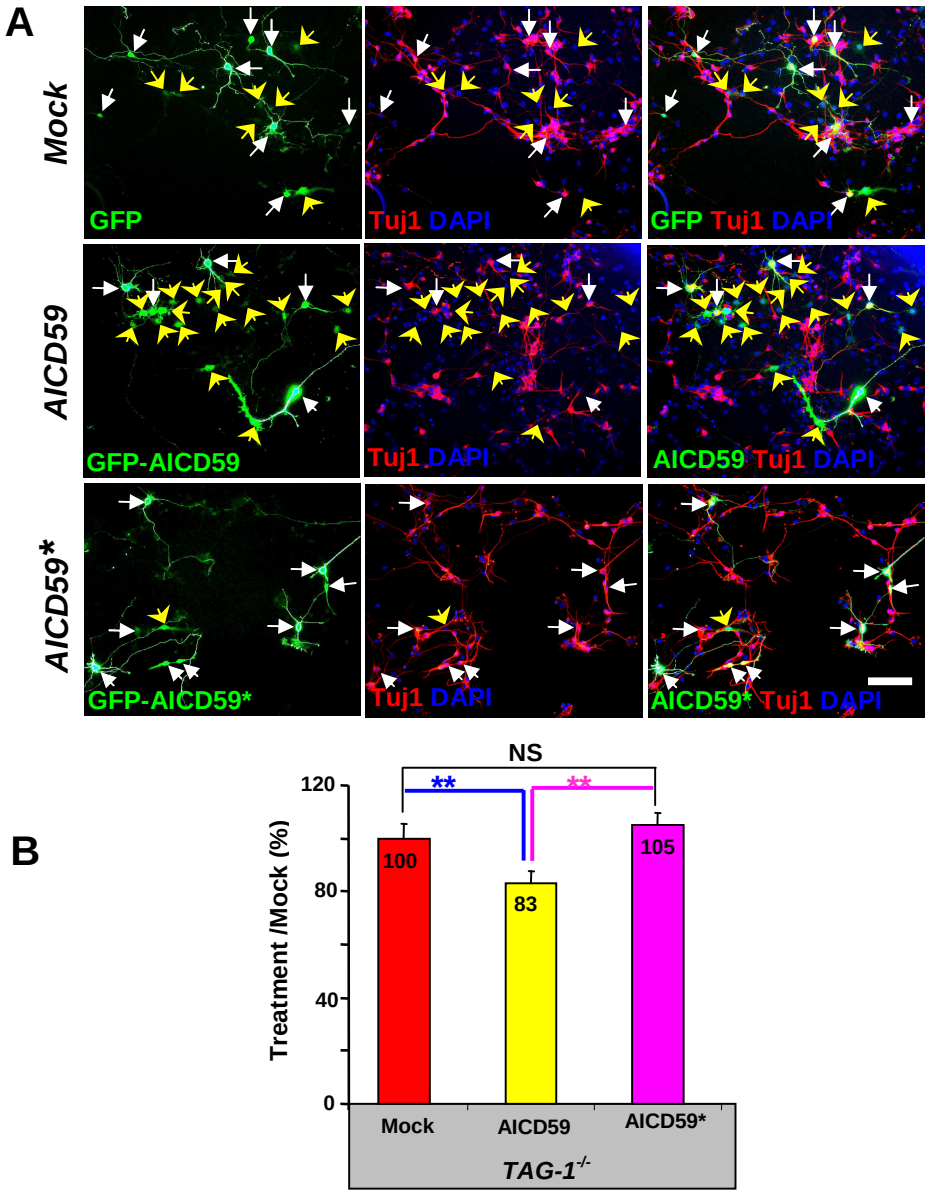

Figure 32. Fe65-AICD binding is required for inhibition of neurogenesis by TAG-1. (**A**) NPCs were isolated from TAG-1$^{-/-}$ and transfected with AICD59 in a pCDF1-MCS1-EF1-copGFP vector (AICD59), an AICD mutant (with the NPTY to NATA mutation; AICD59*) and empty vector containing only GFP (Mock). After 7-8 days *in vitro* differentiation, the cells were double-stained for TUJ1 and DAPI. White arrows indicate neurons infected with AICD59, AICD59* or empty vector and yellow arrows indicate non-neurons infected with AICD59, AICD59* or empty vector. (**B**) TUJ1-positive green cells were quantified relative to the number of all green cells, and normalized to the control group (Mock). Results are means ± s.e.m, n=3, ** p<0.001.

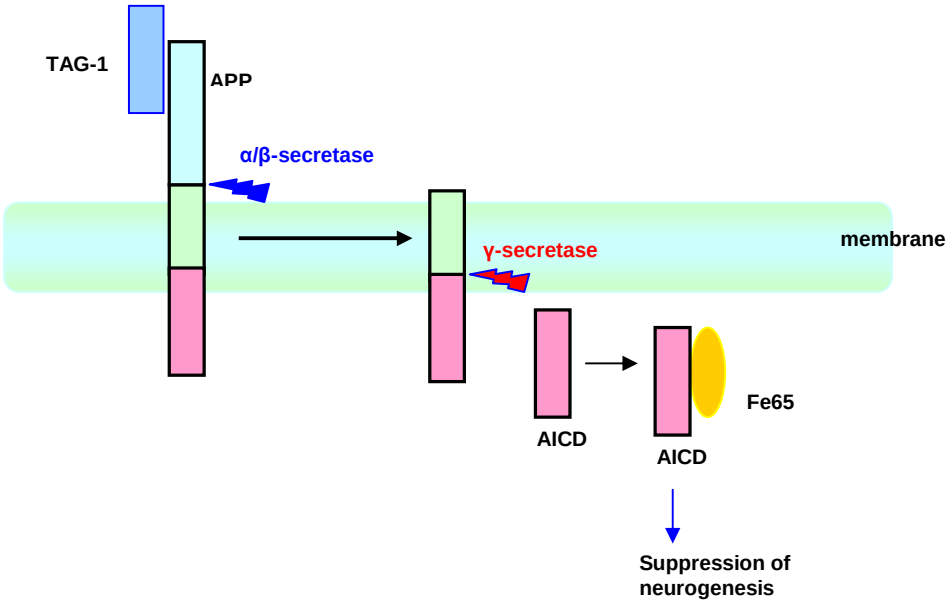

Figure 33. A diagrammatic summary of a working model of TAG-1/APP signalling. TAG-1 is a ligand of APP. When TAG-1 binds to APP, this stimulates or facilitates ectodomain sheding by α- or β-secretase. Once the ectodomain has been shed, γ-secretase cleavage of the membrane bound stub can proceed. Thus, TAG-1 leads to γ-secretase-dependent cleavage releasing AICD intracellularly. AICD interacts with the scaffolding protein, Fe65. This results in an Fe65-dependent suppression of neurogenesis.

5 Discussions

5.1 TAG-1 is a functional ligand of APP that triggers RIP of APP

Regulated-intramembrane proteolysis (RIP) is an important molecular process during development. Several molecules have been found to be processed by RIP, to release their intracellular activated fragments from full-length receptors to participate biological development (Ebinu and Yankner, 2002). Several features of APP are consistent with the characteristics of RIP, suggesting the possibility that APP is also processed by RIP. **i**) APP is a type I transmembrane protein, with a large extracellular domain (Suh and Checler, 2002). **ii**) APP is cleaved by α/β-secretase and γ-secretase, and its released fragments have been already identified, such as α-C-terminal fragments (CTFs), β–CTFs and AICD in intracellular and sAPP α/β, P3 and A β in extracellular of cells (Suh and Checler, 2002). **iii**) AICD has been suggested to be involved in regulation of transcriptional activity (Cao and Südhof, 2001). One of the features of RIP is that a receptor has to bind to its ligand and that this binding causes the extracellular domain of the receptor to be first removed by extracellular cleavage, which is a prerequisite for the second intramembrane cleavage (Brown et al., 2000). Therefore, identification of the ligand of APP is important for APP signalling. Several binding partners of APP have been identified, such as Low density lipoprotein (LDL) receptor-related protein (LRP) (Cam et al., 2005) and its homologous protein, low density lipoprotein (LDL) receptor-related protein 1B (LRP1B) (Cam et al., 2004), F-spondin (Ho and Sudhof, 2004; Hoe et al., 2005), PDGF (Gianni et al., 2003), laminin (Kibbey et al., 2003) and furin (Hwang et al., 2006). All of these Interact with APP and modulate the proteolytic cleavage of APP. Both LRP and LRP1B modulate α- or β- cleavage of APP via regulating the trafficking of APP (et al., 2005; Cam et al., 2004), since the retention of APP on the cell surface decreases Aβ generation but increases sAPPα (Cescato et al., 2000). Furin enhances the α-cleavage of APP via increasing the activity of α-secretase (Hwang et al., 2006). PDGF and F-spondin, are reported to regulate

the generation of C-terminal fragements of APP in an artifical luciferse system (Gianni et al., 2003; Ho and Südhof, 2004; Hoe et al., 2005). However, previous studies have provided little evidence to conclude that any of these ligands of APP are functional ligands of APP capable triggering RIP of APP.

GPI-linked proteins are anchored to the outer leaflet of the plasma membrane and mediate the dynamic remodelling of membranes during cell-cell interactions. F3/contactin and its homologue NB-3 are functional ligands of notch receptor, which trigger the RIP of notch, releasing its intracellular domain (NICD), forming a transcriptional complex with Deltex1 to modulate gene expression of MAG (Hu et al., 2003; Cui et al., 2004). In present study, we have identified TAG-1, another member of the F3/contactin family, as a binding partner of APP. We have confirmed the interaction between TAG-1 and APP using cell adhesion assays and co-immunoprecipitation, Fc-pull down and GST-pull down analysis. In extracts from wild-type brain, antibodies to TAG-1 could precipitate APP, and vice versa. There was no co-immunoprecipitation in samples from $APP^{-/-}$ and $TAG\text{-}1^{-/-}$ mice suggesting that the interaction was specific. TAG-1 is a member of a subfamily of GPI-anchored molecules containing six Ig domains and four fibronectin type III (FNIII) repeats (Karagogeos, 2003). The results from cell adhesion assays and GST precipitation assays, in which Ig domains and fibronectin type III (FNIII) repeats of human TAG-1(TAX) were used as the bait to prey APP, indicate that both domains are required for the binding to APP. We also indentified that the extracellular domain of APP may contain the motif binding to TAG-1. In contrast to APP-Gal4, which can be activated in response to TAG-1 in the luciferase system, C99-Gal4, the fragment of APP which lacks the extracellular domain, failed to respond to TAG-1 in the same luciferase system. This is consistent with the characteristics of extracellular domain of APP. Various binding motifs are found in the extracellular domain of APP, such as inhibitor domain, heparin binding domain (Mok et al., 1997), Cu^{2+} and Zn^{2+} binding domain (Hesse et al., 1994; Bush et al., 1993; Gralle, 2007). Additionally, an N-

glycosylation site has been found in the extracellular domain of APP (Pahlsson and Spitalnik, 1996; Gralle, 2007). However, more investigation is required to further identify the exact amino acids on APP that binds to TAG-1.

To identify whether TAG-1 can trigger RIP of APP, we performed luciferase assays using an artificial GAL4-fused luciferase system. In this system, the yeast Gal4 DNA-binding domain was inserted into the intracellular tail of full-length APP at the cytoplasmic boundary of the transmembrane region (Cao and Südhof, 2001). Therefore, the luciferase reporter activities can basically reflect the amount of AICD-Gal4 released from full-length APP-Gal4. We found that TAG-1 can enhance the activity of this luciferase reporter in both CHO cell lines and neural stem cells in a γ-secretase-dependent manner. To further confirm the idea that TAG-1 can trigger RIP of APP, we performed Western blot analysis. Using CHOAPP cells transfected with cDNAs of Fe65, PS1 and TAG-1 and CHO cells co-transfected with cDNAs of APP-V5, Fe65, BACE1 and TAG-1, we have shown that TAG-1 significantly increases AICD release, which could be blocked by a γ-secretase inhibitor. Moreover, we also showed that endogenous AICD production in MEF cells is dose-dependently increased by transfection with TAG-1 protein or treatment with TAG-1 but not by treatment with F3 protein. Consistently, endogenous AICD production was reduced in embryonic TAG-$1^{-/-}$ mouse brains compared to wild-type mice. In addition to AICD, production of α-CTF and β-CTF also has been observed increased in response to treatment of TAG-1. These results are consistent with one of the features of RIP, ectodomain removal is prerequisite for the following intramembrane cleavage. Therefore, all of this evidence indicates that TAG-1 is the functional ligand of APP, which triggers the proteolytic cleavages of APP by series secretases, and then release the intracellular fragment of APP from the full-length APP.

5.2 TAG-1/APP signalling in neural stem cells

Discussions

Increasing evidence suggests potential roles for both APP and TAG-1 in the development of neural stem cells. APP mRNA transcripts can be detected in mouse ocytes and early in mouse embryogenesis (Fisher et al., 1991), such as in the mouse neural tube on E9.5, a stage when the neural stem cells and RC2-positive radial glia are actively dividing (Salbaum et al., 1994). APP is expressed by neuroepithelial cells of the cortical ventricular zone, particularly in the apical portion where mitosis takes place at E14 to E16 (Lopez-Sanchez et al., 2005). Moreover, secreted N-terminal nonamyloidogenic APP acts as an EGF cofactor to stimulate proliferation of the cells from embryonic neurospheres *in vitro* and its major binding sites locate on the surface of type-A and -C cells in the SVZ (Caillé et al., 2004). Similarly, the expression of TAG-1 appears early in development, for example on the cell bodies of motor neurons in spinal cord at E10.5 and during their lateral migration from the ventricular zone at E13 (Karagogeos, 2003). Thus, our data link the existing evidence for roles for TAG-1 and APP in neural development, showing that TAG-1 and APP are involved in negative modulation of neurogenesis. Notably, if TAG-1 and APP suppressed neurogenesis by independent mechanisms, it might be expected that the effects of knocking out TAG-1 and APP would be additive. However, NPCs from TAG-1/APP double null mice showed a similar enhancement in neurogenesis to NPCs from single TAG-1 and APP null mice, suggesting that the mechanisms by which TAG-1 and APP reduce neurogenesis are convergent. We have further identified APP as the receptor to transduce signals from TAG-1 to inhibit neurogenesis. TAG-1 protein could rescue the abnormal increased neurogenesis in NPCs derived from TAG-1 null mice, but failed to do so in NPCs derived from TAG-1/APP double null mice, indicating that APP acted as the receptor of TAG-1 in neurogenesis inhibition. This result is also consistent with the one that TAG-1 only binds to APP, but not binds to other members of APP family, APLP1 and APLP2, although they share homologous structures with APP and has been suggested involved in RIP as well (Naruse et al., 1998). APP and APLP1, APLP2 are known to compensate functionally for one another as evidenced by the

perinatal lethality of APP/APLP2 and APLP1/APLP2 double null mice that is not observed in deletions of the individual genes (Zheng et al., 1995; von Koch et al., 1997; Heber et al., 2000). However, our data suggested that they are not necessary to share same ligands with APP despite of their similar structures to APP. Their functional compensation may due to same intracellular fragments releasing after distinct ligand binding.

TAG-1 triggers the proteolytic cleavage of APP in a γ-secretase-dependent manner. A γ-secretase inhibitor has been found to enhance the neurogenesis of wild-type NPCs, suggesting that TAG-1/APP inhibit neurogenesis may require γ-secretase. However, the γ-secretase inhibitor has same function on $APP^{-/-}$ NPCs. That may be caused by reduced activity of notch signalling pathway by γ-secretase inhibitor in absence of APP. Presenilin1 (PS1), the core enzyme of γ-secretase complex, has been reported to enhance neurogenesis during embryogenesis. Abnormal premature neurons increased in $PS1^{-/-}$ fetal cortex, meanwhile a down-regulation of Hes5, the down-stream effector of notch signalling pathway, has been observed (Handler et al., 2000). Hes5 has a repressive function on neurogenesis during embryogenesis (Louvi and Artavanis-Tsakonas, 2006). In addition, lack of γ-secretase activity affects the activities of β-catenin, which is involved in neurogenesis as well (Lie et al., 2005).

Consistent with the finding that AICD release is triggered by TAG-1, AICD could rescue the abnormally increased neurogenesis in NPCs derived from $TAG\text{-}1^{-/-}$. Therefore, TAG-1/APP/AICD signalling inhibits neurogenesis. In addition to AICD, the cleavage of APP generates various fragments such as sAPP, Aβ. sAPPα/β can stimulate proliferation of neural progenitor cells from C-type in adult SVZ and embryonic neurospheres (Caillé et al., 2004). Aβ can trigger a Fas- and caspase independent apoptotic pathway in cultured neural progenitor cells and promotes the neuronal differentiation of neural precursor cells (Millet et al., 2005). TAG-1 triggers the cleavage of APP in a α-secretase cleavage dominant

way. Whether sAPPα/β and Aβ are also involved in neurogenesis remain to be further investigated.

APP has various adaptor proteins via binding to its cytoplasmic c-terminal, such as Fe65, X11, mDabl, Jib (Russo et al., 2005). Fe65 has one ww domain and two PTB domains. Fe65 binds to cytoplasmic terminal of APP via its second PTB domain. The interaction between APP and FE65 is reported to have an important role in brain development (Ikin et al., 2007; Guénette et al., 2006). To identify whether FE65 is involved in inhibition of neurogenesis by the TAG-1/APP/AICD signalling pathway, we analysed the neurogenesis of NPCs derived from E14 $Fe65^{+/+}$ and $FE65^{-/-}$ telencephalic brain. The results indicated that Fe65 had the same function on neurogenesis as AICD/APP and TAG-1. The interaction between Fe65 and APP is very important in the transduction of APP signalling after its proteolytic cleavage. So we used another artificial luciferase system that Fe65 was fused a Gal4 at its cytoplasmic terminal and transfected APP or APP* (cannot bind to Fe65), and luciferase reporter in cell lines. With the treatment of TAG-1, APP could increase the luciferase activity of FE65-Gal4, but APP*, failed to do so. TAG-1 could inhibit neurogenesis in NPCs derived from E14 $Fe65^{+/+}$ mice, while this function was abolished in presence of Fe65. Thus, Fe65 is a downstream molecule of TAG-1/APP signalling. As mentioned, the Fe65 family has other two proteins, FE65L1 and Fe65L2, in addition to Fe65. All three proteins share homologous structure and all of them bind to APP (McLoughlin and Miller, 2008). However, the interaction between Fe65L1 or Fe65L2 and APP cannot transactivate gene expression in a luciferase system (Tanahashi and Tabira, 2002; Chang et al., 2003). This finding is consistent with our result that in absence of Fe65, TAG-1 has no function on neurogenesis although both Fe65L1 and Fe65L2 are present. Moreover, AICD*, which cannot bind to FE65, failed to rescue the abnormal increased neurogenesis in $TAG\text{-}1^{-/-}$ NPCs. All these results further confirmed that Fe65 was a downstream element of TAG-1/APP signalling

pathway in neurogenesis inhibition, and binding AICD to Fe65 is necessary for transduction the signals from TAG-1 in NPCs.

Therefore, we have identified a novel signalling pathway, TAG-1/APP, in modulation of neurogenesis. TAG-1/APP signalling pathway, similar to notch signalling pathway, inhibits neurogenesis via releasing its intracellular fragment of the receptor. Notch signalling pathway provides cells a mechanism to control the time and the extent of neurogenesis via “lateral inhibition”. It seems that APP could control neurogenesis in a similar way via modulation by TAG-1. As mentioned in the introduction, neurogenesis is initiated by proneural genes, and later can be modulated by various factors. The mRNA of APP695 can be detected as early as E9 in the mesodermal cells (Sarasa et al., 2000), which is later than the expression of proneural genes. Thus, we can exclude the possibility of that TAG-1/APP signalling has some proneural function. It more seems that TAG-1/APP signalling, like other signalling pathway such as notch and MEK-C/EBP, act only as differentiation factors responsible for initiating the differentiation of neural progenitor cells, rather than as key determination factors in the same sense as bHLHs to determine the cell fate. Gliogenesis is silenced during neurogenic period (Miller and Gauthier, 2007). Some modulators in neurogenesis modulate gliogenesis as well. For example, Notch signalling inhibits neurogenesis while enhances gliogenesis (Louvi and Artavanis-Tsakonas, 2006). Proneural genes such as mash1 and Ngn, initiate neurogenesis while repress gliogenesis (Bertrand et al., 2002). Gliosis has been observed in the hippocampus of two-week old APP null mice (Zheng et al., 1994). Therefore, the function of TAG-1/APP signalling in gliogenesis may help us understand how the neurogenesis is affected when this pathway is disrupted.

APP has reported involved in various physiological processes in addition to on neurogenesis such as cell adhesion, neurite outgrowth, and axonal transport (Breen et al., 1992; Salinero et al., 2000; Schubert and Behl, 1993; Yamazaki et

al., 1997). Our study has indicated a novel function of APP on neurogenesis and has for the first time linked ligand triggered-RIP of APP to a biological process.

5.3 Is transcriptional activity of AICD or Fe65 involved in neurogenesis?

AICD and Fe65 are suggested to form a molecular complex together with other nuclear proteins, histone acetyltransferase (Tip60) or CP2/LSF/LBP1, modulating transcription activity of target gene (Cao and Südhof, 2001; 2004). However, these studies are based on a very artificial system, where a GAL4 binding motif on the luciferase report gene attracts the GAL4-fused AICD or Fe65. Whether endogenous AICD or Fe65 could form a transcriptional complex with other nuclear proteins remains to be further investigated. The nuclear translocation of AICD and Fe65 is controversial. AICD, like NICD (intracellular domain of Notch) has been supposed to translocate into the nucleus after releasing from full-length receptor. Indeed, nuclear localisation of transfected-AICD, which is fused with a green fluorescence protein (GFP) has been observed (Cao and Südhof, 2001; 2004). Fe65 can stabilize AICD and colocalize with it in the nucleus (Kimberly et al., 2001; Walsh et al., 2003). However, some studies have suggested that some AICD, after releasing from full-length APP, is still tethered in the membrane. The membrane-tethered AICD further activates Fe65 via causing its conformational change; the latter enters into the nucleus to regulate the transcription of downstream genes together with Tip60 and other coactivator, while full-length APP inhibits the nuclear location of Fe65 via tethering Fe65 on the membrane (Cao and Südhof, 2004). Moreover, a subsequent study has suggested that Fe65 alone is sufficient for transcriptional activation and that APP and Tip60 play positive and negative modulatory roles, respectively (Yang et al., 2006). Recent study suggests that Fe65 can stimulate the proteolytic liberation of AICD from full-length APP in a γ-secretase-dependent manner (Wiley et al., 2007). Yet, despite the growing evidence that AICD is necessary for neither the transcriptional activity of Fe65 nor its translocation to the nucleus, several recent studies have suggested that AICD can regulate transcription of endogenous

genes, including KAI1, GSK-3β, APP, BACE, neprilysin, α2-actin, transgelin, and EGFR (Müller et al., 2007; Baek et al., 2002; Kim et al., 2003; Pardossi-Piquard et al., 2005; von Rotz et al., 2004; Zhang et al., 2007), although the transcriptional control of KAI1, GSK-3β, APP and neprilysin have been controversial (Chen and Selkoe, 2007; Hébert et al., 2006; Pardossi-Piquard et al., 2005). In the case of the epidermal growth factor receptor (EGFR), direct binding of endogenous AICD to the EGFR promoter is reported (Zhang et al., 2007). It has also been suggested that AICD can enhance the transcriptional activation of another transcription factor, p53 (Ozaki et al., 2006). Thus, despite the controversy over whether AICD is itself a transcription factor in the AICD-Fe65-Tip60 system, there is growing evidence that AICD can influence gene transcription. It is therefore of interest to understand the regulation of AICD cleavage. In our study, we have found that TAG-1 could enhance the transcriptional activity of AICD and Fe65 in a luciferase system. However, like in other studies, the luciferase systems we used are very artificial. This artificial reporter assay system can detect release of AICD tagged with Gal4 but, as the response element is Gal4, activity in this system does not reflect endogenous transcriptional activity of AICD. From the luciferase results, we can only draw the conclusion that TAG-1 trigger AICD release from full-length APP and the binding between APP and Fe65 is necessary for this functional triggering. Whether the transcriptional activities of AICD or Fe65 play the role in neurogenesis inhibition by TAG-1/APP signalling pathway remains to be further investigated. In addition to Tip60 or CP2/LSF/LBP1, FE65 binds to distinct proteins such as mena, c-Abl, LRP (Ermekova et al., 1997; Zambrano et al., 1998; Kinoshita et al., 2001). Thus, the downstream molecules of Fe65 and the further direction of the TAG-1/APP signalling pathway remain to be further identified.

We cannot exclude the possibility that crosstalk between APP and other signalling pathway may be involved in the inhibition of neurogenesis. Crosstalk between the APP and notch signalling pathways has been suggested (Fassa et

al., 2005; Fischer et al., 2005). TAG-1 shares the homology with the structure of F3/contactin and NB-3, the ligands of Notch. However, we have found that TAG-1 did not modulate the transcriptional activity of Hes1 as F3 did in a luciferase assay (**Fig. 34**). Similarly, the expression of the down-stream effectors of the notch pathway such as NICD1, NICD2, Hes1 and hes5 were not detected different in TAG-$1^{-/-}$ fetal brain compared to their wild-type littermate control (**Fig. 34**). However, we cannot exclude the possibility of that the TAG-1/APP signalling pathway cross-talked with other pathways to modulate neurogenesis, such EGFR. The EGFR pathway can activate and phosphorylated erk. Phosphorylated erk can further activate MEK-C/EBP pathway, which promotes neurogenesis in presence of growth factors. As mentioned, EGFR is indicated to be transcriptional regulated by AICD. Therefore, we could suspect from this evidence that cross-talk between TAG-1/APP and MEK-C/EBP signalling pathways may underlie the mechanism of inhibition of neurogenesis by TAG-1/APP.

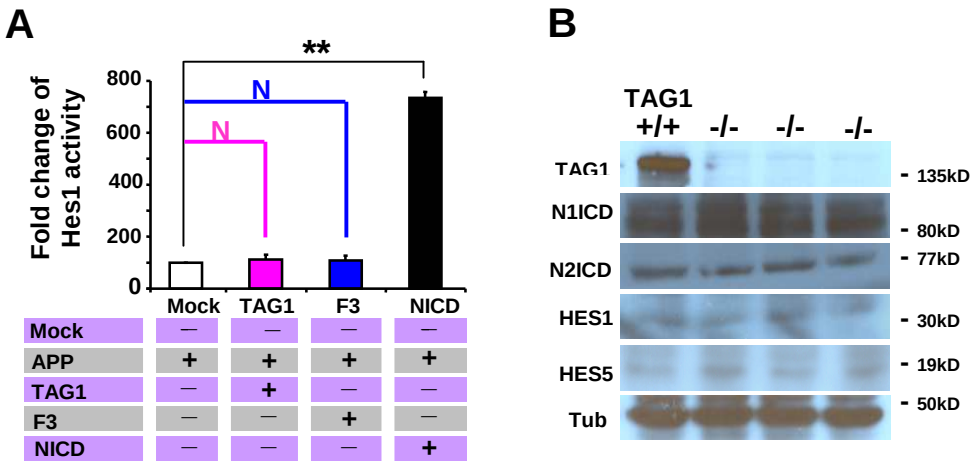

Figure 34. TAG1 fails to trigger NICD activity-dependent Hes1 activity.
Hes1 luciferase reporter activity was affected by neither TAG1 nor F3. NICD transcriptional activity is not triggered by TAG1. CHO cells were transiently co-transfected in 24-well culture dishes with pGVB-Hes1 luciferase reporter plasmid, luciferase internal control plasmid and TAG1 cDNA, F3 cDNA or NICD cDNA as well as empty vector as a control (Mock). Normalized luciferase activities in whole-cell lysates were determined in triplicate and expressed relative to activity in lysates prepared from mock-transfected CHO cells (**A**). There were also no detectable differences between the brains of $TAG1^{+/+}$ and $TAG1^{-/-}$ mice in Western blot analysis of the expression levels of NICD of Notch1 and Notch2, Hes1 and Hes5. Brain lysates of $TAG1^{+/+}$ (WT) and $TAG1^{-/-}$ (KO) mice were Western blotted using antibodies against TAG1 (TG1), Notch1 NICD (N1ICD, ab8925), Notch2 NICD (N2ICD, ab8926), Hes1 (AB5702), Hes5 (ab25374) and γ-Tubulin (**B**). Experiments in A were performed by Dr. Wu-ling yang. Experiments in B were perfomed by Mr. Toshi Futagawa.

5.4 TAG-1/APP signalling and Alzheimer's disease

Increased neurogenesis has been reported in the brains of some Alzheimer's disease (AD) cases (Jin et al., 2004ab). Although it was not replicated in some presenile patients (Boekhoorn et al., 2006), it suggested study of neurogenesis in the animal models of AD. Similar to the studies in human AD patients, the studies in the animal models are controversial. In mice overexpressing the mutant APP (Tg2576), neurogenesis in the hippocampal dentate gyrus is decreaed. The decrease in neurogenesis was found to be caused by Aβ deposition (Dong et al.,

2004; Donovan et al., 2006). Same phenomena have been observed in 11-14 month-old transgenic mice overexpressing APP with Swedish mutation (APP695(K595N/M596L)) (Haughey et al., 2002a;b). However, in another two animal models, which overexpress APP either with mutation of APP751 (K670N/M671L) or with the Swedish mutation, neurogenesis has been found to be increased in both hippocampus and subventricular zone (SVZ) (Sturchler-Pierrat et al., 1997; Jin et al., 2004a). We have shown that the TAG-1/APP signalling pathway inhibits neurogenesis during embryogenesis. However, whether and how this pathway functions on adult neurogenesis and, whether and how it contributes to the development and pathology of Alzheimer's disease remain to be further investigated.

6 Summary

In summary, we have shown that TAG-1, a member of the F3/contactin family, is a functional ligand of APP. Similar to F3/contactin triggered NICD activity (Hu et al., 2003; Cui et al., 2004), this ligand binding promotes AICD release in a γ-secretase-dependent manner. Moreover, we have demonstrated that the TAG-1/APP signalling pathway through Fe65 negatively modulates neurogenesis. Importantly, the increase in neurogenesis observed in neural stem cells isolated from TAG-1 null mice was reversed by expression of AICD, confirming that negative modulation of neurogenesis is a physiological role of cleaved AICD. These findings are important in the context of Alzheimer's disease because abnormal processing of APP could also lead to aberrant AICD generation, which may be linked to abnormal intracellular signalling. Further research is required to understand the details of the mechanisms by which the TAG-1/APP signalling pathway as well as its downstream elements modulate neural stem cells. Knowledge of these mechanisms will provide insights into the cellular processes of neurodegenerative disease and may also offer unique opportunities for pharmacological intervention.

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8 Publication

1. **Q.H. Ma**, S. Li, W.L. Yang, H.B. Li, T. Futagawa, M.H. Mohajeri, X.D. Jiang, X.F. Zhou, D. Bagnard, M. Schachner, G.S. Dawe and Z.C. Xiao. (2008) APP clustering at CNS nodes of Ranvier modulates the currents of Nav1.6 sodium channel depending up the APP668T phosphorylation. (**in preparation**).

2. D.Y. Nie, Q.D. Hu, **Q.H. Ma**, M. Schachner and Z.C. Xiao. (2008) Neurite outgrowth inhibitors at the node of Ranvier. **Nervous Diseases and Mental Hygiene**. 8(1), 5-16.

3. **Q.H. Ma**, T. D. Bagnard, Z.C. Xiao and G.S. Dawe. (2008) A TAG on to the neurogenic functions of APP. **Cell Adhesion and Migration**. 2(1), 2-8.

4. **Q.H. Ma**, T. Futagawa, W.L.Yang, X.D. Jiang, L. Zeng, Y. Takeda, R.X. Xu, D. Bagnard, M. Schachner, A.J. Furley, D. Karagogeos, K. Watanabe, G.S. Dawe, and Z.C. Xiao. (2008) A TAG-1/APP signaling pathway through Fe65 negatively modulates neurogenesis. **Nature Cell Biology**. 10(3), 283-294.

5. **Q.H. Ma**, D. Y. Nie, G. S. Dawe and Z. C. Xiao. (2007) Physiological roles of neurite outgrowth inhibitors at myelinated axons in the central nervous system – implications for the therapeutic neutralization of neurite outgrowth inhibitors. **Current Pharmaceutical Design**. 13, 2529-1537.

6. D.Y. Nie, **Q.H. Ma**, J.W.S. Law, C.P. Chia, N.K. Dhingra, G. Xu, N. Gong, W.L. Yang, Y. Shimoda, Q.W. Chen, Q.D. Hu, A.Y.W. Lee, P.K.H. Chow, Y.K. Ng, E.A. Ling, K. Watanabe, T.L. Xu, A. Habib, M. Schachner and Z.C. Xiao. (2006) Oligodendrocytes regulate formation of nodes of Ranvier via the recognition molecule OMgp. **Neuron Glia Biology**. 2, 151-164.

7. Q.D. Hu, **Q.H. Ma**, G. Gennarini and Z.C. Xiao. (2006) Cross-talk between F3/Contactin and Notch at axoglial interface: a role in oligodendrocyte development. **Developmental Neuroscience**. 28(1-2), 25-33.

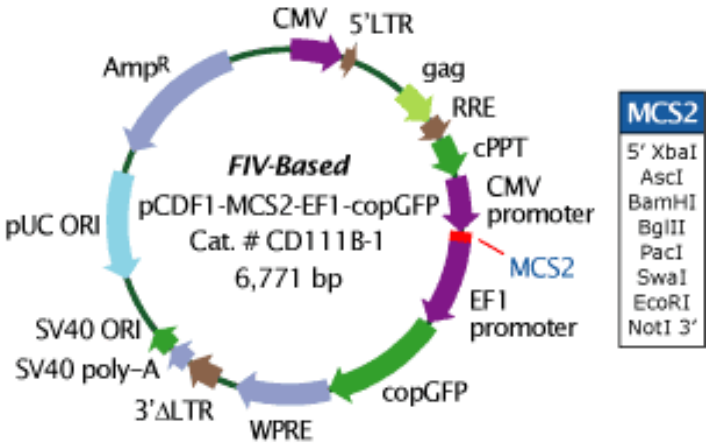
9 Appendix

Abbreviations

µ	Micro (10^{-6})
℃	Degree celcius
g	g-force
bp	Base pairs
kb	Kilo base pairs
kDa	KiloDalton
Amp	Ampicillin
BSA	Bovine serum albumin
FCS	Fetal calf serum
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
GST	Glutathione S transferase
EDTA	Ethylene diamine tetra acetic acid
FNIII	Fibronectin III
Ig	Immunoglobulin
IgG	Immunoglobulin subclass G
GPI	Glycosylphosphotidylinositol
GFP	Green fluorescent protein
AD	Alzheimer's Disease
APP	Amyloid precursor protein
APLP	Amyloid precursor like protein
Aβ	Amyloid beta peptide
CTFs	C-terminal fragments
RIP	regulated intramembrane proteolysis
NPC	Neural progenitor/precursor cells
GOF	Gain of Function
LOF	Loss of Function

Plasmid

Map for PCDF1-MCS2-EF1-CoGFP



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