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Chapter 4

γ-Secretase — Regulated Signaling and Alzheimer's Disease

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1. Introduction

Alzheimer's disease (AD) is an incurable and progressive neurodegenerative disorder and the most common form of dementia that occurs with aging. The main hallmarks of this disease are the extracellular deposition of amyloid plaques and the intracellular aggregation of tangles in the brain [1, 2]. Although the causes of both the onset and progression of AD are still uncertain, much evidence, including results of genetic analysis, indicates that amyloid precursor protein (APP) itself and its proteolytic processing are responsible for AD. Indeed, familial forms of AD (FAD) have mutations [3] or a duplication of the *APP* gene [4] or mutations in the presenilin1 or 2 (*PS1* or *PS2*) genes [5-7] that code for a catalytic component of the γ-secretase complex [8].

Although APP plays a central role in AD [1, 2], the physiological function of this membrane protein is not clear [9]. On the other hand, γ-secretase was first identified as a protease that cleaves APP within the transmembrane domain and produces amyloid-β (Aβ) peptides [10], which are the main constituent of amyloid plaques and are thought to be involved in AD pathogenesis. However, similar to the physiological functions of APP, those of γ-secretase are also still unclear [11, 12].

The signaling hypothesis suggests that the primary function of γ-secretase is to regulate signaling of type 1 membrane proteins (the amino terminus is extracellular, and the carboxy terminus is cytoplasmic); this was proposed by analogy of Notch signaling [13-15]. Notch is a family of evolutionarily conserved type 1 membrane proteins that mediate the fates of numerous cells in both invertebrates and vertebrates [16-18]. The molecular mechanism of the Notch signaling pathway is unique because it is controlled by proteolytic cleavage reactions [19, 20]. In the canonical Notch signaling pathway, ligands bind to the extracellular domain of Notch expressed

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on neighboring cells and trigger sequential proteolytic cleavage. Finally, the intracellular domain (ICD) of Notch (NICD) is released from the cell membrane by γ-secretase; NICD then translocates into the nucleus where it modulates gene expression through binding to transcription factors. Therefore, γ-secretase plays a central regulatory role in Notch signaling.

Recently, more than five dozen type 1 transmembrane proteins, including Notch and APP, have been reported as substrates for γ-secretase [21]. The ICDs of these proteins are also released from the cell membrane [13-15, 22]. Furthermore, it has been shown that some of these ICDs exist in the nucleus. These processes are very similar to those involved in Notch signaling. Thus, the common enzyme γ-secretase modulates the proteolysis and turnover of putative signaling molecules; this suggests that mechanisms similar to the Notch signaling pathway may widely contribute to γ-secretase–regulated signaling [13-15, 23]. Indeed, it has been shown that the ICD of APP (AICD), which is released from the cell membrane by γ-secretase, also translocates to the nucleus [24-26] and may function as a transcriptional regulator [27, 28]. These observations suggest the existence of APP signaling.

To test the hypothesis that APP has a signaling mechanism similar to that of Notch, we established embryonic carcinoma P19 cell lines that overexpressed AICD [29], which may mimic signaling mechanisms. Although neurons differentiated from these cell lines, AICD expression induced dynamic changes in gene expression profile and neuron-specific apoptosis [30]. These results suggest that APP also has a signaling mechanism, which may be closely related to AD.

In this chapter, we first summarize current research progress regarding Notch, APP, and γ-secretase. We also focus on the signaling hypothesis; γ-secretase–regulated mechanisms similar to Notch signaling may widely play roles in signaling events involving type 1 transmembrane proteins, including APP. Next, we review recent evidence supporting the existence of APP signaling. Furthermore, we discuss the possibility that APP signaling is involved in the onset and progression of AD.

2. γ-Secretase controls Notch signaling

Notch is a family of evolutionarily conserved type 1 membrane proteins with a mass of about 300 kDa [31] that mediates fates of numerous cells in both invertebrates and vertebrates [16, 17]. For example, cells expressing the major ligand Delta inhibit the neural differentiation of neighboring Notch-expressing cells during neurogenesis. Disruption of or disorder in Notch signaling leads to developmental defects or cancer in mammals [18].

While *Drosophila* has only one Notch gene, four Notch isoforms (Notch1 to 4) have been identified in mammals. The typical Notch protein contains 36 tandem epidermal growth factor (EGF)-like repeats in its extracellular domain, and six tandem ankyrin-like (CDC10) repeats, a nuclear localization signal, and a PEST sequence in its intracellular domain [31]. The 11th and 12th EGF-like repeats are essential for binding to its ligands [32]. Notch is cleaved in the trans-Golgi network, apparently by furin-like covertase, and is expressed on the cell surface as a heterodimer [33].

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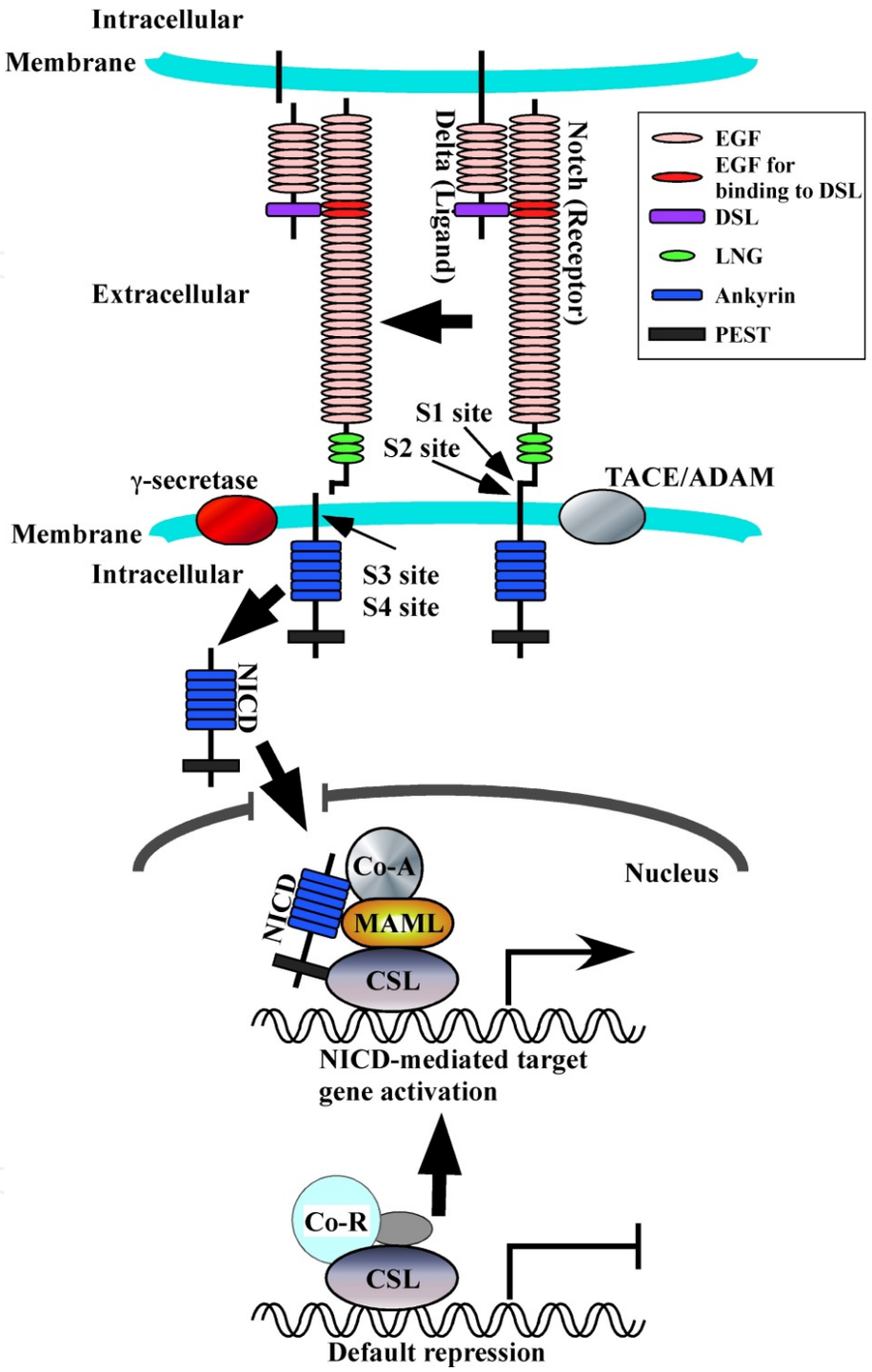

Figure 1. Notch signaling pathway. Notch proteins are expressed on the cell surface as heterodimers after cleavage at the S1 site by furin. The binding of Notch to the ligand triggers sequential proteolytic cleavage of the regulated intramembrane proteolysis (RIP). When Notch binds to the ligand, Notch is cleaved at the S2 site in the juxtamembrane region by TACE or ADAM protease. Next, the remaining protein stub is further cleaved by γ-secretase at the S3 and S4 sites within the transmembrane domain and NICD is released from the membrane. Then, NICD translocates into the nucleus and binds to the CSL together with MAML. The resultant CSL–NICD–MAML complex removes co-repressors (Co-R) from CSL transcription factor and recruits a co-activator (Co-A), resulting in conversion from repressor to activator. Finally, the complexes of CSL-NICD-MAML-Co-activators promote transcription of the target genes.

Drosophila has two different Notch ligands, Delta [34] and Serrate [35]. In mammals, two families of Notch ligands, Delta-like protein family (Dll1, 3, and 4) [36-38] and Jagged family (Jagged 1 and 2) [39, 40], have been identified. The extracellular domains of all these ligands also contain variable number of EGF-like repeats, e.g., *Drosophila* Delta has nine, while most vertebrate Deltas have eight, and *Caenorhabditis elegans* Lag-2 has two repeats. All these ligands share a single copy of a 2nd cysteine-rich conservative motif called the DSL (**D**elta: **S**errate: **L**ag-2) domain [41], which is essential for binding to Notch [42].

As shown in Fig.1, in the canonical Notch signaling pathway, ligands bind to the extracellular domain of Notch proteins on neighboring cells and trigger sequential proteolytic cleavage reactions; this is called the regulated intramembrane proteolysis (RIP) mechanism [43]. Precise steps of Notch processing are mentioned in section 4.2 of this chapter. In brief, first, Notch is cleaved within the juxtamembrane (JM) domain by metalloproteases to remove most of the extracellular region [44, 45]. Next, the remaining protein stub is further cleaved by γ-secretase within the transmembrane (TM) domain and NICD is released from the membrane [46-48]. The released NICD translocates to the nucleus and controls the expression of certain genes through binding to transcription factors. Thus, γ-secretase plays a central regulatory role in Notch signaling.

Members of the CSL transcription factor family (CBF1/RBP-jκ in mammals, Su(H) in *Drosophila*, and Lag-1 in *C. elegans*) are major downstream targets of Notch signaling [19]. NICD binds to CSL transcription factors, and six tandem ankyrin-like repeats in NICD are essential for binding to CSL transcription factors [49]. NICD also binds to Mastermind-like proteins (MAML family in mammals) [50], thus forming the CSL-NICD-MAML complex. The formation of these complexes results in removal of co-repressors from CSL and recruitment of co-activators, such as P/CAF and P300 [50, 51]. Through this process, the CSL complex is converted from a transcriptional repressor to an activator. Finally, these complexes bind to the *cis*-acting DNA sequences of target genes, such as Hes (Hairy/Enhancer of split in *Drosophila*), which encode the basic helix-loop-helix (bHLH) transcription factors, and promote their transcription [52].

3. Amyloid Precursor Protein (APP)

3.1. Overview of APP

APP was identified as a cDNA cloned using a partial amino acid sequence of the Aβ fragment isolated from the amyloid plaque of AD brains [53]. This cDNA coded for an evolutionarily conserved type 1 transmembrane protein. Fig. 2 shows schematic diagram of APP protein. Although APP is expressed in many tissues, this protein especially accumulates in the synapses of neurons. The human *APP* gene is about 240 kb in length containing at least 18 exons [54] and is localized on the long arm of chromosome 21 [53], an extra copy of which is present in patients with Down's syndrome (trisomy 21). Several alternative splicing isoforms of APP have been found, which differ mainly in the absence (APP-695, predominantly

expressed in neurons) or presence (APP-751 and APP-770) of the Kunitz protease inhibitor (KPI) domain toward the N-terminus of the protein [55].

As described below, APP undergoes sequential proteolytic cleavage reactions to generate the extracellular fragment, intracellular fragment (AICD), and Aβ fragment that is located in the membrane-spanning region. Note that both the extracellular fragment and AICD are generated at the same time as Aβ. Extensive post-translational modifications of APP, such as glycosylation, phosphorylation, and tyrosine sulfation, have been observed.

Mammals have two other members of APP family called APP-like protein 1 (APLP1) and 2 (APLP2) [56]. APLP1 expression is restricted to neurons. On the other hand, expression of APLP2 is detected in many tissues, although it is highly enriched in the brain. These APP family proteins share conserved domains, such as the E1 and E2, in the extracellular region. The E1 domain contains several subdomains, such as a growth factor-like domain and a metal-binding motif [57]. The E2 domain has a coiled coil dimerization motif and may bind proteoglycans in the extracellular matrix [58].

Interestingly, the amino acid sequence of the Aβ fragment is not highly conserved and is unique to APP; on the other hand, the highest degree of sequence conservation is found in the ICD not only within the APP homologues [29] but also within the APP family [9]. This strong sequence conservation most likely reflects functional importance of the ICDs in the APP family proteins.

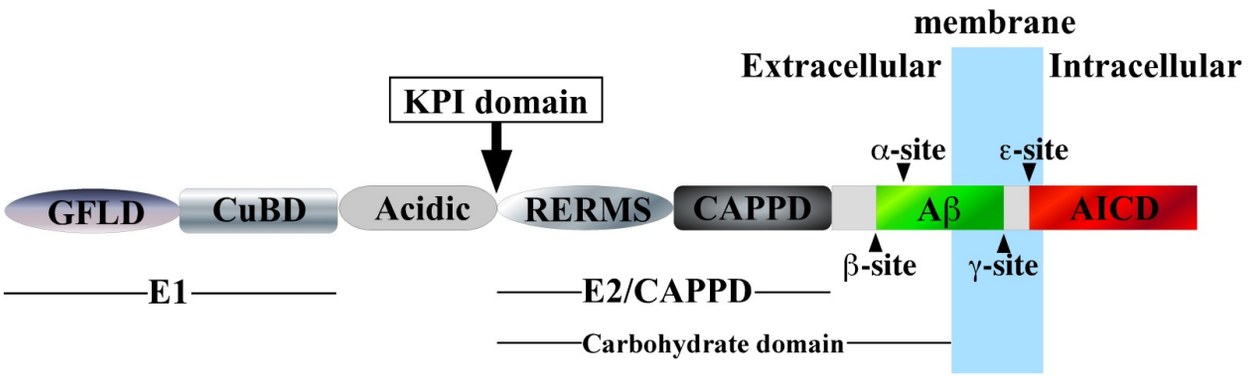

Figure 2. Schematic domain structure of APP. APP protein family shares the conserved E1 and E2 domains in their extracellular region. The E1 domain contains N-terminal growth factor-like domain (GFLD) and copper-binding domain (CuBD). The E1 domain is linked via acidic domain to the carbohydrate domain including E2 domain, which consists of RERMS sequence and central APP domain (CAPPD). E2 domain is followed by the Aβ region, and the intracellular domain (AICD) which is the most conserved region. Although the Kunitz protease inhibitor (KPI) domain is present at the indicated site in APP-751 and APP-770, APP-695 lacks this domain.

3.2. Proposed APP functions

Although the physiological functions of APP are not clear, several possibilities have been proposed. The most considerable functions are synapse formation and repair [59, 60]. Indeed, APP expression is upregulated after neural injury as well as during neuronal differentiation [59, 60]. After translation in the soma, APP is transported in an anterograde manner to the synaptic region, where the amount of APP is correlated with synaptogenesis. APP knockout mice show impaired long-term potentiation and declined memory without remarkable neuronal loss [61]. This evidence also supports this idea.

It has also been suggested that APP acts as a cell adhesion molecule and plays a role in cell–cell interaction. Indeed, the E1 and E2 domains can interact with extracellular matrix proteins and heparan sulfate proteoglycans [57, 58]. In addition, it has also been shown that extracellular domains of APP family proteins can interact with each other *in trans*. Therefore, APP family proteins may bind to each other in a homophilic or heterophilic manner to enhance cell–cell adhesion [62].

As APP may have a signaling mechanism, as described in detail below, the idea that APP is a cell-surface receptor is interesting. Indeed, several candidates of ligand for APP have been proposed. For example, F-spondin [63] and Nogo-66 [64] receptor bind to the extracellular domain of APP and regulate the production of Aβ. In addition, Aβ itself can also bind to the extracellular domain of APP [65].

3.3. Aβ amyloid

Aβ is the main constituent of an amyloid plaque, which is thought to be the hallmark and a major cause of AD pathogenesis in the brain. Thus, the amyloid hypothesis is generally accepted as the mechanism of the onset and progression of AD. The traditional amyloid hypothesis is that overproduced Aβ forms insoluble amyloid plaques, which are commonly observed in the AD brain and are believed to be the toxic form of APP and responsible for neurodegeneration [66].

As detailed in section 4.2., Aβ is generated after sequential cleavage of APP by β- and γ-secretases. Although these fragments range from 36 to 43 amino acid residues in length, Aβ40 and Aβ42 are the most common isoforms. Aβ40 is predominant over Aβ42, but Aβ42 is more amyloidogenic [67] and is, therefore, thought to be closely associated with AD. Furthermore, similar amyloid plaques are found in particular variants of Lewy body dementia [68] and in the muscle disease inclusion body myositis [69]. Aβ also forms aggregates that coat cerebral blood vessels in cerebral amyloid angiopathy (CAA), which is observed in over 90% of AD patients [70].

Deposition of Aβ in the AD brain is thought to be formed due to imbalances between the production of Aβ and its removal from the brain through various clearance mechanisms, including enzyme-mediated degradation [71]. Therefore, mechanisms of not only production but also degradation of Aβ have been studied extensively. As a result, several candidates for Aβ degradation enzymes are proposed. Neprilysin (NEP) and insulin-

degrading enzyme (IDE) are expressed in neurons as well as within the vasculature and the levels of both these enzymes are reduced in AD [71]; therefore, these enzymes have been well studied in relation to AD. Interestingly, it has been reported that *APOE* e4, which is the most-established genetic risk factor for the onset of AD and CAA, is associated with reduced levels of both enzymes [72, 73]. Furthermore, other candidates for Aβ degradation enzymes have been proposed, including endothelin-converting enzymes 1 and 2 (ECE-1 and ECE-2) [74] and angiotensin-converting enzyme (ACE) [75]. The levels of plasmin and plasminogen activators (uPA and tPA) and ECE-2 have also been shown to be reduced in the AD brain [71].

4. γ-Secretase

4.1. Overview of γ-secretase

γ-Secretase was first identified as a protease that cleaves APP within the TM domain and produces Aβ peptides [10], which is thought to be a major cause of the pathogenesis in the AD brain.

γ-Secretase is a complicated complex composed of PS, nicastrin (NCT), anterior pharynx defective-1 (Aph-1), and PS enhancer-2 protein (Pen-2) [8, 11, 12]. Two PS genes, *PS1* located on chromosome 14 [5] and *PS2* located on chromosome 1 [6, 7], have been identified by genetic linkage analyses as the genes responsible for early-onset FAD. The *PS1* and *PS2* genes encode proteins with eight or nine transmembrane domains of 467 and 448 amino acids, respectively, with about 65% sequence identity between the two proteins. Both proteins are the catalytic components of the γ-secretase complex. Although both PS1 and PS2 are expressed ubiquitously in the brain and peripheral tissues of adult mammals, PS1 expression level is significantly higher than that of PS2 [76]. NCT is a single-pass membrane protein and may recognize the substrate proteins of γ-secretase [77]. Indeed, the extracellular domain of NCT resembles an aminopeptidase, but lacks catalytic residues. Thus, this domain can interact with the free N-terminal of stubs of γ-secretase substrates generated by ectodomain shedding [78]; hence, shedding of γ-secretase substrates may be essential for the production of free N-termini of these proteins retained in the membrane to be recognized by NCT. Aph-1 may act as a scaffold during the process of γ-secretase complex assembly, and Pen-2 may act as a trigger for the proteolytic cleavage of PS in order to activate it [11, 12].

The physiological functions of γ-secretase have not been clarified. However, this protease can cleave a surprisingly large number of transmembrane proteins [79]. Indeed, more than five dozen proteins, most of which are type 1 membrane proteins, have been reported as γ-secretase substrates [21]. Interestingly, these substrates have a wide range of biological functions. Representative γ-secretase substrates are shown in Table 1.

Substrate	Function	PS or ICD function
ApoER2	Lipoprotein receptor, neuronal migration	Activates nuclear reporter
APP	Precursor to Aβ, adhesion, trophic properties, axonal transport?	Ab generation, release of ICD, Complex with Fe65/Tip60, Cell death?
APLP1/2	Cell adhesion?	Forms complex with Fe65 and Tip60
β-Catenin	Transduce Wnt signals stabilize adherens junctions	Facilitates phosphorylation
CD43	Signal transduction	Signaling molecule?
CD44	Cell adhesion	Activates TRE-mediated nuclear transcription
CSF1-R	Protein tyrosine kinase	Unknown
CXCL16 & CX3CL1	Membrane chemokine ligands	Unknown
DCC	Axon guidance, tumor suppressor	Activates nuclear reporter
Delta	Notch ligand	Transcriptional regulation
E-cadherin	Cell adhesion	Promotes disassembly of adhesion complex
ERBB4	Receptor tyrosine kinase	Regulates heregulin-induced growth inhibition
HLA-A2	MHC class I molecule	Unknown
IFN-αR2	Subunit of type I IFN-α receptor	Transcriptional regulation
Insulin receptor	Receptor tyrosine kinase	Accumulates in nucleus
IGIF-R	Receptor tyrosine kinase	Unknown
IL-1RI	Cytokine receptor	Unknown
IL-1RII	Cytokine receptor	Unknown
Jagged	Notch ligand	Modulates AP-1-mediated transcription
LAR	Receptor tyrosine phosphatase	Accumulates in nucleus
LDLR	Lipoprotein receptor	Unknown
LRP	Scavenger and signaling receptor	Activates nuclear reporter
Na channel β-subunit	Cell adhesion, an auxiliary subunit of voltage-gated Na channel	Alters cell adhesion and migration
N-cadherin	Cell adhesion	Promotes CBP degradation
Nectin-1α	Adherens junction, synapse receptor	Remodeling of cell junctions?
Notch1-4	Signaling receptor	Transcriptional regulation
NRADD	Apoptosis in neuronal cells	Modulates glycosylation/maturation of NRADD
P75NTR	Neurotrophin co-receptor, dependence receptor	Modulates p75-TrkA complex? Nuclear signaling?
γ-Protocadherin	Cell adhesion, neuronal differentiation	Regulation of gene transcription?
Syndecan-3	Cell surface proteoglycan co-receptor	Regulation of membrane-targeting of CASK
Telencephalin	Cell adhesion	Turnover of telencephalin
Tyrosinase,Tyrosinase-related protein 1/2	Pigment synthesis	Intracellular transport of Post-Golgi Tyr-containing vesicles
Vasorin	TGF-β inhibitor	Unknown

Table 1. Substrates for γ-secretase

4.2. Some γ-secretase substrates share a common proteolytic process

Fig.3 shows the proteolytic processes of Notch, APP, and CD44. There are surprising similarities between these processes and all of these processes follow the RIP mechanism. For example, in the canonical Notch signaling pathway, ligands bind to the extracellular domain of Notch on neighboring cells and trigger sequential proteolytic cleavage reactions (the RIP mechanism) and shedding at the S2 site by TACE or ADAM protease making the truncated Notch [44, 45]. Truncated Notch is further cleaved by γ-secretase in at least two sites within the TM domain [46-48], i.e., at the S3 site to release NICD and at the S4 site to release the remaining small peptide (Nβ). As mentioned above, NICD, which is released from the cell membrane to the cytoplasm by γ-secretase, translocates to the nucleus where its activity is expressed through binding to transcription factors.

The proteolytic process of APP is very similar to that of Notch and also follows the RIP mechanism. Cleavage of APP by α- secretase [80] or β-secretase [81] at the α- or β-site, respectively, within the JM region results in shedding of almost the entire extracellular domain and generates membrane-tethered α- or β-carboxy terminal fragments (CTFs). Several zinc metalloproteinases, such as TACE and ADAM [82, 83], and the aspartyl protease BACE2 [84] can cleave APP at the α-site, while BACE1 (β-site APP cleaving enzyme) cleaves APP at the β-site [81]. Once the extracellular domain has been shed, the remaining stub is further cleaved at least twice by γ-secretase within the TM domain at γ- and ε-sites resulting in production of either non-amyloidogenic p3 peptide (in combination with α-secretase) or amyloidogenic Aβ (in combination with BACE1), respectively, and AICD [11, 12]. As discussed in the next paragraph, although a large proportion of AICD is rapidly degraded in the cytoplasm, a small amount of the remaining AICD may translocate to the nucleus.

It has been reported that several other type 1 membrane proteins also follow the RIP mechanism and their ICDs are released from the cell membrane [13, 14, 22]. For example, as shown in Fig.3, the process of sequential proteolytic cleavage of CD44, which is important for immune system function, is very similar to those of Notch and APP [22]. In addition, the ICD of this protein (CD44ICD) also translocates to the nucleus (Fig.3).

As discussed here, several γ-secretase substrates follow the RIP mechanism. The ICDs of these substrates are released from the cell membrane by γ-secretase, and these ICDs translocate to the nucleus. These processes are very similar to those involved in Notch signaling. Therefore, the observations that the common enzyme, γ-secretase, modulates proteolysis and the turnover of possible signaling molecules led to the attractive idea, the signaling hypothesis, which suggests that mechanisms similar to those occurring in the Notch signaling pathway may contribute widely to γ-secretase–regulated signaling mechanisms.

Actually, Dll1, a major ligand of Notch, is cleaved sequentially by metalloproteases and γ-secretase, and ICD of Dll1 (Dll1IC) is released from the cell membrane and then translocates to the nucleus [85, 86]. Furthermore, we have shown that Dll1IC then binds to Smad 2 and 3, which are transcription factors involved in the TGF-β/activin signaling pathway, and may alter transcription of specific genes that are involved in neuronal differentiation [87]. These results suggest that Dll1 also has a signaling mechanism similar to that of Notch.

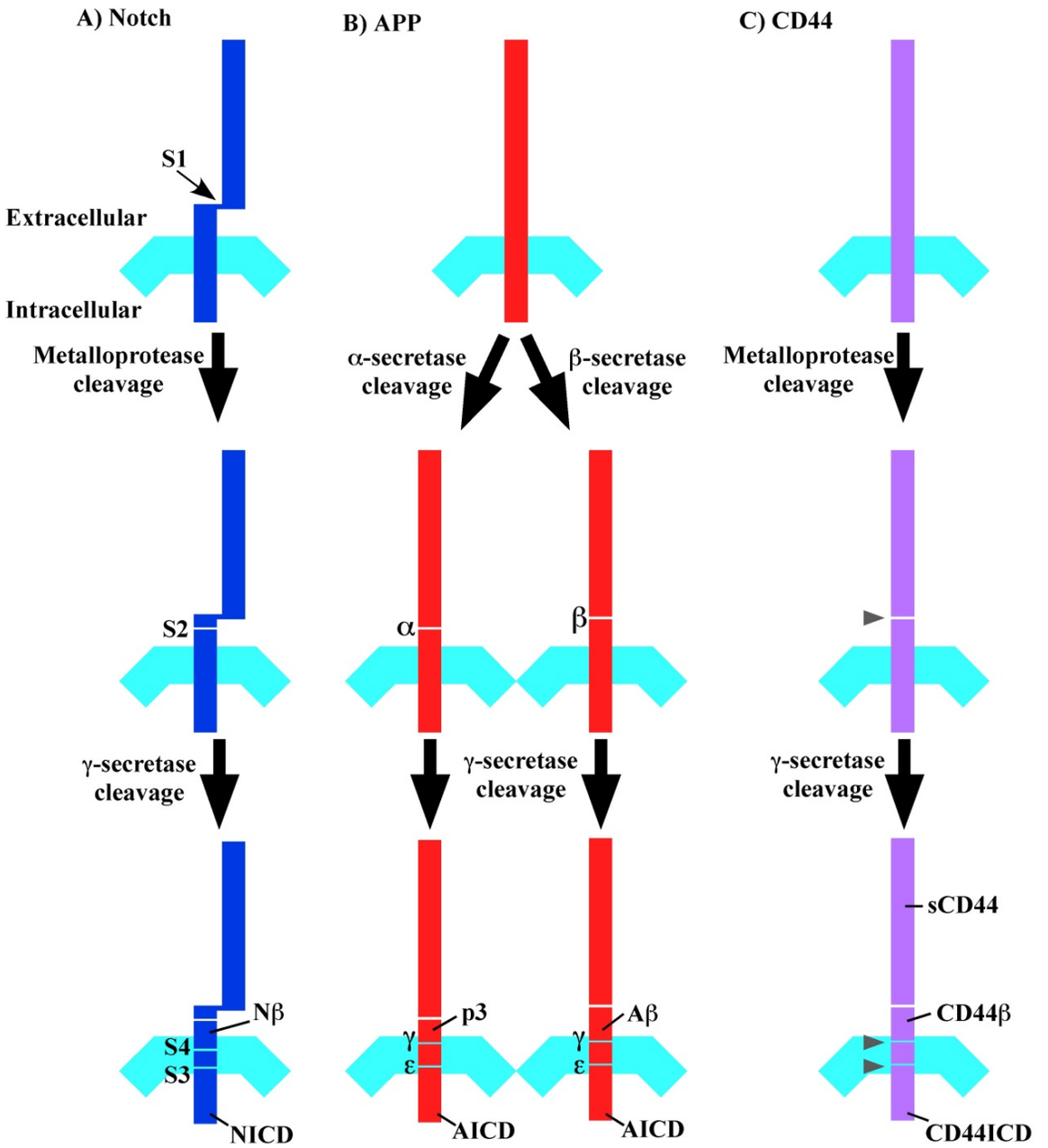

Figure 3. Similarities in the proteolytic processes among Notch, APP, and CD44. (A) In response to ligand binding, Notch undergoes shedding due to metalloprotease cleavage at the S2 site within the juxtamembrane domain. After shedding the extracellular domain, the remaining Notch stub is further cleaved by γ-secretase at S3 and S4 sites within the transmembrane domain. This sequential proteolysis produces NICD and Nβ fragment. (B) Cleavage of APP by α-secretase or β-secretase at the α-site or β-site, respectively, within the juxtamembrane domain results in shedding of almost the entire extracellular domain and generates membrane-tethered α- or β-carboxy terminal fragments (CTFs). Several zinc metalloproteinases and BACE2 can cleave APP at the α-site, while BACE1 cleaves APP at the β-site. After shedding the extracellular domain, the remaining stub is further cleaved at least twice within the transmembrane domain at γ- and ε-sites by γ-secretase, producing either p3 peptide (in combination with α-secretase) or Aβ (in combination with BACE1), respectively, and AICD. (C) Several stimuli, such as PKC activation and Ca^{2+} influx, trigger ectodomain cleavage of CD44 by a metalloprotease at the site within the juxtamembrane domain, resulting in the secretion of soluble CD44 (sCD44). After shedding the extracellular domain, the remaining CD44 stub is further cleaved by γ-secretase at two sites within the transmembrane domain. This sequential proteolysis produces the CD44ICD and CD44β, an Aβ-like peptide.

4.3. Is γ-secretase a proteasome of the membrane?

As mentioned above, more than five dozen γ-secretase substrates, most of which are type 1 membrane proteins, have been reported. This raises the simple question against the signaling hypothesis, why so many membrane proteins can transmit signals to the nucleus. In reply to this question, another possibility that γ-secretase acts as a proteasome of the membrane has been proposed [11, 12]. Indeed, as the ICDs of these substrates including AICD, which are released by γ-secretase, are rapidly degraded [24, 88], it is usually difficult to detect their ICDs by western blotting. Furthermore, ectodomain shedding seems to be constitutive for some substrates, and ligand binding has been reported to enhance only cleavage of Notch [47], Delta [87], Syndecan-3 [89], and ERBB4 [90]. In addition, much evidence supporting the signaling hypothesis was obtained in overexpression assays that differ somewhat from normal physiological conditions. Based on these observations, the proteasome hypothesis suggesting that the primary function of γ-secretase is to facilitate the selective disposal of type 1 membrane proteins has been proposed [11, 12].

Although the proteasome hypothesis for γ-secretase is reasonable and potent, there is no doubt that the certain signaling mechanisms regulated by γ-secretase, such as Notch signaling, exist. Therefore, it is likely that different functions of γ-secretases reflect their variant complexes in different combinations with multiple components, such as Aph-1, Pen2, and/or PS isoforms, with different cellular functions, such as roles in signaling or degradation.

In addition, it seems that a small proportion of ICDs of these substrates that are released by γ-secretase are sufficient for signaling mechanisms. Generally, γ-secretase substrates like APP are considerably more abundant than transcription factors, which are usually rare molecules. Although a large proportion of ICDs of these substrates are rapidly degraded, a small amount of the remaining ICDs may be sufficient for their signaling functions with small quantities of transcription factors. Thus, the majority of ICDs of these substrates may be degraded, and only a small proportion may play roles in signaling.

In relation to this issue, an attractive idea has been proposed in which a certain stimulus controls APP signaling through phosphorylation and dephosphorylation of AICD. Since AICD is stabilized [91] and translocated into the nucleus by Fe65 [26], it is thought that Fe65 is essential for the signaling function of AICD. Non-phosphorylated AICD can bind to Fe65 and form a complex; thus, this complex is stabilized and immediately translocates to the nucleus, where it mediates the expression of target genes in association with Tip60. On the other hand, phosphorylated AICD cannot bind to Fe65. Therefore, phosphorylated AICD without Fe65 cannot translocate to the nucleus. Phosphorylated AICD that remains in the cytosol is rapidly degraded by degradation enzymes such as the proteasome and/or IDE. Indeed, it has been reported that phosphorylation at Thr^{668} in the APP-695 isoform strongly inhibited binding to Fe65 [92, 93].

5. The role of AICD

5.1. Signaling functions of AICD

As mentioned above, the observations that the common enzyme, γ-secretase, modulates proteolysis and the turnover of possible signaling molecules led to the signaling hypothesis. This suggests that mechanisms similar to the Notch signaling pathway may contribute widely to γ-secretase–regulated signaling mechanisms, including APP signaling. If APP signaling exists, it may be closely related to AD.

Actually, there is accumulating evidence for the existence of APP signaling and its contribution to the onset and progression of AD. As mentioned above, the highest degree of sequence conservation within the APP homologues is found in the ICD [9, 29]. This sequence conservation suggests the functional importance of AICD, which may reflect the existence of APP signaling. In addition, several AICD-interacting proteins, which may regulate AICD stability, cellular localization, and transcriptional activity, have been identified. Based on this, several models of APP signaling have also been proposed. As mentioned above, it has been suggested that AICD recruits Fe65 proteins and translocates into the nucleus where the AICD-Fe65-Tip60 ternary complex may control transcription of target genes [27]. Furthermore, *NEP* gene expression requires binding of the AICD to its promoter [94].

Transgenic mice overexpressing both AICD and Fe65 showed abnormal activity of glycogen synthase kinase 3 beta (Gsk3b protein) [95], leading to hyperphosphorylation and aggregation of tau. This results in microtubule destabilization and the reduction of nuclear β-catenin levels causing a loss of cell-cell contact that may contribute to neurotoxicity in AD. Subsequent neurodegeneration and working memory deficits were also observed in these transgenic mice [96]. In other experiments, similar transgenic mice exhibited abnormal spiking events in their electroencephalograms and susceptibility to kainic acid-induced seizures independent of Aβ [97]. Furthermore, the function of c-Abl kinase in the transcriptional regulation of AICD was reported and c-Abl was shown to modulate AICD-dependent cellular responses, transcriptional induction, as well as apoptotic responses [98]. Interestingly, elevated AICD levels have also been reported in AD brains [96]. In addition, AICD was detected in the nucleus in injured brains [99]. Taken together, it is likely that APP signaling changes the expression of certain genes, which may cause AD pathology.

To explore APP signaling, we established several AICD-overexpressing embryonic carcinoma P19 cell lines [29]. Undifferentiated AICD-overexpressing cells retained epithelial cell-like morphology and healthy as well as control cells. Although neurons were differentiated from these cell lines after aggregation culture with all-*trans*-retinoic acid (RA) treatment, AICD expression induced neuron-specific cell death. Indeed, as shown in Fig.4, while neurons from control cells that carried the vector alone were healthy, almost all neurons differentiated from AICD-overexpressing P19 cells showed severe degeneration, becoming spherical with numerous vacuoles and detaching from the culture dishes 4 days after the induction of differentiation.

Since DNA fragmentation was detected, these cells died by apoptosis. In addition, all terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end-labeling (TUNEL)-positive cells were also Tuj1-positive neurons. Taken together,

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we concluded that AICD could induce neuron-specific apoptosis [29]. The effects of AICD were restricted to neurons, with no effects observed in non-neural cells.

Thus, although further studies are required, these results strongly suggest that AICD plays a role in APP signaling and induces neuronal cell death, which may closely relate to the onset and progression of AD.

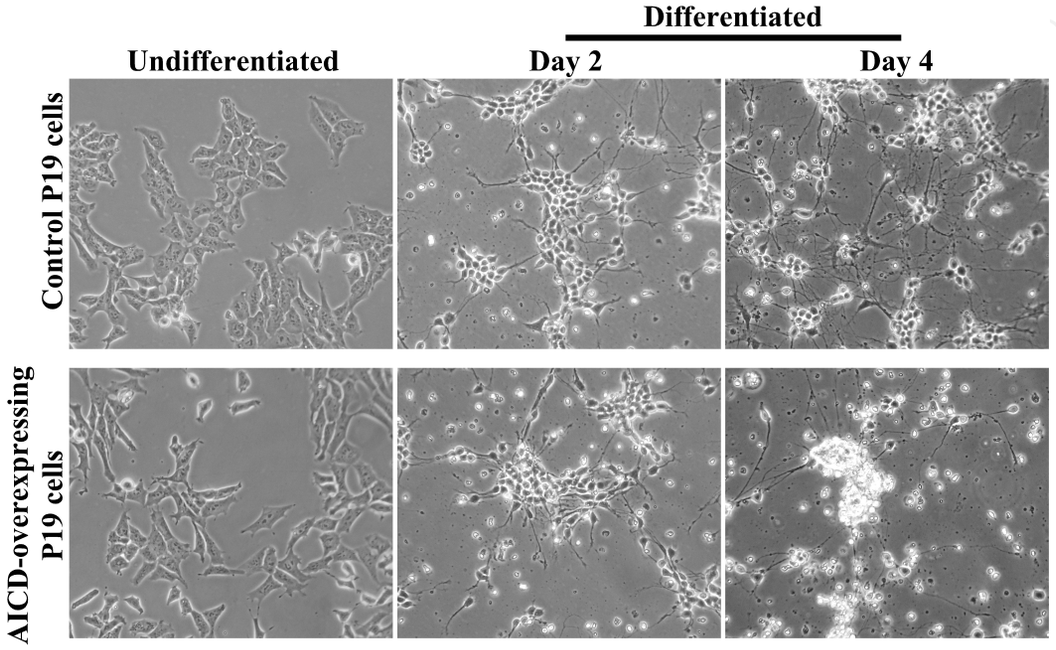

Figure 4. Expression of AICD in P19 cells induced neuronal cell death. After aggregation culture with RA, AICD-expressing P19 and control P19 cells carrying vector alone were replated and cultured for the indicated periods on dishes and allowed to differentiate. Undifferentiated AICD-expressing P19 cells retained epithelial cell-like morphology similar to control cells, while the differentiated cells became round and showed a bipolar morphology with neurite extension. Two days after replating (Day 2), all cell lines grew well and neurons with long neurites appeared. Four days after replating (Day 4), control cells still grew well as clusters and many neurons had differentiated from these cells. However, many AICD-expressing P19 cells showed severe degeneration, becoming spherical with numerous vacuoles and detached from the culture dishes.

5.2. AICD changes the gene expression profile

If APP signaling exists, AICD should change the expression of certain genes. To examine this possibility and identify the genes involved in this neuron-specific apoptosis, we performed DNA microarray analyses to evaluate the changes in the expression of more than 20,000 independent genes induced by AICD through the process of neuron-specific apoptosis [30]. Gene expression levels were deduced by hybridization signal intensity on the DNA microarrays, and the data from AICD-overexpressing cells were compared to data from control cells at the same 3 points during culture: 1) the undifferentiated state; 2) after 4 days of aggregation with RA (aggregated state); and 3) 2 days after replating (differentiated state). According to our expectations, AICD was shown to alter the expression of a great many genes; in the presence of AICD, the expression levels of 277 genes were upregulated by more than 10-fold, while those of 341 genes were downregulated to less than 10% of the original level [30].

Gene Symbol	Gene Name	Function	Relative Expression Levels (fold)		
			Undifferentiated	Aggregated	Differentiated
Non-regulated genes (housekeeping genes)					
Actb	β-actin	cytoskeleton protein	-1.2	1.2	1
Sdha	succinate dehydrogenase subunit A	electron transporter in the TCA cycle and respiratory chain	-1.1	-1.6	-1.2
Eef1a1	eukaryotic translation elongation factor-1 alpha 1	essential component for the elongation phase during protein translation	1	-1.1	1.2
Upregulated genes					
Ptprt	protein tyrosine phosphatase receptor T	protein tyrosine phosphatase that regulates STAT3 activity	906	204	116
Cpb1	carboxypeptidase B1	hydrolysis of C-terminal end of basic amino acid peptide bond	16	296	222
Nr2e1	tailless homolog	transcription factor that is essential for neural stem cell proliferation and self-renewal	5.8	244	54
Myh1	myosin heavy chain 1	one of the components of the motor protein myosin	-4.2	259	-1.1
Dnahc7c	axonemal dynein heavy chain	essential for motility of cilia and flagellae	133	41	43
Alkbh3	alkylation repair homolog 3	AlkB enzyme that repairs methylation damage in DNA and RNA	69	80	43
Ctgf	connective tissue growth factor	skeletogenesis/vasculogenesis by modulating BMP, Wnt, and IGF-I signals	90	54	40
Downregulated genes					
Hes5	hairy and enhancer of split 5	transcription factor that inhibits neurogenesis	-8.7	-3039	-2515
Slc10a6	sodium-dependent organic anion transporter	transport of sulfoconjugated steroid hormones and bile acids	-145	-785	-1212
Nid1	nidogen-1	extracellular matrix linker protein	-304	-165	-507
LOC213332	analog of Na^+-dependent glucose transporter 1	putative glucose transporter	-232	-325	-306
Dtx1	Deltex1	regulator of Notch signaling pathway	-30	-85	-691
Rbp4	retinol-binding protein 4	retinol transporter from the liver to extrahepatic tissues	-525	-100	-24
Col3a1	collagen type III alpha 1	extracellular matrix protein	4.1	-29	-234

Relative expression levels (fold) were estimated from the intensities of hybridization signals. Housekeeping gene expression was unaltered in AICD-overexpressing P19 and control P19 cells in all states, suggesting that these genes are not affected by AICD. These results also indicated that the observed differences in expression were not due to technical problems, such as uneven hybridization or poor RNA quality.

Table 2. Expression levels of 7 upregulated and 7 downregulated genes, as well as 3 housekeeping genes

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AICD strongly induced expression of several genes, representative examples of which are listed in Table 2. For example, AICD-overexpressing P19 cells showed strong expression of the protein tyrosine phosphatase receptor T (*Ptprt*) gene at all sampling points: 906-fold, 204-fold, and 116-fold upregulation, in undifferentiated, aggregated, and differentiated states, respectively, compared with control cells. In contrast to these upregulated genes, the expression of several genes was strongly inhibited by AICD. Although *Hes5* expression was markedly increased through the process of neural differentiation, with an increase of almost 300-fold in control cells, AICD inhibited this induction. As shown in Fig.5, these results were confirmed by RT-PCR. Thus, AICD may induce both upregulation and downregulation of certain genes, suggesting that AICD plays an important role in APP signaling.

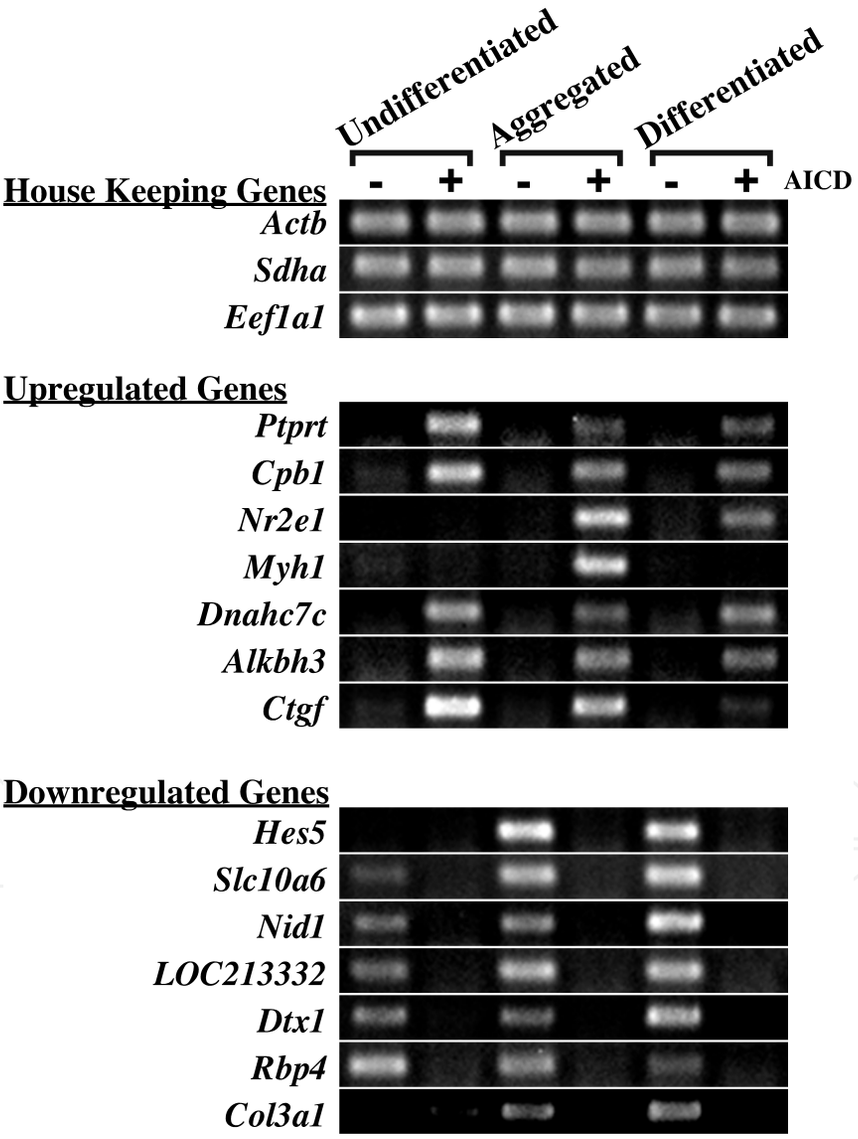

Figure 5. RT-PCR analysis of representative 7 upregulated genes and 7 downregulated genes, as well as 3 housekeeping genes, in P19 cells overexpressing AICD. The RNA samples same as applied to DNA microarray analysis was used in this RT-PCR analysis.

We performed Gene Ontology (GO) analysis and classified these upregulated and downregulated genes according to the GO terms [30]; however, we did not find genes that were significantly related to cell death among those with altered expression. Furthermore, we evaluated AICD-induced changes in the expression of genes thought to be involved in cell death in AD [30]; however, we found no significant changes in expression of these genes. Thus, it is likely that AICD does not directly induce the expression of genes involved in cell death, but the extreme dynamic changes in gene expression disrupt the homeostasis of certain neurons and thus give rise to neuron-specific cell death. Taken together, these results strongly suggest the existence of APP signaling.

6. Can Aβ clarify all aspects of the onset and progression of AD?

Autosomal dominant mutations in and around the Aβ region of the *APP* gene, which accelerate proteolytic processing, are responsible for hereditary early-onset AD [3]. The human *APP* gene is located on the long arm of chromosome 21 [53], an extra copy of which is present in individuals with Down's syndrome (trisomy 21). Patients with Down's syndrome develop AD by 40 years of age, most likely due to this gene dosage effect [4]. In addition, both PS1 and PS2, which are catalytic components of the γ-secretase complex, were identified by genetic linkage analyses as the genes responsible for FAD [5-7]. In many cases, familial diseases can provide an understanding of the sporadic ones. Therefore, both APP itself and its proteolytic processing may be responsible for the onset and progression of not only FAD but also sporadic AD.

As mentioned above, Aβ is the main constituent of amyloid plaque, which is thought to play a major role in the pathogenesis of AD; its presence is a hallmark of the AD brain. Thus, the amyloid hypothesis is generally accepted as the mechanism of the onset and progression of AD. Although an alternative hypothesis has also been proposed, which suggests that soluble Aβ oligomers rather than insoluble amyloid plaques are responsible for the onset and progression of AD because the soluble form of the Aβ oligomer is toxic for neurons [100, 101], Aβ still plays a central role in this idea.

However, several doubts have recently been raised regarding the amyloid hypothesis that Aβ plays a central role in the onset and progression of AD. One of the most critical arguments against this hypothesis is the presence of high levels of Aβ deposition in many non-demented elderly people [102], suggesting that Aβ amyloid plaques are not toxic. Indeed, transgenic mice overproducing Aβ show amyloid deposition mimicking that seen in the AD brain but do not show neurodegeneration [61]. Furthermore, several anti-Aβ drugs and vaccines have failed to show efficacy in phase III clinical trials [103]. Surprisingly, long-term follow-up studies showed unexpected problems [104]. Immunization of AD patients with the anti-Aβ vaccine, AN-1792, cleared Aβ amyloid plaques. Actually, patients with high titers of antibody against Aβ showed virtually complete plaque removal. However, there was no evidence of improvement in survival and/or cognitive function, even in patients with high titers of anti-Aβ antibody [104]. Although several interpretation for this lack of improve-

ment have been proposed, these results lead to the idea that both soluble and insoluble forms of Aβ may not be involved in the onset and progression of AD.

Based on these observations, it has been suggested that AD may be caused by an APP-derived fragment, just not necessarily Aβ [105]. As both extracellular fragments and AICD are generated at the same time as Aβ, acceleration of proteolytic processing leads to overproduction of not only Aβ but also of both the extracellular fragments and AICD. Therefore, it is likely that the extracellular fragments and/or AICD are responsible for the onset and progression of AD. Indeed, AICD has been shown to induce neuron-specific apoptosis, which leads to AD pathology, as mentioned above.

In addition, it has also been proposed that APP is a ligand of Death receptor 6 (DR6) [106], which mediates cell death and is expressed at high levels in the human brain regions most affected by AD. APP is cleaved by β-secretase, releasing the extracellular domain (sAPPβ), which is further cleaved by an as yet unknown mechanism to release a 35 kDa N-terminal fragment (N-APP). This N-APP fragment binds DR6 to trigger neurodegeneration through caspase 6 in axons and caspase 3 in cell bodies [106]. These results suggest that N-APP may also be involved in the onset and progression of AD.

7. The model of APP signaling

Through this chapter, we discussed the possibility of the existence of APP signaling. It is likely that disorders of this signaling mechanism are involved in the onset and progression of AD. As AICD is generated at the same time as Aβ, acceleration of proteolytic processing leads to overproduction of not only Aβ but also AICD in AD brain as discussed above. Furthermore, we showed that AICD alters the expression of certain genes and induces neuron-specific apoptosis [29, 30].

If the APP signaling hypothesis is correct, certain molecules involved in APP signaling may be attractive candidates for the targets of drug discovery for treating AD. Fig.6 is a schematic model of APP signaling. As mentioned above, after cleavage within the JM domain by α- or β-secretase, AICD is released from the membrane by γ-secretase. Inhibiters for these proteases are being studied extensively.

As mentioned in section 4.3, non-phosphorylated AICD can bind to the nuclear adaptor protein Fe65 [92, 93], which is essential for translocation of AICD to the nucleus. However, phosphorylated AICD cannot bind to Fe65. These results suggest the possibility that a certain stimulus controls APP signaling through phosphorylation and dephosphorylation of AICD. It has also been shown that the majority of cell membrane-associated APP is phosphorylated specifically at Thr^{668} in neurons [107]. Therefore, phosphorylated AICD, which is released from the cell membrane to the cytoplasm by γ-secretase, cannot bind to Fe65 and thus cannot translocate to the nucleus. Phosphorylated AICD left in the cytosol is rapidly degraded, probably by the proteasome and/or IDE [88]. However, if AICD is dephosphorylated by certain phosphatase, AICD can binds to Fe65. Thus, AICD/Fe65 complexes may im-

mediately translocate to the nucleus, where they mediate expressions of certain target genes in association with histone acetyltransferase Tip60 [27]. Besides dephosphorylation of AICD, if phosphorylation of membrane-associated APP is inhibited, non-phosphorylated AICD may also increase. Therefore, it is likely that non-phosphorylated AICD is involved in the onset and progression of AD.

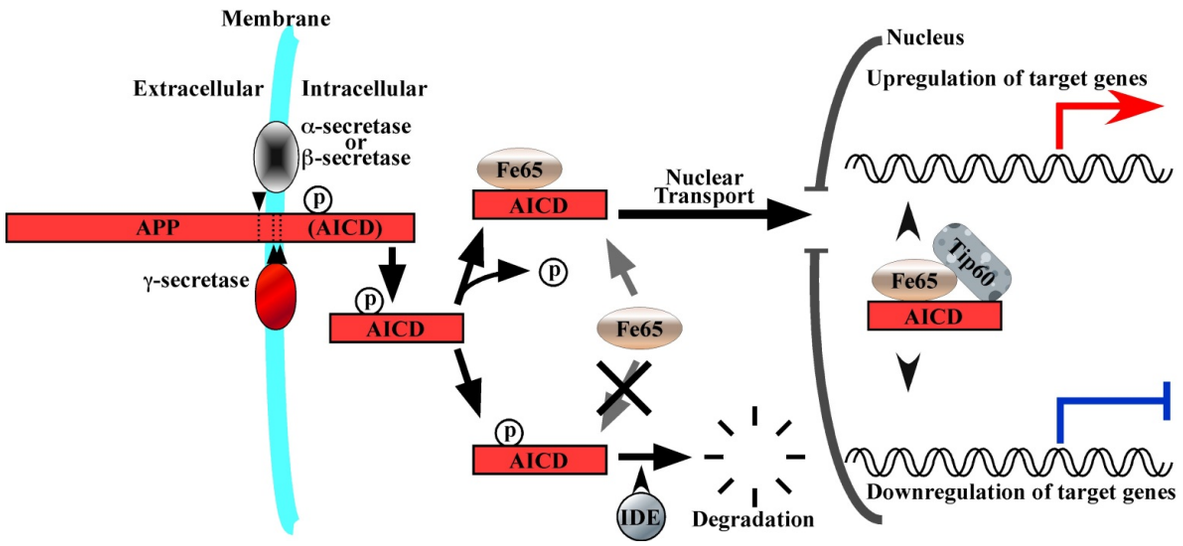

Figure 6. Model of APP signaling pathway. The majority of cell membrane-associated APP is phosphorylated within its ICD in neurons. After cleavage of JM domain by α- or β-secretase, AICD is released from the membrane by γ-secretase. Phosphorylated AICD cannot bind to the nuclear adaptor protein Fe65, which is thought to be essential for translocation of AICD to the nucleus, and thus cannot translocate to the nucleus. Phosphorylated AICD left in the cytosol is rapidly degraded, probably by the proteasome and/or insulin-degrading enzyme (IDE). On the other hand, dephosphorylated AICD binds to Fe65. Therefore, dephosphorylated AICD/Fe65 complexes immediately translocate to the nucleus, where they meidate up- and downregulation of certain target genes in association with Tip60.

In addition to these possibilities, it is also likely that AICD is ineffective in the normal brain, because almost all AICD is degraded rapidly, and APP signaling cannot be transmitted. However, both AICD and Aβ are overproduced in the AD brain compared to normal brain. Thus, although the majority of AICD is degraded, a small amount of the remaining AICD may play a role in signaling and cause neuron-specific cell death in the AD brain. In addition, if the degrading activity of AICD is reduced or lost in the AD brain, APP signaling, which leads to neuron-specific cell death, may be enhanced. Thus, compounds that inhibit translocation of AICD to the nucleus will be good candidates for AD therapy. From this point of view, protein phosphatase inhibitors and chemicals that impair the interaction between AICD and Fe65 may be potential ones.

8. Conclusion

γ-Secretase was first identified as a protease that cleaves APP within the transmembrane domain and produces Aβ peptides, which are the main hallmark of AD and are thought to be

involved in the pathogenesis in the AD brains. However, the physiological functions of this protease remain to be clarified.

The signaling hypothesis for γ-secretase suggesting that its primary function is to regulate the signaling of type 1 membrane proteins was proposed by analogy of Notch signaling. In the canonical Notch signaling pathway, ligands bind to the extracellular domain of Notch expressed on neighboring cells, and trigger sequential proteolytic cleavage. Finally, NICD is released from the cell membrane by γ-secretase and translocates into the nucleus where it modulates gene expression through binding to transcription factors. Thus, γ-secretase plays a central regulatory role in Notch signaling.

While APP is thought to play central roles in the onset and progression of AD, the physiological functions of this protein also have not yet been fully elucidated. However, it has been shown that AICD, which is released from the cell membrane by γ-secretase, also translocates to the nucleus and may function as a transcriptional regulator. These observations suggest the existence of a signaling mechanism similar to that of Notch.

In this chapter, we focused on the signaling aspects of APP and its pathological roles in AD. Indeed, we showed that AICD alters gene expression and induces neuron-specific apoptosis. Thus, it is likely that APP has a signaling mechanism similar to that of Notch and that APP signaling is at least partially responsible for the onset and progression of AD. If the APP signaling hypothesis is correct, several molecules involved in APP signaling may be attractive candidates for the targets of drug discovery for treating AD. Thus, extensive studies about this issue are expected.

Abbreviations

AD, Alzheimer's disease;

Aβ, amyloid-β;

APP, amyloid precursor protein;

AICD, the intracellular domain of APP;

Aph-1, anterior pharynx defective-1;

CAA, cerebral amyloid angiopathy;

Dll, Delta-like protein

Dll1IC, the intracellular domain of Dll1;

EGF, epidermal growth factor;

FAD, familial AD;

Hes, Hairy/Enhancer of split;

ICD, intracellular domain;

IDE, insulin-degrading enzyme;

JM, juxtamembrane;

KPI, Kunitz inhibitor domain;

NICD, the intracellular domain of Notch;

NCT, nicastrin;

NEP, neprilysin;

PS, presenilin;

Pen-2, PS enhancer-2 protein;

RIP, regulated intramembrane proteolysis;

RA, all-trans-retinoic acid;

TM, transmembrane;

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