

# Trafficking and Proteolytic Processing of APP

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Accumulations of insoluble deposits of amyloid  $\beta$ -peptide are major pathological hallmarks of Alzheimer disease. Amyloid  $\beta$ -peptide is derived by sequential proteolytic processing from a large type I trans-membrane protein, the  $\beta$ -amyloid precursor protein. The proteolytic enzymes involved in its processing are named secretases.  $\beta$ - and  $\gamma$ -secretase liberate by sequential cleavage the neurotoxic amyloid  $\beta$ -peptide, whereas  $\alpha$ -secretase prevents its generation by cleaving within the middle of the amyloid domain. In this chapter we describe the cell biological and biochemical characteristics of the three secretase activities involved in the proteolytic processing of the precursor protein. In addition we outline how the precursor protein matures and traffics through the secretory pathway to reach the sub-cellular locations where the individual secretases are preferentially active. Furthermore, we illuminate how neuronal activity and mutations which cause familial Alzheimer disease affect amyloid  $\beta$ -peptide generation and therefore disease onset and progression.

## PROTEOLYTIC PROCESSING OF APP

### APP Processing: The Amyloidogenic and Anti-Amyloidogenic Pathways

The 37–43 amino acid amyloid  $\beta$ -peptide ( $A\beta$ ) is generated by proteolytic processing from its precursor, the  $\beta$ -amyloid precursor protein (APP) in a physiologically normal pathway (Haass et al. 1992, 1993a; Seubert et al. 1992; Shoji et al. 1992; Busciglio et al. 1993; Haass and Selkoe 1993). APP is a type-I oriented membrane protein with its amino terminus within the lumen/extracellular space and its carboxyl terminus within the cytosol (Kang et al. 1987; Dyrks et al. 1988). Although APP

is initially targeted into the secretory pathway (see below), it is proteolytically processed at several different subcellular sites (Weidemann et al. 1989). Three protease activities called  $\alpha$ -,  $\beta$ -, and  $\gamma$ -secretase are involved in specific processing steps (Haass 2004). The name “secretases” refers to the secretion of the proteolytically cleaved substrates. All three protease activities have been identified and are described below.

We discriminate two principal processing pathways: the amyloidogenic pathway, which leads to  $A\beta$  generation; and the anti-amyloidogenic pathway, which prevents  $A\beta$  generation (Fig. 1).  $A\beta$  is produced in the amyloidogenic

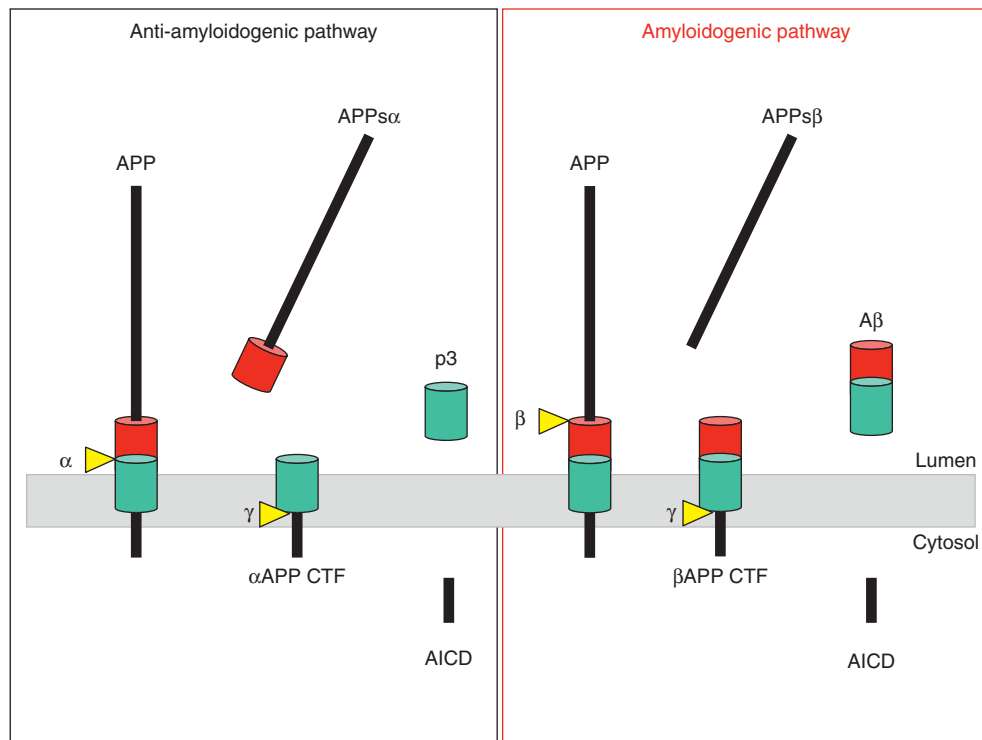
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**Figure 1.** Proteolytic processing of APP within the anti-amyloidogenic (*left*) and amyloidogenic (*right*) pathways.

pathway by the consecutive action of  $\beta$ - and  $\gamma$ -secretase (Haass 2004). The  $\beta$ -secretase activity initiates  $A\beta$  generation by shedding a large part of the ectodomain of APP ( $APPs\beta$ ) and generating an APP carboxy-terminal fragment ( $\beta$ CTF or C99), which is then cleaved by  $\gamma$ -secretase. The latter cleavage occurs within the hydrophobic environment of biological membranes. Consecutive shedding and intramembrane proteolysis is now summarized under the term “regulated intramembrane proteolysis” (Brown et al. 2000; Rawson 2002; Lichtenthaler et al. 2011), a cellular process, which is frequently involved in important signaling pathways (Selkoe and Kopan 2003; see DeStrooper et al. 2011). On  $\gamma$ -secretase cleavage,  $A\beta$  is liberated and then found in extracellular fluids such as plasma or cerebrospinal fluid (Seubert et al. 1992). In the anti-amyloidogenic pathway, APP is cleaved approximately in the middle of the  $A\beta$  region by the  $\alpha$ -secretase activity (Esch

et al. 1990; Sisodia et al. 1990). This processing step generates a truncated APP CTF ( $\alpha$ CTF or C83), which lacks the amino-terminal portion of the  $A\beta$  domain. The subsequent intramembrane cut by  $\gamma$ -secretase liberates a truncated  $A\beta$  peptide called p3 (Haass et al. 1993b), which apparently is pathologically irrelevant.  $\gamma$ -Secretase not only liberates  $A\beta$  (from C99) and p3 (from C83) but also generates the APP intracellular domain (AICD) (Gu et al. 2001; Sastre et al. 2001; Weidemann et al. 2002), which is released into the cytosol and which may have a function in nuclear signaling (Cao and Sudhof 2001; von Rotz et al. 2004). The amyloidogenic and the anti-amyloidogenic processing pathways compete with each other at least in some subcellular loci, since enhancing  $\alpha$ -secretase activity in animal models of Alzheimer disease (AD) or in cultured cells can significantly lower  $A\beta$  generation and even amyloid plaque formation (Nitsch et al. 1992; Postina et al. 2004).

### Familial Alzheimer Disease–Associated Mutations within the APP Gene Affect A $\beta$ Generation and Aggregation

A number of familial Alzheimer disease (FAD)-associated mutations have been found within and around the A $\beta$  domain (Chartier-Harlin et al. 1991; Selkoe 2001; discussed in detail in Schenk et al. 2011). These mutations accelerate disease progression via diverse mechanisms. The Swedish mutation at the amino terminus of the A $\beta$  region (Mullan et al. 1992) results in a significant increase of total A $\beta$  production (such as A $\beta$ 40 and A $\beta$ 42) by providing a better substrate for the  $\beta$ -secretase activity (Citron et al. 1992; Cai et al. 1993). Mutations located just beyond the carboxyl terminus of A $\beta$  (such as the so-called Austrian, Iranian, French, German, London, and Florida mutations) cause the increased production of longer A $\beta$  species (A $\beta$ 42), which aggregate more rapidly and are believed to be the major neurotoxic A $\beta$  species (Suzuki et al. 1994). Mutations in the mid region, such as the Arctic (Nilsberth et al. 2001) and Dutch mutations (Levy et al. 1990), affect the primary sequence of A $\beta$  and apparently change the structure of A $\beta$ , resulting in its enhanced aggregation propensity. Some of these intra-A $\beta$  mutations can lead to mixed amyloid pathologies: marked cerebral angiopathy and marked amyloid plaque formation. For the Flemish mutation, an unexpected pathological mechanism was described recently. This mutation is located in an apparent substrate inhibitory domain that negatively regulates  $\gamma$ -secretase activity by binding to an unknown allosteric site within the complex. The Flemish mutation can reduce the activity of this inhibitory domain and consequently increase A $\beta$  generation (Tian et al. 2010).

### The Amyloidogenic Proteases: $\beta$ - and $\gamma$ -Secretase

#### $\beta$ -Secretase

$\beta$ -Secretase mediates the initial and rate-limiting processing step during A $\beta$  generation (Vassar 2004). Expression cloning or biochemical purification led to the identification of a

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unique  $\beta$ -secretase enzyme (Sinha et al. 1999; Vassar et al. 1999; Yan et al. 1999; Hussain et al. 2000; Lin et al. 2000). Although many different names were originally used to describe this activity, such as memapsin, aspartyl protease 2, or BACE1 ( $\beta$ -site APP cleaving enzyme-1), BACE1 is now the generally accepted term for the enzyme harboring  $\beta$ -secretase activity. BACE1 is a membrane-bound aspartyl protease with its active site in the lumen/extracellular space and with structural similarities to the pepsin family (Hong et al. 2000). Besides BACE1, a homologous protease called BACE2 was identified (Vassar 2004). However, BACE2 is not involved in amyloidogenesis and may rather exert an anti-amyloidogenic activity in non-neuronal cells somewhat similar to  $\alpha$ -secretase (Bennett et al. 2000a; Farzan et al. 2000; Fluhner et al. 2002; Basi et al. 2003). BACE1 is the sole  $\beta$ -secretase, because its knockout completely blocks A $\beta$  generation (Cai et al. 2001; Roberds et al. 2001; Luo et al. 2003). The protease is ubiquitously expressed, with highest levels in brain and pancreas; the physiological relevance of high pancreatic expression is currently not understood. Because APP is also expressed at very high levels in the brain, the concomitant high levels of BACE1 and APP make the brain the primary tissue for high A $\beta$  generation and help explain why AD is a brain disease even though APP is expressed ubiquitously.

BACE1 is an important therapeutic target (Citron 2004), because its inhibition not only reduces A $\beta$  levels but also prevents the accumulation of  $\beta$ CTFs, which contain the entire A $\beta$  domain and serve as the final substrate for A $\beta$  production (see Fig. 1). This is an important issue, because accumulation of such CTFs may cause additional, poorly understood toxic effects. Progress has been made toward the generation of BACE1 inhibitors, and clinical studies are on the way (Citron 2004; Schenk et al. 2011). However, one must be aware that such an approach also inhibits the physiological function of BACE1. So far, only very few physiological substrates have been validated whose cleavage by BACE1 is associated with a clear biological function. BACE1 knockout mice are

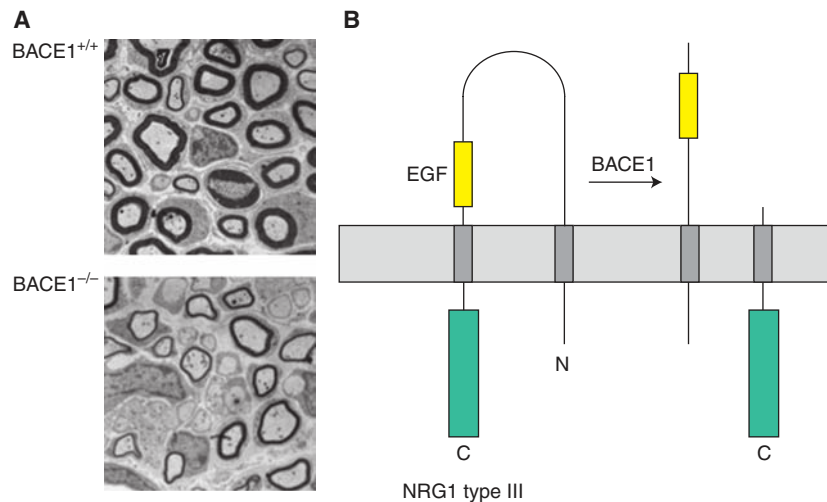
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viable and fertile and do not show any major behavioral, morphological, or developmental deficits (Cai et al. 2001; Roberds et al. 2001; Luo et al. 2003). However, subtle behavioral phenotypes such as some memory impairment and changes in spontaneous activity (Harrison et al. 2003; Dominguez et al. 2005) indicate that a loss of function of BACE1 can have detrimental consequences. Very high postnatal expression levels of BACE1 (Willem et al. 2006) revealed a function of BACE1 in myelination, a process which occurs after birth. Indeed all available BACE1 knockout mice show a significant hypomyelination phenotype in the peripheral nervous system (Fig. 2A; Hu et al. 2006; Willem et al. 2006). Whether myelination within the CNS is also under the control of BACE1, as described by Hu et al. (2006), is currently under debate. Schwann cell-mediated myelination in the peripheral nervous system is regulated via the Neuregulin-1 (NRG1) signaling pathway (Birchmeier and Nave 2008). Interestingly, proteolytic processing of NRG1 (Fig. 2B) is believed to facilitate its signaling activity. Indeed, in the BACE1 knockout animals, uncleaved NRG1

accumulates. Thus, NRG1 is a physiological substrate for BACE1, and at least one of the physiological functions of BACE1 concerns myelination. BACE1 has also been shown to be involved in the regulation of voltage-dependent sodium channels (Kim et al. 2007). Moreover, other substrates such as Type II  $\alpha$ -2,6-sialyltransferase, platelet selectin glycoprotein ligand-1, APP-like proteins, A $\beta$  itself, and the interleukin-like receptor type II have also been shown to be processed by BACE1 (summarized in Willem et al. 2009). However, the physiological consequences of these cleavages are unclear, and one should keep in mind that most substrates were identified on overexpression of BACE1 and/or the substrate, which is likely to generate conditions allowing artificial substrate/protease interactions.

### *$\gamma$ -Secretase*

The A $\beta$ -liberating cleavage of APP is mediated by  $\gamma$ -secretase and occurs within the transmembrane domain (TMD).  $\gamma$ -Secretase structure and function is discussed in De Strooper et al. (2011). Here, we will briefly introduce

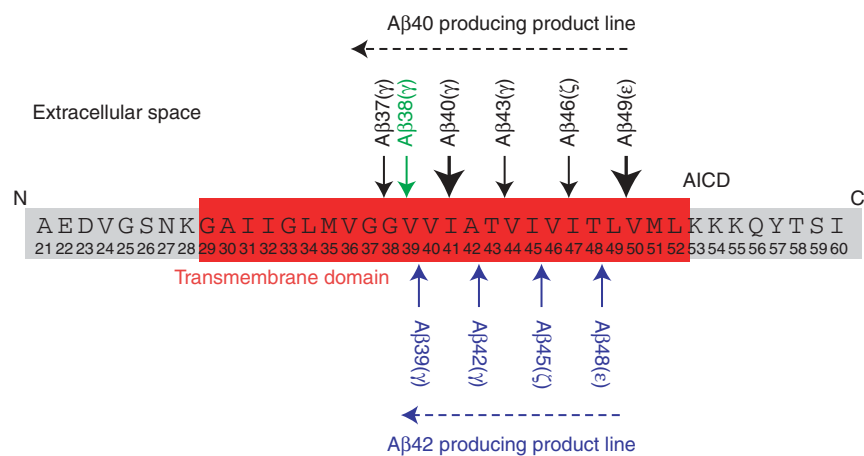


**Figure 2.** Biological function of BACE1 in myelination. (A) A BACE1 knockout in mice results in a hypomyelination phenotype within the peripheral nervous system. Cross-sections through the sciatic nerve of wild-type mice and BACE1 knockout mice are shown. (B) Proteolytic processing of NRG1 type III. NRG1 type III is cleaved by BACE1. This processing step leads to the exposure of EGF-containing domain and facilitates signaling via ErbB4 in Schwann cells.

$\gamma$ -secretase and then focus on the cellular assembly of  $\gamma$ -secretase and its subcellular sites of activity.

$\gamma$ -Secretase is a protease complex consisting of four subunits (reviewed in Steiner et al. 2008). Presenilin (PS) 1 or PS2 contain the two critical aspartyl residues within TMDs 6 and 7, which are part of the catalytic domain of the aspartyl protease activity of  $\gamma$ -secretase (Wolfe et al. 1999). Additional complex components are nicastrin (NCT), anterior pharynx defective (APH)-1a or APH-1b, and the PS enhancer (PEN)-2 (Yu et al. 2000; Francis et al. 2002). These four components are necessary and sufficient for full  $\gamma$ -secretase activity (Edbauer et al. 2003). Little is known about the biological function of NCT, APH-1, and PEN-2. NCT is probably required as a size-selecting substrate receptor (Shah et al. 2005; Dries et al. 2009), although recent findings may challenge such a function (Chavez-Gutierrez et al. 2008; Martin et al. 2009). PEN-2 apparently facilitates PS endoproteolysis into its active heterodimeric state and stabilizes PS within the  $\gamma$ -secretase complex (Hasegawa et al. 2004; Prokop et al. 2004). No specific function has so far been assigned to APH-1, although it may act as a scaffold for the initial binding of NCT and assembly of the complex (LaVoie et al. 2003).

The intramembrane processing of APP by  $\gamma$ -secretase is not restricted to a single site. Rather, it appears that  $\gamma$ -secretase substrates are cleaved several times within their TMDs. Cleavages at the so-called  $\epsilon$ -,  $\zeta$ -, and  $\gamma$ -sites that are separated by approximately three amino acids are postulated (Fig. 3; Sastre et al. 2001; Weidemann et al. 2002; Qi-Takahara et al. 2005; Takami et al. 2009). To make things even more complicated, the final  $\gamma$ -cleavage is also not precise and can occur under physiological conditions at least between amino acids 37 and 43 of the A $\beta$  domain. This difference is of greatest relevance for the understanding of AD pathology, because the longer A $\beta$ 42 species is more aggregation prone and believed to be the toxic building block of A $\beta$  oligomers, which affect memory and cell survival (Haass and Selkoe 2007). How can these multiple cleavages be explained? Although it is not yet finally proven, it is likely that this phenomenon is due to a stepwise cleavage mechanism performed by one and the same  $\gamma$ -secretase. Moreover, stepwise endoproteolysis may be a general phenomenon of intramembrane proteolysis mediated by all  $\gamma$ -secretase-like proteases (Fluhrer et al. 2006, 2008, 2009). Apparently, once APP (or another substrate) is bound to the active site of the  $\gamma$ -secretase complex, intramembrane proteolysis begins with the  $\epsilon$ -cleavage after amino acids



**Figure 3.** Sequential processing of APP by  $\gamma$ -secretase.

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49 or 48. This is then followed by cleavage after amino acids 46 or 45 ( $\zeta$ -cleavage) and terminates with the cleavage at the  $\gamma$ -site mostly at amino acids 42 or 40 (but also after amino acids 37, 38, 39, and 43; Fig. 3). Two product lines are discussed (Fig. 3): one leading predominantly to A $\beta$ 42 generation (starting with the  $\epsilon$ -cleavage after amino acid 48 and followed by cleavages after amino acids 45 and 42); and the other leading predominantly to A $\beta$ 40 (starting with the  $\epsilon$ -cleavage after amino acid 49 and followed by cleavages after amino acids 46 and 43). As discussed in Schenk et al. (2011), these cleavages may be therapeutically modulated to selectively prevent A $\beta$ 42 generation.

### The Anti-Amyloidogenic $\alpha$ -Secretase

As mentioned above, the anti-amyloidogenic processing of APP occurs within the A $\beta$  domain between residues Lys16 and Leu17 (Esch et al. 1990; Sisodia et al. 1990; Wang et al. 1991) and results in the secretion of the large APP amino-terminal domain and the generation of  $\alpha$ -CTF (C83). This cleavage is performed by a set of proteases termed  $\alpha$ -secretases. Shortly after the identification of the  $\alpha$ -secretase cleavage site, it was noted that in cultured cells this cleavage predominantly occurs at the cell surface, suggesting that  $\alpha$ -secretases are plasma membrane (PM)-bound proteases (Sisodia 1992). Activation of protein kinase C by phorbol esters stimulates  $\alpha$ -secretase processing and secretion of the APP ectodomain (Buxbaum et al. 1990). This protein kinase C-dependent APP processing was dubbed “regulated  $\alpha$ -secretase cleavage” of APP. Several zinc metalloproteinases that are members of the “a disintegrin and metalloprotease” family such as ADAM9, ADAM10, TACE/ADAM17 and ADAM19 can function as  $\alpha$ -secretase (Allinson et al. 2003). Targeted disruption of individual genes that encode ADAM10, TACE/ADAM17, or ADAM19 has no effect on constitutive  $\alpha$ -secretase processing of APP, indicating that  $\alpha$ -secretase activity is shared by a set of ADAM proteases (Buxbaum et al. 1998b; Merlos-Suarez et al. 1998; Hartmann et al. 2002; Weskamp et al. 2002). However, recent evidence suggests that, at least in neurons,

the principal constitutive  $\alpha$ -secretase activity is exerted by ADAM10 (Kuhn et al. 2010). Besides APP, Notch receptors and ligands, tumor necrosis factor  $\alpha$ , cadherins and IL-6 receptor, EGF receptor ligands, and several other type I transmembrane proteins are cleaved by  $\alpha$ -secretases to release their extracellular domain. Consequently, the process of ectodomain shedding mediated by  $\alpha$ -secretases appears to be largely sequence independent. At a minimum,  $\alpha$ -secretase cleavage of APP is determined by an  $\alpha$ -helical conformation and the distance (12–13 residues) of the hydrolyzed bond from the membrane (Sisodia 1992).

Amyloidogenic processing appears to be the favored pathway of APP metabolism in neurons, largely because of the greater abundance of BACE1, whereas anti-amyloidogenic pathway is predominant in all other cell types. Overexpression of ADAM10 and other putative  $\alpha$ -secretases in cultured cells as well as in transgenic mice increases the secretion of the APP ectodomain ending at the  $\alpha$ -secretase site. Interestingly, neuronal overexpression of ADAM10 in transgenic mice reduces BACE1 processing of APP and amyloid deposition (Postina et al. 2004). This finding is of physiological relevance because ADAM10 is expressed throughout the cortex and hippocampus in the adult central nervous system, and APP, BACE1, and ADAM10 are co-expressed in human cortical neurons (Marcinkiewicz et al. 2000). Other putative  $\alpha$ -secretases such as ADAM9, TACE/ADAM17 and ADAM19 are also expressed in the adult brain. Thus, up-regulation of  $\alpha$ -secretase activity to promote anti-amyloidogenic processing of APP is potentially of therapeutic value (Postina et al. 2004).

Commitment of APP to amyloidogenic and anti-amyloidogenic pathways can be differentially modulated by the activation of cell-surface receptors such as the serotonin/5-hydroxytryptamine (5-HT<sub>4</sub>) receptor, metabotropic glutamate receptors, muscarinic acetylcholine receptors, and platelet-derived growth factor receptor (Allinson et al. 2003). Signaling downstream from these receptors regulates APP $\alpha$  and A $\beta$  secretion by engaging intermediates including protein kinase C, protein kinase A,

phosphatidylinositol-3-kinase, mitogen-activated protein kinase kinase, extracellular signal-regulated kinase, Src tyrosine kinase, small GTPase Rac, inositol 1,4,5-trisphosphate, cAMP, and cytosolic calcium (reviewed in Gandy et al. 1994; Allinson et al. 2003). Lowering cholesterol levels in cultured cells stimulates  $\alpha$ -secretase cleavage of APP through mechanisms involving impaired APP endocytosis and increased steady-state levels of ADAM10 (Kojro et al. 2001). The effect of cholesterol depletion is not specific to APP cleavage, because the shedding of the human interleukin-6 receptor by ADAM10 and TACE/ADAM17 is also stimulated under these conditions (Matthews et al. 2003).

### CELLULAR TRAFFICKING OF APP

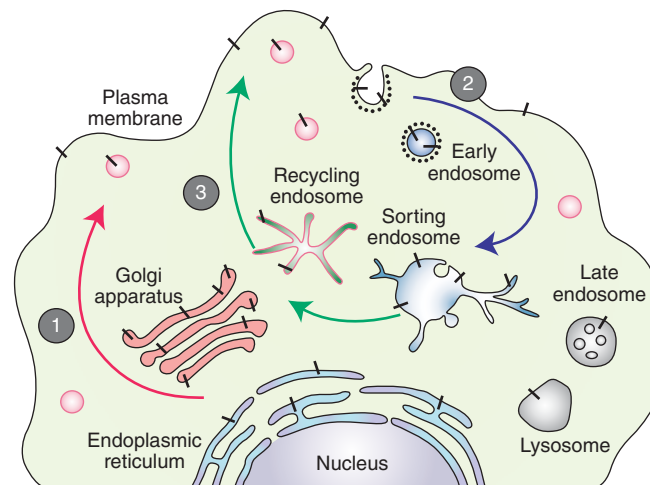
#### Biosynthesis and Trafficking through the Secretory Pathway

The pathways of APP trafficking in a nonpolarized mammalian cell are depicted in Figure 4. During its transit from the ER to the PM, nascent APP is posttranslationally modified by N- and O-linked glycosylation, ectodomain and

cytoplasmic phosphorylation, and tyrosine sulfation. Only a small fraction of nascent APP molecules reach the PM (estimated at ~10% based on APP overexpression in cultured cells), whereas the majority of APP at steady-state localizes to the Golgi apparatus and trans-Golgi network (TGN). APP which is not shed from the cell surface is internalized within minutes of arrival at the cell surface because of the presence of its "YENPTY" internalization motif near the carboxyl terminus of APP (residues 682–687 of APP695 isoform) (Lai et al. 1995; Marquez-Sterling et al. 1997). Following endocytosis, APP is delivered to endosomes, and a fraction of endocytosed molecules is recycled to the cell surface. Measurable amounts of internalized APP also undergo degradation in lysosomes (Haass et al. 1992).

#### Endocytic APP Sorting and $A\beta$ Production

Although attempts to characterize the role of endocytic APP trafficking by expression of dominant-negative dynamin mutants resulted in discrepant findings (Chyung et al. 2003; Ehehalt et al. 2003; Carey et al. 2005), mutations



**Figure 4.** Intracellular trafficking of APP. Nascent APP molecules (black bars) mature through the constitutive secretory pathway (1). Once APP reaches the cell surface, it is rapidly internalized (2) and subsequently trafficked through endocytic and recycling organelles to the TGN or the cell surface (3). A small fraction is also degraded in the lysosome. Nonamyloidogenic processing mainly occurs at the cell surface where  $\alpha$ -secretases are present. Amyloidogenic processing involves transit through the endocytic organelles where APP encounters  $\beta$ - and  $\gamma$ -secretases.

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within the APP cytosolic YENPTY motif selectively inhibit APP internalization and decrease A $\beta$  generation (Perez et al. 1999). This motif and the flanking region serve as the binding site for many cytosolic adaptors that have phosphotyrosine-binding domains, including Fe65, Fe65L1, Fe65L2, Mint 1 (also called X11 $\alpha$ ), Mint 2, Mint 3, Dab1, sorting nexin 17, and c-Jun amino-terminal kinase-interacting protein family members. Overexpression of Mint 1, Mint 2, or Fe65 causes reduction in A $\beta$  generation and less deposition in the brains of APP transgenic mice, strongly suggesting a physiological role for these adaptors in regulating amyloidogenic processing of APP in the nervous system (Miller et al. 2006). In addition to binding APP, Mint proteins can directly bind ADP-ribosylation factors, raising the intriguing possibility that Mints may regulate vesicular trafficking of APP by serving as coat proteins (Hill et al. 2003). A conformational change introduced by phosphorylation at Thr-668 (14 amino acids proximal to the YENPTY motif) interferes with Fe65 binding to APP and facilitates BACE1 and  $\gamma$ -secretase cleavage of APP in cultured cells (Ando et al. 2001; Lee et al. 2003). However, analysis of Thr-668-Ala knock-in mice indicates that the phosphorylation status of Thr-668 does not affect physiological processing of APP into A $\beta$  peptides in vivo (Sano et al. 2006). In addition to its potential role in APP endocytosis, Fe65 stabilizes the highly labile AICD, which may serve as a regulatory step in modulating the physiological function of AICD. Fe65 is capable of interacting with APP and LRP (a multifunctional endocytosis receptor containing two NPXY motifs) via distinct protein interaction domains (Trommsdorff et al. 1998). Lack of LRP expression causes reduced APP internalization and A $\beta$  secretion (Ulery et al. 2000; Pietrzik et al. 2002; Cam et al. 2005), leading to the conclusion that endocytosis of LRP is coupled to APP internalization and processing, and Fe65 acts as a functional linker between APP and LRP in modulating endocytic APP trafficking (Pietrzik et al. 2004). In addition, Ran-binding protein 9 promotes APP interaction with APP and facilitates APP internalization in a Fe65-independent

manner (Lakshmana et al. 2009). Finally, the type I transmembrane protein sorLA/LR11 (a member of the VPS10p-domain receptor family), which functionally interacts with cytosolic adaptors GGA and PACS-1, regulates A $\beta$  production by acting as a Golgi/TGN retention factor for APP (Andersen et al. 2005; Offe et al. 2006; Schmidt et al. 2007). A $\beta$  levels are reduced on overexpression of sorLA/LR11 in cultured cells and increased in the brains of sorLA/LR11 knockout mice (Andersen et al. 2005; Offe et al. 2006). sorLA/LR11 is also genetically associated with AD and its steady-state levels are markedly reduced in the brains of patients with AD, further implicating this sorting molecule in the physiological regulation of APP metabolism (Andersen et al. 2005; Offe et al. 2006; Rogaeva et al. 2007).

### Polarized Trafficking of APP in Non-Neuronal Cells

Obviously, neurons are of pivotal interest for the analysis of APP trafficking and processing. Because of the polarized nature of neurons, a strong interest emerged in understanding how APP is targeted to selected destinations and where during its cellular transport APP is processed into its cleavage products by the three secretase activities described above. Cellular model systems have been developed that allow analysis of polarized sorting in relatively simple peripheral cells. Madin–Darby canine kidney (MDCK) cells are a suitable cell system, as they form polarized monolayers with defined apical and basolateral surfaces on culturing in Transwell chambers. This widely used tool in cell biology not only allows the separate collection of apically and basolaterally secreted proteins but also the detection of membrane-bound proteins on either surface, for example, via biotinylation.

### Polarized Trafficking of APP via Two Independent Sorting Mechanisms

Using MDCK cells, it has been shown that a number of cleavage products of APP including soluble APP ( $\alpha$ -secretase generated APP;  $\alpha$ APPs), A $\beta$ , and p3 are selectively targeted to



the basolateral compartment (Haass et al. 1994; De Strooper et al. 1995a). In addition, surface APP also accumulates on the basolateral surface, and APPs $\alpha$  shed from surface APP accumulates in the basolateral media (Fig. 5). These findings suggested that APP contains a sorting signal that selectively targets it to the basolateral side of polarized cells. Deletion of the last 42 amino acids of the cytoplasmic domain of APP (aa 654–695 according to the numbering of APP695) causes a random (default) transport of the resultant APP- $\Delta$ C to both surfaces (Fig. 5C). Moreover, Tyr 653 was identified as a critical amino acid required for efficient basolateral APP sorting (Haass et al. 1995a). Interestingly, this signal acts independently of a re-internalization signal between aa 684–687 (see above). Surprisingly, when a Tyr653-mutant APP was expressed, the bulk of  $\alpha$ APPs was still sorted efficiently to the basolateral compartment, although the membrane-bound holoprotein was transported equally to both surfaces (De Strooper et al. 1995a; Haass et al. 1995a). This result indicates two independent sorting mechanisms, one for membrane-bound APP and one for soluble APPs $\alpha$  (Fig. 5A–C). Whereas the first sorting pathway is dependent on a cytoplasmic sorting signal similar to other polarized sorted proteins, the soluble ectodomain of APP apparently contains an independent basolateral sorting signal (De Strooper et al. 1995b; Haass et al. 1995a). Indeed, when APPs $\alpha$  was expressed as a recombinant protein, it was still rather efficiently sorted to the basolateral compartment (Haass et al. 1995a). Evidence exists that alternative splicing of APP can affect polarized secretion of soluble APP (Hartmann et al. 1996). APP variants lacking the exon 15 encoded domain are sorted equally into both compartments. It is assumed that the 3D structure by itself, not a linear sequence motif, determines polarized sorting of secreted APPs $\alpha$ . Cellular fractionation studies show that a large amount of APPs $\alpha$  is already generated by  $\alpha$ -secretase within the Golgi compartment long before it reaches the cell surface of MDCK cells (Haass et al. 1995a). Based on previous findings it was assumed that sorting of soluble proteins occurs within a pH-sensitive

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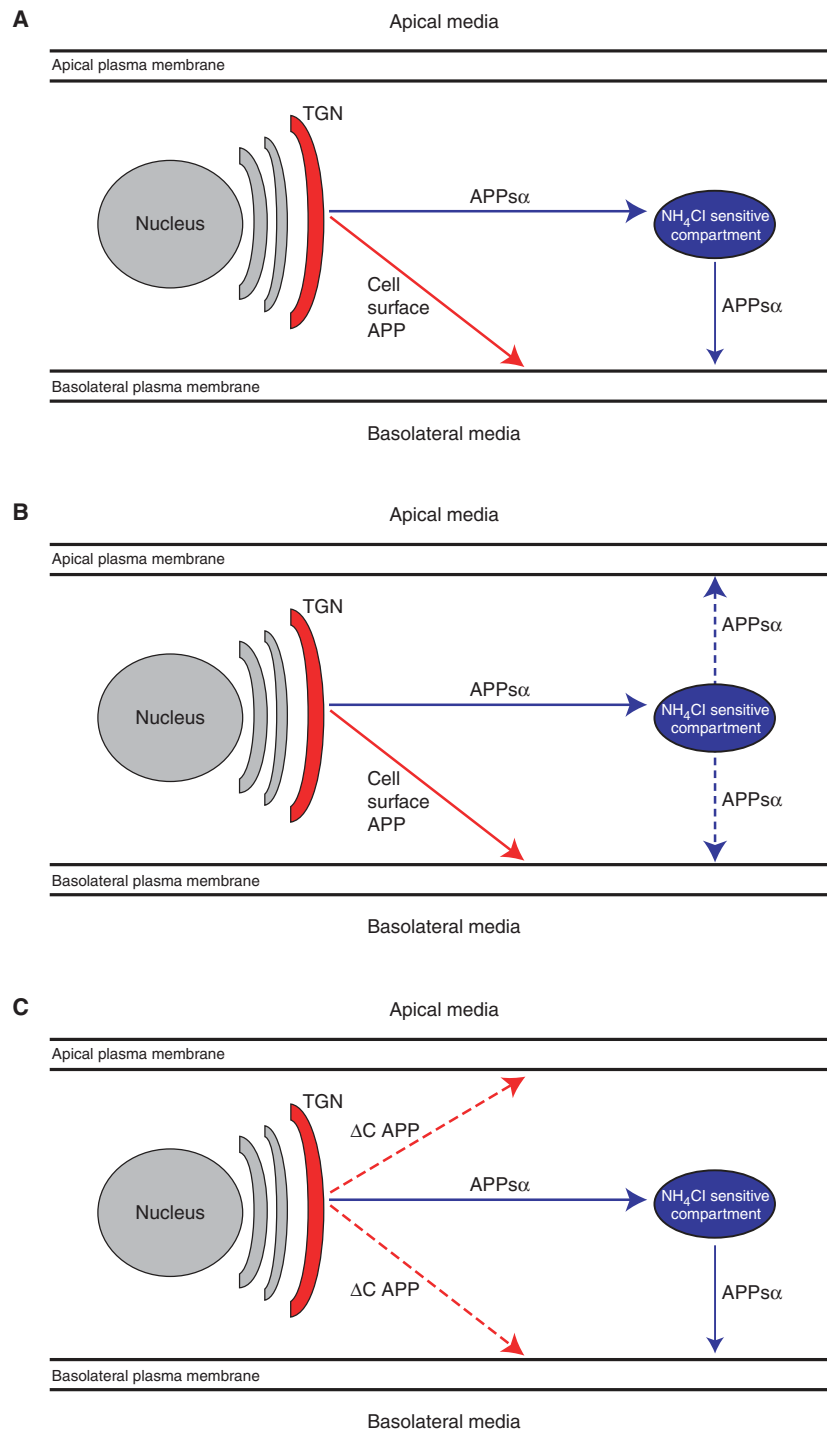
compartment (De Strooper et al. 1995a; Haass et al. 1995a). Indeed NH<sub>4</sub>Cl treatment randomizes polarized secretion of  $\alpha$ APPs. Thus, two distinct pathways mediate polarized sorting of APP in MDCK cells (Fig. 5A–C).

### *Polarized Sorting of Secretases*

The above findings have major implications for the polarized trafficking of all three secretase activities. As described in the first publication on polarized secretion of A $\beta$ , only small amounts of authentic A $\beta$  beginning with Asp1 of the A $\beta$  sequence are observed in the basolateral media (Haass et al. 1994). This is consistent with the finding that BACE1, which cleaves at Asp1, is predominantly targeted to the apical surface of MDCK, a process which markedly limits A $\beta$  generation in polarized cells (Capell et al. 2002). Indeed, when APP is mis-sorted to the apical surface, it is almost exclusively processed by BACE-1. This also indicates that little  $\alpha$ -secretase activity is present in the apical sorting pathway, because apparently there is almost no competition for BACE-1 cleavage of APP on the apical side. Indeed ADAM-10, the major  $\alpha$ -secretase activity (see above), is targeted basolaterally (Wild-Bode et al. 2006); here, it competes efficiently with the rather small amount of BACE1 present within the basolateral pathway. ADAM-10, like many other members of the ADAM family, contains two cytoplasmic Src homology 3 (SH3)-binding domains. Sequential deletion revealed critical Pro residues within the juxtamembrane SH3-binding domain. This trafficking signal was required to target ADAM-10 to adherens junctions and to support its function in cell migration and E-cadherin processing (Wild-Bode et al. 2006). Therapeutic strategies aimed at increasing  $\alpha$ -secretase-mediated anti-amyloidogenic processing of APP have to take this important physiological function—and numerous other functions which depend on  $\alpha$ -secretase-mediated shedding—into consideration.

In contrast to BACE1 and ADAM-10,  $\gamma$ -secretase activity is found on both surfaces (Capell et al. 2002), a finding, which is consistent with the observation that A $\beta$  and p3 can be found

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**Figure 5.** Polarized sorting of APP in MDCK cells. (A) Two independent basolateral sorting pathways for  $\alpha$ APPs via a NH<sub>4</sub>Cl-sensitive compartment (blue) and full-length APP (red). (B) Inhibition of vesicular acidification by NH<sub>4</sub>Cl leads to random secretion of  $\alpha$ APPs whereas full-length APP is still sorted to the basolateral membrane. (C) APP lacking a cytoplasmic signal for basolateral sorting ( $\Delta$ C APP) undergoes random surface transport.

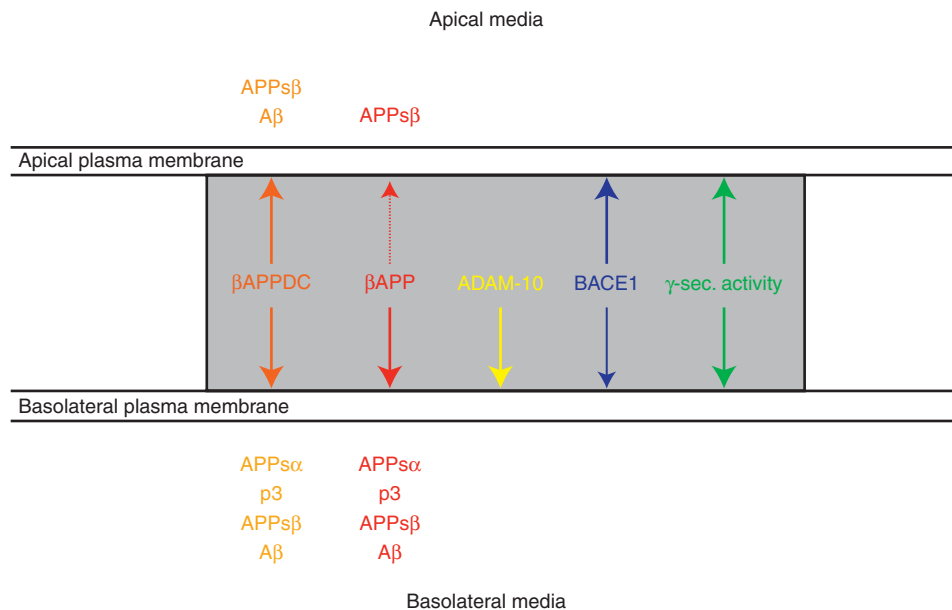
in both compartments, dependent on the APP variant expressed (i.e., with or without a basolateral sorting sequence) (Haass et al. 1995a). Thus the two competing proteases, ADAM-10 and BACE1, are sorted differentially to the basolateral or apical surface, whereas  $\gamma$ -secretase is found on both sides (Fig. 6).

As described above, several familial autosomal dominant mutations were linked to APP. One of these mutations, the so-called Swedish mutation (Mullan et al. 1992) occurs immediately adjacent to the BACE1 cleavage site (Met-Asp). This mutation strongly facilitates BACE-1-mediated processing of APP and therefore results in enhanced A $\beta$  generation (Citron et al. 1992; Cai et al. 1993). In parallel, a significantly enhanced amount of the shorter variant of sAPP (APPs $\beta$ ) is secreted (Haass et al. 1995). Interestingly, when Swedish mutant APP was expressed in MDCK cells, APPs $\beta$  was found to be secreted apically, while the endogenous APPs (mainly consisting of APPs $\alpha$ ) and membrane-bound holoAPP still underwent basolateral sorting (Lo et al. 1994; De Strooper et al. 1995a). Based on the findings described above, this observation may reflect the Swe-

dish mutation-induced processing of the small amounts of APP targeted to the apical surface by the predominantly apically targeted BACE1.

### Trafficking of APP in Neurons

Although MDCK cells have yielded important insights into the polarized trafficking and processing of APP, these findings had to be confirmed in neurons, which are presumably the primary source of A $\beta$  production in vivo. Neurons are highly polarized into soma, axons, and dendrites, all of which perform different functions and therefore are equipped with distinct sets of proteins and lipids that regulate protein trafficking. To complicate matters further, axons and dendrites are subdivided into separate compartments, e.g., dendritic shafts, dendritic spines, axonal shaft, axonal presynaptic endings, and others. An elaborate system of tracks (microtubules), trucks (kinesin and dynein motor proteins), and address labels (specific sorting signals) ensures proper delivery of proteins to their respective destinations. Disturbances in this system could affect APP processing and have been linked to AD



**Figure 6.** Polarized sorting APP, its processing products, and its secretases.

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pathogenesis (De Vos et al. 2008; Morfini et al. 2009). It is therefore essential to understand in detail the trafficking and processing of APP in neurons.

The transport from ER to Golgi and TGN described earlier is thought to be similar in non-polarized cells and in the neuronal soma. But after leaving the TGN in neurons, APP is transported to axons and dendrites in post-Golgi transport vesicles (reviewed in Kins et al. 2006). APP delivery to the axon makes use of the fast axonal transport system (Koo et al. 1990; Sisodia et al. 1993), with kinesin-1 as the microtubule motor protein (reviewed in Kins et al. 2006). As visualized by GFP-tagged fusion proteins, APP is transported in vesicular and often elongated tubular structures which move with special characteristics along the axon (Kaether et al. 2000; Stamer et al. 2002; Goldsbury et al. 2006; Szodorai et al. 2009). Whereas most other proteins are axonally transported in vesicles that display a saltatory movement, often changing directions, the APP tubules continuously move unidirectionally, with an average speed of 4.5  $\mu\text{m/s}$ , reaching maximal speeds up to 10  $\mu\text{m/s}$  (Kaether et al. 2000). This is among the fastest transport velocities measured in cultured neurons. Significant retrograde transport with slightly slower kinetics was also observed (Kaether et al. 2000; Stamer et al. 2002). Little is known about the fate of the axonal transport carrier vesicles. Where do they fuse with the axonal PM? What are their fusion kinetics? Where do the retrograde carriers go? A small fraction of the axonal APP has been suggested to undergo transcytotic transport to dendrites, but the significance and kinetics of this process need to be determined (Simons et al. 1995; Yamazaki et al. 1995). Likewise a detailed study of dendritic transport kinetics of APP is lacking.

What are the sorting signals mediating axonal and/or dendritic transport? An axonal sorting signal was mapped to a juxtamembrane domain which includes the A $\beta$ -domain (Tienari et al. 1996); however, a recent report showed that APP is transported into axons and dendrites without apparent sorting signals (Back et al. 2007). Along this line, APP lacking

the cytoplasmic domain ( $\Delta\text{C}$ ) (where the basolateral sorting signal is located; see above) is transported along neurites with characteristics indistinguishable from wild-type APP (Fig. 7a; Back et al. 2007; Szodorai et al. 2009). Therefore, the basolateral sorting signal in the carboxy-terminal region of APP documented in MDCK studies seems to have no function for polarized sorting in neurons. What emerges at this point is that the sorting of APP in neurons is fundamentally different from that of other polarized cells, and much more work has to be done to fully understand polarized trafficking of APP in neurons.

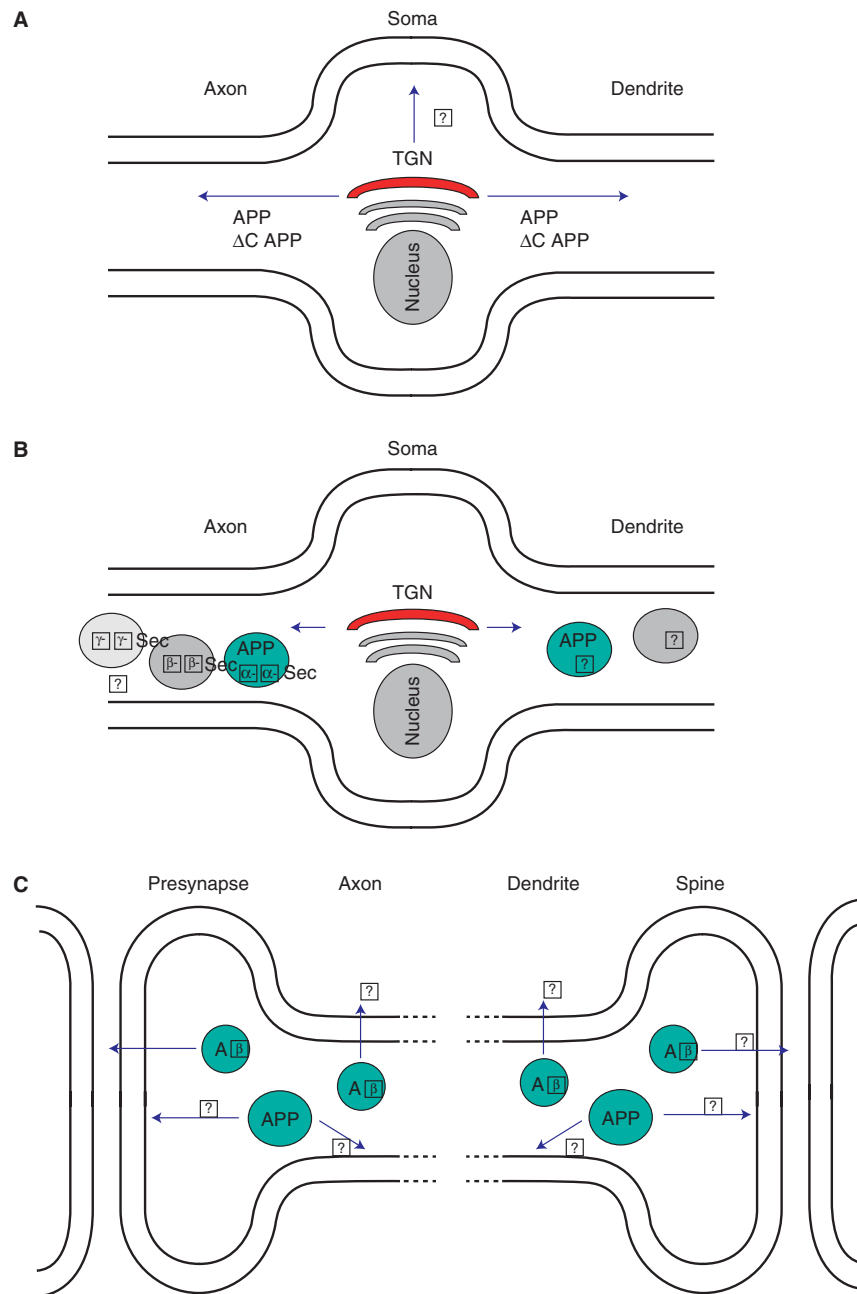
## SUBCELLULAR SITES OF APP PROCESSING

### Subcellular Sites of $\alpha$ -Secretase-Mediated APP Processing

As described above,  $\alpha$ -secretase-mediated shedding occurs predominantly on the cell surface. However, substantial cleavage by  $\alpha$ -secretase has also been reported to occur within TGN, for example in MDCK cells (see above).

### Subcellular Sites of $\beta$ -Secretase-Mediated APP Processing

As described above, BACE1 is a type-1 membrane protein that is co-translationally translocated into the ER as an immature pro-enzyme. During maturation, BACE1 undergoes a number of post/co-translational modifications, including N-glycosylation, disulfide bridge formation, and palmitoylation (Bennett et al. 2000b; Capell et al. 2000; Huse et al. 2000; Benjannet et al. 2001). The propeptide of immature BACE1 is removed by Furin and related proteases during maturation (Capell et al. 2000; Creemers et al. 2001). Surprisingly, the propeptide seems not to significantly affect BACE1 proteolytic function (Creemers et al. 2001). BACE1 reaches the PM and becomes enriched in lipid rafts (Riddell et al. 2001; Cordy et al. 2003). Rafts are discussed as potential sites for efficient A $\beta$  generation, as APP and BACE1 apparently come into immediate contact within this compartment (Ehehalt et al. 2003; Abad-



**Figure 7.** Neuronal APP transport and processing. (A) Post-Golgi sorting of APP. APP as well as APP $\Delta$ C is transported by fast axonal transport along the axon and by as yet uncharacterized transport into dendrites. Transport to and fusion with the plasma membrane in the neuronal soma is likely, but has not been characterized. (B) Transport and sorting of APP and secretases. APP and  $\alpha$ -secretase are transported in axonal carriers that do not contain  $\beta$ - or  $\gamma$ -secretase. Whether  $\beta$ - and  $\gamma$ -secretase are transported together is not known. Colocalization of APP and secretases in dendritic transport vesicles has not been studied. (C) Release of A $\beta$  occurs at presynaptic sites, but whether it is also released from axon shafts is unknown. A $\beta$  is released from dendrites, but the exact location has not been determined. Where APP fuses with the axonal and dendritic plasma membrane and where processing by secretases occurs remains elusive.

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Rodriguez et al. 2004). This would be consistent with the finding that BACE1 undergoes palmitoylation. However, inhibition of palmitoylation and raft localization still permits relatively normal A $\beta$  production (Vetrivel et al. 2009). Thus, the role of rafts in A $\beta$  generation is still under debate. On reaching the PM, BACE1 undergoes internalization. Internalization and targeting to endosomes is dependent on a dileucine motif located within the short cytoplasmic domain of BACE1 (Huse et al. 2000; Pastorino et al. 2002). From endosomes, BACE1 can recycle to the TGN (Walter et al. 2001). A single phosphorylation at serine 498 within the carboxy-terminal domain of BACE1 is sufficient for its transport from endosomes to the trans-Golgi. Recycling requires the Golgi-localized, gamma-ear containing ADP (GGA) ribosylation-binding factor (He et al. 2005; Wahle et al. 2005, 2006), and two such GGA proteins are involved in BACE1 trafficking, GGA1 and GGA2. GGA1 is involved in endosomal retrieval of BACE1 and its recycling to the PM, whereas GGA2 targets BACE1 to lysosomes for its final degradation.

As an aspartyl protease, BACE1 has an acidic pH optimum (around pH 4.5) (Vassar et al. 1999), which is consistent with a major  $\beta$ -secretase activity within endosomes. Indeed,  $\beta$ CTFs accumulate in this compartment on inhibition of endosomal/lysosomal protein degradation (Golde et al. 1992; Haass et al. 1992). Selective processing of APP by BACE1 within endosomes has been used to design and generate highly effective BACE1 inhibitors. To ensure their accumulation within endosomes, a membrane-anchored BACE1 transition-state inhibitor was linked to a sterol moiety. Because of its selective accumulation within endosomes, the membrane-bound inhibitor reduced BACE1 activity much more efficiently than a nonmembrane anchored version (Rajendran et al. 2008). However, BACE1 activity is not exclusively restricted to endosomes. For example, Swedish mutant APP which, because of its missense mutation at the Met-Asp cleavage site is more efficiently processed by BACE1, can already be cleaved within late Golgi compartments (see above).

### Subcellular Sites of $\gamma$ -Secretase-Mediated APP Processing

Early studies on APP processing indicated that  $\gamma$ -secretase cleaves APP to generate A $\beta$  at or near the PM. However, initial characterization of PS subcellular localization—before it was clear that it is not only implicated in  $\gamma$ -secretase processing but is its catalytic subunit—suggested that PS is mainly localized to the ER. This apparent contradiction was coined the spatial paradox by Annaert and De Strooper (1999). Although still not resolved completely, the work of many labs now indicates that mature, proteolytically active PS/ $\gamma$ -secretase is principally localized not in ER, Golgi, or post-Golgi transport vesicles but rather at the PM and in the endosomal/lysosomal system, including phagosomes and autophagosomes (reviewed in Pasternak et al. 2004; Kaether et al. 2006a; Nixon 2007; Dries and Yu 2008). The  $\gamma$ -secretase subunits found in the ER/early secretory pathway most likely represent unassembled or partially assembled subcomplexes, but not the active enzyme. The evidence supporting such a model is manifold and, because of space constraints, only key arguments will be highlighted: (1) APP/C99/C83 are not cleaved by  $\gamma$ -secretase in the ER (Cupers et al. 2001; Maltese et al. 2001; Grimm et al. 2003; Kaether et al. 2006b); (2) by immuno-electron microscopy, PS1 is not detected in Golgi or TGN but in ER and PM (Rechards et al. 2003); whereas the ER pool most likely reflects unassembled PS1, the PM pool has assembled, active complex; (3) only the mature, glycosylated NCT, not immature unglycosylated NCT, is present in the fully assembled, active  $\gamma$ -secretase complex (Edbauer et al. 2002; Kaether et al. 2002), and therefore  $\gamma$ -secretase-associated NCT must have passed the Golgi; (4)  $\gamma$ -secretase has been shown by various methods, including cell surface biotinylation, binding of biotinylated inhibitors specific for the active complex, and microscopic techniques, to be present at the PM (Kaether et al. 2002; Tarassishin et al. 2004; Chyung et al. 2005). It has been shown that the  $\epsilon$ -cleavage of APP differs in endosomes and PM (Fukumori et al. 2006), suggesting that

$\gamma$ -secretase has different properties depending on its subcellular localization, maybe because of differences in pH or lipid composition. One can speculate that changing the ratio of  $\gamma$ -secretase present in the different subcellular compartments might have an impact on AD pathology. Taken together,  $\gamma$ -secretase cleaves APP on the surface and in endosomes/lysosomes, but the relative contribution of the two remains to be determined and may vary among cell types.

Where in neurons do secretases process APP? Given the complex morphology of neurons, it is not surprising that even less is known about the precise subcellular site of  $\gamma$ -secretase processing in neurons (axonal or somal or dendritic endosomes/lysosomes? dendritic or axonal autophagosomes? other organelles?) than in non-neuronal cells (Fig. 7B). A major secretion site of A $\beta$  seems to be distal axons/synapses (Lazarov et al. 2002; Sheng et al. 2002), but recently it was reported that A $\beta$  can be secreted from axons and dendrites and can elicit local effects on neighboring neurons (Fig. 7C; Wei et al. 2010). A detailed analysis aimed at determining the ratio of axonally versus dendritically secreted A $\beta$ , using for example cultured neurons in compartmentalized chambers, has not been performed. It has been suggested that APP is transported in axonal vesicles which harbor  $\beta$ - and  $\gamma$ -secretase and that consequently A $\beta$  is produced in these vesicles (Kamal et al. 2001). This view has been challenged, and several experiments indicate that APP is not processed in axonal transport vesicles, with the exception of limited processing by  $\alpha$ -secretase. Ligation experiments in sciatic nerve indicated that APP, but not PS as component of the  $\gamma$ -secretase, accumulated at the ligation site, indicating completely different transport kinetics of these proteins (Lazarov et al. 2005). In addition, using video microscopy, it was shown that APP and  $\beta$ -secretase are not transported in the same vesicles (Goldsbury et al. 2006). Also, by analyzing immuno-isolated APP-carrying transport vesicles, only  $\alpha$ - but not the other secretases were found to colocalize in these vesicles (Szodorai et al. 2009).  $\gamma$ -secretase is present in synapses/distal axons (Behr et al. 1999; Ri-

## Trafficking and Proteolytic Processing of APP

baut-Barassin et al. 2003; Inoue et al. 2009; Frykman et al. 2010); therefore A $\beta$  could be produced there, but the precise subcellular site(s) of APP processing by  $\beta$ - and  $\gamma$ -secretase in neurons remains to be determined.

### Degradation of APP

It should be noted that alternative, secretase-independent processing of APP exists. Early studies showed that the half-life of APP is very short and that not all APP is secreted as APPs, suggesting secretase-independent processing pathways (Weidemann et al. 1989). Later it was shown that APP is degraded in lysosomes to amyloidogenic and nonamyloidogenic fragments (Golde et al. 1992; Haass et al. 1992). In addition, APP was shown to be a caspase substrate (Weidemann et al. 1999; Lu et al. 2003), but the impact of this processing on A $\beta$  generation and/or AD pathology is probably minor (Harris et al. 2010).

### Activity-Dependent APP Processing

Emerging evidence from a variety of human studies has suggested that A $\beta$  levels and metabolism might be regulated by neuronal activity. In this regard, some patients with temporal lobe epilepsy, who experience elevated neuronal activity, develop A $\beta$ -containing plaques as early as 30 years of age (Mackenzie and Miller 1994; Gouras et al. 1997). Moreover, regions of the brain that develop the highest levels of A $\beta$  plaques, including the frontal and parietal lobes and posterior cingulate cortex, exhibit the highest baseline metabolic activity in the so-called "default network" (Gusnard et al. 2001; Raichle et al. 2001; Buckner et al. 2005). This high metabolic activity is a reflection of elevated neuronal and synaptic activity. Parallel studies in animal models and cell culture have linked APP transport, neuronal activity, and A $\beta$  metabolism. APP is axonally transported from the entorhinal cortex to the hippocampal formation via the perforant pathway (Buxbaum et al. 1998a), and lesions of this pathway in transgenic mice overexpressing FAD-linked mutant APP and PS1 transgenes results in substantially

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less A $\beta$  deposition within the hippocampus (Lazarov et al. 2002). Early studies showed that electrical depolarization increases the release of soluble APP- $\alpha$  in rat hippocampal brain slices (Nitsch et al. 1993), and it appears that these effects are mediated by activation of muscarinic M1 acetylcholine receptors which leads to parallel decreases in A $\beta$  levels (Beach et al. 2001; Hock et al. 2003). On the other hand, specific stimulation of NMDA receptors up-regulates APP, inhibits  $\alpha$ -secretase activity, and promotes A $\beta$  production (Lesne et al. 2005).

To determine the effects of neuronal activity on A $\beta$  secretion, Kamenetz et al. (2003) prepared hippocampal slices from transgenic mice overexpressing APP harboring the FAD-linked Swedish mutation (APP<sub>SWE</sub>) and maintained these preparations in the presence of pharmacological agents that either decrease neuronal activity [tetrodotoxin (TTX), high magnesium, or flunitrazepam than in non-neuronal cells (Fig. 7B). A major secretion site of A $\beta$  seems to be distal axons/synapses [a GABA-A receptor potentiator] or increase it (picrotoxin [a GABA-A channel blocker]). Agents that decreased or increased activity resulted in significant reductions or elevations, respectively, in levels of A $\beta$  (both A $\beta$ 40 and A $\beta$ 42) detected in the slice medium, indicating that the secretion of A $\beta$  from neuronal cells that overexpress APP can be controlled by neuronal activity. Western blot analyses revealed that increasing neuronal activity significantly enhanced the levels of  $\beta$ -CTF, the penultimate precursor of A $\beta$  peptides, and elevated levels of secreted APPs $\beta$ , findings which suggested that the level of BACE cleavage can be controlled by neuronal activity. To address whether APP or its products can control synaptic function, wild-type rat hippocampal slice neurons were transduced with viruses expressing APP or APP mutants, and synaptic responses were evoked onto side-by-side pairs of simultaneously recorded postsynaptic neurons where only one neuron expresses the exogenous protein. Excitatory AMPA and NMDA responses onto neurons expressing recombinant APP were significantly depressed, whereas inhibitory (GABA) currents were unaffected. Neurons expressing APP showed

a significant decrease in the frequency of miniature EPSCs, with no change in their amplitude nor in paired-pulse facilitation, suggesting that the depressive effects of APP overexpression are due to a decrease in the number of functional synapses. Indeed, expression of an APP variant harboring an experimental mutation (APP<sub>MV</sub>) just before A $\beta$  Asp1 that blocks  $\beta$ -secretase cleavage produced no significant depression of transmission. Furthermore, transduced slices treated with a highly potent and selective  $\gamma$ -secretase inhibitor (L-685,458) failed to show synaptic depression onto nearby control cells. These results indicate that  $\gamma$ -secretase processing of APP is required for the depressive effects of APP and that this phenotype is independent of the formation of the large ectodomain of APP following either  $\alpha$  or  $\beta$  cleavage events. The results indicate that processing of APP into A $\beta$  is dependent on neuronal activity and that formation of A $\beta$  results in synaptic depression. Treatment of transduced slices with NBQX (an AMPA receptor antagonist) or D,L-AP5, an agent that blocks NMDA-Rs, prevented the synaptic depression caused by APP overexpression. Finally, Kamenetz et al. (2003) tested whether A $\beta$  produced from overexpressing neurons can affect neighboring neurons in a cell nonautonomous manner. Here, synaptic function in two uninfected cells, one from a region containing many infected cells and another from a region with no infected cells, was examined. Uninfected neurons surrounded by APP<sub>SWE</sub>-infected neurons had significantly depressed transmission when compared to distant control neurons, suggesting that uninfected neurons in infected regions were responding to the local high concentrations of A $\beta$ . These results are consistent with the notion that high A $\beta$  levels may disrupt synaptic function and, more importantly, that A $\beta$  may also have a normal negative feedback function: increased neuronal activity produces more A $\beta$ ; the enhanced A $\beta$  production depresses synaptic function; the depressed synaptic function will decrease neuronal activity.

How do high levels of released A $\beta$  mediate synaptic depression? To address this issue, Hsieh and colleagues (2006) examined the effects of





increased A $\beta$  levels on the structure of dendritic spines of CA1 pyramidal dendrites in organotypic slices that acutely overexpress APP. In this setting, spine density in APP-expressing cells was decreased compared to cells expressing the APP(MV) variant that undergoes much less processing by  $\beta$ -secretase. Similar to earlier studies showing that application of synthetic A $\beta$  reduces the levels of surface NMDA and GluR1 receptors in dissociated cultured hippocampal neurons (Almeida et al. 2005; Snyder et al. 2005), Hsieh et al. (2006) confirmed that A $\beta$  generated in situ also leads to decreases in surface and synaptic AMPA receptors on both spines and dendrites. Using an AMPA-R subunit tagged on its amino terminus with a pH-sensitive GFP variant, Hsieh revealed that APP overexpression led to a significant decrease of spine and dendritic surface AMPA receptors compared to cells expressing the APP(MV) variant.

Although these studies made clear that APP overexpression and resultant A $\beta$  secretion can lead to synaptic dysfunction, the subcellular sites from which A $\beta$  acts remained uncertain. To examine this issue, Wei and colleagues (2010) isolated the sites of increased A $\beta$  production by selectively expressing APP in pre- or postsynaptic neurons. Using two-photon laser-scanning imaging to monitor the synaptic deficits caused by such dendritic or axonal A $\beta$ , Wei and colleagues found that either dendritic or axonal A $\beta$  overproduction was sufficient to cause local spine loss and compromise synaptic plasticity in the nearby dendrites of neurons that did not overexpress APP. The A $\beta$ -mediated synaptic dysfunction could be pharmacologically ameliorated by blockade of either neural activity, NMDA receptors, or nicotinic acetylcholine receptors.

Extending these latter studies to an *in vivo* setting, Cirrito et al. (2005) asked whether synaptic activity influences the levels of A $\beta$  in brain interstitial fluid (ISF). A microdialysis probe with concurrent hippocampal electrophysiological recording was used to assess whether there are dynamic changes in ISF A $\beta$  levels in conjunction with differing levels of neuronal activity in awake, behaving mice. Here, a re-

ording electrode was attached to a microdialysis probe to monitor electroencephalographic (EEG) activity (extracellular field potentials) and simultaneously collect ISF A $\beta$  in the hippocampus of Tg2576 mice at 3–5 months of age. Neuronal and synaptic activity within the hippocampus was enhanced by electrically stimulating the perforant pathway, the major afferent projection from the entorhinal cortex to the hippocampus, conditions that created transient epileptiform discharges within the hippocampus noted on hippocampal EEGs. During these electrical seizures, ISF A $\beta$  levels increased, showing a direct relationship between increased neuronal activity and increases in ISF A $\beta$  *in vivo*. To determine if endogenous neural activity of the perforant pathway modulates ISF A $\beta$  levels within the hippocampus, a selective metabotropic glutamate receptor 2/3 agonist, LY354740, was infused into the hippocampus via reverse microdialysis, and ISF A $\beta$  levels were monitored. This treatment caused a decrease in ISF A $\beta$  within the hippocampus, consistent with the hypothesis that modulation of endogenous neuronal activity in this pathway can alter hippocampal A $\beta$  production. Indeed, strong depression of local activity using the sodium channel blocker TTX revealed that a decline in EEG amplitude was paralleled by a concomitant decrease in A $\beta$  levels. Taken together with the studies of Kamenetz and colleagues, these studies by Cirrito et al. establish that neuronal or synaptic activity can directly influence A $\beta$  levels.

The question remained as to the mechanism by which increased synaptic activity elevates A $\beta$  secretion. One possibility is that the half-life of extracellular A $\beta$  is extended; alternatively, this effect may involve increased APP processing. Using a  $\gamma$ -secretase inhibitor in TTX or vehicle-treated mice, Cirrito et al. (2005) showed that A $\beta$  half-life was unaltered by the depression of neuronal activity. Additionally, expression of the A $\beta$ -degrading enzyme neprilysin was unchanged in TTX-treated mice.

In further attempts to delineate the mechanism(s) responsible for activity-dependent elevations of ISF A $\beta$ , Cirrito and colleagues (2008) hypothesized that A $\beta$  released into the

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brain ISF following synaptic transmission requires an intermediate event involving APP endocytosis, analogous to that shown by Koo and Squazzo (1994) in cultured immortalized mammalian cells. To assess the influence of endocytosis on ISF A $\beta$  levels under normal conditions, a myristylated, cell-permeable peptide (dynamin-DN) which inhibits clathrin-mediated endocytosis was infused into hippocampus by reverse microdialysis. As an *in vivo* control, the dynamin-DN reduced biotinylated transferrin uptake into cells within the dentate gyrus. Importantly, ISF A $\beta$  levels were significantly decreased. Because inhibition of synaptic activity (Cirrito et al. 2005) and inhibition of endocytosis (Cirrito et al. 2008) each reduce ISF A $\beta$  levels, there remained the question of the extent as to which (or both) contributes to ISF A $\beta$  levels. Cirrito et al (2008) co-administered TTX and dynamin-DN. Administration of dynamin-DN first led to a decrease in ISF A $\beta$  levels by 70%, but if the microdialysis perfusion buffer was then switched to contain dynamin-DN and TTX, no additional change in ISF A $\beta$  levels were observed. These findings suggested that all of the ISF A $\beta$  that is produced by synaptic activity requires endocytosis. On the other hand, if animals were pretreated with TTX followed by dynamin-DN, ISF A $\beta$  levels were further reduced when endocytosis was inhibited, a finding that strongly suggested that a “releasable” stored pool of ISF A $\beta$  that is generated via endocytosis is distinct from an A $\beta$  pool that is acutely released by synaptic activity.

Although these studies using hippocampal slices and *in vivo* microdialysis provide compelling support for activity-dependent A $\beta$  production, most such studies have been performed in acute settings. Studies by Tampellini and colleagues (2010) chronically reduced synaptic activity *in vivo* via unilateral ablation of whiskers or chronic diazepam treatment. For the whisker ablation experiments, bulbs were unilaterally removed in 2- to 3-month-old Tg19959 mice that express the FAD-linked Swedish (KM-NL) and London (V717F) APP mutations and develop plaques by age 3 months. At 6 months, cytochrome oxidase (COX) stain-

ing was reduced in the deafferented versus the synaptically active (control) barrel cortices, indicating that the lesion reduced synaptic activity. The barrel cortices with reduced activity showed a striking decrease in both the number of A $\beta$  plaques and the area covered by plaques compared to the control side. Parallel studies in which the transgenic mice were chronically treated with diazepam for 1 month beginning at age 3 months also led to reduced plaque burden in cortex and hippocampus.

Despite evidence from these elegant *in vitro*, *ex vivo* and *in vivo* studies for an important role for synaptic activity in modulating A $\beta$  production and subsequent synaptotoxicity, there remain gaps in our understanding of the underlying neurobiology. For example, does synaptic activity alter the trafficking of APP and/or the secretases to axonal and/or dendritic compartments? How does synaptic activity elevate the steady-state levels of  $\beta$ CTF, the precursor of A $\beta$ ? What are the pre- and postsynaptic receptor(s) involved in binding A $\beta$  and/or its oligomeric forms (see Mucke and Selkoe 2011)? What are the signaling mechanisms by which activated postsynaptic A $\beta$  receptor(s) lead to removal of AMPA and NMDA receptors and how do these changes alter structural plasticity? Finally, is there selectivity in specific neuronal circuits that underlies selectivity vulnerability of certain neuronal populations early in the disease process (i.e., preclinically)?

## CONCLUSIONS

APP is the central protein involved in AD pathology as it serves as the precursor for A $\beta$  generation. Intensive analysis of the cell biology of this protein and its proteolytic processing led not only to a detailed understanding of A $\beta$  generation but also allowed the generation of therapeutically relevant secretase inhibitors. Moreover, understanding the biology of secretases allowed major progress in other fields of cell biological research. For example, research on secretases paved the road toward the understanding of RIP and the signaling pathways triggered by this process.



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