Chapter 10
Proteases in the Nervous System

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10.1 Proteases in Alzheimer's Disease

Alzheimer's disease (AD) is characterized by the massive accumulation of the short cleavage product (beta-amyloid, Aβ) liberated from the transmembrane amyloid precursor protein (APP). The stepwise cleavage of APP is accomplished by membrane-bound β- and γ-secretase. This pathway competes with a non-amyloidogenic pathway characterized by processing of APP by α-secretase. Although the mechanism of Aβ-toxicity is still not well understood, its liberation from APP is considered as the central event in AD pathogenesis underlined by numerous mutations around the secretase cleavage sites in APP or in leading to early-onset AD.

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10.1.1 α-Secretase

α-secretase cleavage occurs within the Aβ domain of APP and, thus, has the potential to prevent Aβ generation. α-secretase cleavage of APP occurs constitutively (constitutive α-secretase activity) (Esch et al. 1990), but can additionally be stimulated above its constitutive level (regulated α-secretase) by different pharmacological agents (reviewed in Bandyopadhyay et al. 2007; Lichtenthaler 2011), as first shown for muscarinic agonists (Nitsch et al. 1992).

The constitutive α-secretase cleavage of APP is mediated by the metalloprotease ADAM10 (A disintegrin and metalloprotease 10) [EC 3.4.24.81]. Other proteases, such as ADAM9 [EC 3.4.24.–] and ADAM17 [EC 3.4.24.86] were recently ruled-out as constitutive α-secretases (Kuhn et al. 2010). Knock-out or knock-down of ADAM10 in primary neurons as well as in several cell lines largely prevents APP α-secretase cleavage (Jorissen et al. 2010; Kuhn et al. 2010). Conversely, overexpression of ADAM10 in cell lines as well as in mouse brain increases APP α-secretase cleavage and consequently, reduces Aβ generation (Lammich et al. 1999; Postina et al. 2004). Additionally, ADAM10 cleaves APP derived peptides in vitro at the correct peptide bond (Lammich et al. 1999). The initial protease cleavage occurs between amino acids lysine16 und leucine17 of the Aβ sequence and appears to be followed by an as yet unidentified carboxypeptidase cleavage selectively removing lysine16 (Esch et al. 1990; Lammich et al. 1999; Kuhn et al. 2010).

ADAM10 has 748 amino acids and is a member of the large ADAM protease family. ADAMs are type I membrane proteins of the metzincin family requiring a zinc ion for proteolytic activity (reviewed in Edwards et al. 2008; Reiss and Saftig 2009). The large ectodomain of ADAM10 consists of an N-terminal signal peptide, followed by the prodomain, the metalloprotease domain with the conserved zinc-binding amino acid motif HEXGHXXGXXHD, a disintegrin domain and a cysteine-rich domain. In contrast to other ADAM proteases, an EGF-like domain is missing in the ADAM10 ectodomain (Janes et al. 2005). The ectodomain is followed by the transmembrane domain and a proline-rich cytoplasmic domain, which provides binding sites for SH3 domain containing proteins, such as SAP-97 (Marcello et al. 2007). A more detailed description of the ADAM protease domains, their structure and specific functions can be found in recent reviews (Edwards et al. 2008; Reiss and Saftig 2009). ADAM10 is post-translationally modified by complex N-glycosylation (Escrevente et al. 2008), and the prodomain is removed by the proprotein convertases furin and PC7 (Anders et al. 2001). Prodomain removal results in the active protease, which mediates proteolysis in late compartments of the secretory pathway and at the plasma membrane. Interestingly, the ADAM10 ectodomain can be released from the cell by ADAM9 and ADAM15 [EC 3.4.24.–] (Parkin and Harris 2009; Tousseyn et al. 2009). The ectodomain shedding is followed by γ-secretase mediated intramembrane proteolysis and translocation of the ADAM10 intracellular domain into the nucleus, where it is found in nuclear speckles, which are assumed to be involved in gene regulation (Parkin and Harris 2009; Tousseyn et al. 2009). This raises the possibility that ADAM10 may function as a signaling protein in addition to its role as protease.

APP is not the only substrate for ADAM10. By now over 30 different ADAM10 substrates have been identified, including cell adhesion proteins (e.g. N-cadherin), receptors (e.g. Notch) and growth factors (e.g. epidermal growth factor, EGF) (Pruessmeyer and Ludwig 2009; Reiss and Saftig 2009). This demonstrates that ADAM10 has a general function in the ectodomain shedding of membrane proteins. Importantly, ADAM protease-mediated shedding occurs for substrates on the same cell surface, but can also happen in trans as shown for ephrin-Eph receptor signaling (Janes et al. 2005). In vivo, the cell surface receptor Notch is a particularly important ADAM10 substrate and requires ADAM10-mediated cleavage for its signal transduction (Bozkulak and Weinmaster 2009; van Tetering et al. 2009). ADAM10 knock-out mice die embryonically due to a loss of Notch signaling (Hartmann et al. 2002). Likewise, mice with a conditional ADAM10 knock-out in the brain die perinatally, again due to a loss of Notch signaling and a major defect in brain development (Jorissen et al. 2010). An ADAM10 knock-out specifically in B cells demonstrated an essential function of ADAM10 in Notch2-mediated B cell development (Gibb et al. 2010).

ADAM10 possesses a broad substrate specificity and can cleave after distinct amino acids (Caescu et al. 2009). In the case of APP, the cleavage site seems to be located in an α-helical structure and appears to be determined by the distance from the membrane surface (Sisodia 1992), which is in agreement with ADAM10 being a membrane-bound protease.

Expression of ADAM10 is controlled at the level of transcription and translation. Posttranslational regulation of ADAM10 activity occurs through the activation of numerous signaling pathways (reviewed in Bandyopadhyay et al. 2007; Lichtenthaler 2011). Additionally intracellular protein transport is increasingly recognized as a major mechanism to control the localization of ADAM10, the access to its substrates and consequently the substrate turnover by ADAM10 (reviewed at the example of APP in Lichtenthaler 2011).

In contrast to the constitutive α-secretase the identity of the regulated α-secretase remains to be fully clarified and may be mediated by different proteases. At least ADAM10 and ADAM17 can act as regulated α-secretases after specific stimuli, such as PACAP peptides and the phorbol ester PMA (Buxbaum et al. 1998; Kojro et al. 2006). Many other stimuli, such as neurotransmitters, growth factors and cytokines, are also known to increase APP α-secretase cleavage, but it remains unclear whether the increased α-secretase cleavage occurs through ADAM10 or ADAM17 or yet other metalloproteases. In fact, overexpression of ADAM8 [EC 3.4.24.–], ADAM9 and several matrix metalloproteases also increases α-secretase cleavage of APP. This suggests that the activation or increased expression of a variety of different metalloproteases may be tested as a means to increase α-secretase cleavage and reduce Aβ generation in AD. Indeed, muscarinic agonists increase APP α-secretase cleavage and reduce amyloid pathology in an AD mouse model, presumably due to enhanced expression of ADAM17 (Caccamo et al. 2006). The therapeutic potential of ADAM10 and other metalloproteases has been reviewed elsewhere in further detail (Endres and Fahrenholz 2010; Lichtenthaler 2011).

Taken together, constitutive α-secretase cleavage of APP is mediated by ADAM10, whereas the regulated α-secretase cleavage occurs through both ADAM10 and ADAM17 and possibly additional metalloproteases.

10.1.2 β-Secretase

In contrast to α-secretase, β-secretase catalyzes the first step in Aβ generation and, thus, is a major drug target for Alzheimer's disease. β-secretase was identified as the transmembrane aspartyl protease BACE1 (β-site APP cleaving enzyme) [EC 3.4.23.46] (Hussain et al. 1999; Sinha et al. 1999; Vassar et al. 1999; Yan et al. 1999; Lin et al. 2000), which is related to the pepsin and retroviral aspartic protease families. Aβ-generation is largely reduced in BACE1 knock-out mice (Cai et al. 2001; Luo et al. 2001; Roberds et al. 2001; Dominguez et al. 2005). A close homolog, called BACE2 [EC 3.4.23.45], was identified shortly thereafter (Saunders et al. 1999; Yan et al. 1999; Solans et al. 2000). Much less is known about BACE2 compared to BACE1.

BACE1 is an N-glycosylated type I membrane protein with 501 amino acids. The N-terminal signal peptide is followed by a prodomain, the protease domain, a transmembrane domain and a short cytoplasmic tail. The catalytic domain comprises the two catalytic aspartic acid residues [amino acids 93–96 (DTGS) and amino acids 289–292 (DSGT)]. In the secretory pathway, the propeptide is cleaved by furin, leading to the active BACE1 protease (Bennett et al. 2000; Capell et al. 2000; Huse et al. 2000; Creemers et al. 2001). BACE1 forms dimers, which have a higher activity than the monomers (Schmechel et al. 2004; Westmeyer et al. 2004). The crystal structure of the BACE1 ectodomain shows a conserved general folding of aspartyl proteases (Hong et al. 2000).

In contrast to the ADAM proteases, which seem to be less dependent on specific amino acid motifs around the cleavage site, BACE1 has a more pronounced substrate specificity and prefers a leucine at the P1 position (Citron et al. 1995; Gruninger-Leitch et al. 2002).

BACE1 is ubiquitously expressed. The highest BACE1 expression level is found in neurons (Vassar et al. 1999), which explains why neurons are particularly vulnerable in AD due to increased Aβ generation. In mice, BACE1 expression is very high in the nervous system shortly after birth and then decreases to much lower levels (Willem et al. 2006). BACE1 localizes to the Golgi, the trans-Golgi network and to the endosomes (Vassar et al. 1999; Capell et al. 2000; Huse et al. 2000). BACE1 has an acidic pH-optimum and seems to be specifically active within acidic cellular compartments such as the late Golgi and endosomes. BACE1 activity has also been observed in other compartments, but this seems to occur only upon overexpression of the protease (Huse et al. 2002).

BACE1 is a major drug target for AD, as its inhibition lowers Aβ generation. Potent BACE1 inhibitors have been developed, but mostly do not reach sufficiently high concentrations in the brain (Vassar et al. 2009). A new generation of BACE1 inhibitors with significantly improved pharmacokinetics has been developed recently, and is currently being tested in patients. Other preclinical strategies to inhibit BACE1 activity consist of targeting BACE1 with antibodies and with modified drugs, which are specifically delivered to endosomes, where BACE1

cleaves APP (Chang et al. 2007; Rajendran et al. 2008; Mitterreiter et al. 2010; Zhou et al. 2011).

In addition to APP several other BACE1 substrates have been identified over the past few years, namely neuregulin-1 type III (NRG1), the P-selectin glycoprotein ligand-1, the sialyltransferase ST6Gal I, β-subunits of voltage-gated sodium channels, the amyloid precursor-like proteins 1 and 2, the interleukin-1 receptor 2, and the LDL receptor-related protein (Kitazume et al. 2002; Lichtenthaler et al. 2003; Li and Sudhof 2004; von Arnim et al. 2005; Wong et al. 2005; Willem et al. 2006; Kuhn et al. 2007). Additionally, a proteomic study identified further proteins as potential BACE1 substrates (Hemming et al. 2009). However, most of these substrates were not confirmed under BACE1 knock-out or knock-down conditions and should be discussed with some care. The most prominent phenotypic change in the BACE1 knock-out is a hypomyelination in the peripheral nervous system during postnatal development, which stems from the reduced cleavage of the BACE1 substrate NRG1 (Hu et al. 2006; Willem et al. 2006). Remyelination also appears to be affected (Hu et al. 2008; Farah et al. 2011). Moreover, BACE1-deficient mice show behavioral changes related to schizophrenia, which may also be due to the reduced NRG1 cleavage (Savonenko et al. 2008). Additional functions of BACE1 in the central nervous system, such as epileptic seizures, are less well understood (Hitt et al. 2010; Hu et al. 2010).

BACE1 protein expression is controlled at the transcriptional level, for example by the transcription factors NFkB, PPARγ and YY1 (reviewed in Rossner et al. 2006). Additionally, distinct mechanisms control the translation of the BACE1 mRNA, including the 5′ non-translated region, a naturally occurring antisense transcript and microRNAs (De Pietri Tonelli et al. 2004; Lammich et al. 2004; Rogers et al. 2004; Faghihi et al. 2008; Hebert et al. 2008; O'Connor et al. 2008; Wang et al. 2008). The translational mechanisms seem to be dysregulated in AD, providing an explanation for the 2–5-fold increase in BACE1 protein levels observed in AD brains.

10.1.3 Alternative β-Secretases

10.1.3.1 Problems with BACE1 as Sole β-Secretase

BACE 1 was identified as major β-secretase in vivo since genetic deficiency in mice dramatically reduced the amount of generated Aβ-peptides (Vassar et al. 1999; Cai et al. 2001). However, some concerns have been raised for BACE1 being the only protease possessing β-secretase activity in humans.

A major criticism is the exceptionally low catalytic specificity of BACE1 for cleavage of wildtype APP with k_{cat}/K_M between 40 and 62 M^{-1} s^{-1} in solution (Lin et al. 2000; Shi et al. 2001) compared to, e.g. artificial BACE1 substrates being cleaved with k_{cat}/K_M 3.42 × 10^5 M^{-1} s^{-1} (Turner et al. 2001).

A second concern is the spectrum of N-terminal Aβ variants generated by BACE1, which differs significantly from the Aβ-composition found in human sporadic Alzheimer's disease (Kawarabayashi et al. 2001). BACE1 dominantly generates full-length Aβ-peptides starting with an N-terminal aspartate in vitro and in vivo, whereas a heterogeneous mixture of N-terminally truncated and modified Aβ peptides has been identified in human brain extracts (Saido et al. 1996). Among others, pyroglutamate (pGlu)-modified Aβ-peptides are a major species found in human AD brain (Saido et al. 1995). The truncated Aβ peptides might be generated by subsequent aminopeptidase cleavage by, e.g. aminopeptidase A (Sevalle et al. 2009) however, other studies suggest a direct liberation of Aβ-peptides differing from the full-length variants by alternative processing (Cynis et al. 2008).

A third concern is the identification of BACE1 using screening techniques based on a rare human familial Alzheimer's disease modification, namely the "Swedish" mutation (KM595/596/NL) (Vassar et al. 1999). Introduction of the "Swedish" mutation into the APP sequence makes it a much better substrate for BACE1 (k_{cat}/K_M 1.03 × 10^5 M^{-1} s^{-1}) than wildtype APP (Hook et al. 2008a, b) and. From the present perspective it is not surprising that BACE1 was identified using the APPswedish variant for beta-secretase screening, however, it might have precluded the identification of other putative candidates with higher preference for wildtype APP.

Although information on putative alternative β-secretases besides BACE1 are limited and frequently contradictory, considerable evidence exists, that also other proteases might at least exert some β-secretase activity in vitro and/or in vivo.

10.1.3.2 Cathepsins

Among the first proteases suspicious for β-secretase activity, several cathepsins were studied. These investigations included the cysteine proteases cathepsin B (CatB) [EC 3.4.22.1], cathepsin L (CatL) [EC 3.4.22.15] and cathepsin S (CatS) [EC 3.4.22.27] belonging to the papain family, in addition to the aspartic protease cathepsin D (CatD) [EC 3.4.23.5] belonging to the pepsin A family of proteases (Cataldo and Nixon 1990; Cataldo et al. 1997; Ladror et al. 1994; Munger et al. 1995). The rationale for investigating lysosomal proteases was the finding of internalization of APP from the cell surface and its degradation in the endosomal-lysosomal compartment. Among the tested cathepsins only CatS showed considerably increased Aβ production in co-transfection experiments in 293 cells. Neither CatB and CatL nor CatD showed an effect on APP processing under these conditions (Munger et al. 1995). CatD was later ruled out as relevant β-secretase since the gene knock out did not change Aβ generation in mice (Saftig et al. 1996). In contrast, CatB remained a putative alternative β-secretase candidate (Hook and Reisine 2003). The findings are mainly based on the sub-cellular distribution of BACE1 and CatB in primary chromaffin cells as model system for constitutive and regulated secretion. CatB seems to be localized in the regulated

secretory pathway, whereas BACE1 was found to be primarily present in vesicles of the constitutive secretory pathway. Since neurons and neuronal-like cells like bovine chromaffin cells release neurotransmitters and neuropeptides as well as Aβ in a regulated fashion, it was postulated, that CatB accounts for the majority of Aβ released from chromaffin vesicles. The approach was substantiated by application of E64d as CatB-specific inhibitor to wildtype APP and "Swedish" APP mice as well as the analysis of the respective gene knock outs crossed with APP overexpressing mice (Hook et al. 2008a, b, 2009, 2011).

However, it has to be noticed, that these findings are controversial. Mueller-Steiner et al. found CatB to be involved in Aβ degradation rather than Aβ generation (Mueller-Steiner et al. 2006) and the role of CatB as alternative β-secretase has not been confirmed by other groups so far.

10.1.3.3 Caspases

Caspases play an essential role in apoptosis by cleaving a subset of cellular polypeptides at Asp-X bonds (Nicholson and Thornberry 1997). It has been demonstrated, that apoptotic cells secrete higher amounts of Aβ compared to controls (LeBlanc 1995). Therefore, the idea was raised, that activated caspases might play a role in APP turnover under apoptotic conditions. Especially caspase 3 [EC 3.4.22.56] from peptidase family C14 was found to be able to cleave APP but not in a β-secretase like fashion (Gervais et al. 1999). Interestingly, besides the absence of direct β-secretase activity of caspase 3, increased amounts of Aβ in the cell culture supernatant have been demonstrated under apoptotic conditions suggesting a shift of substrate into the amyloidogenic pathway after caspase activation. Therefore, apoptosis in general and caspase 3 in particular might play an indirect role for Aβ generation.

In addition to caspase 3, caspase 6 [EC 3.4.22.59] from peptidase family C14 was shown to be able to cleave Aβ directly at the β-secretase site. As it has already demonstrated for BACE1 and CatD, caspase 6 preferentially cleaves the "Swedish" mutant of APP with k_{cat}/K_M 2.2×10^4 M^{-1} s^{-1}, a specificity only 4–5 times lower than the specificity constant of BACE1 for "Swedish" APP. Notably, also the wildtype APP sequence was processed by caspase 6 with a specificity constant of k_{cat}/K_M 0.8×10^4 M^{-1} s^{-1}, which makes wildtype APP a 14–20 times better substrate for caspase 6 than for BACE 1 in vitro (Hook et al. 2008a, b; Gervais et al. 1999). However, the sub-cellular localization of caspases in the cytosol and their restricted activation upon apoptosis excludes them as relevant β-secretases, since the physiological turnover of APP was found to be approx. 8 %/h (Bateman et al. 2006). Nevertheless, under certain conditions, caspases could increase the heterogeneity of N-terminal Aβ-species, since caspase-activation was found to lead preferentially to the generation of truncated Aβ(2–x) via caspase 6 (Gervais et al. 1999) or to Aβ(5–x) via an yet unidentified mechanism (Takeda et al. 2004).

Summarizing, compelling evidence exist, that BACE1 is not the sole enzyme possessing β-secretase activity. Instead it appears that a number of proteases are

able to cleave APP around the β-secretase cleavage site leading to the generation of a heterogeneous mixture of N-terminal truncated Aβ variants later found in brains of patients suffering from Alzheimer's disease.

10.1.4 γ-Secretase: A Hetero-tetrameric Intramembrane Protease Complex

γ-secretase cleaves the C-terminal fragments arising through the initial α- or β-secretase cleavage and results in the secretion of the p3 and Aβ-peptides, respectively. γ-secretase is an unusual aspartyl protease, in that it cleaves its substrate proteins within the phospholipid bilayer of the membrane. γ-secretase belongs to the larger family of GXGD proteases, which also comprises signal peptide peptidase and its homologs (reviewed in Fluhrer et al. 2009). γ-secretase forms a hetero-tetrameric protein complex (Seeger et al. 1997; Capell et al. 1998; Thinakaran et al. 1998; Yu et al. 1998; Li et al. 2000a) composed of its catalytic subunit presenilin (PS) [EC 3.4.23.–] (Steiner et al. 1999; Wolfe et al. 1999; Esler et al. 2000; Kimberly et al. 2000; Li et al. 2000b; Seiffert et al. 2000) and three other proteins nicastrin (NCT), APH-1 and PEN-2 (Yu et al. 2000; Lee et al. 2002; Steiner et al. 2002; Edbauer et al. 2003; Kimberly et al. 2003; Takasugi et al. 2003). These four proteins are necessary and sufficient for γ-secretase activity, as shown by reconstitution experiments in yeast (Edbauer et al. 2003).

All four γ-secretase subunits are integral membrane proteins. NCT is a type I membrane protein and is the largest subunit of the complex (Yu et al. 2000). PS has nine transmembrane domains (TMDs) and harbors the two catalytically active aspartyl residues within its TMDs 6 and 7 (Henricson et al. 2005; Laudon et al. 2005; Oh and Turner 2005a, b; Spasic et al. 2006). APH-1 spans the membrane seven times (Fortna et al. 2004), while PEN-2 contains two TMDs and is the smallest subunit of the γ-secretase complex (Crystal et al. 2003). Mammalian cells have two homologs of PS (PS1 and PS2) as well as of APH-1 (APH-1a and APH-1b) (Francis et al. 2002). Additionally, APH-1a exists as a short and a long splice variant (Lee et al. 2002). PS1 and PS2 as well as APH-1a and APH-1b do not coexist in the same γ-secretase complex (Yu et al. 1998; Steiner et al. 2002; Hebert et al. 2004; Shirotani et al. 2004). As a consequence up to six different γ-secretase complexes can exist. Whether these differ in their proteolytic properties, specifically in their ability to produce Aβ42 remains controversial (Shirotani et al. 2007; Serneels et al. 2009). The γ-secretase complex is assumed to contain one protein of each subunit (1:1:1:1 ratio) (Sato et al. 2007), but there is also evidence for a dimeric γ-secretase complex (Schroeter et al. 2003).

Several proteins have been described as γ-secretase interactors (Wakabayashi et al. 2009; Winkler et al. 2009). They are not integral subunits of the complex, but appear to be transient interactors, which may modulate the activity or the intracellular trafficking of γ-secretase. Examples are transmembrane protein 21 (TMP21)

(Chen et al. 2006a, b) and the recently identified γ-secretase activating protein GSAP (He et al. 2010).

A high-resolution structure of the γ-secretase complex is not yet available, but biochemical experiments demonstrated that the aspartyl-containing TMDs 6 and 7 of PS face each other and form a hydrophilic pore or cavity (Sato et al. 2006; Tolia et al. 2006), in which a substrate is assumed to be cleaved. Cross-linking experiments revealed several amino acids, which are part of this cavity including residues of the catalytic GXGD motif (Sato et al. 2006; Tolia et al. 2006). Amino acids in other TMDs of PS also contribute to formation of the cavity. In addition, the evolutionarily conserved amino acids PAL, which are very close to TMD6, are part of the cavity (Sato et al. 2008; Tolia et al. 2008).

γ-secretase cleaves over 80 different type I membrane proteins (Haapasalo and Kovacs 2011), demonstrating a broad role in regulated intramembrane proteolysis. Similar to the α-secretase ADAM10, the major γ-secretase substrate during development is the Notch receptor. Mice deficient in PS1 die embryonically from a loss-of-Notch signaling phenotype (De Strooper et al. 1999). Binding of a ligand induces ADAM10-mediated ectodomain shedding of Notch. This is followed by γ-secretase-mediated intramembrane proteolysis, which results in the translocation of the Notch intracellular domain into the nucleus, where it acts as a transcriptional activator of Notch target genes. Given the broad spectrum of γ-secretase substrates, a complete inhibition of γ-secretase is associated with severe side effects in mice and men and, thus, is not longer considered a viable approach to therapeutically reduce Aβ levels in AD patients.

The γ-secretase complex assembles in the endoplasmic reticulum. After assembly is complete, PS is cleaved in its large cytoplasmic loop domain between TMDs 6 and 7 into characteristic N- and C-terminal fragments. This cleavage is considered to activate γ-secretase and occurs autocatalytically (Fukumori et al. 2010).

γ-secretase does not cleave the full-length proteins, but only after their ectodomain was shortened by ectodomain shedding (e.g. α- or β-secretase cleavage). The shortened substrates seem to first bind to an exosite, before getting access to the active site. Although evidence was presented that NCT serves as an initial substrate receptor, recognizing the free N-terminus of the substrate (Shah et al. 2005), follow-up studies have yielded both additional supporting data (Dries et al. 2009) as well as further conflicting data (Chavez-Gutierrez et al. 2008; Martin et al. 2009; Zhao et al. 2010). γ-secretase cleavage occurs in a stepwise manner. This has been elucidated in detail for APP and seems to occur in a similar manner for other substrates, such as Notch and CD44. APP is first cleaved by γ-secretase at the C-terminal end of the transmembrane domain. This initial cleavage, called the ε-cleavage site (Gu et al. 2001; Sastre et al. 2001; Yu et al. 2001; Weidemann et al. 2002), is followed by further γ-secretase cleavages, each removing three or four amino acids from the C-terminus (Takami et al. 2009), until the resulting peptide is short enough to be released from the membrane. The ε-cleavage site is located after amino acid 48 or 49 of the Aβ-sequence. This gives rise to two distinct "product lines". The major product line is Aβ49–Aβ46–Aβ43–Aβ40–Aβ37 from which Aβ40 is the principal end product (Qi-Takahara et al. 2005). The minor

product line is Aβ48–Aβ45–Aβ42–Aβ38 giving rise to the pathogenic Aβ42 and the non-pathogenic Aβ38 in comparable amounts. Mutations in PS, which are linked to familial forms of AD, affect the product lines and result in more of the pathogenic Aβ42 relative to Aβ40 and Aβ38 (reviewed in Lichtenthaler et al. 2011).

γ-secretase has a broad substrate specificity (Tischer and Cordell 1996; Lichtenthaler et al. 1999, 2002; Murphy et al. 1999), but amino acids in the juxtamembrane domains and within the transmembrane domains can affect the total amount of γ-secretase cleavage as well as the sites of γ-secretase cleavage (Zhang et al. 2002; Ren et al. 2007; Hemming et al. 2008). Additionally, dimerization of the APP TMD has been suggested to modulate the final cleavage sites of γ-secretase (Munter et al. 2007, 2010). In this model, APP dimerization sterically prevents γ-secretase from further shortening the Aβ42 peptides to Aβ38. Reducing APP dimerization—by site-directed mutagenesis or by using γ-secretase modulatory drugs—allows γ-secretase to continue cleavage, resulting in more Aβ38 and less Aβ42.

Taken together, γ-secretase is an unusual aspartyl protease, which acts as a heterotetrameric complex and cleaves APP and other substrates within the membrane.

10.1.5 Aβ-Degrading Proteases

10.1.5.1 Rationale

Due to the pivotal role of β- and γ-secretase in generation of Aβ-peptides, Alzheimer's disease was considered as a result of abnormal proteolysis followed by misfolding of the excessively produced Aβ molecules. However, it has been shown, that Aβ is a product of physiological APP turnover (Haass et al. 1992), rather than a per se pathophysiological side-product. Turnover of APP was found to be approx. 8 %/h throughout the life span of a human and impaired Aβ-degradation rather than increased Aβ-generation has been identified in AD patients (Bateman et al. 2006; Mawuenyega et al. 2010). Therefore, catabolism of Aβ peptides and identification of catabolic enzymes is crucial for understanding AD. To date a number of potential Aβ-degrading proteases have been identified.

10.1.5.2 Neprilysin and Endothelin-Converting Enzymes

Neprilysin (NEP) [EC 3.4.24.11] also known as neutral endopeptidase or CD10 is a zinc-dependent, membrane-bound metalloendopeptidase from the peptidase family M13 playing a pivotal role in Aβ-degradation in vivo. NEP was identified by investigating the degradation of radio-labeled Aβ(1–42) in the rat brain (Iwata et al. 2000). In this study, it could be shown, that a thiorphan and phosphoramidon-sensitive protease later identified as NEP accounts for the

majority of the Aβ-degradation potential in vivo. The important role was further underlined by investigations using NEP knockout mice showing elevated A-β-deposition (Iwata et al. 2001).

In addition, the NEP-related endothelin-converting enzymes 1 and 2 (ECE-1 and -2) [EC 3.4.24.71] are able to degrade Aβ in vitro (Eckman et al. 2001). Similar to NEP, ECEs are membrane-bound zinc-dependent metalloendopeptidases of peptidase family M13. Especially ECE-1 is present at the cell membrane and within compartments of the secretory pathway and might be able to degrade Aβ molecules in vivo. First evidence comes from ECE-1 heterozygous knockout mice showing increased Aβ-deposition (Eckman et al. 2003). In addition, NEP and ECE-1 knockouts possess a more pronounced Aβ-deposition, than the single knockouts underlining a concerted proteolysis of Aβ in vivo (Eckman et al. 2006).

10.1.5.3 Insulin-Degrading Enzyme

The insulin-degrading enzyme (IDE) [EC 3.4.24.56] is another example of an Aβ-degrading metalloendopeptidase. IDE belongs to the peptidase family M16 and was described to be primarily localized in the cytosol with a prominent function in insulin degradation (Duckworth et al. 1998). In addition, IDE was found to degrade Aβ-peptides in cell culture (Vekrellis et al. 2000), however, the localization of IDE in the cytosol raised some skepticism about the relevance for extracellular Aβ degradation. Recently, IDE was shown to be secreted via an "unconventional" pathway into the extracellular space (Zhao et al. 2009). This finding is in line with increased Aβ deposition in IDE knock-out animals underlining the importance of IDE for Aβ metabolism (Farris et al. 2003, 2004). Together with NEP and the mitochondrial presequence protease "PreP", IDE possesses a characteristic catalytic chamber, which is able to encapsulate peptides of a length of 70 amino acids or less. This might explain the selectivity of these proteases for their respective substrates including Aβ (Malito et al. 2008; de Strooper 2010).

10.1.5.4 Other Aβ-Degrading Proteases

Besides NEP and IDE, a number of different proteolytic enzymes have been shown to be able to degrade Aβ peptides in vitro. Among them the metalloendopeptidase Matrix Metalloproteinase 2 (MMP-2) [3.4.24.24], MMP-9 [EC 3.4.24.35] and the serine protease Plasmin [3.4.21.7], have been identified to possess a potential for Aβ degradation.

MMP-2 and MMP-9 belong to the peptidase family M10 and degrade large macromolecules of the extracellular matrix such as collagens (e.g. IV, V, VII, X). They are expressed at low levels in brain but can be induced by stress in cell culture. Of notice is the ability of MMP-9 to degrade fibrils in contrast to most other Aβ degrading proteases (Yan et al. 2006; de Strooper 2010).

Plasmin is a serine protease of peptidase family S1, which is activated by proteolytic cleavage with uPA or tPA from its precursor protein plasminogen. Plasmin is primarily responsible for degradation of fibrin aggregates in blood (Takada and Takada 1988) and the ability of degrading fibrin aggregates makes it an interesting protease for Aβ aggregate turnover in AD. Indeed, the expression of plasminogen and its activator proteins uPA and tPA have been linked to the central nervous system (Sappino et al. 1993). In addition, uPA and tPA can be induced under certain conditions such as ischemic insults or excitotoxicity (Tsirka et al. 1995). Plasmin has been shown to degrade fibrillar Aβ in vitro with approximately 1/10th of the rate of plasmin degrading fibrin (Tucker et al. 2000). However, the knockout of plasminogen does not show altered steady-state levels of Aβ (Tucker et al. 2004). Obviously, there is no significant contribution of plasmin to the physiological catabolism of Aβ, which is conceivable in the light of plasmin activation as prerequisite step for its proteolytic function. However, under defined experimental conditions such as traumatic brain injury, e.g. by intra-cortical application of Abeta peptides, the expression of uPA and tPA might be induced, which eventually leads to plasmin activation and, therefore, to significant turnover of Aβ peptides (Melchor et al. 2003).

In addition, Angiotensin-converting enzyme (ACE) [EC 3.4.15.1] has been shown to possess some potential of Aβ degradation. ACE is able to degrade monomeric Aβ in vitro (Hu et al. 2001; Zou et al. 2007), however, this finding could not be corroborated in vivo. Neither genetic inactivation (Eckman et al. 2006) nor treatment with anti-hypertensive drugs inhibiting ACE, such as captopril (Hemming et al. 2007) showed an effect on Aβ levels in mouse brain. Therefore, despite the genetic linkage of ACE polymorphisms to the risk of developing AD (Hu et al. 1999; Farrer et al. 2000), a direct role of ACE in Aβ-catabolism could not be provided.

Finally, in spite of its discussed role as putative alternative β-secretase, CatB also has been implicated in Aβ degradation in vivo. It has been found that CatB deficiency in mice expressing the "Swedish" and "Indiana" mutant of APP leads to an increased plaque load in hippocampus and cortex. Furthermore, the ability of CatB for degradation of monomeric and fibrillar Aβ could be demonstrated and the concentration of Aβ peptides generated from wildtype APP in CatB-deficient neurons was increased suggesting a catabolic activity of CatB for Aβ (Mueller-Steiner et al. 2006). As mentioned above, especially the role of CatB in AD is highly contradictory and needs further elucidation.

10.2 Proline-Specific Peptidases in Brain and Neurodegeneration

Nature has evolved a number of neuropeptides, neurotrophic peptide hormones and cardiovascular peptides with a proline residue determining their structural conformation and biological activity. In general, the proline peptide bond has shown to be resistant to proteolytic cleavage and therefore an exclusive number of proline specific peptidases have emerged to regulate these peptides (Yaron and Naider 1993). These include two endopeptidases, five dipeptidyl peptidases, two aminopeptidases, two carboxypeptidases and two dipeptidases. They are either serine peptidases of clan SC or metallopeptidases of clan MG or MH. Except for the three members of clan MG, all of them are post-proline peptidases, whereas soluble aminopeptidase P (sAmpP), membrane aminopeptidase P (mAmpP) and prolidase from subfamily M24B are able to hydrolyze the imide peptide bond. Interestingly, out of the 12 peptidases, 9 require proline at the penultimate position and 7 of them truncate at the N-terminus. Thus, a great number of neuropeptides contain a penultimate proline at their N-termini and their truncation results either in altered receptor selectivity or inactivation (Table 10.1). The best-characterized post-proline dipeptidyl aminopeptidase is dipeptidyl peptidase 4 (DP 4), followed by dipeptidyl peptidase 2 (DP 2) (Lambeir et al. 2003; Maes et al. 2007). However, only recently three additional post-proline dipeptidyl aminopeptidases have been discovered (Abbott and Gorrell 2002; Abbott et al. 2000; Ajami et al. 2004; Scanlan et al. 1994; Gorrell 2005). The resulting X-Pro dipeptides readily cross the cell where they are cytosolically metabolized by prolidase (Cunningham and O'Connor 1997; Mitsubuchi et al. 2008).

10.2.1 Serine Peptidases of Clan SC

Serine peptidases of the SC clan have a unique catalytic triad in the order of Ser, Asp and His located in an α/β-hydrolase fold compared to the chymotrypsin catalytic triad of His, Asp, Ser. They are comprised of both exopeptidases as well as endopeptidases and include the families S9, S10, S15, S28, S33 and S37. However, only S9 and S28 contain eukaryotic proline-specific peptidases, whereas X-Pro dipeptidyl peptidase (S15.001) of S15, prolyl aminopeptidase (S33.001) of S33 as well as prolyl tripeptidyl peptidase (S09.017) of S9 are only distributed in microbial species (http://www.merops.sanger.ac.uk).

10.2.1.1 Prolyl Oligopeptidase Family S9

The S9 family, also referred to as prolyl oligopeptidase family, consists of four subfamilies represented by their respective enzymes prolyl endopeptidase (9A),

Table 10.1 Summary of known and potential substrates of proline-specific peptidases in brain

Substrate	Sequence	Functions	Cleavage by proline-specific peptidases	Comment
Neuropeptides				
Neuropeptide Y	YPSKPDNPGEDAP-AEDMARYYSALRHYINLITRQRY-NH_2	Food-intake, stress	DP4, AmpP1, AmpP2	Alteration
Peptide YY	YPIKPEAPREDASPE-ELNRYYASLRHYL-NLVTRQRY-NH_2	Food-intake	DP4, AmpP2	Alteration
Substance P	RPKPQQFFGLM-NH_2	Nociception, stress	DP4, PEP, AmpP1, AmpP2	Alteration
GALP	APAHRGRGGWTL-NSAGYLLGPVLHLPQMGDQDGKRE-TALEILDLWKAI-DGLPYSHPPQPS	Food-intake, reproduction	DP4	Inactivation
Endomorphin-1	YPWF-NH_2	↓ Nociception	DP4, AmpP1	Inactivation
Endomorphin-2	YPFF-NH_2	↓ Nociception	DP4, AmpP1	Inactivation
PACAP	HSDGIFTDS-YSRYRKQMAV-KKYLAAVLGKRYKQRVKNK	Neurotroph, behavior, circardian	DP4	Inactivation
VIP	HADGVFTSDFS-KLLGQLSAKK-YLESLM	Circardian, sleep	DP4	Inactivation
Thyroliberin	PEHP-NH_2	↑ TSH	PEP	Inactivation
Arg-vasopressin	CYFQNCPRG-NH_2	Aggression	PEP	Inactivation
Oxytocin	CYIQNCPLG-NH_2	Neuromodular, anxiety	PEP	Inactivation
Neurotensin	pELYENKPRRPYIL	Behavior	PEP, neurolysin	Inactivation
Galanin	GWTLNSAGYLL-GPHAVGNHRS-FSDKNGLTS	Food-intake, stress	PEP	Inactivation
Human orexin B	RSGPPGLQGR-LQRLLQASGN-HAAGILTM-NH_2	Food-intake, stress	PEP	Inactivation
Rat orexin B	RPGPPGL-QGRLQRLLQANG-NHAAGILTM-NH_2	Food-intake, stress	DP4	Inactivation
b-Endorphin	YGGFMTSEK-SQTPLVTLFKNAIIK-NAYKKGE	↓ Nociception	PEP	Inactivation
CLIP	PVKVYP-NGAEDESAEAF-PLEF	Circardian rhythm	PEP, AmpP1	Inactivation
MSH-a	Ac-SYSMEHFRWGKPV-NH_2	Anorexic, behavior	PEP, PCP	Inactivation
Tyr-MIF-1	YPLG-NH_2	↓ Nociception ↓ aMSH	DP4, AmpP1	Inactivation

Morphiceptin	YPFP-NH_2	↓ Nociception	DP4, AmpP1	Inactivation
Kentsin	TPRK	↓ Nociception	DP4, AmpP1	Inactivation
b-Casomorphin	YPFVEPI	↓ Nociception	DP4, AmpP1	Inactivation
Carnosine	b-Ala-His	Neuroprotection	Prolidase	Inactivation
Neurotrophic growth factors				
GLP-1	HAEGTFTSDVS-SYLEGQAAKEFIA-WLVKGRG-NH_2	Anorexic, memory	DP4	Inactivation
IGF-1	GPETLCGAEL-VDALQFVCGDRGFYFNKPTG-YGSSSRRAPQT-GIVDECCFRSCD-LRRLEMYCAPLKPAKSA	Neurotroph	DP4	Inactivation
GPE	GPE	Neuroprotective	DP2	Inactivation
Cardiovascular peptides				
Bradykinnin	RPPGFSPFR	Vasodilator	mAmpP, sAmP, PEP	Inactivation
[DesArg] bradykinin	RPPGFSPF		PCP	
Angiotensin I	DRVYIHPFHL	Vasoconstrictor	PEP	
Angiotensin II	DRVYIHPF	Vasoconstrictor	PEP, PCP	Activation
Angiotensin III	RVYIHPF	Vasoconstrictor	PEP, PCP	Inactivation

dipeptidyl peptidase 4 (9B), acyl aminoacyl peptidase (9C) and glutamyl endopeptidase (9D) as well as several peptidases and homologues not assigned to any of the above subgroups (Abbott and Gorrell 2002; Barrett and Rawlings 1992; Gorrell 2005; Polgar 2002; Rosenblum and Kozarich 2003; Yu et al. 2010) (http://www.merops.sanger.ac.uk).

PEP Gene Family S9A

Prolyl endopeptidase (PEP) [EC 3.4.21.26] is the only true proline-specific endopeptidase, representing the S9A subfamily. It is also the only monomer, consisting of 710 amino acids with a molecular weight of approx. 80 kDa (Shirasawa et al. 1994). The human gene of PEP is located on 6q22 (Garcia-Horsman et al. 2007). PEP is located cytosolically in the perivascular space and is associated with the microtubulin cytoskeleton (Schulz et al. 2005; Rossner et al. 2005). It is ubiquitously expressed with the highest expression found in brain, kidney, testis and thymus, whereas very low levels of PEP could be detected in the liver (Myohanen et al. 2008c). In brain it is mainly expressed in neurons of the cerebral cortex, CA1 of hippocampus and Purkinje cells of cerebellum. In cerebral cortex and hippocampus, PEP was specifically expressed in glutameric pyramidal cells, while it also co-localized with gamma-aminobutyric acid (GABAergic) and cholinergic interneurons of the cortex and thalamus. However, no expression of PEP could be detected in nigrostriatal dopaminergic neurons or in astrocytes (Myohanen et al. 2008b). Altered activity and expression of PEP were found in aged wildtype mice, APP transgenic mice and human brains of patients with Alzheimer's disease (AD), revealing increased levels of PEP in the hippocampus of aged wildtype and APP transgenic mice, whereas lower PEP levels were found at the amyloid-beta plaques in brains of human AD and APP transgenic mice (Rossner et al. 2005). Application of PEP selective inhibitors in vivo suggested PEP to play an important role in learning as well as memory formation and PEP has been reported to be involved in the maturation and degradation of several peptide hormones and neuropeptides (Table 10.1), with substance P (SP), arginine vasopressin (AV), thyroliberin, gonadoliberin and alpha melanocyte stimulating hormone (α-MSH) implicated to be physiological substrates according to in vivo animal models (Garcia-Horsman et al. 2007; Perroud et al. 2009; Toide et al. 1995a, b, 1996; Shinoda et al. 1995; Morain et al. 2002; Bellemere et al. 2003, 2005; Schneider et al. 2002; Yamanaka et al. 1999). The crystal structure of PEP revealed a two-domain structure comprised of a α/β-hydrolase domain typical for the SC clan with catalytic triad Ser554, Asp641 and His680 and an open seven bladed propeller domain. Since the catalytic triad is covered by a central tunnel of an unusual beta propeller, the size of the peptide substrates is restricted up to approximately 30 amino acids (Fulop et al. 1998). Nonetheless, although the presence of a membrane-bound PEP has been reported in the brain, PEP currently generally considered to be located cytosolically associated with the perinuclear cytoskeleton, where it has no access to hydrolyze secreted or vesicular neuropeptides (O'Leary

et al. 1996; O'Leary and O'Connor 1995; Schulz et al. 2005; Tenorio-Laranga et al. 2008). Recently PEP has been reported to hydrolyze fragments of α-synuclein, thereby stimulating aggregation of α-synuclein (Brandt et al. 2005, 2008). In addition, PEP has been shown to be involved in the regulation of inositol-3-phosphate (IP3) signaling by its activity and association with SP, NK1 and IP3-1R (Myohanen et al. 2008a; Schulz et al. 2002). Furthermore, PEP has also been described to bind to growth-associated protein 43 (GAP43, neuromodulin) (Di Daniel et al. 2009). Finally, PEP has been associated with various neurodegenerative diseases such as Huntington's Parkinson's, Alzheimer's and Lewis body disease (Mannisto et al. 2007).

Dipeptidyl Peptidase 4 (DP4) Gene Family S9B

DP4

DP4 [EC 3.4.14.5] belongs to the serine peptidase clan SC, subfamily 9B. Currently, four members have been identified belonging to the dipeptidyl peptidase subfamily 9B, including dipeptidyl peptidase 4 (DP4), fibroblast activation protein alpha (FAP), dipeptidyl peptidase 8 (DP8) and dipeptidyl peptidase 9 (DP9) (Lambeir et al. 2003; Scanlan et al. 1994; Abbott et al. 2000; Olsen and Wagtmann 2002; Ajami et al. 2004; Abbott and Gorrell 2002).

DP 4 as representative member of the DP4 gene family is the best-characterized post-proline-dipeptidyl peptidase with most known in vivo substrates (Table 10.1) (Lambeir et al. 2003). The human gene location of DP4 is 2q24.2, encompassing 81.8 kb, spanning 26 exons, that code for two mRNAs of 2.8 kb and 4.2 kb, respectively (Abbott et al. 1994). Interestingly, the nucleotides coding for the residues of the catalytic triad are found on three different exons. The resulting protein has 766 amino acids and the primary structure consists of a short six amino acid cytoplasmic tail, a 22 amino acid transmembrane, a 738 amino acid extracellular portion comprised of a flexible stalk, glycosylation rich region, cysteine rich region and catalytic region with the catalytic triad. Although DP4 is a type II transmembrane glycoprotein, it has also a soluble shedded form in the blood circulation as illustrated in Fig. 10.1 (De Meester et al. 1999; Mentlein 2004; Cordero et al. 2009). The human crystal structure of DP4 reveals two domains, an eight bladed propeller and a catalytic α/β-hydrolase domain. The active site is composed of the catalytic triad Ser630, Asp708 and His740, two anchoring residues Glu204, Glu205 as well as substrate stabilizing residues Arg125, Asn710 and Tyr457. The propeller is open and consists of two subdomains made up of blades II–V and VI–VIII, I, respectively. Each blade has four anti-parallel β-sheets, except for blade IV that has an additional α-helix and two β-sheets forming an extended arm. There are two openings, a side opening and a propeller tunnel (Rasmussen et al. 2003). In a crystal structure, the substrate NPY suggested an entry at the side opening (Aertgeerts et al. 2004b). DP4 is reported to be a homodimer with glycosylation contributing to 23 % of the molecular weight of 110 kDa per subunit

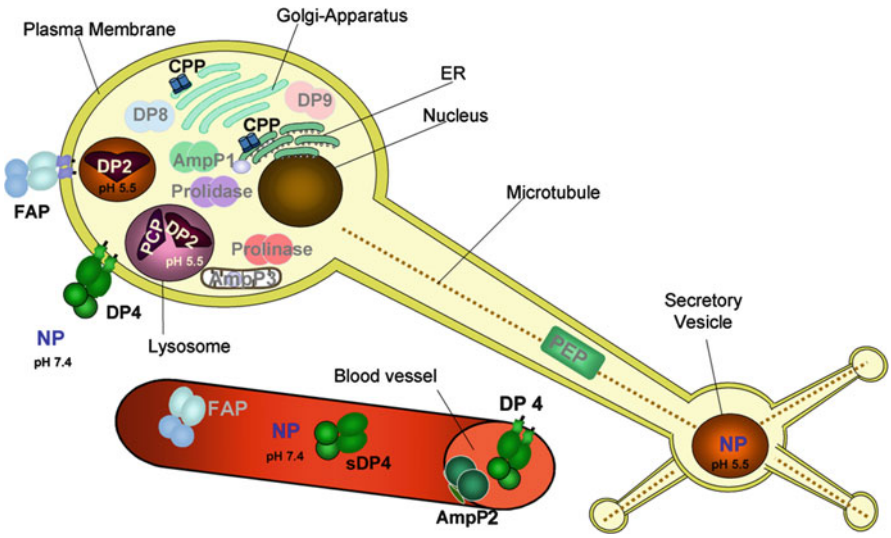

Fig. 10.1 Subcellular localization of proline-specific peptidases. Schematic representation of a neuron and a supplying blood microcapillary including pH-values in different compartiments. Further depicted are the discussed proline-specific peptidases and their sub-cellular localization. Note, that DP4 and FAP exist as membrane bound form present at the plasma membrane. In addition, a soluble form can be found in circulation. NP represents the sub-cellular localization of processed neuropeptides

(Lambeir et al. 2003). Tetramerization has also been described in porcine DP4 (Engel et al. 2003). Post-transcriptional modifications include nine N-terminal glycosylation sites and five disulfide bonds, though O-glycosylation and phosphorylation have also been reported (Rasmussen et al. 2003; Engel et al. 2003; Fan et al. 1997; Aertgeerts et al. 2004a; Alfalah et al. 2002; Tansi et al. 2010; Bilodeau et al. 2006). DP4 is known to cleave neuropeptides such as NPY, substance P, endomorphin 1 and 2; peptide hormones including GLP-1, GLP-2, GIP, glucagon, IGF-1, GHRH as well as various chemokines (Lambeir et al. 2003). According to kinetic analysis, DP4 has a very high selectivity for the neuropeptides NPY and PYY (Mentlein 1999; Medeiros and Turner 1993, 1994, 1993; Lambeir et al. 2003). Thus, it is involved in food up-take, anxiety, stress, cardiovascular, nociception, glucose homeostasis and chemotaxis. Furthermore, it functions as an extracellular adhesion molecule by binding to collagen, fibronectin and plasminogen. In addition, it is implicated in various immune responses via its interaction with several immunological molecules such as ADA or CD45 and acts as a marker for activated T-cells (De Meester et al. 1999; Ohnuma et al. 2008a, b). It is ubiquitously distributed with the highest expression in kidney, lung, liver and small intestine, whereas low expression is found in brain, heart and skeletal muscle (Lambeir et al. 2003; Gossrau 1979; Dikov et al. 1999; Frerker et al. 2007; Yu et al. 2009). However, though low levels of DP4 are found in brain parenchyma, elevated

activity and expression of DP4 could be detected in the meninges, brain capillaries, choroid plexus and circumventricular organs (CVOs) (Aimes et al. 2003; Barnes et al. 1991; Bourne et al. 1989; Nagy et al. 1996; Frerker et al. 2007; Chappa et al. 2006, 2007). These results imply that DP4 is at the interphase between the CNS and the periphery via the blood circulation and CSF respectively, thereby modulating and inactivating neuropeptides and neurotrophic growth factors (Table 10.1). This already explains the possible involvement of DP4 in social and stress-related behavior as observed in DP4−/− knock-out mice, DP4-deficient rats and after in vivo application of DP4 inhibitors, since the CVO's median eminence and area postrema link the CNS with the endocrine and immune system, thereby integrating the two stress axis hypothalamic-pituitary adrenal axis (HPA) and neuro-sympathico axis and resulting in the release of stress hormones and altered cytokines (El Yacoubi et al. 2006; Frerker et al. 2009; Karl et al. 2003; Lautar et al. 2005; Sewards and Sewards 2003; Elenkov et al. 2000). Aging and neurodegeneration is associated with a dysfunctional blood brain barrier (BBB) causing it to become more leaky and eventually disintegrate (Zlokovic 2008, 2010; Pahnke et al. 2009; Altman and Rutledge 2010; Dickstein et al. 2010; Ujiie et al. 2003; Bell et al. 2010; Bell and Zlokovic 2009; Deane et al. 2009; Deane and Zlokovic 2007; Mackic et al. 1998, 2002; Martel et al. 1996; Zlokovic et al. 1990). Thus, DP4 at the altered microvascular may regulate by its enzymatic activity the bioavailability of neuropeptide, neurotrophic growth hormone, cardiovascular peptides and immunological substrates and thus contribute to the pathogenesis of neurodegeneration such as vascular dementia, brain infarcts and Alzheimer's disease.

FAP

FAP [EC 3.4.21.–] also referred to as seprase, has the highest sequence identity to DP4 and is believed to arise from gene duplication due to its gene proximity. FAP is localized on 2q24.3, encompassing 72.8 kb, spanning 26 exons and yielding a 2.8 kb mRNA, that codes for 760 amino acids. Introns are also found between the exon coding for the catalytic residues. Similar to DP4, FAP is a type II glycoprotein. In addition to a low DP4 activity, FAP also exhibits gelatinase and collagenase activity, which is collagen type I specific. Its endopeptidase activity requires the sequence Xaa-Gly-Pro-Yaa (Edosada et al. 2006a, b). Recently, the crystal structure of FAP has been elucidated and comparison with the crystal structure of DP4 points to a lower anchoring of substrates by Glu203–Glu204 due to shielding effects of surrounding hydrophobic residues and lack of Asp663. This in turn, results in a lower exopeptidase activity and enables its endopeptidase activity as confirmed by site-directed mutagenesis with subsequent kinetic studies (Aertgeerts et al. 2005). Although low levels of FAP mRNA have been detected in healthy tissue, expression at protein level is restricted to pathogenic tissues such as human meningioma and astrocytoma, in particular in close vicinity to extracellular matrix. Interestingly, while the contributions of DP8 and DP9 were higher in meningioma compared to FAP and DP4, increased levels of DP4/FAP were associated with

malignancy (Stremenova et al. 2007, 2010; Mentlein et al. 2011). Thus, the expression of FAP has been investigated in several types of cancers, as to identify it as a pharmaceutical target (Garin-Chesa et al. 1990; Henry et al. 2007; Ariga et al. 2001; Iwasa et al. 2003, 2005; Jin et al. 2003; Huber et al. 2003; Mori et al. 2004; Okada et al. 2003; Goodman et al. 2003; Huang et al. 2004; Skubitz and Skubitz 2004; Wesley et al. 2004).

DP8

DP8 [EC 3.4.14.5] consists of 882 amino acids, has a molecular weight of 100 kDa and its human gene localization is 15q22, encompassing 71 kb, spanning 20 exons, encoding four alternatively spliced mRNA products between 3.8 and 4.1 kb and yielding a 882 amino acid protein (Abbott et al. 2000). Although DP8 has previously been reported to be monomeric, current data gave strong evidence for a dimeric structure with an apparent molecular weight above 200 kDa (Bjelke et al. 2006a). So far, it has been suggested to be located in the cytoplasm as a soluble protein and up to now, there has been no evidence for any secretion, though recently loose association of extra-cellular DP8 on plasma membrane was reported (Abbott et al. 2000; Chen et al. 2003a, b; Bank et al. 2011). Recent proteomic screening has revealed phosphorylation of Tyr331 and Thr334 (Yu et al. 2007). Using several chromogenic substrates, DP-8 was shown to display post-proline dipeptidyl aminopeptidase activity similar to that of DP4 (Abbott et al. 2000; Bjelke et al. 2006a). Hydrolysis of NPY, GLP-1, GLP-2, PYY, ITAC, IP-10, SDF-1α and SDF-1β, but not of MIG, Gro-β and Eotaxin could be demonstrated in vitro, though the rate of cleavage was slower compared to DP-4, in particular for PYY (Bjelke et al. 2006a, b; Ajami et al. 2008). The mRNA of DP-8 is ubiquitously distributed with its highest expression in brain and peripheral tissues, such as testis and ovaries (Abbott et al. 2000; Wagner et al. 2006; Qi et al. 2003). In baboon brains, DP 8 was shown to be highly expressed in Purkinje cells and neuronal cells in the granular layer, as well as neurites in the molecular layer, but not in cortex, forebrain or midbrain as shown by ISH. However, homogenates of mice cortex as well as pooled mid and hindbrain showed high levels of DP8 and/or DP9 activity (Yu et al. 2009). Nevertheless, its physiological function is presently unknown and still awaits further studies.

DP9

DP9 [EC 3.4.14.5] has previously been reported to be active as a cytosolic monomer, comprised of 863 amino acids with a molecular weight of approximately 100 kDa (Ajami et al. 2004). It lacks a transmembrane domain and is found intracellular near the Golgi complex, though secretion from transfected cells has not been observed yet. The gene is located on chromosome 19p13.3, encompassing 48 kb, spanning 23 exons and encoding for two alternatively spliced mRNA isoforms of 4.4 kb and 5.0 kb, respectively, with different tissue distribution (Frerker et al. 2007; Abbott and Gorrell 2002; Ajami et al. 2004; Bjelke

et al. 2006a). The enzymatical active protein contains 892 amino acids (Bjelke et al. 2006a). This variant was shown to be active as homodimer with an estimated molecular weight above 200 kDa, whereas no activity could be detected for the shorter variant comprised of 863 amino acids (Olsen and Wagtmann 2002). Using several chromogenic substrates, DP9 exhibited post-proline dipeptidyl aminopeptidase activity similar to that of DP4 and it was shown to truncate NPY, GLP-1, GLP2 and to a far lesser extent PYY in vitro (Table 10.1) (Bjelke et al. 2006a, b). However, very recently the cytoplasmic proteasome-derived antigenic peptide RU134-42 could be identified as the first natural substrate of DP9, suggesting DP9 to play an important role in peptide turnover and antigen presentation (Geiss-Friedlander et al. 2009). DP9 contains an Arg-Gly-Asp cell attachment motif and two potential glycosylation sites, though SDS-analysis of expressed DP9 revealed no mass differences based on deglycosylation (Olsen and Wagtmann 2002; Qi et al. 2003). Similar to mRNAs of DP4 and DP8, mRNA of DP9 is ubiquitously distributed however, with its highest expression in liver, heart and skeletal muscle (Qi et al. 2003; Ajami et al. 2004; Olsen and Wagtmann 2002; Yu et al. 2009). Its physiological function has not been elucidated yet, though an up-regulation of DP9 mRNA was detected in human meningioma (Stremenova et al. 2010).

So far, one cannot differentiate between DP8 and DP9 enzymatic activity due to lack of selective inhibitors, however DP8/DP9 activity could be detected in rodent brain (Frerker et al. 2007). Furthermore, brain and testis have been the only organs in which DP8/DP9 activity precedes over DP4 activity (Yu et al. 2009; Frerker et al. 2007; Dubois et al. 2008). Interestingly, there seems to be a difference between the distribution of DP8 and DP9 in primates such as baboon compared to rodents, expressing low and high levels in cortex, respectively (Yu et al. 2009; Frerker et al. 2007; Ansorge et al. 2009). Similarly, histochemical enzymatic staining of brain sections obtained from DP4 deficient Fischer rats as well as their respective wildtype, revealed ubiquitous staining of DP8 and/or DP9 in both DP4-deficient as well as DP4 wildtype rats, whereas DP4 was only found at the meninges in wildtype rats. Using a DP4-selective inhibitor as well as a DP4-like inhibitor P32/98 in both fluorogenic activity assay with Ala-Pro-AMC as well as NPY hydrolysis by MALDI-TOF-MS, high levels of DP8 and/or DP9 were found in extracts of primary neuronal cells from rat compared to glial cell extracts.

Yet, the suggested cytotoxicity of DP8/DP9 inhibition is currently controversially discussed (Lankas et al. 2005; Burkey et al. 2008; Kirby et al. 2010; Wu et al. 2009; Bank et al. 2011; Ansorge et al. 2011). Non-enzymatic functions of DP8/DP9 include cell adhesion, migration and apoptosis (Yu et al. 2006). Interestingly, similar to PEP, but unlike DP4 and FAP, DP8 and DP9 are reversibly inactivated by H_2O_2 oxidation involving two cysteins in each monomer (Park et al. 2008). Up to now, there are no crystal structures of DP8 and DP9 available, although molecular modeling based on DP4 and FAP crystal structures indicate similar overall structures comprised of a β-propeller and a α/β-hydrolase domains, with the active site being located at the interphase of the two domains. However, two loops and one helix of the propeller domain extending to the interphase cavity

appear to play a role at the active site. While the R125-loop and the E205/E206-helix have been described in the crystal structures of DP4, the P2-loop, containing F357 and R358, seems to be unique to DP8 and DP9 and is suggested to influence substrate and inhibitor binding to the P2-pocket (Rummey and Metz 2007; Engel et al. 2003; Rasmussen et al. 2003; Thoma et al. 2003; Oefner et al. 2003). Due to the shortest gene sizes, lowest numbers of exons, the active site being located in one exon in comparison to DP4 and FAP as well as their closest phylogenetic relationship with respect to prokaryotic members of the family, DP8 and DP9 are believed to be the ancestral genes of the DP4 gene family (Abbott and Gorrell 2002).

Although all of these enzymes described above display DP4-like activity, they exhibit distinct features with respect to cellular compartmentation and glycosylation as illustrated in Fig. 10.1.

Dipetidyl Peptidase-Like Proteins

DP-like proteins 1 (DPL1, DP6, DPP X, S09.973) and 2 (DPL2, DP10, DPP Y, S09.974) were previously assigned to the DP4-gene family S9B based on their sequence homologies to DP4 (Abbott and Gorrell 2002; Gorrell 2005). However, since they lack DP4-like activity due to mutations at the active site, they were moved to S9 family unassigned to any subfamily (http://merops.sanger.ac.uk/cgi-bin/famsum?family=S9). Both of them are type II membrane-bound glycoproteins, suggested to interact with the voltage-gated potassium channel Kv4 (Abbott and Gorrell 2002; Strop et al. 2004; Wada et al. 1992; Chen et al. 2003a, b, 2006a, b; Nadal et al. 2003; Kin et al. 2001; Zagha et al. 2005; Qi et al. 2003). While DPL1 is exclusively expressed in the brain as two variants, i.e. a short and a long form, DPL2 is found in brain, pancreas and adrenal gland (Qi et al. 2003; Chen et al. 2006a, b). The crystal structure of DPL1 resembles that of DP4 (Strop et al. 2004). DPL2 has additionally been associated with asthma.

Ex-DP4-Like Enzymes

In addition, enzymes structurally unrelated to the DP4 gene family have also been reported to display DP4 activity. These include attractin (Duke-Cohan et al. 1996) and N-acetyl alpha-linked acidic dipeptidases (NAALADases I, NAALADases II and NAALADases L) from the metalloprotease clan MH, family M28B (Pangalos et al. 1999). However, elevated DP4-like activity of the NAALADases has previously been detected only in crude extracts after cloning and expression, whereas detailed kinetic analysis of expressed and purified NAALADase I did not reveal any DP 4-like activity (Pangalos et al. 1999; Barinka et al. 2002). Likewise, the DP 4-like activity of attractin in the serum has been controversially discussed for several years (Friedrich et al. 2003; Duke-Cohan et al. 1995, 1996, 2004; Durinx et al. 2000) and only recently been disproved (Friedrich et al. 2007).

10.2.1.2 Family S28

As a member of clan SC, the S28 family has its catalytic residues in the order of Ser, Asp and His located in a α/β hydrolase fold. Peptidases of S28 contain exopeptidases that hydrolyze prolyl bonds, and are known only from eukaryotes. Human members include Prolyl carboxypeptidase (PCP), dipeptidyl peptidase 2 (DP2) and thymus-specific serine protease (TSSP), though the catalytic specificity of latter enzyme has not been elucidated yet. Intriguingly, while DP2 displays post-proline dipeptidyl aminopeptidase activity like the DP4-gene family, its high sequence homology to prolyl carboxypeptidase (PCP) designates it to family S28 (Soisson et al. 2010; Kozarich 2010).

Prolylcarboxypeptidase

PCP [EC 3.4.16.2] (prolycarboxypeptidase, Proline carboxypeptidase, lysosomal Pro-X carboxypeptidase, S28.001) hydrolyzes amino acids from the C-terminal end of oligopeptides having proline at the penultimate position (Skidgel et al. 1981). It is found soluble in lysosomes, having a pH optimum of 5.5, but its enzymatic activity is still retained at neutral pH (Odya and Erdos 1981; Odya et al. 1978). The gene location of PCP is 11q14, encompassing 147 kb covering nine exons that code for a 2.2 kb mRNA. The resulting protein contains 496 amino acids, composed of a signal peptide (1–21), propeptide (22–45) and mature PCP (46–496) (Tan et al. 1993; Watson et al. 1997; Abeywickrema et al. 2010). The enzyme is functional as a homodimer and each subunit has a molecular weight of 58 kDa (Tan et al. 1993). PCP is heavily N-glycosylated, containing six N-glycosylation sites, and the enzyme has also four disulfide bonds (Chen et al. 2009; Soisson et al. 2010). The crystal structure (pdb: 3N2Z) has only recently been elucidated, revealing the typical catalytical α/β-hydrolase domain as well as a novel SKS domain composed of five α-helices forming a novel bundle that caps the catalytical α/β-hydrolase domain. Interestingly, the active site holding the catalytic triad Ser179, Asp430 and His455 has apparently an additional charge-relay system that links the catalytic His455 with His456 and Arg460 and might even suggest a kind of dual catalytic triad bifurcated off Ser179, the ultimate nucleophile. Such His456/Arg460 diad may explain the acidic pH optimum of PCP (Soisson et al. 2010; Kozarich 2010). PCP is expressed in hypothalamus but also in lymphocytes, fibroblasts, endothelial cells, kidney and lung. It is also found soluble in CSF (Zhao et al. 2010a, b; Wardlaw 2011; Palmiter 2009; Kumamoto et al. 1981; Odya and Erdos 1981; Odya et al. 1978). Substrate hydrolysis include inactivation of Angiotensin II and III, desArg-bradykinnin and α-MSH, activation of prekallikrein and degradation of YPRPIHPA peptide fragment of human endothelin B-receptor-like protein 2 in CSF (Shariat-Madar et al. 2002, 2004; Moreira et al. 2002; Wallingford et al. 2009; Zhao et al. 2010a, b; Yang et al. 1968). Hence, it has been implicated in cardiovascular functions such as hypertension,

blood coagulation, food-intake, tissue proliferation and smooth-muscle growth and proposed as a pharmaceutical target for obesity, hypertension and anti-inflammatory therapy (Adams et al. 2011; Mallela et al. 2009; Shariat-Madar et al. 2010; Ngo et al. 2009; Diano 2011; Wang et al. 2006b; Zhou et al. 2010; Zhu et al. 2010; Hagedorn 2011; Javerzat et al. 2009).

Dipeptidyl Peptidase 2 (DP2)

DP2 [EC 3.4.14.2] (DP7, quiescent proline cell dipeptidase, QPP, EC 3.4.14.2, S28.002) is a proline specific serine protease, that hydrolyzes dipeptides from the N-termini of tripeptides and small peptide fragments if proline, norisoleucine or to a lesser extent alanine are at the penultimate position (Leiting et al. 2003; Maes et al. 2005; Mentlein and Struckhoff 1989). DP2 has also been identified with quiescent proline cell dipeptidase (QPP) based on genetic homology and kinetic parameters (Araki et al. 2001; Leiting et al. 2003; Maes et al. 2005). The human gene is located on chromosome 9q34.3 corresponding to 3p13 in rat and the human gene encompasses 2,850 kb comprised of 13 exons. The soluble serine protease contains a proform and has a length of 492 amino acids with a molecular weight of 58 kDa (Underwood et al. 1999; Chiravuri et al. 2000a; Maes et al. 2007). Glycosylation and dimerization are required for the catalytic activity and latter occurs via a leucine zipper motif, which is novel for proteases (Chiravuri et al. 2000a, b). Recently, the crystal structure of DP2 was published in the Protein Data Bank (Bezerra et al. 2012), revealing a α/β-hydrolase domain as well as a novel SKS domain, comprised of 5 α-helices arranged in a helix bundle fold, capping the active site. An insertion from the SKS domain to the active site results in steric hindrance of larger substrates and contains Asp334 for anchoring N-terminus of the peptide substrate.

The homodimer is located either in cellular vesicles that are distinct from lysosomes with secretion being regulated by an increased Ca^{2+} flux, in lysosomes and parts of Golgi complex or in lipofuscin granules (Chiravuri et al. 2000a; Gorenstein and Ribak 1985; Gorenstein et al. 1985; Maes et al. 2007; McDonald et al. 1968). In addition, in some brain fractions a membrane-associated form of DP2 has also been reported and northern blot analysis of rat DP2 revealed two spliced variants in the brain (Araki et al. 2001; Mentlein and Struckhoff 1989). DP2 is ubiquitously distributed with high expression in kidney, brain, testis, heart, resting lymphocytes and differentiated macrophages (Gossrau and Lojda 1980; Maes et al. 2006, 2007; Chiravuri et al. 1999; Underwood et al. 1999). Within various brain regions, high enzyme activities were reported in rat pituitary, human cerebellum and rat human hypothalamus (Maes et al. 2005). In contrast, others found the highest level of DP2 activity in rat brain extracts from the circumventricular organs and meninges, followed by cerebellum, hypothalamus, hippocampus, striatum, subventricular zone (SVZ), amygdala, cortex and spinal cord (Wagner et al. 2008). Furthermore, real-time RT-PCR analysis revealed the highest expression of DP2 mRNA in CVOs followed by cerebellum, moderate levels in

hypothalamus, hippocampus, amygdala, striatum, SVZ and spinal cord, whereas cortex had the lowest mRNA (Wagner et al. 2008). Since it was previously thought to be a lysosomal enzyme, its physiological function to date is unknown. However, altered serum activities of DP2 have been associated with various pathogenic conditions, such as Sjörgen Syndrome, rheumathoid arthritis, Lupus erythematosus, various cancers and Parkinson's disease (Maes et al. 2007). Lately, neurogenin 3-specific DP2-/- deficiency revealed a phenotype with impaired glucose tolerance, insulin resistance and visceral obesity (Danilova et al. 2009). Using chromogenic substrates, DP2 displays post-proline dipeptidyl aminopeptidase activity similar to DP4, however over a broad pH range with an acidic to neutral pH optimum (Underwood et al. 1999; Mentlein and Struckhoff 1989; Leiting et al. 2003; Maes et al. 2005, 2007). While DP2 readily hydrolyses tripeptides, its activity decreases rapidly with increasing chain length of peptide. Thus, it was shown to cleave only fragments of substance P1–4, bradykinin1–3 or bradykinin1–5 (Mentlein and Struckhoff 1989; Brandt et al. 2006; Frerker et al. 2007). Recently the N-terminal truncated tripeptide Gly-Pro-Glu (GPE) of IGF-1 was found to be a substrate of DP2. GPE has been reported to be neuroprotective by stimulating acetylcholine secretion and it has been associated with various neurodegenerative diseases such as Huntington's disease and AD. DP2 has been reported to be involved in apoptosis as a decrease of DP 2 activity caused cells to exit their G0-phase in quiescent lymphocytes and fibroblasts, resulting in an induction of apoptosis by up-regulation of p53 and c-Myc as well as a down-regulation of Bcl-2 (Chiravuri et al. 1999; Chiravuri and Huber 2000; Mele et al. 2009). Nevertheless, another study reported participation in necrosis rather than apoptosis (Maes et al. 2006). Interestingly, ADA was recently discovered to also bind to DP2 through with an order of magnitude lower potency compared to DP4 (Sharoyan et al. 2008).

10.2.2 Metallo-peptidases of Clan MG

Peptidases of the clan MG are described as being co-catalytic having water as nucleophile bound by two cobalt or manganese ions that are ligated by Asp, Asp, His, Glu, Glu. The metal ligands are pentahedrally coordinated by a Glu and the catalytic residues are three histidines. The MG clan contains only the family M24 that is divided into subfamilies M24A and M24B, respectively. The peptidases of the two subfamilies are grouped together on the basis of a common 'pitta-bread fold' comprised of both α-helices and an anti-parallel β-sheets within two structurally similar domains that are thought to be derived from an ancient gene duplication (Bazan et al. 1994). The active site is located between the two domains. The subfamily M24B holds the unique proline-specific peptidases aminopeptidase P1 (AmpP1), aminopeptidase P2 (AmpP2), aminopeptidase P3 (AmpP3) and Prolidase (PEPD), that are able to cleave the imidopeptide bond N-terminally of proline (http://merops.sanger.ac.uk/cgi-bin/famsum?family=m24b).

10.2.2.1 X-Prolyl Aminopeptidases

AmpP1

AmpP1 [EC 3.4.11.9] (X-prolyl aminopeptidases 1, XPNPEP1, M24.009) is a soluble cytosolic protein, lacking the hydrophobic signal sequence at the N-terminus and the GPI-anchor at the C-terminus (Cottrell et al. 2000a). The human gene location is 10q25.3, spans 59 kb with 19 exons and yields a 2.5 kb mRNA (Sprinkle et al. 2000). The resulting protein is a homodimer with each subunit comprised of 623 amino acids and a molecular weight of 71 kDa (Vanhoof et al. 1997a, b; Cottrell et al. 1998; Sprinkle et al. 2000). AmpP1 is not glycosylated, but has N6-acetylation at Lys304 (Choudhary et al. 2009). Recently, a 1.6 Å resolution structure of AmpP1 (pdb: 2CTZ) was elucidated, revealing an atypical three domain structure, compared to two domains in bacterial X-Pro aminopeptidase (pdb: 1A16) and prolidase (pdb: 2OKN_A). The three domains are referred to as N-terminal domain I, middle domain II and C-terminal domain III, containing the active site. While domain I and II are composed of a six-stranded mixed β-sheet, flanked by six α-helices, domain III contains three β-sheets of variable strands covered by six α-helices. The active site consists of three nucleophilic water molecules bound to two Mn^{2+} ions ligated by Asp415, Asp426, H489, Glu523 and Glu537. The side-chains of His395, His485, His498 and Glu41, surrounding the two Mn^{2+} ions are suggested to play a role in substrate recognition and catalysis (Li et al. 2008). Due to its proline specificity, AmpP1 is suggested to hydrolyze peptide hormones, neuropeptides such as tachykinins and otherwise resistant dietary protein fragments, as deficiency of AmpP1 results in excretion of large amounts of imino-oligopeptides. Thus, in vitro substrates of AmpP1 include bradykinin, NPY, β-casomorphin, substance P (SP), [Tyr1]-melanostatin, corticotropin-like intermediate lobe peptide (CLIP), IL-6, morphiceptin and kentsin (Harbeck and Mentlein 1991; Rusu and Yaron 1992; Frerker et al. 2007). Unlike the remaining proline-specific peptidases, AmpP1 is able to cleave Xaa-Pro-Pro-Yaa peptides, such as bradykinin, catalyzing it in a divalent cation-dependent manner (Griswold et al. 1996, 1999). AmpP1 is found in the brain parenchyma, particularly in astrocytes, though its physiological function in brain has not been elucidated yet. In addition, AmpP1 is ubiquitously distributed in peripheral tissues, with its highest expression in pancreas, followed by heart and muscle (Mentlein et al. 1990; Frerker et al. 2007).

AmpP2

AmpP2 [EC 3.4.11.9] (X-prolyl-aminopeptidase 2, XPNPEP2, EC 3.4.11.9, M24.005) is a GPI-anchored membrane-bound aminopeptidase encoding for 673 amino acids with a molecular mass of 75.5 kDa. The human gene localization is Xq25 encompassing 31 kb, containing 20 exons and a mRNA of 3.5 kb (Sprinkle

et al. 1998; Venema et al. 1997). In addition to lipidation at Ala649 by GPI-amidation, AmpP2 is also heavily N-glycosylated, with five sugar chains contributing to about 15 % of the molecular weight, thereby increasing the predicted molecular weight to about 90 kDa per subunit. However, the functional enzyme forms a homooligomer of 217–360 kDa (Ersahin et al. 2005). Prior to GPI-anchoring to the plasma membrane, the C-terminal propeptide has to be removed from the precursor to yield the mature form of AmpP2. Currently, there is no crystal structure of AmpP2 available, however, model building of the catalytic C-terminal domain and site directed mutagenesis suggest an active site with two Mn^{2+} ions ligated to Asp450, Asp461, His524, Glu555 and Glu569 as well as the catalytic residues His430, His524 and His533, all located at the typical 'pitta-bread fold' (Cottrell et al. 2000b). AmpP2 is highly expressed in kidney, followed by lung, heart, placenta, liver, small intestine, colon as well as endothelial and smooth muscle cells of capillaries and lymphatic vessels, but not in brain, skeletal muscle, pancreas, spleen, thymus, prostate, testis, ovary, or leukocytes (Venema et al. 1997; Cottrell et al. 2000b; Mentlein and Roos 1996; Ersahin et al. 2003; Taylor-McCabe et al. 2001; Frerker et al. 2007). It hydrolyzes NPY, PYY, SP and bradykinin (Medeiros and Turner 1994, 1996; Taylor-McCabe et al. 2001; Ersahin and Simmons 1997; Orawski and Simmons 1995; Orawski et al. 1987; Maggiora et al. 1999; Abid et al. 2009; Chappa et al. 2007). Though not expressed in brain parenchyma, N-terminal truncation of NPY and SP by AmpP2 was detected in vitro BBB studies, brain perfusion and sub-cellular membrane fraction, and therefore implies expression of AmpP2 in the brain microvasculature (Frerker et al. 2007; Chappa et al. 2006, 2007). Similar to AmpP1, AmpP2 is also able to cleave Xaa-Pro-Pro-Yaa peptides such as inactivating bradykinin. Hence, it is predominantly involved in cardiovascular diseases, also associated with angioedema induced by ACE inhibitors and the selective inhibitor Apstatin has been investigated as potential drug candidate (Adam et al. 2002; Blais et al. 1999; Ersahin et al. 1999; Ersahin and Simmons 1997; Taylor-McCabe et al. 2001; Maggiora et al. 1999; Prechel et al. 1995; Wolfrum et al. 2001).

AmpP3

AmpP3 [EC 3.4.11.9] (X-prolyl aminopeptidase 3, XPNPEP3, M24.026) has been discovered by nucleotide sequencing recently. Its gene location is 22q13.2, encompasses 70.2 kb with 10 exons translating a 3 kb mRNA (O'Toole et al. 2010). Two alternative spliced isoforms have been reported, determining mitochondrial and cytosolic sub-cellular localization of AmpP3, respectively (Ersahin et al. 2005). The resulting protein is composed of 507 amino acids with a molecular weight of 51 kDa for the mature mitochondrial isoform and 57 kDa for the immature cytosolic one. AmpP3 is ubiquitously expressed both at mRNA as well as protein level with highest expression in heart followed by pancreas, kidney, and testis then T cells, B cells, and monocytes. Transcripts encoding the predicted mitochondrial protein predominated in all samples (O'Toole et al. 2010). Sequence

analysis with AmpP1/2 and Prolidase points to two domains composed of an N-terminal Aminopeptidase P domain and a C-terminal Prolidase domain, which is also the catalytic domain (O'Toole et al. 2010). The enzyme is suggested to display similar activities as AmpP1 and AmpP3, having a proposed active site of two Mn^{2+} ions ligated by Asp331, Asp342, His424, Glu475 and Glu475 as determined by similarity. Substrates of AmpP3 were shown to be ciliary proteins. Mutation of AmpP3 results in nephronophthisis-like nephropathy 1 (NPHPL1), an autosomal recessive kidney disease with a phenotype of nephropathy, kidney cysts, cardiomyopathy, hypertension, seizure, tremor, mental retardation, subarchnoidal cysts, cerebellar vermis aplasia, retinal degeneration, hearing loss and liver fibrosis (O'Toole et al. 2010).

10.2.2.2 Prolidase

Prolidase [EC 3.4.13.9] (imidodipeptidase, peptidase D, PEPD, EC3.4.13.9, M24.007) catabolizes the resulting Xaa-Pro/Hyp dipeptides derived from the various DP4-like enzymes to their respective amino acids (Cunningham and O'Connor 1997). The enzyme belongs to the clan MG, subfamily M24B, requires two Mn^{2+} ions to coordinate to Asp276, Asp287, His370, Glu412 and Glu452, and has the catalytic residues H255, H366 and H377 (Besio et al. 2010). Its crystal structure revealed the typical 'pitta-bread fold' comprised of 21 α-helices and 5 anti-parallel β-sheets within two structurally similar domains, contributing 36 % and 16 % to the overall crystal structure, respectively (pdb's: 2OKN, 2IW2). It is ubiquitously distributed in the cytosol of many tissues such as brain, kidney, heart, liver, muscles, thymus, spleen, prostate, testis and placenta, where it exists as a homodimer with a molecular weight of 54.3 kDa per subunit. Posttranscriptional modifications include N-acetylation at Ala2 as well as phosphorylation at Thr188 and Thr487, respectively (Gevaert et al. 2005; Gauci et al. 2009; Beausoleil et al. 2004). Tyr-phosphorylation was shown to activate the enzyme (Surazynski et al. 2001). The gene is located at 19q12-q13.11, spans 130 kb and has 15 exons. Natural mutations of PEPD, comprised of several point mutations and deletions, result in prolidase deficiency (PD), a very rare autosomal recessive disorder associated with massive iminodipeptiduria. The clinical phenotype of PD includes skin ulcers, mental retardation, recurrent infections and abnormalities in collagenous tissues. However, these features are incompletely penetrant and highly variable in both age of onset and severity. There is a tight linkage between the polymorphisms of prolidase and the myotonic dystrophy trait as well as an increased risk factor for developing Lupus erythematosus (Tanoue et al. 1990a, b, c, 1991a, b; Endo et al. 1989a, b, 1990; Kikuchi et al. 2000; Ledoux et al. 1994, 1996; Forlino et al. 2002; Wang et al. 2006a; Falik-Zaccai et al. 2010; Lupi et al. 2004, 2006; Shrinath et al. 1997). Treatment of PD involves administration of L-proline and L-glycine containing ointments for skin, dietary supplementation with L-proline and essential amino acids, as well as erythrocytes transfusion. In addition to its Xaa-Pro dipeptidase activity, human recombinant prolidase also

displays organophosphoric acid anhydrolase (OPAA) activity and is therefore suggested as a treatment for organophosphorous toxins such as soman (Wang et al. 2004, 2005, 2006c).

10.2.3 Metallo-peptidase of Clan MH

10.2.3.1 Prolinase

Prolinase [EC 3.4.13.18] (cytosol non-specific dipeptidase, carnosine dipeptidase 2, carnosinase 2, Pro-X-dipeptidase, peptidase A, EC3.4.13.18, M20.005) catabolizes Pro-Xaa dipeptides into respective amino acids, displaying a pH optimum between 8.0 and 9.5. However, amongst all the proline-specific peptidases described above, it has the broadest specificity, since P1 can be occupied by any amino acids (Teufel et al. 2003). The enzyme belongs to the clan MG, subfamily 20A, having the catalytic residues Asp101 and Glu166 and requiring two Mn^{2+} ions to coordinate to His99, Asp132, Glu167, Asp195 and His445 (Unno et al. 2008). The enzyme is strongly inhibited by p-hydroxymercurybenzoate and bestatin with IC50s of 13 μM and 7 nM, respectively (Teufel et al. 2003). A 1.7 Å crystal structure revealed a homodimer and each subunit is comprised of two domains referred to as A and B. While domain A contains the metal and bestatin-binding site, domain B provides the major interface for dimerization. Since bestatin bound to domain A of one subunit also interacts with the domain B of the other subunit, the dimer interphase is suggested to be involved in the substrate hydrolysis, which would also require structural flexibility between the domain A and B (Unno et al. 2008). The CNDP2 gene is located at 18q22.3, encompasses 27 kb containing 14 exons. The 5 kb mRNA encodes for 475 amino acids with a molecular weight of 53 kDa per subunit (McDonough et al. 2009; Wanic et al. 2008; Teufel et al. 2003). Three potential N-glycosylation sites were identified in the protein sequence and post-transcriptional modifications include N-acetylation at Ala2 as well as N6-acetylation at Lys9 (Choudhary et al. 2009). The homodimer is found in the cytosol, ubiquitously distributed in various tissues both at mRNA as well as protein levels (Teufel et al. 2003). In brain, its protein is highly expressed in the parafascicular nucleus of the thalamus, tuberomammillary nucleus of the hypothalamus and the mitral cell layer of the olfactory bulb, while in striatum only the neuronal process, but not cell bodies are stained. No expression could be detected in glial cells (Otani et al. 2005, 2008). In CNS and autonomic nervous system, the major substrate of prolinase is L-carnosine, a pseudodipeptide comprised of β-alanyl-L-histidine that has neuroprotective functions by intra-cellular buffering, chelating capabilities, anti-oxidant properties and free radical scavenger (Balion et al. 2007; Min et al. 2008; Unno et al. 2008; Stvolinsky et al. 1999; Boldyrev 1993, 1994; Boldyrev et al. 1999a, b, 2010; Klebanov et al. 1997, 1998). It is also reported to have neurotransmitter properties in the olifactory system and hypothalamic network (Unno et al. 2008; Bonfanti et al. 1999; De Marchis et al. 2000a, b;

Tabakman et al. 2002). L-carnosine is secreted via ependymal and glial cells and it was shown to protect β-amyloid induced cell death in granular neurons of the cerebellum by free radical scavenging (Bonfanti et al. 1999; De Marchis et al. 1997, 2000b; Biffo et al. 1990a, b; Boldyrev et al. 2004b). Methylation and acetylation results in the formation of = beta-Alanyl-N(pi)-methyl-L-histidine and N-acetyl carnosine, respectively, being less neuroprotective though (Boldyrev 2000; Bonfanti et al. 1999; Boldyrev and Abe 1999; Boldyrev et al. 1995). Based on its neuroprotecting properties, L-carnosine, its respective analogues as well as prolinase-resistant analogues have been investigated as pharmaceutical target for neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, ischemia and aging (Boldyrev et al. 1999a, 2001, 2004a, b, 2010; Boldyrev 2005; Pegova et al. 2000; Chasovnikova et al. 1990; Gallant et al. 2000; Stvolinsky et al. 1999, 2000, 2003, 2010; Quinn et al. 1992; Min et al. 2008; Orioli et al. 2011; Vistoli et al. 2009). Polymorphism of carnosinase alias prolinase is associated with diabetic nephropathy in type 1 diabetes, though this is currently controversially discussed (Ahluwalia et al. 2011; McDonough et al. 2009; Wanic et al. 2008).

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