21 Neuropeptidases

K.-S. Hui

Abstract: Neuropeptides are neurotransmitters and modulators distributed in the central nervous system (CNS) and peripheral nervous system. Their abnormalities cause neurological and mental diseases. Neuropeptidases are enzymes crucial for the biosynthesis and biodegradation of neuropeptides. We here focus on the peptidases involved in the metabolism of the well-studied opioid peptides. Bioactive enkephalins are formed from propeptides by processing enzymes—prohormone thiol protease, prohormone convertase 1 and 2 (PC 1 and 2), carboxypeptidase H/E, and Arg/Lys aminopeptidase. After they exert their biological effects, enkephalins are likely to be inactivated by degrading enzymes—angiotensin-converting enzyme (ACE), aminopeptidase N (APN), puromycin-sensitive aminopeptidase (PSA), and endopeptidase 24.11. Recently, a neuron-specific aminopeptidase (NAP), which was a putative enkephalin-inactivating enzyme at the synapses, was found. Neuropeptidases are useful drug targets and their inhibitors can be therapeutic. Synthetic anti-enkephalinases and anti-aminopeptidases are being developed. They are potent analgesics but have fewer side effects than the opiates.

List of Abbreviations: AD, Alzheimer's disease; APN, aminopeptidase N; AVP, arginine-vasopressin; ACE, angiotensin-converting enzyme; CPE, carboxypeptidase E; CSF, cerebrospinal fluid; CM, conditioned medium; DAT, dementia of Alzheimer type; DAP, dipeptidyl aminopeptidase; Leu-Enk, leucine-enkephalin; Leu βNA, leucine β-naphthylamide; MHC, major histocompatibility complex; Met-Enk, methionine-enkephalin; MSA, multiple system atrophy; βNA, β-naphthylamide; NAP, neuron-specific aminopeptidase; NPY, neuropeptide Y; NEP, neutral endopeptidase; NE, norepinephrine; PD, Parkinson's disease; pLAP, placental leucine aminopeptidase; PE, proenkephalin; PC1 and PC2, prohormone convertase 1 and 2; POMC, proopiomelanocortin; PSA, puromycin-sensitive aminopeptidase; CCK-8S, sulfated cholecystokinin-8; TRH, thyrotrophin-releasing hormone; VIP, vasoactive intestinal peptide

1 Introduction

Neuropeptidases are defined as peptidases that are active in the brain or as enzymes that degrade neuropeptides, peptide neuromodulators, or peptide hormones found in the brain. Brain peptidases participate in the general metabolism of peptides (proteins). In addition, they are possibly involved in neurotransmission. The neuropeptidases that have been actively studied in the last 15 years are emphasized here. For the other peptidases, please refer to the author's former review (Hui and Lajtha, 1983).

Neuropeptides play important roles in mental and neurological diseases (Beal et al., 1986; Vecsei and Widerlov, 1988, 1990; Wahlestedt et al., 1989). They are widely distributed in the central nervous system (CNS) and peripheral nervous system, where they serve as neurotransmitters and neuromodulators. Deficiency of neuropeptides, for example, ACTH, can cause various neurological signs (Sato et al., 1991). The neurophysiological abnormalities include slow wave activity on electroencephalograms, delayed conduction velocity of the peripheral nerves, and low amplitude of muscle action potentials. Recent interest has focused on the role of neuropeptides in degenerative neurological diseases. Understanding this role could help define what is specifically vulnerable to the pathological processes, thus leading to improvement in diagnosis and therapy.

2 Abnormalities of Neuropeptides and Neuropeptidases

2.1 Neuropeptides in Degenerative Diseases

In the CNS, neuropeptides are co-stored with catecholamines, especially norepinephrine (NE). Their involvement in pathologies is characterized by a noradrenergic impairment (Martignoni et al., 1992). In Parkinson's disease (PD), and in multiple system atrophy (MSA) as well, a central noradrenergic deficit has been demonstrated, and in dementia of Alzheimer type (DAT), impaired noradrenergic transmission has been found. PD, MSA, and DAT patients showed a significant reduction in cerebrospinal fluid (CSF) neuropeptide Y (NPY) and NE levels (Beal and Martin, 1986; Martignoni et al., 1992).

In Huntington's disease, there are reduced concentrations of substance P, methionine-enkephalin (Met-Enk), and cholecystokinin in the basal ganglia; in contrast, the concentrations of somatostatin and thyrotrophin-releasing hormone (TRH) are increased (Sagar et al., 1984). The basal ganglia neurons in which somatostatin and NPY are co-localized are selectively preserved. A marked increase of somatostatin has been reported in the striatum in Huntington's chorea. In another study, neurotensin was increased in the pallidum, while in Parkinson's disease no significant changes in neurotensin content were observed (Beal et al., 1986; Palacios et al., 1990).

In Alzheimer's disease (AD) and in dementia associated with Parkinson's disease, concentrations of cortical somatostatin are reduced (Jankovic and Maric, 1987). NPY and corticotrophin-releasing factor are also reduced in the cerebral cortex of patients with AD. The reduced cortical concentrations of somatostatin and NPY in the cerebral cortex of patients with AD reflect a loss of neurons or terminals in which these two peptides are co-localized (Beal and Martin, 1986). Somatostatin concentration is reduced in the hippocampus and neocortex of patients dying with Alzheimer's type dementia. The levels of TRH and gonadotrophin-releasing hormone neuropeptides are significantly reduced in patients with senile dementia. But, no changes were found in the telencephalic neurotensin content in senile dementia of the Alzheimer type (Sagar et al., 1984).

Receptors of the neuropeptides are also changed in neurological diseases. Marked reductions in the density of somatostatin binding sites were observed in the caudate and putamen of patients with Huntington's chorea. However, these receptors were well preserved in the nucleus accumbens and in the ventral aspects of the anterior putamen. No alteration of somatostatin receptors was observed in other brain areas. These findings suggest that somatostatin receptors in the human striatum are markedly downregulated or are localized where a population of neurons is at risk.

Mice with megencephaly due to brain cell hypertrophy exhibit neurological and motor disturbances with seizure-like activity, and disturbances in the insulin-like growth factor system as well (Petersson et al., 1999). Its enkephalin messenger RNA expression is upregulated in the dentate gyrus granular layer and in ventral cortices, but downregulated in the CA1 pyramidal layer (Petersson et al., 2000). Enkephalin-like immunoreactivity is elevated in mossy fibers of the hippocampus and the ventral cortices. Cholecystokinin has region-specific up- and downregulation in the hippocampal formation and increased levels in ventral cortical regions. Galanin and NPY expression are increased in several layers and interneurons of the hippocampal formation, and in ventral cortices as well. In contrast, galanin-like immunoreactivity is reduced in nerve terminals in the forebrain. It is not clear whether the mainly increased peptide levels contribute to the excessive growth of the brain or represent a consequence of this growth and/or of the neurological and motor disturbances.

Following intracerebral innoculation of prions in mice, NPY mRNA expression is specifically upregulated in CA3 pyramidal neurons, whereas its expression in hilar neurons remains unaltered. Neuropeptide alterations preceding neurological dysfunction and neuronal death play a possible role in prion diseases (Diez et al., 1996). NPY may regulate glutamate release at the Schaffer collateral-CA1 synapses in scrapie-infected mice.

2.2 Neuropeptides in Cerebrospinal Fluid

In the lumbar CSF of patients with AD, high levels of neurotensin are detected (Martignoni et al., 1992). There is a good correlation between plasma and CSF arginine-vasopressin (AVP) values in most patients with Parkinson's disease, dementia, cerebrovascular disease, multiple sclerosis, or other, mostly peripheral, neurological disorders (Neuser et al., 1984; Sorensen et al., 1985; Cramer et al., 1988; Vecsei and Widerlov, 1988). Significantly higher CSF-AVP values were found in patients with cerebrovascular disease, whereas lower CSF values were found in patients with dementia and Parkinson's disease (Reid and Morton, 1982; Sundquist et al., 1983). However, CSF/plasma gradients in patients with dementia and Parkinson's disease were decreased to about 0.30, compared with 0.98 in patients with peripheral neurological disorders. In hypoxic-ischemic encephalopathy, a significant elevation of plasma β-endorphin concentration was

observed (Sankaran et al., 1984). The association of increased plasma β-endorphin concentration is linked with ongoing hypoxemia.

2.3 Neuropeptides in Neurological Diseases

Met-Enk, but not leucine-enkephalin (Leu-Enk), inhibits neurological signs and markedly diminishes the occurrence and intensity of histological lesions in the brain, and in the cervical, thoracic, and lumbar spinal cord in the development of experimental allergic encephalomyelitis (EAE) (Jankovic and Maric, 1987). Met-enk, a potent immunomodulator/regulator, is important in the pathogenesis and prevention of the inflammatory autoimmune disease.

N-Acetylaspartylglutamate (NAAG), a neuropeptide found in millimolar concentrations in brain, is localized in subpopulations of glutamatergic, cholinergic, GABAergic, and noradrenergic neuronal systems. NAAG is released upon depolarization by a Ca^{++}-dependent process and is an agonist at mGluR3 receptors and an antagonist at *N*-methyl-D-aspartate (NMDA) receptors. NAAG exerts neuroprotective effects in a neonatal rat model of hypoxia–ischemia (Cai et al., 2002). The effects are largely associated with activation of the mGlu2/3 receptor. NAAG and β-NAAG protect against injury induced by NMDA and hypoxia in primary spinal cord cultures (Yourick et al., 2003).

Vasoactive intestinal peptide (VIP), a neuropeptide with a potent Leu-Enk anti-inflammatory effect, protects from inflammatory disorders. In most neurodegenerative disorders, including multiple sclerosis, Parkinson's disease, and Alzheimer's disease, massive neuronal cell death occurs as a consequence of an uncontrolled inflammatory response. VIP also has a neuroprotective effect by inhibiting the production of microglia-derived proinflammatory factors (tumor necrosis factor α, interleukin-1β, nitric oxide). It prevents neuronal cell death following brain trauma by reducing the inflammatory response of neighboring microglia. VIP is thus a valuable neuroprotective agent for the treatment of pathologic conditions in the CNS where inflammation-induced neurodegeneration occurs (Delgado and Ganea, 2003). TRH induced neurological improvement in 17 of the 23 patients with amyotrophic lateral sclerosis (ALS) but little or none in the other ALS patients or in patients with other neurological diseases (Congia et al., 1991).

Neurotropic murine coronavirus MHV-JHM (JHMV) causes encephalitis and paralytic-demyelinating disease in susceptible strains of mice and rats (Congia et al., 1991). It is a good model for human demyelinating diseases such as multiple sclerosis. Intracerebral administration of β-endorphin reduced the incidence of JHMV-induced paralytic-demyelinating disease in mice. Protection from the disease was accompanied by significantly reduced virus replication in the brain (Gilmore et al., 1993). The data suggest that β-endorphin engages immune mechanisms of host resistance to JHMV infection to protect the mice from disease.

Peptides derived from ACTH and MSH help post-lesion repair mechanisms in the peripheral nervous system by enhancing the early sprouting response of the damaged nerve. These peptides prevent cisplatin neuropathy in women suffering from ovarian cancer. Treatment based on nonendocrine fragments of ACTH/MSH could be a therapeutic option in cisplatin neuropathy (Gispen et al., 1992).

2.4 Neuropeptide Degradation in Neurological Diseases

Mutations in tripeptidyl peptidase (TPP-I) have recently been associated with a lysosomal storage disease, late infantile neuronal ceroid lipofuscinosis (CLN2) (Tomkinson, 1999; Junaid et al., 2000; Golabek et al., 2003). This disease is characterized by the accumulation of proteinaceous and auto-fluorescent material within the lysosomes of neurons, which undergo massive cell death during the course of the disease (Bernardini and Warburton, 2002; Warburton and Bernardini, 2002; Wujek et al., 2004). TPP-I is required for the partial or complete digestion of certain neuropeptides by brain lysosomes. Dipeptidyl peptidase-I expressed in other tissues has extensive activity on peptides and can compensate for the loss of TPP-I (Bernardini and Warburton, 2002).

The levels of NAAG and the activity of carboxypeptidase II are altered in a regionally specific fashion in several neuropsychiatric disorders (Coyle, 1997). In Alzheimer's postmortem brain, somatostatin-28

degradation is increased in Brodmann area 22 whereas substance P degradation is increased in the temporal cortex. Changes in the degradation of these neuropeptides that are affected in AD correlate with alterations in the activity of specific neuropeptidases. Trypsin-like serine protease activity is increased in Brodmann area 22 in AD. It parallels the increased degradation of somatostatin-28. The decreased activity of neutral endopeptidase 24.15 (NEP; EC 3.4.24.15) in the temporal cortex corresponds to the decreased degradation of substance P (Waters and Davis, 1995, 1997).

2.5 Exopeptidases in Aging and in Neurological Diseases

The process of aging involves alterations in the activity of peptidases and proteases, although the precise changes have not yet been fully characterized. The activity of the soluble fractions of prolyl endopeptidase was reduced in the lungs of aged animals (Waters and Davis, 1997). Reduced activity of soluble pyroglutamyl peptidase I and aminopeptidase N (APN) were detected in the aged kidney and heart, respectively. In contrast, increased activity of particulate prolyl endopeptidase was detected in the brain stem. Most of these changes can be correlated with known alterations in the levels of peptides controlled by each enzyme.

Puromycin-sensitive aminopeptidase (PSA) functions as a trimming enzyme in the major histocompatibility complex (MHC) class I pathway, which is activated in brains of patients with AD. In these brains, intensely stained cells were found to be rich in the cerebral cortex. Double immunofluorescence studies confirmed that PSA-positive cells were reactive microglia. Such PSA-positive reactive microglia tended to be located in and around senile plaques and were observed to be associated with neurons containing neurofibrillary tangles. The microglia PSA may be associated with the pathological conditions of AD (Minnasch et al., 2003).

Alanyl aminopeptidase activity was lower in the CSF of patients with AD, whereas no differences in CSF were detected in regard to the remaining aminopeptidases (Montes et al., 1998). No changes were found in the levels of amino acids in CSF or plasma. The plasma/CSF ratio for aminopeptidase activities was higher in patients with AD, although the difference was significant only for alanyl aminopeptidase (Iribar et al., 1998). In brains of patients with sporadic AD, decreased neuronal expression of a brain-specific carboxypeptidase B (CPB) and clusters of microglia with peptidase immunoreactivity associated with its extracellular deposition were detected (Matsumoto et al., 2000). Brain CPB has a physiological function in APP processing and may have significance in AD pathophysiology.

2.6 Endopeptidases in Neurological Diseases

A DNA polymorphism at the angiotensin-converting enzyme (ACE) gene has been linked to the risk for late onset Alzheimer's disease (Alvarez et al., 1999). Increased frequency of the ACE-I allele has been found in patients with AD. In the distribution of an insertion (I)/deletion (D) polymorphism of ACE in patients with AD, an association between AD and ACE genotypes or alleles was found. The frequency of II genotypes in AD was 1.4 times higher than in controls, while that of DD genotypes was only 0.4 times as high. The altered distribution of ACE alleles in patients appeared to be independent of apolipoprotein E (Hu et al., 1999).

Conditioned medium (CM) of NEP 24.15 antisense-transfected neuroblastoma has a significantly higher level of amyloid β (Aβ) (Yamin et al., 1999). Furthermore, synthetic Aβ-degradation is increased or decreased following incubation with CM of sense- or antisense-transfected cells, respectively. Soluble $A\beta_{1-42}$ is degraded more slowly than soluble $A\beta_{1-40}$, while aggregated $A\beta_{1-42}$ showed almost no degradation. Pretreatment of CM with serine proteinase inhibitors completely inhibits Aβ degradation. Additionally, a serpin family inhibitor tightly associated with plaques and elevated in brains of patients with AD blocks Aβ degradation. Recombinant NEP 24.15 alone does not degrade Aβ. ^{14}C-Diisopropyl fluorophosphate-radiolabeled CM from NEP-overexpressing cells contains increased levels of several active serine proteinases suggesting that NEP activates one or more Aβ-degrading serine proteases. The serpin inhibitor

causes Aβ accumulation by inhibiting an Aβ-degrading enzyme or by direct binding to Aβ, rendering it degradation resistant.

Insulin-degrading enzyme is the main soluble Aβ-degrading enzyme at neutral pH in the human brain. The highest Aβ protein-degrading activity in the soluble fraction occurs between pH 4 and 5, and this aspartyl protease is inhibited by pepstatin. Synaptic membranes have much lower Aβ protein-degrading activity than the soluble fraction. EDTA inhibits the degrading activity but inhibitors of NEP 24.11, -24.15, -24.16, ACE, aminopeptidases, and carboxypeptidases have little or no effect (McDermott and Gibson, 1997). A novel Zn^{++}-dependent metalloprotease activity associated with a Golgi apparatus- and plasma membrane-enriched fraction can degrade endogenous APP to generate Aβ containing C-terminal fragments. This protease generates amyloidogenic fragments of APP that can serve as precursors for Aβ (Mok et al., 1997).

3 Classification of Exo- and Endopeptidases

Exo- and endopeptidases are involved in the breakdown of larger (>30 amino acids) peptides into smaller (<30 amino acids) and then into free amino acids. The distinction between the two groups of peptidases is by no means absolute, since some endopeptidases can exhibit exopeptidase activity (Orlowski, 1983). The exopeptidases classified by the IUPAC (3.4.11–17) are divided according to their specificity into those hydrolyzing single amino acids from the N-terminal of the peptide chain (3.4.11), those hydrolyzing single amino acids from the C terminus (3.4.16–17), those specific for dipeptide substrates (3.4.13), and those splitting off dipeptides from the N terminus (3.4.14) or C terminus (3.4.15). The group hydrolyzing single amino acids from the C terminus is subdivided into serine carboxypeptidases (3.4.16) and metallocarboxypeptidases (3.4.17).

4 Enkephalin-Processing Enzymes

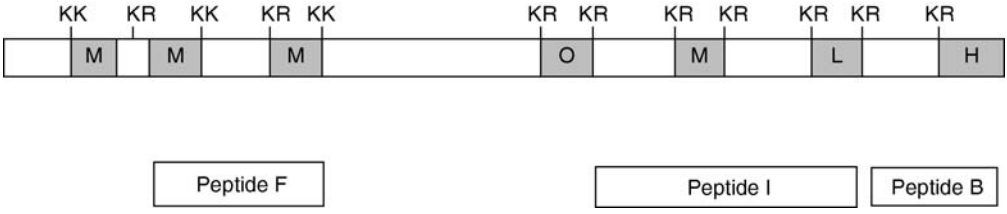
Enkephalins, endorphins, dynorphins, and orphanin, the families of opioid peptides found in the brain, are neurotransmitters and neurohormones (Nakanish et al., 1979; Gubler et al., 1982; Boileu et al., 1983; Yoshikawa et al., 1984; Civelli et al., 1985; Molleraeu et al., 1996; Nothacker et al., 1996; Hook and Reisine, 2001). Their close structural relationship shows considerable overlap in their receptor-mediated actions: analgesia (Frenk et al., 1978; Herman et al., 1985), catalexy/epilepsy (Frenk et al., 1978), memory (De Wied et al., 1978), hypothermia (Holaday et al., 1978), appetite (Brands et al., 1979; Morley, 1980), sexual behavior (Meyerson and Terenius, 1977), hormones (Morley, 1981), mental diseases, behavior, and cell function (Bloom et al., 1976, Jacquet and Marks, 1976). Most recently the involvement of enkephalins in pain perception and behavior was demonstrated in transgenic Enk-knockout mice (Konig et al., 1996). To evaluate the significance of neuropeptides, it is necessary to study the mechanisms underlying and controlling their synaptic formation and inactivation.

Production of endogenous enkephalin opioid peptides requires proteolytic processing of proenkephalin (PE). It takes part in the secondary pathway for production of active enkephalins that are secreted upon stimulation of enkephalinergic neurons. PE and enkephalins belong to the opioid precursor gene family that encodes protein precursors whose proteolytic products are enkephalin, β-endorphin, dynorphin, and orphanin (Gubler et al., 1982). Proteolytic processing enzymes are required for generating active opioid peptides that are secreted to activate opioid receptors (Bzdega et al., 1993; Chen et al., 1993; Reinscheid et al., 1995; Noda et al., 2000; Cahill et al., 2001; King et al., 2001; Przewlocki and Przewlocka, 2001; Suzuki et al., 2001).

Enkephalins and other neuropeptides, including NPY, somatostatin, VIP, and galanin, are synthesized as precursors that require proteolytic processing at paired basic residues to generate active peptides (Steiner et al., 1992; Hook et al., 1994; Hook and Yasothotnsrikul, 1998; Seidah et al., 1998). PE contains multiple copies of the pentapeptide Met-Enk, Leu-Enk, and enkephalin-related peptides (*Figure 21-1*). PE requires proteolytic processing at paired basic residue sites (Lys-Arg, Arg-Arg, and Lys-Lys), and at monobasic arginine sites as well, to liberate active enkephalins.

Figure 21-1

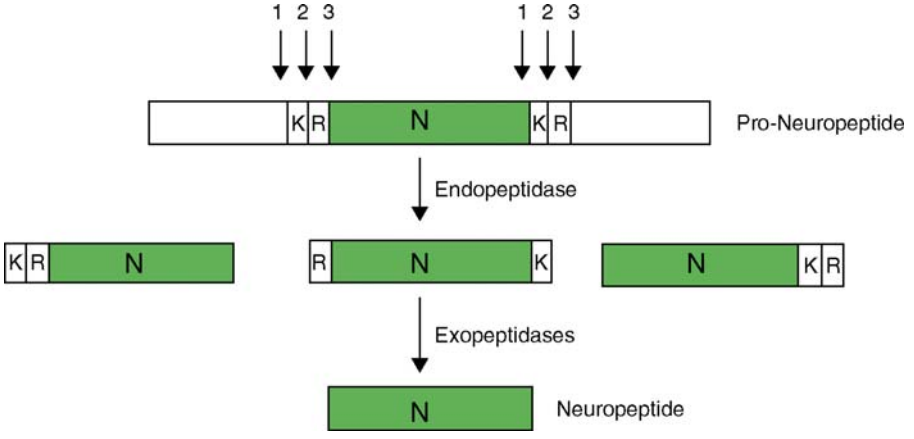
Structure of proenkephalin (PE). PE contains Met-enkephalin (M), Leu-enkephalin (L), Met-Enk-Arg-Gly-Leu (O), and Met-Enk-Arg-Phe (H). K represents lysine and R arginine



Processing of PE begins with removal of the NH_2-terminal signal peptide at the rough endoplasmic reticulum (RER) during protein translation (Steiner et al., 1992; Hook et al., 1994; Hook and Yasothotnsrikul, 1998; Seidah et al., 1998; Acher et al., 2002). The PE is routed through the RER and Golgi apparatus, where they are packaged into secretory vesicles. There, endopeptidases cleave at the Lys-Arg and other paired basic residue sites (Lys-Lys, Arg-Arg, Arg-Lys) that flank the enkephalins within the precursor. The resultant peptide intermediates contain basic residue extensions at the COOH- and/or NH_2-termini that are later removed by carboxypeptidase E/H (Fricker, 1991; Hook and Yasothotnsrikul, 1998) and Arg/Lys aminopeptidase (Hook and Yasothotnsrikul, 1998; Yasothornsrikul et al., 1998) respectively. This multistep proteolytic pathway generates bioactive enkephalins (*Figure 21-2*).

Figure 21-2

Processing of a model proneuropeptide. This model proneuropeptide contains one copy of the processed peptide neurotransmitter or hormone. Cleavage at the dibasic site occurs at its NH_2 terminus, between the dibasic residues, or at the COOH terminus of the dibasic residue site, represented by *arrows* at positions 1, 2, or 3, respectively. Removal of basic residues at COOH- and NH_2-termini are carried out by carboxypeptidase E/H and Arg/Lys aminopeptidase, respectively



Four proteases consisting of the cysteine protease known as "prohormone thiol protease" (PTP) (Yasothornsrikul et al., 1999), the subtilisin-like prohormone convertase 1 and 2 (PC1 and PC2) (Azaryan et al., 1992), and a 70-kDa aspartyl protease (Azaryan et al., 1995c), were found using full-length enkephalin precursor as substrate (Krieger and Hook, 1991). In chromaffin granules, PTP is the major, protease having PE-cleaving activity, PC1 and PC2 have comparably lower activities, and the 70-kDa aspartyl protease has the least PE-cleaving activity (Hook and Eiden, 1985; Azaryan et al., 1995c; Hook et al., 1996). PTP shows preference for processing PE, with minimal processing of POMC in vitro (Azaryan et al., 1995b; Schiller et al., 1996).

4.1 Prohormone Thiol Protease

PTP generates PE products in vitro that resemble those in vivo (Ungar and Phillips, 1983; Schiller et al., 1995; Hook et al., 1997). PTP has cleavage specificity for dibasic and monobasic processing sites that are cleaved within enkephalin-containing peptide substrates. Native PTP cleaves PE and enkephalin-containing peptide substrates at paired basic residue cleavage sites, and at monobasic Arg sites as well. The cleavage specificities of PTP for cleavage between and at the NH_2-terminal side of the dibasic residue sites indicate that resultant peptide intermediates possess basic residue extensions at their NH_2-termini (Loh et al., 1985; Azaryan and Hook, 1994a, b; Azaryan et al., 1995a). Studies of PE-derived peptides demonstrate the presence of enkephalin peptides with basic residue extensions at their NH_2-termini (Hook and Eiden, 1984; Kreiger et al., 1992). These results indicate the necessity for an aminopeptidase that removes Arg and Lys residues from NH_2-termini of peptide intermediates as one of the later steps in PE processing.

PTP has not been fully purified and characterized because of the trace yield. It was partially purified from bovine medullary chromaffin granules. The soluble protease is a glycoprotein with a pI of 6.0 and a pH optimum of 5.5 (Krieger and Hook, 1991). It is a thiol enzyme as shown by its dependence of dithiothreitol and inhibition by *p*-hydroxymercuribenzoate, mercuric chloride, cystatin C, and E-64. It was reported that PTP possesses a unique NH_2-terminal primary sequence, which is not homologous to other known proteases (Tezaosudusm et al., 1995). However, further work is needed to show that PTP is a novel enzyme.

Recently, Yasothornsrikul et al. reported that secretory vesicle cathepsin L is the responsible cysteine protease of chromaffin granules for converting PE to the active enkephalin peptide neurotransmitter (Yasothornsrikul et al., 2003). The cathepsin L activity was identified by affinity labeling with an activity-based probe for cysteine proteases followed by mass spectrometry for peptide sequencing. Production of Met-Enk by cathepsin L occurred by proteolytic processing at dibasic and monobasic prohormone-processing sites. Co-localization of cathepsin L with Met-Enk in secretory vesicles of neuroendocrine chromaffin cells was shown by immunofluorescent confocal and immunoelectron microscopy. Cathepsin L was co-secreted with Met-Enk. In cathepsin L-gene-knockout mice, significant reduction in Met-Enk levels in brain occurred with an increase in the relative amounts of enkephalin precursor (Yasothornsrikul et al., 2003).

4.2 Prohormone Convertase 1 and 2

PC1 and PC2 are proprotein convertase members of the mammalian subtilisin-like family. They cleave proinsulin and other prohormones primarily at the COOH-terminal side of paired basic residues, with some cleavage at the NH_2-terminal side of a single arginine residue of a peptide (Hwang et al., 2000). PTP cleaves at the NH_2-terminal side of paired basic residues and between the two basic residues, whereas PC1 and PC2 cleave pro-opiomelanocortin (POMC) between the two basic residues and at the COOH-terminal side of the dibasic residues. The proteases have highly conserved primary sequences with respect to signal sequence, pro-segment, catalytic domain, and P domain. The mammalian PC1 and PC2 contain catalytic triad residues Asp, His, and Ser. The bovine PC1 contains Asn as the oxyanion hole residue and PC2 contains Asp as the oxyanion hole residue. Each of them possesses the P domain with a functional RRGDL motif (Hwang et al., 2000).

4.3 Carboxypeptidase H/E

The cleavage specificities of the processing enzymes require carboxypeptidase E/H and aminopeptidase for the removal of NH_2- and COOH-terminal basic residues from the peptide intermediates (Dhanvantari et al., 2002; Wei et al., 2003). Carboxypeptidase E (CPE) (EC 3.4.17.10; carboxypeptidase H) removes basic amino acids from the COOH terminus of peptides to make them biologically active. A variety of neuropeptide-processing endopeptidases cleave at specific cleavage sites, at the COOH ends of the basic residues, generating intermediates with the COOH-terminal amino acids. However, a single carboxypeptidase has been implicated in the processing of mammalian neuropeptides (Wei et al., 2002).

CPE is present within secretory granules in both a soluble form and a form that is membrane bound at pH 5.5 but soluble at neutral pH (Fricker et al., 1990). Combined polar and hydrophobic interactions of the COOH-terminal peptide appear to be responsible for the reversible pH-dependent association of CPE with membranes (Silva et al., 1995; Dhanvantari et al., 2002).

4.4 Arg/Lys Aminopeptidase

Arg^0-Met-Enk was found and isolated from bovine striatum (Hui et al., 1994). The structure of the purified material was identified by microsequencing and mass spectrometry as the hexapeptide Arg-Tyr-Gly-Gly-Phe-Met. Ninety percent of the purified peptide was Arg^0-[O]Met-Enk, consisting of equimolar Met(*R*)- and (*S*)-sulfoxide. The existence of this enkephalin intermediate indicates that PTP is the putative processing enzyme. It will be interesting if there is an enzyme that specifically removes the N-terminal basic amino acid to turn on enkephalin activity.

There is an aminopeptidase activity in neurosecretory vesicles that converts Arg^0-Met-Enk to Met-Enk (Gainer et al., 1984; Hook and Eiden, 1984). Both arginine aminopeptidase (RAP) and lysine aminopeptidase (KAP) activities were found in neurosecretory vesicles of chromaffin granules (Yasothornsrikul et al., 1998). They are involved with reduced cysteinyl residues. The majority of the RAP/KAP activity resides with the soluble component of chromaffin granules, rather than the membrane component. RAP, but not KAP, is stimulated with NaCl. The RAP and KAP activities have pH optima at 6.7 and 7.0, respectively. RAP has a lower K_m. Both enzymes, possible metalloproteases, are inhibited by the specific aminopeptidase inhibitors bestatin, amastatin, and arphamenine. KAP activity is partially inhibited by Ni^{++} and Zn^{++}, whereas RAP activity is affected less. The chromaffin granule RAP/KAP resembles rat aminopeptidase B, which specifically removes basic residues from NH_2-termini of peptides.

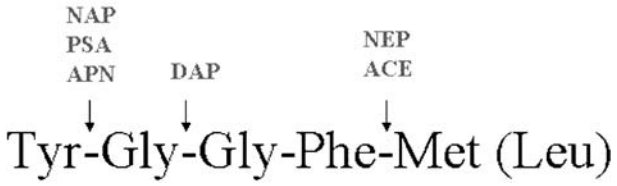
Recently we found that KAP is different from RAP (Hui and Hui, unpublished observations). KAP and RAP can be physically separated by FPLC with Mono Q where KAP is eluted with less NaCl. The molecular weight of KAP was determined by gel-filtration to be 62,000 daltons, which is 10,000 more than RAP. Using aminoacyl β-naphthylamides (βNA) as substrate, KAP prefers lysine five times more than arginine, but RAP prefers arginine one time more than lysine. NaCl can activate KAP. RAP is inhibited by thiol blocking agents and is most sensitive to arphamenine B. In contrast, KAP is most sensitive to bestatin.

Leukocyte-derived RAP is a 960-amino-acid protein with significant homology to placental leucine aminopeptidase (pLAP) and adipocyte-derived leucine aminopeptidase (aLAP/ERAP1) (Tanioka et al., 2003; Bolumar et al., 2003). It contains the HEXXH(X)18E zinc-binding motif, a characteristic of the M1 family of zinc metallopeptidases. It is a subfamily with pLAP and aLAP/ERAP1 in the M1 family. L-RAP located in the lumenal side of the endoplasmic reticulum and has a preference for arginine with synthetic substrates. Its substrate specificity is restricted. It cleaves angiotensin III, kallidin, and the N-terminal extended precursors of MHC class I-presented antigenic peptides.

5 Enkephalin-Degrading Enzymes

Study of neurotransmitter enzymes has made a substantial contribution to our understanding of synaptic biochemistry. Some of their inhibitors turn out to be valuable therapeutics. Enkephalin binds to the opiate receptors on the neuronal membrane to trigger intracellular functions. Its action is apparently terminated by synaptic degradation, since no uptake, internalization mechanism, *N*-acetylation, *O*-sulfation, phosphorylation, or glycosylation has been found (Patey et al., 1981; Goodman et al., 1983, Schwartz et al., 1985). In the CNS, several sets of peptidases are capable of cleaving enkephalins (Tye-Gly-Gly-Phe-Met [Leu]) at different sites: aminopeptidases (APN, PSA, neuron-specific aminopeptidase (NAP)) at the Tyr-Gly amide bond, dipeptidyl aminopeptidase (DAP) at the Gly-Gly bond, carboxylpeptidase at the Phe-Met bond, and ACE and NEP, both at the Gly-Phe bond (*Figure 21-3*).

Figure 21-3
Degradation of enkephalin by peptidases at different sites. NAP, neuron-specific aminopeptidase; APN, aminopeptidase N; PSA, puromycin-sensitive aminopeptidase; DAP, dipeptidyl aminopeptidase; ACE, angiotensin-converting endopeptidase; NEP, endopeptidase 24.11



5.1 Endopeptidase 24.11

Endopeptidase 24.11 (NEP 24.11) (neprilysin; EC 3.4.24.11) participates in the postsecretory processing and metabolism of synaptically released neuropeptides (Turner et al., 1996). Inhibition of NEP by thiorphan (Patey et al., 1981) induces analgesia (Llorens et al., 1980), but it has been controversial whether it is neuronal (Horsthemke et al., 1983; Barnes et al., 1988). NEP 24.11 is a cell-surface zinc metallopeptidase with specificity directed toward the hydrolysis of peptide bonds at the N-terminal side of amino acids of hydrophobic residues: Phe, Tyr, Leu, and Trp (Turner and Barnes, 1995; Hooper and Turner, 1988). NEP 24.11 is sensitive to the chelating agents EDTA and 1,10-phenanthroline. It is highly susceptible to phosphoramidon ($K_i = 2$ nM) and thiorphan. The enzyme is distributed widely and in the CNS serves to inactivate enkephalins, tachykinins, and somatostatin (Turner et al., 1996). In vitro, the enzyme hydrolyzes neutrophil chemotactic peptide, atrial natriuretic peptides, vasoactive intestinal polypeptide, and calcitonin gene-related peptide. NEP 24.11 was found to be co-localized on the plasma membrane of substance P-rich boutons in the substantia nigra, suggesting its probable role as a neuropeptidase (Turner et al., 2001). Molecular cloning of NEP 24.11 revealed its identity with the common acute lymphoblastic leukemia antigen (CD10) (Howell et al., 1991), implying its primary role in the immune system. Nonetheless, this underscores the similarities of signal mechanisms in, and interaction between, the immune and nervous systems.

5.2 Angiotensin-Converting Enzyme

ACE (EC 3.4.15.1, peptidyl dipeptidase A), a peptidyl carboxypeptidase, is responsible for the conversion of angiotensin I to the potent vasoconstrictor angiotensin II and for the degradation of bradykinin (Chai and Mendelsohn, 1995). It hydrolyzes a range of peptides including opioid peptides, neurotensin, bombesin, tachykinins, and luteinizing hormone-releasing hormone (Chai and Mendelsohn, 1995). ACE is a membrane-bound ectoenzyme of 146 kDa that consists of a large extracellular domain with a transmembrane anchor and a small intracellular carboxyl terminus (Soubrier et al., 1988). Its extracellular domain contains two regions of high sequence homology, each containing an active site sequence. Although the two sites are catalytically active, the C-terminal one is more responsible for most of the hydrolysis of angiotensin I and for the binding of inhibitors (Wei et al., 1991; Perich et al., 1992). The N-terminal site has structural constraints that limit the binding of some substrates and drugs (Wei et al., 1992). In human beings, ACE has two forms and two functions. Testicular ACE (tACE) has only one active site and has a sequence identical to the C terminus of the somatic ACE. Somatic ACE exists in most cells, and tACE, which is half the size of somatic ACE, is found only in the testis (Ehlers and Riordan, 1991; Perich et al., 1992).

The enzyme is composed of α-helices for the most part, and incorporates a zinc ion and two chloride ions (Schullenk and Wilson, 1988). Chloride ions activate tACE, and they interact with the substrate as well. However, the structure of tACE places the chloride ions outside the active site. Therefore, they play an

indirect role in substrate activation (Wei et al., 1991). The zinc ion lies in the active site and interacts directly with inhibitor lisinopril (Wei et al., 1992). The structure of tACE shows it to be similar to ACE forms found in other species, and in other zinc-containing metallopeptidases as well.

Testicular ACE is roughly an ellipsoid in shape, divided into two subdomains by a central groove. The active site is toward the bottom of the groove and is capped by an N-terminal lid, which prevents large molecules from fitting into the active site. Somatic ACE consists of two parts (called the N- and C-domains), each with a different function. The newly described tACE structure, which is identical to the C-domain structure of somatic ACE, now serves as a template for the search of specific domain-selective ACE inhibitors (Wei et al., 1991).

5.3 Aminopeptidases

The liberation of Tyr is the major mode of enkephalin inactivation with intact-cell and cell-free preparations (Hui and Lajtha, 1983; Lentzen and Palenker, 1983). The aminopeptidase inhibitors exert a dose-dependent, naloxone-reversible, analgesic effect when administered to mice (Zhang et al., 1982; De La Baume et al., 1983; Herman et al., 1985). In addition, aminopeptidases that are sensitive to puromycin underlie a variety of specialized CNS functions: memory (Herman et al., 1985), amnesia (Eisenstein et al., 1983), apoptosis (Tobler et al., 1997), and schizophrenia (Hui et al., 1995). Brain aminopeptidase activity can be simply classified into PSA (gene)-dependent; PSA-independent; but puromycin-sensitive, and puromycin-insensitive. Earlier, it had been suggested that enkephalin binding to the opiate receptor was coupled to subsequent aminopeptidase degradation (Knight and Klee, 1978). APN was copurified with opiate receptors (Hui et al., 1985), though APN was later found localized exclusively in the blood microvessels (Solhonne et al., 1987; McLellan et al., 1988).

More than 20 aminopeptidases (EC 3.4.11) have the capability to liberate various amino acid residues from the NH_2-termini of peptide substrates. These ubiquitous enzymes are mostly concentrated in brain and kidney. They are classified according to the preference for the NH_2-terminal amino acid of the substrates, their location, the susceptibility to inhibitors, the metal ion content, the residues that link the metal to the enzyme, and the pH for maximal activity. These enzymes are considered to have their original structures, substrate specificities, and specific locations depending on their physiological roles.

5.3.1 Aminopeptidase N

Leu-Enk is readily hydrolyzed to free tyrosine and Gly-Gly-Phe-Leu by APN on the surface of microglia (Lucius et al., 1995). APN activity in microglia is higher than in rat peripheral monocytes and macrophages. Sequence data has shown its strong homology to CD13, a 150-kDa cell-surface glycoprotein (Look et al., 1989; Razak and Newland, 1992). Its nucleotide sequence predicts a 967-amino-acid integral membrane protein with a single, 24-amino-acid hydrophobic segment near the amino terminus. Amino-terminal protein sequence analysis of CD13 molecules indicated that the hydrophobic segment is not cleaved, but rather serves both as a signal for membrane insertion and as a stable membrane-spanning segment. The remainder of the molecule consists of a large extracellular carboxy-terminal domain, which contains a pentapeptide consensus sequence characteristic of members of the zinc-binding metalloprotease superfamily. APN, a membrane-bound glycoprotein, is involved in the metabolism of regulatory peptides by diverse cell types, including small intestinal and renal tubular epithelial cells, macrophages, granulocytes, and synaptic membranes prepared from cells of the CNS (Look et al., 1989).

5.3.2 Puromycin-Sensitive Aminopeptidase

The concentration of PSA is highest in the brain (McLellan et al., 1988), 100-fold that of APN (Solhonne et al., 1987). Eighty percent of the brain PSA is cytosolic. Using Ala-β-naphthylamide as substrate for rat brain PSA, V_{max} was shown to be pH independent over the range of 5.5–9.0, while the K_m exhibited a pK_a

of 7.7. This latter value corresponds to the pK_a of the amino group of the substrate. Using X-Ala and X-Leu to examine the specificity of the P1 site, it was found that Arg and Lys exhibit the highest affinity, followed by Met, Val, Leu, Trp, and Phe, which bind congruently 5- to 20-fold less. Although the K_m varied more than 20-fold within this series, V_{max} showed considerably less variation. Significantly weaker binding was observed with P1 Gly, Ala, Ser, or Pro, with no binding detectable with P1 Glu. The presence of P′1 Leu compared to P′1 Ala results in an approximate tenfold decrease in K_m with little change in V_{max}. The effect of varying P′1 residues was examined with the series Leu-X. In this case, basic and hydrophobic amino acids, with the exception of Val, all exhibit nearly the same K_m. The binding of Arg-Arg and Lys-Lys showed the same K_m as obtained for Arg-Leu or Lys-Leu, respectively. When Leu-Ser-Phe was compared with Leu-Ser, the P′2 residue led to a 100-fold decrease in K_m and slightly less than a fivefold increase in V_{max}. In contrast, the addition of a P′2 Met to Leu-Trp resulted in only a threefold decrease in K_m and a threefold increase in V_{max}. PSA prefers a basic or hydrophobic residue in the P1 and P′1 sites and the subsite–subsite interactions primarily affect binding (Johnson and Hersh, 1990).

Brain has soluble and membrane-associated forms of PSA. All tissues contained significant levels of the soluble enzyme form, with this enzyme accounting for greater than 90% of the arylamidase activity in brain, heart, and skeletal muscle. In contrast to the results obtained with the soluble enzyme form, brain was the only tissue found to contain the membrane-associated enzyme form. The brain membrane-associated enzyme could be distinguished from the membrane-associated aminopeptidase activity in other tissues on the basis of its sensitivity to inhibition by puromycin (McLellan et al., 1988).

Each membrane-bound and cytosolic PSA is composed of a single polypeptide of a molecular mass of 100 kDa. The anti-soluble aminopeptidase antiserum reacts with both enzyme forms on immunoblots and inhibits both with nearly identical inhibition curves. The isoelectric points (pI = 5.0) of the two forms were shown to be identical. N-terminal sequencing yielded a common sequence (P-E-K-R-P-F-E-R-L-P-T-E-V-S-P-I-N-Y) for the two enzyme forms, and peptide mapping yielded 26 peptides that also appeared to be identical between the two enzyme forms. The membrane form of the PSA is identical to the soluble enzyme; it associates with the membrane by interactions with other integral membrane proteins (Dyer et al., 1990).

A membrane-bound aminopeptidase was purified from the rat brain (Hui et al., 1983c). The enzyme was extracted with 1% Triton X-100 and purified by chromatography successively on DEAE-Sepharose CL-6B, Bio-Gel HTP, and Sephadex G-200 columns. The purified enzyme showed one band on disc gel electrophoresis and two bands on sodium dodecyl sulfate (SDS) electrophoresis with molecular weights of 62,000 and 66,000. The aminopeptidase has a pH optimum of 7.0, a K_m of 0.28 mM, and a V_{max} of 45 μmol/mg of protein/min for Met-Enk. It releases tyrosine from Met-Enk, but it does not split the byproduct, GlyGlyPheMet. It hydrolyzes neutral and basic aminoacyl βNA, but not γ- or β-endorphin, or dynorphin. The enzyme is inhibited by the specific aminopeptidase inhibitors amastatin, bestatin, and bestatin-Gly. Its subcellular localization, substrate specificity, pH optimum, and molecular weight distinguish it from leucine aminopeptidase, aminopeptidase A, aminopeptidase B, aminopeptidase M, and the soluble aminopeptidase for enkephalin degradation.

PSA is inhibited by enkephalin-containing polypeptides derived from proenkephalin A, proenkephalin B, and proopiomelanocortin (Hui et al., 1983a). Of the peptides, Arg^0-Met-Enk was the most potent inhibitor with an IC_{50} of about 0.6 μM; it was more effective than bestatin. This inhibition was partly due to substrate competition. PSA hydrolyzed Arg^0-Met-Enk to Arg, Tyr, and Gly-Gly-Phe-Met in a substrate-inhibited manner. The hexapeptide also inhibited the breakdown of Arg- and Tyr-βNA by the membrane PSA. Since Arg^0-Met-Enk did not inhibit leucine aminopeptidase, it was a more selective inhibitor than bestatin of Met-Enk breakdown by aminopeptidases. Arg^0-Met-Enk also inhibited enkephalin breakdown by synaptosomal plasma membranes but not by brain slices.

PSA Gene The human PSA gene is composed of 23 exons and 22 introns and spans approximately 40 kb of chromosome 17 at the interval 17q12–21 (Thompson et al., 1999). The gene (NPEPPS) was physically mapped to q21.2→q21.32 of chromosome 17 using fluorescence in situ hybridization (Bauer et al., 2001). PSA is 27–40% homologous to several known Zn^{++}-binding aminopeptidases including APN

(Constam et al., 1995). An analysis of the 5′-end of the human PSA transcript reveals that the translational start site corresponds to nt 210 of the human PSA cDNA. A comparison of the exon/exon boundaries of the human PSA gene with those of the human APN gene shows little conservation, suggesting that the two genes, which are closely related in protein sequence, diverged early during evolution (Thompson et al., 1999). The tissue distribution of PSA is a polymorphism within the coding region and the complete 3′-UTR (Bauer et al., 2001).

Putative catalytic residues of PSA, Cys146, Glu338, and Lys396, were mutated and the resultant mutant enzymes ApPS C146S exhibited normal catalytic activity (Thompson and Hersh, 2003). ApPS E338A exhibited decreased substrate binding, and ApPS K396I exhibited decreases in both substrate binding and catalysis. ApPS K396I and ApPS Y394F were analyzed with respect to transition-state inhibitor binding. No effect was seen with the K396I mutation, but ApPS Y394F exhibited a 3.3-fold lower affinity for RB-3014, a transition-state inhibitor. Thus, Tyr394 is involved in transition-state stabilization.

Conversion of glutamate 309 to glutamine resulted in a 5,000- to 15,000-fold reduction in catalytic activity (Thompson et al., 2003). Conversion of this residue to alanine caused a 25,000- to 100,000-fold decrease in activity, while the glutamate to valine mutation was the most dramatic, reducing catalytic activity 300,000- to 500,000-fold. In contrast to the dramatic effect on catalysis, all three mutations produced relatively small (1.5- to 4-fold) effects on substrate binding affinity. Mutation of a conserved tyrosine, Y394, to phenylalanine resulted in a 1,000-fold decrease in k_{cat}, with little effect on binding. Glutamate 309 acts as a general acid/base catalyst. Its mutation E309V converts the enzyme into an inactive binding protein. The effect of mutating tyrosine 394 is consistent with involvement of this residue in transition-state stabilization (Thompson et al., 2003).

Homozygous goku mice generated by gene-trap mutation showed dwarfism, a marked increase in anxiety, and an analgesic effect (Osada et al., 1999). The function of PSA is disrupted in transcriptional arrest of the PSA gene and a drastic decrease of aminopeptidase activity. Because the PSA gene is strongly expressed in the brain, especially in the striatum and hippocampus, the PSA gene is required for normal growth and for behavior associated with anxiety and pain (Osada et al., 1999).

PSA Functions In patients with schizophrenia, prefrontal cingulate and frontal cortices, thalamus, hippocampus, hypothalamus, and outer globus pallidus contained significantly less PSA as quantified by Western blot analysis than the corresponding areas from control subjects (Hui et al., 1995). Aminopeptidases may play an important role in the processes of tolerance and withdrawal associated with morphine administration. Increased activity of PSA was found in the brain cortex of heroin addicts in humans (Larrinaga et al., 2005). In rats treated with morphine, the activity of soluble PSA was found to be higher in the frontal cortex (Irazusta et al., 2003). In contrast, rats experiencing withdrawal symptoms presented decreased levels of aminopeptidase activity in certain brain areas. The activity of APN in the hippocampus and soluble PSA in the frontal cortex were lower in rats experiencing naloxone-precipitated withdrawal symptoms. However, the activity of the aminopeptidases in vitro was unaltered by incubation with morphine, suggesting an indirect action of this opioid upon the aminopeptidases.

PSA participates in proteolytic events essential for cell growth and viability (Constam et al., 1995). Proteolysis involves a cascade of enzymes including 26S proteasome. PSA is localized to the cytoplasm and to the nucleus and is associated with microtubules of the spindle apparatus during mitosis. Puromycin and bestatin both arrested the cell cycle, leading to an accumulation of cells in the G2/M phase, and ultimately induced cells to undergo apoptosis at concentrations that inhibit PSA.

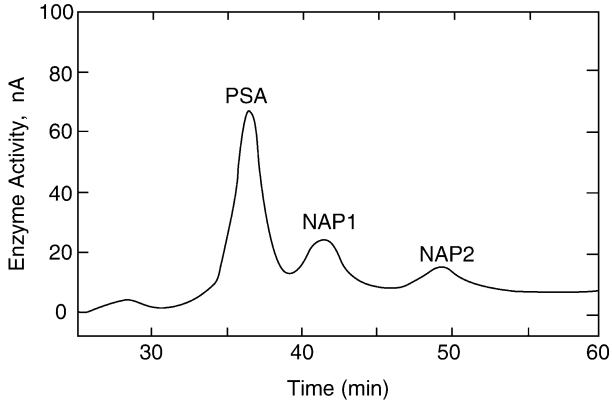
PSA in Development PSA activity increases twofold in the synaptosomal and mitochondrial fractions during the period of axonal and dendritic growth. This enzyme also has significant age-related changes in the nuclear fraction. Significant developmental changes of APN are found only in the myelinic and microsomal fractions and they are less significant than those found for PSA (de Gandarias et al., 1999). PSA is mainly transported by anterograde axonal flow and plays a role in the metabolism of neuropeptides in nerve terminals or synaptic clefts (Yamamoto et al., 2002).

5.3.3 Neuron-Specific Aminopeptidases

Strong aminopeptidase activity has been found in the CNS neuronal cell bodies by using histochemistry and in situ hybridization (Constam et al., 1995). Synapses, channels of communication between neurons, are sites of storage of information that is encoded by genes and by experience—memories. The nerve terminal is a site of integration where the signals it receives can modify the secretory response to an action potential. The release of neuropeptides (neurotransmitters) is the final response of a nerve to the excitatory and inhibitory inputs that converge upon it. Because it is the site of final signal output, the nerve terminal is an especially sensitive and critical point of control for neurons. To understand how these modifications occur by studying the neuropeptide metabolism in nerve terminals presents a neural anchor to decipher how the brain works. If anatomical distribution (specific location) associated with limited substrate specificity does constitute a "functional" specificity, it would be interesting to find an aminopeptidase specific for the CNS synapse or neuron (Shaw and cook 1978). Knowledge of specific enkephalin-inactivating aminopeptidases will facilitate our understanding on how the neuropeptide functions at the synapses.

The widely distributed aminopeptidases in brain possess overlapping substrate specificity. In order to quantify accurately an aminopeptidase in biological samples, a post-column continuous-flow aminopeptidase detector was developed (Hui and Hui, 1996). Its conjugation with an FPLC provides a fast, sensitive, specific, and reliable method for brain aminopeptidase screening and quantitation. An enzyme of interest is separated from interfering peptidases, activators, and endogenous inhibitors in the samples (Hui and Hui, 1996). Using leucine β-naphthylamide (Leu βNA) as substrate, two novel neuron-specific enkephalin-degrading aminopeptidases (NAP1 and NAP2) are separated from PSA and from each other with a FPLC Mono Q (*Figure 21-4*). They are present only in the mammalian CNS and not in peripheral tissues, serum, or sciatic nerve (*Table 21-1*). The two aminopeptidases are present exclusively in neurons, not in other nerve cells and neuroblastomas. The regional distribution of NAP1 and NAP2 is different. The highest NAP1 was found in the hippocampus, whereas the highest NAP2 was in the colliculus. Hypothalamus has the highest ratio of NAP1:NAP2. Both aminopeptidases are enriched in the synaptosomes with NAP1 > NAP2.

Figure 21-4
Zymogram of aminopeptidases by FPLC-aminopeptidase analyzer



In rat brains, 85% of NAP1 is soluble and the rest is associated with membranes despite their relationship not being established (Hui et al., 1998). It is higher in the synaptosomes, of which the lysate has a specific activity 350% of that of the cytosol (S_2) fraction, and 200% that of the membrane fraction. In rat cerebrocortical neuron cultures, its activity is 33% of the total aminopeptidase activity; in cerebellar granule cells, it is 12%, and it is absent in astrocytes. NAP1 cannot be found in glioma C_6 and neuroblastoma

Table 21-1
Distribution of NAPs and PSA in different rat tissues

	Activity (units*)		
	NAP1	NAP2	PSA
CNS			
Whole brain	82	40	400
Brian regions: cortex, striatum, corpus callosum, hypothalamus, midbrain, hippocampus, cerebellum, medulla oblongata, superior and inferior colliculus	40–123	14–58	140–430
Spinal cord (cervical, sacral)	32–40	16–30	141–165
Olfactory bulb	21	7.8	230
Other tissues			
Kidney, spleen, heart, intestinal mucosa, skeletal muscle, testis, liver, adrenal gland, pituitary, sciatic nerve, retina	n.d.	n.d.	34–250
Serum	n.d	n.d.	2.5

50 μl of the S_3 fraction of 2.5 mg of tissue or serum was submitted to the automatic FPLC-aminopeptidase analyzer
*arbitrary fluorescence units
n.d.: not detectable

SK-N-SH cells. Its predominance in brain synaptosomes suggests that NAP1 plays a significant role in neurotransmission and synaptic differentiation.

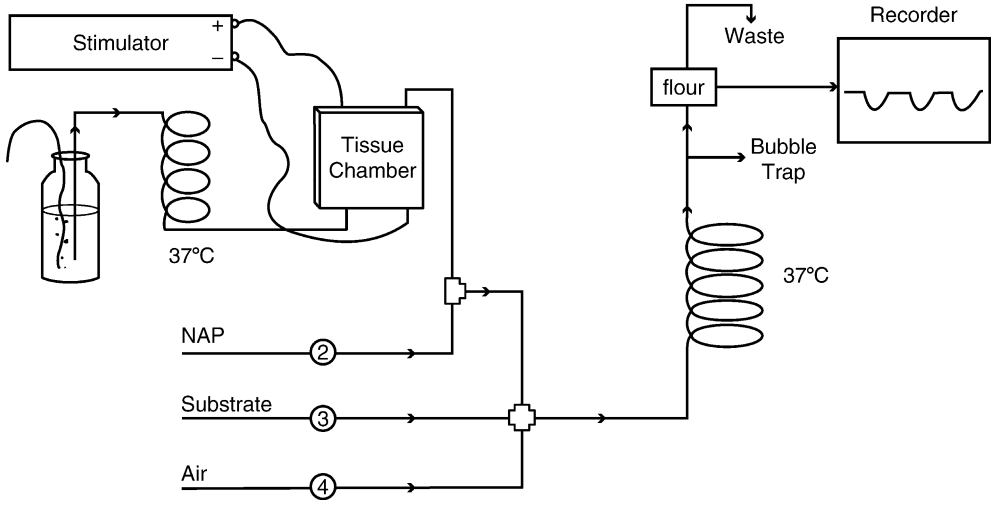
Purification and Characterization of NAP NAP1 was purified from rat brain to homogeneity by ammonium sulfate fractionation, followed by column chromatography, successively on phenyl-Sepharose, Sephadex G-200, and twice on Mono Q FPLC (Hui et al., 1998). The purified single-chain enzyme was estimated to be 110 kDa. It has a pI of 5.25 and a pH optimum of 7.0. Only Mg^{++} restores the activity of the apoenzyme. The neutral aminopeptidase hydrolyzes βNA of amino acids with aliphatic, polar uncharged, positively charged, or aromatic side chains. It has a *K*m of 95 μM and a k_{cat} of 7.8 s^{-1} on Met-enk, releasing only the N-terminal tyrosine. The thiol-dependent metalloenzyme is most sensitive to amastatin inhibition with a K_i of 0.04 μM and is the aminopeptidase most sensitive to puromycin. Its properties are different from those of the ubiquitous PSA obtained from the same enzyme preparation. The blocked N terminus, substrate and inhibitor specificity, hydrolytic coefficiency, metal effects, pI, molecular weight, and catalytic site show that this enzyme is distinct from all other known aminopeptidases (Hui et al., 1998).

Using an anti-PSA IgG to screen a rat brain cDNA expression library a 1,561-bp cDNA was isolated. Probing with this cDNA, we cloned a candidate 1,404-bp cDNA (63.2% identity to mouse PSA) encoding the N-terminal section of neural aminopeptidase. The nucleotide segment position at 875–1,404 is homologous to position 1,087–1,613 of PSA (96.4% identity), containing a sequence encoding a divalent metal-binding motif, $HEXXH(X)_{18}E$, of aminopeptidases (Shannon et al., 1989). The sequence at the 5′-end 1–874, with an identity 44.9% to PSA, is novel. It is void of the sequence encoding a universal N-terminal PENKRPFERLPTEVSPINY of PSA (Dyer et al., 1990; Constam et al., 1995; Tobler et al., 1997). The blocked N-terminal residue, though, waits to be identified. The cloning data imply that the purified enzyme is a unique aminopeptidase and a possible member of the PSA superfamily.

NAP1 During Neuron Growth NAP1 was found in the rat hippocampus in all ages (Hui and Hui, 2003). It was lower in immature rat; the 19th embryonic-day fetus contained the least. NAP1 increased steeply during the prenatal through the early post-natal period, nine-fold by the first month. The rate of increase diminished subsequently, increasing 20% in the second month and 13% in the third. The age-dependent increase in NAP1 activity was parallel to its protein expression. The specific hydrolytic activity/NAP1 antigenicity in newborn, 15-day-old, and 30-day-old were 1.00, 0.88, and 1.00, respectively. Its growth profile was distinct from that of PSA. A similar difference between them was also found in the developing primary cerebellar granule cells. Puromycin (1–5 μM) blocked neurite outgrowth and caused apoptosis by non-antibiotic effects.

NAP1 Is a Putative Synaptic Enzyme If NAP1 is a synaptic enzyme, it could be released by electrical stimulation or inhibited by synaptic factors. Using a modified aminopeptidase analyzer (*Figure 21-5*), we found a NAP1 inhibitor released by electric stimulation from slices of brain, but not in kidney or liver. Five hippocampal (or other tissue) slices (1 mg, 450 µm thick) were rinsed, equilibrated, and perfused with Krebs solution at 0.1 ml/min in a microvolume (0.5 ml) glass chamber. The perfusion effluent was mixed with NAP1 (*Figure 21-5*) at a flow rate of 0.05 ml/min, and joined with Leu βNA (0.15 ml/min). The enzyme reaction proceeded in a delaying coil (37°C) for 3 min before the detection of the released

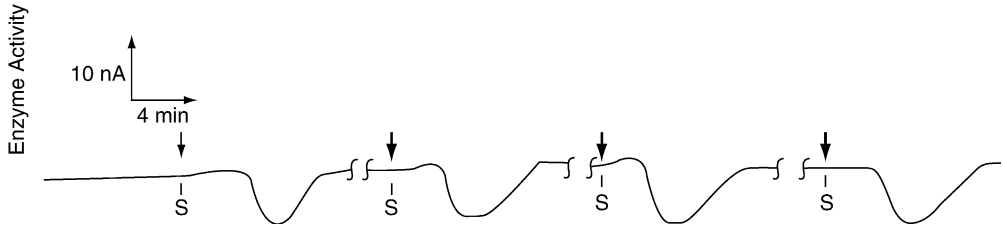
Figure 21-5
Flow diagram of the automated analyzer for NAP inhibitor released from tissue slices



β-naphthylamide by fluorescence. The inhibitor activity was monitored continuously by measuring the decrease of free β-naphthylamide.

The inhibitor was released by electric stimulation at optimal conditions for neuropeptide release (20V, 20 Hz, 20ms, 300 shocks) (Milusheva et al., 1992). *Figure 21-6* shows a typical tracing of the NAP1 activity inhibited by electric stimuli on brain slices. With each stimulus, a 4-min inhibitory effect, independent of its intensity (10–40 V) and frequency (10–40 Hz), was observed. The potency of the released inhibitor by a stimulus was equivalent to 1 nmol of bestatin. The potent inhibition was not due to substrate (100 µM Leu βNA) competition. It was due neither to serotonin, catecholamines, acetylcholine, amino acids, nor Met-Enk, which had no inhibitory effect on NAP1. The perfusate showed no effect

Figure 21-6
Synaptic aminopeptidase activity inhibited by factors released by electric stimulation (S) from hippocampal slices



on radioreceptor bindings at δ, μ, or κ sites indicating that the inhibition was not by enkephalins or enkephalin-like peptides. The ninhydrin-positive inhibitor could be retained on a reverse-phase C_{18} Sep-Pak column and eluted by methanol. Its activity disappeared after treatment with 6N HCl at 110°C for 16 hr. Our data indicate that the small-peptide inhibitor was vesiculated in synapses. The inhibitor(s) was estimated to be 1,000 daltons by Sephadex G-10.

6 Other Peptidases

6.1 Tripeptidyl Peptidase (TPP)

Protein degradation is essential for the life and death of every cell. Proteins are broken down to their constitutive amino acids by a succession of peptidases, both in lysosomes and in the cytosol. TPP-I and TPP-II are enzymes that can "count to three" and release N-terminal tripeptides from oligopeptides generated by different endopeptidases. The tripeptides are then degraded by other exopeptidases to release amino acids and dipeptides.

The molecular weight of TPP-I was calculated to be 280,000 and 290,000 by non-denaturing polyacrylamide electrophoresis (PAGE) and gel filtration respectively, and to be 43,000 and 46,000 on SDS-PAGE in the absence and presence of β-mercaptoethanal, respectively. The enzyme is composed of six identical subunits. Human TPP-I has five potential *N*-glycosylation sites at Asn residues 210, 222, 286, 313, and 443. A dual role of oligosaccharide at Asn-286 in folding and lysosomal targeting could contribute to the unusual, but cell type-dependent, fate of misfolded TPP-I conformer and represents the molecular basis of the disease process in subjects with naturally occurring missense mutation at Asn-286 (Wujek et al., 2004). Although TPP-I zymogen is capable of auto-activation in vitro, a serine protease that is sensitive to AEBSF participates in the processing of the proenzyme to the mature, active form in vivo (Golabek et al., 2003).

TPP-I is inhibited by PCMBS, DFP, and $HgCl_2$. It is an exo-type serine peptidase that is regulated by SH reagent. TPP-I releases the tripeptide Arg-Val-Tyr from angiotensin III more rapidly than from Ala-Ala-Phe-MCA, and also releases Gly-Asn-Leu from neuromedin B with the same velocity as from Ala-Ala-Phe-MCA (Du et al., 2001). TPP-I degrades small peptides with an extended N-terminal domain, but not structured peptides. In general, this cut off occurs between masses of 4.5 and 6 kDa. Reference to the structures of other peptidases suggests a mechanism for this size selectivity (Bernardini, Warburton, 2001). The order of TPP-I mRNA expression is as follows: kidney > or = liver > heart > brain > lung > spleen >> skeletal muscle and testis (Du et al., 2001).

TPP-I is largely responsible for the degradation of sulfated cholecystokinin-8 (CCK-8S), which enters the cell by receptor-mediated endocytosis through the cell surface, whereas TPP-II is responsible for regulating extracellular CCK-8S levels (Warburton and Bernardini, 2002; Breslin et al., 2003). TPP-II (EC 3.4.14.10) is a serine peptidase apparently involved in the inactivation of cholecystokinin octapeptide (Rose et al., 1996). TPP-II was mostly detected in neurons and also in ependymal cells and choroid plexuses, localizations consistent with a possible participation of the peptidase in the inactivation of cholecystokinin circulating in the CSF. It was also detected at the ultra-structural level in the cerebral cortex and hypothalamus. The peptidase mainly associated with the cytoplasm of neuronal somata and dendrites, often in the vicinity of reticulum cisternae, Golgi apparatus, or vesicles, and with the inner side of the dendritic plasma membrane (Facchinetti et al., 1999).

6.2 *N*-Acetylated α-Linked Acidic Dipeptidase (NAALADase)

NAAG is catabolized to *N*-acetylaspartate and glutamate primarily by glutamate carboxypeptidase II (NAALADase), which is expressed on the extracellular surface of astrocytes. NAALADase has been cloned from human brain (Luthi-Carter et al., 1998a), rat brain cDNA (Luthi-Carter et al., 1998b), and rat hippocampal cDNA library (Bzdega et al., 1997). NAALADase inhibition prevents cocaine-kindled seizures (Witkin et al., 2002; Rojas et al., 2003). NAALADase inhibitor 2-(phosphonomethyl)pentanedioic acid (2-PMPA) produced dose-dependent protection (10–100 mg/kg) against both the development of seizure

kindling and the occurrence of seizures during the kindling process without observable behavioral side effects (Thomas et al., 2001). Its inhibition also protects motor neurons from death in familial amyotrophic lateral sclerosis models (Ghadge et al., 2003).

6.3 Bestatin-Insensitive Aminopeptidase

Bestatin-insensitive aminopeptidase (BIA), a thiol aminopeptidase, was resistant to puromycin and bestatin inhibition (Neidle et al., unpublished observations). It was purified 744-fold to homogeneity from rat brain cytosol. BIA was separated from aminopeptidase B (APB) and PSA and isolated by DEAE chromatography. It has a molecular weight of 280 kDa and is composed of six 45-kDa subunits. It releases Tyr from enkephalin, but not from dipeptides. The enzyme is inhibited by heavy metal ions and thiol-binding reagents including leupeptin and E-64. The aminopeptidase can be readily differentiated from enzymes with partially overlapping specificity such as leucine aminopeptidase; aminopeptidases A, B, and N; or PSA by its resistance to inhibition by bestatin and to metal-chelating agents.

7 Therapeutic Uses of Neuropeptidase Inhibitors

ACE inhibition reduces morbidity and mortality in patients with hypertension, diabetes mellitus, atherosclerosis, heart failure, and nephropathy. ACE inhibitors could be useful in the management of a wide range of cardiovascular pathologies (Stanton, 2003). Patients at risk of cardiovascular events but having normal left ventricular function demonstrate clear benefits of an ACE inhibitor. Patients with chronic left ventricular dysfunction or postmyocardial infarction show reduction of ischemic events (Stanton, 2003). The use of ACE inhibitors to treat hypertension indicates that neuropeptidases do make good drug targets (Docherty et al., 2003). With genome research yielding many possible new drug targets, neuropeptidases that are causally responsible for disease processes might therefore make better targets, especially if it leads to the development of drugs that can be administered orally. Besides inhibitors, antibodies for peptidases can also be useful therapeutics.

7.1 Synthetic Anti-Enkephalinases

Inhibition of aminopeptidases and NEP with bestatin and thiorphan exerts strong and long-lasting analgesia in terminal cancer patients; a similar effect was found with two other active inhibitors, acetorphan and carbaphethiol (Noble and Roques, 1992). Kelatorphan, a mixed inhibitor of PSA, APN, NEP, and DAP, is as active as morphine in many tests (Thorsett and Wyvratt, 1987). Another mixed inhibitor, phelorphan, affects the morphine withdrawal syndrome (Van Amsterdam et al., 1987). SCH-34826, an orally active NEP inhibitor that produces analgesia in mice, does not alter gastrointestinal movement or respiratory function (Chipkin et al., 1988). In addition, it is inactive in tests measuring potential antianxiety, antidepression, and antipsychotic effects demonstrating that the inhibitors have more specific pharmacological effects than the opiates. Since the enkephalinergic pathway of analgesia is unique, it is feasible that the NAP inhibitor may be a non-addictive analgesic without psychotropic side effects.

7.2 Synthetic Anti-Aminopeptidase

The reversible and irreversible inhibitors of aminopeptidases are designed to be small, stable, of higher affinity, and blood–brain barrier permeable (Tieku and Hooper, 1992). The reversible mercaptoethylamine with a hydrophobic side chain (2-amino-4-methyl-1-pentanethiol) is a potent dentate inhibitor of APN (Pickering et al., 1985). The reversible carbaphethiol, a parenterally active form of phethiol, was also

developed. The structural features necessary for interaction of mercaptoamines with aminopeptidases are specific. Aminopeptidases contain subsites that contribute to specific substrate binding: the "*R*" group of the N-terminal amino acid and the "*R′*" group of the peptide chain (Bryce and Rabin, 1964; Van Amsterdam et al., 1987). Irreversible inhibitors resistant to metabolic inactivation are of greater value for in vivo studies. Diazomethyl ketone, which specifically attacks the active site cysteine residue (Fujiwara et al., 1982), has been shown to be an extraordinarily potent inhibitor of pyroglutamyl aminopeptidase (Wilk et al., 1985). It is highly effective in in vivo studies and has low toxicity. By substituting the functional groups of these synthetic compounds, potent and selective inhibitors for ENK inactivation may become available.

7.3 Endogenous Peptidase Inhibitors

Naturally available inhibitors frequently have higher selectivity and potency than synthetic ones. Snake venom peptide inhibitors played a crucial role in establishing the clinical value of Capoten in hypertension. Microbial inhibitors were shown to be highly active for a spectrum of peptidases and proteinases (Wilk et al., 1985). The discovery of phosphoramidon as a NEP inhibitor has been very helpful in developing novel inhibitors (Kenny, 1977). It is of interest that all the above-mentioned synthetic and natural inhibitors are modified peptides (Umezawa, 1972; Umezawa and Aoyagi, 1977). Recently one of the most potent ACE inhibitors, designated converstatin, was characterized to be a tryptic fragment of a plasma protein with a K_i value in the picomolar range (Okuda and Arakawa, 1985). Peptide inhibitors for enkephalinases would be superior because of their non-toxicity (Rapaka, 1986). They can be metabolized to amino acids that do not cause liver and kidney damage as the opiates do. These inhibitors of cerebral peptidases cannot cross the placental barrier, which is an additional advantage for their use as analgesics for pregnant women.

7.4 Endogenous Anti-Aminopeptidases

Arg^0-Met-Enk was purified and characterized as an endogenous PSA inhibitor in calf striatum (Hui et al., 1994). It is likely that in addition to their possible role as opioids, the enkephalin-containing polypeptides may be regulators of enkephalin levels (Hui et al., 1982, 1983a). It is the most potent one and is stronger than bestatin and puromycin (Hui et al., 1983b, 1994). The inhibitor can be inactivated by a specific enzyme, DAP-V, specifically releasing the N-terminal Arg-Tyr (Hui, 1988). Although NAP is sensitive to small-peptide inhibition in vitro, its substrate and inhibitor specificity is different from that of PSA (Hui et al., 1998).

7.5 Inactivation of Arg^0-Met-Enk

Arg^0-Met-enk can be degraded by a new type of DAP. The enzyme was purified about 2,100-fold with 7% recovery from the rat brain membrane by column chromatography, successively on Cellux D, Arg-Tyr-AH-Sepharose 4B, hydroxylapatite, and Sephadex G-75, after the membrane was solubilized with Nonidet P40 (Hui, 1988). The enzyme activity was assayed by HPLC using Arg^0-Met-Enk as substrate in the presence of bestatin, thiorphan, and captopril. In SDS-PAGE, the purified enzyme was apparently homogeneous with a molecular weight of 64,000 daltons. This thiol enzyme is optimally active at pH 7 and is selectively activated by Mn^{++}, Co^{++}, and Zn^{++}. It splits Arg^0-Met-Enk into equal amounts of Arg-Tyr and Gly-Gly-Phe-Met with a K_m of 100 μM and V_{max} 3.8 μmol/mg protein per min. DAP does not hydrolyze the model substrates for DAP-I, DAP-II, DAP-III, DAP-IV, amino acid βNAs, actin, desmin, tubulin, glial fibrillary acidic protein, and cytoskeletonal neurofilament proteins. The enzyme is insensitive to puromycin, but is inhibited by several neruropeptides; angiotensin III is the most potent, with a K_i of 0.3 μM. Its substrate specificity, pH optimum, molecular weight, activators, and catalytic sites demonstrate that this enzyme is distinct from other DAPs.

8 Neuropeptidases Regulated by Inhibitors

The presynaptic Enks have to cross the synaptic cleft before acting on the dendrites, axons, or neuronal perikarya (Pasquini et al., 1992). The blood-borne Enks indicate that some peptide receptors are distant from the synapses. During their travel, attack by peptidases is unavoidable. Recent findings indicate that the pharmacological effects of puromycin, bestatin, and thiorphan are due to their protection of Enk from destruction before its receptor binding and their inhibition of the postsynaptic peptidases (Graf et al., 1982). Bestatin and thiorphan increase the release of Enk by K^+ stimulation (Patey et al., 1981) indicating that peptidase inhibitors play a major regulatory role in the process.

The physiological function of insulin is controlled by degradation in its releasing neuron (Halban, 1980). The presynaptic regulation allows the neurons to store releasable peptide that is relatively independent of axoplasmic transport. It is believed that Enks and neurotransmitters alike are constantly released at basal levels, which increase with stimulation. They are continuously produced at a high level and are degraded after production, with degradation being inhibited during stimulation of release. The inhibitor is released together with Enks into the synaptic cleft. Indeed, Enk is co-released with the peptide (Winkler et al., 1987). A NAP inhibitor is released in rat hippocampal slices by electric stimulation. The enzyme is found to be sensitive to a small-peptide soluble fraction extracted from bovine brains (Hui and Hui, unpublished observations).

The study of the synaptic peptidases and their endogenous inhibitors is important from both a theoretical and practical point of view. First, it enables the design of inhibitors of the relevant peptidase that may mimic, to a large extent, the effects of exogenous opioids. Second, inhibitors may potentiate Enk biological actions, and thereby contribute to the delineation of their functional roles. In addition, it will provide new insight into the regulatory mechanism of peptide neurotransmission in the living brain that will lead to the development of novel inhibitors that are innovative analgesics without the unwanted side effects experienced with narcotics.

9 Conclusion

Neuropeptidases play a critical role in the biosynthesis and metabolism of neuropeptides that are crucial for health. Abnormalities of neuropeptidases have been found in numerous mental, degenerative, and neurological diseases. Opioid peptide enkephalin, the most well studied neuropeptides is synthesized with a group of peptidases acting in an orderly sequence. In contrast, the inactivation of enkephalin is likely to be controlled by a single peptidase—NAP. Study of the neuropeptidases will broaden our knowledge on how the nervous system functions. That will ultimately lead to the development of better diagnosis and treatment, targeted at the gene or enzyme level, in mental and neurological diseases. Therapeutics developed by this approach will be safer, more efficient, and cost effective.

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