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A New Type of Neuron-specific Aminopeptidase NAP-2 in Rat Brain Synaptosomes

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Abstract

A novel neutral aminopeptidase (NAP-2)¹ was found exclusively in the rat central nervous system (CNS). It was separated from the ubiquitous puromycin-sensitive aminopeptidase (PSA) and the neuron-specific aminopeptidase (NAP) by an automated FPLC-aminopeptidase analyzer. The activity of the neuronal aminopeptidase enriched in the synaptosomes is different from NAP and PSA in distribution and during brain development. The enzyme was purified 2,230-fold to apparent homogeneity from rat brain cytosol with 4% recovery by ammonium sulfate fractionation, followed by column chromatography successively on Phenyl-Sepharose, Q-Sepharose, Sephadex G-200, and Mono Q. The single-chain enzyme with a molecular mass of 110 kDa has an optimal pH of 7.0 and a pI of 5.6. It splits β -naphthylamides of amino acid with aliphatic, polar uncharged, positively charged, and aromatic side chain. Leucyl β -naphthylamide (Leu β NA) is the best substrate with the highest hydrolytic efficiency followed by Met β NA = Arg β NA = Lys β NA > Ala β NA > Tyr β NA > Phe β NA. The cysteine-, metallo-, glyco-aminopeptidase releases the N-terminal Tyr from Leu-enkephalin with a K_m 82 μ M and a k_{cat} of 1.08 s⁻¹, and Met-enkephalin with a K_m of 106 μ M and a k_{cat} of 2.6 s⁻¹. The puromycin-sensitive enzyme is most susceptible to amastatin with an IC₅₀ of 0.05 μ M. The data indicate that the enzyme is a new type of neuron-specific aminopeptidase found in rodent. Its possible function in neuron growth, neurodegeneration, and carcinomas is discussed.

INTRODUCTION

Aminopeptidase activities are well known for the inactivation of enkephalins, neuropeptides, and neurohormones (Taylor, 1993a; Taylor, 1993b; Hui, 2007a). Recently, they are found cleaving amino acid residues from the N-terminal of larger brain proteins (Yao and Cohen, 1999; Lendeckel et al., 2000; Stoltze et al., 2000; Karsten, 2006; Sengupta et al., 2006). Their

¹The abbreviations used are: ACE, angiotensin-II convertase; Bicine, N, N-bis (2-hydroxyethyl) glycine; CNS, central nervous system; CP, carboxypeptidase; DAP, dipeptidyl aminopeptidase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, Fast-Protein Liquid Chromatography; HPLC, high-pressure liquid chromatography; KAP, lysine aminopeptidase; LAP, leucine aminopeptidase; LEK, leucine enkephalin; MEK, methionine enkephalin; β NA, β -naphthylamide; NAP, neuron-specific aminopeptidase; NAP-2, neuron-specific aminopeptidase type 2; NEP, endopeptidase 24.11; PAGE, polyacrylamide gel electrophoresis; PSA, puromycin-sensitive aminopeptidase; RAP, arginine aminopeptidase; SDS, sodium dodecyl sulfate. All amino acids are in the L-configuration unless otherwise mentioned.

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degradation of neurodegenerative proteins broadens interest in their neural functions. Although aminopeptidases have been shown to play a role in amyloid deposition and tau pathology in Alzheimer's disease (AD) (Schonlein et al., 1994; Minnasch et al., 2003), their functions in the brain are not fully understood.

Highly expressed in neuron cell bodies (Constam et al., 1995), aminopeptidases are the most abundant peptidases in the CNS (Hui, 2007a). They are required for the enkephalin inactivation by liberating its N-terminal Tyr (Lentzen and Palenker, 1983; Hui, 2007b). While aminopeptidase inhibitors exert a dose-dependent, opiate antagonist-reversible analgesia (Zhang et al., 1982; De La Baume et al., 1983; Herman et al., 1985), elevated brain enkephalin could contribute to cognitive impairments in hAPP mice and possibly in humans with AD (Meilandt et al., 2008).

Using cross-species functional genomics, we recently found that PSA suppresses tau-induced neurodegeneration (Karsten et al., 2006). In addition, aminopeptidases sensitive to puromycin inhibition participate in a number of CNS mechanisms, namely pain (Hui, 2007a), schizophrenia (Hui et al., 1995), neuron growth (Hui and Hui, 2003), anxiety (Osada et al., 1999), amnesia (Eisenstein et al., 1983), and apoptosis (Tobler et al., 1997). It is unclear what and how puromycin-sensitive aminopeptidases contribute to the diverse brain functions and its pathological changes.

We report here the separation of a neural aminopeptidase from PSA and NAP in the rat CNS by an FPLC-aminopeptidase analyzer. The enzyme purified to apparent homogeneity from rat brain is characterized. The aminopeptidase that splits enkephalin and peptide substrates has a distinct developmental profile.

MATERIALS AND EXPERIMENTAL PROCEDURES

Separation and Identification of Aminopeptidases in Subcellular Fractions

Adult male Sprague-Dawley rats (250–300 g) and mice were bred in our animal facility. Human brain samples of short postmortem delays were kindly provided by the Harvard Brain Tissue Resource Center, McLean Hospital, Belmont, MA. Chicken and calf brains were obtained from local slaughter houses. The cultures of rat cerebrocortical neurons, mouse astrocytes, cerebellar granule cells, glioma C6, and neuroblastoma were prepared and harvested as described (Hui et al., 1998). Subcellular fractions, synaptosomes, synaptic lysates, and synaptic membranes were prepared from rat brains. The post-microsomal (S_3) fractions were prepared from the tissues or culture cells in Bicine buffer (50 mM bicine buffer, pH 7.0, complimented with 0.2 mM dithiothreitol, DTT) (Hui et al., 1998).

The S_3 fraction (100 μ l) from 0.5 mg wet weight of tissues or culture cells was injected directly into an automatic FPLC-aminopeptidase analyzer equipped with a Mono Q column (5 $\times$ 50 mm; Amersham Pharmacia Biotech) (Hui and Hui, 1996; Hui et al., 1998). The sample was washed for 1 min with Bicine buffer at a flow rate of 0.5 ml/min, before eluted with a linear NaCl gradient that increased from 0 to 0.21 M at 12 min, 0.23 M at 26 min, 0.24 M at 36 min, 0.29 M at 56 min, and 0.5 M at 61 min. The elution from the FPLC was joined and mixed on-line with a 0.32 ml/min flow of 0.05 mM Leu β NA. The reaction took place while passing through a coil at 37 $^{\circ}$ C for 3 min. Finally, the sample was monitored with a Kratos FS 970 spectrofluorometer (excitation 250 nm/emission 389 nm). The method detected as little as 1.0×10^{-4} of a hydrolyzed substrate, with a sensitivity of 100 pg of aminopeptidase.

Purification of the Peak III Aminopeptidase

The neural enzyme, purified at 4 $^{\circ}$ C, was assayed by incubating aliquots (2–25 μ l) of the elution fractions with 20 μ M Leu β NA in Bicine buffer at 37 $^{\circ}$ C for 5–30 min. After the reaction was

terminated with 1.8 ml of 0.1 N HCl, the sample was measured for fluorescence with excitation at 229 nm and emission at 300 nm. By the end of each purification step, an aliquot (10–100 μ l) of the combined active fractions was submitted to an aminopeptidase analyzer for purity check.

Rat brains (50 g) were homogenized in 9 volumes (w/v) of Bicine buffer then centrifuged at 30,000 g for 1 h. The supernatant was precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 40–70% saturation. The particulate, dissolved in 75 ml of Bicine buffer containing 35% saturation of $(\text{NH}_4)_2\text{SO}_4$, was loaded onto a Phenyl-Sepharose column (2.5×10 cm). Subsequent to the wash with 100 ml of the same buffer solution, the column was eluted with 200 ml of a reversed gradient of $(\text{NH}_4)_2\text{SO}_4$ (35 – 0% saturation) at a flow rate of 0.5 ml/min and collected in 2.5-ml fractions,

The pooled active fractions were desalted with an Amicon UM-10 and loaded onto a Q-Sepharose column (1.5×25 cm). The sample was washed with 90 ml of Bicine buffer then eluted at a flow rate of 0.5 ml/min with a gradient of 0 – 0.5 M NaCl in 200 ml of Bicine buffer. The combined active 2.5-ml fractions were concentrated to 3 ml with a 2-ml TosoHaas DEAE-650 column. The sample, directly loaded onto a Sephadex G-200 (1.5×85 cm) column, was eluted at a flow rate of 0.1 ml/min, and collected in 1.5-ml fractions. The combined active fractions (< 20 ml) were submitted to FPLC purification on a Mono Q column (5×50 mm).

The loaded sample was washed with 10 ml of Bicine buffer at a flow rate of 1 ml/min. It was then eluted with a linear NaCl gradient consisting of 0 – 0.21 M for 12 min, 0.21 – 0.29 M for 44 min, 0.29 – 0.5 M for 5 min, and 0.5 M for 5 min. The active 0.5-ml fractions were pooled and kept in 10% sucrose at -80°C for further study and characterization.

Aminopeptidase Assays for Enzyme Characterization

The breakdown of amino acyl β NAs was assayed as described (Hui et al., 1998). In brief, amino acyl β NA (20 μ M) was incubated with 15 ng of the purified aminopeptidase in 0.2 ml of Bicine buffer at 37°C for 10 min. The reaction was stopped with 1.8 ml of 0.1N HCl and the fluorescence of the released β -naphthylamine was measured. For peptides, 22.5 nmoles of the substrate was incubated with 30 ng of the purified aminopeptidase in 40 μ l of Bicine buffer. After the reaction was terminated with 10 μ l of 20% trichloroacetic acid, the sample was cleared by centrifugation and submitted to HPLC analysis (Hui et al., 1998).

Assays for Dipeptidyl Aminopeptidases (DAPs), Neutral Protease, and Cathepsin D

The purified aminopeptidase (15 ng) was incubated with 20 μ M Gly-Arg β NA (model substrate for DAP-I), Lys-Ala β NA (DAP-II), Arg-Arg β NA (DAP-III), or Gly-Pro β NA (DAP-IV) in 0.2 ml of Bicine buffer at 37°C for 10 min. The reaction was terminated with 1.8 ml of 0.1 N HCl and the fluorescence was assessed (Hui, 1988). Neutral protease activity was assayed with alkali-treated casein and fluorescamine. Cathepsin D activity was assessed with hemoglobin and alkaline copper reagent (Hui et al., 1998).

Kinetic Studies

Kinetic constants were calculated with Lineweaver-Burk analysis. The inhibitor IC_{50} values were determined with semi-log plots using six different test concentrations. The enzyme was compared with PSA and NAP that were prepared as described (Hui et al., 1998).

Electrophoresis, Isoelectrofocusing, and Western Immunoblots

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (Hui et al., 1993). The pIs were estimated on PhastGel IEF 3–9 gels with a Pharmacia PhastSystem (Hui et al., 1998). For Western blots, proteins on the SDS-PAGE gels were electro-transferred to Immobilon-P membranes (Millipore) before immunological detection.

Preparation of Mono-specific Anti-PSA Polyclonal Antibodies

IgG of the heat-treated goat anti-rat PSA antiserum was purified by column chromatography on Protein-G Sepharose followed by solid-phase affinity chromatography using rat brain PSA as a ligand (Hui et al., 1993).

Glycoprotein Identification and Protein Determination

Glycoproteins were identified with biotin hydrazide/avidin phosphorylase and periodate oxidation (Hui et al., 1998). Protein contents were quantified with the Bio-Rad Protein Assays or by UV detection. In the latter method, the sample was loaded onto a Sephadex G-10 column (0.5 × 20 cm) in a Waters HPLC with UV detection at 280 nm and eluted with 0.1 M potassium phosphate buffer containing 0.2 M NaCl at a flow rate of 0.2 ml/min (Hui, 1988).

RESULTS

Identification of a Novel Aminopeptidase in Rat Brain

Three neutral aminopeptidase peaks, Peak I, Peak II, and Peak III, were separated from the rat brain post-microsomal (S_3) fraction with the automatic aminopeptidase analyzer (Figure 1A). The S_3 fraction contained > 90% of the three peaks in the brain (data not shown). The largest Peak I eluted at 0.24 M NaCl was the PSA and the smaller Peak II at 0.25 M NaCl the NAP (Hui et al., 1998). Peak III eluted at 0.27 M NaCl with the highest salt concentration, was the most acidic aminopeptidase to our knowledge (Hui and Hui, 1996). The tiny peaks of the basic lysine aminopeptidase (KAP) and the arginine aminopeptidase (RAP; aminopeptidase B) were eluted before Peak I. The KAP was eluted with a much lower concentration of NaCl at 0.11 M and the RAP at 0.15 M (Hui and Hui, 2006). The dimensions of Peak I, Peak II, and Peak III changed when assayed with various amino acyl β NA substrates. Adding 0.5 μ M puromycin or bestatin to the substrate solution drastically reduced the three peaks to almost disappear. The data put forward that Peak III is a type of puromycin-sensitive aminopeptidase.

Peak III was the smallest amongst the three peaks, 15% of the PSA and 50% of the NAP (Figure 1A). It could not be found in the cerebral cortex samples from human, calf, and chicken. In addition, it was not detected in the human Brodmann area 9, cerebellum with dentate, and anterior hippocampus from 5 control and 9 AD subjects. The enzyme was only found in the rodent species and the peak size of the rat was twice of the mouse. Like the NAP, Peak III was present just in the CNS. It was absent in the peripheral tissues including kidney, liver, spleen, heart, intestinal mucosa, testes, skeletal muscle, adrenal gland, serum, sciatic nerve or pituitary gland (Figure 1B).

Distribution of Peak III Aminopeptidase in Rat Brain

Peak III aminopeptidase was distributed in the brain heterogeneously, with the highest activity in the cerebral cortex and the lowest in the olfactory bulbs (Table 1). The retina resembled the peripheral tissues, rather than the brain, in having little, if any of the enzyme. Except for the hypothalamus, the activity in the brain regions was higher than the spinal cord (cervical and sacral sections). Peak III aminopeptidase was less active than NAP in all brain regions, despite the two activities were neither parallel nor complimentary. The highest activity of the Peak III aminopeptidase was found in the cerebral cortex and the NAP in the hippocampus. The highest Peak III aminopeptidase:NAP ratio was in the corpus callosum and the lowest in the hypothalamus.

Subcellular distribution of Peak III aminopeptidase

The majority (85%) of the Peak III aminopeptidase activity was found in the cytosol (S_2 fraction) and the rest in the membranes. After the Peak III aminopeptidase, NAP, and PSA

were released by hypotonic shock from the brain crude synaptosomal (P_2) fraction, their activities were 15:30:55 in ratio. The Peak III aminopeptidase:PSA ratio, similar to the NAP:PSA, was 70% higher than in the S_2 fraction (Table 2). The Peak III aminopeptidase activity in the synaptic lysates of the cerebral cortical and the hippocampal synaptosomes was 82–136% higher and in the synaptic membranes 36–45% higher than the S_2 fractions. Conversely, the PSA activity in the synaptic lysates was 38–45% and synaptic membranes 6–18% lower than the S_2 fractions.

In cells cultured *in vitro*, Peak III aminopeptidase represented 7% of the total aminopeptidase activity in the cerebrocortical neurons and the cerebellar granule cells. It was absent in the astrocytes. Peak III aminopeptidase, like NAP, was not detected in the glioma C6 and the neuroblastoma SK-N-SH cells. The findings suggest that Peak III aminopeptidase, along with NAP, is expressed exclusively in differentiated CNS neurons.

Developmental Changes of Peak III Aminopeptidase

Peak III aminopeptidase activity was low in the immature rat brain as shown in the hippocampus. It began barely detectable one day before birth (0.01 unit; Figure 2). At birth the activity increased 4 folds to 0.05 units. Afterward, it increased further to 0.45 units (44 folds) on day 30. The rise of Peak III aminopeptidase paralleled to the surging brain growth after birth and continued through infancy. Its increase during early development was larger than the 9-fold-increased NAP and the 1.5-fold-increase of the rate of general protein synthesis in brain (Hui and Hui, 2003). The developmental pattern of Peak III aminopeptidase also differed from the PSA with activity higher in the fetus (Figure 2).

Purification and Characterization of Peak III Aminopeptidase

In the early steps of the purification, Peak III aminopeptidase was isolated together with PSA and NAP by the ammonium sulfate fractionation and the sequential column chromatography successively on Phenyl-Sepharose, Q-Sepharose, and Sephadex G-200. It was separated from the PSA and the NAP by the Mono Q in the FPLC. The purified enzyme showed a single peak (Figure 1C) whose mobility was distinct from the NAP (Figure 1D) but identical to the rat brain Peak III aminopeptidase (Figure 1A) in the aminopeptidase analyzer. The overall purification was estimated to be 2,230 folds with a final yield of 4%.

In the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the purified enzyme showed a solitary band with a mobility corresponding to a molecular mass of 110 kDa (Figure 3A). Separately, the molecular mass of the enzyme was also estimated to be 110 kDa by HPLC size-exclusion chromatography with TSK-Gel G3000 SW, or by FPLC with Sepharose 12 HR (Hui et al., 1998). Both electrophoresis and chromatography results indicate that the Peak III aminopeptidase is a single-chain polypeptide of 110 kDa.

The extracted Peak III aminopeptidase after the un-denatured PAGE (without SDS) was active against Leu β NA. The enzyme activity was also detected *in situ* with stabilized diazonium salts following the Leu β NA incubation (Hui et al., 1998). In the un-denatured PAGE, Peak III aminopeptidase ran slightly faster than NAP towards the anode and NAP ran a little faster than PSA. The pI of the purified Peak III aminopeptidase was estimated to be 5.6 as compared to 5.25 for the NAP and 5.1 for the PSA with a Pharmacia PhastSystem.

Hydrolytic Kinetics and Substrate Specificity of Peak III Aminopeptidase

At the optimal pH (7.0), Peak III aminopeptidase split β NAs of amino acid with aliphatic, polar uncharged, positively charged, and aromatic side chain. Leu β NA was the best substrate with the highest hydrolytic efficiency (k_{cat}/K_m) followed by Met β NA = Arg β NA = Lys β NA > Ala β NA > Tyr β NA > Phe β NA (Table 4). The enzyme was least active on Trp-, γ -Glu-, Pro-,

His-, Cys-, Gly-, Glu-, Thr-, Ile β NA, and inactive on Ser-, pGlu-, D-Ala-, β -Asp-, D-Leu-, α -Asp-, Val β NA. The hydrolytic coefficients of the Peak III aminopeptidase on the amino acyl β NAs were approximately half of the NAP's and 10% of the PSA's.

As opposed to the PSA and the NAP, the affinity of Peak III aminopeptidase to Leu-enkephalin (LEK) was higher than to Met-enkephalin (MEK). For LEK, the K_m was 82 μ M with a k_{cat} of 1.08 s^{-1} ; for MEK, the K_m was 106 μ M with a k_{cat} of 2.6 s^{-1} . Peak III aminopeptidase released only the N-terminal Tyr of the enkephalins and left the residual tetrapeptides intact. The lack of activities on di- and tripeptide with N-terminal tyrosine (Tyr-Gly, Tyr-Gly-Gly) indicated that the aminopeptidase activity depended on the length of the peptide substrates. The enzyme did not release the N-terminal dipeptide (Arg-Arg-, Gly-Arg-, Gly-Pro-, and Lys-Ala-) from the model dipeptidyl aminopeptidase substrates for DAP-I, -II, -III, and -IV. Therefore, it is not expected to be a dipeptidyl aminopeptidase. Peak III aminopeptidase that possessed neither cathepsin D activity on hemoglobin nor neutral protease activity on alkali-treated casein excludes it to be an endopeptidase.

Catalytic Classification and Inhibitor Profile of Peak III Aminopeptidase

Peak III aminopeptidase was inhibited 95% by *p*-chloromercuriphenylsulfonic acid (a model cysteine protease inhibitor) at 20 μ M and 90% by 1,10-phenanthroline (metallo-protease inhibitor) at 1 mM. It was not affected (< 5%) by pepstatin (aspartic protease inhibitor) at 100 μ M and phenylmethyl sulfonyl fluoride (serine protease inhibitor) at 500 μ M. In addition, the aminopeptidase was insensitive to the inhibitors for carboxypeptidase (CP; guanidinoethyl mercaptosuccinic acid at 100 μ g/ml), angiotensin-II convertase (ACE; captopril at 100 μ M), and endopeptidase 24.11 (NEP; thiorphan at 0.3 μ M). Because in the catalytic and inhibitor classification 5 to 10 times the inhibitor K_i constants were applied, Peak III aminopeptidase is neither a CP, nor an ACE, or a NEP.

Peak III aminopeptidase was highly sensitive to aminopeptidase inhibitors. It was most susceptible to amastatin with an IC_{50} of 0.05 μ M. In addition to amastatin, Peak III aminopeptidase, NAP, and PSA were also equally sensitive to bestatin ($IC_{50} = 0.2 \mu$ M) and proctolin ($IC_{50} = 0.8 \mu$ M). The enzyme was inhibited by puromycin with an IC_{50} of 2.6 μ M as compared to 1.0 μ M for NAP and 5.4 μ M for PSA. Arg⁰-enkephalin inhibited NAP ($IC_{50} = 5.0 \mu$ M) > Peak III aminopeptidase ($IC_{50} = 5.2 \mu$ M) > PSA ($IC_{50} = 5.6 \mu$ M).

Western Blots and Glycoprotein Identification of Peak III Aminopeptidase

Peak III aminopeptidase strongly reacted to the mono-specific polyclonal anti-PSA IgG (Figure 3B). In the Western blots, the antibody was purified by affinity chromatography using rat PSA as a ligand. The crossed immunological reactivity shows that Peak III aminopeptidase and PSA share some common epitopes. After SDS-PAGE and treated with hydriodic acid hydrazide, Peak III aminopeptidase bound to biotin indicating it contains carbohydrates.

DISCUSSION

Although neutral aminopeptidases are the most abundant and widely distributed proteases in the brain, their neural functions are not fully explored. The new development on their possible role in neurodegeneration calls for a better understanding of brain aminopeptidases. Peak III aminopeptidase, NAP, and PSA were separated from each other by ion-exchange Mono Q in the FPLC (Figure 1). In rat brain, Peak III aminopeptidase and NAP co-existed but of varying activities suggesting their separate functions (Table 1). The highest activity of Peak III aminopeptidase found in the cerebral cortex and the hippocampus suggests its upper role in brain functions.

Peak III aminopeptidase, like NAP, is absent in the peripheral organs and neural tissues: sciatic nerve, retina, and pituitary gland. It was not found in chicken and other mammals. The exclusiveness in rat and mouse indicates a distinctive purpose in the species. The findings further support the existence of species-specific enzymes in animals. A bestatin-insensitive aminopeptidase was found only in rat and mouse (Neidle and Hui, unpublished observations). However, whether the Peak III aminopeptidase is rodent specific, needs further study with additional species.

Peak III aminopeptidase's activity detected in the CNS, nerve endings, and neuron cultures, but not in the peripheral tissues, astrocytes, glioma, and neuroblastoma indicate its neuron specificity. The regional and subcellular distributions of Peak III aminopeptidase and NAP are not identical, parallel, or complimentary (Table 2). Therefore, their functions are unlikely to be the same. Since hypothalamus is neuropeptide-rich whereas corpus callosum is axon-rich, the highest Peak III aminopeptidase:NAP ratio in the corpus callosum and the lowest ratio in the hypothalamus suggest that Peak III aminopeptidase prefers brain proteins rather than neuropeptides for substrates. It also suggests its plausible participation in axonal transportation.

The developmental profile of Peak III aminopeptidase is different from NAP, PSA, prolidase, and general protein synthesis (Hui and Hui, 2003). Its activity increases markedly during the early brain development (Figure 2). Its rise after birth indicates a possible role in neuron differentiation. The difference from NAP and PSA in developmental changes, as well as its distinctive chromatographic behavior, occurrence, tissue and subcellular distribution, prompted us to Peak III aminopeptidase's purification and characterization.

The longer retention of the Peak III aminopeptidase than the NAP and PSA in the Mono Q facilitated its purification (Table 3). The purified neutral exopeptidase differs from PSA and NAP on splitting the synthetic substrates and peptides (Table 4). The disparate substrate specificities indicate that these three aminopeptidases are not isozymes. Peak III aminopeptidase is obviously not an enkephalin (neuropeptide)-specific enzyme. However, if anatomical location associated with limited substrate specificity constitutes a "functional" specificity, its synaptic location could contribute to neuropeptide-specific functions.

The potent inhibition by chloromercuriphenylsulfonic acid and the chelating 1,10-phenanthroline indicates that Peak III aminopeptidase is a cysteine- and metallo-aminopeptidase. The metal identity, however, needs a further metal analysis for verification. The low susceptibility to other peptidase inhibitors distinguishes Peak III aminopeptidase from aminopeptidase B, CP, NEP, and ACE. Nor is the enzyme a dipeptidyl aminopeptidase since it is inactive towards model DAP substrates. The sensitivity and selectivity of Peak III aminopeptidase to aminopeptidase inhibitors demonstrates that it is a neuronal aminopeptidase sensitive to puromycin (Table 5).

Aminopeptidase inhibitor is rarely mono-specific to a single enzyme. For instance, bestatin inhibits several structurally and functionally unrelated aminopeptidases: LAP, PSA, NAP, KAP, and RAP (Hui, 2007a). Peak III aminopeptidase, PSA, and NAP could be different enzymes while they are all sensitive to puromycin. Peak III aminopeptidase is most sensitive to amastatin, indicating its relation to the M1 family of zinc aminopeptidases (gluzincin). Like most aminopeptidases (Constam et al., 1995), Peak III aminopeptidase is apparently a glycoprotein. Although the carbohydrate moieties of Peak III aminopeptidase are not known, they could serve as signals that target the aminopeptidase to different intracellular sites. Or, they might lead the aminopeptidase binding to different organelles for intracellular transportation.

NAP and Peak III aminopeptidase both bind to the rat anti-PSA antibody (Hui et al., 1998). The cross-immunoreactivity indicates that the epitopes of Peak III aminopeptidase, NAP, and

PSA are similar (Figure 3). Peak III aminopeptidase differs from NAP and PSA in development, as well as chromatographic behavior, occurrence, tissue and subcellular distribution, hydrolytic kinetics, and substrate and inhibitor specificities imply that Peak III aminopeptidase is a new type of NAP (Table 5). We designate it as *neuron-specific aminopeptidase type 2* (NAP-2) since it is the brain-specific enzyme found after NAP (Hui, 2007b).

Aminopeptidase activities in the CNS are tentatively categorized into three groups: Genep_{SA}-dependent puromycin-sensitive, Genep_{SA}-independent but puromycin-sensitive, and puromycin-insensitive (Osada et al., 1999). In the mouse brain, Genep_{SA}-dependent puromycin-sensitive is most abundant (59%), followed by the Genep_{SA}-independent but puromycin-sensitive (29%), and puromycin-insensitive aminopeptidase(s) (12%). NAP-2 seemingly belongs to the PSA subfamily. A better classification of NAP-2 could be achieved by molecular cloning and mass spectrometry. Using a rat brain expression library, we have cloned 4 novel PSA-like cDNAs. Two have new 5' end segments, one has a new 3' end segment, and one has a new inserted segment (Hui et al., unpublished observations). The findings indicate that a NAP-2 mRNA is likely to be unique in rat brain.

Puromycin induces amnesia and memory loss that are typical AD symptoms. Recently PSA was identified to hydrolyze human full-length tau as a putative protector against tauopathy (Karsten et al., 2006; Sengupta et al., 2006). Because of their enzymatic similarities to PSA, NAP and NAP-2 could have an implication in neurodegenerative diseases. In brain regions that are most affected by AD, we recently found an 80% loss of NAP as compared to the control counterparts (Hui et al., unpublished observations). At present, reason for the absence of NAP-2 in human brain and whether that relates to human neurodegeneration (AD) remain unclear.

Many cell-specific aminopeptidases participate in explicit targeted functions. Aminopeptidase Q regulates the key peptides at the embryo-maternal interface (Maruyama et al., 2007). Placental LAP/oxytocinase prevents premature delivery and pre-eclampsia (Nomura et al., 2005). Adipocyte-derived LAP and leukocyte-derived RAP (Hattori et al., 1999; Tanioka et al., 2003) processes the major histocompatibility complex class I molecules (Saric et al., 2002; Saveanu et al., 2005). The absence of NAP-2 (and NAP) in the neuroblastoma implies its plausible role for neuron differentiation. Furthermore, mature neurons do not multiply (Rakic, 2006). No malignancy of mature neurons has occurred spontaneously or been introduced by carcinogens, although glioma and other types of brain tumors are frequent. Since NAP-2 and NAP are found only in neurons but not in glioma and neuroblastoma, it will be very interesting to study if the neuron-specific enzymes prevent any unwanted cell divisions in carcinomas.

In conclusion, a new type of neuron-specific aminopeptidase, NAP-2, was isolated, identified, purified, and characterized from rat brain cytosol. It is the second brain-specific enzyme found. Further study of NAP-2 together with NAP and PSA will give new insights in neurodegeneration, memory, and carcinomas.

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Figure 1. Aminopeptidase zymograms of the FPLC-aminopeptidase analyzer. Post-microsomal fraction of 1.25 mg of rat brain (A) was studied with an automatic aminopeptidase analyzer. It was compared with a same amount of sample from the kidney (B), 0.5 μ g of the purified Peak III aminopeptidase (C), and 0.39 μ g of the purified neuron-specific aminopeptidase (D). The enzyme activity was monitored by the released fluorescence as described in the Methods.

Figure 2.

Ontogeny of aminopeptidases in rat hippocampus. Post-microsomal fraction from 0.5 mg hippocampus at different ages of rat was studied by an aminopeptidase analyzer (see Methods for details). Each point represents the Mean $\pm$ S.D. of 4 to 6 experiments. The activity of Peak III aminopeptidase (▲), neuron-specific aminopeptidase (•), and puromycin-sensitive aminopeptidase (●) are expressed in arbitrary fluorescence units μ A generated by per mg of protein.

Figure 3. SDS-PAGE (A) and Western immunoblots (B) of the purified Peak III aminopeptidase. Purified Peak III aminopeptidase (7 μ g) was used in each run. Molecular mass markers are myosin, 200 kDa; phosphorylase *b*, 97.4 kDa; and bovine serum albumin, 69 kDa. In Western blots analysis, Peak III aminopeptidase was resolved by SDS-PAGE, transferred to Immobilon P membrane, reacted with mono-specific goat anti-rat brain PSA polyclonal IgG, and visualized by alkaline phosphatase color reaction.

Table 1

Peak III Aminopeptidase and Neuron-Specific Aminopeptidase Distribution in Rat Central Nervous System

	Peak III [*]	NAP	Peak III/NAP ^{**}
Cerebral cortex	66.0	132	0.50
Hippocampus	59.2	140	0.42
Colliculus	58.4	130	0.45
Striatum	46.4	98.7	0.47
Medulla oblongata	30.4	60.8	0.50
Cerebellar cortex	29.6	77.9	0.38
Corpus callosum	28.4	47.3	0.60
Cervical spinal cord	24.6	49.2	0.50
Hypothalamus	21.4	71.3	0.30
Sacral spinal cord	18.7	49.2	0.38
Olfactory bulb	11.5	30.3	0.38

* Enzyme activity in arbitrary fluorescence units.

** Peak III aminopeptidase (Peak III) as compared with neuron-specific aminopeptidase (NAP).

50 μ l of S₃ fraction of 2.5 mg of tissue was submitted to the automated FPLC-aminopeptidase detector. Each value represents an average of 3–4 experiments with $\pm$ 5% variations.

Table 2
Subcellular Distribution of Three Puromycin-Sensitive Aminopeptidases in Rat Brain

	Enzyme Specific Activity (μ A/mg protein) [*]		
	PSA	NAP	Peak III
Cerebral cortex			
S ₂	1.73	0.16	0.11
Synaptic lysate	0.96	0.56	0.20
Synaptic membranes	1.42	0.31	0.16
Hippocampus			
S ₂	1.32	0.17	0.11
Synaptic lysate	0.82	0.59	0.26
Synaptic membranes	1.24	0.35	0.15

* Enzyme activity in arbitrary fluorescence units for puromycin-sensitive aminopeptidase (PSA), neuron-specific aminopeptidase (NAP), and Peak III aminopeptidase (Peak III). Each value represents an average of 3–4 experiments with $\pm$ 5% variations.

Table 3
Typical Purification of Peak III Aminopeptidase from Rat Brain Cytosol

	Protein (mg)	Total activity (μmol/min)	Specific activity (nmol/mg/min)	Purification (fold)	Yield (%)
1. Homogenate	7,500	107	0.01	1	100
2. Supernatant	3,045	68.1	0.02	1.57	64
3. Ammonium sulfate	634	55.7	0.09	6.16	52
4. Phenyl-Sepharose	45	42.0	0.93	65.6	39
5. Q-Sepharose	24	28.5	1.19	83.4	27
6. Sephadex G-200	10.1	15.5	1.52	107	14.5
7. FPLC Mono Q	0.19	4.24	22.3	2,230	4.0

The data are taken from a single enzyme preparation starting with 50 g of rat brain and carried through each purification step.

Table 4
Kinetic Constants of Puromycin-Sensitive aminopeptidases on Hydrolysis of β -Naphthylamides and Peptides

	$k_{cat} (s^{-1})^*$			$k_{cat}/K_m (s^{-1}M^{-1} \times 10^6)$			$K_m (\mu M)$		
	I**	II	III	I	II	III	I	II	III
Aminoacyl βNA ***									
Leu	72.3	12.3	4.13	36.2	11.2	4.13	2	1	1
Met	33.8	15.4	5.17	10.2	4.82	1.03	3.3	2.9	5
Arg	27.2	30.8	6.88	9.07	5.60	1.03	3	5	6.7
Lys	36.0	30.8	5.17	7.2	5.60	1.03	5	5	5
Ala	27.2	15.4	5.17	2.72	1.40	0.52	10	10	10
Tyr	18.7	11.2	3.43	2.08	0.93	0.45	9	10	7.7
Phe	43.3	17.5	5.17	0.87	0.80	0.21	50	20	25
Enkephalin									
MEK	10.3	7.80	2.6	0.13	0.08	0.02	80	95	106
LEK	12.9	3.24	1.08	0.11	0.05	0.01	117	74	82

* The MW of NAP and Peak III aminopeptidase are 110 kDa and PSA is 100 kDa for the calculations.

** I: Puromycin-sensitive aminopeptidase; II: Neuron-specific aminopeptidase; III: Peak III aminopeptidase; MEK: Met-enkephalin; and LEK: Leu-enkephalin.

*** Peak III aminopeptidase was inactive ($K_m > 250 \mu M$ Tyr-Gly, Tyr-Gly-Gly, Gly β NA, Pro β NA, Ile β NA, Val β NA, Cys β NA, Ser β NA, Thr β NA, His β NA, α -Asp β NA, β -Asp β NA, Glu β NA, pGlu β NA, γ -Glu β NA, D-Ala β NA, D-Leu β NA, ArgArg β NA, GlyArg β NA, GlyPro β NA, and LysAla β NA).

Table 5

Comparison of Neuron-Specific Aminopeptidase Type 2, Neuron-Specific Aminopeptidase, and Puromycin-Sensitive Aminopeptidase

	NAP-2	NAP	PSA
Occurrence	Rat & mouse	Mammals	Mammals & avian
Distribution	CNS	CNS	Ubiquitous
Regional activity	Cerebral cortex>hippocampus	Hippocampus>cerebral cortex	Hippocampus>cerebral cortex
Subcellular location	Synaptosomes	Synaptosomes	Cytoplasm
NaCl (M) in Mono Q elution	0.27	0.25	0.24
MW	110 kDa	110 kDa	100 kDa
pI	5.6	5.25	5.1
N-terminal sequence	Unknown	N-blocked	PEKRPFERLPTEVSPINY
IC₅₀ (μM) of puromycin	2.6	1.0	5.4
K_m	MEK>LEK [*]	LEK>MEK	LEK>MEK
k_{cat}/K_m^{**}	Leu>Met=Arg=Lys>Ala	Leu>Lys=Arg>Met>Ala	Leu>Met>Arg>Lys>Ala
Higher activity during brain development	Postnatal	Postnatal	Prenatal
Reported in references	This study	Hui et al., 1998 Hui & Hui, 2003	Hui et al., 1998 Hui & Hui, 2003

NAP-2: Neuron-specific aminopeptidase type 2; NAP: Neuron-specific aminopeptidase; PSA: Puromycin-sensitive aminopeptidase.

* MEK: Met-enkephalin; LEK: Leu-enkephalin;

** Amino acyl β-naphthylamides as substrates.