A Novel Neuron-specific Aminopeptidase in Rat Brain Synaptosomes
ITS IDENTIFICATION, PURIFICATION, AND CHARACTERIZATION

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A specific aminopeptidase localized exclusively in neurons of the central nervous system was identified with an automated continuous-flow aminopeptidase analyzer developed recently in this laboratory. The enzyme was purified from rat brain 4933-fold to homogeneity with 9.5% recovery by ammonium sulfate fractionation, followed by column chromatography successively on phenyl-Sepharose, Sephadex G-200, and twice on Mono Q FPLC. The purified single-chain enzyme was estimated to be 110 kDa in molecular mass. It has a pI of 5.25 and a pH optimum of 7.0. Only Mg(II) restores the activity of the apoenzyme. The neutral aminopeptidase hydrolyzes β-naphthylamides 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 methionine-enkephalin, 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 puromycin-sensitive aminopeptidase obtained from the same enzyme preparation. The blocked N terminus, substrate and inhibitor specificity, hydrolytic efficiency, metal effects, pI, molecular weight, and catalytic site show that this enzyme is distinct from all other known aminopeptidases. Its enrichment in the synaptosomes suggests that this first reported neuron-specific peptidase plays a role in neurotransmission and synaptic differentiation.

Neuropeptides are an essential class of chemical messengers in the central nervous system (CNS), yet the role of specific peptidases in regulating their formation and disposition is equivocal. Along with their involvement in protein turnover, CNS aminopeptidases mediate a variety of specialized functions. Inhibition of cerebral aminopeptidase by puromycin has been shown to induce analgesia (1), apoptosis (2), and amnesia (3). Recently, we found fewer aminopeptidase antigens in brain regions of postmortem schizophrenic than control brains (4).

Aminopeptidases (EC 3.4.11.-) are classified according to the number of amino acids cleaved from the N terminus, the relative efficiency with which residues are removed, the location of the aminopeptidase, the susceptibility to inhibitors, the metal ion content or the residues that link the metal to the enzyme, and the pH for maximal activity. Several aminopeptidases from brain have been purified and characterized; however, their specificity to neural function is poorly understood.

Neutral aminopeptidases are the most abundant peptidases in the brain (5, 6), with amounts 100-fold that of aminopeptidase N. Different molecular weights and variable subcellular localization in brain have been found for them. Implicated in the inactivation of many neuropeptides including enkephalins (5), they are amphitropic enzymes, with 80% found in the cytosol and the rest in the membrane fraction (6).

Peptidases are found to be differentially localized in neurons and glia (7). Strong arylamidase activity has been detected histochemically in CNS neuronal cell bodies (8). Most recently, transcripts of the ubiquitous puromycin-sensitive aminopeptidase (PSA), could be detected by in situ hybridization in rat CNS neurons, but not in surrounding glial cells or in blood vessels (9). Despite all these compelling findings, a neuron-specific peptidase has not been identified. Knowledge of such aminopeptidase(s) would facilitate our understanding of the neuropeptide function in the synapses.

Using our newly developed FPLC aminopeptidase analyzer (10), we identified a neutral aminopeptidase enriched in brain synaptosomes. The neuron-specific enzyme was purified to apparent homogeneity from the soluble fraction of rat brain. The purified enzyme degrades enkephalins, and its properties are distinct from all known aminopeptidases.

EXPERIMENTAL PROCEDURES

Assay of Neural Aminopeptidase in Cultures and Tissues—Primary cultures of rat cerebrocortical neurons, mouse astrocytes (11), and cerebellar granule cells (12) were collected by centrifugation. The postmicrosomal (S$_i$) fractions were prepared from cells or tissues of adult male Sprague-Dawley rats (250–300 g). The samples were injected directly into an automated FPLC aminopeptidase analyzer equipped with an Amersham Pharmacia Biotech Mono Q column (5 × 50 mm) (10).

After the injection of the S$_i$ fraction (100 μl) extracted from 1.25 mg of wet tissue, the sample was eluted with a NaCl gradient increasing linearly from 0 to 0.21 M at 12 ml and then to 0.39 M at 56 ml. A stream of the enzyme eluate (0.05 ml/min) from the FPLC was first mixed with 50 μl Bicine buffer, pH 7.0, in the presence of 0.2 μM DTT (Bicine buffer, 0.05 M/ml), and later mixed with 0.2 ml Leu βNA in the same buffer (0.23 M/ml). The enzyme reaction took place at 37 °C in a coil of Teflon tubing for 4 min. Finally, the sample was measured by excitation at 250 nm and emission at 389 nm in a Kratos FS 970 spectrophluo-
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orometer (Kratos, Westwood, NJ). The method detected when > 1.0 x 10^(-10) of the substrate was hydrolyzed, with a sensitivity of 100 pg of aminopeptidase (10).

Preparation of Synaptosomes, Synaptic Lysates, and Solubilized Synaptic Membranes—Rat brains were homogenized in 10 volumes (w/v) of 0.32 M sucrose containing 1 mM EDTA and homogenized with a Teflon plunger. The homogenate was centrifuged at 700 g for 20 min. The pellet was re-homogenized in 10% sucrose and centrifuged as before. The two supernatants were combined and centrifuged at 10,000 x g for 30 min. The pellet (P2), separated from the supernatant (S2), was re-suspended in a small volume of 10% sucrose. It was layered on 20% sucrose layers in a centrifuge tube and centrifuged at 100,000 x g for 2 h. The synaptosomal fraction at the 27%/41% interface was isolated and collected by centrifugation at 100,000 x g for 1 h. The fraction was re-suspended in 10% sucrose and pelleted by centrifugation.

The synaptosomal fraction was incubated in 5 ml Tris-HCl buffer, pH 8.1, for 30 min at 0 °C. The synaptically soluble fraction was separated from the particulate (membranes) by centrifugation at 100,000 x g for 30 min. The particulate fraction was washed with Tris-HCl buffer containing 1 mM DTT and incubated with 0.5 ml of 0.1% Nonidet P-40 in Bicine buffer at 37 °C for 45 min. The supernatant was collected by centrifugation at 50,000 x g for 1 h.

Purification of Neural Aminopeptidase—The enzyme was purified at 4 °C by column chromatography. The sample was assayed by incubating aliquots (2–25 μl) of the eluate fractions with 20 μM Leu βNA in Bicine buffer at 37 °C for 5–30 min. The reaction was stopped by adding 1.8 ml of 0.1 M HCl, and the liberated βNA was measured automatically with the aid of a batch sampler (Technicon Instruments Corp.) and a peristaltic pump. Its fluorescence was measured at an excitation at 229 nm and an emission at 300 nm. After each purification step, the active fractions were pooled. An aliquot of the combined sample was studied for enzyme purity with the automated FPLC aminopeptidase analyzer.

Rat brains (50 g) were homogenized in nine volumes (w/v) of Bicine buffer and centrifuged at 30,000 x g for 1 h. The supernatant was precipitated with (NH4)2SO4 between 40 and 70% saturation. The pellet was dissolved in 75 ml of Bicine buffer. The sample, after adding (NH4)2SO4 to 35% saturation, was loaded onto a phenyl-Sepharose column (2.5 x 10 cm) that had been equilibrated with Bicine buffer containing the same strength of (NH4)2SO4. The column was washed with two volumes of the starting buffer, then eluted with 200 ml of a gradient of 35–0% (NH4)2SO4 in Bicine buffer at a flow rate of 0.5 ml/min; the eluate was collected in 2.5-ml fractions.

The active fractions were pooled, desalted with Amicon UM-10, and loaded on a Q-Sepharose column (1.5 x 25 cm). The column was washed with two volumes of Bicine buffer and eluted with a 200-ml gradient of 0–0.5 M NaCl in Bicine buffer at a flow rate of 0.5 ml/min. The 2.5-ml fractions containing the enzyme activity were combined and concentrated to 3 ml with a 2-ml Tosohaas DEAE-650 column. The enzyme was loaded directly onto a Sephadex G-200 (1.5 x 85 cm) column, eluted at a flow rate of 0.1 ml/min, and collected in 15-ml fractions. After the active fractions were pooled (20–25 ml), the sample was submitted to FPLC (Amersham Pharmacia Biotech) separation on a Mono Q column (5 x 50 mm).

The FPLC column was washed with 10 ml of Bicine buffer after the sample was injected. It was then eluted at a flow rate of 1 ml/min by the same buffer with a linear gradient consisting of 0–0.2 M NaCl for 12 min, 0.21–0.29 M NaCl for 44 min, 0.29–0.5 M NaCl for 5 min, and 0.5 M NaCl for 5 min. The active 0.5-ml fractions were pooled, desalted with Amicon UM-10, and submitted to a second Mono Q FPLC with conditions identical to those of the first FPLC. After the final purification step, the active fractions were pooled and kept at −80 °C in 10% sucrose until further characterization.

SDS-PAGE, Isoelectrofocusing, and Western Immunoblots—SDS-PAGE was carried out as described previously (4). The pl’s were estimated using an Amersham Pharmacia Biotech PhastSystem with precast PhastGel IEF 3–9 gels. For Western blots, proteins in SDS-PAGE gels were electrotransferred to Immobilon-P membranes (Millipore) and detected immunologically (4).

Molecular Mass Determination by HPLC and FPLC—The HPLC size-exclusion chromatography was performed with a TSK G3000 SW column (15). In FPLC, 25 μl of the purified sample was loaded onto a Superose 12 HR 10/30 column (Amersham Pharmacia Biotech), before being eluted with Bicine buffer complemented with 0.15 M NaCl at 0.4 ml/min.

Purification of Monospecific Anti-PSA Polyvalent Antibodies—IgG of heat-treated goat anti-rat PSA antisera (a generous gift from Dr. L. Hersh) was purified by column chromatography on Protein G-Sepha-
hypo- or hypertonic shock with NaCl. In subcellular distribution, the enzyme activity was higher in the synaptosomes, of which the lysate had a specific activity 350% of that of the cytosol (S2) fraction, and the membranes, 200%. On the other hand, the specific activity of the total aminopeptidase was 80% in the former and 90% in the latter.

The enrichment of the neural aminopeptidase in synaptosomes prompted us to study its activity in culture cells. In primary cultures, its activity was 33% of the total aminopeptidase activity in rat cerebrocortical neurons, 12% of that in cerebellar granule cells, but absent in mouse astrocytes. Neural aminopeptidase could not be found in glioma C6 and neuroblastoma SK-N-SH cells. The findings indicate that the enzyme is unique to the CNS neurons.

Purification of Neural Aminopeptidase—The enzyme was purified from rat brain for further characterization. Its soluble extract was first concentrated and purified by (NH4)2SO4 precipitation (Table II). The subsequent hydrophobic interaction chromatography on phenyl-Sepharose gave a 10-fold purification of the enzyme. The step also reduced the salt content of the sample, making it suitable for the succeeding ion-exchange chromatography on Q-Sepharose. Gel filtration chromatography on Sephadex G-200 gave a slight purification, but it changed the sample buffer for the later FPLC. Neural aminopeptidase and PSA with the activity in a 1:5 ratio were co-purified from purification step 1 through 6. The two aminopeptidases were resolved from each other by Mono Q FPLC, with neural aminopeptidase (fractions 43–50) eluted after PSA (fractions 33–40). The isolated neural aminopeptidase was further refined with an additional Mono Q FPLC. In the second Mono Q FPLC, a single enzyme peak (fractions 38–45) was found to coincide with the only protein peak that was detected by UV. The results of our purification of the aminopeptidase, with a complete purification taking 6–7 days, are summarized in Table II. The overall purification of the enzyme through step 8 was 4933-fold with 9.3% recovery.

Enzyme Stability—The purified enzyme completely lost its activity upon freezing and thawing. DTT was essential to maintain the enzyme activity; in Bicine buffer, it retained 85% of the activity at 4 °C for 3 days. When the purified enzyme was kept in 10% sucrose at −80 °C, more than 95% of the enzyme activity could be recovered after 6 months of storage.

Analysis of Enzyme Homogeneity—Neural aminopeptidase was purified to homogeneity as analyzed by polyacrylamide gel electrophoresis. A solitary protein species was observed for the purified enzyme after electrophoresis on a 10% polyacrylamide slab gel under denatured or undenatured conditions (Fig. 2). In undenatured PAGE, identical RF values were found for the protein and the aminopeptidase activity detected after being extracted from the excised gel, or by in situ detection with stabilized diazonium salt following incubation with βNA substrates.

The purified enzyme has a symmetrical pH profile between 5.5 and 8.5 with an optimum of 7.0. After the enzyme was

![Fig. 1. Chromatograms of aminopeptidases by automated FPLC aminopeptidase detection. Purified neural aminopeptidase (0.39 μg; A) was submitted to FPLC coupled with an automated aminopeptidase detector. The enzyme activity was measured by fluorescence of the released βNA as described under “Experimental Procedures.” Neural aminopeptidase (NAP) was compared with purified PSA (0.45 μg; C) or soluble fraction of 1.25 mg of rat brain (B) and kidney (D).]
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**Distribution of neural aminopeptidase and puromycin-sensitive aminopeptidase in different rat tissues**

| Tissues                  | Enzyme activity* |
|--------------------------|------------------|
| Whole brain              | NAP 400          |
| Hippocampus              | 538              |
| Cerebral cortex          | 430              |
| Hypothalamus             | 320              |
| Striatum                 | 380              |
| Corpus callosum          | 375              |
| Midbrain                 | 240              |
| Cerebellum               | 140              |
| Medulla oblongata        | 142              |
| Spinal cord              |                  |
| Cervical                 | 141              |
| Sacral                   | 165              |
| Olfactory bulb           | 230              |
| Kidney                   | 250              |
| Spleen                   | 88               |
| Heart                    | 67               |
| Intestinal mucosa        | 67               |
| Skeletal muscle          | 55               |
| Testis                   | 45               |
| Liver                    | 42               |
| Adrenal gland            | 34               |
| Serum                    | 2.5              |

* Arbitrary fluorescence units; ND, not detectable (<0.1 unit). 50 μl of S3 fraction of 2.5 mg of tissue or serum was submitted to the automated FPLC-aminopeptidase detector. Each value represents the average of three experiments with ±5% variations. NAP, neural aminopeptidase; PSA, puromycin-sensitive aminopeptidase.

Molecular Mass Determinations—In SDS-PAGE, the molecular mass of the purified enzyme was estimated to be 110 kDa. By HPLC size-exclusion chromatography on TSK-Gel G3000 SW, or by FPLC on Sepharose 12 HR, the molecular mass of the enzyme was also estimated to be 110 kDa. The results indicate that neural aminopeptidase is a single-chain polypeptide of 110 kDa in molecular mass.

Metal Effects—Divalent metals (0.05–1 mM) inhibited neural aminopeptidase in a dose-dependent manner, and with an effect proportionately correlated to their atomic weights. At 1 mM, Co(II) and Cu(II) each inhibited the activity by 92%, Mn(II) 75%, Zn(II) 63%, Ca(II) 18%, and Mg(II) 0%. When neural aminopeptidase was dialyzed against 5000 volumes of 2 mM FPLC-aminopeptidase detector. Each value represents the average of PSAs, puromycin-sensitive aminopeptidase.

Inhibitor Profile—Neural aminopeptidase was insensitive to the aspartic protease inhibitor pepstatin and the serine protease inhibitor phenylmethylsulfonyl fluoride (Table IV). The cysteine protease inhibitor p-chloromercuriphenylsulfonic acid strongly inhibited the purified enzyme, followed by cystatin > E-64 > N-ethylmaleimide. That neural aminopeptidase is a thiol-dependent enzyme is further supported by the necessity of DTT for optimal enzyme activity, preparation, and storage. The enzyme is sensitive to chelating agents; at 1 mM, EDTA and 1,10-phenanthroline inhibited 90% of the enzyme activity.

Neural aminopeptidase was susceptible to aminopeptidase inhibitors including actinin, araphenine B, probestatin, and H-boroPheC6H12 (Table IV). Amastatin was the most potent inhibitor with a Ki of 0.04 μM, followed by bestatin (0.19 μM), proctolin (0.76 μM), puromycin (0.95 μM), and Arg⁴-enkephalin (4.75 μM). The enzyme was insensitive to inhibitors of endopeptidase 24.11, angiotensin II convertase, and carboxypeptidase.

Western Blot Studies, Glycoprotein Detection, and N-terminal Determination—In Western blots, neural aminopeptidase reacted equimolarly to the monospecific polyclonal anti-PSA IgG purified by affinity chromatography using PSA as a ligand (Fig. 3). After SDS-PAGE and treatment with hydriodic acid, the purified enzyme firmly bound to biotin hydrazide and presumably glycoprotein. In peptide microsequencing with Edman degradation, PTH-amino acids could not be detected for 20 cycles. We then directly submitted 25 μg of the purified enzyme, loaded onto a small piece (1 × 10 mm) of Immobilon-P membrane, to microsequencing. PTH-amino acid could not be detected either. However, when we used either procedure for the purified PSA, we could detect and identify PTH-amino acids from the first up to the 18th cycle. These results show that the N terminus of the purified enzyme is blocked, which is different from the naked ones of all known aminopeptidases (GenBank).

Difference between Neural Aminopeptidase and PSA—To further clarify that the purified aminopeptidase is not the common PSA, we compared it directly with the PSA purified from the same enzyme preparation. After the first Mono Q FPLC, the active PSA fractions (fractions 33–40) were combined. The sample was desalted with Amicon UM-10, then submitted to a second Mono Q FPLC with the chromatographic conditions identical to the first one. The active fractions were pooled and used for the comparison studies.

PSA was purified to homogeneity by 1423-fold with a 7.7% yield. The purified PSA, with a specific activity of 70 nmol/mg protein per min, was free of neural aminopeptidase. The molecular mass of PSA, as determined by SDS-PAGE and size-exclusion HPLC, was estimated to be 100 kDa. It has a pI of 5.1 and a neutral pH optimum of 7.0. Despite the fact that it was glycoprotein-positive as detected with the GlycoSystems Glyco Track kit, its N-terminal sequence was found to be KRPFERLPTEVSPINY, identical to that of the rat, human, and mouse brain PSA (2, 6, 9). These results show that the purified PSA is the well known puromycin-sensitive aminopeptidase.

The divalent metal effects on the holoenzyme and the apon-
zyme of neural aminopeptidase and PSA are distinct. In contrast to neural aminopeptidase, Mg(II) activated the PSA enzyme activity in a dose-dependent mode, by 30% at 1 mM. In contrast to neural aminopeptidase, Mg(II) activated the PSA enzyme activity in a dose-dependent mode, by 30% at 1 mM. Whereas Ca(II) was ineffective. Third, the EDTA-treated PSA lost more (86%) of its activity, which Mg(II) was unable to restore, whereas neither Co(II), Zn(II), nor Ca(II) up to 1 mM was unable to restore, whereas neither Co(II), Zn(II), nor Ca(II) up to 1 mM inhibited the PSA apoenzyme.

Leu βNA was the best substrate for PSA followed by Met βNA > Arg βNA > Lys βNA > Ala βNA > Tyr βNA > Phe βNA (Table III). Its \( k_{\text{cat}}/K_m \) was 3 times that of neural aminopeptidase with Leu βNA and 1.5–2 times with enkephalins. Compared with neural aminopeptidase, PSA was more susceptible to certain aminopeptidase inhibitors: proestatin, actininon, araphenine, and H-boroPhe\( _6 \)H\(_{12} \) (Table IV). But it was less sensitive to the inhibition of puromycin (\( K_i = 4.9 \mu M \)), amastatin (0.06 \( \mu M \)), and Arg\(^\text{\textbeta} \)-enkephalin (5.1 \( \mu M \)). The log-dose effect curve of puromycin inhibition of PSA shifts to the right of that of the neural aminopeptidase (Fig. 4). Neural aminopeptidase is the aminopeptidase most sensitive to puromycin, 4 times more than PSA.

**DISCUSSION**

Brain function depends upon the synaptic interactions between neurons. The particular distribution of the CNS peptidase neurons and the high peptide concentration in the nerve terminals indicates that a mechanism specifically mediates neuropeptide biosynthesis and catabolism. Although the exact mechanism is unknown, it is generally agreed that enkephalins are inactivated by enzymatic degradation. Several peptidases present in CNS are able to cleave enkephalins at different sites: aminopeptidases hydrolyzing the Tyr-Gly amide bond, dipeptidyl aminopeptidase cleaving the Gly-Gly bond, and angiotensin II convertase and endopeptidase 24.11 both splitting the Gly-Phe bond. The enzyme kinetics and the ubiquitous distribution indicate that angiotensin II convertase (19), dipeptidyl aminopeptidase (20), and endopeptidase 24.11 (21) are nonspecific peptidases in neural tissues. The major mode of enkephalin inactivation in vitro and in vivo is the liberation of Tyr by neutral aminopeptidases, which are particularly abundant in brain (22).

We have developed a specific and sensitive assay for individual brain aminopeptidases to study their function (10). The method exploits the specificity of a continuous flow enzyme assay with Leu βNA and the high resolution FPLC separation. With this method, neural aminopeptidase is separated from all other aminopeptidases, as well as endogenous inhibitors and substrates that interfere in peptidase assays in crude tissue extracts (10).

Neural aminopeptidase was found to be present only in the CNS. It could not be detected in peripheral tissues, serum, or sciatic nerve. The culture study further indicates that the aminopeptidase is a neuron-specific enzyme since it is absent in other nerve cells. Interestingly, the enzyme is also not found in the undifferentiated neuroblastoma SK-N-SH. Its heterogeneous distribution in the CNS, with the highest activity in the hippocampus, suggests its involvement in regional, specific neural function. The enrichment of the enzyme in the synaptosomes shows that neural aminopeptidase is a nerve-ending enzyme. Since the neuroblastoma clone is unable to establish functional synapses, there is a possible relationship between the expression of neural aminopeptidase and the capability for advancing in the differentiation and achieving a fully differentiated state. However, immunohistochemistry studies are desired to determine its specific location in axon or dendrite.

The absence of neural aminopeptidase in a non-mammalian species, the chicken, may be significant. Since neural aminopeptidase is also absent in the peripheral nerve, retina, and pituitary gland, it may have arisen late in evolution and be associated with higher brain function in mammals. Further examination of the phylogenetics of neural aminopeptidase heterogeneity would be of interest.

Our final preparation of the enzyme appeared to be homogeneous by PAGE and isoelectrofocusing. The molecular weight estimated by HPLC and FPLC gel filtration and by SDS-PAGE suggests that neural aminopeptidase is a monomer with a molecular mass of 110,000 daltons. The data suggest that the peptidase is a metallo-enzyme; however, the identity of the metal awaits a metal analysis of the purified enzyme. Whether the thiol-dependent aminopeptidase is a cysteine enzyme containing a catalytic dyad of cysteine and histidine is unknown.

Neural aminopeptidase has a neutral pH optimum and a selective substrate specificity. It is a neutral arylamidase that is most sensitive to amastatin. The purified enzyme is neither a dipeptidase, tripeptidase, nor dipeptidyl aminopeptidase I–V, nor an endopeptidase, because it is inactive on their specific substrates. Since the enzyme is insensitive to their specific
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### Table III

| Substrate | NAP | PSA |
|-----------|-----|-----|
| aa βNA    |     |     |
| Leu       | 12.3| 72.3|
| Lys       | 30.8| 36.0|
| Arg       | 30.8| 27.2|
| Met       | 15.4| 33.8|
| Ala       | 15.4| 27.2|
| Tyr       | 11.2| 18.7|
| Phe       | 17.5| 43.3|
| Ile       | 3.33 |6.18 |
| Enkephalins | | |
| MEK       | 7.80 |10.3 |
| LEK       | 3.24 |12.9 |

| k<sub>cat</sub>/K<sub>m</sub> | NAP | PSA |
|---------------------------|-----|-----|
| aa βNA                   |     |     |
| Leu                      | 11.2| 36.2|
| Lys                      | 5.60| 7.2 |
| Arg                      | 5.60| 9.07|
| Met                      | 4.82| 10.2|
| Ala                      | 1.40| 2.72|
| Tyr                      | 0.93| 2.08|
| Phe                      | 0.80| 0.87|
| Ile                      | 0.003|0.006|
| Enkephalins              |     |     |
| MEK                      | 0.08 |0.13 |
| LEK                      | 0.05 |0.11 |

| K<sub>m</sub> | NAP | PSA |
|---------------|-----|-----|
| aa βNA        |     |     |
| Leu           | 1   | 2  |
| Lys           | 5   | 5  |
| Arg           | 5   | 3  |
| Met           | 2.9 | 3.3|
| Ala           | 10  |10  |
| Tyr           | 10  | 9  |
| Phe           | 20  | 50 |
| Ile           | 909 |1000|

* The mass of neural aminopeptidase (NAP) is 110 kDa and puromycin-sensitive aminopeptidase (PSA) 100 kDa for the calculations.

### Table IV

| Addition         | Concentration | % of control<sup>a</sup> | NAP | PSA |
|------------------|---------------|--------------------------|-----|-----|
| None             |               |                          | 100 | 100 |
| Aspartic         |               |                          | 100 | 98  |
| Pepstatin        | 100 μM        |                          | 109 | 58  |
| Serine           |               |                          | 100 | 101 |
| SBTI             | 100 μg/ml     |                          | 59  | 109 |
| Aprotinin        | 250 μg/ml     |                          | 60  | 18  |
| PMSF             | 500 μM        |                          | 100 | 108 |
| Serine/cysteine  |               |                          |     |     |
| Chymostatin      | 1 μg/ml       |                          | 100 | 101 |
| Leupeptin        | 20 μM         |                          | 72  | 73  |
| Antipain         | 100 μM        |                          | 14  | 2   |
| Cysteine         |               |                          |     |     |
| Cystatin         | 12.5 μg/ml    |                          | 68  | 101 |
| N-Ethylmaleimide | 100 μM        |                          | 81  | 103 |
| Iodoacetate      | 20 μM         |                          | 132 | 114 |
| PCMPS            | 20 μM         |                          | 6   | 1   |
| E-64             | 2 μM          |                          | 80  | 100 |
| Metallo          |               |                          |     |     |
| S-OH-Quinoline   | 1 mM          |                          | 100 | 104 |
| 1,10-Phenanethione | 1 mM |                   | 9   | 13  |
| EDTA             | 1 mM          |                          | 5   | 15  |
| APN              |               |                          |     |     |
| H-boroPheC<sub>6</sub>H<sub>12</sub> | 1 μg/ml | | 10 | 0 |
| APN/LAP          | 20 μM         |                          | 3   | 0   |
| Actinonin        |               |                          |     |     |
| APN/APB          | 1 μg/ml       |                          | 30  | 0   |
| Probestin        |               |                          |     |     |
| APB              | 20 μM         |                          | 23  | 0   |
| Arphamenine B    |               |                          |     |     |
| CP               | 100 μg/ml     |                          | 100 | 100 |
| ACE              | 100 μM        |                          | 85  | 90  |
| NEP              |               |                          |     |     |
| Thiopran         | 0.3 μM        |                          | 100 | 100 |

* Each value represents the average of three experiments with ±5% of variations. The inhibitors were incubated with 15 ng of neural aminopeptidase (NAP) or 12 ng of puromycin-sensitive aminopeptidase (PSA) with 20 μM of Bicine buffer for 15 min. The reactions were terminated with 1.8 ml of 0.11 M HCl and measured by fluorometry. ACE, angiotensin II convertase; APB, aminopeptidase B; APN, aminopeptidase N; CP, carboxypeptidase; GEMSA, guanidino-ethyl mercaptosuccinic acid; LAP, leucine aminopeptidase; NEP, endopeptidase 24.11; PCMPS, p-chloromercuriphenylsulfonic acid; PMSF, phenylmethyl sulfonyl fluoride; SBTI, soybean trypsin inhibitor.

Inhibitors, it is obviously not an endopeptidase 24.11, an angiotensin II convertase, or a carboxypeptidase. Its molecular mass also excludes the purified enzyme from being a cytosol alanyl aminopeptidase, leucine aminopeptidase, aminopeptidase N, aminopeptidase A or B (23), or aspartyl aminopeptidase (24).

In view of the extensive work that has been carried out on brain aminopeptidases, it is surprising to find an enzyme specific to the CNS. The failure to observe this enzyme in the past was probably due to its resemblance to PSA. Neural aminopeptidase, a minor enzyme, could be easily overlooked and lost during brain PSA purification by preparative column chromatography.

Neural aminopeptidase and PSA can be physicially resolved by ion-exchange chromatography. Their distribution in tissues,
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TABLE V
Comparison of neural aminopeptidase and puromycin-sensitive aminopeptidase

| Distribution | NAP | PSA | PSA |
|--------------|-----|-----|-----|
| Subcellular location | CNS | Ubiquitous | Ubiquitous |
| Mass | 110 kDa | Cytoplasm | Cytoplasm |
| pH | 5.25 | PEKRPERLPTEVSPINY | PEKRPERLPTEVSPINY |
| N-terminal sequence | N-blocked | Mg(II) | Mg(II) |
| IC₅₀ (µM) of puromycin | 1.9 | Co(II) > Zn(II) | Co(II) |
| Metal inhibition | None | Mg(II) | Mg(II) |
| Metal activation | Mg(II) | Mg(II) > Co(II) | Mg(II) |
| Restoration from EDTA treatment | Co(II) > Mg(II) > Zn(II) | Co(II) > Mg(II) > Mn(II) |
| kₑ₋₆₄ | Leu > Lys > Arg > Met > Ala | Leu > Met > Arg > Lys > Ala |
| Reported in references | This study | This study | 2, 6, 9 |

* Amino acyl β-naphthylamides were used as substrates. NAP, neural aminopeptidase; PSA, puromycin-sensitive aminopeptidase.

brain regions, cells, and subcellular fractions is unmatched (Table I). The difference in molecular mass, pH, metal effects, inhibitor specificity, and hydrolytic coefficient demonstrates that neural aminopeptidase is distinct from PSA. Although both enzymes are inhibited by puromycin, neural aminopeptidase is 4 times more sensitive than PSA (Fig. 4). Thus, neural aminopeptidase is the conceivable in vivo target enzyme for puromycin to exert its effect in analgesia (1), apoptosis (2), and amnesia (3).

The N-terminal sequences of the neural aminopeptidase and PSA are different; the latter is identical to that of PSA of rat (6) and the deduced PSA sequence of mouse neuroblastoma (2) and human brain (9). Although both are glycoproteins, neural aminopeptidase is not a possible post-translational modification of PSA, or vice versa. Neural aminopeptidase is a highly active enzyme in rat brain, with a specific activity 70% and protein amounts one-third of PSA. In addition, because their activities in different tissues are not parallel (Table I), neural aminopeptidase is not a PSA precursor. Further, the cDNA sequence shows that active PSA is not processed from a zymogen (2, 9).

As judged by the cross-immunoreactivity, neural aminopeptidase and PSA share some common epitopes. We used the anti-PSA IgG to screen a rat brain cDNA expression library and isolated an 1561-base pair cDNA. It has a 95.8% sequence homology to the mouse neuroblastoma PSA.2 Using this cDNA as a probe, we cloned a candidate 1404-base pair cDNA (63.2% homology to the mouse neuroblastoma PSA) encoding the N-terminal section of neural aminopeptidase. The nucleotide segment position at 875–1404 is homologous to position 1087–1613 of PSA (96.4% identity), containing a sequence encoding a divalent metal-binding motif, HEXXXH(X)ₓE, of aminopeptidases (25). 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 PEKRPERLPTEVSPINY of PSA (2, 6, 9). The blocked N-terminal residue, though, awaits to be identified. The cloning data imply that the purified enzyme is a unique aminopeptidase and a possible member of the PSA superfamily. Hence, the study on the genetic relationship of neuron-specific aminopeptidase and the nonspecific PSA should be fruitful.

The primary structure, the physicochemical nature, and biochemical character demonstrate that the purified enzyme is a novel aminopeptidase, whereas the purified PSA is similar to the well documented PSA (Table V). Aminopeptidases sensitive to puromycin are believed to be involved in the inactivation of enkephalins, dynorphins, cholecystokinin, somatostatin, oxytocin, angiotensin, procolitin, and vasopressin (5). Neural aminopeptidase and PSA act differently toward synthetic substrates. They may contrast in their activity toward certain physiologically important neuropeptides and endogenous inhibitors. The enrichment of neural aminopeptidase in rat nerve endings and its exclusiveness in rat primary neurons imply a functional role for neural aminopeptidase different from that of the housekeeping PSA.

Although peptidases of high specificity do exist, a neuropeptide-specific peptidase has not been found. A site-specific peptidase is therefore the candidate regulator of neuropeptide activity. In the model for “synaptic peptidases,” it is desirable to have an ecto-enzyme that is near the synaptic cleft and recognizes only a specific bond in a specific peptide, which it could cleave to effect the inactivation of the synaptic effect of the peptide. Indeed, part (15%) of the neural aminopeptidase is membrane-associated. But its major (85%) intracellular localization argues against its being a classic model synaptic peptidase. It has been recently shown that endogenous opioid peptides primarily act nonjunctionally on the plasma membranes of dendrites, axons, and probably, neuronal perikarya (26). Thus, regulation of peptide degradation in the neuropeptide-releasing neuron and the neuron responsive to the peptide could both be important determinants of the physiological roles of peptidergic neurons. It is also possible that peptides are continuously produced at a high level and are degraded after production, with degradation inhibited during stimulation of release. The pre-synaptic terminal regulation of degradation would allow the peptidergic neurons to store releasable peptides that could be relatively independent of axoplasmic transport. Furthermore, degradation of synaptic neuropeptides could also occur through the actions of soluble, secreted peptidases (27). The exact role of neural aminopeptidase in opioid peptide regulation of synapse needs further investigation.

In summary, we have identified and purified a novel aminopeptidase localized exclusively in the CNS. The thiol-dependent metallo-enzyme releases N-terminal amino acids from enkephalins and βNAs. It is the aminopeptidase most sensitive to puromycin inhibition. The enzyme is the first peptidase found to be specific to neurons. The peptidase, enriched in nerve endings, may have an important role in different synaptic functions. These are under investigation in our laboratory.

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