Neuropeptide Y (NPY) is a 36-residue-long neuropeptide which has been implicated in the regulation of feeding behavior and modulation of the circadian rhythm. We identified the primary structure of the endogenous NPY-immunoreactive material in the rat hippocampus using a combination of chromatographic techniques and nanospray mass spectrometry. The major component in the brain tissue corresponded to the authentic amidated form of NPY(1–36). The fate of NPY in the central nervous system was studied by subjecting pure peptide to the protease(s) present in hippocampal synaptic clefts in vitro. The enzyme revealed properties of aspartic protease, being blocked by pepstatin and having a pH optimum between 4 and 5. The results indicate that NPY and its inactivation pathway in the brain, which is different from that found in the peripheral, may have important consequences in vivo.

Neuropeptide Y (NPY) is a 36-amino-acid-long peptide with an amidated C terminus, first isolated by Tatemoto and coworkers in 1982. The peptide belongs to the pancreatic polypeptide family (1) and is one of the most abundant found in the central nervous system (2). NPY has been implicated in several central regulatory functions such as circadian rhythm (3) and feeding behavior (4). Stimulation of either the central noradrenergic or NPY pathways activates the hypothalamic-pituitary-adrenocortical axis in the rat and in conscious sheep (5). In a recent study (6) we found increased concentrations of NPY-like immunoreactivity (NPY-LI) in specific rat brain regions following repeated electroconvulsive shocks. The measured NPY-LI consisted of intact NPY(1–36) and a component anticipated to be NPY sulfoxide. This finding was thought to be of importance due to different biological properties of NPY(1–36) compared to the effects of its C-terminal homologues (7, 8) on the different receptor types for NPY (8–11). To characterize the existing peptide pool in a tissue, various chromatographic techniques combined with immunochemical methods are usually used. However, one limiting factor in the identification of the peptide homologues in biological material is the specificity of the antisera used.

Little is known about the fate of NPY once it is released into the synaptic cleft in the central nervous system. The C terminus of NPY is amidated, protecting the molecule against an attack of carboxypeptidases. So far, it has only been shown that NPY is degraded in the periphery by endopeptidase-2 (12) and dipeptidyl peptidase IV (13). In a study on cultivated neurons and glial cells (14) it has been found that serine protease can play an important role in extracellular processing of NPY. In the present study we intended to reveal the structure of existing endogenous NPY in the rat brain and to investigate its inactivation using nanospray mass spectrometry (ESI-MS). This technique, based on the very low flow rates, utilizes extremely low amounts of biological material which can be unambiguously characterized for a relatively long time (15). Hippocampus was chosen in this study because it contains high concentrations of NPY and because this tissue is the major site for the Y2 receptor, which has a high affinity to C-terminally truncated homologues of the peptide (16, 17).

EXPERIMENTAL PROCEDURES

Materials—Synthetic human/rat NPY was purchased from Peninsula (St. Helens, United Kingdom). The C1-NPY antisera was obtained from professor Elvar Theodorsson, University Hospital, Linköping, Sweden. Proteinase inhibitors and 1-heptanesulfonic acid were purchased from Sigma-Aldrich (Stockholm, Sweden), and CoCl2, ZnCl2, HgCl2, and CaCl2 were from ICN Pharmaceuticals (Costa Mesa, CA). Laboratory reagents of the analytical grade were purchased from Merck unless indicated otherwise.

Characterization of Endogenous NPY Tissue Preparation—Ten male Sprague-Dawley rats (ALAB, Sollentuna, Sweden) weighing 250 g were sacrificed by decapitation. Immediately after death, the hippocampi were dissected according to the method of Glowinski and Iversen (18), weighed, and stored at −80 °C until extraction. The frozen hippocampi (approximately 1.2 g) were cut into small pieces and boiled for 10 min in 10 volumes of 1 M acetic acid. After homogenization, the sample was centrifuged at 2500 × g for 10 min. The supernatant was lyophilized and stored at −80 °C until used.

Radioimmunoassay—NPY-LI was analyzed using the C1 antisera which cross-reacts with human/rat NPY (100%), porcine NPY (107%), sulfonlated NPY (88%), peptide YY (63%), including their C-terminal fragments, except (Leu11-Pro14)-NPY and NPY(1–21) (6). HPLC-purified 125I-Bolton-Hunter-labeled NPY was used as radioligand and human/rat NPY as a standard.

Size-Exclusion Chromatography—The sample was redissolved in 2.5 ml of H2O and passed through a 0.45-μm Millipore filter before being applied onto a Sephadex G-50 Superfine column (2.5 × 95 cm, Pharmacia Biotech Inc.). The column was eluted with 720 ml of 1 M acetic acid at a flow rate of 0.5 ml/min. Four-ml fractions were collected, and 10-μl aliquots of each fraction were tested for NPY-LI with a competitive radioimmunoassay. The column was calibrated in a separate run.
with 1 pmol of synthetic human/rat NPY(1–36).

Reversed-phase High-performance Liquid Chromatography (RP-HPLC)—Two different columns were subsequently used for HPLC purifications. A Delta Pak RP-HPLC column (0.9 × 150 mm, Waters Sverige AB, Sweden) was connected to the SMART micropurification system (Pharmacia) and eluted with a 40-ml linear gradient of acetonitrile (20–50%) supplemented with 0.1% trifluoroacetic acid. Flow rate was maintained at 0.5 ml/min. One-ml fractions were collected and 10-μl aliquots of each fraction were tested for NPY-LI. Active fractions were pooled and applied on a µRPC C2/C18 column (2.1 × 100 mm, Pharmacia) connected to the SMART system. The column was eluted with a 5-ml linear gradient of acetonitrile (32–43%) supplemented with 0.1% trifluoroacetic acid. Flow rate was adjusted to 0.1 ml/min. 100-μl fractions were collected and the NPY-LI was assessed in 5 μl of each fraction, as described above.

Ion-pair Reversed-phase HPLC—A PepRPC HR 5/5 column (5 × 50 mm, Pharmacia) connected to the SMART system via a short column holder (Pharmacia) was used. Immunoreactive fractions collected from the µRPC column and corresponding to the intact NPY were pooled, and a part of it was subjected to the ion-pair chromatography. The column was eluted with a 40-ml linear gradient of methanol (20–100%) supplemented with 0.1% trifluoroacetic acid and 0.1% heptanesulfonic acid at a flow rate of 1 ml/min. 0.5-ml fractions were collected and the NPY-LI content was measured in 5-μl aliquots of each fraction.

Electrospray Ionization Mass Spectrometry—The system used was a Finnigan MAT 95Q (Finnigan MAT, Bremen, Germany) equipped with an atmospheric pressure ionization source, as described previously (19). Immunoreactive samples containing endogenous NPY material were introduced via a Valco injector equipped with a 5-μl loop. The loop was loaded with the analyte and the material was eluted at a flow rate of 200 nl/min. No sheath gas was applied in this case.

Liquid chromatography-mass spectrometry was performed essentially as described in a previous paper (19) using the SMART system connected to the ESI interface. The samples were separated on a reversed-phase column µRPC C2/C18 (2.1 × 100 mm) using a 3-ml linear gradient (0–60%) of acetonitrile/0.1% trifluoroacetic acid. The flow rate was maintained at 0.1 ml/min.

Tryptic Digestion of Endogenous Material—The NPY sample was desalted on a fast desalting column (Pharmacia). After evaporating to near dryness, the sample was digested overnight with 0.5 μg of modified trypsin (Promega, Madison, WI) in 0.1 m ammonium bicarbonate at 30 °C. The generated peptides were isolated by RP-HPLC on a µRPC C2/C18 SC prototype column (1 × 100 mm, Pharmacia) connected to the SMART system, using a 4-ml acetonitrile/0.3% trifluoroacetic acid gradient (0–40%) at a flow rate of 25 μl/min. By comparing with the SwissProt (updated 8/18/96) and NCBI (version 9/5/96) data bases available on the Internet, the unique pattern of trypptic fragments detected by ESI-MS was identified (20). The searching programs were PepptideSearch (EMBL Protein and Peptide Group, Heidelberg, Germany) and the MS-Fit (UCSF Mass Spectrometry Facility, San Francisco, CA). The monoisotopic mass tolerance was set to 0.5 Da, and the number of missed cleavages was set to 1. The charge states of the particular ions were determined using isotope distribution measured at a moderate resolution of 5000 (10% valley) and by applying PepMatch software supplied by the Finnigan MAT.

Characterization of Enzymatic Activity—Synaptosomes were isolated from four hippocampi following a procedure described elsewhere (21). The synaptosomal pellet was resuspended in 500 μl of 10 mM Tris-HCl buffer (pH 7.4) and kept at –80 °C until used. The synaptosomal preparation was sonicated before use. The typical assay mixture consisted of 10 μl of the synaptosomal preparation (diluted 20 times with the appropriate buffer) and 5 μg of human/rat NPY as a substrate in a total volume of 20 μl. Aliquots were withdrawn after various time intervals, adjusted to 10 μl with 30% acetonitrile/0.1% formic acid, and subjected to analysis by ESI-MS. Inhibitory studies were performed under the above described conditions at optimal pH (pH 4), but the enzyme preparations were preincubated with the respective inhibitor for 30 min at 37 °C followed by addition of the substrate. Appropriate blanks were added where necessary.

RESULTS

Characterization of Endogenous NPY in Rat Hippocampus—The extracted sample from rat hippocampi was submitted to size-exclusion chromatography on a Sephadex G-50 Superfine column and eluted as two major components (fractions 71–78 and 145–150) and one minor component (fractions 104–112) of NPY-LI (Fig. 1A). The first component co-eluted with synthetic human/rat NPY(1–36). Further purification of the NPY-LI fractions was achieved by two consecutive RP-HPLC runs. In the first step, fractions 71–78 and 145–150 from the size-exclusion chromatography were pooled and applied on the HPLC column in separate runs; however, only the first pool possessed NPY-LI (Fig. 1B). Fractions 16–19, co-eluting with authentic NPY, were analyzed by radioimmunoassay. The dotted lines indicate the gradients used. The elution position of synthetic NPY(1–36) is indicated with an arrow in A.

Fig. 1. Chromatographic profile of NPY-like immunoreactive material extracted from 10 rat hippocampi. Material was extracted after size-exclusion chromatography on a Sephadex G-50 column (2.5 × 50 cm) (A), RP-HPLC on a Delta Pak column (0.9 × 150 mm) eluted with a gradient of 20–50% acetonitrile/trifluoroacetic acid (B), RP-HPLC on a µRPC C2/C18 SC 2.1/10 (2.1 × 100 mm) eluted with a gradient of 32–43% acetonitrile/0.1% trifluoroacetic acid (C), and ion-pair RP-HPLC on a PepRPC HR 5/5 (5 × 50 mm) column eluted with a gradient of 20–100% methanol/trifluoroacetic acid/heptanesulfonic acid (D). Fractions were collected and NPY-LI analyzed by radioimmunoassay. The dotted lines indicate the gradients used. The elution position of synthetic NPY(1–36) is indicated with an arrow in A.
present in fractions 13–14 from the second RP-HPLC step were pooled, evaporated, and then redissolved in 25 μL of 30% acetonitrile/0.1% formic acid. This material was directly analyzed (two times at 400 fmol) by nanospray mass spectrometry. Fig. 2A shows the obtained mass spectrum containing the multiply charged species of rat NPY at m/z of 610.1 (+7), 712.9 (+6), and 855.7 (+5). The spectra were accumulated and deconvoluted as shown in Fig. 2B, revealing the molecular mass of 4270 corresponding to the intact sequence of rat/human NPY(1–36). The content of fractions 8–9 (Fig. 1C) was analyzed in a similar way by the ESI-MS. This resulted in detection of the salt adducts (data not shown), and no peptide could be seen in this sample. The purity of the preparation subjected to nanospray analysis was estimated using intensities of the contaminating ion shown in Fig. 2A (integrated area of each component in the ion chromatogram). The molecular masses of the contaminant(s) did not correspond to any pro-NPY-derived fragment. The NPY preparation was approximately 80% homogenous; therefore it was further purified by ion-pair RP-HPLC (Fig. 1D) before tryptic digestion. The NPY structure was verified by searching the protein databases (SwissProt and NCBI) for the unique set of tryptic fragments prepared from the additional 4 pmol of endogenous material (Fig. 1D). Three fractions were identified along the chromatogram: fragment T2 at m/z 772.4 (NPY 20–25), T3 at m/z 1029.6 (NPY 26–33), and a triply protonated ion T1 + T2 at m/z 938 (NPY 1–25) (molecular mass 2812.3). The MassSearch program does not list the number of entries searched. In contrast, the MS-Fit program has selected 5,500 of 209,493 entries, using a combination of molecular weight (0.1–100 kDa) and defined species (Rattus norvegicus). The results from both searching procedures suggested only one solution to this combination and thus was consistent with the intact NPY molecule (SwissProt, P09640; NCBI, 100226 3 128120).

**Metabolism of NPY in the Rat Hippocampus**—Metabolism of NPY in the rat hippocampus was studied using both membrane preparations (synaptosomal) and cytosolic extracts at various pH values. An on-line liquid chromatography-mass spectrometry experiment was performed to disclose any suppressed fragments which could be missed during direct analysis (Fig. 3A). Human/rat NPY was efficiently cleaved by the proteinase present in the synaptosomal fraction of the hippocampus. ESI-MS revealed that NPY was processed between Leu30-Ile31. The cleavage gave rise to two fragments, NPY(1–30), with a molecular mass of 3454, and its C-terminal counterpart NPY(31–36), with a molecular mass of 835 (Fig. 3B).

The proteolytic activity cleaving NPY was studied using various proteinase inhibitors. Table I summarizes the data, which indicate that pepstatin, Zn2++, and Hg2++ were the most effective. Time-dependent studies using pepstatin showed that this inhibitor was effective even during a prolonged incubation time (data not shown). The pH optimum for the proteolytic enzyme was between 4 and 5 (Fig. 4).

**DISCUSSION**

This study, based on the mass spectrometric verification of the peptide identity, indicates the presence of an intact NPY(1–36) molecule in the rat brain hippocampus. Our earlier findings, based on an immunological technique, anticipated that only NPY(1–36)-immunoreactive material could be detected in this tissue (6), which consisted of NPY and its sulfoxidated form. The limiting factor in such studies is the specificity of the

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**Fig. 2. Characterization of endogenous NPY by nanospray MS.** A, accumulated mass spectrum showing the multiply charged species of endogenous NPY. m/z, mass-to-charge ratio. B, deconvoluted ESI spectrum revealing the molecular weight of intact amidated NPY.
antiserum used which, in our study, was directed toward the C-terminal portion of the NPY molecule (6). Sulfoxidated NPY (if existing) should have been eluted around fractions 8–9 (Fig. 1C) from the reversed-phase column under the conditions tested. The estimated amount of material present in this immunoreactive pool is low (approximately 25%) in relation to the total NPY-LI content, and this observation is consistent with our previous experiments (6). A relatively low amount of material and high salt content could simply mask the presence of another peptidergic component. The low molecular weight component (fractions 145–150, Fig. 1A) was subjected to further separation on the RP-HPLC column. Apparently, this immunoreactive pool lost its reactivity against the NPY antiserum after further purification. One explanation could be that the fractions are eluted near the total volume of the column (fractions 148–155) and that low molecular weight contaminants may contribute to the false-positive NPY-like immunoreactivity.

Using the nanospray MS technique, we were able to verify that the fractions showing NPY-LI and co-eluting with synthetic peptide in the last chromatographic step mainly consisted of a component having a molecular mass of 4270, corre-
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responding to the intact sequence of amidated rat/human NPY(1–36). This also indirectly confirms the specificity of the antiserum used in this study. Furthermore, the identity of the endogenous NPY was verified using a set of peptide fragments produced after limited digestion with trypsin. Mass spectrometric peptide mapping combined with protein data base search is a very efficient technique for the rapid identification of isolated peptides and proteins (22, 23). Here, we applied this strategy to confirm the identity of NPY(1–36) in the rat hippocampus.

Several proteinases cleaving NPY have been described, including aminopeptidase P, dipeptidylpeptidase IV (13, 24), and a phosphoramidon-insensitive endopeptidase (endopeptidase-2) (12). All of these enzymes originated from the peripheral systems. The metabolic pathway of NPY in the central nervous system, however, is yet unknown. In a recent study, Ludwig and co-workers (14) showed that cultivated neurons and microglia digested NPY with a cleavage pattern resembling the one observed when NPY was degraded by purified urokinase-type plasminogen activator, plasmin, thrombin, and trypsin. Thus, it was suggested that serine proteinases might play an important role in extracellular neuropeptide processing under physiological conditions. The present study shows that human/rat NPY was efficiently cleaved at an acidic pH by the proteinase present in the synaptosomal fraction of rat hippocampus. The cleavage pattern revealed that NPY is processed between Leu30–Ile31, thus giving rise to two fragments, NPY(1–30) at molecular mass 3454, and its C-terminal counterpart NPY(31–36), at molecular mass 835. The proteolytic activity was completely blocked by 1 μM pepstatin, thus classifying the enzyme as belonging to the family of aspartic proteinases. The enzyme was also partially blocked by the presence of Zn2+ and Hg2+. An intact C-terminal end of the NPY molecule is essential for its action upon the Y1 and Y2 receptors. For a full recognition by the NPY Y1 receptor, both the C- and N-terminal parts of the molecule are necessary. Elongated C-terminal homologues are more potent on the Y2 receptor than shorter ones (25). Thus, the C-terminal truncation of NPY observed in the present study would totally hamper the action of NPY. The cleavage pattern causing formation of NPY(1–30) and NPY(31–36) described in this work is new and cannot be caused by the presently known peptidases. Dipeptidylpeptidase IV liberates N-terminal dipeptide Tyr-Pro (13, 24), and the specificity of endopeptidase-2 was tested using a radioactive substrate without structural identification of the fragments formed during the enzymatic process (26). Nevertheless, this latter enzyme is optimally active at neutral pH and was efficiently inhibited by chelating agents, whereas the enzyme described in this paper was active at acidic pH and was classified as an aspartic protease. The physiological function of the described enzyme is not known. One possible explanation is that NPY is internalized into the neuron after it has been bound to its receptor, followed by its termination by vesicular enzymes. In this context it is interesting to mention the recent work on neurotensin (27), demonstrating the existence of a retrograde axonal transport for neurotensin in the rat brain. This peptide can be retrogradely transported in dopaminergic nigrostriatal neurons, and such transport first involves binding of neurotensin to its receptors presynaptically located on dopamine nerve terminals of the nigrostriatal pathway. Then the ligand-receptor complex can be rapidly internalized. Finally, the complex enters the perikaryon and is processed along a variety of intracellular pathways, including lysosomal inactivation.

In the present study we applied various liquid chromatographic techniques in combination with electrospray ionization mass spectrometry. This strategy was used for characterization of endogenous NPY in central nervous system tissue and also for a rapid and unambiguous verification of the cleavage pattern of the neuropeptide in selected brain structures. The detailed knowledge on NPY metabolism in the central nervous system may lead to the design and synthesis of specific inhibitors having potential therapeutic applications in, e.g., problems with food intake and metabolism, which are at least partially dependent on NPY biosynthesis and release (17, 28, 29).

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