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Review

Mass spectrometric analysis of neuropeptidergic systems in the human pituitary and cerebrospinal fluid

Dominic M. Desiderio*

Departments of Neurology and Biochemistry, Charles B. Stout Neuroscience Mass Spectrometry Laboratory, The University of Tennessee, Memphis 956 Court Avenue, Room A218, Memphis, TN 38163, USA

Abstract

Neuropeptidergic systems have been studied in human tissues and fluids, which include the pituitary and lumbar cerebrospinal fluid, respectively. This paper reviews the qualitative and quantitative mass spectrometric analytical data obtained from three areas of study. Methionine enkephalin (ME) and β-endorphin (BE) were quantified in the human pituitary by liquid secondary ion mass spectrometry (LSI MS)–tandem mass spectrometry. Corresponding stable isotope-incorporated synthetic peptide internal standards were used. Proenkephalin A and proopiomelanocortin produce ME and BE, respectively. The analysis of neuropeptides in macroadenomas demonstrated a decrease in both of those neuropeptidergic systems relative to controls. An analysis of prolactin-secreting microadenomas showed an increase in the proenkephalin A system. Mass spectrometry was also used to detect opioid peptide-containing proteins in the pituitary. Enzymes that process the precursors of proenkephalin A and tachykinin (substance P) neuropeptides were studied in human lumbar cerebrospinal fluid. Electrospray ionization mass spectrometry was used to characterize the molecular mass of each peptide product.

Keywords: Reviews; Neuropeptides; Methionine enkephalin; β-Endorphin; Proenkephalin A; Proopiomelanocortin; Tachykinin

Contents

*Tel.: +1-901-448-5488; fax: +1-901-448-7842.
E-mail address: ddesiderio@utmem.edu (D.M. Desiderio)

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1. Introduction

Neuropeptidergic systems provide an excellent window into the elucidation of several different homeostatic and pathologic conditions in human tissues and fluids. For example, neuropeptides that participate in pain [1,2], stress [3,4], tumor formation [5,6], senile dementia of the Alzheimer type [7], and other pathologies [8] have been specific targets of research. Enzymes that biosynthesize and degrade opioid and tachykinin peptides were also studied [9,10].

Opioid peptides have been the primary focus in these studies because they are generally inhibitory in cellular regulatory processes; tachykinins, a secondary focus, are generally excitatory.

This review discusses the MS–MS measurement of ME and BE in macroadenomas and in prolactin-secreting microadenomas; proteins that include opioid peptides; and enzymes in human lumbar cerebrospinal fluid (CSF) that process precursors, and that biosynthesize and degrade neuropeptides.

Mass spectrometry (MS) was used to optimize the molecular specificity of the analytical measurement during this study of endogenous human neuropeptidergic systems.

The hypothesis under study is that metabolic defects in neuropeptidergic systems contribute to macroadenoma (non-secreting tumors) formation and to idiopathic low back pain. These two pathologies were selected as model systems because they have no known mechanism.

The rationale for this type of study is that knowledge of the amount, and of the amino acid sequence, of each pertinent peptide or protein, and any difference in those parameters between controls and pathologies, can be used to elucidate the molecular basis of a pathology and possibly for effective pharmacological intervention.

2. Experimental

2.1. Acquisition of human pituitary control tissue

Post-mortem human pituitary control tissue was obtained from individuals who died from severe wounds that did not involve the head. Gunshot wounds to the chest were generally the cause of death. In the autopsy room, a portion of the pituitary was sent for pathologic evaluation, and the remaining tissue was frozen rapidly and stored at −70°C until processed. The anterior lobe of each post-mortem human pituitary was used as a control tissue [11] because macroadenomas derive from the anterior lobe.

The post-mortem time between death and tissue acquisition ranged between 12 and 16 h. An analysis of the post-mortem stability of pituitary neuropeptides [12] demonstrated that the concentration of ME, substance P (SP), and BE are stable within this time-frame, after an increase and a decrease during the initial 15–30 min.

2.2. Acquisition of adenoma tissue

Adenoma tissue was acquired during neurosurgery. A portion of the tissue was sent for pathologic evaluation, and the remaining tissue was frozen immediately in liquid nitrogen and stored (−70°C) until processed.

2.3. Acquisition of human lumbar cerebrospinal fluid

CSF obtained by lumbar puncture from patients during their clinical evaluation to determine the cause of their chronic low back pain [1,13] was frozen immediately in liquid nitrogen and stored (−70°C) until analyzed. Standard clinical laboratory tests were performed on each CSF sample, and the remaining volume of CSF (a few milliliters) was made available for this study.

Each patient was categorized according to their physiological response (pain reduction) to the injection of a spinal anesthetic [1,13–15]. A patient who was relieved of pain with either the lumbar puncture, or with an injection of a volume of physiological saline equivalent to the volume of CSF removed, was classified as a control (C). If the patient was relieved of pain with one of the successive levels of medication (0.5, 1.0, 1.5 and 2.0% lidocaine, respectively) that was injected into the lumbar region, then that patient was classified as a physiologic responder (PR), and became a candidate for surgery. A patient who was not relieved of pain by lidocaine and a full mid-thoracic spinal anesthetic was classified as a physiologic non-responder (PNR). Each PNR patient underwent a psychological test to remove malingerers, hypochondriacs, etc. The remaining patients were classified as idiopathic low back pain patients (no known cause for their pain), and were the targets for this study. Each CSF sample used in this study was obtained before any lidocaine injection.

Each CSF sample was divided into two portions: one portion remained frozen (pre-incubate). The second portion (post-incubate) was incubated (37°C, 1 h) to allow endogenous CSF peptidases to metabolize precursors to the various products, which include intermediate-sized precursors, neuropeptides, and inactive metabolites. Those experimental conditions were chosen because it has been shown [16] that synthetic ME added to human CSF was metabolized to GGFM by an endogenous aminopeptidase (see also [17]).

2.4. Processing of pituitary tissue

Each frozen pituitary sample was weighed quickly and homogenized individually (10 ml, 1.0 *M* acetic acid, 4°C) with a Polytron microhomogenizer (Brinkmann Instruments, Westbury, NY, USA; setting of 6=13 000 rpm). The two stable isotope-incorporated synthetic peptide internal standards, 5 μg [($^2H_4$) Ile^{22}] $BE_{1-31,\ human}$ and 1 μg [($^2H_5$) Phe] ME, were added to each tissue sample before tissue homogenization. The internal standards were added at the beginning of the analytical process to compensate for the loss of the endogenous neuropeptides BE and ME during tissue processing and peptide purification. Each internal standard displayed the same chemical, biochemical, and physical behavior as the corresponding endogenous peptide during these procedures. The homogenate was incubated (4 h, 4°C) to ensure that the endogenous and exogenous peptides experience the same microenvironment during

the precipitation of protein and cellular debris. An aliquot (0.2 ml) of the homogenate was taken for total protein determination by the modified Lowry method (microcolorimetric total protein kit 690A, Sigma) after centrifugation (31 000 *g*, 30 min., 4°C) of the homogenate.

2.5. Chromatography

2.5.1. Solid-phase extraction

A solid-phase extraction cartridge (contains 360 mg C_{18} sorbent; Sep-Pak; Millipore–Waters, Bedford, MA, USA) was used to remove water-soluble solutes, saccharides, and salts from a tissue homogenate and to prepare a peptide-rich fraction [1]. A cartridge was fitted to the end of a 10-ml glass syringe, and was activated by eluting, in sequence, with methanol (4 ml), water (4 ml) and TFA (8 ml, 0.1%). The entire supernatant from each tissue homogenate was applied to the cartridge. The sample-loaded cartridge was rinsed with TFA (0.1%, 4 ml), and the peptide-rich fraction was removed with a bolus of acetonitrile (100%, 3 ml). The acetonitrile was evaporated, and the sample was lyophilized to dryness.

2.5.2. Gradient RP-HPLC separation of peptides

Gradient RP-HPLC was performed (Varian model 5000, Varian, Palo Alto, CA, USA) with a μ-Bondpak octadecylsilyl analytical column (10×3.9 mm; particle size 10 μm; pore size 125 Å Millipore–Waters). The peptide-rich fraction from the solid-phase extraction was dissolved in acetonitrile (500 μl, 12%) in triethylamineformate buffer (TEAF; 40 m*M*, pH 3.0), and that entire volume was injected onto the analytical column. The flow-rate was 1.5 ml/min. The gradient was: 12–30% acetonitrile between 0 and 70 min; 30–100% between 70 and 80 min; and 100% between 80 and 90 min. The percentage of the organic modifier was returned to its initial value (12%) within 10 min, and another 10 min was taken to reestablish the UV baseline. Each ME, SP, and BE fraction was collected at the respective retention time; each fraction was lyophilized. After applying any synthetic peptide and before any sample separation, the column was cleaned rigorously, and the column cleanliness was verified by collecting each appropriate fraction for a radioimmunoassay (RIA) measurement of peptide-like immunoreactivity (-li) [2].

2.5.3. Isocratic RP-HPLC separation of peptides

Isocratic RP-HPLC [18] was performed on a polymer analytical column (150×4.6 mm, particle size 5 μm, pore size 100 Å; Polymer Laboratories, Amherst, MA, USA).

The SP fraction from the gradient RP-HPLC was dissolved in acetonitrile (18%, 500 μl) in TEAF (40 m*M*, pH 3.0), which was also the eluant for the isocratic HPLC of SP. The entire volume was injected onto the RP-HPLC column, which was pre-equilibrated with that organic modifier–buffer eluant. The fraction that eluted at the pre-determined SP retention time was collected and lyophilized for RIA.

The BE fraction from the gradient RP-HPLC was treated with trypsin to produce the tryptic peptide BE_{20-24}=NAIIK [19]. The eluant for the isocratic HPLC of NAIIK was acetonitrile (2%) in TEAF (40 m*M*, pH 3.0). The lyophilized mixture of tryptic peptides was dissolved in of that eluant (500 μl), and the entire volume as applied to the HPLC column, which had been pre-equilibrated with that eluant. The NAIIK fraction was collected, and lyphilized to dryness.

The ME fraction collected from the gradient HPLC was dissolved in acetonitrile (15%, 500 μl) in TEAF (40 mM, pH 3.0) — the eluant for the isocratic HPLC of ME. The entire volume was injected onto the HPLC column, which was pre-equilibrated with that eluant. The ME fraction was collected and lyophilized to dryness.

2.5.4. Use of HPLC to study the enzymolysis of SP

RP-HPLC was used to separate synthetic SP from its enzyme-produced fragments. An HPLC system (Varian, Walnut Creek, CA, USA; model 5000), variable wavelength detector (Waters, Milford, MA, USA model 450), and strip-chart recorder (Houston, Austin, TX, USA; OmniScribe B-500) were used. A guard column preceded the analytical column (Millipore–Waters Chromatography, Bedford, MA, USA; μBondpak C_{18}, 300×3.9 mm I.D., 10 μm particle size, 125 Å pore size). The volatile buffer system was TEAF (30 m*M*, pH 3.15), and the organic modifier was acetonitrile. The flow-rate of the mo-

bile phase was 1.5 ml/min, and peptide bond UV absorbance (200 nm) was monitored.

2.6. Trypsinolysis of β-endorphin

The solution of peptide standards that was used to produce the calibration curve (not shown), and the gradient HPLC BE lyophilizate of the human pituitary extracts, were treated separately with trypsin. The trypsin powder (Sigma: HPLC-purified) was dissolved in TFA (0.1%; 100 mg/ml). Samples were dissolved in NH_4HCO_3 (0.8 ml, 0.1 *M*, pH 8.0), 20 μl trypsin (2 mg) was added, and the mixture was incubated (37°C, 1 h). A second aliquot (20 μl) of trypsin solution was added to ensure complete trypsinolysis, and the mixture was incubated (1 h). During trypsinolysis, the enzyme-to-substrate ratio was targeted towards 1/20 (w/w); the substrate amount ranged between ≈50 and 150 μg BE. The reaction was stopped after 2 h by immersing the mixture into liquid nitrogen and lyophilizing the reaction mixture to dryness.

2.7. Radioimmunoassay of neuropeptides

Commercial RIA kits from IncStar (Stillwater, MN, USA) were used to measure SP-li. Briefly, either one of the several known amounts of peptide (for obtaining the calibration curve) or an appropriately diluted unknown sample reacted with an anti-peptide antiserum and a ^{125}I-labeled peptide in a reaction buffer (2–8°C, 16–24 h), followed by reaction with the secondary antibody and precipitation with $(NH_4)_2SO_4$. The radioactivity in the pellet was counted in a gamma-counter (LKB-Wallac 1282 Compugamma; Gaithersburg, MD, USA). All measurements were performed in duplicate. RIA data are expressed as peptide-like immunoreactivity (peptide-li). The suffix-li is necessary until the amino acid sequence of the peptide is determined [21]. The sensitivity range for the SP RIA kit was ≈6–200 pg, based on the volume (0.2 ml) used in the assay.

2.8. Mass spectrometric methods used to analyze neuropeptides

All MS–MS quantitative analyses were performed on a tandem mass spectrometer (AutoSpec Q, VG-Fisons Analytical, Antrincham, UK). The LSI MS Cs^+ ion gun was operated at an energy of 35 keV. Glycerol was used as the matrix. The accelerating voltage was set at 8 kV. All samples and standards were reconstituted into acetic acid (100 μl, 0.1%) in water–methanol (1:1, v/v), and an aliquot (10 μl) of that solution was applied to the LSI MS probe tip, which contained glycerol (≈0.5 μl).

The tandem mass spectrometer was also operated with an electrospray ionization (ESI) source to determine the molecular mass (M_r) of each HPLC-separated peptide in the enzymolysis experiments. HPLC fractions were lyophilized to dryness and redissolved in the ESI solvent [methanol–water (1:1, v/v) plus 1% acetic acid]. The solution was delivered to the ESI source with a syringe pump (model 22, Harvard Apparatus, Woburn, MA, USA) at a flow-rate of 3 μl/min. In general, $(M+2H)^{2+}$ and $(M+H)^+$ ions were observed for each peptide, except for SP_{1-9}, where the $(M+2H)^{2+}$ ion was observed.

2.9. Internal standards used to quantify neuropeptides

A corresponding deuterated synthetic peptide was used as the internal standards for the MS–MS quantification of BE and ME. Because a deuterated peptide, compared to its corresponding native peptide, has the same susceptibility to degradation and loss during tissue processing and peptide purification, and the same behavior during MS analysis (ionization and fragmentation), it is the best internal standard for the quantification of a peptide.

[($^2H_4$) Ile^{22}] $BE_{1-31,\ human}$ [20] and [($^2H_5$) Phe] ME [18] were synthesized by the solid-phase peptide synthesis method.

2.10. Tandem mass spectrometric methods to analyze peptides

2.10.1. MS–MS of BE and ME

Before the quantification of a neuropeptide, the amino acid sequence of that peptide was established to ensure optimal molecular specificity. MS–MS obtained the amino acid sequence of a peptide. In an MS–MS instrument, MS-1 selects the precursor ion, generally the LSI MS-produced $(M+H)^+$ of the peptide (558 for NAIIK and 574 for ME), and MS-2

collects the corresponding product-ion spectrum, which contains the amino acid sequence-determining fragment ions from only that peptide $(M+H)^+$ ion. MS-2 was scanned at a rate of 5 s per scan. The pressure of the collision-activation dissociation gas, He, was adjusted to attenuate (by 50%) the intensity of the precursor ion.

2.10.2. LSI MS–MS–MS of BE and ME

To date, the highest level of molecular specificity for the quantification of endogenous peptides has been achieved with MS–MS, which monitors the metastable transition that links the selected precursor ion with a unique product ion [6,21]. Generally, the $(M+H)^+$ of the peptide is chosen as the precursor ion, one of its unique amino acid sequence-determining fragment ions is chosen as the product ion, and the transition of the precursor ion to the product ion is monitored. Therefore, for the quantification of BE, the transition $NAIIK^+$ to NAI^{-+} (m/z 558 to 299) was monitored, and for the ME, the transition $YGGFM^+$ to $YGGF^{-+}$ (m/z 574 to 425) was monitored. The ion current obtained from the endogenous peptide was compared to the ion current obtained from the internal standard, and the ratio of the ion currents was calculated. From a calibration curve, the ratio of the endogenous peptide to the internal standard was obtained; that ion current ratio was multiplied by the amount of internal standard added before tissue homogenization to provide the amount of endogenous peptide.

2.11. SP metabolism and enzyme saturation and kinetics

The rate of decrease of the synthetic SP that was added to human CSF was measured. The CSF acted as its own buffer. To a series of CSF samples (20 μl each), a volume (80 μl) of various solutions of synthetic SP was added to produce the final concentrations (0, 124, 247, 495, 989, and 1980 nmol SP/μl). The mixture was vortexed and incubated (37°C, 48 h) in a gently shaking water bath. Pilot studies showed that this time period provided the optimum amount of enzymatically produced peptide fragments. Samples were removed from the incubator, and were stored immediately (−70°C) until they were analyzed by HPLC. An aliquot (30 μl) of the incubated sample was injected onto the HPLC analytical column. Gradient I, which was used to analyze endogenous SP and its metabolites [1], was 10% acetonitrile at 0 min, 0.28%/min from 0–18 min, 0.10%/min from 18–48 min, 0.5%/min from 48–72 min, 30% at 72–80 min, and 1.0%/min from 80 to 150 min. The rate of decrease of [SP], and the rate of increase of the metabolic products, was measured by the HPLC peak area (peak-height× peak-width at half-height). Duplicate measurements agreed within ±1.7% ($n=10$). Memory effects, which could be caused in part by contamination of the column by biologic or synthetic peptides, were minimized with a column rinse of 100% acetonitrile (10 min) between each injection. The rate of disappearance ($-d[SP]/dt$), or velocity (v)=nmol/ml/min, of the synthetic SP, which was caused primarily by the action of one or more endogenous CSF enzymes, was plotted vs. [SP]. The corresponding double reciprocal (Lineweaver–Burk) plot, from which the Michaelis–Menten parameters were calculated, was plotted.

The identity of each metabolite was suggested first by the RP-HPLC retention time of each peptide fragment, and ESI-MS confirmed the M_r of each peptide. For example, with gradient I, the following retention times were obtained: SP_{1-11} at 60 min, SP_{1-4} at 2 min, SP_{1-7} at 5 min, SP_{1-9} at 21 min, SP_{3-11} at 66 min, and SP_{6-11} at 68 min. Because SP_{1-4} elutes so close to the solvent front in gradient I, the more shallow gradient II was used [10], in which SP_{1-4} eluted at 5 min and SP_{1-7} at 29 min. Gradient II was 0% acetonitrile at 0–2 min, 0.42%/min from 2 to 40 min, and 16% at 40–60 min.

Therefore, in this experiment the four metabolites identified by RP-HPLC retention time and ESI-MS M_r determination were SP_{3-11}, SP_{1-4}, SP_{1-7}, and SP_{1-9}.

2.11.1. Two-dimensional gel electrophoresis

Briefly, isoelectric focusing (IEF) tube gels were used for the first-dimension separation, and a constant voltage was used (200 V for 2 h, 500 V for 2 h, 800 V for 16 h). The tube gels were extruded and embedded onto the second dimension gel. Second-dimension gel electrophoresis was performed at constant current, and the proteins were transferred to

a PVDF membrane. A Western blot was performed with a BE antibody.

3. Analysis of neuropeptides in the pituitary

3.1. Quantification of opioid neuropeptides in human pituitary macroadenomas

ME and BE, which derive from the proenkephalin A and POMC neuropeptidergic systems, respectively, were measured in pituitary macroadenoma tissue [11].

The measurement of the steady state amount of those two opioid neuropeptides from two different neuropeptide families is an important experiment to accurately address the question of whether peptides from different precursors co-regulate or co-vary with each other in the human pituitary, and whether a defect in the metabolic biosynthesis of those peptides could contribute to the formation of a human pituitary macroadenoma.

The MS–MS quantification of a native peptide is based on the comparison of the amount of the ion current from the endogenous peptide with the current from the stable isotope-incorporated synthetic peptide internal standard. The internal standard contains five deuterium atoms on the phenylalanine ring; that location is metabolically and chemically stable. For example, d_5-ME was used to measure endogenous ME. MS–MS linked the peptide's protonated molecule ion, $(M+H)^+$, to a unique amino acid sequence-determining product ion [5,6,18,22,23].

Fig. 1 contains the MS–MS measurement of ME

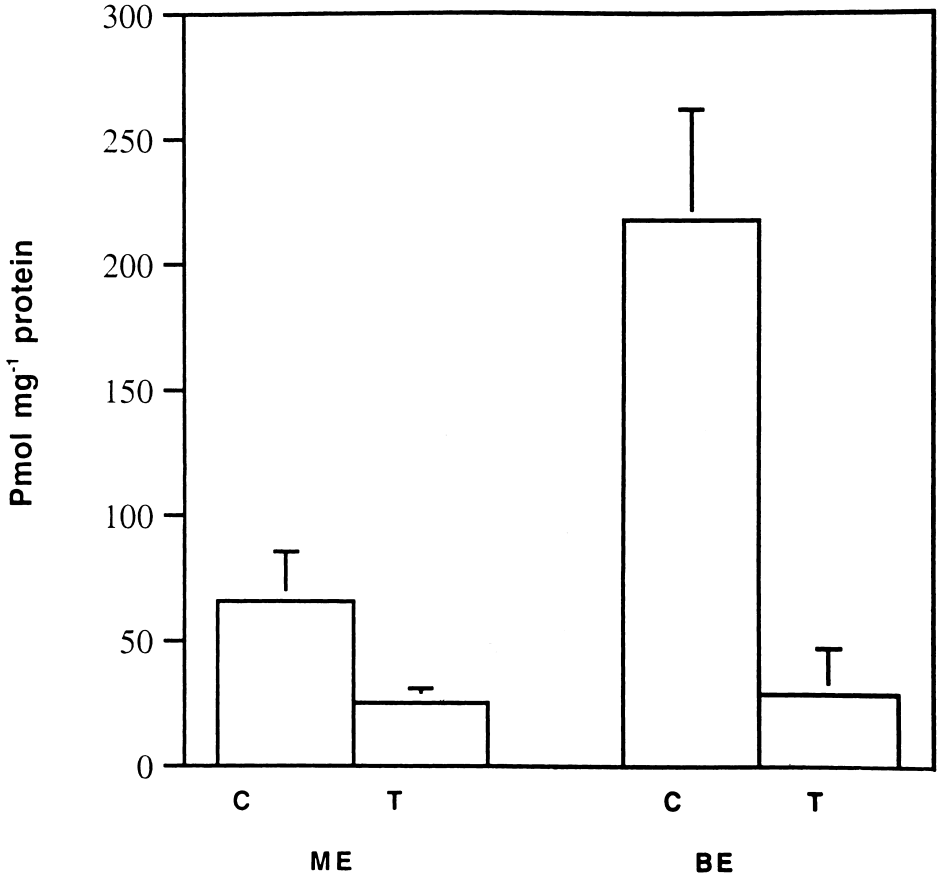

Fig. 1. MS–MS measurement of methionine enkephalin (ME) and of β-endorphin (BE) in control pituitaries (C) and in macroadenomas (T) [11]. ME decreased 2.6-fold and BE decreased 7.6-fold [C versus T].

and of BE in control (C) pituitaries and in macroadenomas (T=tumors). Values are plotted as $\bar{x}\pm$S.E.M. The ME concentration decreased 2.6-fold [C (65.8±19.8 pmol/mg protein) versus T (25.0±5.7)] and BE decreased 7.6-fold [C (217.7±44.2) versus T (28.8±18.3)]. The range (24 to 62%) of the coefficients of variation of these measurements reflects the normal biological variations observed in the peptide content in human tissues; that biological variation is much higher than the variation of a tandem mass spectrometry measurement, which is <5% [18].

The data in Fig. 1 demonstrate a decrease in the concentration of the two opioid neuropeptides, ME and BE in the macroadenomas compared to controls. Thus, two major opioid inhibitory systems have been lost in those tumors.

Either a down-regulation of the synthesis of the proenkephalin A and of the POMC neuropeptidergic systems, or an increased level of degradation of ME and of BE, or a combination of those two processes, has occurred in these macroadenomas. An accurate analysis of the opioid proteins and enzymes in the proteome will identify the specific metabolic defect.

Because corticotrophs contain BE, a down-regulated (fewer cells; defective production or metabolism) POMC system in pituitary corticotrophs may have contributed to the formation of these macroadenomas. Furthermore, the proenkephalin A system in gonadotrophs, somatotrophs, and thyrotrophs is down-regulated, and that process may have contributed to the formation of these macroadenomas.

These experimental data are compatible with other studies. For example, others [24] have shown that human anterior pituitary medial tissue (recalculated, assuming 10% protein by weight) contains ME-li=45±4.0 and lateral tissue contains 32±4.0 pmol/mg protein; and BE-li=1,990±370 and 350±110 pmol/mg, respectively. (Note the biological variation in these measurements.) Thus, these experimental data and the Roth study provide a mutual corroboration of the endogenous content of ME and BE. However, MS–MS provides a much higher level of molecular specificity compared to immunoassay [6,22].

Because human pituitary macroadenomas and microadenomas might begin with the unimpeded proliferation of a particular cell type, it is possible that the removal of an inhibitory factor [25], an increase in an excitatory factor, or a combination of those two mechanisms could lead to that proliferation. In general, as described above, opioid peptides are inhibitory, and tachykinin peptides (such as SP) are excitatory, in cellular processes. Thus according to our data, the measured decrease of two separate and major opioid inhibitory neuropeptidergic systems may have led to an increase in overall excitatory processes and a macroadenoma.

Peptides are also involved in cell proliferation [26–29], and a dysfunction in neuropeptide regulation may contribute to pituitary macroadenoma formation.

Therefore, these experimental data indicate that two potentially new mechanisms, decreases in the proenkephalin A and of the POMC neuropeptidergic systems, may be involved in the formation of pituitary macroadenomas.

3.2. Quantification of opioid neuropeptides in prolactin-secreting human pituitary adenomas

Opioid peptides were also measured in prolactin-secreting microadenomas. The two opioid neuropeptides, ME and BE, were quantified in six well-characterized prolactin-secreting human microadenomas and in ten control human pituitaries [5] to test the hypothesis that an altered metabolism of these two neuropeptide systems may have led to the transformation of adenohypophyseal (anterior pituitary) tissue [30]. Prolactin-secreting tumors are characterized by an abnormally high serum prolactin level that is usually above the levels that might be attributed merely to the ‘stalk effect’, and by a positive immunohistochemical staining of the tumor cell for prolactin. A normal serum prolactin-li level is 3–23 ng/ml [31].

The serum prolactin-li measurements ranged from 103–880 ng prolactin-li/ml, with $\bar{x}\pm$S.E.M.=313±324 ng prolactin-li/ml ($n=5$). The average age of this group of three males and eight females was 34±13 years.

Fig. 2 contains the tandem MS measurements of the steady state amount of ME and of BE in the controls and in the prolactin-secreting tumors. The error bar in the ME-control measurement is too small to be noted. The concentration of ME increased 13-fold [C (77±6 pmol/mg protein) versus T

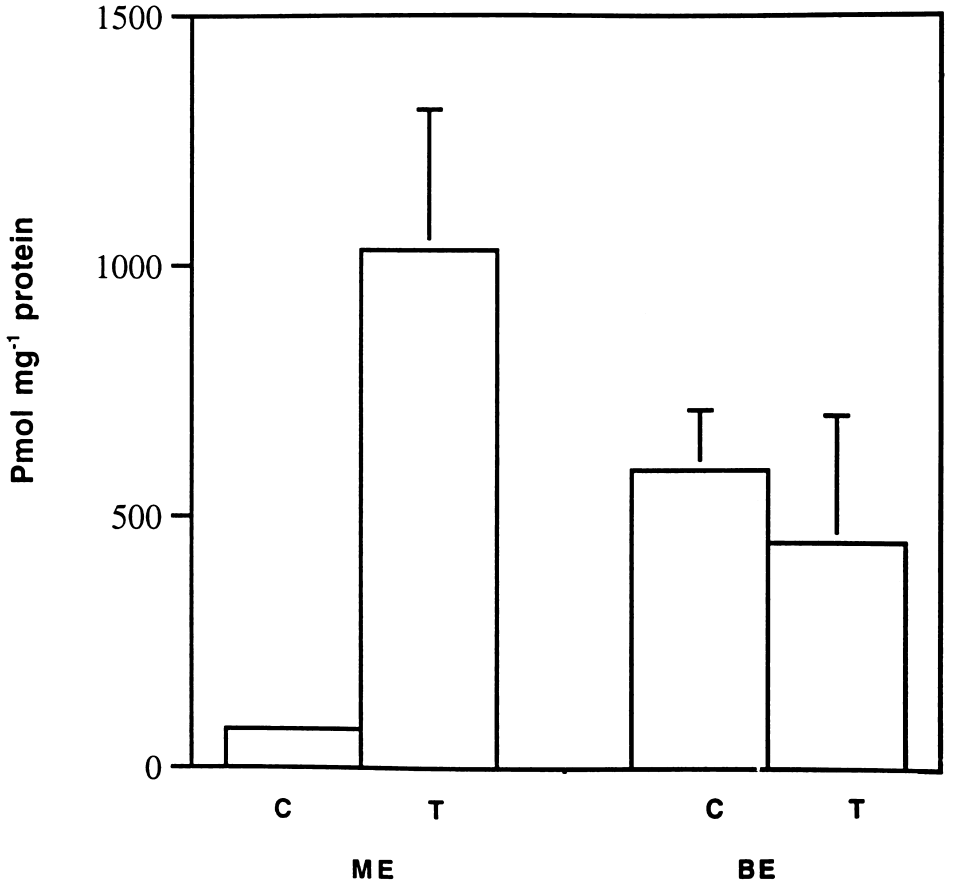

Fig. 2. MS–MS measurement of methionine enkephalin (ME) and of β-endorphin (BE) in pituitary controls (C) and in prolactin-secreting tumors (T) [5]. ME increased 13-fold and BE decreased 25% [C versus T].

(1032±278)] and BE decreased 25% [C (594±121) versus T (450±253)]. The coefficients of variation of these measurements ranged from 8 to 57%; see the similar comment made above.

Thus, an elevated level of ME, which is a product of the proenkephalin A neuropeptidergic system, may play a role in the formation of prolactin-secreting tumors.

The significant increase in the level of ME could stimulate the release of prolactin from the pituitary mammotroph cells. The source of that increased ME could derive from an increased production of proenkephalin A, an increased amount of prohormone convertases 1 and 2, or other factors that participate in those synthesis and degradation systems.

3.3. Two-dimensional gel electrophoretic analysis of the human pituitary proteome

The individual neuropeptide measurements that have been described above extends our knowledge of possible contributing factors of tumor formation in the pituitary. A higher level of information is needed to correlate and extend those findings. Proteome analysis [32–34] is now being used to provide a more comprehensive view of all of the proteins that are contained in a biological extract. Enzymes, receptors, proteins, precursors, intermediate-sized precursors, and other proteins are contained in the proteome. The combination of two-dimensional gel electrophoresis and mass spectrometry provides a

very powerful method to rapidly analyze the proteome with a high level of detection sensitivity and molecular specificity.

Fig. 3 contains a photograph of the two-dimensional gel electrophoretic separation of the proteins contained in the human pituitary proteome. One

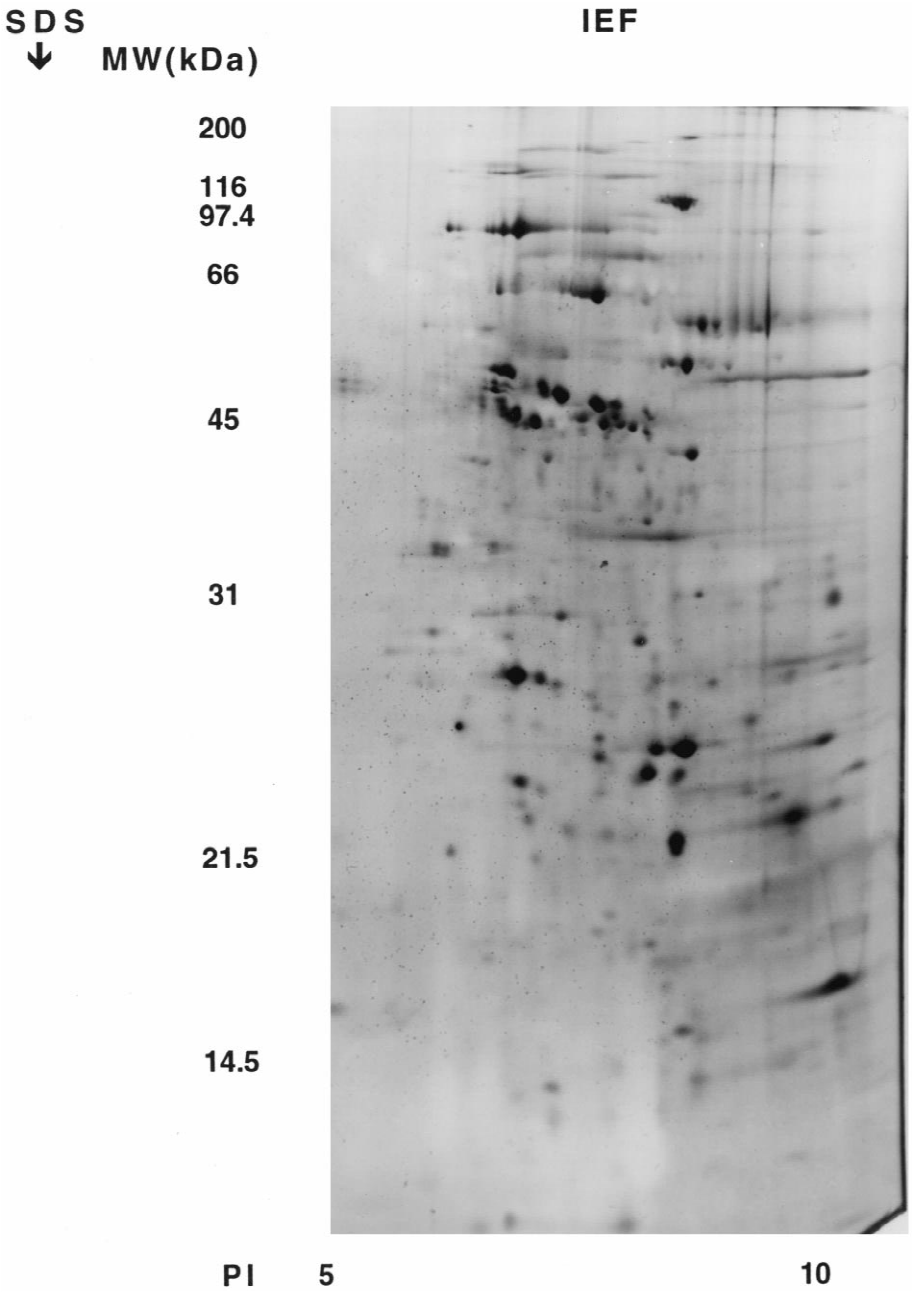

Fig. 3. Two-dimensional gel electropherogram of the human pituitary proteome.

human pituitary (0.53 g) was homogenized, and an aliquot (10%) of that homogenate was analyzed. The human eye resolves the protein spots in the gel electropherogram better than this photograph.

IEF in the first dimension separated the proteins by their charge, and polyacrylamide gel electrophoresis in the second dimension separated proteins by their molecular mass. The gel was silver-stained. The p*I* range is 5→10 (left→right), and the molecular mass range is 14 500→200 000 (bottom→top).

Clearly, a need exists to reduce the complexity of the analysis, and to accurately and preferentially select only those individual proteins and protein families of interest to this study.

Towards that end, Fig. 4 contains the Western blot data of the proteome separation shown in Fig. 3. A commercially available BE antibody was used to detect only POMC-derived/BE-containing proteins. Only one POMC-protein (indicated by the arrow) was detected in that gel; that protein had a p*I* of ≈8.0 and a M_r of ≈15 000.

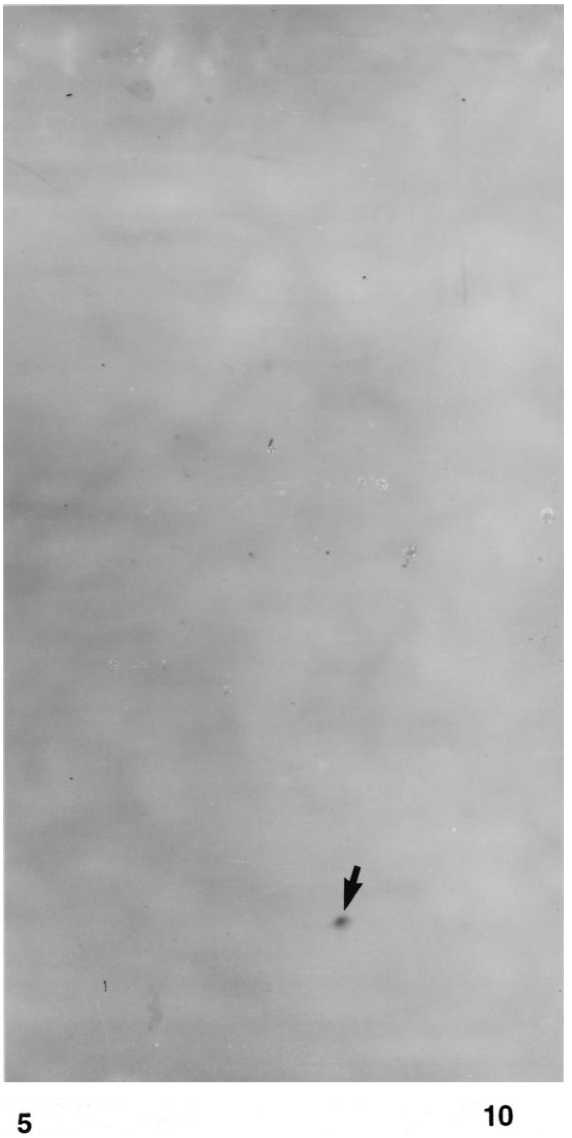

Fig. 4. Western blot with a BE antibody of the proteome in Fig. 3.

3.4. MS analysis of opioid proteins in the pituitary

Mass spectrometry was used to detect and the characterize opioid proteins in bovine and human pituitaries [35–39].

LSI MS was used to detect the unique set of 'marker' peptides that are produced by trypsin treatment of an opioid protein. Opioid proteins have been found in human lumbar CSF [15,40], BE-proteins in the human pituitary [41], and ME-proteins in the bovine pituitary [36].

For example, an HPLC-purified opioid protein fraction that contained BE-li, but that eluted at a different retention time to BE, was studied (data not shown) [36]. ESI-MS analysis of this immunoreactive fraction showed the presence of a major component with an M_r of 9862. This fraction was HPLC-purified, and digested with trypsin. The digest was analyzed by LSI MS. The ions at m/z 558 and 1134, which correspond to the $(M+H)^+$ ion of the two marker tryptic peptides, BE_{20-24} ($=POMC_{254-258}$) and BE_{10-19}($=POMC_{244-253}$), were observed in the mass spectrum. These data indicated that the component with an M_r of 9862 was a new BE-protein in the bovine pituitary.

The ion at m/z 558 was analyzed by tandem MS. The product-ion spectra of the native peptide and of synthetic NAIIK (BE_{20-24}) are equivalent. Thus, the $(M+H)^+$ ion at m/z 558 and the amino acid sequence confirm unambiguously that the tryptic peptide with an M_r of 557 from bovine pituitary is NAIIK (BE_{20-24}), and that the corresponding protein with an M_r of 9862 is a new BE-protein that contains the C-terminal portion of POMC.

3.5. Other measurements of native neuropeptides in the pituitary

Brief mention is made here of related studies of neuropeptides.

The alteration of opioid peptide concentration in the rat pituitary was studied after a survivable, closed-head injury [42]; the effects in the posterior pituitary of space-flight stress were studied on the POMC, proenkephalin A, and tachykinin neuropeptidergic systems [43]; the post-mortem stability of pituitary opioid and tachykinin peptides was studied [12], and leucine enkephalin (LE) was analyzed in the bovine pituitary by a combination of capillary zone electrophoresis [44–48] and MS [49].

Native BE_{1-31} [37,50,51] was analyzed, and a discrepancy was found between the molecular mass of the cDNA-deduced peptide and the MS-measured endogenous peptide [50]. That study was recently confirmed in an independent study [52]. Such discrepancies demonstrate the importance of accurately analyzing the proteome and of carefully characterizing with MS each opioid/tachykinin-protein. Most of the amino acid sequence data in protein databases are only calculated from the DNA sequence, and therefore must be experimentally confirmed, especially in human tissue, to accurately locate the type of metabolic defects that are described here.

4. Analysis of neuropeptides in human lumbar cerebrospinal fluid

This section reviews neuropeptide research in idiopathic low back pain. CSF is used for this study because it is used for clinical diagnosis, and it contains neuropeptides, opioid proteins, and enzymes that synthesize and degrade neuropeptides. These experiments are designed to test the hypothesis that defects in the metabolism of opioid and tachykinin neuropeptidergic systems play a role in idiopathic low back pain, which has no known molecular basis for its pathology.

4.1. Description of patient populations

The differential spinal test with lidocaine distinguishes among three different patient populations that were used in this study. Patients relieved of pain with either the lumbar puncture itself or with an injection of physiological saline (equivalent to the volume of CSF that had been removed) were classified as placebo responders, or controls (C). If a patient was relieved of pain with any one of the second through fifth successive levels of medication (0.5, 1.2, 1.5 and 2.0% lidocaine, respectively) injected into their lumbar region, then they were classified as physiologic responders (PR), and they became candidates for surgery. Those patients who were not relieved of pain by lidocaine or by a full mid-thoracic spinal anesthetic were classified as nonphysiologic responders (NPR), and those patients were not candidates for surgery. The NPR category contained malingerers (readily identified with an MMPI test), idiopathic low back pain patients, and others. The idiopathic low back patients were the focus of this study.

This section briefly summarizes the results that were obtained from six different studies of CSF peptides, proteins, and enzymes.

4.2. Study of opioid and tachykinin peptides, and their precursors and precursor-processing enzymes, in human CSF

Opioid and tachykinin neuropeptides, which derive from two biological sources (intact, and released from their corresponding precursors by the action of endogenous human CSF neuropeptidases), were characterized in human CSF with a combination of post-HPLC detection techniques [40]. RIA measured the ME-li, LE-li, SP-li, and BE-li. Trypsin and carboxypeptidase B were used to release ME and BE from their corresponding precursors. Human CSF also served as a source of endogenous neuropeptides. MS data corroborated the amino acid sequence of the ME and SP that was derived from both sources (intact, precursors). These results demonstrated the presence of endogenous intact neuropeptides, several different neuropeptide-containing precursors, and appropriate precursor-processing enzymes in human CSF for the precursors of ME, LE, BE and SP.

4.3. Study of proenkephalin A and POMC peptides in human CSF

Precursors to BE and to ME, and the proteolytic enzymes that cleave those precursors to the neuro-

peptides BE and ME, were determined in several milliliters of human CSF [15]. Endogenous peptides were HPLC-purified (90-min gradient), and were detected with receptorassay, RIA and MS. Total opioid receptor activity measurements and the profile of HPLC-receptoractivity of human CSF samples were used to monitor the metabolism of neuropeptides. MS data linked the $(M+H)^+$ ion of ME to a unique fragment ion. A later-eluting fraction at 84 min that appeared in all HPLC-receptorassay profiles contained opioid receptoractivity that displaced [^{3}H] etorphine. The quantitative and qualitative patterns of opioid receptoractivity in those profiles changed within the few minutes that elapsed between acquiring the first and second CSF samples. That 84-min fraction that contained opioid proteins was fractionated further with a more shallow (120-min) gradient into three HPLC sections that displayed δ-opioid receptor-preferring activity, using [^{3}H]-ME as the ligand. These three HPLC sections were hydrolyzed separately with human CSF, the source of endogenous neuropeptidases, to yield products that correlated to BE-li in one section of the chromatogram and to ME-li in another section.

4.4. Analysis of proenkephalin A, POMC, and protachykinin neuropeptides in human lumbar CSF by HPLC, RIA, and enzymolysis

In a cell, tissue, and CSF, the amount of each neuropeptide reflects the balance between the synthesis from its precursor and its subsequent degradation to inactive metabolites; therefore, any aberrations in that neuropeptide metabolism will be reflected in that neuropeptide measurement. The three patient population C, PR, and NPR were studied.

Furthermore, it is important to measure not only a single peptide, but also other peptides that derive from several different precursors such as proenkephalin A (enkephalins), POMC (endorphins), and tachykinin (SP) neuropeptides because several different neuropeptides may play a role in idiopathic low back pain and because those neuropeptide systems may interact or may co-vary with each other. The concentration was measured of the free, native peptide relative to the amount of the peptide that had remained encrypted within its precursor (the opioid/tachykinin peptide-containing protein) because that ratio (free/encrypted) may be considered a parameter that reflects the overall balance among the precursor, the neuropeptide, metabolites, and enzymes [2].

To demonstrate the amount of free neuropeptide, the amount of neuropeptide-containing proteins, and the effect that trypsin plus carboxypeptidase enzymolysis plays, one HPLC–RIA profile obtained from CSF (1 ml) is shown in Fig. 5. Those data show the different amounts of endogenous peptide, the presence of opioid and tachykinin proteins, the presence of enzymes, and the relatively large changes in the peptide content following enzymolysis.

Table 1 collects the measurements from a set of patients (4 C, 8 PR, and 8 NPR), and contains the measured peptide-li due to each intact neuropeptide (ME-li, LE-li, SP-li, and BE-li) and also due to that ME-li that is produced by the enzymolysis of the precursor fraction (Prec-ME). Two groups of samples (pre- and post-incubation), and three clinical groups (C, PR, NPR) were studied.

Three significant differences were observed in the post-incubation samples, and those differences indicated that the amount of a neuropeptide that remained encrypted within its precursor may play a role in idiopathic low back pain.

- LE-li in the controls (0 ± 0) was significantly lower ($P=0.001$) versus the PR samples (6.8 ± 1.7). That difference indicates a more extensive metabolism of LE in combination with an extensive synthesis and or less metabolism of its precursor.
- ME-li was significantly lower ($P=0.05$) in the control (3.4 ± 3.5) than the ‘cryptic’ ME-li (158 ± 2.5); that latter is the amount of ME that was liberated by trypsin plus carboxypeptidase B treatment of the precursor fraction. Even though ME-li values are not quantitatively much different from the corresponding pre-incubation values, incubation did significantly increase that difference.
- SP-li was significantly lower in the PR group (1.8 ± 0.6) versus the NPR group (73 ± 0.5; $P<0.001$); that difference indicates a higher synthesis-to-metabolism ratio of the tachykinin system in the NPR versus the PR.

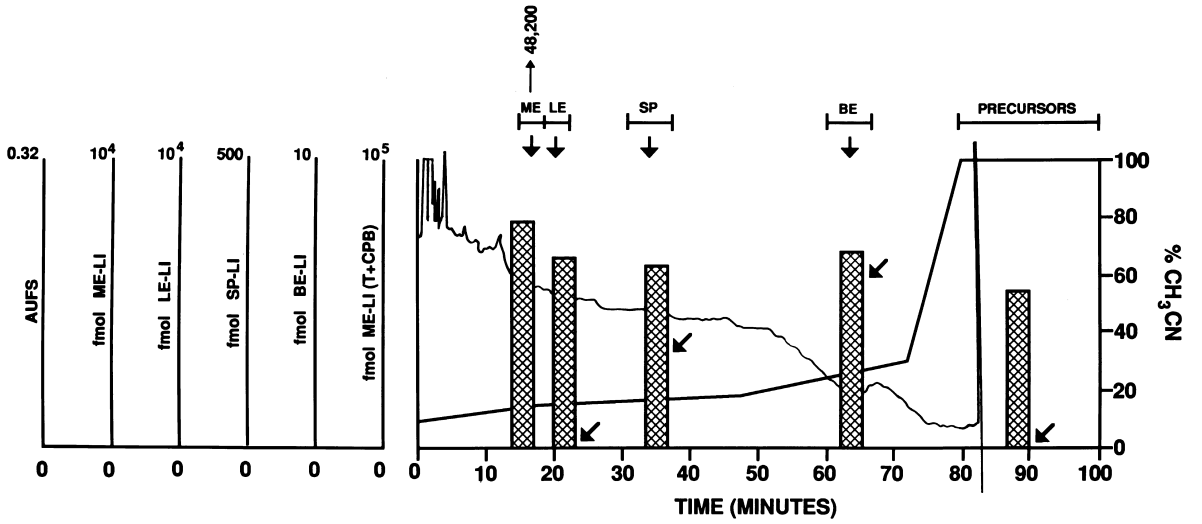

Fig. 5. HPLC–RIA profile of a representative human lumbar CSF sample (1 ml; post-incubate) from an NPR patient. Absorbance units full-scale (AUFS) (200 nm) are listed on the far-left, the percentage of acetonitrile on the right, and retention time (min) on the bottom. The retention time of each synthetic peptide is indicated by the vertical arrow below the labels ME, LE, SP, and BE. The fractions collected for ME, LE, SP, BE, and precursors are indicated by the horizontal bars along the top. The mobile phase flow-rate was 1.5 ml/min; the organic modifier was acetonitrile; and the buffer was TEAF (pH 3.5). The height of each vertical bar corresponds to the amount of peptide-li measured in each indicated peptide fraction. The arrow on the right-hand, side (or top, for ME-li) of each hatched bar indicates the corresponding pre-incubation values. The different RIA sensitivities for the neuropeptides ME-li, LE-li, SP-li, and BE-li, and for the ME-li released after trypsin plus carboxypeptidase B treatment, are shown on the set of separate scales on the left. Reprinted from Ref. [2] with permission.

These data demonstrate that LE, ME, and SP are three important neuropeptide 'markers' in the lumbar CSF of idiopathic low back pain patients. Therefore, an alteration in the metabolism of the preproenkephalin A, prodynorphin, and tachykinin precursors may be important physiological factors in idiopathic low back pain.

The NPR category is very important to consider in a study on idiopathic low back pain, because it contains two different sets of patients:

Table 1
Measurement of immunoreactivity due to intact neuropeptides (ME-li, LE-li, and BE-li) and due to the ME-li that derives from proteolysis of the 'precursor' fraction (prec-ME)[a]

Group	*n*	ME-li	LE-li	SP-li	BE-li	Prec-ME
Pre-incubation						
C	4	12±3.5	2.7±2.8	37.0±0.2	2±0.9	82±3.5
PR	8	173±3.4	44±2.5	50±0.5	3.8±0.5	177±0.6
NPR	8	20±3.5	7.7±1.9	45±0.8	3.9±0.2	114±2.0
Post-incubation						
C	4	3.4±3.5	0±0	53±1.7	3.6±0.7	158±2.5
PR	8	104±3.2	6.8±1.7	18±0.6	2.0±0.5	203±1.7
NPR	8	80±2.9	10±2.5	73±0.5	3.6±0.2	50±2.6

[a] Reprinted from Ref. [2] with permission. Amounts are given as fmol peptide-li/ml CSF. Each entry is the mean±S.E.M. C=control; PR=physological responder; NRP=physiologic non-responder; *n*=number of samples.

- Hypochondriacs, malingerers, etc. This sub-group is readily identified, and excluded from any further study by a psychological test (MMPI).
- Idiopathic low back pain patients, who may have a defective neuropeptidergic system and who truly experience pain, not because of a mechanical problem such as a pinched nerve, etc., which could be relieved by surgery, but rather due (possibly) to a defective neuropeptidergic processing system. Clinicians estimate that ≈30% of their low back pain patients fall into this group. For example, the opioid and tachykinin neuropeptidergic systems appear to be defective in these patients.

4.5. Analysis of preproenkephalin A-processing enzymes in human lumbar CSF

Kinetic parameters were measured for the endogenous neuropeptidases that are important for ME homeostasis in humans [9]. Those neuropeptidases in human lumbar CSF were allowed to act upon several synthetic, biologically significant peptides that are also contained within preproenkephalin $A_{human,1-267}$. The amount of endogenous ME-li in human lumbar CSF is 74.1±5.7 fmol ME-li/ml CSF ($n=56$; $\bar{x}\pm$S.E.M.). The kinetic parameters of the various enzymes that inactivate synthetic ME (YGGFM) and that also produce ME from two different portions of the preproenkephalin $A_{human,1-267}$ precursor molecule were determined. The enzyme that inactivates synthetic ME to PheMet, and that correlates to the rate of decrease of ME, has a $V_{max}=560\pm43.3$ nmol/ml/min and a $K_M=4514\pm373$ μM ($n=56$; $\bar{x}\pm$S.E.M.). The octapeptide precursor preproenkephalin $A_{human,186-193}$ (YGGFMRGL) was added to CSF samples to characterize those processing and converting enzymes that produce the ME pentapeptide. The rate of the decrease of the octapeptide precursor to produce YGGFMR, was V_{max} 0.192±0.038 nmol/ml/min, with a K_M of 513±121 μM ($n=10$; $\bar{x}\pm$S.E.M.). Similarly, a bovine tridecapeptide precursor analog $PPE_{bovine,125-137}$ to preproenkephalin $A_{human,\ 128-140}$ (PPE_{human}=GSEILAKRYGGFM; $PPE_{bovine,\ 125-137}$=GGEVLGKRYGGFM) was used to characterize that enzyme system that produces ME from an N-terminally extended ME peptide. That endopeptidase had a V_{max} of 0.120±0.048 nmol/ml/min and a K_M of 734±296 μM ($n=10$). Those endogenous enzymes in human CSF may relate to the prohormone convertase (PC) enzymes.

4.6. Analysis of SP-inactivating enzymes in human CSF

Kinetic parameters were determined for enzymes in human lumbar CSF that inactivate SP (RPKPQQFFGLM-NH_2) [10]. For the human lumbar CSF samples analyzed in this study, that inactivating enzyme had a $K_M=2.24\pm0.93$ mM and a $V_{max}=0.113\pm0.035$ nmol/ml/min ($n=10$; $\bar{x}\pm$S.E.M.) for the rate of decrease of SP. HPLC analysis (Fig. 6) of the incubated synthetic SP fragments demonstrated that the primary enzyme-produced fragment is SP_{3-11}, with minor amounts (in decreasing order) of SP_{1-4}, SP_{1-7}, and SP_{1-9}. ESI MS measured the M_r of the four peptides SP_{3-11} (Fig. 7), SP_{1-4}, SP_{1-7}, and SP_{1-9}. Fig. 8 summarizes the metabolism data measured for SP_{1-11}. These data demonstrate that the primary enzyme in human lumbar CSF that acts on synthetic substance P is a post-proline cleaving enzyme.

4.7. Analysis of neuropeptides in the CSF obtained from Alzheimer patients

Opioid-receptoractive peptides were measured in the CSF of human controls and compared to patients diagnosed with senile dementia of the Alzheimer's type [7]. [^{3}H]Etorphine was the ligand used to detect in the HPLC fractions the presence of those endogenous peptides that preferentially intact with several different opioid receptors. The receptorassay used a receptor-rich P_2 fraction that was extracted from a canine limbic system. The total opioid peptide content found in the HPLC fractions 6–20 (to avoid the salts contained in fractions 1–5) of Alzheimer's patients' CSF (383±187 pmol ME-like receptoractivity per ml CSF) is significantly higher than the corresponding total from patients with no known neurological disorders (89.1±46.3 pmol ME-like receptoractivity per ml).

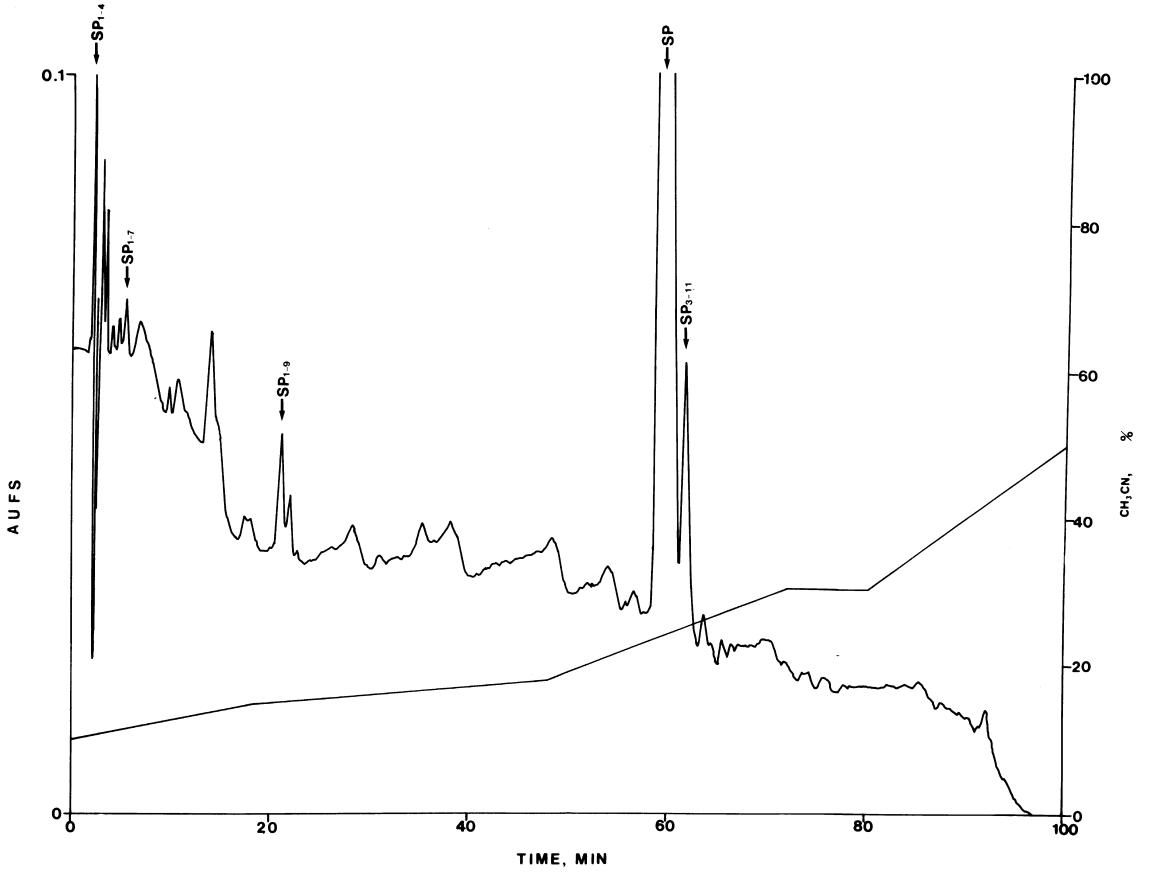

Fig. 6. (A, see chromatogram above) RP-HPLC chromatogram, using gradient I, of the CSF sample 103– to which synthetic SP_{1-11} was added. The retention times of SP_{1-11} and of the four enzyme-produced peptide fragments (SP_{3-11}, SP_{1-4}, SP_{1-7}, and SP_{1-9}) are noted on the chromatogram. Reprinted with permission. (B, see chromatogram on next page) RP-HPLC chromatogram of the same CSF sample shown in (A), using the shallower gradient II to shift the retention times of SP_{1-4} and SP_{1-7}. Reprinted from Ref. [10] with permission.

5. Conclusions

Mass spectrometry was used to analyze opioid peptides, proteins, and enzyme-produced neuropeptides in human tissues and fluids. MS combines a high level of detection sensitivity with molecular specificity for peptide quantification and protein characterization. When the amino acid sequence of a peptide is determined in qualitative or quantitative analysis, the molecular specificity is optimal. The use of MS–MS with a stable-isotope incorporated internal standard optimizes the molecular specificity for peptide quantification.

Two opioid neuropeptidergic systems (proenkephalin A and POMC) were decreased in macroadenomas compared to controls. To date, no mechanism is known for those non-secreting tumors. These measurements indicate that the loss of two separate inhibitory in the anterior pituitary signals may be a contributing factor to macroadenoma formation.

Idiopathic low back pain also has no known mechanism. These measurements of neuropeptides and opioid proteins may contribute to the elucidation of that pathology.

More research is required in both pathologies. The recent developments in proteomics, two-dimensional gel electrophoresis, and mass spectrometry (matrix-assisted laser desorption-ionization; quadrupole ion trap) will combine to significantly advance our

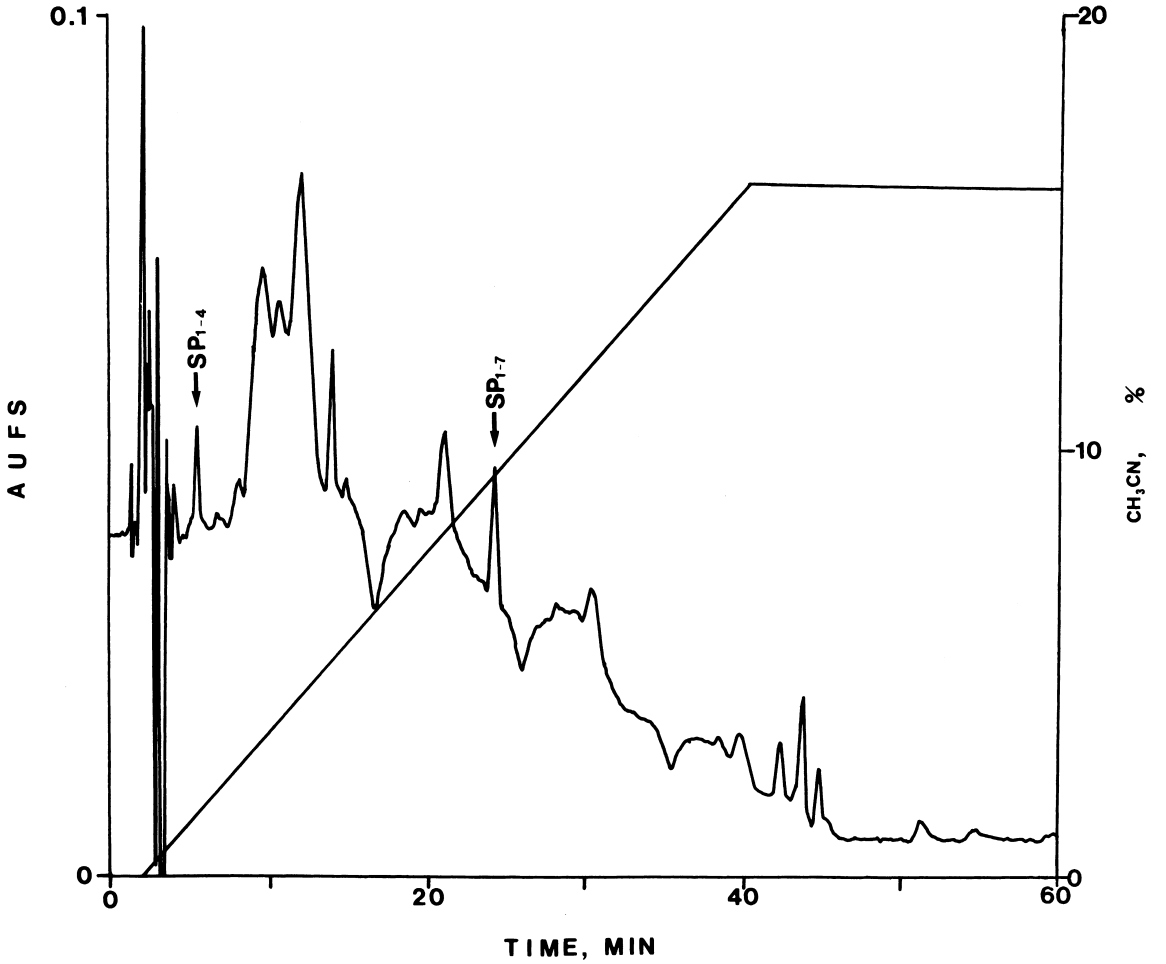

Fig. 6. (*continued*)

understanding of the molecular bases of those two pathologies.

6. List of abbreviations

$(M+H)^+$	protonated molecule ion
-li	-like immunoreactivity
A	alanine (Ala)
Å	angstrom
BE	beta endorphin$_{\text{human, 1–31}}$
C	control
CSF	cerebrospinal fluid
ESI	electrospray ionization
F	phenylalanine (Phe)
G	glycine (Gly)
HPLC	high-performance liquid chromatography
I	isoleucine (Ile)
IEF	isoelectric focusing
K	lysine (Lys)
L	leucine (Leu)
LE	leucine enkephalin (YGGFL)
LSI MS	liquid secondary ion mass spectrometry
M	methionine (Met)
ME	methionine enkephalin (YGGFM)
MMPI	Minnesota multiphase inventory
MS	mass spectrometry
MS–MS	tandem mass spectrometry
M_r	molecular mass

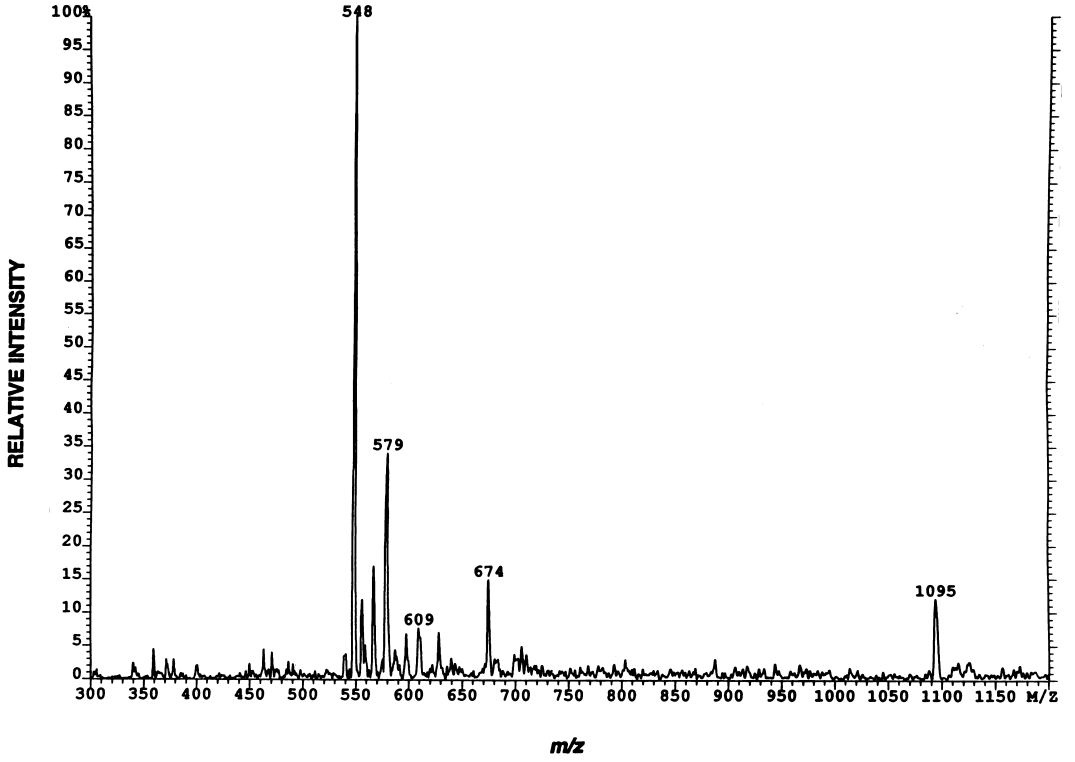

Fig. 7. Electrospray ionization mass spectrum of the HPLC fraction that contains the peptide fragment SP_{3-11}. The $(M+H)^+$ ion occurs at m/z 1095, and the $(M+2H)^{2+}$ ion at m/z 548. Reprinted from Ref. [10] with permission.

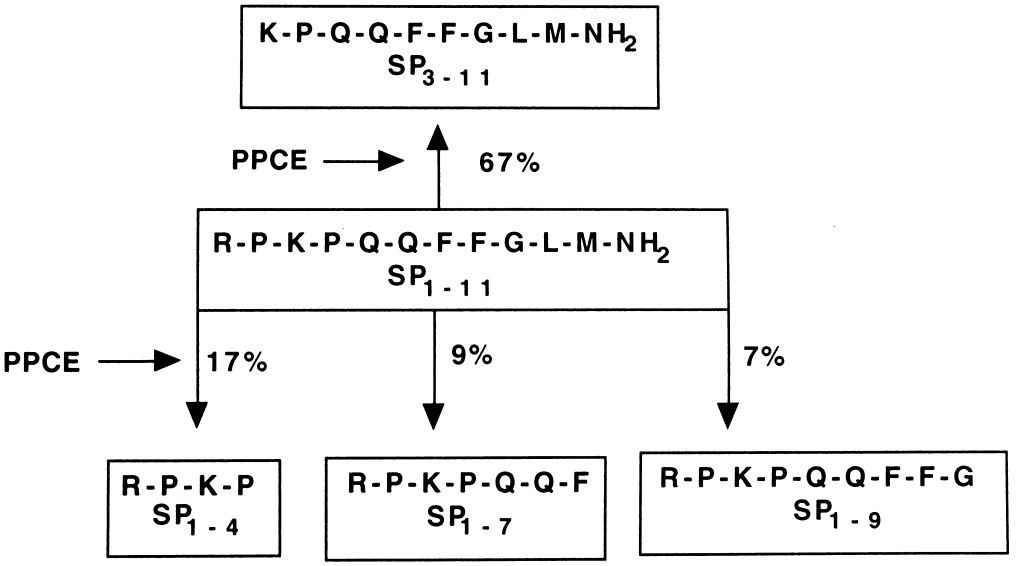

Fig. 8. Peptide products produced from synthetic substance P by enzymes in human lumbar CSF. The percentage indicates the relative amount of each product measured in the 96-h incubation sample. Reprinted from Ref. [10] with permission.

N	asparagine (Asn)
NPR	physiologic non-responder
OPCP	opioid peptide-containing protein
P	proline (Pro)
POMC	proopiomelanocortin
PR	physiologic responder
PVDF	polyvinylidene difluoride
Q	glutamine (Gln)
R	arginine (Arg)
RIA	radioimmunoassay
RP	reversed phase
SP	substance P (RPKPQQFFGLM-NH_2)
T	macroadenoma (tumor)
TEA	triethylamine
TFA	trifluoroacetic acid
UV	ultraviolet
v	velocity (nmol/ml/min)
Y	tyrosine (Tyr)

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