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Analysis of proenkephalin A, proopiomelanocortin and protachykinin neuropeptides in human lumbar cerebrospinal fluid by reversed-phase high-performance liquid chromatography, radioimmunoassay and enzymolysis

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ABSTRACT

A comprehensive high-performance liquid chromatographic, radioimmunoassay, and enzymatic degradation scheme has been developed to analyze several intact neuropeptides and the corresponding peptides created by *in vivo* enzymolysis of precursors to study neuropeptides in human lumbar cerebrospinal fluid (CSF) and to test the hypothesis that defects in the metabolism (synthesis, degradation) of neuropeptide precursors, neuropeptides, and metabolites play a role in low back pain. CSF samples were obtained from three different patient groups: controls (C), whose low back pain was relieved without lidocaine; pharmacological responders (PR), whose pain was relieved by lidocaine and who were candidates for surgery; and pharmacological non-responders (PNR), whose pain was not relieved by lidocaine and a mid-thoracic anesthetic, and who were not candidates for surgery. The metabolic acitivity involved during synthesis and degradation of the peptides was assessed by measuring intact, native neuropeptide immunoreactivity in pre-incubated and post-incubated CSF samples, where samples were incubated at 37°C for 1 h. Pre-incubation radioimmunoassay measurements reflected the content of intact peptides present in lumbar CSF at the time of sampling, and post-incubation measurements assayed the amount of peptide that had remained embedded within its precursors [cryptic methionine enkephalin (ME)] and that had been released by the action of CSF peptidases. Significant differences were found in post-incubation samples for the amount of proenkephalin A [ME, leucine enkephalin (LE)] and tachykinin [substance P (SP)] peptides. For example, significant differences were observed for ME-like immunoreactivity (C *versus* cryptic), SP-like immunoreactivity (PNR *versus* PR), and LE-like immunoreactivity (PR *versus* C). No significant differences were observed among the peptides within the pre-incubation samples. The significant differences observed within the post-incubation samples demonstrated that the neuropeptides that had remained encrypted within their precursors in the corresponding pre-incubated samples may reflect an altered metabolism of the proenkephalin A (ME, LE) and tachykinin (SP) systems, and therefore those neuropeptide families may be significant factors in idiopathic low back pain.

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INTRODUCTION

Several different neuropeptide systems [1] have been suggested to play a role in human idiopathic chronic low back pain (LBP) [2–5] and in other pathophysiological conditions where opioid and tachykinin peptides are important [6–10]. For example, opioids modulate the firing rate of substance P (SP)-containing neurons [11]. Previous data indicated that the amount of total opioid receptor (TOR) activity in lumbar cerebrospinal fluid (CSF) correlated with the amount of lidocaine required to relieve LBP [3], and also that the amount of TOR was extraordinarily high in one particular LBP patient [2]. Tachykinin immunoreactivity in LBP CSF correlated with the response of patients to lidocaine [4]. However, notwithstanding the research aimed towards understanding pain in general and idiopathic LBP in particular, many questions remain on the detailed molecular processes involved in LBP. Whereas it is true that many other systems such as excitatory and inhibitory amino acids may also play a role in LBP, we have focused in this study on neuropeptides [12], which exist in much lower concentration and which provide markedly more information than amino acids [13].

We developed a comprehensive analytical system [high-performance liquid chromatography (HPLC), radioimmunoassay (RIA), enzymolysis] to test the hypothesis that neuropeptide metabolism is different in controls *versus* idiopathic LBP patients, and that the opioid (proenkephalins A and B, proopiomelanocortin = POMC) and tachykinin systems are involved. Molecular processes involved in LBP should be clarified experimentally by measuring peptide-like immunoreactivity [methionine enkephalin (ME)-, leucine enkephalin (LE)-, SP-, and β-endorphin (BE)-like immunoreactivity] in lumbar CSF samples because the amount of those neuropeptides will reflect the balance between their synthesis from precursors and their subsequent degradation to inactive metabolites, and therefore will reflect any aberrations in metabolism. For this reason, peptides were measured in a control (C) population and were compared to data from pharmacological responders (PR) and pharmacological non-responders (PNR). Furthermore, it was important to measure not only one peptide but also several other peptides derived from several different precursors such as proenkephalin A (ME, LE), POMC (BE), and tachykinins (SP) because several neuropeptides may play a role in LBP, and those neuropeptide systems may interact or co-vary with each other. The concentration of free native peptide relative to the amount of the peptide that remained encrypted within its precursor (for example, free ME *versus* the amount of ME released enzymatically *in vitro* from precursors) may be considered a parameter that reflects the overall activity of the precursor, the neuropeptide, and metabolites. These data were obtained readily from our analytical system because these neuropeptides are collected from a gradient reversed-phase (RP) HPLC elution, and because the late-eluting region in an HPLC chromatogram contains several different precursors [5]; other intermediate-sized precursors may also elute elsewhere in a chromatogram. Because RIA cannot establish the amino acid sequence data of a peptide [13], the term "-like immunoreactivity (-LI)" is used in this paper.

This study was designed to elucidate some of the diverse molecular processes involved in the metabolism of neuropeptides in idiopathic LBP by measuring the following.

(1) The amount of immunoreactivity corresponding to four different HPLC-purified free neuropeptides in each CSF sample obtained from three clinical categories (C, PR, PNR) [4]. Immunoreactivity was measured in the HPLC fractions corresponding to methionine enkephalin (ME = YGGFM) and to leucine enkephalin (LE = YGGFL), which both derive from the proenkephalin A precursor, substance P_{1-11} (SP = RPKPQQFFGLM-NH_2), which derives from the α-, β- and γ-preprotachykinin precursors, and β-endorphin ($BE_{1-31,human}$ = YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE), which derives from POMC (Y = Tyr, G = Gly, F = Phe, M = Met, L = Leu, R = Arg, P = Pro, K = Lys, Q = Gln, T = Thr, S = Ser, E = Glu, V = Val, N = Asn, A = Ala, and I = Ile).

(2) The amount of intact ME-LI *versus* the amount of ME-LI produced by *in vitro* treatment of the precursor HPLC fraction with the two proteolytic enzymes, trypsin (T) and carboxypeptidase B (CPB).

(3) The amount of immunoreactivity of these neuropeptides measured after incubation (37°C, 1 h) of the CSF sample. Incubation allows endogenous CSF peptidases to cleave neuropeptides from their precursors.

EXPERIMENTAL

The experimental scheme used to incubate, separate, and analyze the neuropeptide content in these LBP CSF samples is shown in Fig. 1. The pre-incubation and post-incubation samples were eluted with an RP-HPLC gradient (see Fig. 2) to separate ME, LE, SP, BE, and the precursor region. The four native peptide fractions (ME, LE, SP, BE) were analyzed directly by RIA [5], and the precursor fraction was treated with T and CPB before measurement of ME-LI [14,15].

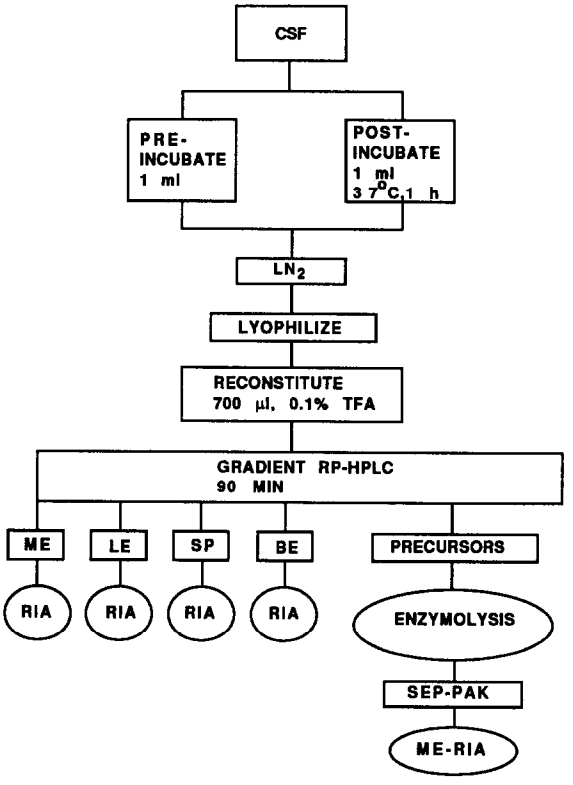

Fig. 1. Experimental scheme used to incubate, separate, and analyze endogenous neuropeptides in lumbar CSF samples, including intact ME-LI, LE-LI, SP-LI, and BE-LI, and ME-LI produced by enzyme treatment (T plus CPB) of the HPLC precursor fraction. Pre-incubate and post-incubate samples were purified with gradient RP-HPLC.

Human cerebrospinal fluid

Samples of human CSF, which were obtained by lumbar puncture from patients during their clinical evaluation to determine the cause of their chronic LBP [3,4], were frozen immediately in liquid nitrogen and stored at −70°C until analyzed. Standard clinical laboratory tests were performed on all CSF samples, and the remaining volume of CSF (generally a few milliliters) was made available to this study.

Patients were categorized according to their physiological response (pain reduction) to the injection of a spinal anesthetic [2–5,16]. Patients relieved of pain with either the lumbar puncture alone or with an injection of a volume of physiological saline equivalent to the volume of CSF removed were classified as C. If the patients were relieved of pain with one of the second through fifth successive levels of medication (0.5, 1.0, 1.5, and 2.0% lidocaine, respectively) injected into their lumbar region, then they were classified as PR and became candidates for surgery. Patients who were not relieved of pain by lidocaine and a full mid-thoracic spinal anesthetic were classified as PNR and were not candidates for surgery. All CSF samples used in this study were 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 permit endogenous CSF peptidase activity to lead to more extensive (but not necessarily complete) metabolism of precursors to intermediate precursors, neuropeptides, and inactive metabolites. Those experimental conditions were chosen because we [17] and others [18] have shown that synthetic ME added to human CSF was metabolized by an endogenous aminopeptidase to GGFM.

RP-HPLC purification of endogenous neuropeptides

A Varian Model 5000 HPLC system (Walnut Creek, CA, USA) was used. A guard column preceded an RP polymer analytical column (Polymer Labs., Amherst, MA, USA; PLRP-S, 150 mm × 4.6 mm I.D.). The volatile ion-pairing buffer was triethylamine–formic acid [TEAF, 40 m*M*, pH 3.15; triethylamine (TEA), Pierce, Rockford, IL, USA; formic acid (F), Mallinckrodt, Paris, KY, USA] [19], and the organic modifier was acetonitrile. Gradients of 90 min and 120 min have been described elsewhere [5]. The flow-rate of the mobile phase was 1.5 ml min^{-1}. A portion (1 ml) of a CSF sample was lyophilized, reconstituted into 0.7 ml of TEAF buffer, and injected onto the HPLC analytical column. Fractions were collected manually at each neuropeptide retention time, and the acetonitrile was evaporated with nitrogen gas; the remaining volume was lyophilized, and the residue was reconstituted in the RIA buffer.

The HPLC retention time of each synthetic peptide (ME, LE, SP, BE) was calibrated in a separate experiment to avoid any column contamination. An HPLC analytical column must be cleaned of any neuropeptide due to any possible incomplete elution after the injection of a synthetic peptide or a biologic extract [16], and thus a rigorous column-cleaning procedure was developed and used in this study. Because an HPLC column could retain picomole amounts of peptides that could interfere with subsequent RIA [16], the column was cleaned ten times with the 90-min gradient, and then with a 3-h 100% acetonitrile wash. The next day, the column was cleaned again with the 90-min gradient, and a CSF sample was injected (for example, pre-incubation sample No. 1) and eluted with the gradient. A second sample (a repeat of pre-incubation sample No. 1) was injected following the first gradient, and the sample was eluted with the same gradient. Therefore, the column was cleaned twice with the 120-min gradient and three times with the 90-min gradient. The next day, the column was cleaned again with the 90-min gradient, and another sample was injected (for example, post-incubation sample No. 2) and eluted with the gradient. A second sample was injected (post-incubation sample No. 2) and eluted. The column was cleaned again. This procedure was repeated each following day for each CSF sample.

Fractions were collected manually at the individual calibrated retention times. Synthetic ME eluted at 16.8 ± 0.04 min, LE at 20.0 ± 0.13 min, SP at 32.5 ± 1.2 min, and BE at 62.8 ± 0.85 min. To insure that we collected each peptide from a biological sample (a shift in a few tenths of a minute generally occurs in the HPLC retention time of a peptide in a biological extract), the fractions eluting within the range of ± 1.5 min of the expected retention time were collected manually in polypropylene tubes for intact ME and LE and within ± 3.5 min for intact SP and BE. Therefore, either a 3-min (4.5 ml total volume) or a 7-min (10.5 ml) fraction was collected. The precursor fraction was collected for 20 min (30 ml) from 81 to 100 min. Manual collection was used to minimize the collection of background matrix (column bleed, buffers) that might interfere with RIA. All samples were lyophilized.

Radioimmunoassay

Commercially available RIA kits for ME, LE, SP, and BE were used (IncStar, Stillwater, MN, USA). Borosilicate glass tubes were used for RIA. [^{125}I]Peptide tracers were used in each kit, and radioactivity was measured in a gamma counter (LKB Compugamma 1282, Turku, Finland). Cross-reactivities (on a molar basis) for each antibody were as follows: for the ME kit, LE 2.8%, α-endorphin 0.10%, SP <0.002%, and α-neo-endorphin <0.002%; for the LE kit, ME 1.1%, BE 1.0%, α-neo-endorphin 0.5%, dynorphin$_{1-13}$ 0.5%, α-endorphin <0.02%, and γ-endorphin <0.02%; for the SP kit, ME <0.002%, LE <0.002%, BE <0.008%, eledoisin <0.002%, and physalemin <0.002%; and for the BE kit, LPH (lipotropin) 5.6%, dynorphin <0.1%, α-neo-endorphin <0.1%, α-endorphin <0.1%, LE <0.1%, ME <0.1%, β-LPH$_{61-76}$ <0.1%, β-LPH$_{61-77}$ <0.1%, ACTH$_{1-39}$ (adrenocorticotropic hormone) <0.01%, α-MSH

(melanocyte stimulating hormone) <0.1%, β-MSH <0.1%, prolactin <0.1%, LH (luteinizing hormone) <0.1%, FSH (follicle stimulating hormone) <0.1%, TSH (thyrotropin stimulating hormone) <0.1%, vasopressin <0.1%, oxytocin <0.1%, and N-acetyl-BE 100%. The sensitivity of each kit was as follows: for the ME kit 10 fmol per tube, LE 36 fmol per tube, BE 0.6 fmol per tube, and SP 3 fmol per tube. RIA data are given as fmol peptide-like immunoreactivity ml^{-1} CSF (for example, fmol ME-LI ml^{-1} CSF).

The blanks for each neuropeptide measurement were obtained by measuring the immunoreactivity of lyophilized samples at the respective retention times of ME, LE, SP, and BE after an extensive cleaning of the column at the beginning of a day and before any sample injection. The blank values ($n = 2$) measured were equivalent to 0 fmol ME-LI ml^{-1} CSF, 140 ± 99 fmol LE-LI, 56 ± 0.6 fmol SP-LI, and 3.2 ± 1.1 fmol BE-LI (average ± S.E.M.). Each neuropeptide measurement reported below was corrected by subtracting its corresponding blank value.

Enzymolysis experiments

Late-eluting HPLC fractions that contained proenkephalin precursors yielded ME [5,14] when treated with T [20] and CPB [21]. Therefore, to measure the amount of total ME-LI that could be derived from late-eluting ME-containing precursors in lumbar CSF samples, the samples were treated with T to produce ME-K or ME-R. Subsequent CPB treatment removed the C-terminal basic residue to produce ME.

The late-eluting CSF precursor HPLC fraction was lyophilized, the residue was redissolved in Tris (50 m*M*, 2 ml, pH 7.4) and incubated with immobilized trypsin beads (Pierce, Bellefonte, PA, USA; 1.7 U, 30 min, ambient temperature). The mixture was centrifuged to remove the trypsin beads, and the supernatant was treated within 3 min with CPB (Sigma, St. Louis, MO, USA; 9.7 U, 30 min, ambient temperature). Enzymolysis was stopped by acidification by adding a sufficient amount of 1% trifluoroacetic acid (TFA) to change the pH from 7.4 to 3. The mixture was frozen in liquid nitrogen and lyophilized; the residue was redissolved in TFA (0.1%, 3 ml). Peptides were separated with a Sep-Pak [22], eluted with 25 ml of acidified acetonitrile (0.1% TFA–acetonitrile, 50:50, v/v), and ME-LI was measured.

Data analysis

The individual neuropeptide-LI measurements were log-transformed [$y = \log(y + 1)$] [23] and tested for significance with the Student's t-test (unpaired, one-tailed). Significance was defined at the $p = 0.05$ level.

RESULTS

An analytical scheme (Fig. 1) was developed and used in this study to test the hypothesis that metabolic defects in neuropeptidergic systems are one of the contributing factors to LBP. This paper describes the RP-HPLC–RIA analysis of intact and cryptic neuropeptides in human lumbar CSF. Fig. 2 contains a representative gradient RP-HPLC chromatogram obtained from the analysis of one of the human lumbar CSF samples (patient BR, category PNR, volume = 1 ml, post-incubation sample). The horizontal bars along the top of the chromatogram indicate the fractions that were collected for the four intact neuropeptides (ME, LE, SP, and BE) and the precursors. The connected solid-line indicates the acetonitrile gradient, and the trace indicates the UV absorbance measured at 200 nm. In general, UV detection sensitivity (200 nm) for peptides is at the nanomolar level and RIA at the femtomolar level [13,24,25].

The amount of immunoreactivity measured in this post-incubation sample is represented by the height of the vertical hatched bar at the neuropeptide's and the precursor's retention times, and the five corresponding ranges of RIA measurements are given in the set of left-hand vertical axes. Note the wide range of amounts (10^4, 10^4, 500, 10, and 10^5) for ME-LI, LE-LI, SP-LI, BE-LI, and precursor ME-LI, respectively. The arrow associated with the right-hand side (or top, for ME-LI) of each vertical hatched bar denotes the amount of neuropeptide-LI measured for the

TABLE I

MEASUREMENTS OF IMMUNOREACTIVITIES DUE TO INTACT NEUROPEPTIDES (ME-LI, LE-LI, SP-LI, AND BE-LI) AND DUE TO ME-LI DERIVED FROM PROTEOLYSIS OF THE PRECURSOR FRACTION (Prec-ME)

Amounts are given as fmol peptide-LI ml^{-1} CSF. Each table entry is the mean ± S.E.M.

Group	*n*	Pre-incubation					Post-incubation				
		ME	LE	SP	BE	Prec-ME	ME	LE	SP	BE	Prec-ME
C	4	11.9 ± 3.5	2.7 ± 2.8	37.0 ± 0.2	2.1 ± 0.9	82.2 ± 3.5	3.4 ± 3.5	0 ± 0	52.7 ± 1.7	3.6 ± 0.7	157.5 ± 2.5
PR	8	172.8 ± 3.4	43.7 ± 2.5	50.3 ± 0.5	3.8 ± 0.5	176.8 ± 0.6	103.7 ± 3.2	6.8 ± 1.7	18.1 ± 0.6	2.0 ± 0.5	203.2 ± 1.7
PNR	8	20.4 ± 3.5	7.7 ± 1.9	44.7 ± 0.8	3.9 ± 0.2	113.8 ± 2.0	80.3 ± 2.9	10.0 ± 2.5	73.1 ± 0.5	3.6 ± 0.2	50.3 ± 2.6

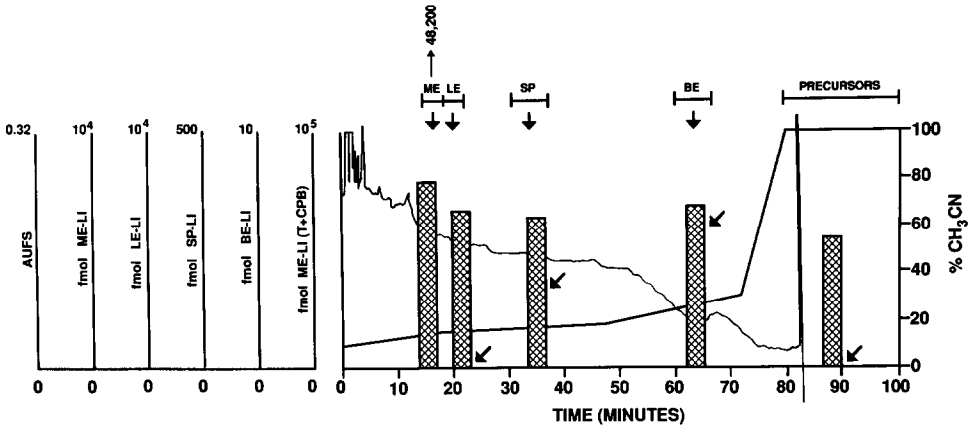

Fig. 2. Gradient RP-HPLC chromatogram of a representative human lumbar CSF sample (1 ml; post-incubate) from patient BR (PNR). Absorbance units full scale (AUFS) (200 nm) are listed on the far left scale, the percentage of acetonitrile on the right scale, and retention time (min) on the bottom scale. The retention times of synthetic peptides are indicated by the vertical arrows below the labels ME, LE, SP, and BE. The fractions collected for ME, LE, SP, BE, and precursors are indicated by the horizontal bars. The mobile phase flow-rate was 1.5 ml min^{-1}; the organic modifier was acetonitrile; and the buffer was triethylamine formate. The height of each vertical bar corresponds to the amount of peptide-like immunoreactivity measured in each indicated peptide fraction. The arrow on the top, side, or bottom of a bar indicates corresponding pre-incubation values. The five different RIA sensitivities for ME-LI, LE-LI, SP-LI, BE-LI, and ME-LI (after T plus CPB) are shown on the set of separate scales on the left (and to the right of the AUFS scale).

corresponding pre-incubation sample for this sample; for example, the ME-LI in the pre-incubate sample was 48 200 fmol ml^{-1} CSF.

This representative set of data for one CSF sample demonstrates that incubation alters the amount of peptide in the five HPLC fractions, that this lumbar CSF sample contains a significant amount of ME-containing precursors, and that HPLC plus RIA is an appropriate analytical method for this LBP study. Of course, amino acid sequence data are needed for each neuropeptide-LI measured [13].

Table I contains the immunoreactivity measurements of the native ME-LI, LE-LI, SP-LI, and BE-LI in pre-incubation and post-incubation samples, and the measurement of ME-LI in pre-incubation and post-incubation samples after proteolytic enzyme treatment of the precursor fraction. In the pre-incubated samples, only one set of meaurements was significant: LE-LI, pre-incubate C *versus* post-incubation C ($p = 0.001$). However, only one patient (No. 4) had a non-zero value, and thus we do not consider that this pair of measurements is significant.

The three significant differences that were observed in the post-incubation samples indicated that the amount of neuropeptides that remained encrypted within their precursors may play a role in this population of LBP patients. For example, LE-LI in the C category was significantly lower ($p = 0.001$) in the C *versus* the PR samples, indicating the more extensive metabolism of LE in combination with extensive synthesis and/or less metabolism of proenkephalin A. Second, ME-LI was significantly lower ($p = 0.05$) than the cryptic ME-LI (amount of ME liberated by T plus CPB treatment of precursor fraction). Even though ME-LI values are not quantitatively much different from the corresponding pre-incubate values, the incubation did increase significantly the difference. Third, SP-LI was significantly lower in PR *versus* PNR samples ($p < 0.001$), indicating a higher synthesis-to-metabolism ratio of the tachykinin system in the PNR group *versus* the PR group.

DISCUSSION

This paper demonstrates that LE, ME, and SP may be three important neuropeptide markers in the lumbar CSF of LBP patients, and that altered metabolism of the proenkephalin A and tachyki-

nin precursors may be important physiological factors in LBP. These conclusions are obtained by analyzing neuropeptides in pre-incubation and post-incubation samples and by analyzing the precursor fraction in an HPLC gradient.

The data in Fig. 2 demonstrate that immunoreactivity was measured in that representative lumbar CSF sample after HPLC separation (patient BR; post-incubate) at the retention times of the four intact neuropeptides and in the enzyme-treated precursor fraction. The neuropeptide-LI data in Table I demonstrate metabolic features of these LBP CSF samples. First, a wide range of neuropeptide-LI amounts is manifested within each category and among the neuropeptides. Second, significant differences in amounts occur among neuropeptides in pre-incubation *versus* post-incubation samples, and those differences reflect the fact that proenkephalin A and tachykinin peptides remain encrypted within their respective precursors and are excised by the action of CSF peptidases.

The PNR category is important to consider because it apparently contains two sets of patients: (a) hypochondriacs, malingerers, etc., and (b) patients with a defective neuropeptidergic system who truly experience pain, not because of a mechanical problem such as a pinched nerve, but rather due to a defect in neuropeptide processing [26]. For example, this study demonstrated that in the post-incubation samples the SP-LI is significantly increased in PNR *versus* PR patients. This difference is compatible with out hypothesis and may indicate that some of the PNR LBP patients studied here have a defect in the metabolism of SP. The increased amount of PNR SP-LI could reflect an increased production of SP precursors, a decreased metabolism of SP, or a combination of both factors.

During *in vitro* incubation with CSF peptidases, some neuropeptides are released from their corresponding precursors, and some of those neuropeptides are metabolized further to inactive metabolites. The enzymes required for the synthesis of a C-terminal peptide carboxamide such as SP [27] are different from the T-like and CPB-like enzymes that produce free carboxyl terminal neuropeptides. It is tempting to speculate that the difference observed here may offer clues to clarify the molecular processes involved in idiopathic PNR LBP (as opposed to LBP relieved by placebo or surgery), and also may be the reason why patients in that PR group responded to lidocaine to relieve LBP. SP is assumed to be a neurotransmitter in the primary afferent neuron, and ME decreases the firing rate of SP-containing neurons [11].

It is instructive to inspect in greater detail the analytical measurements obtained from one patient (also shown in Fig. 2). The ratio of the pre-incubation to post-incubation amount of the peptide-LI varied over a wide range. For example, ME-LI decreased 600%. LE-LI remained unchanged, SP-LI increased 200%, BE-LI increased 128%, and the ME-LI from its precursors increased 2730%. The peptidases contained in the CSF of these patients clearly have acted upon the proenkephalin A, tachykinin, and POMC precursors, and have excised the neuropeptides from those precursors. Those changes indicate that CSF contains those intact neuropeptide-LI and that incubation metabolizes (ME-LI decreased) and synthesizes (LE-LI, SP-LI, BE-LI, and precursor-ME-LI increased) neuropeptides. Thus, these multiple neuropeptide systems display a wide range of metabolic activity.

Because of the rapid velocity of various neurochemical events it was important in this study to obtain a lumbar sample very quickly in the clinic and to freeze it immediately in liquid nitrogen to stop all metabolic processes. The endogenous neuropeptide content is a reflection of two different processes in lumbar CSF, namely, synthesis and degradation. On one hand, synthesis is a process by which neuropeptides are released from different-sized intermediate precursors. For example, ME derived from proenkephalin A; LE could be formed from the proenkephalin A and proenkephalin B precursors; and SP derived from several different precursors (α-, β-, γ-preprotachykinins). BE derived only from the POMC precursor. On the other hand, neuropeptide degradation occurred by the action of various proteolytic enzymes including aminopepti-

dases [17], dipeptidyl aminopeptidases, enkephalinases, dipeptidylcarboxypeptidases, CPB-like, and T-like activity.

It was important to distinguish among three separate types of patient populations that provided the lumbar CSF. The control category includes patients in whom the clinician determines that no surgery is required. Either the lumbar puncture itself or the injection of saline relieved the pain. The transient LBP could have derived from physical or emotional stress and not from a defective neuropeptidergic system.

PR, however, do require surgery to relieve their LBP. Their pain derives from spinal cord nerve problems and probably not from a defective neuropeptide metabolism. However, the different neuropeptide levels may reflect the attempt of the neuropeptidergic pathways to relieve the pain and to re-establish homeostatic conditions.

The PNR do not require surgery. Indeed, the clinician requires an objective criteria to identify this class of patients, and this rational study combining chromatographic, analytical, and enzymatic methods may be a step in that direction. It is clear that some of these PNR patients may be hypochondriacs or malingerers, and thus an appropriate test (Minnesota MultiPhasic Inventory, MMPI) and work history may help to identify those patients. However, a very important subgroup of idiopathic LBP patients may also be included in this PNR group. For example, we hypothesize that some of the PNR have a defective neuropeptidergic system associated with the synthesis or degradation of precursors and/or a synthesis or degradation problem in the metabolizing of the neuropeptides. Clearly, the tachykinin (SP) and proenkephalin A (ME, LE) data in Table I show that those two neuropeptidergic pathways operate at different levels in these LBP patients; specifically, SP-LI is much higher in the PNR *versus* PR patients. Thus, the data in this study are compatible with our hypothesis.

Neuronal events consist, in part, of the synthesis of precursor molecules in the cell body, anterograde axonal transport and metabolism of precursors, pre-synaptic vesicular storage of neuropeptides, synaptic release, metabolism, and diffusion of neuropeptides and post-synaptic receptor binding of neuropeptides. Furthermore, neuropeptides that were released into the CSF derive from synaptic diffusion, pre-synaptic and post-synaptic receptor binding and release, and free nerve-endings. The brain is considered to be the site of synthesis of enkephalins and endorphins, and the spinal cord dorsal root ganglia to be one of the main sites of the synthesis of tachykinins. Within the CSF, large and intermediate-sized precursors are metabolized by enzymes to neuropeptides and to inactive metabolites. Therefore, each CSF sample reflects the steady-state equilibrium of these multiple dynamic processes.

The measurements in this present work are in agreement with these general concepts and with data found in previous studies [3–5,16,28], wherein it was suggested that the amount of TOR correlated with clinical findings [3] and that tachykinins play a role in LBP [4]. It is important to study human CSF rather than animal CSF because of known species differences in neuropeptide processing [29].

Other workers have studied the presence of neuropeptides in CSF [18,30], but in another clinical population such as psychiatric patients; they used a much larger volume of CSF. In another study, SP was not detected by HPLC–RIA, but rather, an N-terminally extended SP was detected [31].

CONCLUSIONS

Chronic idiopathic human LBP involves the metabolism of several neuropeptides, including protachykinin, which produces SP, and proenkephalin A, which produces ME and LE.

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