Identification of neuropeptide FF-related peptides in human cerebrospinal fluid by mass spectrometry

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Received 16 September 2002; revised 4 November 2002; accepted 4 November 2002

First published online 14 November 2002

Edited by Guido Tettamanti

Abstract Several neuropeptide FF (NPFF)-related peptides, known as modulators of the opioid system, have been previously characterized in bovine and rodent brain. Reverse-phase high pressure liquid chromatography (HPLC) fractions of a human with normal pressure hydrocephalus cerebrospinal fluid (CSF), co-migrating with NPFF-related synthetic peptides, were characterized by capillary HPLC coupled on-line to nanospray ion trap tandem mass spectrometry. Two peptides present in the pro-NPFF$_A$ precursor, NPAF (AGEGLNSQFWSLAAPQRF-NH$_2$) and NPSF (SLAAPQRF-NH$_2$), were identified. The monitoring of NPFF-related peptides in human CSF can be helpful to understand their roles in pain sensitivity.

Key words: Neuropeptide FF;
Electrospray tandem mass spectrometry;
Human cerebrospinal fluid;
Nanospray ion trap tandem mass spectrometry

1. Introduction

The knowledge of the mammalian endogenous anti-opioid neuropeptide FF (NPFF, FLFQPQRF-NH$_2$) system has recently been greatly improved by the identification of two receptors, NPFF$_1$ [1,2] and NPFF$_2$ [1–3], and by the characterization of two precursor proteins (pro-NPFF$_A$ and pro-NPFF$_B$ [2]) predicted to generate peptides carrying a homologous sequence in their C-terminal part [1,2,4,5]. Among those peptides, NPFF and neuropeptide AF (NPAF, AGEGLSSPFWSLAAPQRF-NH$_2$) have been identified in bovine brain and have been shown to modulate the analgesic effects of morphine in rodents [6]. Both peptides possess the C-terminal sequence PQRF-NH$_2$, essential for their interactions with NPFF receptors [7]. More recently, on the basis of the difference in the sequences of precursors between species [4,5], several NPFF-related peptides containing either the NPFF or the NPAF C-terminal sequence (NPSF, SLAAPQRF-NH$_2$) have been isolated in rodents [8,9]. Moreover, a second precursor belonging to this family has been described also exhibiting a PQRF-NH$_2$ C-terminus [2] and a corresponding octadecapeptide has recently been identified in rat brain [10]. The affinity of this peptide to NPFF receptors, however, remains to be determined.

The human genes encoding pro-NPFF$_A$ and pro-NPFF$_B$ have been cloned [1,2,4,5]. According to their amino acid sequence and to the presence of putative cleavage motifs, two peptides ending in PQRF-NH$_2$ may be predicted to be matured from human pro-NPFF$_A$ and one from human pro-NPFF$_B$ in NPFF neurons and should be released in the extracellular space. The first one from pro-NPFF$_A$ corresponds to the NPFF sequence extended by SQA in the N-terminus (SQA-NPFF, SQAFLFQPQRF-NH$_2$) (Fig. 1). The second one is an octadecapeptide (hNPAF, AGEGLNSQFWSLAAPQRF-NH$_2$), which differs by two amino acids from the bovine NPAF. An octapeptide (NPVF, VPNLPQRF-NH$_2$) is expected from pro-NPFF$_B$ (Fig. 1) [1].

In the present study we have investigated the presence in human cerebrospinal fluid (CSF) of NPFF-related peptides ending in PQRF-NH$_2$ and predicted to be generated from pro-NPFF$_A$ and pro-NPFF$_B$. CSF was obtained during the changing of a ventriculo-peritoneal shunting valve in a patient with normal pressure hydrocephalus to improve clinical symptoms by evacuating CSF. Therefore, a large volume of CSF could be obtained which could contain several NPFF-related peptides originating from different regions of the brain. In fact, whereas the tissue localization of both precursors is largely unknown in human brain, pro-NPFF$_B$ as well as pro-NPFF$_A$ mRNA have been detected in rat, particularly in hypothalamus [4,11].

High performance liquid chromatography (HPLC) fractions of CSF, co-migrating with peptide standards, were subjected to further characterization by capillary HPLC coupled on-line to nanospray ion trap tandem mass spectrometry (nanospray MS/MS) which has proven to be an efficient tool for the characterization of neuropeptides in biological samples [10,12].

2. Materials and methods

2.1. Chemicals

NPFF-related peptides were synthesized by the solid-phase method using FMOC chemistry as previously described [13]. FMOC amino acid derivatives were purchased from Bachem, France. Iodination of [D-Tyr^1,(NMe)Phe^3]NPFF was performed according to Dupouy and Zajac [14].

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Abbreviations: CSF, cerebrospinal fluid; HPLC, high pressure liquid chromatography; RIA, radioimmunoassay; MS, mass spectrometry; MS/MS, tandem mass spectrometry

0014-5793/02/$22.00
PII: S0014-5793(02)03686-4

2.2. CSF sample

CSF was collected from a 74 year old patient with normal pressure hydrocephalus during the changing of a shunting ventriculo-peritoneal valve for a valve dysfunction with the informed consent of the patient. CSF was collected from an external catheter at 08.00 h and immediately frozen at −20°C. After centrifugation of a 20 ml CSF sample (2000×*g* for 10 min at 4°C), supernatant was lyophilized and stored at −80°C until HPLC analysis. Protein concentration in CSF before lyophilization was 0.84 mg/ml.

2.3. HPLC procedures

The HPLC system consisted of a solvent delivery pump (ICS, France) and an injection valve model rheodyne with a 500 μl loop. Dried CSF was dissolved in mobile phase in order to get a protein concentration of 2 mg/ml.

Diluted CSF samples (500 μl per run, 12 runs) were applied to a C_8 Aquapore RP300 Brownlee (4.6×200 mm) (HPLC-1). The column was equilibrated with 70% mobile phase A (0.09% trifluoroacetic acid in water) and 30% mobile phase B (0.09% trifluoroacetic acid in 75% acetonitrile) at a flow rate of 400 μl/min. Elution was achieved by 30% B for 6 min, followed by a linear gradient of 30–60% B during 50 min. HPLC fractions of 1 min (0.4 ml), starting at the injection time, from 12 separate runs were pooled and lyophilized.

Fractions co-migrating with NPSF and NPVF were subjected to a second HPLC separation (HPLC-2). The column C_8 Aquapore RP300 Brownlee (4.6×200 mm) was equilibrated with 94% mobile phase A (0.09% trifluoroacetic acid in water) and 6% mobile phase B (0.09% trifluoroacetic acid in 75% acetonitrile) at a flow rate of 400 μl/min. Elution was achieved by a linear gradient of 6–60% B during 56 min. Fractions of 0.4 ml were collected.

Fractions co-migrating with SQA-NPFF, NPFF and hNPAF were subjected to a second HPLC separation on a Spheri-5 RP-8S 5 μm Brownlee (2.1×220 mm) (HPLC-3). The column was equilibrated with 98% mobile phase A (0.09% trifluoroacetic acid in water) and 2% mobile phase B (0.09% trifluoroacetic acid in 75% acetonitrile) at a flow rate of 400 μl/min. Elution was achieved by 2% B for 6 min, followed by a linear gradient of 2–80% B during 50 min. Fractions of 0.4 ml were collected.

2.4. Radioimmunoassay (RIA)

RIA was performed on samples analyzed by mass spectrometry using a NPFF antiserum. The NPFF-related peptide concentration in samples was determined according to calibration curves of the corresponding synthetic peptide. The method of obtaining NPFF antiserum and the RIA procedure have been previously described [9].

2.5. On-line capillary HPLC/nanospray MS/MS

HPLC fractions collected using either the HPLC-2 or the HPLC-3 procedure were concentrated under vacuum and analyzed by on-line capillary HPLC/nanospray MS/MS. The sample was injected onto a C18 PepMap™ (LC Packings) column (75 μm×150 mm). The separation was achieved using an isocratic elution at 0% B for 2 min followed by a linear gradient of 0–40% B in 38 min at a flow rate of 150 nl/min. Solvent A was 0.1% formic acid in H_2O/CH_3CN (99/1 v/v) and solvent B was 0.1% formic acid in H_2O/CH_3CN (10/90 v/v). The eluent was introduced into an LCQ Deca ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) through a nanoflow needle (New Objective, Cambridge, MA, USA) at 2.0 kV. MS/MS data were acquired using a three *m/z* unit ion isolation window and a relative collision energy of 35%.

3. Results

3.1. HPLC separation of NPFF-related peptides

In order to identify NPFF-related peptides in CSF, the corresponding synthetic peptides were first separated by successive HPLC and their fractions of elution were determined (Fig. 1 and Table 1). Inter-day coefficient of variation in retention time was less than 5% for all peptides in all HPLC conditions except for NPSF and NPVF in HPLC-1 conditions (less than 10%). CSF was subjected to the same HPLC runs and fractions of interest corresponding to the fractions of elution of synthetic peptides (Table 1) were collected. Adjacent fractions were combined to take into account inter-day variations in peptide migration and were lyophilized before MS analysis.

3.2. Identification of hNPAF and NPSF by mass spectrometry

Synthetic hNPAF (doubly charged ion at *m/z* 990.0) and NPSF (singly charged ion at *m/z* 888.5) were first analyzed by on-line capillary HPLC/nanospray MS/MS and showed retention times of 42.2 min and 28.6 min in the capillary HPLC system, respectively (Fig. 2A,C). Analyses of the HPLC fractions collected from the CSF sample, the retention time of which corresponded to that of hNPAF and NPSF, were then performed, taking care to avoid any carry over from one injection to the other. In each case, a peak of the appropriate *m/z* value at the retention time of the corresponding synthetic peptide was detected (Fig. 2B,D). The unambiguous identification of each neuropeptide was then given by the MS/MS data.

The MS/MS spectrum of the synthetic hNPAF peptide displayed a series of y and b fragment ions, including an intense y_4 fragment ion at *m/z* 546.3 (Fig. 3A). This fragment corresponds to the C-terminal PQRF-NH_2 sequence, which is a characteristic feature of all expected NPFF-related peptides. MS/MS analysis of the peak detected in the HPLC fraction corresponding to hNPAF of the CSF sample is displayed in Fig. 3B. The MS/MS fragmentation pattern observed is identical to the fragmentation pattern of synthetic hNPAF and thus unambiguously identifies hNPAF in the CSF sample.

Similarly, the MS/MS spectrum of the synthetic NPSF peptide displayed a series of y fragment ions, including the characteristic y_4 fragment ion corresponding to the PQRF-NH_2 C-terminal sequence (Fig. 4A). The MS/MS fragmentation pattern of the peak detected in the HPLC fraction corresponding to NPSF of the CSF sample is identical to the fragmentation pattern of the corresponding synthetic peptide (Fig. 4B). NPSF is thus unambiguously identified in the CSF sample.

The three other NPFF-related peptides (NPVF, SQA-

Table 1
Reliability of the procedure: HPLC fractions of elution, RIA specificity and MS/MS detection limits of NPFF-related synthetic peptides

	HPLC-1 fraction number	HPLC-2 fraction number	HPLC-3 fraction number	RIA IC_{50} (fmol)	MS/MS detection limit (fmol)
SQA-NPFF	35 ± 1	–	43 ± 1	33	10
NPFF	35 ± 1	–	44 ± 1	30	10
hNPAF	41 ± 1	–	45 ± 1	420	20
NPSF	13 ± 1	35 ± 1	–	867	1
NPVF	14 ± 1	38 ± 1	–	1500	1

HPLC fractions corresponded to 1 min elution.
The MS/MS detection limit was defined as the minimum amount of peptide necessary to unambiguously identify its specific MS/MS fragmentation pattern.

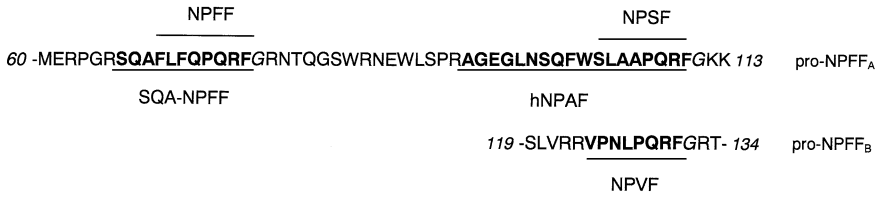

Fig. 1. NPFF-related peptides in human pro-$NPFF_A$ and pro-$NPFF_B$ sequences. *G*: amidation site.

NPFF, and NPFF), which could also be expected, were not detected in the CSF sample using this approach.

The HPLC fractions of CSF analyzed by mass spectrometry were also subjected to a RIA using an antiserum anti-NPFF. The mean IC_{50} values obtained from independent experiments ($n \geq 3$) with synthetic peptides are presented in Table 1. Inter-assay coefficient of variation was less than 15%. hNPAF and NPSF immunoreactivities were estimated to be 35 and 100 fmol/ml CSF, respectively. NPVF immunoreactivity was estimated at 100 fmol/ml CSF, SQA-NPFF at 7 fmol/ml and NPFF 12 fmol/ml. The amounts of hNPAF and NPSF detected during the mass spectrometric analyses were estimated to be around 50 fmol and 5 fmol, respectively, according to the signal intensity observed for 100 fmol of synthetic hNPAF and 60 fmol of synthetic NPSF (Fig. 2). The amounts of hNPAF and NPSF in the CSF were then estimated to be around 20 and 10 fmol/ml, respectively.

4. Discussion

We report here on the availability of a reliable method to identify and quantify NPFF peptides, which led for the first

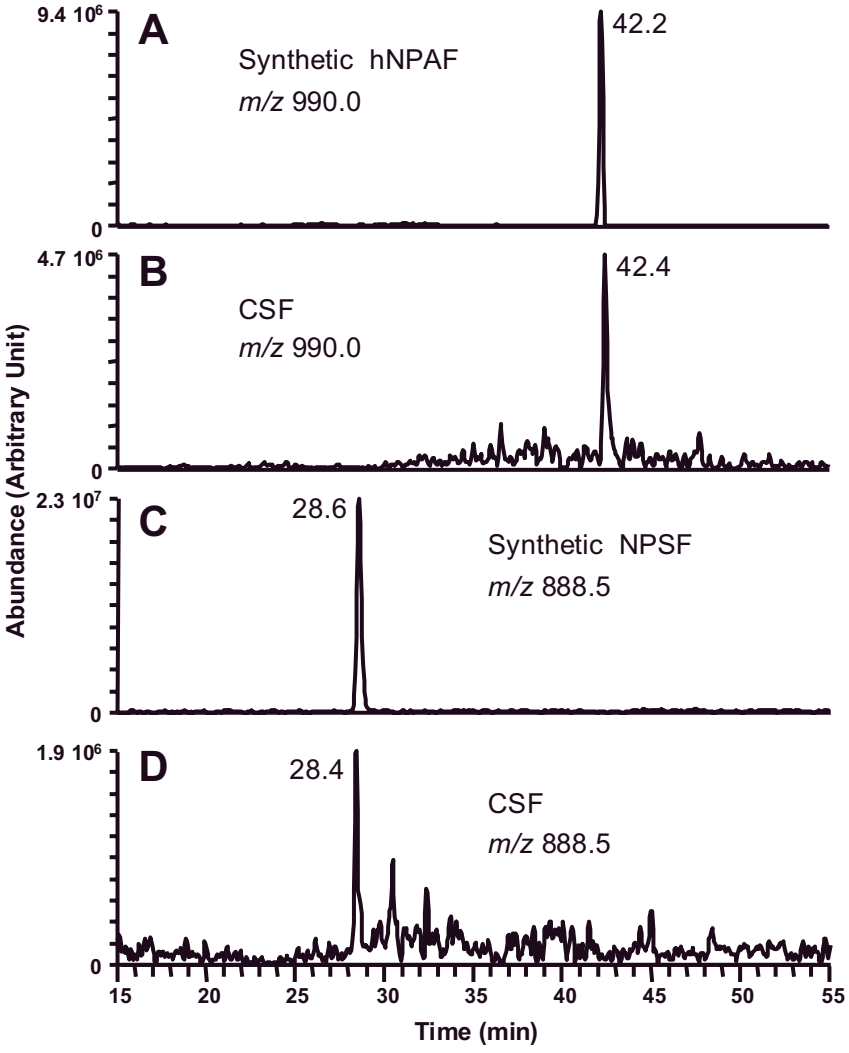

Fig. 2. Capillary HPLC/nanospray MS analyses of hNPAF and NPSF. Reconstructed ion chromatograms of (A) 100 fmol of synthetic hNPAF (doubly charged ion at *m/z* 990.0), (B) collected HPLC fraction (40–42 min HPLC-1 and 41–44 min HPLC-3) of CSF sample at *m/z* 990.0 (doubly charged ion of hNPAF), (C) 60 fmol of synthetic NPSF (singly charged ion at *m/z* 888.5), (D) collected HPLC fraction (12–14 min HPLC-1 and 34–36 min HPLC-2) of CSF sample at *m/z* 888.5 (singly charged ion of NPSF).

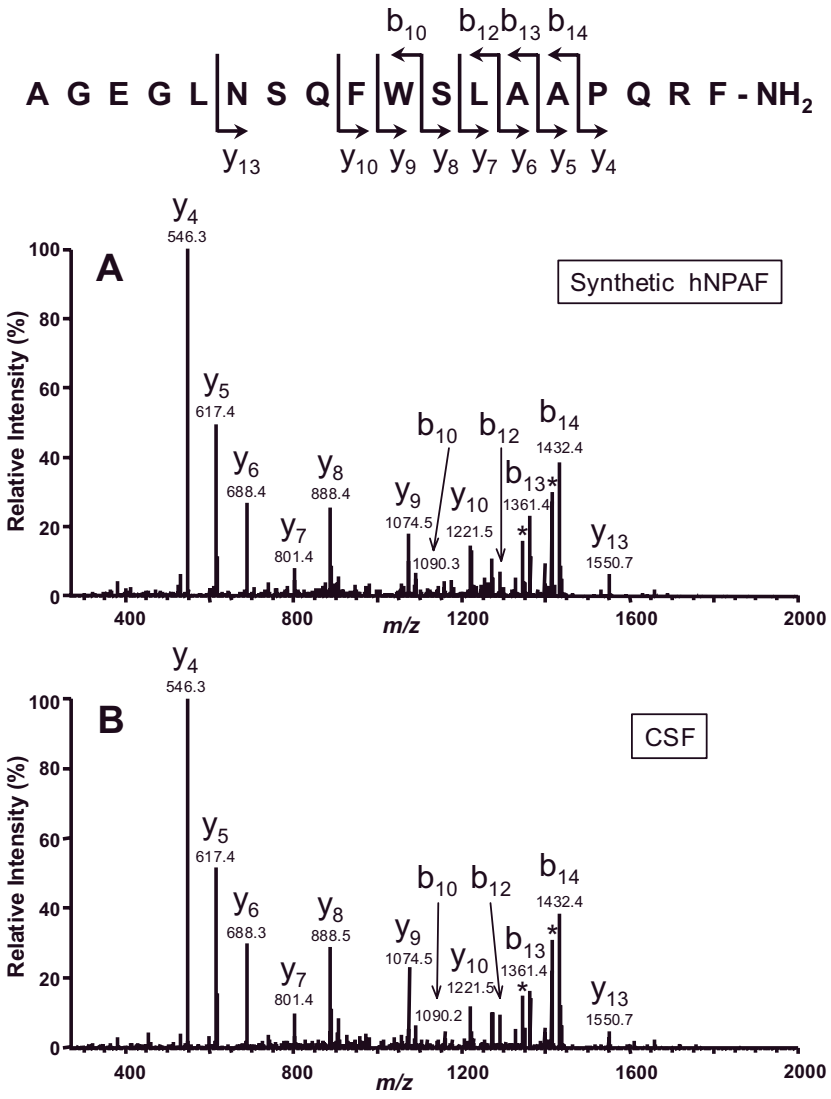

Fig. 3. Identification of hNPAF in CSF. The tandem mass spectra of (A) synthetic hNPAF and (B) hNPAF in an HPLC fraction of CSF sample were acquired from the doubly charged precursor ion at *m/z* 990.0. Fragment ion peaks are labeled according to Biemann's nomenclature [22]. Asterisks correspond to the loss of NH_3 from the b ions. The peptide sequence and fragmentation pattern is indicated at the top.

time to the characterization in human CSF of two peptides, NPSF and hNPAF, derived from pro-$NPFF_A$. The strategy is based on the analysis of untreated CSF by successive HPLC separations followed by on-line capillary HPLC/nanospray MS/MS analyses. This MS/MS procedure allows the unambiguous identification of femtomoles of neuropeptides by their specific fragmentation pattern.

This study was performed on CSF collected from a shunting valve of a patient with normal pressure hydrocephalus in order to obtain a large volume of sample of ventricular origin which could contain peptides issued from both NPFF precursors. Mass spectrometry did not evidence NPVF, SQA-NPFF or NPFF whereas RIA analysis revealed NPFF-like immunoreactivity.

NPFF-like immunoreactivity has been previously evidenced in human plasma and CSF [15–17] suggesting that NPFF-related peptides of neuronal origin circulate in human biological fluids. A major peak of immunoreactivity, coeluting with synthetic NPFF, was observed using HPLC separation [15,16]. It could not be excluded that immunoreactivity co-eluting with NPFF could represent another NPFF-related peptide such as SQA-NPFF. This hypothesis is supported, in our study, by the coelution of SQA-NPFF and NPFF in the conditions of the first HPLC. After a second HPLC 7 fmol/ml SQA-NPFF immunoreactivity and 12 fmol/ml NPFF immunoreactivity were quantified in human CSF. The previous study of Sundblom et al. [16] reported 1.5 fmol/ml NPFF immunoreactivity. This discrepancy may be explained by the origin of the CSF. In the study of Sundblom et al. [16], the CSF was collected by lumbar punction of healthy volunteers or chronic pain patients, whereas in the present study, CSF was of ventricular origin from a patient with normal pressure hydrocephalus. Gradients between ventricular and lumbar CSF of several neurotransmitters and neuropeptides have been reported [18]. Furthermore, changes in CSF concentration of several neuropeptides have been observed in patients with normal pressure hydrocephalus [18].

The presence of SQA-NPFF and NPFF was not confirmed by mass spectrometry analysis but the amount of these peptides estimated by RIA is close to their limit of detection in

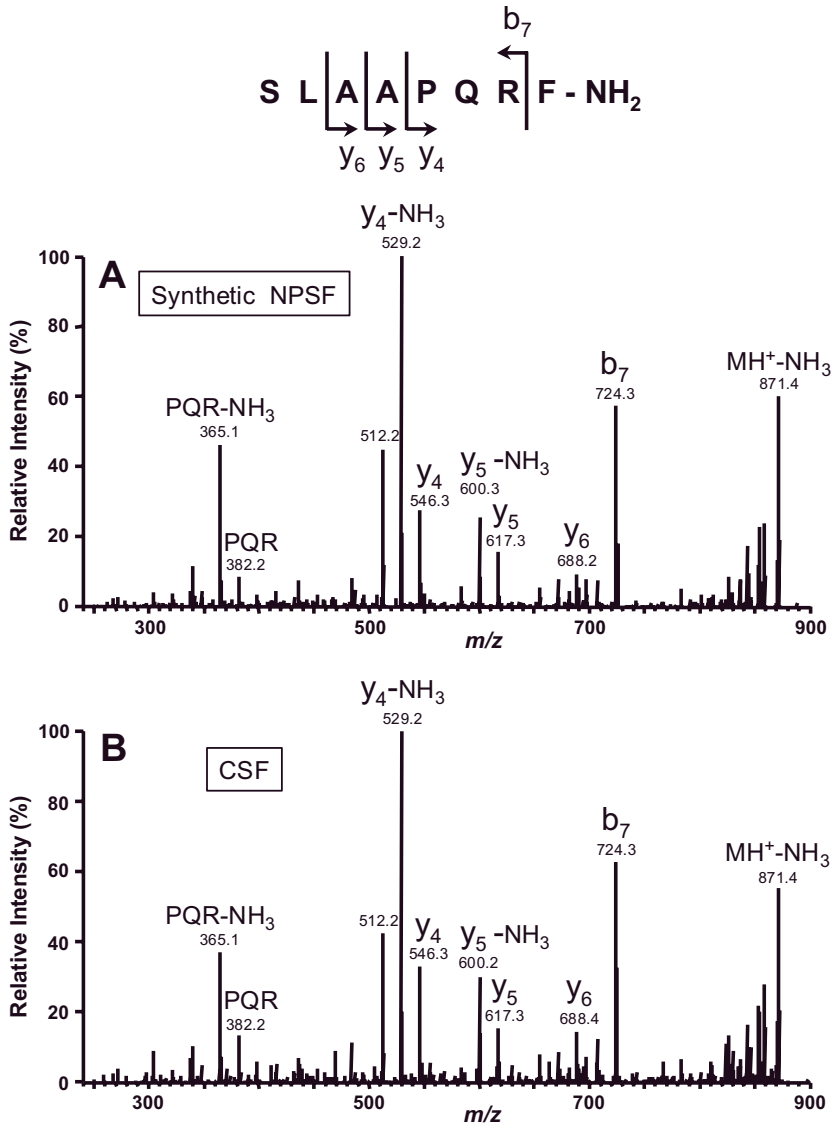

Fig. 4. Identification of NPSF in CSF. The tandem mass spectra of (A) synthetic NPSF and (B) NPSF in an HPLC fraction of CSF sample were acquired from the singly charged precursor ion (MH^+) at *m/z* 888.5. Fragment ion peaks are labeled according to Biemann's nomenclature [22]. PQR corresponds to an internal fragment ion. The peptide sequence and fragmentation pattern is indicated at the top.

mass spectrometry. Another possibility is that the immunoreactivity observed is not due to SQA-NPFF and NPFF but to unknown peptides eluting at the same retention times in our HPLC conditions and cross-reacting with the NPFF antiserum. This latter hypothesis is likely to apply to NPVF, an octapeptide predicted to be matured from pro-$NPFF_B$ in humans. Its amount was estimated to be 100 fmol/ml CSF by RIA, and it was not detected by mass spectrometry. In this case, the estimated amount of NPVF was high enough to allow its characterization by mass spectrometry.

In this study, we identified hNPAF and NPSF by mass spectrometry in human CSF. NPSF represents the C-terminal part of hNPAF but is not preceded in pro-$NPFF_A$ by a cleavage motif suggesting that it is a fragment issued from a longer peptide by enzymatic degradation inside the neurons, in the extracellular liquid of nervous tissue and/or in the CSF. This peptide has also been isolated from nervous tissue of mouse and rat [8,9]. In contrast to hNPAF, NPSF displays a weak affinity for $NPFF_1$ and $NPFF_2$ receptors [19]. However, when intracerebroventricularly injected, these two peptides reverse morphine-induced analgesia in rodents [6,9]. In contrast, intrathecal injection of NPSF enhances morphine-induced analgesia in rat [20]. These results suggest a physiological role of these peptides in the nervous system by modulation of the opioid system.

Receptors for NPFF have been localized in human spinal sensory system by autoradiography [21] and $NPFF_2$ RNA expression has been detected in many brain regions [2]. In the present study, the identification in human CSF of NPFF-related peptides strongly suggests that pro-$NPFF_A$ protein expressed in human nervous system is also matured to generate some peptides resistant to peptidase degradation able to diffuse in the CSF biological fluid and to act at distance of their site of synthesis.

The monitoring of NPFF-related peptides in human CSF can be helpful to understand their roles in pain sensitivity.

Acknowledgements: This work was supported by grants from CNRS, ARC (4523) and the Région Midi-Pyrénées.

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