 ISSN 0172–780X **www.nel.edu**

NEW SECTION
Technical developments and novel technologies*

LEADING RESEARCH PAPER
Direct analysis of neuropeptides by *in situ* MALDI-TOF mass spectrometry in the rat brain

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Submitted: *November 7, 2002*
Accepted: *November 12, 2002*

Key words: ***in situ* Maldi-TOF; neuroimmunity; mass spectrometry; vasopressin**

Neuroendocrinology Letters 2003; ***24****(1/2):9–14 pii: NEL241203A01* *Copyright © Neuroendocrinology Letters* ***www.nel.edu***

Abstract The measure of neuropeptides is an important tool in biology to better define endocrine and neuroendocrine function. Traditionally most methods have relied on the development of specific antibodies. Newer molecular methodologies have used measures of gene expression of neuropeptide precursors, such as Northern blot, PCR or *in situ* hybridization analysis. Matrix-assisted laser desorption/ionization (MALDI) mass analysis is a novel powerful technique for investigation of neuropeptides. Multiple peptides and peptide forms can be detected simultaneously and with great sensitivity in tissue extracts or partially purified samples. We have now adapted a MALDI methodology for the direct measurement of neuropeptides on fresh tissue sections of rat brains. We have validated the method by examining peptidergic mass profiles of the supraoptic nucleus (SON) and caudate putamen hypothalamic regions. Interestingly, mass profiles showed that vasopressin, which is specifically present in the SON, is modulated when animals are treated with lipopolysaccharides. MALDI-MS on brain slides is a novel complementary technique for neurobiologists and endocrinologists in order to investigate the dynamic and regional distribution of neuropeptides during physiological events.

* The note from the Editor-in-Chief:
I would like to express a sincere gratitude to Professor Michel Salzet for his initiative to open a new section in the Neuroendocrinology Letters devoted to technical developments in neuroendocrinology, neurosciences, neuroimmunology, neurogenetic, psychoneuroimmunology, neuropharmacology, neuroproteomic, neuroimage. The aim of this section is to highlight novel technologies for brain research studies, and is opened here with the leading paper by Isabelle Fournier, Robert Day and Michel Salzet.

LEADING RESEARCH PAPER

Introduction

Neuropeptides serve important functions in the brain and in the periphery in cell-to-cell communication. The ability to rapidly and effectively establish peptides levels and forms is a critical aspect of neuroendocrinology. Changes in peptide levels can reflect their importance in specific events and can lead to establish their direct functions. The measure of various neuropeptides can be a long and complicated process that often involves the measure of only one peptide at a time, after sometimes lengthy and arduous purification procedures. Mass spectrometry techniques have offered a novel rapid and sensitive methodology for the detection and measure of multiple peptides within one sample. Research employing mass spectrometry for proteomic studies *i.e.* to characterize a protein pattern in normal or in pathological tissues has been intense. Of particular interest has been the development of direct analyses on fresh tissues using Matrix Assisted Laser Desorption/Ionization (MALDI) mass spectrometry. Since the last five years, the use of invertebrate models for direct analysis of neuropeptides secreted by neurons using fresh tissues dissected and deposited in vial with matrix has been in constant development [1–6]. Sweedler *et al* have recently studied the mollusc *Aplysia californica* using the direct profiling of neuropeptide precursor processing in a single cell [7–8]. The use of MALDI allows obtaining *in situ* amino acids sequence using the fragmentation method of post-source decay (PSD) [9–11]. This is a strategy for peptide identification and characterization of post-translational modifications. However, Caprioli and collaborators examined a completely novel approach. They have performed the direct spatial mapping of peptides in cells using mass spectrometry [12–13]. This group developed the used of MALDI-MS to image fresh tissues slices after coating the sample with matrix. Molecular ion images are then successfully generated from area of rat pituitary where over than 50 different peptides as well as their precursors, isoforms and metabolic fragments can be observed [9]. The development of new imaging computer software allowing both instrument control and data imaging acquisition and processing for MALDI-MS of thin tissue sections has now opened the door of a new world for mass spectrometry application on biological tissue [12–13].

In the present study, we performed for the first time a comparative study from rats subjected to a bacteria-like challenge using direct MALDI-MS analysis on rat fresh tissue slices in order to investigate neuropeptides by *in situ* MALDI-TOF mass spectrometry. We specifically focused our interest on peptides in the supraoptic nucleus, such as vasopressin and demonstrated that mass spectrometry can also be a tool for *in situ* and dynamic studies.

Material And Methods

MALDI mass spectrometry

MALDI-TOF mass spectra were performed on a Voyager-DE STR mass spectrometer (Applied Biosystems, Framingham, MA, USA) MALDI-TOF with delayed extraction and operating with a pulsed nitrogen laser. Mass spectra were recorded in the linear or refletron positive mode using a delay time of 150–1000 ns, depending on the mass range. Individual external calibrations were performed on each slide using standard neuropeptides (Substance P, ACTH18–39, ACTH 7–38, Bovine Insulin and Bovine ubiquitin). Each spectrum is the results of 400 laser shots averaged on the area of interest. Slices were visualized in the mass spectrometer with the CCD camera equipping the instrument. The magnification was high enough to allow an easy orientation on the tissue. Matrix was used as purchased and prepared in standard solvent mixtures (ACN/water 4:1, v/v). Matrix solutions were applied with a micropipette and then allowed to dry at room temperature.

Tissue preparation

Adult female Wistar rats (animal use accreditation by the French Ministry of the Agriculture N° 04860) were used in this study and maintained under standard care. Female rats weighing 300–350g were injected i.p. with 200 μl of lipopolysaccharides (125 μg/ml/100g animal) and sacrificed 9 hours later. Brains were dissected from the animals and fresh frozen sections (12 μm) were obtained on cryostat. In order to localize the supraoptic nucleus some of the sections were transferred to glass slides and stained with toluidine blue. Sections of interest were then transferred to the MALDI-MS sample plate before matrix addition (alpha-cyano-4-hydroxycinnamic acid or sinapinic acid).

Results And Discussion

In situ MALDI analysis of rat brain sections (Figure 1) have demonstrated that good quality mass spectra can be obtained. In fact, different spectra recorded from different rat brain regions i.e. the supraoptic nucleus (SON) and the caudate putamen (CP) region revealed a large number of ions ranging from m/z 500 up to m/z 6000 reflecting the high sensitivity of the technique in order to detect *in situ* neuropeptides (Fig. 1). Moreover, the range of peptide detection depends on the nature of the matrix used. Alpha-cyano-4-hydroxycinnamic acid allows detecting peptides with a molecular mass ranged between 500 to 3000 Da with a 0.5 Da precision (Fig. 2a). By contrast (Fig. 2b), sinapinic acid matrix is more useful for larger peptides (>10 Da). The lack of precision (0.5 Da) comes from the fact that we have used an external calibration. However, in further work we will be able to use the peptides identified in the present work as internal calibrants.

Considering the above technical realization, comparison of mass profiles (Fig. 1) obtained in SON and CP shows different mass pattern reflecting both common masses between both brain region (Table 1) or by

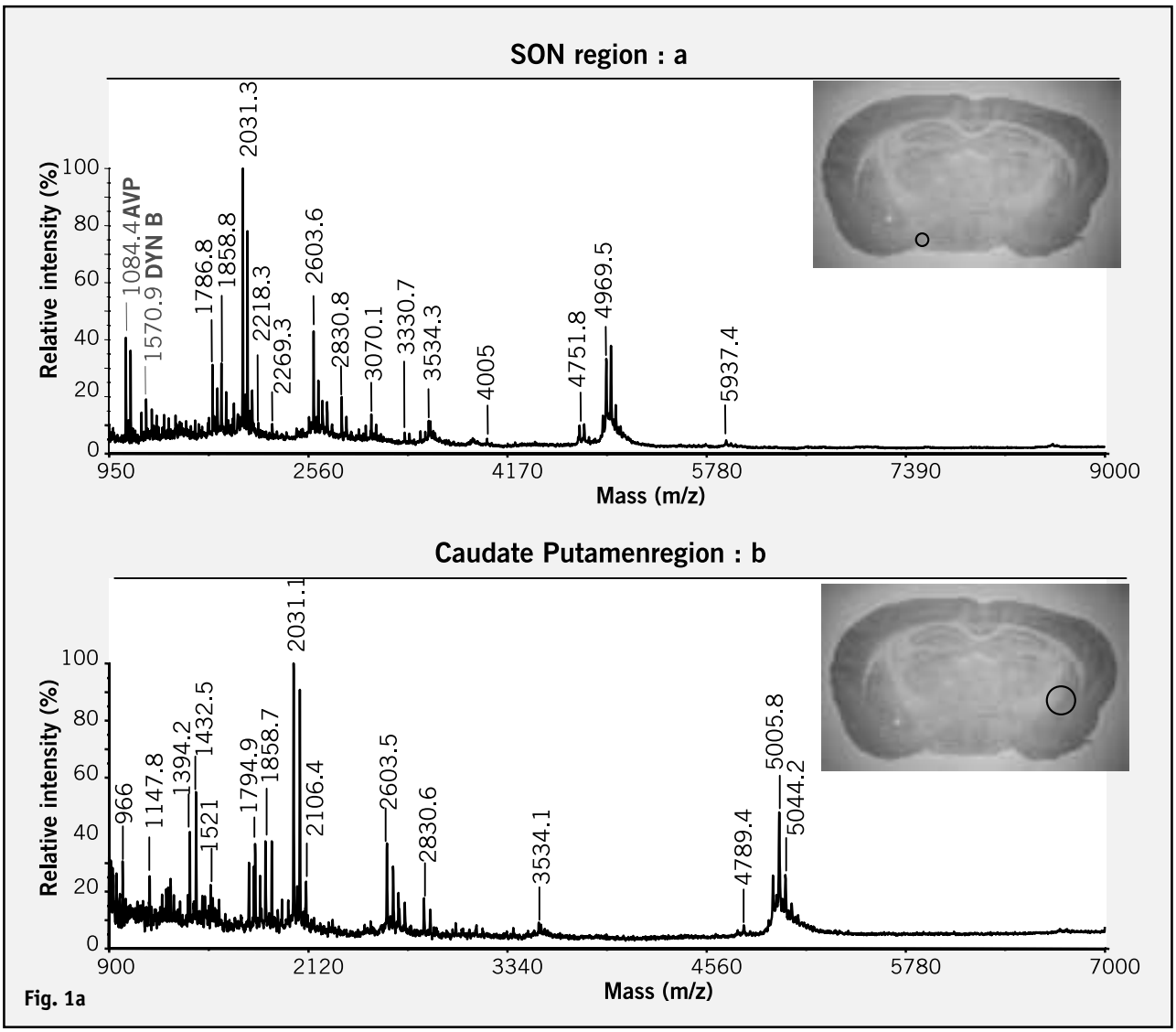

Figure 1. Rat brain sections (12µm) obtained with a cryostat.
a: MALDI-TOF mass spectrum in the positive mode recorded directly from the supraoptical nucleus (matrix: CHCA).
b: MALDI-TOF mass spectrum in the positive mode recorded directly from the Caudate Putamen region (matrix CHCA).

specific peptides to one region e.g. vasopressin (AVP) (1084.4 Da) which is known to be specific of SON and absent in CP (Figs. 1, 3a).

We focused on AVP due to its specific localization in the SON (Fig. 3a). In this context, we attempted to estimate if *in situ* MALDI experiments can be a dynamic tool to follow peptide evolutions directly in the tissue after several physiological changes. Previous studies have shown that AVP levels change during bacteria infection [14]. Using immunochemical techniques it was shown that LPS administered in the medium for 3h increased significantly the AVP release lasting up to 6h after treatment. These results provide evidence that LPS influences AVP secretion in SON neurons. Using *in situ* MALDI, we demonstrate that a previous treatment of rats with LPS in order to mimic bacteria challenge lead to an extensive release of AVP from SON cells (Fig. 3c). No ion corresponding to this peptide can be detected after 9 hours post-injection, even though CCK (10–20) and other peptides are still present. On the contrary, in the control rats injected with a saline solution, AVP is not released and the peak corresponding to this neuropeptide can be observed in the mass spectrum (Fig. 3b). These data are in agreement with those obtained by Nava *et al.* [14]. Moreover, Matsunaga *et al.* [15] confirmed that administration of low dose (5 μg/kg) and high dose (125 μg/kg) of LPS induced

Table 1: Examples of peptides detected by in situ MALDI in SON and CP from rat brain sections based on their known mass.

Peptide	Mass (Da)	Region of expression
Dynorphin A (1-8)	981.75	SON, CP
Vasopressin	1084.4	SON
CCK (1-8) sulfoxyde	1079.86	SON, CP
CCK (10-20)	1248.98	SON, CP
DYnorphin B	1570.27	SON
Unknown	2603.6	SON, CP
Unknown	2830.4	SON,CP
Unknown	3534	SON,CP

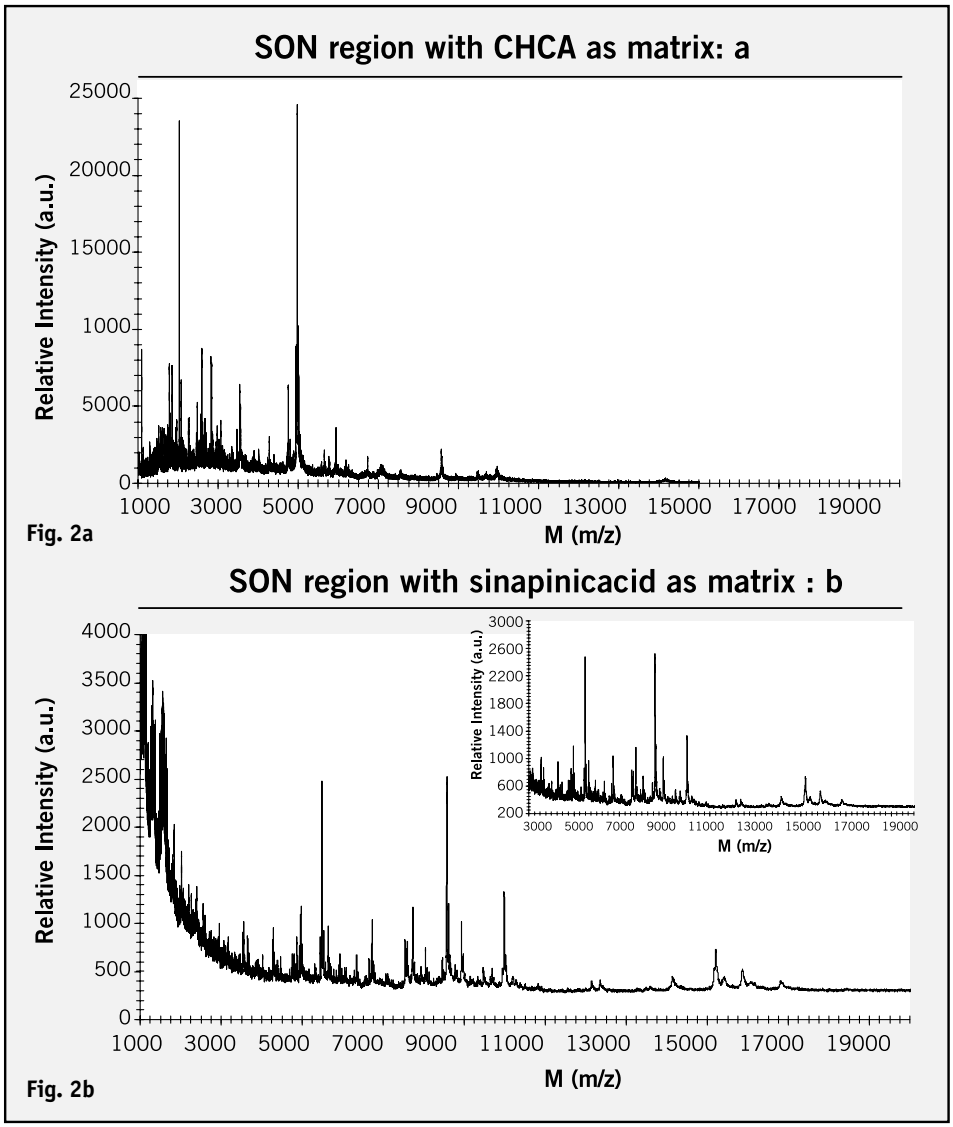

Figure 2 a: MALDI-TOF mass spectrum in the positive mode recorded directly from the supraoptical nucleus (matrix: CHCA acid).
b: MALDI-TOF mass spectrum in the positive mode recorded directly from the supraoptical nucleus (matrix: sinapinic acid).

intense nuclear c-fos Fos immunoreactivity in many oxytocin (OT) and AVP neurons in all the observed hypothalamic regions. The percentage of c-fos positive nuclei in OT magnocellular neurons was higher than that of AVP magnocellular neurons in the SON, the magnocellular neurons in the paraventricular nucleus (magPVN), rostral SON (rSON), and nucleus circularis (NC), whose axons terminate at the posterior pituitary for peripheral release. The percentage of c-fos positive nuclei in AVP parvocellular neurons in the paraventricular nucleus (parPVN) was higher than that of OT parvocellular neurons, whose axons terminate within the brain for central release. Moreover, the percentage of c-fos-positive nuclei in AVP magSON neurons and rSON was significantly higher than that of the magPVN and NC when animals were given LPS via intraperitoneal (i.p.)-injection. This regional heterogeneity was not observed in OT magnocellular neurons of i.p.-injected rats or in either OT or AVP magnocellular neurons of intravenous (i.v.)-injected rats. This suggest that LPS-induced peripheral release of AVP and OT is due to the activation of the magSON, magPVN, NC, and rSON, and the central release of these hormones is in part derived from the activation of parPVN neurons. The sum of these data may explain some central effects observed *in vivo* after lipopolysaccharides administration.

The results presented above confirm that *in situ* MALDI is a potentially powerful methodto investigate such dynamic physiological experiments, Furthermore, most of the peptides identified by their mass like AVP (1084.4 Da) also contained an analogue which is associated with potassium (MK+ : 1122.6) but not with sodium (MNa+) (Fig. 3a). This reflects the fact that *in situ* neuropeptides are complexed with potassium and less with sodium. Also, some peptides are oxidized like the CCK (1–8). This could be explained by the fact that although the peptides are embedded in the MALDI matrix, oxidation can occurred or as we have already found during FMRF-amide purification by reversed-phased HPLC, oxidation can be considered as an inactivation process of active peptide [16].

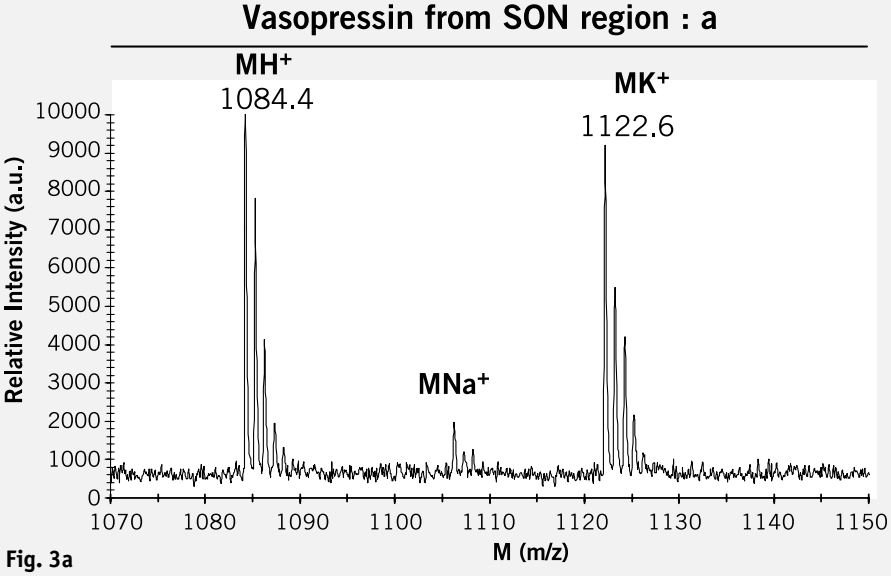

Fig. 3a

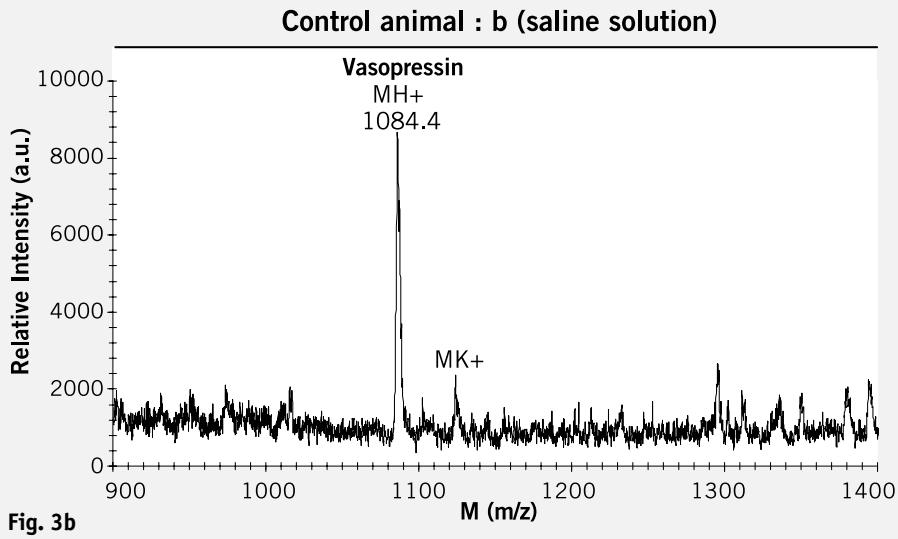

Fig. 3b

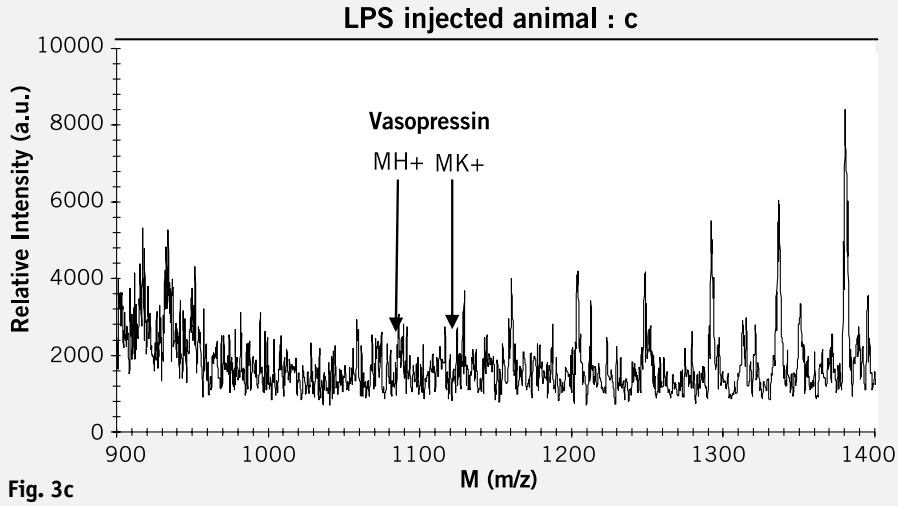

Fig. 3c

Figure 3 a: MALDI-TOF mass spectrum in the positive mode recorded directly from the supraoptical nucleus (matrix: CHCA). Zoom on Vasopressin ions.
b, c: Comparison of MALDI-TOF mass spectra recorded in the supraoptical nucleus (matrix CHCA); b: control (saline injection) *vs.* c: challenged animals.

Taken together these results reflect the high sensitivity and discrimination power of the MALDI-MS technique. These data are also in agreement with those of Caprioli *et al.* [17]. In the rat pituitary, using MALDI-MS, they detected all the pro-opiomelanocortin derived peptides as well as other neuropeptides which are known to be absent in SON and CP brain regions as confirmed by MALDI-MS profiles obtained in the present study (Figs. 1 and 2). Thus, over 50 neuropeptides have been detected in brain slides based on their molecular masses measurement. As suggested by Chaurand *et al.* [12] truncated analogs with either N-terminal or C-terminal residues, partially acetylated and unacetylated forms and in several cases possible phosphorylated and sulphated forms have been detected as previously by HPLC, immunoassays, microsequencing and electrospray mass spectrometry [18]. Thus, *in situ* MALDI-MS allows us to investigate the distribution pattern of neuropeptides in brain and can also be used for dynamic studies.

In terms of present limitations, it is difficult to perform mass spectrometry peptide fragmentation employing MALDI post-source decay (PSD) fragment ion mass analysis directly on the tissue. The ability to rapidly identify peptides *in situ* studying fragmentation mass spectra to obtain the primary structure would permit the determination of the many unknown peptide observed. The low level of peptides or the energy necessary to breakdown *in situ* peptides could be one explanation for the present limitations. However, the use of infrared laser in such experimental conditions may provide the required alternative solution [19,20].

Acknowledgement

The Centre National de la Recherche Scientifique (CNRS), the Ministere de l'Education National, de la recherche et des Technologies (MENRT) , the Fondation de La Recherche Medicale (FRM), the Genopole of Lille, the Fonds Europeen de Dévelopement Economique et Regional (FEDER), the Conseil Regional Nord-Pas de Calais and the Canadian Institutes of Health research (CIHR) supported this work. Robert Dayis a scholar (chercheur boursier Senior) of the Fonds de la Recherche en Santé du Québec (FRSQ) and invited researcher from the CNRS and the université of Lille I. The authors would also like thanks students of the Neuroimmune schools (Diana Colon, Rudy Murillo and Michel Numez) for their skill work during the school.

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