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Practical Aspects of in Vivo Detection of Neuropeptides by Microdialysis Coupled Off-Line to Capillary LC with Multi-stage MS

Qiang Li[a], **Jon-Kar Zubieta**[c,d], and **Robert T. Kennedy**[a,b,*]

[a] Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109

[b] Department of Pharmacology, University of Michigan, Ann Arbor, Michigan 48109

[c] Departments of Psychiatry and Radiology, University of Michigan, Ann Arbor, Michigan 48109

[d] Molecular and Behavioral Neuroscience Institute, University of Michigan, Ann Arbor, Michigan 48109

Abstract

A method using capillary LC-MS^3 to determine endogenous opioid peptides in microdialysis samples collected *in vivo* was developed, validated, and applied to measurements in the rat striatum. Peptides in dialysate rapidly degraded when stored at room temperature or −80 °C. Adding acetic acid to a final concentration of 5% stabilized the peptides for 5 days allowing storage of fractions and off-line measurements which proved more convenient and reliable than previously used on-line methods. Study of the effect of dialysis flow rate from 0.2 to 2 μL/min and column inner diameter (i.d.) from 25 to 75 μm on the relative signal obtained for peptides revealed that lowest flow rates and smallest column i.d. gave the highest relative signal. The method was tested for 10 different neuropeptides and LODs were from 0.5 pM to 60 pM (4 μL samples) for most. β-endorphin had an LOD of 5 nM when detected directly, but it could be quantitatively determined by detecting a characteristic peptide produced by tryptic digestion with an LOD of 3 pM. This approach may prove useful for other large neuropeptides as well. The method was used to determine met-enkephalin, leu-enkephalin, dynorphin $A_{1–8}$, and β-endorphin *in vivo*. Endomorphin 1 and 2 were below the detection limit of the method *in vivo*. Quantitative determination of leu-enkephalin using external calibration was verified by standard addition experiments. The improvements over previous approaches using capillary LC-MS^n make *in vivo* neuropeptide monitoring more practical and feasible for a variety of neuropeptides.

Introduction

Neuropeptides constitute a large family of over 100 intercellular signaling molecules in the central nervous system (CNS). An important route to studying the function of peptidergic neurotransmitters and neuromodulators is to determine their concentration dynamics in the brain. Although measurements of mRNA or peptide content in tissue can be used for some studies, *in vivo* measurements allow direct detection of extracellular peptide rather than only peptide content, enable direct correlation to behavior, and use fewer animals. Positron emission tomography (PET) is one approach to estimate peptides *in vivo*; however, this method is expensive, not compatible with freely moving animals, and is presently limited to just a few peptides.[1] Microdialysis sampling combined with analysis of collected fractions is the most

*Corresponding Author Contact Information: Mailing Address: Robert Kennedy, Department of Chemistry, University of Michigan, 930 N. University Ave, Ann Arbor, MI 48109-1055, Phone: 734-615-4363, E-mail: rtkenn@umich.edu.

popular approach for *in vivo* neuropeptide studies;[2, 3] however, use of this method is challenging because of low *in vivo* concentration of peptides (1–100 pM) and low recovery of peptides by microdialysis (< 20% typically). Thus the assay method for neuropeptides must be sensitive enough to quantify picomolar concentrations in microliter volumes and robust enough to quantify peptide in many fractions collected.

Determination of endogenous peptides in microdialysis samples is often performed using immunologic methods, in particular radioimmunoassay (RIA).[4–6] Although RIAs can be developed with the requisite detection limits (100 amol), maintaining this sensitivity for routine measurements can be difficult. Immunoassays also have questionable specificity because of cross-reactivity between the target peptides and related peptides such as precursors and metabolites.[7, 8] Furthermore, RIAs are usually limited to measurement of a single peptide per sample. For these reasons, other approaches to peptide assay have been sought for dialysis samples.

One promising method for peptide determinations in dialysate is capillary LC coupled with multi-stage mass spectrometry (MS^n).[9–12] This method has been shown to yield attomole detection limits, sequence specificity, and multi-analyte capability. Although LC-MS^n is promising, it has several limitations and unknowns for routine use. First, peptides in dialysates are unstable resulting in decreasing concentration as samples are stored. A second problem has been that the most sensitive peptide detection has been achieved on small bore columns (25 μm i.d.) which are not commercially available. Larger columns may be used if larger volumes of dialysate are collected; however, the relative merits and interaction of different dialysis flow rates and column inner diameters have not been quantified. Finally, the only endogenous peptides that have been quantified *in vivo* using capillary LC-MS are met and leu-enkephalin,[12–14] neurotensin (NT)[9] and angiotensin[15], although several others have been detected.[14, 16] The potential for monitoring of other peptides in brain has not been reported. We have addressed these issues by: 1) developing a sample storage method that stabilizes dialysate peptide, 2) quantitatively evaluating the effect of dialysis flow rate and column inner diameter on peptide detection in dialysate, and 3) exploring sensitivity for different neuropeptides. The latter study revealed that a large neuropeptide, β-endorphin (BE), had high LODs when detected directly but much better LODs could be achieved by digesting the peptide with trypsin and detecting a characteristic fragment. This approach may prove useful for other large neuropeptides as well. Overall, this collection of advances increases the practicality of using capillary LC-MS^n for *in vivo* neuropeptide measurements.

The method is demonstrated and validated for *in vivo* measurements of opioid peptides. The most important opioid peptides are enkephalins,[17] dynorphin,[18, 19] and BE[20]. These peptides, which are derived from the protein precursors proenkephalin, prodynorphin, and proopiomelanocortin respectively, exert their effects through binding to the μ, δ, and κ opioid receptors. Endomorphins are another set of putative endogenous opioid peptides with more obscure precursors.[21] Endogenous opioids have been implicated in many important functions including pain transmission, reward mechanisms, stress responses, food intake, and learning and memory. The ability to monitor this family of peptides *in vivo* may prove useful in elucidating their role in these processes.

Experimental Section

Chemicals and Reagents

Capillary LC solvents were purchased from Burdick & Jackson (Muskegon, MI). Enkephalins, Dynorphin $A_{1–8}$ (Dyn $A_{1–8}$), other neuropeptides, and proteomics grade Trypsin were from Sigma-Aldrich. BE was from AnaSpec, Inc. (San Jose, CA). Ethanol, acetic acid, and hydrofluoric acid were purchased from Fisher Scientific (Pittsburgh, PA). The chemicals to

prepare macroporous photopolymer frits, including isooctane, toluene, trimethylolpropane trimethacrylate, glycidyl methacrylate, and benzoin methyl ether (BME) were purchased from Sigma-Aldrich. Arificial cerebral spinal fluid (aCSF) used for microdialysis perfusion consisted of 145 mM NaCl, 2.68 mM KCl, 1.10 mM $MgSO_4$, 1.22 mM $CaCl_2$, 0.50 mM NaH_2PO_4, and 1.55 mM Na_2HPO_4, adjusted pH to 7.4 with 0.1 M NaOH. High K^+ aCSF solution was the same as aCSF but with 75 mM KCl and NaCl reduced by a comparable amount to maintain ionic strength. Mobile phases and aCSF were prepared weekly and were filtered with 0.02 μm-pore filters (Whatman, Maidstone, England) to remove particulates. Fused silica capillary was from Polymicro Technologies (Phoenix, AZ).

Animals and *in vivo* Microdialysis

Adult male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing between 295 and 455 g were used for all experiments. The rats were kept in a temperature and humidity controlled room with 12 h light/dark cycles with food and water freely available. Each rat was used only once and euthanized on conclusion of the experiment. All animals were treated as approved by the University of Michigan Unit for Laboratory Animal Medicine (ULAM) and in accordance with the National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Animals were initially given an intraperitoneal injection of 65 mg/kg ketamine (from Sigma) prepared in an isotonic salt solution with 33 mg/kg booster injections given as needed to maintain the surgical plane of anesthesia. 4 mm long microdialysis probes with polyarylethersulphone (PAES) membrane (CMA Microdialysis, North Chelmsford, MA, USA) were positioned and inserted into the striatum (1 mm anterior and 2.8 mm lateral of bregma to a depth of 8 mm from dura) using a stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA). The probes were perfused for at least 1 h after insertion prior to data collection.

For experiments requiring stimulation for neuronal depolarization, the aCSF perfusion syringe was switched by a connection with Teflon tubing to a 75 mM K^+ aCSF solution. All aCSF solutions were syringe filtered (0.02 μm, Anotop 10, Whatman, Maidstone, England) immediately preceding use. Following a recording session the animal was euthanized and the brain immediately removed and frozen at −80 °C until histology. Probe position was verified by visual examination of 35 μm sections taken via microtome (Leica, SM2000R, Bannockburn, IL).

Trypsin Digestion of BE

0.5 μL of 870 pM trypsin dissolved in NH_4HCO_3 buffer (pH = 7.8) was added to 5 μL of standard or dialysate sample. BE in dialysate was circa 100 pM, so the enzyme:substrate molar ratio was near 1:1 in the reaction mixture. (This ratio is higher than general trypsin digestion reaction for larger samples, but was found to yield best digestion with minimal background for the small samples with low concentrations.) The mixture was incubated at 37 °C for 6 hours and then frozen at −80 °C following addition of 0.5 μL glacial acetic acid until detection.

Neuropeptide Detection with Capillary LC-MS^3

Chromatography columns with integrated electrospray emitter tips were prepared in-house from 28 cm lengths of 25 μm i.d. fused silica capillary. To prepare columns, a ~500 μm long section of polyimide coating was removed 5 cm from one end of the tubing by rotating within the electric arc created it between two tungsten electrodes with 7.6 kV applied between them. An additional 1 cm coating nearer to the tip side was removed with a flame. Capillaries were filled with a photopolymer solution as described before[14] using He gas pressure and placed inside PEEK sleeves (Upchurch Scientific, Oak Harbor, WA) so that only the 500 μm section

of bare fused silica was exposed. Polymerization was achieved by illuminating the exposed region of the capillary with a UV lamp (Spectronics, Westbury, NY) for 30 min. (In practice, 20 capillary frits were prepared simultaneously.) Frits were flushed with acetone and dried for storage by passing He gas through the capillaries for 2 min at 500 psi. A P-2000 CO_2 laser puller (Sutter Instruments, Novato, CA) was used to create integrated electrospray emitters near the frit. The settings used on the laser puller were cycled once (line 1: heat 300, velocity 30, delay 128, pull 0; line 2: heat 300, velocity 30, delay 128, pull 125). The resulting pulled columns were etched with 49% hydrofluoric acid for 45 seconds to create sharp-edged electrospray emitters with 4 μm i.d., verified by scanning electron microscopy. Pulled columns were packed with an acetone slurry (5 mg/mL) of 5 μm Alltima C18 reversed-phase particles (Alltech, Deerfield, IL) at 500 psi as described elsewhere.[22] 4 cm of the total 20 cm length of fused silica was packed. Peptide samples were injected in weak mobile phases to allow the analytes to stack at the head of the column; therefore, the void at the head of the column did not contribute to extracolumn band broadening.

The capillary LC system utilizes a high pressure (4000 psi) pump (Haskel Inc., Burbank, CA) for sample loading and desalting, and a lower pressure (500 psi) micro HPLC pump (MicroPro, Eldex Laboratories, Napa, CA) for gradient separation (Figure 1). Switching between the two pumps gives a higher flow rate for loading and desalting and a lower flow rate for efficient separation and electrospray ionization without requiring equilibration of the pumping system at the different flow rates. Typically a 7 min sample loading time and 8 min desalting time was used for a 5 μL sample. The gradient program for separation consisted of an isocratic step of 10% B for 1 min, linear increase to 95% over 2.5 min, followed by isocratic at 95% B for 0.5 min. Mobile phase A consisted of water with 2% acetic acid and mobile phase B consisted of methanol with 2% acetic acid. The final flow rate in the separation column (after flow splitting) was 90 nL/min, as measured using a SLG1430 l flow meter from Sensirion (Stäfa, Switzerland). The column was coupled to either a Finnigan™ nanospray ionization source (Thermo Fisher Scientific, Waltham, MA, USA) or a PV-550 nanospray ESI source (New Objective, Woburn, MA, USA) interfaced to an LTQ XL linear ion trap (LIT) MS (Thermo Fisher Scientific). A +3.0 kV potential was applied to a liquid junction prior to the column for electrospray.

Samples were injected using a WPS-3000TPL autosampler (Dionex, Sunnyvale, CA), which has the capability of operating different injection modes according to sample requirements. To inject 4 μL from a 5 μL volume the “microliter pickup” mode was used which sandwiches the sample volume between transport liquid (water) in a 10 μL sample loop. With this method, most of the *in vivo* sample could be injected with little waste. A washing program for the sample loop and injection needle between injections prevented carry-over between injections for concentrations < 1 nM.

The LIT-MS was operated in positive mode. All measurements were made with the following settings: automatic gain control (AGC) on, collisionally induced dissociation (CID), q = 0.25, isolation width 3 *m/z*, activation time 0.25 ms, micro scan number = 1 and default target count values. Collision energies were optimized for best sensitivity during constant infusion of each peptide. The MS^3 pathways for the 4 opioid neuropeptides were: 574→397→278, 323, 380 for Met-enkephalin (ME), 556→397→278, 323, 380 for Leu-enkephalin (LE), 491.5→435→417, 426, 694 for Dyn $A_{1–8}$, and 568→558→282, 451, 817 for tryptic fragment $BE_{10–19}$. Identification of granddaughter peaks used for quantification was based on the method in a previous study.[23] Optimization of the ion optics for maintaining the best sensitivity of the instrument was achieved by tuning with direct infusion of 18 nM neurotensin$_{1–11}$ in 50% methanol 2% acetic acid at 100 nL/min every 2 weeks.

RESULTS AND DISCUSSION

Neuropeptide Sample Storage and Handling for Reliable Off-line Measurement

Dialysis measurements may be performed off-line, where samples are collected in fractions and stored for later analysis, or on-line, where the analysis of fractions is performed serially as dialysate is removed. Off-line measurements of neuropeptides in dialysate are hampered by severe loss of peptide signals in dialysis samples stored for more than a few hours. The effect of peptide sample deterioration on quantification can be ameliorated by adding isotopically labeled standards to dialysis sample;[15] however, this does not avoid the problem of sample loss and subsequent effect on sensitivity. We previously avoided sample loss by using on-line analysis.[12, 14] Although on-line analysis limits degradation, it has the drawback of limiting temporal resolution to the chromatographic analysis time. Furthermore, a practical effect was that if a problem occurred with the analytical method during an experiment (such as column clogging or leaks), then it was difficult to recover and resulted in loss of the experiment.

In view of these persistent difficulties, we sought storage conditions that would stabilize peptides in dialysate samples and allow off-line measurements. Pilot experiments were performed by storing solutions of 60 pM LE in aCSF under different conditions and monitoring the LE content in the samples over time. As shown in Figure 2A, addition of acetic acid to a final concentration of 5% and storage at −80 °C prevented the rapid degradation seen without this combination (Figure 2A). This method also proved effective for neurotensin$_{1–13}$ ($NT_{1–13}$) and Dyn$A_{1–8}$ standard samples (Figure 2B). We then determined the effectiveness of this procedure for ME, LE, and Dyn $A_{1–8}$ in dialysis samples collected *in vivo*. As shown in Figure 2C, this method allows storage with minimal loss of sample for ~ 5 days. By day 10 however, degradation is significant.

The reason for the effectiveness of the acid treatment in aiding storage at −80 °C was not explored; however, it can reasonably be attributed to inhibition of enzyme activity that might be associated with microbial contamination, minimization of oxidation rates of peptides prone to this reaction, such as those containing tyrosine, cysteine, or methionine residues[24], and prevention of adsorption by minimizing electrostatic attraction by protonating both the peptides and surfaces of capillary tubing and vials.[25]

Effect of Microdialysis Flow Rate and Column Size on Neuropeptide Detection

In designing a microdialysis experiment, the interaction of column i.d., dialysis flow rate, and sample volume must be considered. We have previously advocated use of small bore columns (25 μm i.d.) based on their better sensitivity compared to larger bore columns (e.g., 75 μm i.d.).[26, 27] On the other hand, larger bore columns are commercially available and presumably easier to use. The preferred column may be affected by the dialysis flow rate used. Low flow rates increase the concentration of peptide in dialysate (relative recovery), but also decrease both the total mass of peptide collected (absolute recovery) and the total volume available for analysis. This situation would be more suited to small bore columns. An alternative approach is to use high dialysis flow rates to generate larger volume samples and inject onto larger bore columns to gain the convenience of working with larger collection volumes and bigger columns. This method would be especially advantageous if the sensitivity advantage of smaller columns can be offset by injecting and preconcentrating larger volumes on larger bore columns.

To gain a quantitative understanding of these relationships, we evaluated the recovery and sensitivity under a variety of conditions using LE as a model peptide. As shown in Figure 3A, the absolute recovery increases with flow rate; however, the effect is small with only a 9.5% increase over the flow rate range investigated. In contrast, the concentration collected has a dramatic increase of 3.7-fold with lowering of flow rate. (For these experiments we utilized a

PAES probe, which we found in preliminary experiments to have good recoveries for a variety of peptides.)

We also determined the difference in sensitivity on 25 and 75 μm i.d. columns for a given injection volume and linear flow velocity (Figure 3B). These experiments revealed that the smaller columns had an approximately 10-fold improved sensitivity. The increase in sensitivity with small capillary i.d. can be attributed to: 1) more effective on-column concentration of the mass that is loaded onto the column, 2) reduced mass flow rate of solvents and other background constituents into the ESI source, which allows for greater analyte ionization efficiency,[28, 29] and 3) inherent decreased electrospray tip inner diameter which increases the ionization efficiency resulting from initial smaller droplets formed.[30] Although all these factors likely contribute, it appears that the majority of the enhancement can be accounted for by the 9-fold concentration enhancement expected for a 3-fold column i.d. reduction.

With the sensitivity and recovery data, we then calculated how varying column i.d. and dialysis flow rate would affect the measurements of peptide as summarized in Table 1. For this table, we assumed samples were collected for 20 min. We then calculated the relative amount of sample that could be injected onto the column and the relative signal that would be obtained based on the difference in sensitivity. (All calculated parameters were made relative to the smallest column and lowest flow rate.) Injection volume was a function of both the sample available and how much could be practically injected. With the autosampler used in these experiments it was possible to inject 3 μL from a 4 μL sample and 7 μL from a 10 μL sample. Another consideration was the maximal sample volume that could be loaded quantitatively loaded onto the column. If the injection volume becomes too large, analyte can “breakthrough” and begin to elute from the column before the completion of sample loading resulting in non-quantitative injections.[31] In separate experiments we determined that the volume limit was 10 μL for a 25 μm i.d. column and assumed a 9-fold larger volume for the 75 μm i.d. column. Therefore, at higher dialysis flow rates even though 40 μL was available, only 10 μL was considered to be loaded onto the smaller bore columns.

Based on these considerations, we can see that at lower dialysis flow rates the same amount of sample can be loaded onto both sizes of column (Table 1). At higher dialysis flow rates, more sample can be loaded onto the bigger columns allowing more of the sample available to be used. Despite the better loading capacity of the bigger columns, the enhanced sensitivity of the smaller columns means that they yield the better relative signal under all conditions except at the highest flow rates. Furthermore, this difference can be as large as 10-fold.

These results help quantify the advantages of working with smaller bore columns and lower flow rate microdialysis. The effect observed will depend on the collection time. If longer collection times were used, corresponding to lower temporal resolution for the measurement, then the smaller columns have less of an advantage. Conversely, the advantage of smaller columns will be greater when higher temporal resolution is desired. These results do not mean that only smaller columns and low flow rates can be successful for *in vivo* measurements, but they do illustrate the substantial benefits for sample limited analysis such as peptide dialysates.

25 μm i.d. Column Stability and Reproducibility

As suggested by the above results, 25 μm i.d. columns are preferred for achieving high sensitivity. However, using such small columns leads to higher possibility of clogging which is initially detected as a decrease in peak area due to lower flow rate during column loading (all injections are based on loading for a fixed time). Indeed, in previous work we used a new column each day to prevent variability associated with gradual column clogging over longer periods.[12] We found that with filtering of all solutions that entered the column with every week and adding low dead volume (0.6 μL) in-line filters (Upchurch Scientific, INC.) to all lines

that feed the column, such columns can be stable for 2–4 days with at least 10 injections each day. Another consideration with in-house prepared columns is reproducibility. Column-to-column retention time relative standard deviations (RSDs) were < 10% (n = 50) and this mainly depended upon variation in the length of packed beds, which was not tightly controlled in these experiments. Peak areas were more variable from column-to-column with some batches of columns giving RSDs of ~5% and others over 50%. As all columns were calibrated prior to use, this variation was not problematic.

Detection of a Variety of Neuropeptides with the cLC-MS^3 System

Previous work on *in vivo* detection of endogenous neuropeptides using LC-MS has been limited to ME, LE, $NT_{1–13}$ and angiotensin. Therefore we sought to determine how widely applicable these procedures could be to different peptides by determining detection limits for a selection of 10 neuropeptides in 4 μL samples dissolved in aCSF (Table 2). For these experiments, we utilized MS^3 on a linear ion trap (LIT) MS, and in some cases a quadrupole ion trap (QIT) MS. MS^3 offered an ~5-fold improvement in LOD over MS^2 and was used for all experiments. As shown in Table 2, the LOD was generally better when using the LIT than the QIT, although the difference was only substantial for peptides that had the worst LODs on the QIT.

Most of the peptides detected had LODs of 40 pM or better suggesting the potential to at least detect, if not quantify, peptides in dialysate. Two of the peptides were poorly detected though. Substance P is adsorptive and recent work suggests the possibility of obtaining much better detection limits for this peptide with procedures to mitigate this problem.[32]

BE was also found to have an LOD that would be too high for *in vivo* monitoring applications. The difficulties of detecting BE could be due to its hydrophilicity and size. BE is more hydrophilic than most of the other neuropeptides we examined, as estimated using the Bull and Breese index (B & B)[33, 34] (see Table 2), which tends to reduce ESI efficiency.[35] BE is also large enough that its signal is dispersed across many ions of different charge state reducing the abundance of the dominant ion chosen for collision and multiple stages of mass spectrometry.

We examined the possibility of detecting and quantifying BE based on detection of a characteristic peptide produced after tryptic digest. Of the ions detected in MS analysis of a BE tryptic digest (Figure 4A), the $[M+2H]^{2+}$ ion at *m/z* 567 corresponding to $BE_{10–19}$ (as underlined in the amino acid sequence of $BE_{1–31}$ YGGFMTSEKSQTPLVTLFK-NAIIKNVHKKGQ) is the most abundant. This peak was chosen for MS^3 and quantified for indication of intact BE concentration. Using this procedure we obtained an LOD of 3 pM for BE, representing an ~1,800-fold improvement over detecting the intact peptide. The improvement is likely due to both the smaller size of the fragment (and fewer charge states) and the greater hydrophobicity of the fragment (B & B index = −331 compared to −99 for intact BE). Using this method, it was possible to detect and quantify BE in dialysate as illustrated by the mass chromatograms in Figure 4E. We also found that determination of BE by this method gave comparable reproducibility (RSD = 13% for 58 pM sample, n = 3) to direct detection of other peptides. The calibration curve of BE resulted in an excellent linearity too with correlation coefficients of 0.99 from 58 to 580 pM (n = 3).

A trade-off for obtaining higher sensitivity by detecting the tryptic fragment is that some specificity is lost because the native peptide is not directly detected. As a negative control, we analyzed dialysate without tryptic digestion and found that the peptide was not detected (see Figure 4D) indicating that the peptide is not produced endogenously at detectable levels and therefore would not interfere with detection. A Basic Local Alignment Search Tool (BLAST) search revealed that the $BE_{10–19}$ sequence is only found in proopiomelanocortin (POMC), the protein precursor of BE. Based on these results, we conclude that this peptide is a specific

marker for BE or peptides that contain the BE sequence from POMC. We expect that this approach may also be useful for other larger neuropeptides as well.

In Vivo Monitoring of Opioid Peptides

As a demonstration of this method, we used it to monitor 4 opioid peptides in microdialysis fractions collected off-line at 25 min intervals under basal conditions and during infusion of 75 mM K^+ aCSF through the probe in anesthetized rats. ME, Dyn $A_{1–8}$, and LE could be detected with one chromatographic separation as illustrated in Figure 5. Because BE required trypsin digestion, it was determined in separate experiments. Endomorphins were not detected despite the excellent LODs. These peptides could be produced at lower amounts (if at all in this brain region), more rapidly degraded, or modified resulting in lower concentrations of the actual sequence tested.

The concentration of peptides in dialysate samples was determined by external calibration using standard solutions of peptide dissolved in aCSF as summarized in Table 3. Calibrations were performed by triplicate injections of 3 different concentrations (plus a blank) from 20–200 pM for ME and LE, 68–680 pM for Dyn $A_{1–8}$ and 58–580 pM for BE. Calibrations were linear with correlation coefficients of 0.98 to 0.99. Peak area RSDs for standards were 7–9% across all calibrations for all analytes. To validate the use of external calibration, we performed a standard addition experiment by measuring the LE content of fractions collected *in vivo* before and after spiking 70 pM of standard LE to the dialysate. The spiked concentration increased the peak area of the LE peak by the amount predicted by the external calibration curve to within 9% (n = 6 fractions from 2 animals). Based on this result, we conclude that external calibration is adequate for quantification in the dialysate samples.

Our concentration of BE is ~66% lower than a previous report of 320 pM in dialysate measured by RIA[36]. This difference could be due to differences in sampling conditions and brain regions, but also could reflect the higher specificity of the LC-MS method. No direct measurement of Dyn $A_{1–8}$ has previously been reported for comparison. For ME and LE, our data are in reasonable agreement to previous work in our lab although slightly higher (~15% increase for ME and 42% increase for LE compared to previous results)[12], which may reflect the use of off-line storage or different dialysis probes. Elevated concentrations may also be because dialysis measurements were taken beginning 1 h after probe implantation and insertion of the probe may temporarily raise levels. Arguing against this possibility though, we did not observe decreasing concentrations with time during our measurements. Also, in preliminary experiments, we have not observed lower levels with probes implanted overnight. Our data show a ratio of ME and LE in striatum of ~2:1 under basal conditions, consistent with some[37], but not all[38], previous measurements of ME and LE in striatal tissue. Interestingly, this ratio is different from the ratio of 6:1 for the copies of ME and LE in the precursor proenkephalin A[39] indicating that processing of the precursor proteins (ME and LE can also be produced from other precursor opioid proteins) results in extracellular concentrations not directly predictable from the copy number.

K^+ infusion evoked a substantial increase in all 4 peptides levels as expected due to depolarization of neurons (see Figure 6 and Table 3). The percent increase of ME and LE is comparable to previous reports.[12, 40] The dynamic information in Figure 6 illustrate that the release of peptide is not sustained for the entire time course of K^+ application. This likely reflects a limited supply of peptide available for release from neurons, but the potential that inhibition of secretion occurs over time cannot be ruled out based on this data.

One potential area for improvement for *in vivo* peptide measurements is reproducibility. Replicate measurements of standards had RSDs of 7% at 60 pM for LE. When standard solutions were sampled by microdialysis and collected into fractions, the RSDs were increased

to 14% indicating additional variability associated with sampling, fraction collection, transport through the tubing, and manipulation of the small samples. RSDs measured in replicate fractions from a single animal (4 fractions per animal) under basal conditions averaged 15% for ME, 17% for LE, 24% for Dyn A_{1-8} and 18% for BE (n = 5 animals). For the enkephalins and BE, the RSD is comparable to the RSD of standards indicating that variability is mostly due to the analytical method. The comparable RSDs for BE and enkephalins support the conclusion that the trypsin digestion procedure was reproducible and reliable for quantitative measurement. The higher RSD for Dyn A_{1-8} could be mainly due to the relatively high LOD for that peptide, indicating increased irreproducibility when measuring near the LOD. RSDs, especially for the more abundant peptides, could potentially be improved by using automated fraction collection or internal standards. Also, use of a triple quadrupole MS instead of an ion trap might also allow improvement of RSDs;[41] however, because of the limitation to MS^2 with such instruments, the LOD may not be as good as that achieved using MS^3 on ion traps. Interestingly, the mean basal dialysate concentration of different rats tended to have larger RSDs for the enkephalins (42% for ME, 40% for LE, 15% for Dyn A_{1-8}, and 19% for BE), indicating that inter animal comparisons will have to account for these differences in individuals.

CONCLUSIONS

We have enhanced the potential for monitoring neuropeptides *in vivo* by microdialysis coupled with LC-MS^3. Treatment of collected fractions with acetic acid stabilizes samples so that they can be stored for days allowing off-line analysis. Study of different dialysis flow rates and column inner diameters reveals that best sensitivity is achieved with low flow rates and smaller bore columns. This advantage is not critical at low temporal resolution, but increases with the temporal resolution required. The approach is viable for a variety of peptides, based on sensitivity requirements; although some larger neuropeptides such as BE require detecting a tryptic fragment to obtain the requisite sensitivity. The method is demonstrated for 4 opioid peptides, including two previously undetected by LC-MS allowing initial *in vivo* concentration estimates to be obtained. Further improvements in stability and availability of 25 μm i.d. columns as well as reproducibility are desirable to improve the utility of the method.

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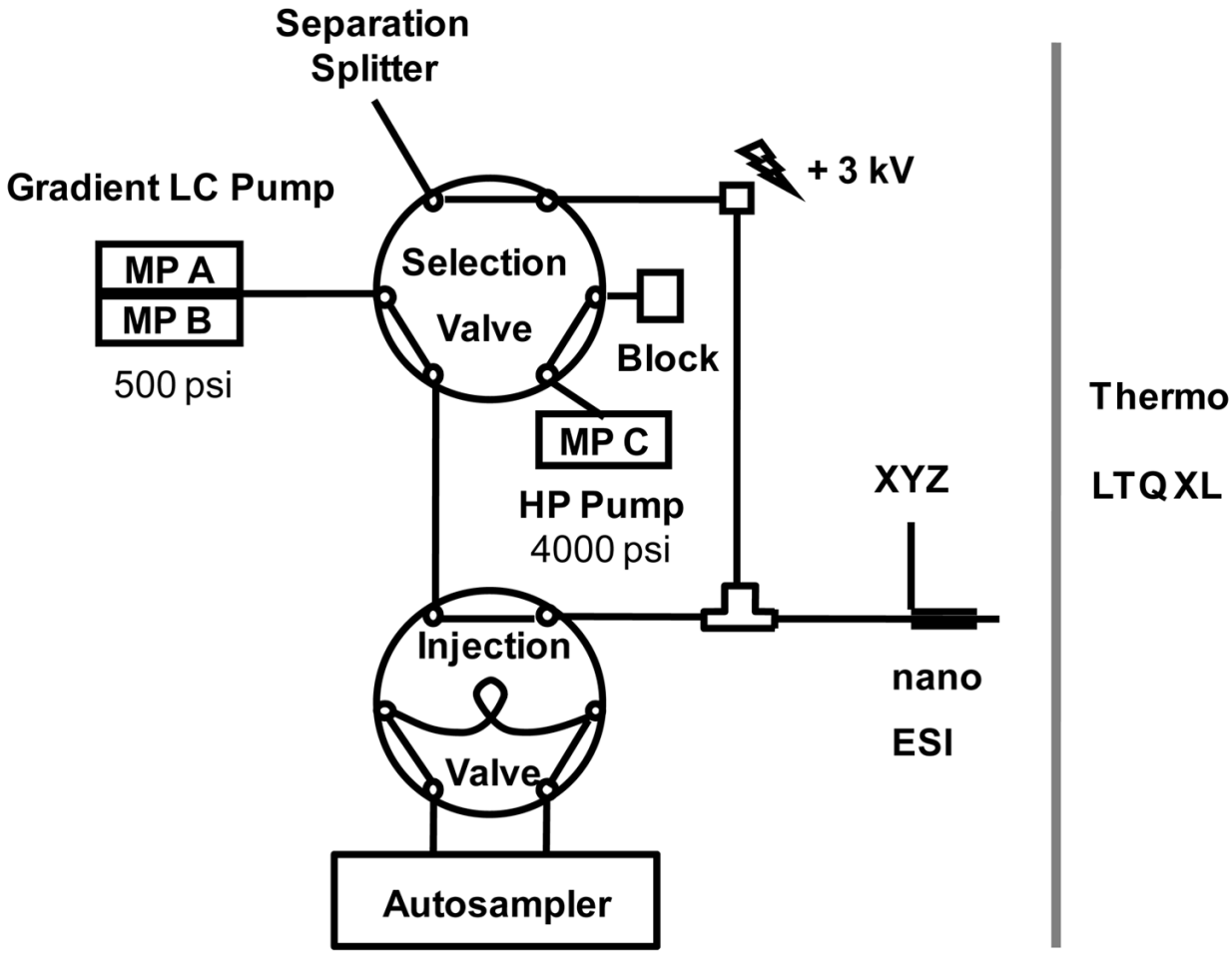

Figure 1.
Diagram of the capillary LC system coupled with a linear ion trap MS. Mobile phase A (MP A) and mobile phase C (MP C) are both 2% acetic acid in H_2O, while mobile phase B (MP B) is 2% acetic acid in methanol.

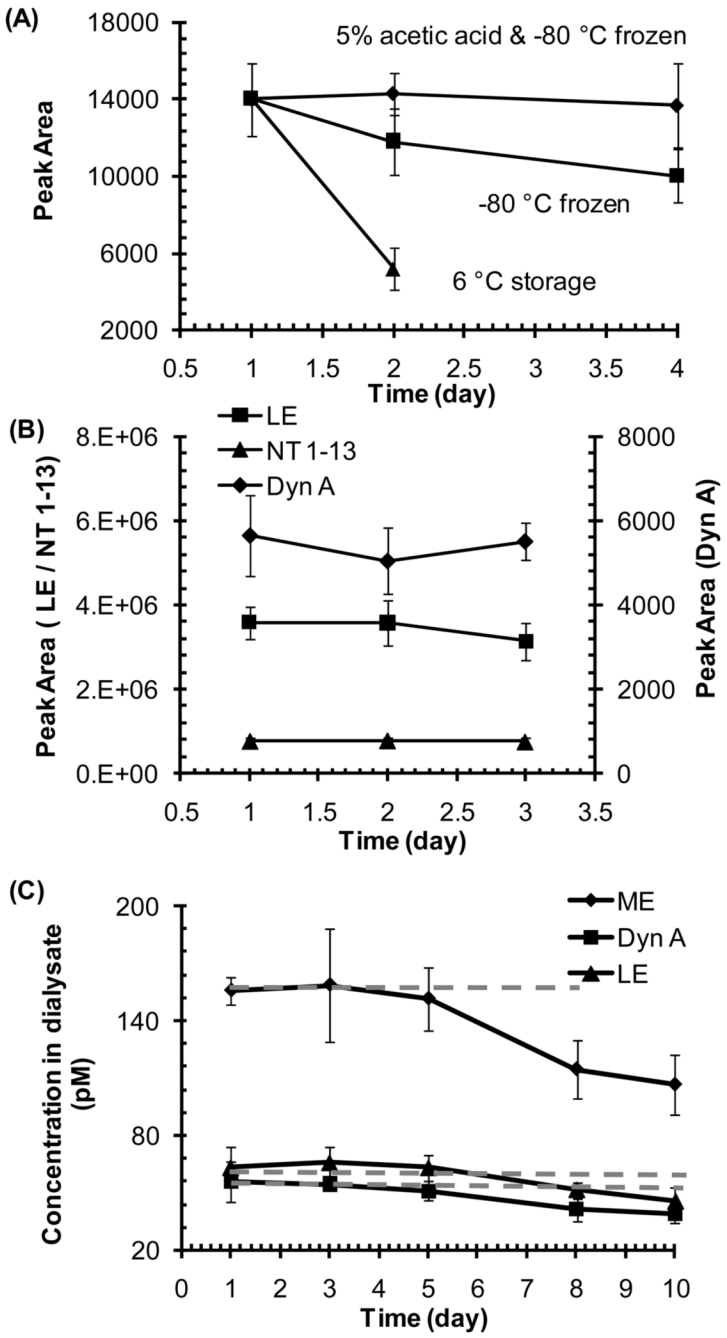

Figure 2.

Effect of storage conditions on peptide detection. (A) Peak areas for 60 pM LE samples stored in aCSF at 6 °C, −80 °C, and −80 °C with 5% acetic acid (n = 3, error bar = ± 1 standard deviation (SD)). (B) Peak areas of 600 pM LE, 660 pM $NT_{1–13}$ and 680 pM Dyn $A_{1–8}$ in aCSF with 5% acetic acid and stored at −80 °C (n = 3, error bar = ± 1 SD). (C) Measured concentration of ME, Dyn $A_{1–8}$ and LE in dialysates stored over 10 days with 5% acetic acid at −80 °C (n = 3, error bar = ± 1 SD). In this experiment, 15 basal *in vivo* dialysis samples were collected from an anesthetized rat at 25 min intervals with 0.2 μL/min aCSF perfusion rate. Acetic acid to a final concentration of 5% was added to the fractions which were then stored at −80 °C. 3 of

the 15 samples were chosen at random for analysis on the 1st, 3rd, 5th, 8th and 10th days after sample collection.

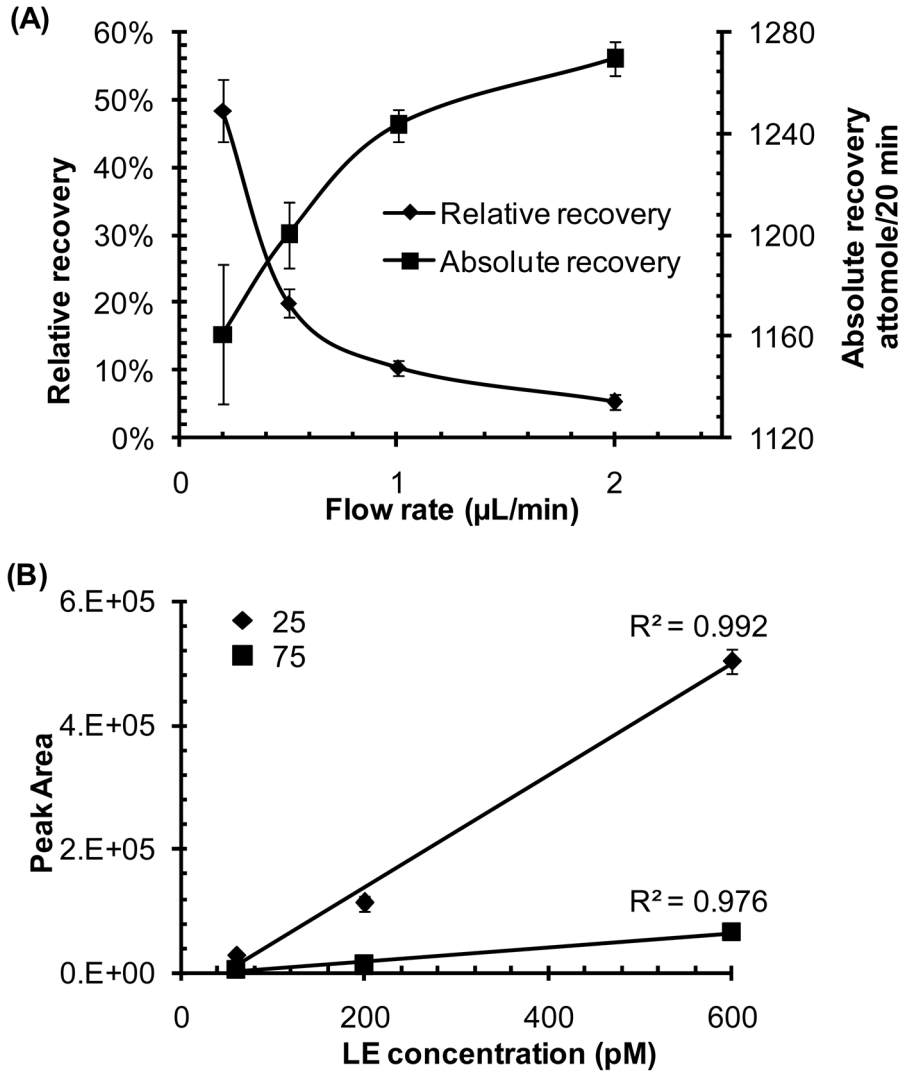

Figure 3.

Effect of dialysis flow rate and column i.d. on detection of neuropeptides. (A) Relative and absolute recovery of microdialysis probe (CMA/12 Elite with PAES membrane, 4 mm long, MWCO 20 kDa) at 37 °C with perfusion flow rate from 0.2 to 2 μL/min for LE. (B) Comparison of the calibration curve of LE on 25 μm and 75 μm i.d. columns (n = 3, error bar = ± 1 SD). Sensitivity is 10 fold higher on the smaller column.

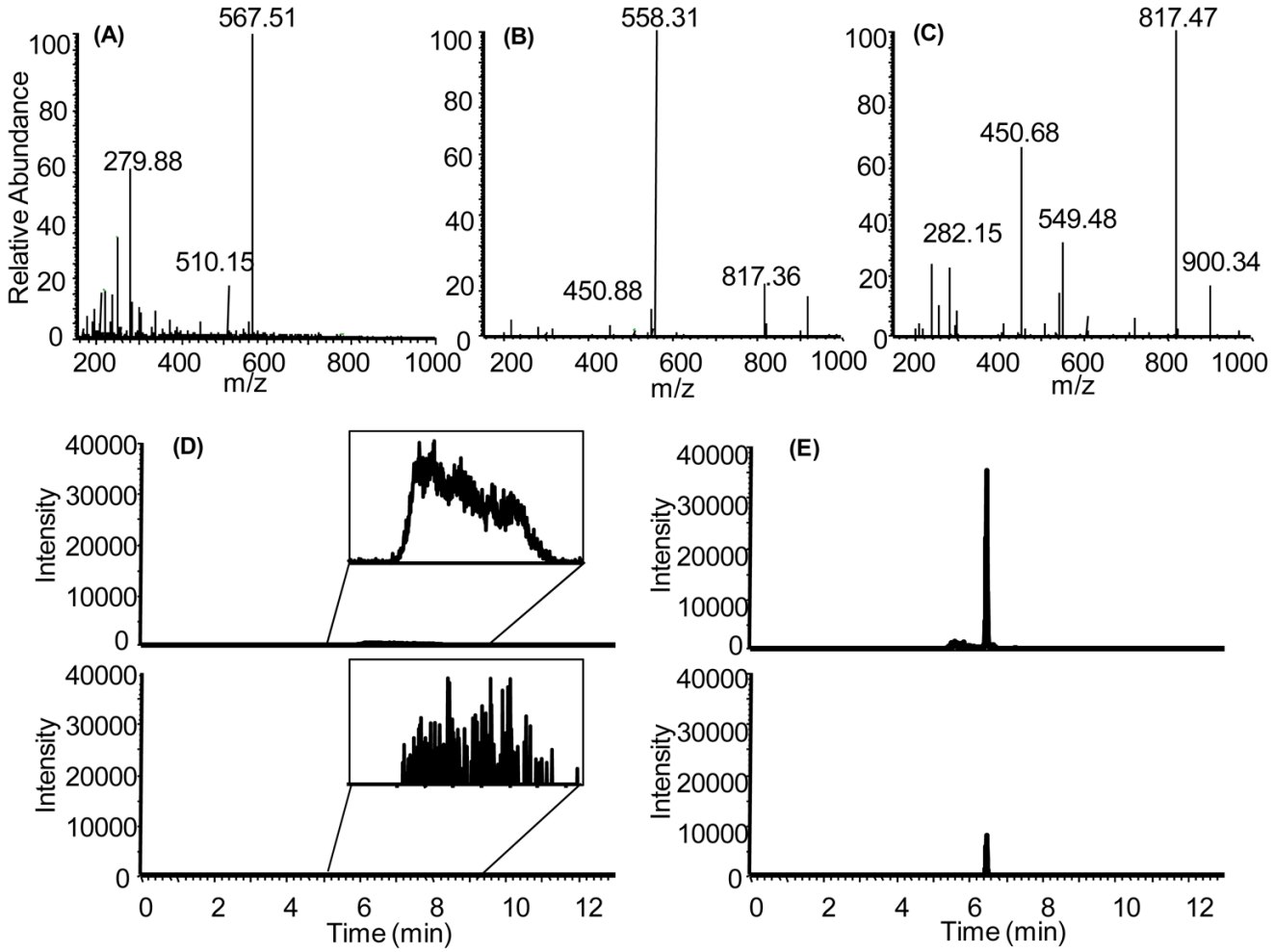

Figure 4.

Detection of BE by a characteristic tryptic fragment. (A), (B), and (C) show MS, MS^2 and MS^3 spectra respectively of BE tryptic fragment $BE_{10–19}$. The ions chosen for CID are 568→558→282+451+817. Total ion chromatogram (TIC) and reconstructed ion chromatogram (RIC) of $BE_{10–19}$ in an *in vivo* dialysate sample are illustrated without (D) and with (E) prior trypsin digestion. TIC shows all ions passing the detector on the mass spectrometer while RIC shows the chromatogram of selected characteristic ions to reduce noises. Lack of signal without digestion illustrates that this peptide is not present endogenously. Chromatograms in (D) and (E) are plotted on the same intensity scale for comparison. Inset in (D) shows the elution window for the peptide at ~1000-magnified scale on y-axis

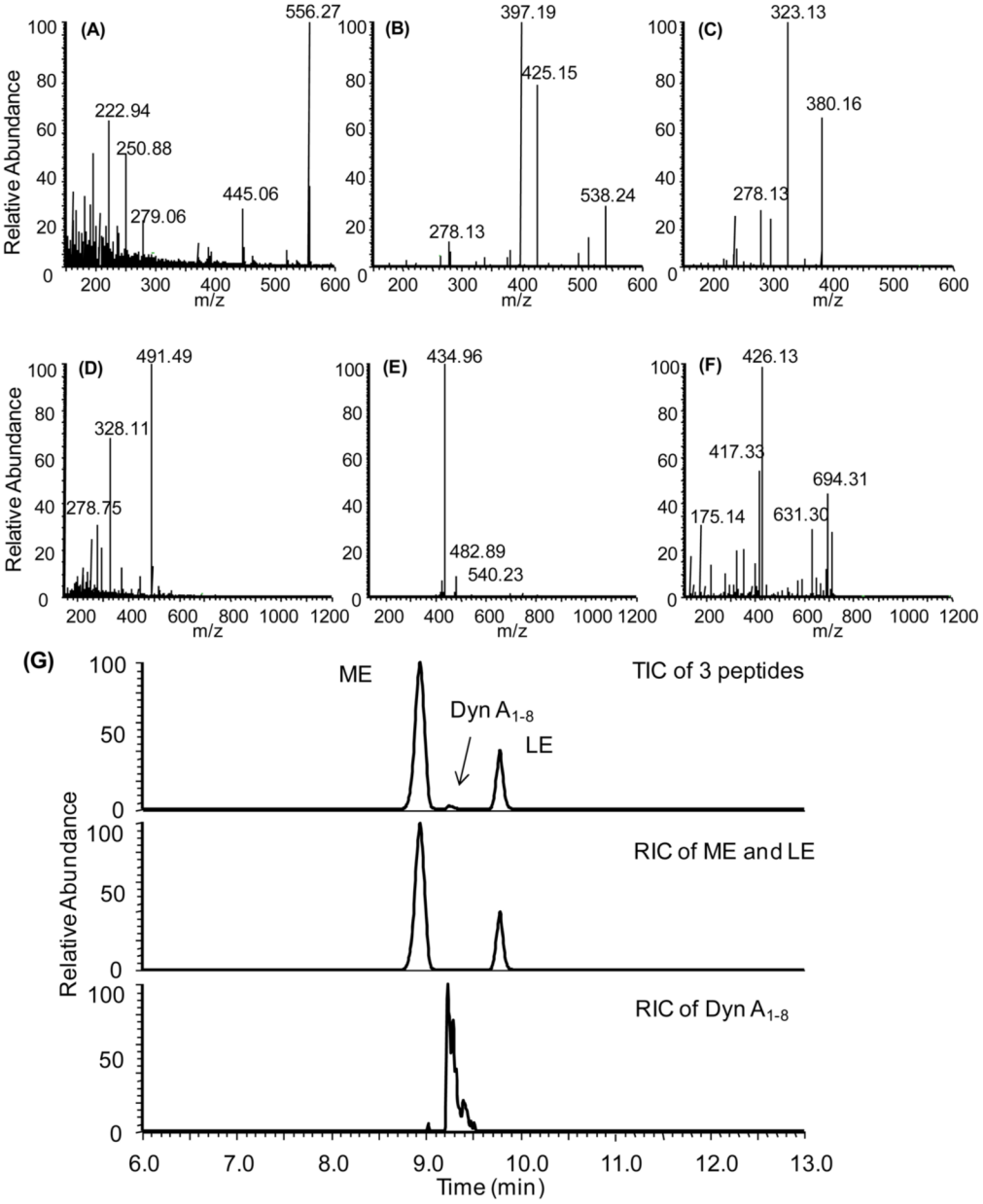

Figure 5.

In vivo detection of enkephalins and Dyn $A_{1–8}$. (A), (B), and (C) show MS, MS^2 and MS^3 spectra of LE respectively. The ions chosen for CID were 556→397→278+323+380. (Spectra for ME are similar except the MH^+ peak is at *m/z* 574 in the first stage spectrum.) (D), (E), and (F) show the MS, MS^2 and MS^3 spectra respectively of Dyn $A_{1–8}$. The ions chosen for CID are 491→435→417+426+694. (G) Total ion chromatogram (TIC) and reconstructed ion chromatogram (RIC) of ME, Dyn $A_{1–8}$, and LE from a 5 μL basal *in vivo* microdialysis sample collected from an anesthetized rat. RIC were derived with the three most abundant MS^3 ions.

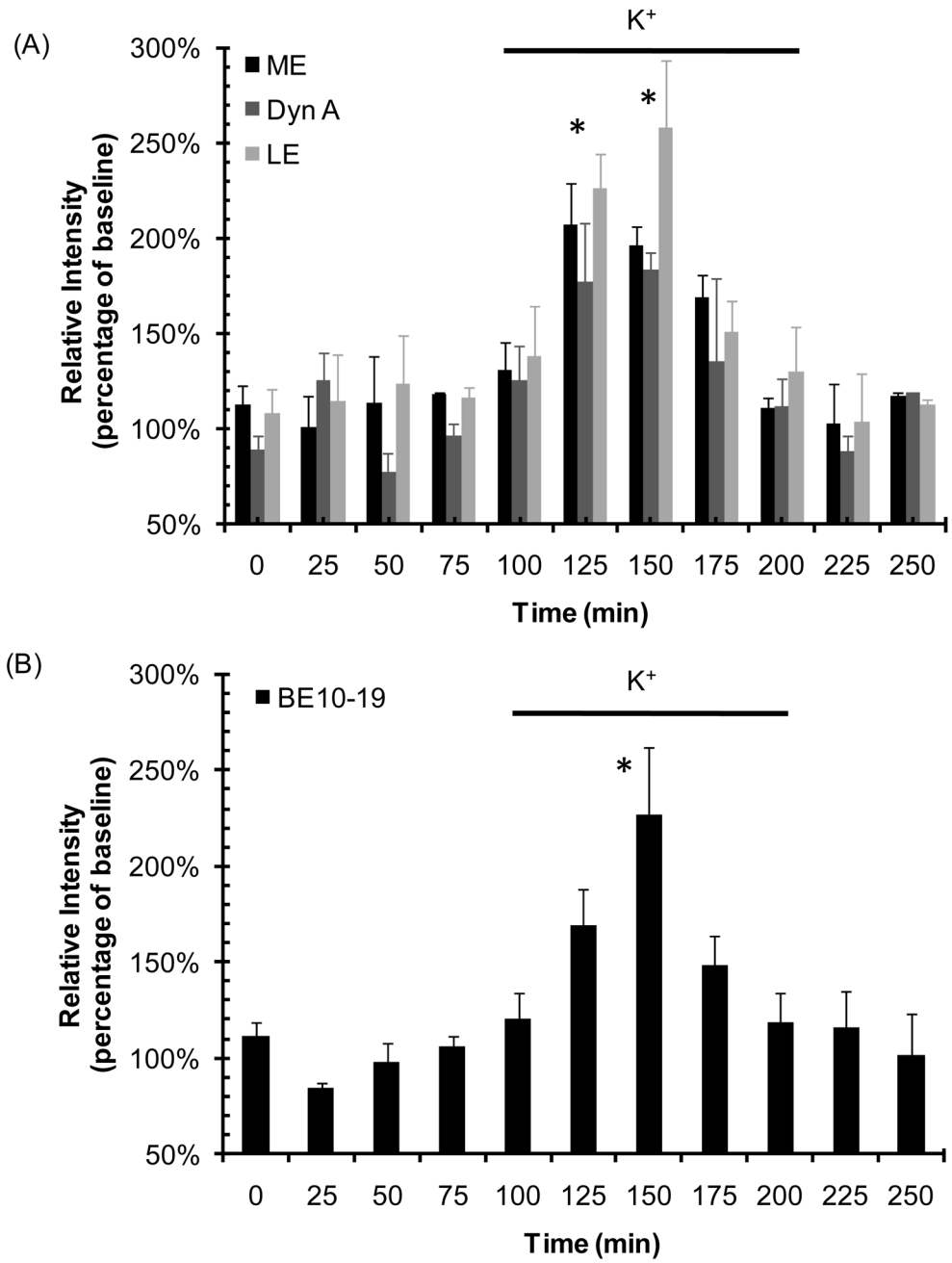

Figure 6.

In vivo monitoring of opioid peptides. ME, Dyn $A_{1–8}$ and LE were detected simultaneously (A) and BE, as a tryptic fragment, in separate rats (B). Fractions were collected at 25 min intervals and analyzed by LC-MS^3 (n = 3 rats, error bars = ±1 SEM). The bar indicates application of 75 mM K^+ to the microdialysis corrected for 25 min delay time due to the dead volume of connection tubing and the probe. The stimulated levels were significantly higher than baseline levels as determined by t-test (* $p<0.025$).

Table 1

Estimate of signal for LE with different microdiaysis flow rate on 25 and 75 μm i.d. columns. Calculations assume 20 min fraction collection times. Relative recoveries were used from Figure 3A. Calculation of amount loaded was based on the volume that could be injected onto a column (a function of column capacity and autosampler capability) and relative recovery. Amount was relative to the smallest column and lowest flow rate. Relative signal was calculated based on the amount loaded and the difference in sensitivity (10-fold as shown in Figure 3B). All values are relative to the lowest flow rate and smallest column.

Flow rate (μL/min)	Sample volume for 20 min (μL)	Relative recovery	Column i.d. (μm)	Injection volume (μL)	Relative amount loaded on- column	Relative signal
0.2	4	48.4%	25	3	1	1
			75	3	1	0.1
0.5	10	20.0%	25	7	0.96	0.96
			75	7	0.96	0.10
1	20	10.4%	25	10	0.72	0.72
			75	17	1.22	0.12
2	40	5.3%	25	10	0.37	0.04
			75	37	1.35	0.14

Table 2

LODs of 10 neuropeptides on our system. (EM 1 = endomorphin 1; EM 2 = endomorphin 2; Orp FQ = orphanin FQ; Oxy = oxytocin). LOD for BE is given for intact peptide and a characteristic tryptic peptide, $BE_{10–19}$, marked by *. Some peptides were also tested on a Finnigan LCQ Deca QIT. Bull and Breese index (B & B index) were calculated based on hydrophobicity scales of their amino acids, representing higher hydrophobicity with more negative value.[33]

Peptide	Amino Acid Number	Molecular Weight	B & B index	LOD(concentrations in 4 μL volume)	
				QIT	LIT
ME	5	573.7	−398	3 pM	1 pM
LE	5	555.6	−596	1 pM	0.5 pM
Dyn $A_{1–8}$	8	981.2	−381		40 pM
BE	31	3466.0	−99	58 nM	5 nM
$BE_{10–19}$			−331		(3 pM)*
EM 1	4	610.7	−1080		8 pM
EM 2	4	571.7	−1160		17 pM
Orp FQ	17	1809.1	261		60 pM
SP	11	1347.6	−121	100 nM	10 nM
$NT_{1–13}$	13	1672.9	−323	4 pM	4 pM
Oxy	9	1007.2	−146	6 nM	60 pM

Table 3

Summary of basal and stimulated dialysate concentration of ME, LE, Dyn A, and BE from striatum of anesthetized rats (n = 3). Stimulated results were obtained by perfusing 75 mM K^+ aCSF solution for 100 min. The stimulated levels were significantly higher than baseline levels as determined by t-test (* $p<0.025$). Corrected concentrations were calculated according to the average *in vitro* recovery and the dilution with addition of acetic acid and trypsin.

Concentration ± SEM (pM)	ME	LE	Dyn A	BE
Basal	127 ± 16	51 ± 9	78 ± 7	109 ± 7
Stimulated	223 ± 48*	106 ± 37*	109 ± 10*	206 ± 72*
Basal (corrected)	308 ± 40	110 ± 19	162 ± 15	299 ± 19
Stimulated (corrected)	542 ± 117	228 ± 80	228 ± 20	565 ± 197