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Sample-Dependent Effects on the Neuropeptidome Detected in Rat Brain Tissue Preparations by Capillary Liquid Chromatography with Tandem Mass Spectrometry

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The effect of sample extraction and preparation on neuropeptidomic analysis of brain tissue by capillary liquid chromatography with tandem mass spectrometry was investigated. In agreement with previous reports, analysis of peptide extracts of brain tissue from animals sacrificed by microwave irradiation, which fixes tissue, allows identification of neuronally derived peptides whereas similar analysis of tissue from animals sacrificed without fixation does not. A comparison of a physical method for cell lysis (sonication) to physical combined with chemical cell lysis (sonication with detergent treatment) revealed that the latter method increased the number of neuronally derived peptides positively identified by ~3-fold, from 16 to 44, for analysis of microwave-fixed rat striatum. Use of synaptosome preparations also allowed detection of neuronally derived peptides (23 positively identified) without a requirement of microwave fixation, suggesting that this method may be a useful alternative for sample preparation. Although numerous peptides were identified in these experiments, several known neuropeptides were not detected including neuropeptide Y and neurotensin. Chemical properties such as hydrophobicity and atypical gas-phase fragmentation were found to account for the inability to detect these peptides. These results suggest that further improvement in sample preparation and automated spectral interpretation are needed to provide better coverage of neuropeptides in mammalian tissues. A total of 39 novel neuronally dervived peptides, including some originating from proenkephalin and phosphatidylethanolamine binding protein, were identified in striatum and synaptosome.

Neuropeptides represent an important class of neurotransmitters and neuromodulators that are involved in many diverse and important functions such as learning, memory, sensory perception, and appetite regulation. These signaling molecules are made from biologically inactive protein precursors (prohormones) by post-translational processing that includes cleavage by specific proteases at select amino acid sites. Peptidergic fragments of other neuronal proteins, while not involved in neurotransmission, are also of interest as they may serve as important biomarkers indicative of brain injury[1,2] and disease.[3]

The importance of neuropeptides in neural function, along with the advent of proteomic methods, has led to the emergence of "neuropeptidomics" wherein analytical techniques are used for the identification of peptides in brain tissue. This approach has been used to identify novel neuropeptides and their posttranslational modifications,[4−9] investigate processing and degradation pathways,[10−13] and determine expression level changes of neuropeptides.[14,15]

Neuropeptidomic analysis of mammalian tissue has frequently relied on using liquid chromatography−electrospray ionization-tandem mass spectrometry (LC−ESI-MS^2) analysis for neuronal samples. MS^2 spectra acquired from such experiments are subjected to protein database searching algorithms for peptide

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sequence determination. This approach is similar to "shotgun" proteomics in which a protein mixture is treated with a protease to form a collection of peptides that are characteristic of the proteins. A significant difference is that the protease used to create peptides in shotgun proteomics is controlled by the experimenter, but for a neuropeptidomic method, the protease is endogenous and not necessarily known.

Sample preparation is a significant consideration in neuropeptidomic analysis. Peptidases released during sample preparation can rapidly degrade neuropeptides, lowering their concentration and resulting in a mix of neuropeptides different from what is normally present in vivo. Enzymatic degradation of other abundant proteins also creates a large background of peptides interfering with neuropeptide detection. For example, analysis of brain tissue from freshly sacrificed animals reveals mostly peptide fragments from blood proteins such as hemoglobin with little or no signal obtained from classical neuropeptides.[6] One approach to avoiding the peptidase problem is to use mice with a mutation that inactivates the enzyme responsible for one of the most common protein cleavages.[11,16] While increasing the concentration of active peptides prior to the subsequent LC−MS^2 analysis, this method is limited to intermediates with C-terminal basic residues and the availability of mice with the mutation. In addition, the mutation could alter the physiological levels of a given peptide. Analysis of dialysate collected from live animals for peptides avoids some of these problems as well.[4,5] While this method yields relatively low numbers of peptides, most of them appear to be of neuronal origin. It has also been found that sacrificing the animal by focused microwave irradiation, a technique that rapidly fixes tissue and terminates enzyme activity by raising the brain temperature to 90 °C in <1 s,[17−19] will practically eliminate detection of blood peptides and yield many more neuropeptides than tissue harvested from animals sacrificed by other methods.[7] Sacrifice by decapitation followed by rapid microwave treatment has also been reported to reduce the background from degradation of abundant proteins.[20]

Despite these improvements, it is evident that current methods do not provide complete coverage of the neuropeptidome because known neuropeptides and expected fragments of prohormones are not always detected by these methods. Additional method development is required therefore to achieve the significant goal of reliably detecting and identifying all the neuropeptides in a sample. To gain insights into the limits of current methods, we further examine the effect of tissue preparation methods on the quality of neuropeptidomic data. Modified extraction procedures and other tissue preparations, such as synaptosomes, are found to improve the yield of neuropeptides and allowed detection of a novel group of neuropeptides demonstrating the significance of their use for neuropeptidomics. Furthermore, we show that some known neuropeptides cannot be identified by current approaches because of poor sensitivity or atypical fragmentation.

EXPERIMENTAL SECTION

Chemicals and Reagents. Capillary LC solvents were purchased from Burdick & Jackson (Muskegon, MI). Peptide standards were from Sigma (St. Louis, MO) except neurotensin, which was from American Peptides (Sunnyvale, CA). Acetic acid and hydrofluoric acid were purchased from Fisher Scientific (Pittsburgh, PA). The following chemicals to make the polymer column frits were from Aldrich (Milwaukee, WI): 2,2,4-trimethylpentane, toluene, trimethylolpropane trimethacrylate, glycidyl methacrylate, and benzoin methyl ether. All other chemicals were from Sigma.

Normal (Unfixed) Tissue Preparation. Male Sprague−Dawley rats, 6−12 weeks of age (Harlan, IN), were sacrificed by decapitation followed by rapid (<90 s) removal of the brain. The brain was immediately frozen in liquid nitrogen. Striatum samples were removed whole by free-hand dissection and stored at −80 °C until peptide extraction. 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.

Microwave Irradiated (Fixed) Tissue Preparation. Male Long Evans rats, 4−8 weeks of age (Harlan, IN), were sacrificed by focused microwave irradiation using a Muromachi Microwave Fixation Applicator, model TMW-4012C (10-kW output). Animals were subjected to an output power of ∼3.5 kW for 1.5 s by exposure of the head to the microwave beam. Striatum or hypothalamus samples were either removed whole by free-hand dissection or pooled from several slices of 1-mm coronal sections (prepared with a rat brain matrix) and stored at −80 °C until peptide extraction. All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health. The animal facility was accredited by the American Association for Accreditation of Laboratory Animal Care.

Peptide Extraction from Brain Tissue by Sonication. Some experiments utilized a rapid peptide extraction from brain tissue that has been described elsewhere.[6] This procedure consisted of suspending tissue in a chilled solution of 0.25% acetic acid that was then homogenized using a sonic dismembrator (Fisher Scientific) on power setting 3 for 10 s. The homogenate (0.1−0.2 mg of tissue/μL) was centrifuged at 20000*g* for 30 min at 4 °C. The supernatant containing both proteins and neuropeptides was removed from the resulting tissue pellet and placed into a Microcon YM-10 centrifugal filter device from Millipore (Bedford, MA). The 10 000 molecular weight cutoff of the membrane inside the filter device retains proteins, and the resulting peptide-rich filtrate was frozen on liquid nitrogen and stored at −80 °C until analysis.

Vigorous Peptide Extraction from Brain Tissue. A more vigorous peptide extraction was also used that combined physical and chemical cell lysis. Tissue was placed into 250 μL of cold (4 °C) homogenization solution containing 145 mM KCl from ICN Biomedicals Inc (Aurora, OH), 8 mM sodium dodecyl sulfate (SDS) from Sigma, and 0.25% acetic acid. The tissue/homogenization solution was set on ice for 1 h. Throughout the 1-h period, tissue homogenization and cell lysis was aided every 10 min by 5-s sonication using a sonic dismembrator followed by 30 s of vortexing. The homogenate (0.1−0.2 mg of tissue/μL) was then

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centrifuged at 20000*g* for 30 min at 4 °C to remove cell debris. The supernatant was then placed in the Microcon YM-10 centrifugal filter device and centrifuged at 14000*g* to separate the peptides from the proteins. The peptide-containing filtrate was frozen on liquid nitrogen and stored at −80 °C until analysis.

Synaptosome Preparation from Rat Brain Tissue. Synaptosomes were prepared from cerebra of male Sprague−Dawley rats (130−150 g) that were sacrificed by decapitation. The brains from four rats were homogenized in 120 mL of 0.32 M sucrose, 0.3 mM EGTA, and 4 mM Tris-HCl, pH 7.4, utilizing a glass Dounce homogenizer fitted with a Teflon pestle from Bellco Glass Inc. (Vineland, NJ). The homogenate was centrifuged at 1000*g* for 10 min, and the supernatant was collected and centrifuged a second time at 12000*g* for 15 min. The resulting supernatant was discarded. The synaptosomal pellets were washed by homogenization in sucrose-containing homogenization buffer and pelleted by centrifugation. The washed pellet was suspended in 120 mL of ice-cold Krebs-Ringer solution (KRB) containing 150 mM NaCl, 2.4 mM KCl, 1.2 mM Na_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.1 mM DTT, and 5 mM HEPES buffered to pH 7.4 with Tris base. KRB-suspended crude synaptosomes were pelleted by centrifugation for 10 min at 4300*g*. The KRB-washed synaptosomal pellets were suspended in oxygenated (90% O_2, 5% CO_2) isotonic KRB solution containing 10 mM glucose and 2.4 KCl at a protein concentration of 2.0 mg/mL and stored at −80 °C until analysis.

Preparation of Capilliary LC Columns with Integrated Electrospray Emittters. Capilliary LC columns with a 25-μm inner diameter (i.d.) were prepared as described previously.[4] Briefly, a macroporous polymer frit was formed inside a 20-cm-long fused-silica capilliary with an i.d. of 25 μm by exposing a 100-μm section of capillary filled with a solution of glycidyl methacrylate (7% v/v) and trimethylolpropane trimethacrylate (16% v/v) to a UV light.[21,22] Prior to UV exposure, the outer polyimide coating of the capillary was removed to a length of 100 μm by burning with a high-voltage arc across two electrodes using a device built in-house. A P-2000 CO_2 laser puller (Sutter Instruments, Novato, CA) was used to pull the column outlet to a fine tip at a distance of ~7 mm from the frit. These pulled capillaries were etched with 50% hydrofluoric acid (use extreme caution and neutralize with $CaCl_2$)[23] to create sharp-tipped electrospary emitters with ~3-μm i.d. The capillary columns were slurry-packed to a length of 4 cm with 5-μm Alltima C18 reversed-phase particles (Alltech, Deerfield, IL) at 5.5 MPa using helium through a high-pressure bomb. The void at the head of the column did not contribute to extracolumn band broadening because all samples were loaded onto the column in weak mobile phases to allow analytes to stack at the head of the column.

Automated Two-Pressure LC−ESI-MS^2 System and Operation. The capillary LC−MS^2 system used consisted of an Eldex Micropro LC pump (Eldex Laboratories, Napa, CA), a high-pressure pneumatic amplifier pump (model DSFH-151, Haskel, Burbank, CA), and two six-port Valco injection valves (model C2, Houston, TX) as previously described.[4] The valves are plumbed so that one functions as an injection valve for standard solutions and sample loading using a 5-μL capillary as the injection loop. The second valve is connected to the pump inlet of the injection valve and is used to select between the LC pump and the high-pressure pump. The high-pressure pump is used to rapidly load samples from the injection loop and desalt samples prior to chromatographic separation as well as rinse the capillaries while the lower pressure pump is selected for the separation.

Peptide samples were loaded onto the column by filling the sample loop through the injection inlet and using the high-pressure pump to flush through the loop for 13.5 min at 370 nL/min. The attached column was desalted by flushing with 1% acetic acid in water for 5 min at the same flow rate. The column flow rate was switched to 100 nL/min to begin separation. Gradient elution consisted of a linear ramp of 100% mobile phase A (water), 0% mobile phase B (methanol), to 100% mobile phase B over 60 min where both mobile phases contained 1% acetic acid. For tissue sample analysis, 5 μL of 0.2 mg/μL samples were loaded onto the column.

An LCQ-Deca quadrupole ion trap (ThermoFinnigan, San Jose, CA) mass spectrometer was used with the following operating parameters: automatic gain control (AGC) on, maximum AGC time 300 ms, $q = 0.25$, isolation width 3 m/z, normalized collision energy 35%, activation time 0.25 ms, and the default number of microscans and target count values. All analyses were performed in "data-dependent" mode in which the mass spectrometer performs tandem mass spectra on ions that are detected above a certain threshold. Data-dependent MS^2 spectra were collected using the precursor ion window of 500−2000 m/z. Dynamic exclusion was enabled with a repeat count of 1 and a repeat duration of 0.5 min. The peak exclusion list size was 30 with an exclusion duration of 5 min and an exclusion mass width of 1.0. The most intense peak from the MS scan was selected by the data-dependent analysis with a minimum MS signal of 10^5 counts and an isolation width of 3.0. The spectrometer was tuned as described previously.[4]

Peptide Sequencing and Protein Precursor Identification. SEQUEST Browser (v3.0, ThermoFinnigan) was used to analyze all the MS^2 spectra obtained with the following settings to create DTA files: bottom MW 500, top MW 2000, grouped scans 1, min number ions 15, minimum TIC 1000. Correlations were performed with the Swiss Prot protein database for *Rattus norvegicus*. Mass spectra that matched a peptide sequence with $\Delta CN > 0.1$ were considered significant.[24] All the MS^2 spectra were analyzed again as SEQUEST DTA files on the on-line version of Mascot (Matrix Science, http://www.matrixscience.com) using the same database and the MS/MS ion search algorithm with the following parameters: peptide tolerance ± 2.5, MS/MS tolerance ± 1.0 consistent with SEQUEST. Sequences were considered confirmed by Mascot if the score indicated a homology with greater than 95% probability. If the confirmed Mascot sequence for a given spectrum was the same as the significant SEQUEST match, then sequence was considered positively identified. This cross algorithm check reduces the number of false positives reported.[5]

Capillary LC with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (LC−MALDI-

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TOF-MS). Columns were prepared similar to those used for capillary LC-ESI but omitting the preparation of the emitter. For off-line interface of capillary LC to MALDI-TOF-MS, a system similar to that previously described was used.[25] Briefly, effluent from the column was mixed with matrix solution (CHCA saturated in acetonitrile/0.1% TFA at 50/50, v/v) pumped at 25 nL/min through a Y-connector. The outlet of the Y-connector consisted of a 50 μm i.d. × 180 μm o.d. × 1.5 cm long piece of fused-silica capillary that was positioned over a MALDI target plate mounted on a motorized $x-y$ positioner. Droplets from the mixed matrix and column effluent were pulled down to the plate at 5-s intervals by application of −2 kV for 300 ms between the capillary tip and MALDI plate. After each fraction was deposited, the motorized stage automatically positioned the MALDI plate for deposition to a new target spot. The dwell time for each spot was 40 s. Mass spectra were acquired from each spot with a MALDI TOF Spec 2E mass spectrometer (Micromass, Milford, MA) equipped with a delayed extraction source and 337-nm pulsed (4 ns) nitrogen laser operated in reflectron mode. All mass spectra were obtained in positive ion mode with 20-kV source voltage, 2.2-kV extraction pulse voltage, 19.98 -kV extraction voltage, 16-kV focus voltage, and suppression mass of 500 m/z with scan extended to 5000 m/z. Mass spectra were generated by summing 10−50 scans (each scan corresponds to 5 laser shots) into a single spectrum in Masslynx software (Micromass).

RESULTS AND DISCUSSION

Microwave Fixed Tissue versus Unfixed Tissue. In a previous study of rat hypothalamus neuropeptidome, it was reported that analysis of microwave-fixed tissue resulted in detection of many more neuroactive peptides than unfixed tissue, which yielded primarily degradation products of blood proteins, when using sonication only for extraction and LC−ESI-MS^2 for identification.[6] We confirmed this observation for analysis of rat striatum samples. For unfixed tissue, an average of 915 ± 252 (n = 2) MS^2 spectra were obtained and for fixed tissue an average of 1827 ± 274 (n = 3) MS^2 spectra. For unfixed tissue, SEQUEST and Mascot returned 71 ± 4 and 38 ± 9 spectra with significant scores, respectively. These significant spectra corresponded to 23 unique peptides after cross-checking the algorithms. For fixed tissue analysis, SEQUEST and Mascot returned 174 ± 22 and 15 ± 5 spectra with significant scores, respectively. These significant spectra corresponded to 14 unique peptides after cross-checking the algorithms. Although analysis of unfixed tissue produced more positively identified peptides than fixed tissue, the unfixed tissue peptides were of little interest because they were primarily fragments of hemoglobin and a few membrane-bound proteins, similar to previous reports.[6,26] We found that addition peptidase inhibitor cocktails during tissue preparation reduced the number of hemoglobin fragments but did not improve neuropeptide detection (data not shown). In contrast, all 14 peptides (Table 1, peptide extraction by sonication) detected in the fixed tissue sample originate from precursors of neurological significance.

Effect of Using a Vigorous Peptide Extraction. The initial study used extraction based on mechanical disruption of tissue by sonication following previous reports;[7] however, it is also possible to use chemical methods, such as treatment with detergents or enzymes, to lyse and disrupt the membrane of cells and vesicles for peptide extraction. While either method can be sufficient independently, it has been shown that using a combination of physical and chemical lysis procedures may be more effective at fully rupturing cells in biological material.[27,28] We therefore tested the use of a combined extraction that involved both sonication and treatment with SDS on fixed tissue samples (see Vigorous Peptide Extraction in Experimental Section). We observed an average of 1667 ± 332 MS^2 spectra per analysis (n = 3) with 185 ± 91 and 51 ± 11 obtaining significant matches in SEQUEST and Mascot, respectively. Cross-checking the algorithms revealed 44 unique peptides positively identified, representing an over 3-fold improvement in peptide detection over the gentler extraction (Table 1). The number of protein precursors represented nearly doubled from 7 to 13.

The improvement in peptide numbers detected can be largely attributed to improved extraction efficiency. It is also possible that more peptides were produced due to protein degradation by proteases or other processes during the longer extraction period; however, extensive enzyme action during extraction seems unlikely given that the extraction occurred after microwave fixation, which is believed to arrest enzymatic action[17−19]and generally increases the levels of active peptides.[29,30] Furthermore, the extraction involved use of surfactants and acidic conditions that would be expected to contribute to denaturation of any proteases. Finally, if protein degradation during the extraction step was a significant contributor to producing the peptides detected, it would be more likely to detect fragments of highly abundant proteins such as hemoglobin or actin. These results suggest that greater coverage of the neuropeptidome can be achieved using this more aggressive extraction procedure combined with microwave fixation. It also suggests that previous neuropeptidome studies have been limited, at least partially, by the extraction methods.

The use of surfactants as an extraction method is well known; therefore, it is surprising that it has yet to be used for neuropeptidomic studies. A likely reason is concern that signal suppression effects from SDS would impair analyte detection by ESI-MS;[31,32] however, the good yield of peptides suggests that this problem was outweighed by improved extraction efficiency. Deleterious effects of surfactant were probably mitigated by the membrane filtration step, which could remove SDS as micelles and as monomer bound to retained proteins.

Synaptosome Preparation. The microwave fixation method has clearly been an important breakthrough in sample preparation for neuropeptidomics.[7,19] One drawback of this method is the expense of equipment required to perform microwave fixation. Other methods of sample preparation that could avoid the problems of overwhelming detection of blood peptides are of interest. Therefore, we also tested the hypothesis that analysis of synaptosomes from unfixed tissue (i.e., tissue acquired from rats

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Table 1. Neuropeptides Detected and Identified from Focused Microwave Irradiated Tissue from the Striatum Region of the Rat Brain Using Capillary LC–ESI-MS^2

Protein Precursor	Sequence	Sonication Extraction (observed)	Vigorous Extraction (observed)
Proenkephalin A	SPQLEDEAKELQ	3	3
	SPQLEDEAKELQ-amide*	1	3
	YGGFMRGL	3	3
	YGGFMRF	1	3
	VGRPEWWMDYQ	3	3
	VGRPEWWMDYQ-amide*		2
	FAESLPSDEEGESYSKEVPEME*		2
	YGGFM	2	3
	YGGFL	1	3
Prodynorphin	PKLKWDNQ*		1
Chromogranin A	WSRMDQLAKELTA		2
Chromogranin B	SFAKAPHLDL	2	3
	AALAAVISAPVDNRDH*	2	3
	GRGREPGAYPALDSRQE*		1
Chromogranin C	QELGKLTGPSNQ		1
	NLQIPPEDLIEMKLKAGE-acetyl (K)*		1
Cerebellin	SGSAKVAFSAIRSTNH	2	3
ProSAAS	SLSAASAPLAETSTPL	1	3
(neuroendocrine peptide)	LENSSPQAPARRLLPP		1
Protachykinin 1	ALNSVAYERSAMQNYE		1
	ALNSVAYERSAMQNYE-amide*		1
Thymosin beta-4	PLPSKETIEQEKQAGES	1	2
Thymosin beta-10	PTKETIEQEKRSEIS	3	3
	EKNTLPTKETIEQEKRSEIS		3
Prohormone convertase 2	IKMALQQEGFD	2	3
Prosomatostatin	SANSNPAMAPRE		1
Phosphatidylethanolamine-binding protein (Neurostimulating peptide)	GLDPGKLYTL*		3
	GLDPGKLYTL-amide*		1
	KGNDISSGTVLSEY*		2
	WDDSVPKLHDQLAGK*		3
	WDDSVPKLHDQLAGK-amide*		2
	QAEWDDSVPKLHDQLAGK*		2
	MNRPSSISWDGL*		2
	PSSISWDGLDPGKLYTL*		3
	PSSISWDGLDPGKLYTL-amide*		3
	YGGVTVDELGKVLTPYQV*		3
	YGGVTVDELGKVLTPYQV-amide*		3
	VDYGGVTVDELGKVLTPYQV-amide*		1
	DYGGVTVDELGKVLTPYQV*		3
	RVDYGGVTVDELGKVLTPYQV*		2
	RVDYGGVTVDELGKVLTPTQVMNR*		3
	RVDYGGVTVDELGKVLTPTQVMNR-amide*		3
	MNRPSSISWDGLDPGKLYTL*		2
	TPTQVMNRPSSISWDGLDPGKLYTL*		2

[a] Three repetitions were performed using either sonication only for extraction or sonication plus treatment with detergents for extraction (vigorous). Peptides listed were found to be significant by both SEQUEST and Mascot as described in the Experimental Section. The number of samples the peptides were observed is given for each method of extraction. Peptide sequences marked (*) are novel endogenous neuropeptides detected by this study.

sacrificed by decapitation) subjected to the vigorous peptide extraction procedure would produce improvements in peptide yield over crude unfixed tissue. Synaptosomes are saclike structures formed by "pinching off" nerve endings during homogenization. As a result, synaptosomes contain mature secretory vesicles but are devoid of blood and much cellular debris. Such preparations would be expected to concentrate neuropeptides relative to whole tissue preparations. Data-dependent analysis produced an average of 2022 ± 54 MS^2 spectra ($n = 3$) with 455 ± 149 and 40 ± 17 spectra returning significant SEQUEST and Mascot scores, respectively. Cross-checking the algorithms gave a total of 23 unique neuropeptides (Table 2).

Peptidomic analysis of synaptosomes, unlike crude tissue, did not yield any blood peptides and instead resulted in detection of mostly neuronally derived peptides including known neurotransmitters and related peptides. Indeed, significant overlap was found with the microwave-fixed tissue analysis (see Figure 1). The method of synaptosome preparation used here required a large amount of tissue (4 whole brains were used for a single synaptosome preparation in this experiment). This preparation may have limited the number of peptides detected because peptides localized to specific brain regions will be diluted by the use of whole brain, making their detection more difficult. In principle, synaptosomes can be prepared from smaller brain regions to improve detection of localized neuropeptides. This result suggests that analysis of synaptosomes can yield neuropeptides without blood peptides and is a potential alternative to microwave fixation. One caveat, however, is that, without microwave fixation, it is unclear if some of the peptides detected were produced by peptidases after sacrifice. Therefore, the microwave fixation method is still preferred.

Table 2. Neuropeptides Detected and Identified in Synaptosomes[a]

Protein Precursor	Sequence	Synaptosome (observed)
Proenkephalin A	SPQLEDEAKELQ	3
	SPQLEDEAKELQ-amide	1
	VGRPEWWMDYQ	3
	YGGFM	3
	YGGFL	2
Chromogranin B	SFAKAPHLDL	3
	GRGREPGAYPALDSRQE*	2
	QYDDGVAELDQLLHY*	1
	LLDEGHDPVHESPVDTA*	2
	AALAAVISAPVDNRDH*	1
Chromogranin C	QELGKLTGPSNQ	2
Cerebellin	SGSAKVAFSAIRSTNH	3
ProSAAS (neuroendocrine peptide)	SLSAASAPLAETSTPLRL	3
	SLSAASAPLAETSTPL	2
	acetylation-LSAASAPLAETSTPLRL*	1
Protachykinin 1	ALNSVAYERSAMQNYE*	1
Neurogranin	KGPGPGGPGGAGGAR*	3
Synapsin IIb	VDAPAPSAASRKA*	3
	NGIAVGPKQVQAS*	2
	QAVKQTAASAGLVD*	3
Prodynorphin	PKLKWDNQ*	1
Synaphin 1	KYLPGPLQDMFKK*	1
	DTVIKLPGPLQDMFKK*	2

[a] The numbers total the number of times a peptide was observed from three repetitions. Peptide sequences marked (*) are novel endogenous neuropeptides detected by this study.

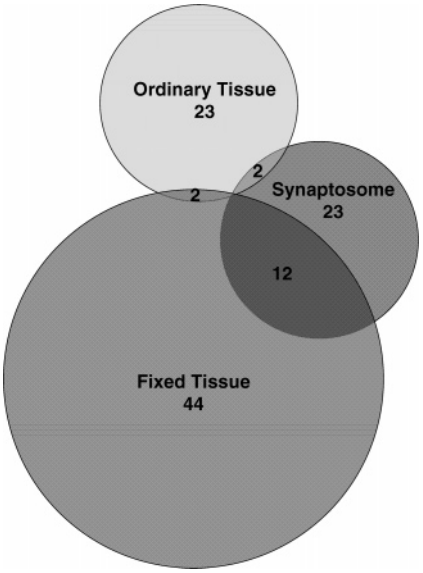

Figure 1. Venn diagram of peptides detected in microwave-fixed tissue, ordinary unfixed tissue, and the synaptosome preparation. The areas of the circles are proportional to the total number of peptides identified in each sample, and the numbers indicate the actual number of peptides in each area. The synaptosome preparation shows the least number of peptides but shares more commonality with the fixed tissue than ordinary tissue. This result is a reflection of the ordinary tissue showing primarily blood-type peptides whereas the other two sample types reveal more neuronally derived peptides.

Effect of Sample Analysis Replication. It has been shown that it is important to replicate sample analysis in order to maximize peptide coverage by data-dependent analysis because this improves the chance of detecting low-abundance peptides.[33] Figure 2 illustrates that a single repetition of analysis improved peptide yield 23−29% for all sample types, but no improvement was found by the third replication. The leveling off of this curve suggests that further significant improvement in neuropeptidome coverage would be unlikely to result from further repetitions of analysis.

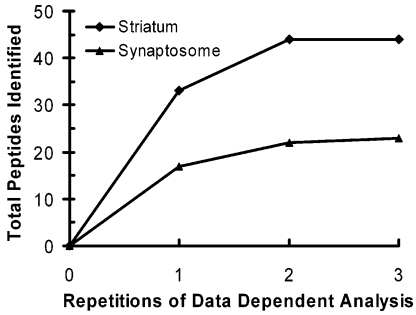

Figure 2. Improvement of neuropeptidome coverage with repetition of analysis. Each sample type was analyzed three times. The total number of peptides positively identified using the dual search algorithm, described in the Experimental Section, was monitored.

Analytical and Chemical Implications of Neuropeptide Type. The improved sample extraction and preparation methods allowed detection of many previously unreported peptides. At the same time, however, we observed that important neuropeptides, such as substance P, neurotensin, and neuropeptide Y, known to be present in this brain region, were not detected in any samples by capillary LC−ESI-MS^2.

To determine whether the reason for not observing these peptides by capillary LC−ESI-MS^2 was methodological, we analyzed samples of striatum from rats sacrificed microwave fixation by capillary LC with off-line MALDI-TOF-MS. Using this method, substance P was routinely detected (4 of 4 attempts) and neurotensin was usually detecteable (3 of 4 attempts). Figure 3 illustrates the mass spectra obtained from spots that were collected at the known elution time of substance P and neurotensin. These peaks have a good match with the mass of the peptides (difference from actual mass is <50 ppm). The identity was further confirmed by spiking brain samples with substance P and neurotensin and observing coelution of the spiked sample (data not shown). These results demonstrate that substance P and neurotensin were present in the samples; therefore, the inability to detect and identify them by LC−ESI-MS^2 was not due to complete degradation of the peptide. Neuropeptide Y was not detected by the MALDI method.

To better understand why capillary LC−ESI-MS^2 did not detect and identify these peptides, we analyzed standard solutions containing the peptides at 600 pM and at 100 nM each using the same LC−ESI-MS^2 and data-dependent conditions as the tissue samples. For the lower peptide concentration solution, none of the three peptides were observed ($n = 3$). In the higher concentration solution, substance P was observed and produced highly significant SEQUEST and Mascot scores.

These results demonstrate that substance P can be detected and identified by our capillary LC−ESI-MS^2 system in simple mixtures. Detection of a particular peptide in a complex mixture by data-dependent analysis is highly dependent on the other peptides that coelute with the peptide. Other peptides of high abundance and high ionization yield can prevent the peptide from being selected for MS^2 analysis. Such an effect is a potential reason for substance P not being detected in this case. It is also possible

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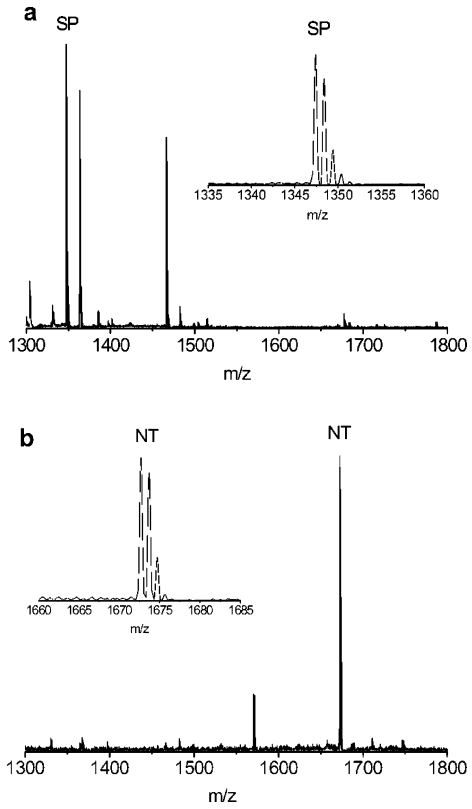

Figure 3. MALDI-TOFMS spectra acquired at the capillary LC retention time of substance P (a) and neurotensin (b) from striatum of rats sacrificed by microwave fixation and treated by the vigorous extraction procedure. Insets show the peaks that match the molecular weight of the peptides.

that the substance P concentration in tissues is below the detection limit of the LC−ESI-MS^2 method.

Neuropeptide Y (NPY) was not detected by data-dependent scanning even at 100 nM. LC separation combined with single ion monitoring analysis, which should be more sensitive, was also unsuccessful at detecting the peptide at 100 nM. The inability to detect NPY was not due to poor ionization efficiency because direct infusion of 600 pM into the mass spectrometer resulted in a peak at 1427.6 m/z, corresponding to the 3^+ charge state of the neuropeptide, that had intensity comparable to peptides such as PKLKWDNQ from prodynorphin and ALNSVAYERSAMQNYE from protachykinin that were identified. Given that NPY was detectable in direct infusion but not during separation, we conclude that NPY is lost during sample loading or chromatography. This reason would also explain why NPY was not detected in brain samples by the MALDI method. It is not clear what chemical property of NPY resulted in this problem; however, NPY is the longest and most hydrophobic peptide we examined. NPY has a hydrophobicity of 40, which was calculated using retention coefficients of the individual amino acids as described elsewhere.[34] For comparison, a recent study of 346 tryptic peptides found that over 80% of the peptides had hydrophobicity of <40 and a range of 3−60.[34] It is possible therefore that the high hydrophobicity or large size of this peptide is incompatible with our procedure.

Neurotensin was also not identified by capillary LC−ESI-MS^2 even at high concentrations in standards solution. In this case, however, the problem appears to be atypical peptide fragmentation. After electrospray ionization, charges migrate from the initial protonation site (such as the N-terminal amino group or basic amino acid side chains) toward an amide carbonyl oxygen atom on the peptide backbone.[35−37] Fragmentation of peptide occurs by nucleophilic attack of the peptide amide bond by a neighboring amide carbonyl group giving rise to b- and y-type product ions. Strong basic sites such as arginine can sequester the proton, preventing its further mobility and limiting fragmentation.[35] In peptides containing one or more arginines, such as neurotensin (see sequence in Figure 4), selective cleavage can occur at acidic residues if the number of protons is equal to or less than the number of arginines present.[37] This effect can explain the rather selective fragment of neurotensin, which has MS^2 spectra dominated by two peaks (579.0 and 780.8 m/z) as seen in Figure 4. Only a few of the other expected b- and y-type ions, calculated using the program MS-Product (http://prospector.ucsf.edu/), which does not account for the enhanced cleavage rule, are observed in the actual neurotensin spectrum (see Figure 4). Supporting the conclusion that limited peptide fragmentation was a factor in preventing detection of neurotensin, we observed the precursor ion peak for the 2^+ charge state of neurotensin (837.2 m/z) in two tissue samples, but the spectra were not correctly identified. Incorrect identities were also assigned for standards of neurotensin. These results suggest that manual interpretation of spectra may be necessary for confirming the presence of select peptides. Ultimately improvements in automated interpretation of spectra will be required to correctly identify neuropeptides with atypical fragmentation patterns. Indeed, previous studies have shown that proton mobility is the most important factor influencing fragmentation and thus the subsequent database search result scores.[38]

Biological Significance of Novel Endogenous Neuropeptides Discovered in This Study. With the improved methods, we have identified 39 novel peptides from 10 protein precursors. We attribute the detection of these new peptides to the combined use of microwave fixation and vigorous extraction. Many of the peptides have properties that may imply biological activity.

Previous work has identified several non-opiod peptides from the proenkephalin precursor,[5,7,8] some of which appear to have neuronal signaling properties.[5] In this study, we have observed three new proenkephalin-derived peptides (see Tables 1 and 2). Two of these peptides are amidated forms of previously observed peptides.[7,8] Amidation is a common modification that can improve peptide stability and modulate peptide activity.[39,40] Another peptide, FAESLPSDEEGESYSKEVPEME, is flanked by dibasic sites in the proenkephalin sequence consistent with specific production by convertases. The production and modification of these peptides are similar to known neurotransmitters, suggesting that they should be further evaluated for possible neuroactivity.

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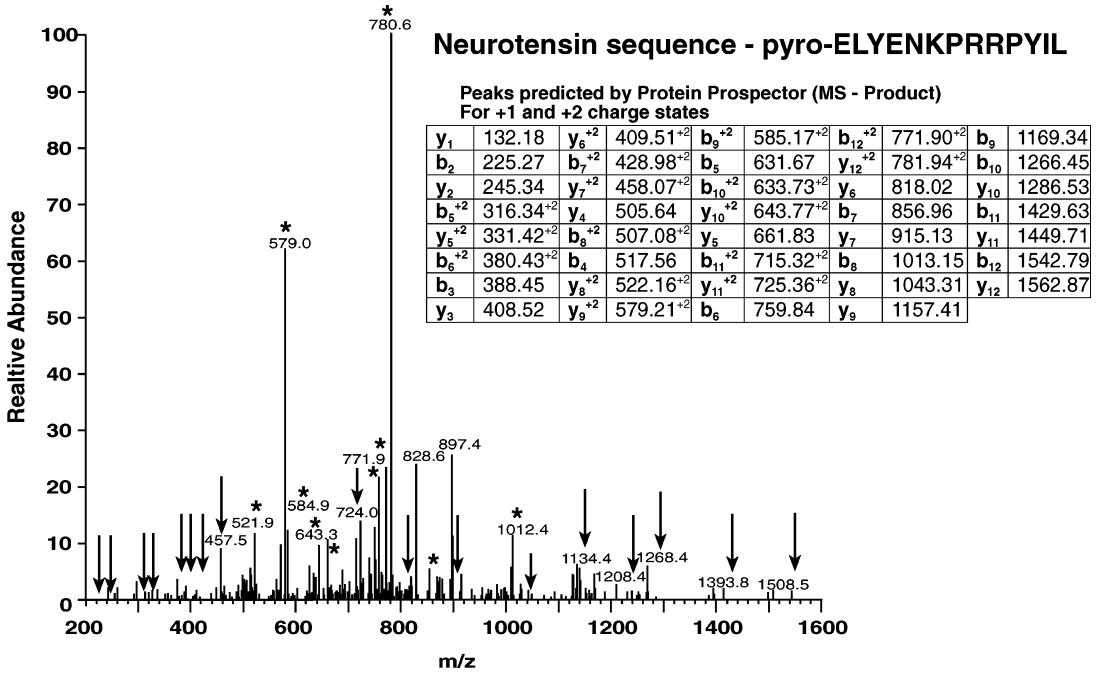

y_1	132.18	y_6^{+2}	409.51^{+2}	b_9^{+2}	585.17^{+2}	b_{12}^{+2}	771.90^{+2}	b_9	1169.34
b_2	225.27	b_7^{+2}	428.98^{+2}	b_5	631.67	y_{12}^{+2}	781.94^{+2}	b_{10}	1266.45
y_2	245.34	y_7^{+2}	458.07^{+2}	b_{10}^{+2}	633.73^{+2}	y_6	818.02	y_{10}	1286.53
b_5^{+2}	316.34^{+2}	y_4	505.64	y_{10}^{+2}	643.77^{+2}	b_7	856.96	b_{11}	1429.63
y_5^{+2}	331.42^{+2}	b_8^{+2}	507.08^{+2}	y_5	661.83	y_7	915.13	y_{11}	1449.71
b_6^{+2}	380.43^{+2}	b_4	517.56	b_{11}^{+2}	715.32^{+2}	b_8	1013.15	b_{12}	1542.79
b_3	388.45	y_8^{+2}	522.16^{+2}	y_{11}^{+2}	725.36^{+2}	y_8	1043.31	y_{12}	1562.87
y_3	408.52	y_9^{+2}	579.21^{+2}	b_6	759.84	y_9	1157.41		

Figure 4. MS^2 spectrum of a 600 pM standard of neurotensin by direct infusion. The inset shows the sequence of neurotensin and the predicted MS^2 peaks according to MS-Product. Peaks in the spectrum marked with asterisks are predicted. Arrows in the spectrum indicate *m*/*z* values that were expected but not detected.

We have found 18 peptides from phosphatidylethanolamine-binding protein (PEBP), also known as neurostimulating peptide. Several of the fragments are "nested" with overlapping sequences differing by single amino acids. It is unclear if these nested peptides are part of normal processing or if they are produced from a larger peptide that became partially degraded by peptidases released during sample preparation. The latter seems unlikely given the use of microwave fixation and harsh conditions of extraction as discussed above. Several of the fragments are amidated and some have intriguing homology (YGGX) with opioid peptides. PEBP fragments, including some that overlap with our own observations, have been detected by proteomic analysis of media conditioned by rat hippocampal cells, suggesting that these peptides can be secreted by neurons.[41] PEBP fragments are involved in enhancing acetylcholine synthesis,[42] and elevated tissue concentration of PEBP has been implicated in Alzheimer's disease.[43] Taken together, these observations suggest the potential for biological activity of the PEBP fragments found here.

ProSAAS peptides appeared in all our sample types. ProSAAS is involved in endoprotease processing of prohormones as an endogenous inhibitor of the precursor cleaving enzyme PC1.[44,45] Thus, the new sample preparation procedures have revealed novel peptides in brain tissue and suggest that further improvements in this step may be important for determining a complete neuropeptidome.

CONCLUSION

A combination of physical and chemical cell lysis methods for extraction significantly improved coverage of the neuropeptidome that can be detected by capillary LC−ESI-MS^2 using a quadrupole ion trap mass spectrometer. Improved coverage allowed novel peptides to be detected and will begin to allow identification of peptides characteristic of discrete brain regions. Neuropeptidomic analysis of synaptosomes was found to have some potential as a sample preparation method for avoiding interference from blood peptides. The ability to detect some peptides by capillary LC−MALDI-TOF-MS that were not detected by LC−ESI-MS^2 suggests that this method may be a necessary complement for gaining better coverage of the neuropeptidome. We have also revealed that complete coverage of the neuropeptidome will require methods that account for diverse fragmentation patterns and chromatographic properties of neuropeptides. Achieving a better understanding of these parameters will allow discovery of more neuropeptides and a greater understanding of brain peptide chemistry.

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