Quantitative Neuropeptidomics of Microwave-irradiated Mouse Brain and Pituitary*

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In neuropeptidomics, the degradation of a small fraction of abundant proteins overwhelms the low signals from neuropeptides, and many neuropeptides cannot be detected by mass spectrometry without extensive purification. Protein degradation was prevented when mice were sacrificed with focused microwave irradiation, permitting the detection of hypothalamic neuropeptides by mass spectrometry. Here we report an alternative and very simple method utilizing an ordinary microwave oven to inhibit enzymatic degradation. We used this technique to identify brain and pituitary neuropeptides. Quantitative analysis using mass spectrometry in combination with stable isotopic labeling was performed to determine the effect of microwave irradiation on relative levels of neuropeptides and protein degradation fragments. Microwave irradiation greatly reduced the levels of degradation fragments of proteins. In contrast, neuropeptide levels were increased about 2–3 times in hypothalamus by the microwave irradiation but not increased in pituitary. In a second experiment, three brain regions (hypothalamus, hippocampus, and striatum) from microwave-irradiated mice were analyzed. Altogether 41 neuropeptides or fragments of secretory pathway proteins were identified after microwave treatment; some of these are novel. These peptides were derived from 15 proteins: proopiomelanocortin, proSAAS, proenkephalin, preprotachykinins A and B, provasopressin, prooxytocin, melanin-concentrating hormone, proenkephalin, preprotachykinins A and B, proproenkephalin, preprotachykinins A and B, provaso-pressin, chromogranins A and B, secretogranin II, proopiomelanocortin; ACTH, adrenocorticotropic hormone. Neuropeptides are involved in a wide variety of systems, including reward mechanisms, pain, memory, food intake and body weight regulation, circadian rhythms, and many others (1, 2). A large number of studies have examined the changes in levels of various peptides upon different treatments or in different physiological states. These studies typically measured peptide levels using radioimmunoassays (RIAs). Although this approach is sensitive, most antisera are not specific for a single peptide and are able to cross-react with N- and/or C-terminally extended peptides and with peptides modified by post-translational modifications such as acetylation, phosphorylation, or sulfation (3). Also RIAs require several months to develop an antiserum for a particular peptide and considerable time and expense to characterize the antiserum with a variety of peptides. Mass spectrometry provides an alternative approach to detect peptides, and when stable isotopic tags are used to label two different samples, the relative levels of a peptide in the two samples can be accurately determined (4, 5). Unlike RIAs, mass spectrometry is not limited to known peptides and also provides information regarding post-translational modifications (6–9).

A major drawback of mass spectrometry-based approaches for analysis of peptides has been the low levels of neuropeptides in brain and most other tissues relative to the levels of peptides that result from protein degradation. The pituitary has sufficient levels of peptide hormones that mass spectrometry-based techniques are capable of detecting these peptides above the protein degradation background (5, 10–15). We have previously used pituitary to optimize the isotopic labels for relative quantitation of peptides and to examine changes in peptides levels in mice with altered levels of the various peptide processing enzymes (5, 10–13). However, previous attempts to perform similar analysis with brain extracts did not detect any neuropeptides; all of the observed peptides were fragments of abundant proteins present in brain or blood. One solution to this problem was to use Cpefastfat mice and to affinity purify the neuropeptide process-

1 The abbreviations used are: RIA, radioimmunoassay; MSH, melanocyte-stimulating hormone; CLIP, corticotrophin-like intermediate lobe peptide; J-peptide, joining peptide; PAM, peptidylglycine α-amidating monooxygenase; PC1, prohormone convertase 1; PC2, prohormone convertase 2; TMAB, [3-(2,5-dioxopyrrolidin-1-yloxycarbon- yl)propyl]trimethylammonium chloride; kW, kilowatts; POMC, proopiomelanocortin; ACTH, adrenocorticotropic hormone.
ing intermediates containing C-terminal Lys and/or Arg residues on anhydrotryps in-agarose (16, 17). These mice lack the active form of carboxypeptidase E, one of the enzymes that process neuropeptides from their precursors, and as a result the Lys- and/or Arg-extended forms of these peptides accumulate (12, 18–20). Many protein degradation fragments were purified on this column and detected in the mass spectrometer, but a comparison of Cpefat/{fat} and wild type mice was performed, and all peptides unique to the Cpefat/{fat} mice were found to correspond to neuropeptides or other peptides generated from the processing of secretory pathway proteins. Although useful, this technique was limited to studies on Cpefat/{fat} mice.

Another approach is to block the postmortem degradation of proteins by sacrificing mice with focused microwave irradiation. Previous studies have found that microwave irradiation blocks the postmortem changes of labile molecules such as cyclic AMP, arachidonic acid, and phosphoproteins (21, 22). The basic principle is thermal inactivation of the enzymes in the tissue within seconds. The levels of neuropeptides recovered from brain are typically higher in animals sacrificed with microwave irradiation (23–25). Svensson et al. (9) were able to detect a number of neuropeptides using a mass spectrometry-based peptidomic approach with hypothalami from rats and mice sacrificed by microwave irradiation. However, all of these previous studies used focused microwave irradiation to sacrifice the animals, and there are two problems with this approach. First, the animals must be placed in a restraining device that is an extremely stressful stimulus that can cause changes in brain chemistry. Second, the microwave devices capable of focused irradiation are not widely available due to their high price (over $40,000 for a basic machine). In contrast, ordinary household type microwave ovens costs around $100 and are widely available. We hypothesized that the postmortem protein degradation seen in conventional studies did not occur immediately after decapitation but after the many seconds or minutes that it took to remove the brain from the skull and dissect the relevant regions. Therefore, we tested whether postmortem protein degradation could be prevented by sacrificing mice using a standard decapitation procedure and then immediately irradiating the head in a conventional microwave oven. In addition, we used differential isotopic labels to compare non-irradiated and irradiated brain and pituitary tissues and to examine whether a 20-s delay prior to the microwave treatment had a large effect on the recovery. The results of these analyses indicate that a conventional microwave oven is sufficient to prevent postmortem protein breakdown, thus enabling peptidomic studies to be performed on wild type mice.

**EXPERIMENTAL PROCEDURES**

**Reagents**—DMSO, disodium phosphate, phosphoric acid, sodium hydroxide, glycine, hydroxylamine, and formic acid were obtained from Aldrich Chemicals. TFA and acetonitrile were purchased from Fisher Scientific. Hydrochloric acid was sequanal grade (6 n, constant boiling) from Pierce. The reagents [3-(2,5-dioxopyrrolidin-1-yl)oxy-carbonyl]propyl(trimethylammonium chloride (TMAB) with either nine hydrogens (H9-TMAB) or nine deuteriums (d9-TMAB) were synthesized as described previously (6). Water was purified with a Milli-Q system (Millipore, Bedford, MA).

**Animals**—All procedures with live animals were approved by the appropriate committee at Albert Einstein College of Medicine, and the animals were obtained from our breeding colony within the barrier facility. Altogether four experiments were performed with different groups of mice. The purpose of the first experiment was to determine whether a conventional microwave oven could be used to rapidly raise the brain temperature of a mouse head following decapitation. For this experiment, 19 adult mice (C57BKS/) were divided into six groups (n = 3–4 per group) and sacrificed by decapitation. The head was placed in a conventional microwave oven (General Electric, 1.38 kW) for 5–13 s at full power. For consistent results, the head was always placed in the same location within the microwave oven. Immediately after microwaving, the temperature of the brain was recorded using a digital probe thermometer. Similar experiments were performed with two other microwave ovens of different power ratings, and these were also found to be capable of raising the brain temperature to >80 °C within 10 s (data not shown).

The second experiment involved 12 adult male mice (C57BKS/) divided into four groups (n = 3 each). For two of the groups, immediately after decapitation the head was microwaved for 5 s to raise the brain temperature to ~60 °C and then allowed to cool for 1 min before removal of the brain and pituitary. The groups that were not microwaved were processed without delay between sacrifice and dissection; in these mice, the pituitaries were typically removed within 1 min of death. For all four groups, the pituitaries were stored at −80 °C until processing as described below. The third experiment used 12 adult female mice (C57BKS/) divided into four groups (n = 3 each) and processed as above for the second experiment except that the tissue was microwaved for 8 s to raise the temperature to ~80 °C prior to removal of the brain, dissection of the hypothalamus, and removal of the pituitary. The fourth experiment used 18 adult female mice (C57BL/6) divided into four groups (n = 4–5). For two of the groups, the decapitated heads were immediately microwaved, whereas those in the other two groups were allowed to sit at room temperature for 20 s before being microwaved. After removal of the brain and pituitary, the brain was dissected into hypothalamus, hippocampus, and striatum. Tissues were stored at −80 °C until processing as described below.

**Peptide Extraction and Labeling**—Each of the experiments examining peptides involved four groups of mice so that duplicate LC/MS analyses could be performed (Fig. 1). These duplicate analyses had the hydrogen and deuterium labeling reversed to reduce the potential artifacts that would result if one of the labeling reagents was less reactive. For example, in some experiments the pool of three non-microwaved pituitaries was labeled with H9-TMAB, and the pool of three microwaved pituitaries was labeled with d9-TMAB; in the reverse labeling paradigm, the pool of three microwaved tissues was labeled with H9-TMAB, and the pool of three non-microwaved tissues was labeled with d9-TMAB. Peptides were extracted in an appropriate volume of 10 mM HCl (100 μl per pituitary; 300–400 μl per hypotha-
amis, striatum, or hippocampus) by sonicking two to three times for 5 s and incubation at 70 °C for 20 min. One hundred microliters of 0.2 M phosphate buffer, pH 9.5 were added, and the homogenate was centrifuged at 50,000 × g for 40 min at 4 °C. The pellet was resuspended in 200–300 µl of 0.2 M phosphate buffer, pH 9.5, and centrifuged again. The second supernatant was combined with the first supernatant, concentrated to 500–700 µl in a vacuum centrifuge, and combined with 200 µl of 0.4 M phosphate buffer, pH 9.5. The pH was adjusted to 9.5 with 1 M NaOH. For the samples that represented extracts of three pituitaries, 7.0 µl of 300 g/ml H_2-TMAB or d_5-TMAB in DMSO was added. After 10 min at room temperature, an appropriate volume of 1.0 M NaOH was added to the reaction mixture to adjust the pH back to 9.5, and the reaction was further incubated — 10 min. The addition of labeling reagent and base was repeated six times over 2 h, and the mixture was incubated at room temperature for another 2 h. The total amount of light or heavy label used was 5 mg per individual pituitary (i.e. 15 mg for the extract of three glands). For the other tissues, the volume and concentration of labeling reagent were adjusted so that the total amount of label used amounted to 4 mg per individual hypothalamus or striatum and 6 mg per individual hippocampus. After the pH was adjusted back to 9.5, 60–80 mg per individual hypothalamus or striatum and 6 mg per individual hippocampus were adjusted so that the total amount of label used amounted to 4 mg for the extract of three glands. The peptides were eluted from the C18 column with 80% acetonitrile, the eluate was concentrated to 20 ml.

RESULTS

Several different microwave ovens were tested, and each was able to rapidly raise the core temperature of a mouse head within several seconds. The weakest of the microwave ovens tested (1.38 kW) required 5 s to raise the temperature of the brain to 60 °C, and 80 °C was reproducibly reached after 8 s (Fig. 2). Longer times resulted in higher temperatures, but when heated to 90 °C or more, the brain appeared greatly altered in appearance, and much morphology was lost. In contrast, the brains heated to 60 or 80 °C were morphologically similar to unheated brain, although there was a clear change in tissue elasticity upon 60–80 °C heating. More powerful ovens were able to achieve these temperatures in shorter times, but there was greater variability among samples. Thus,

LC/MS/MS Analysis—LC and tandem mass spectrometry experiments were carried out on an API Q-Star Pulsar-i™ quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex) equipped with a nanoelectrospray ionization source. An UltiMate™ capillary/nano-LC system connected to a FAMOS microautosampler system was coupled to the mass spectrometer. The sample was loaded on a PepMap™ C18 precolumn (5 µm, 100 Å, 300-µm inner diameter × 5 mm, LC Packings) and desalted for 20 min with 2% acetonitrile and 0.1% formic acid in water. To remove the acetonitrile, the eluate was concentrated to 20 µl in a vacuum centrifuge, 80 µl of water was added, and the solution was concentrated again to 50 µl. Aliquots of sample were subjected to LC/MS/MS analysis as described below. LC/MS/MS Analysis—LC and tandem mass spectrometry experiments were carried out on an API Q-Star Pulsar-i™ quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex) equipped with a nanoelectrospray ionization source. An UltiMate™ capillary/nano-LC system connected to a FAMOS microautosampler system was coupled to the mass spectrometer. The sample was loaded on a PepMap™ C18 precolumn (5 µm, 100 Å, 300-µm inner diameter × 5 mm, LC Packings) and desalted for 20 min with 2% acetonitrile and 0.1% formic acid in water. To remove the acetonitrile, the eluate was concentrated to 20 µl in a vacuum centrifuge, 80 µl of water was added, and the solution was concentrated again to 50 µl. Aliquots of sample were subjected to LC/MS/MS analysis as described below.

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### Table I

**Analysis of pituitary peptides using isotopic labels and mass spectrometry**

Column 2 lists the common name for the observed peptide or the position within the precursor. Column 3 indicates amino acid sequences. The sequence bracketed by parentheses represents a predicted peptide based on the similarity to the mass of a known peptide. All other peptides were identified unambiguously by MS/MS. Column 4 indicates the number of TMAB labels observed on the peptide (No. T). Columns 5 and 6 indicate the observed monoisotopic molecular mass (in Da) (M obs) of the unprotonated peptide (after subtracting the mass of the tags) and the corresponding theoretical monoisotopic mass (in Da) (M theor) of the unprotonated peptide. Column 7 shows the difference (in parts per million) between the observed and theoretical masses. Columns 8 and 9 represent the ratio of the peak height of the peptide in extracts of tissues microwaved to 60 or 80 °C relative to the peak height observed in extracts from tissues without microwave irradiation. Column 10 (Delay/immed) indicates the ratio of the peak height of the peptide in extracts of tissues with 20-s delay prior to microwave irradiation relative to the peak height of the peptide in extracts of tissues with immediate microwave irradiation after decapitation. Avg, average value; dehydroS, dehydroserine; LPH, lipotropin; phosphoS, phosphoserine.

| Precursor Peptidome | Peptide name | Sequence from MS/MS (or predicted) | No. T | M obs | M theor | ppm | Avg ± range |
|---------------------|--------------|-----------------------------------|-------|-------|---------|-----|-------------|
| Chromogranin A      | AYGFRDPGQPL  |                                   | 1     | 1219.60 | 1219.60 | 4   | 0.42 ± 0.20 |
|                     | LDEGYKPV     |                                   | 1     | 928.43  | 928.43  | -   | 0.78 ± 0.13 |
| Chromogranin B      | LLDEGYYPV    |                                   | 1     | 1041.52 | 1041.51 | 9   | 0.24 ± 0.07 |
|                     | LLDEGYYPV    |                                   | 1     | 1910.94 | 1910.94 | 2   | 0.51 ± 0.17 |
| Chromogranin B      | GLQYRRGSSELRAPPR |                              | 2     | 2099.09 | 2009.08 | 5   | 0.80 ± 0.23 |
| Chromogranin B      | GLQYRRG-phosphoS-EEDRAPPR |                      | 2     | 2179.06 | 2179.05 | 3   | 0.72 ± 0.01 |
| Chromogranin B      | KFLASVTLSKVR |                                   | 3     | 1698.96 | 1698.97 | 8   | 0.81 ± 0.05 |
| Hemoglobin α        | LWP-hemorphin-7 |                              | 3     | 1332.72 | 1332.71 | 7   | 0.05 ± 0.01 |
| Proopiomelanocortin | FKNAIKNA     |                                   | 3     | 1017.60 | 1017.60 | 1   | 0.28 ± 0.13 |
| Proopiomelanocortin | FKNAIKNA     |                                   | 3     | 1154.67 | 1154.66 | 9   | 0.43 ± 0.18 |
| Proopiomelanocortin | KYMGHFRRWD  |                                   | 2     | 1337.66 | 1337.64 | 16  | 0.78 ± 0.45 |
| Proopiomelanocortin | AEEAVWGQ-dehydroS-PE |                     | 1     | 1356.59 | 1356.57 | 15  | 0.84 ± 0.08 |
| Proopiomelanocortin | Ac-SMEHFRWKPV-amide |                     | 1     | 1413.72 | 1413.71 | 8   | 1.37 ± 0.50 |
| Proopiomelanocortin | Ac-SMEHFRWKPV-amide |                     | 1     | 1455.77 | 1455.72 | 33  | 0.83 ± 0.08 |
| Proopiomelanocortin | RPVKYPNAENE  |                                   | 2     | 1513.81 | 1513.79 | 14  | 1.29 ± 0.15 |
| Proopiomelanocortin | Ac-SMEHFRWKPV-amide |                     | 1     | 1540.69 | 1540.65 | 24  | 0.81 ± 0.11 |
| Proopiomelanocortin | Ac-SMEHFRWKPV-amide |                     | 1     | 1576.79 | 1576.77 | 11  | 0.89 ± 0.27 |
| Proopiomelanocortin | Ac-SMEHFRWKPV-amide |                     | 1     | 1621.79 | 1621.79 | 2   | 0.71 ± 0.32 |
| Proopiomelanocortin | Ac-SMEHFRWKPV-amide |                     | 1     | 1645.80 | 1645.79 | 3   | 0.89 ± 0.26 |
| Proopiomelanocortin | Ac-SYSMEHFRWKPV-amide |                   | 1     | 1663.80 | 1663.80 | 0   | 1.04 ± 0.47 |
| Proopiomelanocortin | Ac-SYSMEHFRWKPV-amide |                   | 1     | 1705.81 | 1705.81 | 2   | 1.05 ± 0.59 |
| Proopiomelanocortin | Ac-SYSMEHFRWKPV-amide |                   | 1     | 1720.83 | 1720.82 | 2   | 0.95 ± 0.31 |
| Proopiomelanocortin | Ac-YGGFMTEKSGPTVLTV |                  | 1     | 1786.84 | 1786.85 | 4   | 0.13 ± 0.02 |
| Proopiomelanocortin | EAEAVWGQGSSPESPR |                   | 2     | 1811.80 | 1811.80 | 2   | 1.59 ± 0.42 |
| Proopiomelanocortin | EAEAVWGQGSSPESPES-ami |                 | 2     | 1868.84 | 1868.82 | 13  | 1.23 ± 0.04 |
| Proopiomelanocortin | Ac-YGGFMTEKSGPTVLTV |                 | 2     | 1899.89 | 1899.84 | 26  | 0.64 ± 0.31 |
| Proopiomelanocortin | Ac-YGGFMTEKSGPTVLTV |                 | 2     | 1939.86 | 1939.87 | 5   | 0.82 ± 0.23 |
| Proopiomelanocortin | Ac-YGGFMTEKSGPTVLTV |                 | 2     | 1983.97 | 1983.98 | 4   | 0.65 ± 0.03 |
| Proopiomelanocortin | EAEAVWGQGSSPESPES-ami |                 | 1     | 2095.99 | 2095.96 | 14  | 0.87 ± 0.15 |
| Proopiomelanocortin | RPVKYPNAEINESAEAFPL |                | 2     | 2229.14 | 2229.15 | 4   | 0.05 ± 0.01 |
| Proopiomelanocortin | RPVKYPNAEINESAEAFPL |                | 2     | 2305.01 | 2304.99 | 6   | 1.02 ± 0.22 |
| Proopiomelanocortin | RPVKYPNAEINESAEAFPL |                | 2     | 2309.18 | 2309.12 | 24  | 0.05 ± 0.38 |
| Proopiomelanocortin | RPVKYPNAEINESAEAFPL |                | 2     | 2358.17 | 2358.19 | 6   | 0.27 ± 0.02 |
| Proopiomelanocortin | ELEGERPLGEQVEDAEKDD |              | 2     | 2470.20 | 2470.18 | 9   | 0.85 ± 0.22 |
| Proopiomelanocortin | RPVKYPNAEINESAEAFPL |                | 2     | 2505.26 | 2505.26 | 0   | 0.93 ± 0.13 |
| Proopiomelanocortin | RPVKYPNAEINESAEAFPL |                | 2     | 2585.19 | 2585.22 | 12  | 1.43 ± 0.20 |
| Proopiomelanocortin | Ac-YGGFMTEKSGPTVLTFKNAIKNA |        | 3     | 2899.60 | 2899.51 | 31  | 1.05 ± 0.15 |
| Proopiomelanocortin | Ac-YGGFMTEKSGPTVLTFKNAIKNA |       | 3     | 3036.58 | 3036.58 | 0   | 1.12 ± 0.44 |
| Proopiomelanocortin | LEQVLEDAEKDDGPYRHEFRRWSNPKDK |         | 2     | 3455.65 | 3455.64 | 2   | 0.92 ± 0.28 |
| Proopiomelanocortin | LEQVLEDAEKDDGPYRHEFRRWSNPKDK |         | 3     | 4436.14 | 4436.14 | 0   | 0.92 ± 0.22 |
| Proopiomelanocortin | LEQVLEDAEKDDGPYRHEFRRWSNPKDK |         | 5     | 4579.37 | 4579.33 | 9   | 0.95 ± 0.11 |
| Proopiomelanocortin | LEQVLEDAEKDDGPYRHEFRRWSNPKDK |         | 5     | 4659.33 | 4659.33 | 1   | 1.00 ± 0.07 |
**TABLE I** — continued

| Peptide name | Sequence from MS/MS (or predicted) | No. T | M obs | M theor | ppm | Delay/immed |
|--------------|------------------------------------|------|-------|---------|-----|-------------|
| **60 °C/control** | **80 °C/control** |**Delay/immed** |
| Oxytocin | CYIQNCPLG-amide | 1 | 1006.45 | 1006.44 | 5 | 0.68 | 0.22 | 0.76 |
| ProSAAS | GEAAGAVQELARALAHLLEAERQE | 1 | 2531.30 | 2531.31 | 33 | 1.20 | 0.39 | 0.79 |
| ProSAAS | AVPRGEAAGAVQELARALAHLLEAERQE | 1 | 2954.48 | 2954.58 | 32 | 1.16 | 0.25 | 0.97 |
| Provasopressin | GFFRLT | 1 | 739.40 | 739.40 | 4 | 0.85 | 0.19 | 1.00 |
| Provasopressin | CYFQNCPRG-amide | 1 | 1083.45 | 1083.44 | 6 | 0.71 | 0.21 | 0.97 |
| Provasopressin | AGTRESVDSAKPRVY | 2 | 1634.83 | 1634.84 | 6 | 0.69 | 0.07 | 1.00 |
| Provasopressin | VQLAGTRESVDSAKPRVY | 2 | 1975.06 | 1975.06 | 1 | 0.41 | 0.07 | 1.25 |
| Secretogranin II | QELGKLTGPSNQ | 2 | 1270.65 | 1270.65 | 3 | 0.17 | 0.06 | 1.25 |
| Unidentified | | 1 | 1116.50 | | | | |
| Unidentified | | 2 | 1215.56 | | | | |
| Unidentified | | 1 | 1410.66 | | | | |
| Unidentified | | 5 | 1595.77 | | | | |
| Unidentified | | 2 | 1676.78 | | | | |
| Unidentified | | 1 | 1678.82 | | | | |

It was optimal to reduce the power setting to produce a more uniform response as found for the weaker oven. For example, a 1.6-kW oven at power level 5 for 5 s reached a brain temperature of 60 °C, and at power level 6 for 6 s reached 85 °C (data not shown).

To test whether the microwave treatment affected the levels of peptides in pituitary, microwave-heated and non-microwave pituitaries were extracted, labeled with H$_{2}$- or D$_{2}$-TMAB, and analyzed by LC/MS. Some of the peptides detected in this analysis were substantially lower in the samples that had been heated in the microwave oven (Table I). For example, a peptide subsequently identified by MS/MS analysis as β-endorphin-(18–26) was reduced 2–3-fold by the microwave treatment (Fig. 3A). Other peptides greatly reduced by the microwave treatment include a chromogranin B fragment of 1041.51 Da, β-endorphin-(18–27), a secretogranin II fragment of 1270.65 Da, the β-hemoglobin fragment LVV-hemorphin-7, acetyl-α-endorphin, and several fragments of corticotrophin-like intermediate lobe peptide (CLIP) of 2229.15, 2309.12, and 2358.19 Da (Table I). However, many other peptides were not substantially altered by the treatment such as joining peptide (J-peptide), α-melanocyte-stimulating hormone (α-MSH), diacetyl-α-MSH, CLIP, acetyl-β-endorphin-(1–26) and acetyl-β-endorphin-(1–27), and several other peptides (Fig. 3B and Table I). In general, comparable results were obtained for tissue heated to 60 and 80 °C, although the variability in the 60 °C pituitary sample was slightly greater than the variability observed at 80 °C (Table I). Because some enzymes may only be partially inhibited by heating to 60 °C for a short time, it is possible that some of the variability at this lower temperature was caused by incomplete inactivation of the proteases. Therefore, further experiments used only 80 °C.

To test whether small variations in the length of time between the decapitation and the microwave irradiation affected the peptide levels, a 20-s delay was introduced between the time of decapitation and the start of the microwave. These mice were compared with a similar group of mice that were immediately microwaved after decapitation. If postmortem changes occur in the first 20 s after decapitation, then the delay should lead to higher levels of the postmortem fragments and lower levels of the undegraded peptides. However, none of the pituitary peptides detected in this study changed more than 20% in both replicates (Table I, right column). A few peptides showed 30–50% decreases in one of the analyses (Table I), but these peptides were not detected in the duplicate run, and so it was not possible to comment on the reproducibility of these few changes. The finding that some of the peptides (i.e. the 2229.14- and 2358.17-Da CLIP fragments) that were markedly decreased by the microwave irradiation were only slightly affected by the delay in the microwave procedure suggests that these peptides are normally present in the pituitary (although at lower levels than found when non-microwaved samples are used).
During this analysis of pituitary, 49 peptides were identified by MS/MS sequence analysis, and most of these corresponded to previously reported pituitary peptides. One peptide that has not been previously reported is the proopiomelanocortin-derived J-peptide with a novel post-translational modification that adds 365 Da to its mass. The b- and y-series fragment ions clearly match those of the unmodified J-peptide, indicating the modification is labile. Some of the y-series ions (y5 and higher) also are detected with a mass 365 Da heavier, suggesting that the modification is located on Ser15 (Fig. 4). The addition of 365 Da corresponds to Hex-HexNAc where Hex is a six-carbon sugar (mannose, glucose, etc.) and NAc refers to an acetyl-2-amino group (27–29). Signature fragments of Hex-HexNAc are detected at m/z of 186, 204, and 366 (Fig. 4), confirming the assignment (27–29). In addition to the 49 peptides identified in this study by MS/MS sequence analysis another seven were detected; one of these had a mass that was within 1 ppm of the theoretical mass of a known mouse pituitary peptide (phosphorylated ACTH) but that did not have sufficient MS/MS data in the present study to provide an unambiguous assignment; this peptide is indicated in Table I with parentheses surrounding the sequence. Six additional peptides could not be matched to any known pituitary peptide and are listed in Table I as unidentified.

Previous studies on brain extracts prepared from non-microwaved samples showed a large number of protein fragments to be present in the <10-kDa microfiltrate. The quantitative isotopic labeling method was used to further investigate the formation of the protein fragments. Unlike the pituitary samples, which showed a large number of equal intensity peak pairs corresponding to hydrogen- and deuterium-labeled peptides, nearly all of the peptides in hypothalamic extracts were considerably more abundant in the non-microwaved control sample (Fig. 5A). In most cases, the level of the peptide in the microwaved sample was only a few percent of the level of the peptide in the non-microwaved sample; in these cases, it was necessary to compare MS/MS spectra from the two separate runs labeled in reverse order of each other to be sure that these ions represented the same peptide. In all cases, the peptides that were greatly decreased by the microwave treatment were found by MS/MS analysis to be protein fragments (Table II). Altogether 35 peptides that greatly decreased upon microwave treatment were identified from MS/MS sequence analysis. Twenty-five of these peptides corresponded to hemoglobin, either the α subunit (13 peptides) or the β subunit (12 peptides), three peptides matched fragments of the excitatory amino acid transporter EEAT2, two peptides matched malate dehydrogenase, and the other five peptides corresponded to five additional proteins (Table II). In most cases, the levels of the peptide in the microwave-irradiated samples were so low that the precise ratio could not be calculated and was expressed instead as an upper limit based on the signal to noise ratio (Table II).

In addition to the 35 peptides identified by MS/MS sequencing and that showed a dramatic decrease upon microwave irradiation, hundreds of additional peptides were detected that also showed a dramatic decrease upon microwaving; these were not identified. However, a small number of peptides were found that showed the opposite change: an increase upon microwave treatment (Fig. 5B). Five of these peptides that increased upon microwaving were identified from the MS/MS analysis, and in all cases they represented peptides previously found to be produced in the secretory pathway and that were proposed to be neuropeptides. These include four proSAAS-derived peptides and one product of chromogranin B (Table II). All of these five peptides result from cleavages at prohormone convertase consensus sites (KR, RR, and RXXR) or other sites commonly used in prohormone processing (PR) followed by carboxypeptidase action to remove the C-terminal basic residues.

Due to the large number of protein degradation fragments in the non-microwaved extract, it was not possible to detect more than the small number of neuropeptides reported in
Table II. To avoid this problem and to examine the effect of postmortem delay on the peptide levels, we compared H9- and d9-TMAB labeled samples from sets of animals that had either been microwaved immediately after decapitation or microwaved after a 20-s delay. As with the other experiments, this analysis was performed with two separate pools of animals, one labeled in the “normal” direction and the other labeled in the “reverse” direction (see Fig. 1). Rather than average the duplicate analyses, as done in the other experiments presented in Tables I and II, the data for each separate run are shown in Table III; this was done because some of the peptides show a large difference in one of the runs but not the other (discussed further below). In these analyses, a large number of peptides were detected of which 65 were identified from MS/MS sequencing, and another four were tentatively identified based on a match to the mass of a known brain peptide or protein fragment and in some cases partial MS/MS data. A large number of other peptides were detected but not identified in the present study; 22 of the most abundant of these were quantified along with those peptides that were identified (Table III). The majority of the identified peptides correspond to either known neuropeptides or peptides derived from other proteins known to be present in peptide-containing secretory vesicles. Examples of this latter category include fragments of the enzymes peptidylglycine α-amidating monoxygenase (PAM), prohormone convertase 1 (PC1), and prohormone convertase 2 (PC2). The peptide from PAM corresponds to the N-terminal pro region, which is known to be cleaved from PAM at the Lys-Arg site immediately downstream of the 951.53-Da peptide that was detected (Table III). The PC2 fragment also corresponds to a region of the pro peptide of this enzyme and is released by cleavage at Pro-Arg and Arg-Lys-Lys-Arg sequences followed by removal of the C-terminal basic residues by a carboxypeptidase. The PC1 fragment corresponds to a region near the C terminus of the protein (residues 619–628) and represents cleavage at Arg-Arg and Lys-Arg sequences. All of these cleavages are likely to occur within the secretory pathway, and so these peptides are likely to exist in vivo prior to any postmortem degradation. Furthermore the levels of these peptides either do not change
or decrease during the 20-s postmortem delay, indicating that they are not formed during the postmortem period. Nearly all of the known neuropeptides that were detected in the present study were either not affected by the delay in the microwave treatment or showed a decrease in levels, consistent with the idea of postmortem degradation of these peptides (Fig. 6 and Table III). In a few cases, the apparent levels of neuropeptides were elevated by the postmortem delay in one but not both of the duplicate runs. However, rather than interpret this as postmortem formation of the peptide, it is possible that this is due to variability in the tissue dissections (see “Discussion”).

In addition to the 41 neuropeptides or secretory vesicle protein fragments identified in the present study (and three additional tentatively identified peptides), 24 peptides were found that resulted from protein cleavages (Table III). In the majority of these cases the peptide that was detected corresponded to either the N or C terminus of the protein. Furthermore, the cleavage that formed these peptides occurred at an Asp residue with Asp-Pro representing 40% of the cleavage sites (Table III). Asp-Xaa and especially Asp-Pro bonds are sensitive to acid; therefore, these protein fragments may have been produced during the extraction in 10 mM HCl. It is unlikely that these protein fragments were produced during the postmortem period because their levels did not consistently increase during the 20-s postmortem delay (Table III).

Several of the peptides that corresponded to protein degradation fragments showed b- and/or y-series that clearly matched a known protein, but the mass of the observed peptide was larger than that of the known protein fragment. For example, a 1688.88-Da fragment of β-tubulin was found with the same b-series as a 1280.68-Da β-tubulin fragment, indicating that the N-terminal region of the two peptides was identical (Fig. 7). The additional mass (408.10 Da) precisely matches the C-terminal extension of the peptide by a Cys residue (103.01 Da) and the addition of glutathione (305.09 Da) on this Cys residue. Two N-terminal fragments of vesicle-associated membrane protein-associated protein (VAP-33, also known as VAMP-A and VAP-A) were detected (Table III). Whereas the 1206.66-Da fragment represents the peptide with the N-terminal Met removed and the second residue acetylated, the 1781.94-Da peptide represents the N-terminal fragment resulting from an upstream ATG initiation site followed by removal of the Met and acetylation.

**DISCUSSION**

Neuropeptides are known to undergo degradation during the postmortem period (30). A number of previous studies have found that sacrificing animals with microwave irradiation led to higher yields of labile molecules, including peptides (21–25) and if not available, they are so inexpensive that this is not a factor. In contrast, the high cost of the focused microwave irradiation device has limited the availability of this procedure. Second, the focused irradiation device requires placing the mouse in a restraining device prior to sacrifice, and this causes immobilization stress in the animal. In contrast, the technique used in the present study involved standard decapitation to sacrifice the animals, which was immediately followed by the microwave irradiation. Although this introduced a small delay of several seconds before the brain was heated to 80°C, this short delay did not appear to contribute to increase in pituitary peptides (Tables I and II). Thus, it appears that pituitary peptides are more stable to postmortem degradation than hypothalamic peptides.

In addition to the effect of the microwave irradiation on peptide levels, the treatment has a much more profound effect on protein degradation. Our finding, which is in agreement with a previous study on rodent hypothalamus (9), extends this previous study to additional brain regions. More importantly, the present study was done using a conventional microwave oven, which has several advantages over the focused microwave device used in previous studies. First, conventional microwave ovens are common in many laboratories, and if not available, they are so inexpensive that this is not a factor. In contrast, the high cost of the focused microwave irradiation device has limited the availability of this procedure. Second, the focused irradiation device requires placing the mouse in a restraining device prior to sacrifice, and this causes immobilization stress in the animal. In contrast, the technique used in the present study involved standard decapitation to sacrifice the animals, which was immediately followed by the microwave irradiation. Although this introduced a small delay of several seconds before the brain was heated to 80°C, this short delay did not appear to contribute to
Analysis of hypothalamic peptides using isotopic labels and mass spectrometry: effect of microwave irradiation on relative levels of peptides

For abbreviations, see Table I legend.

| Precursor | Sequence from MS/MS | No. | T | M obs | M theor | ppm | 80 °C/control |
|-----------|---------------------|-----|---|-------|---------|-----|---------------|
| Hemoglobin α | VDPVFKLHSLH | 2 | 1267.68 | 1267.69 | −8 | 0.12 ± 0.04 |
| Hemoglobin α | KIGGGHAEYGEALERMF | 2 | 1934.99 | 1934.93 | 31 | <0.05 |
| Hemoglobin α | KIGGGHAEYGEALERMF | 2 | 2006.06 | 2005.98 | 40 | <0.05 |
| Hemoglobin α | LASHHPADTPAVHASLKD | 2 | 2013.02 | 2013.01 | 5 | <0.05 |
| Hemoglobin α | GIGGHHGAEGYAEALERMF | 2 | 2063.05 | 2062.99 | 29 | <0.05 |
| Hemoglobin α | LASHHPADTPAVHASLKD | 2 | 2160.07 | 2160.07 | 0 | <0.05 |
| Hemoglobin α | AAWKGIGGGAEGYAEERALM | 2 | 2173.06 | 2173.04 | 9 | <0.05 |
| Hemoglobin α | VTLASHHPADTPAVHASLKD | 2 | 2213.18 | 2213.12 | 27 | <0.05 |
| Hemoglobin α | LASHHPADTPAVHASLKD | 2 | 2273.16 | 2273.16 | 0 | <0.05 |
| Hemoglobin α | LASHHPADTPAVHASLKD | 2 | 2326.21 | 2326.21 | 0 | <0.05 |
| Hemoglobin α | VTLASHHPADTPAVHASLKD | 2 | 2360.17 | 2360.19 | −8 | <0.05 |
| Hemoglobin α | LASHHPADTPAVHASLKD | 2 | 2473.28 | 2473.28 | 0 | <0.05 |
| Hemoglobin α | LASHHPADTPAVHASLKD | 2 | 2586.34 | 2586.36 | −8 | <0.05 |
| Hemoglobin β | VEPENFRLLY | 1 | 1158.59 | 1158.60 | −9 | 0.12 ± 0.11 |
| Hemoglobin β | ADEVGGEALGRLL | 1 | 1298.68 | 1298.68 | 0 | <0.05 |
| Hemoglobin β | LIVVWCTRY | 1 | 1323.70 | 1323.70 | 0 | <0.11 ± 0.02 |
| Hemoglobin β | AFNDGLNHLDSLK | 2 | 1442.69 | 1442.71 | 14 | <0.05 |
| Hemoglobin β | VYPWTCTRYFD | 1 | 1472.70 | 1472.71 | −7 | <0.05 |
| Hemoglobin β | KVAGVAAALAKHY | 3 | 1533.87 | 1533.88 | −7 | <0.05 |
| Hemoglobin β | VHLDEAASKAVSGLW | 2 | 1652.85 | 1652.85 | 0 | <0.05 |
| Hemoglobin β | KNADEVGGEALRLLV | 2 | 1738.98 | 1738.96 | 12 | <0.05 |
| Hemoglobin β | AFNDGLNHLDSLKGF | 2 | 1747.84 | 1747.85 | −6 | <0.05 |
| Hemoglobin β | FOQVAVAAALAKHY | 3 | 1808.94 | 1809.00 | −33 | 0.08 ± 0.02 |
| Hemoglobin β | FDSFQGLSASAIMGNAKV | 3 | 2043.97 | 2043.99 | −10 | <0.05 |
| Cytochrome c oxidase | EDEVGEGALRRLLVYVPTQRY | 1 | 2491.24 | 2491.29 | −20 | <0.05 |
| Excitative amino acid transporter EEA2 | NHFHBNPPTGYEO | 1 | 1580.69 | 1580.71 | −13 | <0.05 |
| Excitative amino acid transporter EEA2 | LIIHGGIF | 1 | 868.51 | 868.52 | −12 | <0.05 |
| Excitative amino acid transporter EEA2 | LILPPLSSL | 1 | 1080.70 | 1080.72 | −19 | 0.08 ± 0.01 |
| Glutamate/aspartate transporter | IAFFGDL | 1 | 844.44 | 844.47 | −36 | <0.05 |
| Histone 1 | IRNMFPPNLVE | 1 | 1328.71 | 1328.69 | 15 | 0.11 ± 0.02 |
| Malate dehydrogenase | AAVEYLEL | 1 | 777.40 | 777.43 | −39 | <0.05 |
| Malate dehydrogenase | VAVLASHGIGQPLSLLOCK | 2 | 1792.04 | 1792.08 | −22 | <0.05 |
| Malate dehydrogenase | VAVLASHGIGQPLSLLOCKNPLVSR | 2 | 2545.55 | 2545.50 | 12 | <0.05 |
| Peptidyl-prolyl isomerase | FDTADDEPLGRSVFEL | 1 | 1922.93 | 1922.93 | 0 | <0.05 |
| Reticulin 1 | LVQLVDSKFL | 2 | 1275.70 | 1275.71 | −8 | <0.05 |
| Chromogranin B fragment | LGALFNPYDFDPLQWKNDSFE | 2 | 2400.13 | 2400.14 | −4 | 2.13 ± 0.27 |
| ProSAAS (little SAAS) | SLSAASAPLVTSTPRIL | 1 | 1811.98 | 1812.00 | −11 | 3.25 ± 0.24 |
| ProSAAS (PEN) | SVQOLQPVEPPENLQALLR | 1 | 2316.21 | 2316.23 | −9 | 2.84 ± 0.05 |
| ProSAAS fragment | GEAGAVEOELRHALLEAERO | 1 | 2531.33 | 2531.31 | 8 | 1.88 ± 0.06 |
| ProSAAS fragment | AVPGEAGAVEOELRHALLEAERO | 1 | 2954.55 | 2954.57 | −7 | 1.87 ± 0.11 |
| Thymosin β-4 | ACSDKPMMAIEFDFKSKLKTQEQN | 9 | 4963.49 | 4963.54 | −10 | 4.17 ± 1.61 |

Quantitative Peptidomics in Mouse Tissues
## Table III

**Analysis of hypothalamic, striatal, and hippocampal peptides using isotopic labels and mass spectrometry: effect of a 20-s delay prior to the microwave irradiation on relative levels of peptides**

All masses are monoisotopic except the mass of thymosin β-4, which is the average mass. Amino acid sequences with parentheses represent predicted peptides based on the similarity of the mass to that of a known peptide. **Hypo**, hypothalamus; **Hippo**, hippocampus; **NL**, normal labeling scheme (the extracts from immediately microwaved tissues were labeled with light label, and the extracts from the delayed microwaved tissues were labeled with heavy label); **RL**, reverse labeling scheme (the extracts from the immediately microwaved tissues were labeled with heavy label, and the extracts from the immediately microwaved tissues were labeled with light label); **GSH**, glutathione; **Mox**, oxidized Met; **ND**, not detected; **PO**, peak overlap (could not accurately quantify due to another co-eluting peptide with similar m/z). Other abbreviations are the same as in Table I legend.

| Precursor | Peptide name | Sequence from MS/MS (or predicted) | No. T | Delay/immediate microwave irradiation |
|-----------|--------------|------------------------------------|-------|---------------------------------------|
|           |              |                                    |       | Hypos | Hippo | Striatum |
|           |              |                                    |       | NL   | RL   | NL   | RL   |
|           |              |                                    |       | PO   | PO   |
| Chromogranin A | 391–402 | AYGFRDPGQOL | 1 | 1219.60 | 1219.60 | 1 | 0.85 | 0.84 | 0.87 | 1.21 | 0.88 | 0.75 |
| Chromogranin B | 438–446 | LLDEGHYPV | 1 | 1041.51 | 1041.51 | 3 | 0.98 | 0.93 | 0.81 | 1.18 | 0.85 | 0.70 |
| Chromogranin B | 570–578 | (IWWRRPSFEDNQWSYGE) | 2 | 2155.01 | 2154.96 | 21 | 0.88 | 1.14 | 0.77 | 0.64 | 0.91 | 1.11 |
| Chromogranin B | 516–535 | LQALNFYPFDQLOWKNSDFE | 2 | 2400.15 | 2400.14 | 2 | 0.90 | 0.98 | 0.91 | 1.20 | 0.93 | 0.83 |
| Preprotachykinin A | Neurokinin A | HKTDSFVGLM-amide | 2 | 1132.56 | 1132.58 | 18 | 1.08 | 1.50 | ND | ND | 1.00 | 0.98 |
| Preprotachykinin B | Substance P | RPFPQQFFGLM-amide | 2 | 1366.75 | 1364.76 | 12 | 1.05 | 0.98 | ND | ND | 1.13 | PO |
| Preprotachykinin A | C-terminal flanking peptide | ALNSVAYERSAMQNYE | 1 | 1845.85 | 1844.84 | 1 | 0.91 | 1.31 | ND | ND | 1.04 | 0.85 |
| Preprotachykinin B | Neurokinin B | DMHDFVGLM-amide | 1 | 2099.54 | 2099.52 | 21 | 1.03 | 0.63 | ND | ND | ND | ND |
| Proenkephalin | Heptapeptide | YGGFMRF | 1 | 876.40 | 876.40 | 2 | 0.76 | 0.70 | 3.33 | 0.57 | 0.95 | 0.42 |
| Proenkephalin | Met-oxidized | YGGF-Mox-RF | 1 | 892.40 | 892.40 | 5 | ND | PO | PO | ND | 1.01 | 0.46 |
| Proenkephalin | Phosphorylated | FAESLPSDEEGENYSKEVPEIE | 2 | 2346.95 | 2347.10 | 1 | 0.81 | 0.99 | ND | ND | 0.83 | 0.54 |
| Proenkephalin | CLIP | RPVKVYPNVAENESAEAEFLF | 2 | 2585.23 | 2585.23 | 9 | 0.43 | 0.39 | 1.7 | 0.85 | 1.00 | 0.54 |
| Proenkephalin | Phosphorylated | RPVKVYPNVAENESAEAEFLF | 2 | 2278.63 | 2278.64 | 12 | 1.01 | 0.92 | ND | ND | 0.86 | 0.96 |
| Proenkephalin | J-peptide | AEEEAVWGDGSPEPSPRE-amide | 1 | 1939.86 | 1939.86 | 2 | 0.57 | 0.41 | ND | ND | ND | ND |
| Proenkephalin | CLIP | RPVKVYPNVAENESAEAEFLF | 2 | 2585.23 | 2585.23 | 9 | 0.43 | 0.39 | 1.7 | 0.85 | 1.00 | 0.54 |
| Proenkephalin | CLIP | RPVKVYPNVAENESAEAEFLF | 2 | 2585.23 | 2585.23 | 9 | 0.43 | 0.39 | 1.7 | 0.85 | 1.00 | 0.54 |
| Proenkephalin | PEN | SVDQDLGPEVPPENVLGALLRV | 2 | 2316.23 | 2316.23 | 2 | 0.85 | 0.75 | 1.33 | 0.97 | 1.10 | 0.54 |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Proenkephalin | ProSAAS | ASAPVQLESDAEKDDGPYRVEHFRWSNPPKD | 2 | 3455.64 | 3455.64 | 0 | 0.68 | 0.54 | ND | ND | ND | ND |
| Peptide name                                      | Sequence from MS/MS (or predicted) | Delay/immediate microwave irradiation | HIPP | HIPPO | STRIATUM | Daily/Immediate microwave irradiation | Daily/Immediate microwave irradiation |
|--------------------------------------------------|-----------------------------------|--------------------------------------|------|-------|---------|--------------------------------------|--------------------------------------|
| Secretogranin II                                  | 300–316 ESKDQLSEDASKVITYL         | No. T                                 | 3    | 1924.96 | 1924.96 | 2                                  | 1.03                                  |
| Aspartate transaminase N-terminal fragment        | SSWWTHVEMGPPD                    | No. T                                 | 1    | 1527.65 | 1527.67 | 14                               | 0.81                                  |
| ATP synthase, /H9251 -subunit C-terminal fragment | AKLKEIVTNFLAGFEP                 | No. T                                 | 3    | 1775.98 | 1775.98 | 7                                  | 1.07                                  |
| ATP synthase, subunit F C-terminal fragment       | PKFEVIDKPQS                      | No. T                                 | 3    | 1286.69 | 1286.68 | 12                               | 0.57                                  |
| Dihydrolipoamide S- succinyltransferase C-terminal fragment | PRVLLLDL | No. T                                 | 1    | 937.60  | 937.60  | 2                                  | 1.10                                  |
| ES1 C-terminal fragment                            | GIGAMVKNVLELTGK                  | No. T                                 | 3    | 1528.89 | 1528.86 | 20                               | 0.94                                  |
| Hemoglobin /H9251 C-terminal fragment             | KFLASVSTVLTSKYR                  | No. T                                 | 3    | 1699.00 | 1698.97 | 22                               | 0.74                                  |
| Hemoglobin /H9251 Internal fragment              | LPGALSALSDLHAHKLRVD               | No. T                                 | 2    | 2012.12 | 2012.12 | 1                                  | 1.09                                  |
| Hemoglobin /H9251 C-terminal fragment             | (FTPAVHASLDKFLASVSTVLTSKYR)       | No. T                                 | 8    | 2737.49 | 2737.49 | 8                                  | 0.97                                  |
| Microtubule-associated protein tau                | SPQLATLADEVSASLAKQGL             | No. T                                 | 2    | 1998.07 | 1998.07 | 3                                  | 0.92                                  |
| NADH dehydrogenase C-terminal fragment            | PTKEPEPVVHYDI                    | No. T                                 | 2    | 1522.78 | 1522.77 | 1                                  | 0.96                                  |
| Peptidyl-prolyl isomerase Internal fragment        | EDENFILKHTGPGILSM                | No. T                                 | 2    | 1899.95 | 1899.95 | 1                                  | 0.93                                  |
| Seryl tRNA synthetase N-terminal fragment         | VLDLDLFRVDKGGD                   | No. T                                 | 2    | 1560.82 | 1560.82 | 2                                  | 1.00                                  |
| Synaptosomal associated protein                   | PLADLNIKDFL                      | No. T                                 | 3    | 1257.70 | 1257.70 | 3                                  | 0.87                                  |
| Tubulin /H9252 (isoforms 2–6) N-terminal fragment | MREIVH(I/L)QAGQC                 | No. T                                 | 2    | 1688.78 | 1688.76 | 11                               | 1.31                                  |
| Thymosin /H9252 -4 Entire protein except for N-terminal | Ac-SDKPDMAEIEKFDKSKLKKTETQEKNPL | No. T                                 | 9    | 4963.63 | 4963.54 | 18                               | 0.90                                  |
| Unidentified                                     | 3                                 | No. T                                 | 1    | 1272.65 | 1272.65 | ND                                | 0.90                                  |
| Unidentified                                     | 2                                 | No. T                                 | 2    | 1434.67 | 1434.67 | ND                                | 1.36                                  |
| Unidentified                                     | 1                                 | No. T                                 | 3    | 1480.69 | 1480.69 | 11                               | 0.97                                  |
| Unidentified                                     | 2                                 | No. T                                 | 2    | 1494.85 | 1494.85 | ND                                | 1.22                                  |
| Unidentified                                     | 1                                 | No. T                                 | 3    | 1505.75 | 1505.75 | ND                                | 0.82                                  |
| Unidentified                                     | 3                                 | No. T                                 | 1    | 1527.65 | 1527.65 | ND                                | 1.12                                  |
| Unidentified                                     | 2                                 | No. T                                 | 2    | 1647.89 | 1647.89 | ND                                | 0.64                                  |
Although the microwave irradiation greatly reduced the levels of most protein degradation fragments, a number of these fragments were still observed in the present study (Table III). However, these fragments were not the major ones observed without microwaving (Table II). Instead the fragments detected after microwave treatment generally corresponded to either the N- or C-terminal region of the protein after cleavage at an Asp residue. The Asp-Xaa bond is more acid-labile than others, and Asp-Pro bonds are especially sensitive to acid (31). These cleavages may have occurred during the peptide extraction when the tissue was sonicated in 10 mM HCl and heated to 70 °C for 20 min. Alternatively it is possible that the cleavage at Asp-Xaa sites occurred during the microwave heating. It is also conceivable that these cleavages occurred normally in brain, prior to death, and are not a postmortem or extraction artifact. Further studies are needed to address these possibilities.

In addition to greatly reducing protein degradation and elevating brain peptide levels, the microwave treatment also showed another effect: a partial reduction in the levels of some pituitary peptides (Table I). However, many pituitary peptides were not affected by the microwave irradiation. In general, those pituitary peptides that required processing only at conventional sites (KR and RR) were not dramatically affected by the microwave treatment. The formation of these peptides requires the action of one or more prohormone convertases, carboxypeptidases, and in some cases, the amidation enzyme peptidylglycine α-amidating monoxygenase. In contrast, the peptides that were affected by the microwave irradiation required processing at additional sites. For example, the peptides α- and γ-endorphin correspond to the N-terminal portion of β-endorphin and require cleavage between Thr-Leu for α-endorphin and Leu-Phe for γ-endorphin. These peptides as well as the C-terminal portion of β-endorphin produced by cleavage at the Leu-Phe site (i.e., β-endorphin-(18–26) and β-endorphin-(18–27)) were reduced 2–3-fold, but not eliminated, by the microwave treatment. Other peptides partially affected by microwave irradiation include several CLIP fragments that result from cleavage of CLIP at Leu-Glu or Glu-Phe sites near the C terminus. The reason for the partial effect of the microwave treatment is not known. One possibility is that these peptides are formed after secretion from the tissue by extracellular peptidases such as angiotensin-converting enzyme, neutral endopeptidase, endothelin-
converting enzyme, and/or carboxypeptidase A-5 (32–34). Because cells are still alive after decapitation, they will presumably continue to secrete their contents during the time interval between decapitation and the eventual freezing of the tissue. But in the absence of blood flow, the secreted peptides and their extracellular processing products will accumulate in the tissue. In contrast, microwave irradiation to heat the tissue to 80 °C is likely to eliminate secretion, which would cause a decrease in the levels of peptides that result from this extracellular processing. Thus, the levels and forms of the peptides in the microwave-irradiated samples would more accurately represent the in situ levels and forms.

Although the use of microwave irradiation is a major improvement in peptidomic analysis of brain and pituitary tissue, there are still some obstacles to overcome. One major issue concerns the variability between duplicate analyses. In all experiments performed in this study duplicate samples were analyzed in which the hydrogen and deuterium labels were reversed. This provided a control for potential differences in the reactivity of the two different labeling reagents and also provided an overall indication of the variability between samples. In many cases, the duplicate samples were fairly close to each other. However, in a few cases the apparent levels of neuropeptides were much different in the duplicate runs (see hippocampus and striatum data, Table III). There are several potential sources for this variability. It is possible that the differences reflect animal to animal variation. It is also possible that there was variability in the peptide extraction and/or labeling. However, this is unlikely because the extraction techniques used in the present study are similar to those used in many previous studies examining peptide levels by RIA, and our control studies with standard peptides show that the labeling procedure is very accurate and reproducible. One consideration was that the microwave treatment did not completely inactivate the proteolytic enzymes; this was more of an issue with the tissues heated to 60 °C. But there was not a

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**Fig. 7.** MS/MS spectra of N-terminal fragments of β tubulin. A, the monoisotopic precursor mass of the β tubulin fragment was 1280.68 Da after subtraction of the single H<sub>9</sub>-TMAB label. The Ile in position 7 is present in β tubulin isoforms 3, 5, and 6, whereas isoforms 2 and 4 have the isomeric Leu in this position. Thus, the MS cannot distinguish among these five isoforms. B, the monoisotopic precursor mass of the peptide was 1688.88 Da after subtraction of the single H<sub>9</sub>-TMAB label, and the b-series clearly corresponds to that of the β tubulin identified in A. The additional mass of this peptide, 408.10 Da, corresponds to an additional C-terminal Cys (present in all five isoforms of β tubulin) with glutathione attached through a disulfide bond. As in A, the MS/MS data cannot distinguish among β tubulin isoforms 2–6. In both CID spectra, b<sup>−</sup><sub>n</sub> = b<sub>n</sub> − 59, b<sup>−</sup><sub>n</sub> = b<sub>n</sub> − 29.5 due to neutral loss of H<sub>9</sub>-trimethylamine from the TMAB label.
large difference between the 60 and 80 °C results, suggesting that partial inactivation of the enzymes did not occur. Similarly for many peptides the introduction of a 20-s delay between decapitiation and the microwave irradiation did not cause a large (i.e. >30%) change in the peptide levels, suggesting that variability in the procedure was not contributing to the differences between replicates. Because the largest variations between the duplicate runs were observed for proenkephalin-derived peptides in the hippocampus (Table III), it is likely that small errors in the dissection contributed to this variability; proenkephalin peptides are more abundant in the striatum than hippocampus, and so inclusion of a small amount of striatum in the hippocampal fraction for one of the tissues in the pool would cause a large apparent increase in the level of the peptide. The microwave-irradiated brains were more difficult to dissect using standard procedures because the tissue becomes less flexible after microwaving. This is a relatively minor issue, and it should be possible to modify the dissecting technique to provide reproducible dissection of microwaved mouse brain.

An unexpected finding was the identification of novel forms of several peptides or protein fragments. The POMC-derived J-peptide is known to undergo post-translational modifications such as N-terminal acetylation, but to our knowledge the O-glycosylation of Ser15 with a disaccharide has not been previously reported. From the additional mass (365 Da) and breakdown pattern, it is very likely that this modification is a disaccharide of six-carbon sugars (glucose, mannose, etc.) containing an O-acetyl group (27–29). The novel N-terminal fragment of VAP-33 represents initiation at an ATG upstream of that which was predicted (and which was also found in the present study). The upstream site does not contain a Kozak consensus sequence, and so it was not thought to be utilized (35). Our finding that both ATG initiation sites are used raises the issue as to the function of these alternative forms; it is possible that the properties of the longer protein are different from the shorter one. The modification of β tubulin by the addition of glutathione on the Cys near the N terminus also appears to be a novel modification. It is unlikely that the novel forms of β tubulin, VAP-33, and J-peptide are due to the microwave procedure. Instead it is more likely that these modifications were not previously found because the techniques used (RIA, protein gels, and Western blots) would not have detected such subtle modifications. Based on the large number of additional peptides that were detected in the microwave-irradiated brains that have not yet been sequenced, it is possible that many more post-translational modifications will be detected in further peptidomic studies on microwave-irradiated mouse brain.

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REFERENCES

1. Wilding, J. P. (2002) Neuropeptides and appetite control. Diabet. Med. 19, 619–627
2. Strand, F. L. (2003) Neuropeptides: general characteristics and neuropharmaceutical potential in treating CNS disorders. Prog. Drug Res. 61, 1–37
3. Chard, T. (1987) An Introduction to Radioimmunoassay and Related Techniques, 3rd Ed., Elsevier, Amsterdam
4. Tao, W. A., and Aebersold, R. (2003) Advances in quantitative proteomics via stable isotope tagging and mass spectrometry. Curr. Opin. Biotechnol. 14, 110–118
5. Che, F.-Y., and Fricker, L. D. (2005) Quantitative peptidomics of mouse pituitary: comparison of different stable isotopic tags. J. Mass Spectrom. 40, 238–249
6. Schrader, M., and Schulz-Knappe, P. (2001) Peptidomics technologies for human body fluids. Trends Biotechnol. 19, S55–S60
7. Verhaert, M., Utenweiler-Joseph, S., de Vries, M., Loboda, A., Ens, W., and Standing, K. G. (2001) Matrix-assisted laser desorption/ionization quadrupole time-of-flight mass spectrometry: an elegant tool for peptidomics. Proteomics 1, 186–193
8. Cluynen, E., De Loof, A., and Schoofs, L. (2003) The use of peptidomics in endocrine research. Gen. Comp. Endocrinol. 132, 1–9
9. Svensson, M., Skold, K., Svenningsson, P., and Andreu, P. E. (2003) Peptidomics-based discovery of novel neuropeptides. J. Proteome Res. 2, 213–219
10. Che, F.-Y., and Fricker, L. D. (2002) Quantitation of neuropeptides in Cpefat/Cpefat mice using differential isotopic tags and mass spectrometry. Anal. Chem. 74, 3190–3198
11. Che, F.-Y., Epper, B. A., Mains, R. E., and Fricker, L. D. (2003) Quantitative peptidomics of pituitary glands from mice deficient in copper transport. Cell. Mol. Biol. 49, 713–722
12. Che, F.-Y., Biswas, R., and Fricker, L. D. (2005) Relative quantitation of peptides in wild type and Cpefat/fat mouse pituitary using stable isotopic tags and mass spectrometry. J. Mass Spectrom. 40, 227–237
13. Pan, H., Nanno, D., Che, F.-Y., Zhu, X., Salton, S. R., Steiner, D. F., Fricker, L. D., and Devi, L. A. (2005) Neuropeptide processing profile in mice lacking prohormone convertase-1. Biochemistry 44, 4939–4948
14. Jimenez, C. R., Li, K. W., Dreisewerd, K., Mansvelder, H. D., Brussaard, A. B., Reinhold, B. B., Van der Schors, R. C., Karas, M., Hillenkamp, F., Burbach, J. P. H., Costello, C. E., and Geraerts, W. P. M. (1997) Pattern changes of pituitary peptides in rat after salt-loading as detected by means of direct, semiquantitative mass spectrometric profiling. Proc. Natl. Acad. Sci. U. S. A. 94, 9481–9486
15. Desiderio, D. M. (1999) Mass spectrometric analysis of neuropeptidergic systems in the human pituitary and cerebrospinal fluid. J. Chromatogr. B Biomed. Sci. Appl. 731, 1–22
16. Fricker, L. D., Mckinzie, A. A., Sun, J., Curran, E., Qian, Y., Yan, L., Patterson, S. D., Courchesne, P. L., Richards, B., Levin, N., Mzhavia, N., Devi, L. A., and Douglass, J. (2000) Identification and characterization of proSAAS, a granin-like neuroendocrine peptide precursor that inhibits prohormone processing. J. Neurosci. 20, 639–648
17. Che, F.-Y., Yan, L., Li, H., Mzhavia, N., Devi, L., and Fricker, L. D. (2001) Identification of peptides from brain and pituitary of Cpefat/Cpefat mice. Proc. Natl. Acad. Sci. U. S. A. 98, 9971–9976
18. Nagert, J. K., Fricker, L. D., Varlamov, O., Nishina, P. M., Rouille, Y., Steiner, D. F., Carroll, R. J., Paigen, B. J., and Leiter, E. H. (1995) Hyperproinsulinemia in obese fat/fat mice associated with a point mutation in the carboxypeptidase E gene and reduced carboxypeptidase E activity in the pancreatic islets. Nat. Genet. 10, 135–142
19. Fricker, L. D., Berman, Y. L., Leiter, E. H., and Devi, L. A. (1996) Carboxypeptidase E activity is deficient in mice with the fat mutation: effect on peptide processing. *J. Biol. Chem.* **271**, 30619–30624
20. Fricker, L. D., and Leiter, E. H. (1999) Peptides, enzymes, and obesity: new insights from a “dead” enzyme. *Trends Biochem. Sci.* **24**, 390–393
21. Galli, C., and Racagni, G. (1982) Use of microwave techniques to inactivate brain enzymes rapidly. *Methods Enzymol.* **86**, 635–642
22. O’Callaghan, J. P., and Siriram, K. (2004) Focused microwave irradiation of the brain preserves in vivo protein phosphorylation: comparison with other methods of sacrifice and analysis of multiple phosphoproteins. *J. Neurosci. Methods* **135**, 159–168
23. Theodorsson, E., Stenfors, C., and Mathe, A. A. (1990) Microwave irradiation increases recovery of neuropeptides from brain tissues. *Peptides* **11**, 1191–1197
24. Mathe, A. A., Stenfors, C., Brodin, E., and Theodorsson, E. (1990) Neuropeptides in brain: effects of microwave irradiation and decapitation. *Life Sci.* **46**, 287–293
25. Nylander, I., Stenfors, C., Tan-No, K., Mathe, A. A., and Terenius, L. (1997) A comparison between microwave irradiation and decapitation: basal levels of dynorphin and enkephalin and the effect of chronic morphine treatment on dynorphin peptides. *Neuropeptides* **31**, 357–365
26. Che, F.-Y., Yuan, Q., Kalinina, E., and Fricker, L. D. (2005) Peptidomics of Cpe fat/fat mouse hypothalamus: effect of food deprivation and exercise on peptide levels. *J. Biol. Chem.* **280**, 4451–4461
27. Alving, K., Paulsen, H., and Peter-Katalinic, J. (1999) Characterization of O-glycosylation sites in MUC2 glycopeptides by nanoelectrospray QTOF mass spectrometry. *J. Mass Spectrom.* **34**, 395–407
28. Moody, A. M., North, S. J., Reinhold, B., Van Dyken, S. J., Rogers, M. E., Panico, M., Dell, A., Morris, H. R., Marth, J. D., and Reinherz, E. L. (2003) Sialic acid capping of CD68 core 1-O-glycans controls thymocyte-major histocompatibility complex class I interaction. *J. Biol. Chem.* **278**, 7240–7246
29. Cox, A. D., Howard, M. D., Brisson, J. R., van der Z, M., Thibault, P., Perry, M. B., and Inzana, T. J. (1996) Structural analysis of the phase-variable lipooligosaccharide from Haemophilus somnus strain 738. *Eur. J. Biochem.* **253**, 507–516
30. Zhu, X., and Desiderio, D. M. (1993) Methionine enkephalin-like immuno-reactivity, substance P-like immunoreactivity and endorphin-like immuno-reactivity post-mortem stability in rat pituitary. *J. Chromatogr.* **616**, 175–187
31. Allen, G. (1989) *Sequencing of Proteins and Peptides*, 2nd Ed., pp. 73–104, Elsevier, Amsterdam
32. Corvol, P., and Williams, T. A. (1998) Peptidyl-dipeptidase A/angiotensin I-converting enzyme, in *Handbook of Proteolytic Enzymes* (Barrett, A. J., Rawlings, N. D., and Woessner, J. F., eds) 1st Ed., pp. 1066–1076, Academic Press, San Diego, CA
33. Turner, A. J. (2003) Exploring the structure and function of zinc metallopeptidases: old enzymes and new discoveries. *Biochem. Soc. Trans.* **31**, 723–727
34. Wei, S., Segura, S., Vendrell, J., Aviles, F. X., Lanohe, E., Day, R., Feng, Y., and Fricker, L. D. (2002) Identification and characterization of three members of the human metallocarboxypeptidase gene family. *J. Biol. Chem.* **277**, 14954–14964
35. Skelhel, P. A., Fabian-Fine, R., and Kandel, E. R. (2000) Mouse VAP33 is associated with the endoplasmic reticulum and microtubules. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 1101–1106