Anal. Chem. **2008,** *80,* 7128–7136

Quantitative Measurements of Cell−Cell Signaling Peptides with Single-Cell MALDI MS

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Cell-to-cell signaling peptides play important roles in neurotransmission, neuromodulation, and hormonal signaling. Significant progress has been achieved in qualitative investigations of signaling peptides in the nervous system using single cell matrix-assisted laser desorption/ionization mass spectrometry. However, quantitative information about signaling peptides is difficult to obtain with this approach because only small amounts of analytes are available for analysis. Here we describe several methods for quantitative microanalysis of peptides in individual *Aplysia californica* neurons and small pieces of tissue. Stable isotope labeling with *d*0- and *d*4-succinic anhydride and iTRAQ reagents has been successfully adopted for relative quantitation of nanoliter volume samples containing the *Aplysia* insulin C_β peptide. Comparative analysis of the C_β peptide release site, the upper labial nerve, and its synthesis location, the F- and C-clusters, shows that the release site possesses almost three times more of this compound. The method of standard addition permits absolute quantitation of the physiologically active neuropeptide cerebrin from small structures, including nerves and neuronal clusters, in the femtomole range with a limit of detection of 19 fmol. The simplicity of these methods and the commercial availability of the reagents allow quantitative measurements from a variety of small-volume biological samples.

The central nervous system is one of the most chemically, structurally, and functionally heterogeneous systems in an animal. Linking neurochemistry and brain function requires an understanding of the cell−cell signaling peptides (SPs) used in the brain. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) and electrospray ionization (ESI) MS have become the technologies of choice for qualitative investigations of the peptide content in a brain sample. Neuropeptidomics, the detailed investigation of endogenous peptides in the brain and peripheral nervous system, continues to evolve[1−8] (for reviews, see refs 9 and 10). Optimized sampling, extraction, and data-analysis methods are now available for following the multistep processing of prohormones into biologically active peptides.[2,4,5,7,11−13]

Although these approaches are more useful for homogeneous populations of cells, in the nervous system even adjacent neurons have significantly different biochemical profiles that include unique SP complements. The microliter or larger volumes of nervous tissue used for neuropeptidome studies contain hundreds of thousands of cells. As the individual neurons comprising pooled samples can contain unique complements of SPs that are involved in functionally antagonistic networks, averaging the SP content from hundreds of thousands of neurons makes understanding the functional consequences of this neuropeptide complexity difficult. Furthermore, the increased dynamic range inherent to such complex mixtures places constraints on analytical measurement systems. In contrast, interpretation of results is easier when performing qualitative and quantitative microanalyses of individual cells or small representative tissue areas. Not only are these smaller samples chemically simpler, the putative functions of the detected peptides are not obscured by the other cell contents present in larger samples.

In systems biology research, with its emphasis on obtaining, integrating, and interpreting chemical, spatial, and temporal information, investigation of individual cells has become an important focus. With the use of mass spectrometric technologies, tremendous gains in the qualitative measurement of peptides from individual neurons have been made.[14−19] MALDI, in particular, has proven valuable in single cell studies by offering high

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10.1021/ac8010389 CCC: $40.75
Published on Web 08/16/2008

sensitivity, low sample consumption, relatively simple sample preparation, and the ability to investigate small-volume samples over a broad dynamic range. MS-based investigations have enabled a range of prohormones and unusual post-translational modifications to be characterized in a number of invertebrate neuronal model systems, most notably in mollusks[2,15,20] and arthropods.[21,22] Single cell MALDI MS recently has been used to investigate mammalian cells,[23,24] and several reports have demonstrated the ability to perform MS peptide sequencing using single cell samples.[25,26]

Clearly, obtaining qualitative information on signaling molecules at the single cell level is relevant to understanding their function but so also is the acquisition of quantitative information. For example, we would expect behavioral changes to be influenced by neurotransmitter and neuromodulator levels; in point of fact, we recently reported that serotonin levels in the soma of an identified neuron from *Pleurobranchaea californica* varied quantitatively according to the animal's hunger state.[27] Several microanalytical methods are available for quantitative single cell measurements and include, among others, electrophoresis, immunohistochemistry, radioisotope labeling, and reverse transcriptase polymerase chain reaction (see refs 28 and 29 for detailed descriptions of these technologies). Although a number of approaches for assaying individual cells are available, most are less suitable for measuring multiple compounds simultaneously. This is especially true when examining SPs in individual cells because of the wide range of concentrations, varied physicochemical properties of the peptides, high levels of biochemical and morphological compartmentalization in cells, and exceptional complexity of the intracellular and extracellular matrixes. Another issue is that SPs typically are packed in dense core vesicles, which must be lysed for quantitative analysis.

Mass spectrometric methods are often used to obtain quantitative or semiquantitative information on a variety of analytes. A number of elegant MS-based approaches have been demonstrated in recent years that use in situ, in vitro, and in vivo isotope labeling of analytes with ^{15}N, ^{18}O, iTRAQ, ICTL, SILAC, MCAT, *d*0- and *d*4-succinic anhydride-, and Ji et al.[30−36] How well do these approaches work in neuropeptide investigations? The studies by Desiderio et al.[37] and others[38] detail quantitative measurement of neuropeptides, as do more recent approaches using a range of sample sizes, isotope labeling strategies, and mass analyzers.[6,11,13,39−43]

However, even though these quantitative peptide measurement approaches have been well validated, it is uncertain if they may be suitable for minute-volume samples such as individual subcellular regions, single cells, and small tissue regions. Although MALDI MS is capable of profiling small samples for qualitative information,[17,18,24,44,45] adding labeling reagents, performing reactions, and mixing control and experimental samples often has limited the application of stable isotope labeling to many-fold larger samples.

Here we demonstrate several strategies for MS-based measurements of peptides in single cells and small tissue samples that provide both semi- and absolute quantitative information, in this case using individual molluskan neurons from cerebral ganglion F- and C-clusters and nerves known to produce *Aplysia* insulin (AI) and cerebrin prohormones. We previously characterized the neuropeptide content and function of these peptides in these neurons.[46−48] The approaches described here are based on well-known stable isotope labeling as well as use of the standard addition method, employed here on a smaller-volume scale than before. These strategies broaden significantly the application of single cell MALDI MS in neurobiological and physiological research and can be adapted to other types of cells and other small-volume analytical samples.

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EXPERIMENTAL SECTION

Animals. Adult *Aplysia californica* weighing 175−250 g were obtained from Charles M. Hollahan (Santa Barbara Marine Bio., Santa Barbara, CA). Animals were maintained in constantly circulated, aerated artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH), chilled to 14 °C.

Chemicals. Sigma-Aldrich (St. Louis, MO) was the source for all chemicals, unless stated otherwise. Cerebrin was custom-synthesized by Commonwealth Biotechnologies (Richmond, VA). Peptide calibration standard II (angiotensin II, angiotensin I, substance P, bombesin, ACTH clip 1-17, ACTH clip 18-39, somatostatin 28, bradykinin fragment 1-7, renin substrate tetradecapeptide porcine) was obtained from Bruker Daltonics (Bremen, Germany). Acetonitrile (ACN) was obtained from Fisher Scientific (Fair Lawn, NJ).

Sample Preparation. *Aplysia californica* were anesthetized by injection of a solution containing 390 mM $MgCl_2$ dissolved in water, equal by mass to one-third of each animal's body weight, into the vascular cavity. The cerebral ganglia with adjacent nerves were surgically dissected and placed in artificial seawater (ASW) containing (in mM) 460 NaCl, 10 KCl, 10 $CaCl_2$, 22 $MgCl_2$, 26 $MgSO_4$, and 10 HEPES (pH 7.7) supplemented with antibiotics (100 units/mL penicillin G, 100 μg/mL streptomycin, and 100 μg/mL gentamicin). A 1% protease type IX in ASW-antibiotics solution treatment for 60−120 min (depending on animal size) at 34 °C was used to reduce adherence between cells such as glia and neurons. This treatment also helps to remove connective tissue surrounding the ganglia and nerves as well as improves the success of individual neuron isolation. After the protease treatment, the ganglia were washed in ASW and stored in 33% glycerol/67% ASW (v/v) solution at 14 °C until use. This solution helps to stabilize cells during storage and neuron isolation. Isolation of individual neurons and small pieces of tissue was performed manually under visual control assisted by a Leica MZ 7.5 high-performance stereomicroscope with a 7.9:1 zoom (Leica Microsystems Inc., Bannockburn, IL).

Morphology. To investigate cellular morphology, saturated concentrations of sulforhodamine dissolved in 506.2 mM KCl and 5 mM HEPES solution (pH 7.6) were pressure-injected into the neuronal cell body of interest. Next, the cerebral ganglion was incubated in ASW containing 10 mM probenecid overnight.[49] Embedding the cerebral ganglia in sucrose was performed with some modifications as described by Kabotyanski et al.[50] Briefly, the ganglia were transferred onto a glass coverslip where ASW was substituted by a sucrose solution (1 M sucrose, 10 mM $CaCl_2$). After the preparation was dried at room temperature, the neurons were investigated using a Zeiss Axiovert inverted microscope (Carl Zeiss, Oberkochen, Germany) coupled with a CCD camera, driven by the MCID software package (Imaging Research, Ontario, Canada).

Mass Spectrometry. An Ultraflex II matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) mass spectrometer controlled with FlexControl software was employed in this study (Bruker Daltonics). The mass spectra were visualized and processed using FlexAnalysis software.

MS peptide profiling was performed using the following instrument settings: reflector mode, positive voltage polarity, pulsed ion extraction delay 20 ns, ion source voltage "1" 25 kV, ion source voltage "2" 21.7 kV, reflector voltage "1" 26.3 kV, reflector voltage "2" 13.8 kV, reflector detection voltage 1.732 kV, sample rate 0.5 GS/s. In iTRAQ experiments performed to obtain quantitative information, MS/MS peptide sequencing was employed. The mass spectrometer settings in this case were as follows: LIFT TOF/TOF mode,[51] positive voltage polarity, sample rate 0.5 GS/s, reflector detector voltage 1.849 kV, ion source voltage "1" 8 kV; ion source voltage "2", 7.2 kV; LIFT voltage "1", 19 kV; LIFT voltage "2", 3 kV; reflector voltage "1" 29.5 kV, reflector voltage "2" 13.8 kV.

MALDI matrix solution consisted of α-cyano-hydroxycinnamic acid (CHCA) saturated in a 1:1 mixture of ACN/0.1% (v/v) aqueous trifluoroacetic acid (TFA). Mass calibration was performed internally using one or more of the following approaches: using known endogenous peptides, adding angiotensin I to the MALDI matrix, and externally by applying Bruker's Peptide Calibration Standard II to the target. For those experiments using angiotensin I, 160 fmol was deposited on a spot for quantitation of single cells and small pieces of cerebral commissure. The angiotensin I amount was increased 10-fold when extracts of F- and C-clusters and upper labial (ULAB) nerves were characterized.

Peptide Extraction and Stable Isotope Labeling. F- and C-clusters and the ULAB nerves were dissected from a cerebral ganglion preparation (for description of cerebral ganglion morphology see ref 48 and Figure 1) and separately incubated in 50 μL of extraction media containing 2:2:1 water/methanol/acetic acid overnight at 4 °C. Unlike larger samples, homogenization and sonication were not required for efficient analyte extraction. The extracts were used in stable isotope labeling experiments where the amino groups, including free peptide N-termini and lysine residues, were isotopically labeled with *d*0- or *d*4-succinic anhydride. As a result, masses of labeled peptides increased by 100*X* Da when a light form of succinic anhydride was added, and by 104*X* Da when a heavy form was added (*X* represents the number of residues labeled). The labeling procedure used is similar to one described by Che et al.[11] with several modifications. Briefly, a 20-μL aliquot of each sample was supplemented with 70 μL of water, 5 μL of 10 mM phosphate buffer (pH 8), and 4 μL of 1 M NaOH. Either 1 μL of 2 M *d*0- or of *d*4-succinic anhydride in dimethyl sulfoxide (DMSO) was added to the mixtures and allowed to react for 20 min at room temperature. Next, 1 μL of 1 M NaOH was added to the mixture to maintain a pH of ~9. The addition of the succinic anhydride solution, the 20-min incubation period, and the pH adjustment steps were repeated two more times. Quenching of unreacted compounds was performed using 10 μL of 2.5 M glycine in water. Because several components, such as high levels of inorganic salts and DMSO, can interfere with the MALDI measurement, an additional peptide purification step is often required. Therefore, mixtures of 5 μL of labeled ULAB and 5 μL of labeled F- and C-cluster samples were cleaned with ZipTip pipet tips packed with 0.6 μL of C_{18} resin (Millipore, Bedford, MA). Analytes were eluted from the ZipTip pipet tip with 2 μL of CHCA solution onto a prespotted AnchorChip MALDI

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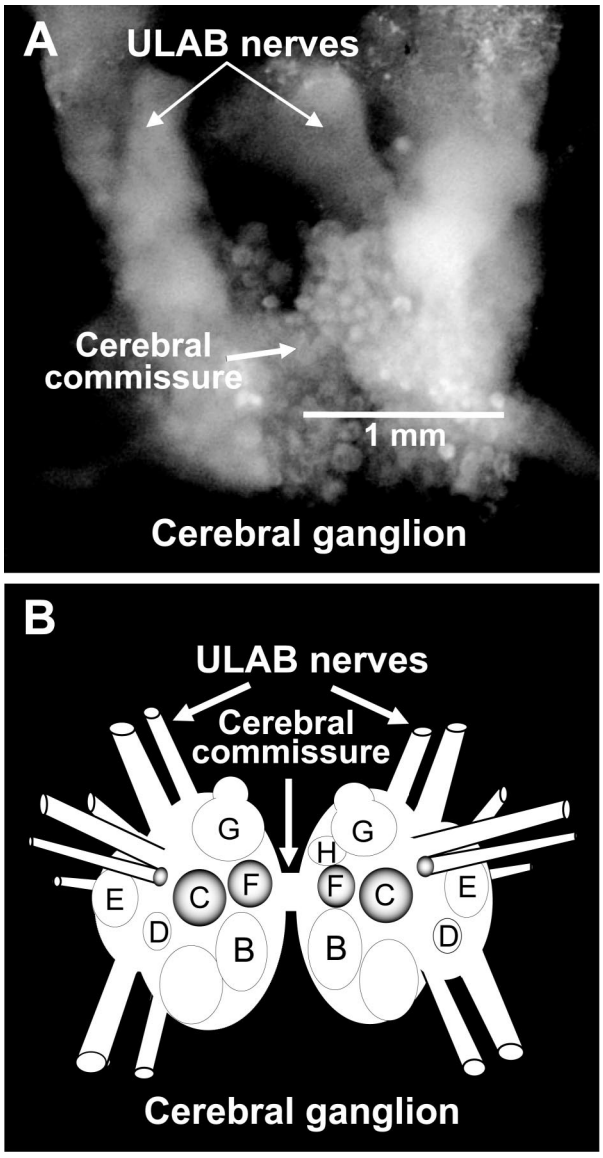

Figure 1. An *Aplysia californica* cerebral ganglion has a well defined but complex structural organization. (A) Microphotograph of the cerebral ganglion, dorsal side, after treatment with protease and removal of its protective sheath. (B) Schematic of the cerebral ganglion with the identified neuronal clusters localized on its dorsal side labeled by the appropriate nomenclature letter.

sample plate (Bruker Daltonics). The AnchorChip's hydrophobic surface reduced sample spreading and therefore increased analyte concentration, yielding higher quality and more consistent data.

Individual neuron labeling with the iTRAQ reagents multiplex kit (Applied Biosystems, Foster City, CA) was performed essentially according to the manufacturer's manual but with several modifications to minimize sample handling and to adjust for the small sample volumes. Individual cerebral ganglion F-cluster top layer neurons[48] were manually isolated and placed onto an AnchorChip sample plate at a region that was not covered with MALDI matrix. Next, ~150 nL of acidified methanol was applied to the cell-containing spot; the small sample size allows for successful and quick analyte extraction. The dried sample was reconstituted in ~100 nL of iTRAQ buffer on the sample plate, followed by immediate addition of ~100 nL of iTRAQ stable isotope labeled reagent, either 117 or 114. The resulting sample was dried for 2−4 min, forming a viscous spot, and reconstituted in 500 nL of CHCA solution. A quality check to test the completeness of the derivatization was performed with MALDI MS, and then the samples were redissolved, combined, recrystallized, and reanalyzed.

A similar procedure was applied in experiments with *d*0-SA and *d*4-SA labeling of analytes from single CF_T neurons. The same step as above for on-plate peptide extraction was used and either acidified methanol or a 0.1% aqueous solution of formic acid supplemented with 0.01% TFA was employed as an extraction media. After spot drying, extracted peptides were reconstituted in a 100 nL solution containing 6 parts water, 1 part triethylamine and 1.2 parts of 2 M *d*0-SA or *d*4-SA, mixed immediately before use. Then, a 600-nL MALDI matrix solution was applied after a 15 min reaction time. The resulting samples were examined with MALDI MS to confirm completeness of labeling. For the final step, samples were reconstituted in 800 nL of a 50/50 ACN/water mixture, and 500 nL of each sample was taken and mixed in pairs on clean spots on a MALDI sample plate for relative quantitative measurements. The last two operations have to be done quickly due to the evaporation of organic solvent. No cleanup of samples using a solid phase extraction material was performed. The quality of labeling using both iTRAQ and succinic anhydride was determined by the disappearance of signals from unlabeled analytes in the mass spectra.

Absolute Quantitation Using Standard Addition. For absolute quantitation of peptides in the F- and C-clusters and in the ULAB nerves, peptides from the cell(s) or tissue were extracted using the acidified methanol solution. Several 200-nL aliquots of a sample were produced and spiked with internal synthetic standard, the physiologically active peptide cerebrin,[20] at the appropriate concentration. Next, 300-nL aliquots of the final solutions were deposited onto a sample plate and mixed with 300 nL of CHCA solution.

In experiments to achieve absolute quantitation of the cerebrin in a small region of the cerebral commissure, ~1/10 of this structure was dissected and placed onto a sample plate and covered with 500 nL of CHCA matrix containing the internal standard angiotensin I. The samples were examined with MALDI MS to determine the signals produced by endogenous peptides. Next, the samples were reconstituted with acidified methanol containing synthetic cerebrin as an internal calibrant. Repetitive cycles of MS measurement, reconstitution/addition of the calibrant and drying, followed by a new MS measurement, allowed the construction of a calibration curve for quantitative measurement of cerebrin in the sample. Importantly, to build this calibration curve, the intensity of the endogenous cerebrin signal detected in control measurements was subtracted from the total signal representing endogenous and spiked cerebrin. Also, intensities of cerebrin and cerebrin sodium adduct peaks were normalized to the intensities of the internal standard signals and intensity values summed. The best-fit line through the calibration curve was used, and the amount of endogenous peptide was determined. In all cases, standard deviations of the measurements are presented. For statistical analysis, three or more individual samples were analyzed and the mean and standard deviation calculated.

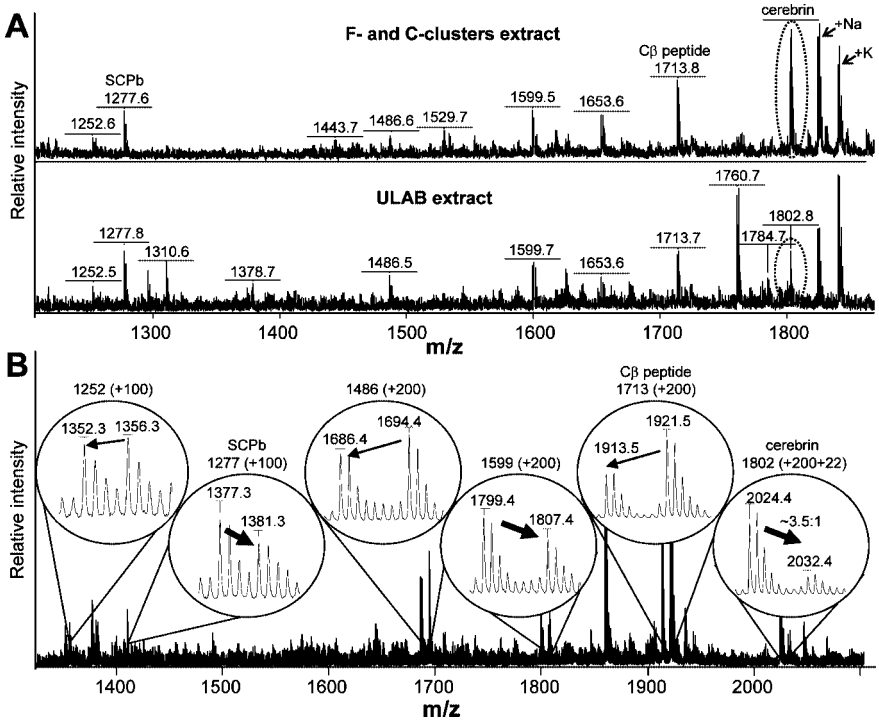

Figure 2. Known SPs can be detected in extracts of F- and C-clusters and ULAB nerves, including cerebrin. The *d*0- and *d*4-succinic anhydride labeling allows the determination of relative quantities of peptides present in different regions of the nervous system. (A) Mass spectra of F- and C-cluster and ULAB extracts. (B) Mass spectrum of a mix of ULAB nerve extract labeled with *d*0-SA and an extract of F- and C-clusters labeled with *d*4-SA. SCPb: small cardioactive peptide.

RESULTS AND DISCUSSION

Optimization of Sample Preparation Protocols. To develop and optimize quantitative MALDI MS approaches, we used several well-characterized AI- and cerebrin-producing neuronal networks partially localized in the F- and C-clusters of the cerebral ganglion as well as in the ULAB nerve and cerebral commissure.[46,48] These networks have distinct morphological elements that can be identified and isolated. The cell bodies of the AI neurons are located in the F- and C-clusters of the *Aplysia* cerebral ganglion (Figure 1). Processes from these neurons form AI-containing structures in the anterior tentacular (AT) and ULAB nerves, regions of putative AI secretion into molluscan blood. Interestingly, the AT nerve structures are significantly larger than the ULAB structures. Cerebrin has a different distribution; the cerebrin-expressing neurons have their cell bodies predominantly in the F-cluster and their terminals in the cerebral commissure and ULAB nerves. As with other neurosecretory cells, AI- and cerebrin-expressing neurons produce significant amounts of intercellular signaling peptides. Despite the multicellular nature of these structures, their relative sizes are small (Figure 1), typically, several tens to hundreds of micrometers across in the longest direction. For example, the cerebral commissure and ULAB nerves have well-developed but small-volume neurohemal release zones that only occupy the surface layers of both structures. These factors make these peptidergic systems suitable models for single cell and small tissue microanalysis method development and optimization.

To verify that the F- and C-clusters, ULAB nerves, and cerebral commissure have enough peptides to characterize, we compared the concentrations of a single peptide from each prohormone, the AI prohormone C_β-peptide and cerebrin. For relative quantitation of the C_β peptide, we used *d*0- and *d*4-succinic anhydride. This approach is well established and based on succinylation of free amino groups where modification of basic sites with acidic carboxyl groups occur.[11,52] Each modification results in an increase of peptide molecular mass by 100 Da for *d*0-SA and 104 Da for *d*4-SA. MALDI MS profiling of the samples revealed that both peptides, C_β and cerebrin, are present in the extracts (Figure 2A). Moreover, several related peptides, C_β (1-14) and C_β (1-13), are also observed. MS/MS experiments confirm that these shortened peptide forms are endogenous and not caused by in-source decay of C_β (Supporting Information Figure 1). These peptides are likely products of enzymatic degradation of C_β.[46] Interestingly, the intensity of the cerebrin peak was relatively low in the ULAB nerve extract compared to the F- and C-cluster extracts (Figure 2A). However, a direct interpretation of a lower MALDI peak intensity as a lower amount of cerebrin present in the ULAB may not be correct, as peak heights can be altered via signal suppression by other constituents in the sample.[53,54] Indeed, analysis of the same samples after stable isotope labeling, mixing, and purification using a solid phase extraction approach demonstrated that the ULAB nerve extract had a higher amount of cerebrin than did the F- and C-cluster samples, whereas the C_β peptide was more prominent in the F- and C-cluster samples (Figure 2B). These results are in agreement with prior immunohistochemical data where AI immunoreactive fibers are located

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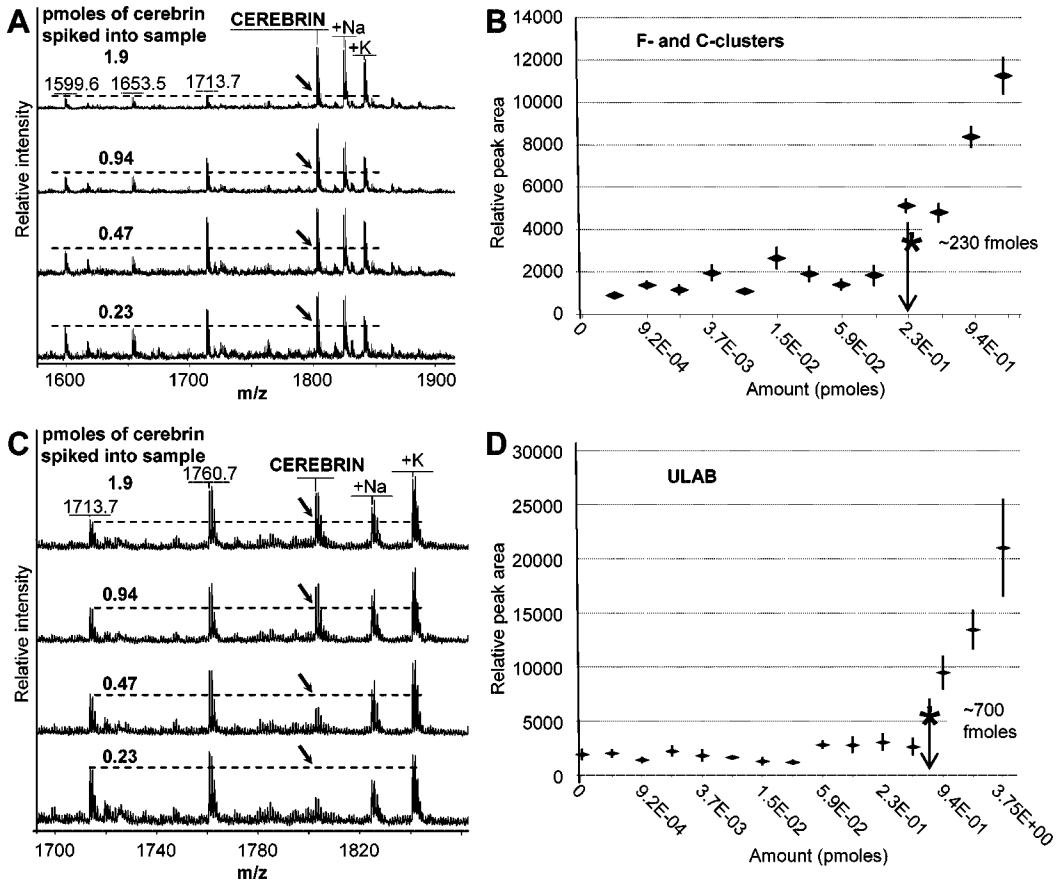

Figure 3. Absolute quantitation using standard addition with cerebrin as the calibrant. (A) Mass spectra of F- and C-cluster extracts spiked with different amounts of cerebrin noted above each mass spectrum. (B) Sum of areas of the cerebrin molecular ion peak and the areas of its sodium and potassium adducts from three different F- and C-cluster extracts measured in triplicate. (C) Mass spectra of ULAB nerve extract spiked with different amounts of cerebrin. (D) Sum of areas of the cerebrin molecular ion peak and the areas of its sodium and potassium adducts from three different ULAB nerve extracts measured in triplicate. Standard deviations are shown.

mostly in the AT nerve, with minor amounts within the ULAB, while cerebrin immunoreactivity is predominantly located in the ULAB.[20,46,48]

Although isotope labeling provides relative quantitation, the majority of methods for absolute quantitation employ a known amount of synthetic or purified calibrant that is identical to an endogenous analyte. In this work, we used synthetic cerebrin. To minimize the influence of factors such as analyte signal suppression, we employed the method of standard addition (Figure 3), even though this approach is not normally applied to so small a volume sample. For our physiological samples, many analytes produced multiple peaks from the molecular ion and ions of their alkali metal adducts because of the high salt concentrations in these biological tissues (Figure 3A,C). Relative intensities of the molecular ion, as well as its individual sodium and potassium adduct signals, varied from sample-to-sample and could not be directly fit to calibration curves created via external calibration. Therefore, our approach included internal spiking with synthetic standard and summation of the intensities of the cerebrin-related peaks. This produced consistent data. Results of the absolute quantitation experiments show that F- and C-clusters, on average, contain 230 fmol of cerebrin (three individual samples measured in triplicate) whereas ULAB nerves have ~700 fmol (three individual samples measured in triplicate). These observations are in agreement with data obtained on the same samples analyzed with the *d*0-SA and *d*4-SA approach (Figures 2 and 3). Both the comparison of cerebrin signals in SA-labeled samples (Figure 2B, far right insert), and analysis of data obtained in the spiking experiments (Figure 3B,D), demonstrate that the ULAB nerve has an approximately 3-fold higher cerebrin amount than that found in the F- and C-clusters.

Relative Quantitation of Signaling Peptides with Single Cell MALDI MS. Can these methods be downscaled to work with individual cells? Individual CF_T neurons (Figure 4A) are a good model for optimization of analytical methods focused on studying endogenous intercellular signaling peptides. These 50−100-μm diameter cells can be easily recognized by their location in the top F-cluster cellular layer and white color, as well as their expression of significant amounts of AI.[46] Larger than many mammalian neurons, their size is approximately the same as some of the largest neurons in the human nervous system, e.g., the magnocellular neuroendocrine neurons and some dorsal root ganglion neurons.

We developed an approach for peptide labeling in single cells using the succinic anhydride method that comprises only one labeling step and does not require peptide purification after derivatization (see Supporting Information). However, this approach produced more than one labeled product for C_β peptide. Both doubly labeled and smaller, triply labeled peaks were

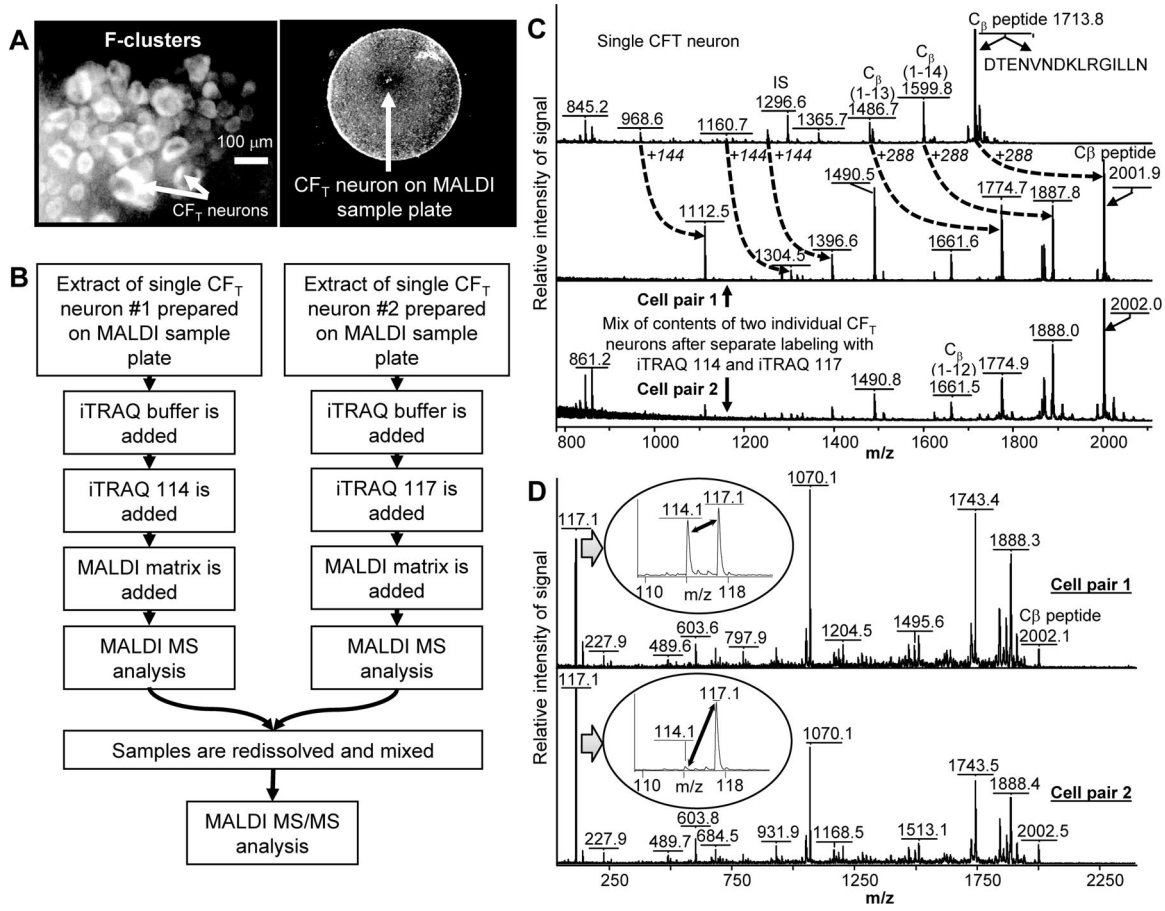

Figure 4. iTRAQ labeling allows the determination of relative quantities of peptides present in single neurons. (A) Sample preparation is a crucial step for successful quantitative MALDI MS measurements of peptides in single cells. Microphotograph of the dorsal view of a F-cluster with individual cell bodies visible (left). Image of an individual CF_T neuron deposited on a MALDI sample plate (right). (B) Steps involved in peptide quantitation in single neurons using iTRAQ reagents are shown. (C) iTRAQ labeling of peptides in single cell preparations resulted in formation of singly or doubly labeled molecules. Top mass spectrum represents the peptide profile of an individual CF_T neuron before labeling. Two bottom mass spectra are mixtures of two different pairs of CF_T cells. (D) Tandem mass spectra of C_β peptide acquired from the mixed contents of two pairs of individual CF_T neurons after separate labeling with iTRAQ 114 and iTRAQ 117. The insets show the mass range containing isotopic labels cleaved from labeled molecular ions during the fragmentation process. Angiotensin I (*m/z* 1296) was added as an internal standard (IS) to the MALDI matrix (top mass spectrum).

detected (Supporting Information Figure 2). This situation introduces added complexity to the quantitative analysis process. Moreover, the presence of multiple peaks from a single analyte may result in lower signal intensity of the derivatized analytes. Another concern is that the formation of succinylated amides at all amine residues of a peptide may reduce the formation of positive charges and therefore analyte ionization in the positive mode.[11] To alleviate the issues of multiple labeling of analytes, we used the commercially available iTRAQ kit for quantitation via MALDI MS. The iTRAQ kit includes a set of four isobaric reagents that react with the amine groups of peptides. The iTRAQ labeling procedure requires only one step, and the resulting mass spectra of a derivatized sample tends to be less complicated because differences in stable isotope labeling are revealed only during MS/MS experiments.

A schematic of the approach used to analyze individual neurons is shown in Figure 4B. There are several important steps to ensure optimal results. The extraction of endogenous peptides should use an appropriate buffer that is compatible both with the MALDI measurement process as well as with stable isotope labeling; a volatile extraction media works well. We used acidified methanol because it is known to efficiently extract peptides localized in biological tissues,[55] its higher viscosity allows manipulations of small solution volumes and deposition in a reasonably small area without spreading, and it evaporates relatively slowly. In contrast, we found that the iTRAQ buffer did not work well as an extraction media for individual neuron samples.

The addition of the standard iTRAQ buffer to samples containing the extraction buffer resulted in a significantly reduced signal intensity of the labeled peptides. Therefore, improved results were obtained when the samples were dried after extraction and before addition of iTRAQ buffer. Surprisingly, direct MS profiling shows that the stable isotope labeling time in such small samples can be reduced to only several minutes as compared to the manufacturer's recommended 1 h (Figure 4C, bottom two traces). This reduction in reaction time may be due to (1) small sample sizes allowing faster mixing, (2) the large excess of labeling reagent, (3) or the rapid reduction in volume after deposition onto the open sample plate concentrating analytes and reagents. After the reaction is complete, the evaporation of volatile solvent leads to

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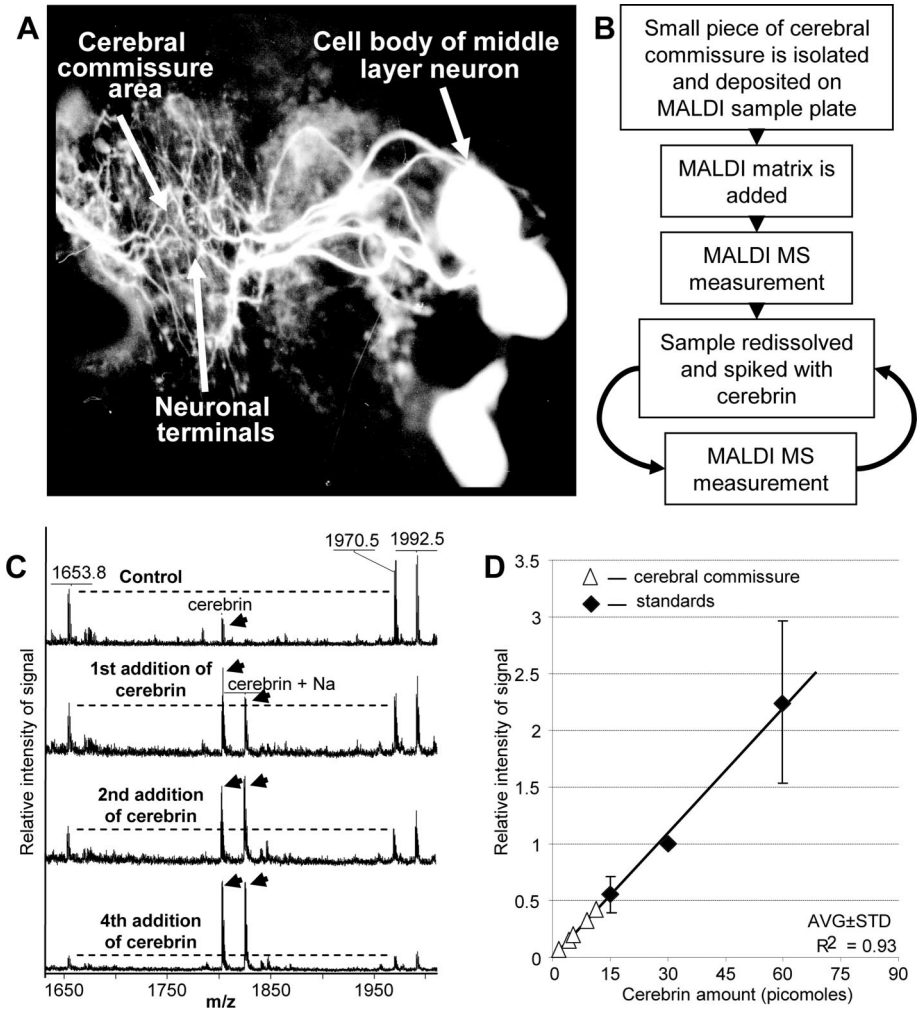

Figure 5. Multiple additions of the calibrant to the same sample spot on the MALDI sample plate allows absolute quantitation of the amount of analyte present in the spot, in this case, from a small fraction (~1/10) of the cerebral commissure. (A) Image of the cerebral commissure, a small structure located between the cerebral hemiganglia containing terminals of cerebrin-expressing neurons. Sulphorhodamine was injected into the neurons shown on the microphotograph. (B) Procedure used for peptide quantitation. (C) Mass spectra of cerebral commissure sample repeatedly spiked with cerebrin. (D) Cerebrin amounts measured in five different pieces of cerebral commissure. △ represent individual values for each sample, with the value being the sum of normalized intensities of the cerebrin molecular ion peak and its sodium adduct peak. ◆ show average values for increases in the sum of cerebrin and its adduct peak intensities after spiking of the samples with known amounts of cerebrin. Standard deviations are shown.

the formation of a small spot of viscous material, which is then reconstituted with CHCA solution containing an organic solvent. Samples were examined with MS before mixing to verify the absence of detectable amounts of unlabeled analytes, allowing samples to be mixed without removal of any excess stable isotope label. Interestingly, labeling with stable isotopes improves the detectability of several peptides. For example, C_β (1-12) is observed in labeled CF_T neurons but not in the unlabeled cell (Figure 4C). Despite the relatively small amount of analytes in single cells, MS/MS analysis can be performed on the iTRAQ-labeled peptides. For example, C_β peptides in two different CF_T neurons were individually labeled with the 114 or 117 iTRAQ reagent and subjected to MS/MS sequencing after sample mixing (Figure 4D). As expected, samples of two similarly sized and located cells have similar concentrations of C_β-peptide (Figure 4D, upper trace), whereas the second mixture containing a larger and smaller cell (Figure 4D, lower trace) shows a less intense signal from the smaller cell.

Absolute Quantitation of Signaling Peptides in Small Tissue Samples with MALDI MS. With the use of MALDI MS, determination of absolute quantities of cerebrin in small samples, such as a single cell or commissure subsection, presents several issues, including the minute analyte amounts and difficulties in small-volume sample handling and manipulation. Dividing samples into aliquots is not feasible for these nanoliter volume samples without employing state-of-the-art microfluidics.

We validated the well-known approach of standard addition by consecutive spiking of the sample spot with known peptide amounts. Although this appears to be a straightforward procedure, standard addition approaches require a nondestructive readout because the sample is spiked and the signal measured, and then an additional aliquot of standard is added and the sample reanalyzed. Standard addition works here because each MALDI measurement process uses only a small fraction of the sample. In essence, most of the analyte is left behind.[56] Additionally, cerebrin signal intensity was normalized to the signal intensity of

Table 1. Comparison of Methods Successfully Used in This Study for Peptide Quantification in Small Tissue Samples and Individual Cells Using MALDI MS

approach	relative quantitation	absolute quantitation	single cell analysis	complexity of sample prep	cost
*d*0- and *d*4-succinic anhydride labeling	yes	no	no	moderate	low
spiking with analyte of interest		yes	yes	easy	variable
iTRAQ	yes		yes	easy	moderate

the internal standard, angiotensin I, which was added with the MALDI matrix at the first preparation step. This normalization can help to compensate for loss of material if cerebrin and angiotensin I are consumed during the sample measurement process. Interestingly, the average intensity of angiotensin I signal did not change significantly over multiple recrystallizations and spot interrogations (control, 28K counts; 1st recrystallization, 30K counts; 2nd recrystallization, 30K counts; and 4th recrystallization, 29K counts). At the same time, average intensity of the cerebrin signal increased during the additions from 200 to 1700 counts. Therefore, samples previously profiled with MALDI MS can be reconstituted and spiked with an additional amount of calibrant. Implementation of this strategy allows one to build a complete calibration curve for a single cell sample to determine the concentration of peptide; here we have done this for five individual small tissue areas containing ~1/10 of a cerebral commissure (Figure 5). With the use of a signal-to-noise ratio of 3 as our detection limit and 10 as the limit of quantitation, the corresponding values were calculated as 19 and 64 fmol. Despite the ability of this MALDI MS instrument to probe high attomole levels of peptide standards, the reduced limit of detection observed here may be because of the high alkali metal concentrations in these samples or because of their biochemical complexity.

CONCLUSIONS

Successful relative and absolute quantitation of signaling peptides can be achieved in a single cell-sized sample using MALDI MS. A combination of appropriate sample preparation methods, analyte labeling with stable isotope techniques, and advanced MALDI MS approaches allows relative and absolute quantitation of analytes in nanoliter volume samples, including single cells (Table 1). Samples previously analyzed with MALDI MS can be further modified by spiking with the analyte of interest, addition of internal standards, and mixing with other sample(s). We expect these approaches will be used in a variety of small-volume investigations, in addition to the neuropeptide application demonstrated here.

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ACKNOWLEDGMENT

We would like to thank Dr. Suresh Annangudi for providing important information on the stable isotope labeling using the succinic anhydride approach, Dr. Elena Romanova for helpful discussion on MS/MS sequencing, and Stephanie Baker for carefully editing this manuscript. This material is based on work supported by the National Institutes on Drug Abuse under Award No. DA018310 to the UIUC Neuroproteomics Center on Cell to Cell Signaling and Award No. DA014879.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review May 21, 2008. Accepted July 14, 2008.

AC8010389