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Molecular histochemistry identifies peptidomic organization and reorganization along striatal projection units

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Abstract

Matrix assisted laser desorption ionization (MALDI) imaging mass spectrometry (IMS) provides a technical means for simultaneous analysis of precise anatomical localization and regulation of peptides. We explored the technical capability of MALDI-IMS for characterization of peptidomic regulation by an addictive substance along two distinct projection systems in the mouse striatum. The spatial expression patterns of Substance P and Proenkephalin, marker neuropeptides of two distinct striatal projection neurons, were negatively correlated at baseline. We detected 768 mass/charge (m/z) peaks whose expression levels were mostly negatively and positively correlated with those of substance P and Proenkephalin-A(218–228), respectively, within the dorsal striatum. Following nicotine administration, there was a positive shift in correlation of m/z peak expression levels with Substance P and with Proenkephalin-A (218–228). Our exploratory analyses demonstrate the technical capacity of MALDI-IMS for comprehensive identification of peptidomic regulation patterns along histochemically distinguishable striatal projection pathways.

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Keywords

nicotine addiction; caudate-putamen; striatum; peptides; peptidomics

Introduction

Peptides, defined as polymers of between 2 and 100 amino acids, are abundantly expressed in the mammalian brain; some 850 peptides are known to exist in the mouse brain (1). Intracellular peptides are associated with the nucleus, lysosome, or membrane and are involved in many processes within and across cells; they are collectively termed neuropeptides. While some neuropeptides contribute to many brain functions underlying behaviors relevant to emotion, motivation, learning and memory (1;2), their functional roles in the brain are still poorly understood. This is due to technical difficulties inherent in identification and characterization of peptides. *In situ* hybridization shows localization of precursor mRNAs of peptides, but does not ascertain how and where cleaved peptides, a biologically functional unit, are expressed and regulated. Immunohistochemical analysis provides good spatial resolution but is not amenable to precise quantitative analysis; radioimmunoassay is suitable for quantitative analysis, but lacks spatial resolution within a given brain region. Both approaches are limited to peptides for which reliable antibodies are available and neither reliably detects the entire population of cleaved products. None of these techniques are suitable for comprehensive, peptidomic characterization on the same tissues or sections. While mass spectrometry provides comprehensive peptide characterization without relying on antibodies, it lacks spatial resolution within a specific brain region.

A peptidomics approach is essential to improving our understanding of neuropeptide functions, as many peptides in the brain are likely to exert concerted regulation. Matrix assisted laser desorption ionization imaging mass spectrometry (MALDI-IMS) quantitatively and comprehensively profiles regulation of known and unknown peptides and their cleaved products with excellent spatial resolution in mammalian tissues (3–6). Because immunohistochemistry and MALDI-IMS cannot be applied on the same sections, the spatial location of peptides is validated by histological means (typically hematoxylin and eosin stain (H&E) and Nissl staining) on the same sections with MALDI-IMS. When anatomical units are immunohistochemically identified on adjacent sections, the expression of peptides coordinated with such anatomical units within a region cannot be determined on the same sections. We explored the capacity of this technique to characterize regulation of neuropeptides along small anatomical units within a target brain region on the same sections.

The dorsal striatum contains two distinct projection pathways. Neurons of one pathway contain Substance P and dynorphin and project to the substantia nigra pars compacta (i.e., striatal direct pathway) and those expressing enkephalins project to the globus pallidus and substantia nigra pars reticulata (i.e., striatal indirect pathway)(7). These two projection units are also distinct in their co-expression of other peptides and their functions (7;8). As these two distinct projection neurons are also relatively segregated in patchy compartments termed

striosomes and the surrounding, extrastriosomal matrix, peptides enriched in either compartment can, in theory, serve as markers for all the other peptides with a certain degree of spatial resolution consistent with striosome/matrix organization. We used nicotine, a highly addictive substance (9;10) that regulates many neuropeptides (11), to evaluate the technical utility of MALDI-IMS to characterize peptidomic regulation together with markers of the two distinct striatal projection pathways.

Methods and Materials

Mice

We used six male C57BL/6J mice (Jackson Lab, Bar Harbor, ME) for MALDI-IMS and two male C57BL/6J mice (Jackson Lab, Bar Harbor, ME) for tandem mass spectrometry (MS/MS) validation. The six mice for MALDI-IMS were divided into saline (N=3) and nicotine (N=3) treatment groups. Two drug-free mice were used for MS/MS validation of peptide identities. We used 5-week old mice, as this age corresponds to the developmental stage at which high levels of vulnerability to addiction-related effects to nicotine are seen in rodents (12–20) and humans (21).

Drug

(–)-Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO) for injection was dissolved in physiological saline at a concentration of 0.1 free base mg ml^{–1} and pH was adjusted to 6.8–7.6. Nicotine injections were administered subcutaneously at a volume of 2 ml kg^{–1}. Mice were sacrificed 48 hrs after nicotine injections (0.2 mg/kg), as our previous data show that the most robust, long-term addiction-related behavioral alteration occurs at this time point (19).

MALDI-IMS and peptide validation

We used MALDI-IMS and tandem mass spectrometry (MS/MS) to identify and validate peptides (see Methods, Fig. S1 and Fig. S2 in Supplement 1).

Statistical analysis When a pair of data had unequal variances or unequal sample sizes, statistical significance was determined by Welch's t-tests; the level of significance was adjusted by Bonferroni correction. Correlation data were analyzed by regression analysis and analysis of covariance (ANOCVA).

Results

With the use of MALDI-IMS, we identified 768 mass/charge (m/z) peaks, of which 417 m/z peaks (54%) were significantly regulated by nicotine (Table S1 in Supplement 2); 47% were up-regulated and 53% were down-regulated by nicotine. The two index peptides were validated by MS/MS (Fig S2 in Supplement 1) as Substance P (58–68, RPKPQQFFGLM, m/z = 1347.74) and Proenkephalin-A(218–228) (VGRPEWWMDYQ, m/z = 1466.65) (Fig. S3 in Supplement 1).

We analyzed the relative intensities of m/z peaks, representing expression levels pooled from the 200 μm × 200 μm sampling units (i.e., pixels) with a 8 μm thickness. Nicotine

downregulated Substance P and upregulated Proenkephalin-A(218–228) (Fig. 1A). Substance P and Proenkephalin-A(218–228) levels negatively correlated with each other at the baseline (see Fig. 1B, **saline**), indicating relative spatial segregation of these two peptides within the dorsal striatum (7). Image data confirmed the largely negative correlation between the two peptides at the baseline (see Fig. 1C, **saline**); relative high expression levels (see orange and yellow pixels) of Substance P correspond to relatively low expression levels (blue pixels) of Proenkephalin-A(218–228) in some areas, thereby establishing the validity of MALDI-IMS for identification and characterization of peptide distribution and regulation among the spatial units within the dorsal striatum.

Nicotine administration eliminated this negative correlation (Fig. 1B, nicotine) by downregulating Substance P and upregulating Proenkephalin-A(218–228) (see Fig. 1A). Nicotine reduced Substance P in the dorsal half, while it slightly raised Proenkephalin-A(218–228) levels (see Fig. 1C). This somewhat spatially independent regulation of the two peptides likely contributed to the disappearance of the overall negative correlation (see Fig. 1B, **nicotine**).

We next sought to identify the expression levels of all detected m/z peaks along the Substance P and Proenkephalin-A(218–228) axis. Correlation of spatial expression patterns between each of the two index peptides and all detected m/z peaks was used to evaluate their basal affiliation with either index peptide and their alteration by nicotine. At the baseline, expression of peaks with high mass values (>1400 m/z) were negatively correlated with Substance P among $200\ \mu\text{m} \times 200\ \mu\text{m}$ spatial units (Fig. 2A **saline**, **blue**), suggesting that Substance P and the majority of large sized peptides are inversely expressed in the spatial units of the dorsal striatum. Nicotine induced more positively correlated expression of m/z peaks among low mass values (<1600 m/z) and slightly shifted correlation coefficients in both positive and negative directions among high mass values between 1400 and 1900 m/z (see Fig. 2A, **orange**). These changes resulted in an overall shift from negative to positive medians with increased variance (Fig. 2B).

Basal expression levels of m/z peaks generally were positively correlated with Proenkephalin-A(218–228) across the entire mass value range (Fig. 2C, **saline**), suggesting that the majority of peptides and Proenkephalin-A(218–228) were similarly expressed in the $200\ \mu\text{m} \times 200\ \mu\text{m}$ spatial units (Fig. 2C, **saline**). Nicotine administration resulted in a positive shift in the number of m/z peaks correlated with this index peptide across the entire mass value range with a sizable number of negative correlation shifts between 1100 to 2000 m/z (Fig. 2C, **nicotine**). The net result was a positive shift in the correlation median with a higher level of variance (Fig. 2D).

Overall, m/z peaks were negatively correlated with Substance P and positively correlated Proenkephalin-A(218–228) at baseline (Fig 2E, see blue dots in the upper left quadrant). Nicotine increased the number of m/z peaks that are positively correlated with both Substance P and Proenkephalin-A(218–228) (see orange dots in the upper right quadrant), while simultaneously increasing a sizable number of m/z peaks more negatively correlated with both index peptides (see orange dots in the lower left quadrant). Taken together, these

analyses, based on the spatial units within the dorsal striatum, revealed a highly coordinated peptidomic expression shift along the two index peptides.

Peptides of which spatial expression levels exhibit high negative correlation values can be used to explore the anatomical, and more importantly, functional heterogeneity within the dorsal striatum. Such new, more reliable markers might identify a novel projection or compartmental organizations independent of or in align with the known projection and striosomal organizations. We thus explored the technical capability to identify peptides that more clearly differentiate the $200\ \mu\text{m} \times 200\ \mu\text{m}$ spatial units. Using correlation coefficients among intensities of all validated peptides (Table S3 in Supplement 2), we characterized their spatial expression patterns within the dorsal striatum (Fig 3A, **Saline**). Peptides with low mass values tended to show high positive correlation coefficients with each other; those with high mass values are similarly positively correlated with each other. By contrast, those with low and high mass values are negatively correlated with each other, indicating that peptides with different mass values are spatially differentiated among the $200\ \mu\text{m} \times 200\ \mu\text{m}$ spatial units. Nicotine altered this pattern by increasing positively correlated peptides particularly (Fig. 3B, **Nicotine**). Validation by MS/MS identified several peptides with high negative correlations. For example, we identified peptide peaks at 1244.63 m/z and 1271.61 m/z and validated them as somatostatin and secretogranin-2, respectively (see Table S2 in Supplement 2). These two peptides are enriched in striosomes and matrix, respectively (7). As expected, these two peptides showed a high negative correlation, reflecting their spatial segregation. Moreover, spatial expression levels of each of these markers was negatively correlated with secretogranin-1 and neurogranin, indicating that different cleaved products of secretogranin are spatially differentiated and neurogranin represents another spatial dimension that negatively correlated with both a striosomal and a matrix marker.

Discussion

We used MALDI-IMS analyses to identify a large number of putative peptides and their cleaved products *in situ* from the dorsal striatum of saline- and nicotine-treated mice. The identities of the two index peptides were confirmed by MS/MS to be Substance P and Proenkephalin-A(218–228). Relative to these two peptides that represent two spatially and functionally separate anatomical units within the dorsal striatum, other putative peptides showed highly coordinated expression patterns and alterations. Our data demonstrate the technical capability of MALDI-IMS for performing peptidomics analyses of expression and regulation along two distinct projection units.

Several technical limitations are worthy of mentioning. *In situ* mass spectrometry, in general, distinguishes peptides that differ by ~ 0.1 Da. It should be cautioned, however, that peaks detected might not represent all different peptides, as the same peptides could be detected at different mass values due to modification (e.g., isotopic ion and oxidation of methionine). Thus we used the term, a “peak”, to simply indicate a signal detected at a given mass value.

As distinct peptides have different ionization efficiencies generating different responses at the detector, comparison of absolute expression levels is achievable only by using an

internal standard and measuring absolute abundance (22). Thus, the primary focus of our study was on relative levels of the same peptide peaks between saline and nicotine conditions in this study. Another general technical limitation of MALDI-IMS is that large normalized peak intensities (e.g., >10) tend to be more reliably detected than those of small normalized peak intensities (<2–3). Among the 768 m/z peaks detected, 39% had normalized peak intensities below 2 (see Table S1 in Supplement 2). Moreover, MALDI-IMS detected even very weak levels of regulation (e.g., 12.3% up-regulation at 1487.69 m/z and 10.3% down-regulation at 1522.74 m/z; see Table S1 in Supplement 2). Given that it is thought that technical variability of peptide expression levels is low (23), this is likely near the detection limit of difference between two experimental groups. These data attest the reliability and sensitivity of our method.

Although the actual resolution limit of the grid size is $10\mu\text{m} \times 10\mu\text{m}$ (24), the grid size of $200\mu\text{m} \times 200\mu\text{m}$ is commonly used to detect peptides because m/z peaks need to be accumulated within each grid sampling to be detectable. We thus chose the $200\mu\text{m} \times 200\mu\text{m}$ grid; the movement of the laser was programmed to cover the entire $200 \times 200\mu\text{m}$ area with randomly generated movements. This resolution was sufficient to detect peptide expression and coordinated peptide regulation. Substance P and proenkephalin-A(218–228) are relatively enriched inside and outside the striosomal compartment, which appear to be irregularly-shaped clusters in the mouse dorsal striatum and are a few hundred micrometers long (25;26). The spatial resolution of the current analysis is sufficient to detect the relative compartmental organization of peptides within the dorsal striatum.

Our analyses revealed a concerted shift in expression levels of many peaks, potentially representing peptides and their cleaved and modified products, along two distinct anatomical and functional units. Such peptidomics analyses suggest that a majority of peptides change their expression in a highly coordinated manner along these two distinct striatal projection pathways. Moreover, our correlation matrix serves as a means to identify novel markers of compartmental organization within a given structure. This analytical approach can be applied to any brain region where index peptides are reasonably segregated. Our analyses open a novel analytical possibility for identification of peptidomic alterations relevant to histochemical subunits within a region of interest.

Our data demonstrate the versatile technical capability of *in situ* mass spectrometry. The hypothesis-testing element of this technique is that it characterizes coordinated expression of peptides along anatomically identifiable units. The same data set can be reanalyzed based on different anatomical units, thereby expanding the ability to test hypotheses in an unlimited fashion. Moreover, this technique has a hypothesis-generating component, as it identifies any peptide that shows coordinated regulation without relying on a known anatomical unit or marker peptide.

Further methodological and technical refinement is needed to validate our MALDI-IMS data. It is generally more difficult to detect neuropeptides than proteins and lipids due to their low abundance. Many methodological steps influence the ultimate detection of neuropeptides, including washing solvent, washing time, type of matrix, matrix

concentration, pH, and organic modifiers of the matrix solution (27). A future challenge is to improve the preparation conditions for validation and peptide loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression levels of Substance P and Proenkephalin-A(218–228) in the dorsal striatum
A: Average intensities (+SEM error bar) of Substance P and Proenkephalin-A(218–228) after saline and nicotine administration. $*p < .001$ (Welch's t-test). **B:** Intensity of the mass/charge (m/z) peaks of Substance P and Proenkephalin-A(218–228) in $200 \mu\text{m} \times 200 \mu\text{m}$ sampling units. Blue; Saline, $R = -.497$, $F_{(1, 39)} = 12.82$, $p < .001$, Orange; Nicotine, $R = -.0393$, $F_{(1, 46)} = .071$, $p = .791$. **C:** *In situ* mass spectrometry images of the dorsal striatum. Upper panel: scanned optical images from a Scanmaker 9800XL. Each dot represents the center of the $200 \mu\text{m} \times 200 \mu\text{m}$ unit from which samples were collected. Heat map of

Substance P ($m/z = 1347.7$, middle panel) and Proenkephalin-A(218–228) ($m/z = 1466.7$, bottom panel). A schematic drawing of the dorsal striatum from which samples were taken is shown on the right. dStr, dorsal striatum; NAc, nucleus accumbens; CC, corpus callosum.

Figure 2. Correlation coefficient for expression levels of peptides with Substance P or with Proenkephalin-A(218–228)

Each dot represents a correlation coefficient (R) of each peptide with either Substance P (**A**) or with proenkephalin (**C**). Blue, saline; Orange, Nicotine. **B and D:** The horizontal line represents the median. Whiskers represent $\pm 1.5 \times$ interquartile range (IQR, 75 to 25 percentile range), and open circles represent outliers that are defined as data points outside the whiskers. Each dot represents the R of each peptide. Blue, saline; Orange, Nicotine. Substance P: $F_{1, 2178} = 39.923$, $p < .001$ (Levene's homogeneity of variance test), $*p < .001$ (Welch's t-test). Proenkephalin-A(218–228): $F_{1, 2178} = 257.13$, $*p < .001$ (Levene's test), $*p < .001$ (Welch's t-test). **E:** R's of peptides with Substance P and Proenkephalin-A(218–228). Blue, Saline, $R = -.412$, $F_{(1, 1096)} = 223.5$, $p < .001$. Orange, Nicotine, $R = -.139$, $F_{(1, 1080)} = 21.41$, $p < .001$. Two regression lines are significantly different ($F_{(1, 2176)} = 134.362$, $p < .001$, ANCOVA).

Figure 3. Correlation matrix (Pearson correlation coefficients) between the peptides acquired from $200\mu\text{m} \times 200\mu\text{m}$ units

The rows and columns represent the mass/charge (m/z) values (**A,B**). Red and blue dots represent positive and negative correlation values. Color coding reflects positive correlation in red, negative correlation in blue, and no correlation in white. The intensity of each hue reflects the degree of correlation.