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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) (MALDI-IMS) provides a technical means for simultaneous analysis of precise anatomic localization and regulation of peptides. We explored the technical capability of matrix-assisted laser desorption ionization mass spectrometry 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 expression levels of substance P and proenkephalin A (amino acids 218–228), respectively, within the dorsal striatum. After nicotine administration, there was a positive shift in correlation of mass/charge peak expression levels with substance P and 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.

*Keywords:* Caudate-putamen, In situ mass spectrometry, Nicotine addiction, Peptides, Peptidomics, Striatum

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Peptides, defined as polymers of 2–100 amino acids, are abundantly expressed in the mammalian brain; ~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. Although 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 situation is due to technical difficulties inherent in identification and characterization of peptides. In situ hybridization shows localization of precursor messenger RNAs 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; conversely, 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 the aforementioned techniques are suitable for comprehensive, peptidomic characterization on the same tissues or sections. Although 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 understanding of neuropeptide functions, as many peptides in the brain are likely to exert concerted regulation. Matrix-assisted laser desorption ionization (MALDI) imaging mass spectrometry (IMS) (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 histologic means (typically hematoxylin-eosin and Nissl stains) on the same sections with MALDI-IMS. When anatomic units are immunohistochemically identified on adjacent sections, the expression of peptides coordinated with such anatomic 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 anatomic 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 neurons expressing enkephalins project to the globus pallidus and substantia nigra pars reticularis (i.e., striatal indirect pathway) (7). These two projection units are also distinct in their coexpression of other



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peptides and their functions (7,8). Because 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 Laboratory, Bar Harbor, Maine) for MALDI-IMS and two male C57BL/6J mice (Jackson Laboratory) 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 because this age corresponds to the developmental stage at which high levels of vulnerability to addiction-related effects of nicotine are seen in rodents (12–20) and humans (21).

### Drug

(−)-Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, Missouri) for injection was dissolved in physiologic saline at a concentration of .1 free base mg/mL, and pH was adjusted to 6.8–7.6. Nicotine injections were administered subcutaneously at a volume of 2 mL/kg. Mice were sacrificed 48 hours after nicotine injections (.2 mg/kg), as our previous data showed 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 MS/MS to identify and validate peptides (Figures S1 and 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.

## 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 upregulated and 53% were downregulated by nicotine. The two index peptides were validated by MS/MS (Figure S2 in Supplement 1) as substance P (amino acids 58–68, RPKPQQFFGLM, m/z = 1347.74) and proenkephalin A (amino acids 218–228, VGRPEWWMDYQ, m/z = 1466.65) (Figure 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 8-μm thickness. Nicotine downregulated substance P and upregulated proenkephalin A (218–228) (Figure 1A). Substance P and proenkephalin A (218–228) levels negatively correlated with each other at baseline (Figure 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 (Figure 1C, saline); relatively high expression levels (see orange and yellow pixels) of substance P corresponded to relatively low expression levels (blue pixels) of proenkephalin A (218–228) in some areas, 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 (Figure 1B, nicotine) by downregulating substance P and upregulating proenkephalin A (218–228) (Figure 1A). Nicotine reduced substance P in the dorsal half and slightly raised proenkephalin A (218–228) levels (Figure 1C). This spatially independent regulation of the two peptides likely contributed to the disappearance of the overall negative correlation (Figure 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 baseline, expression of peaks with high mass values (>1400 m/z) were negatively correlated with substance P among 200 μm × 200 μm spatial units (Figure 2A, saline, blue), suggesting that substance P and most 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 positive and negative directions among high mass values between 1400 m/z and 1900 m/z (Figure 2A, orange). These changes resulted in an overall shift from negative to positive medians with increased variance (Figure 2B).

Basal expression levels of m/z peaks generally were positively correlated with proenkephalin A (218–228) across the entire mass value range (Figure 2C, saline), suggesting that most peptides and proenkephalin A (218–228) were similarly expressed in the 200 μm × 200 μm spatial units (Figure 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 m/z and 2000 m/z (Figure 2C, nicotine). The net result was a positive shift in the correlation median with a higher level of variance (Figure 2D).

Overall, m/z peaks were negatively correlated with substance P and positively correlated with proenkephalin A (218–228) at baseline (Figure 2E, blue dots in upper left quadrant). Nicotine increased the number of m/z peaks that are positively correlated with substance P and proenkephalin A (218–228) (Figure 2E, orange dots in upper right quadrant), while increasing a sizable number of m/z peaks more