APPLICATION NOTE

#58 A Novel Reactive Matrix for Mass Spectrometry Imaging of Neurotransmitters

Application & Background

The complex chemical signaling that occurs in the brain is responsible for almost all normal physiologic function (1). This signaling is carried out primarily by neurotransmitters (NTs), which are substances that carry chemical information between neurons in the brain. Due to their integral role in so many biological processes, researchers are continually striving to better understand how NTs function in various diseased states (1). Given the high degree of correlation between regions of the brain and their function, one very useful analytical method to characterize NT distribution within brain tissue is matrix-assisted laser desorption/ionization- (MALDI-) mass spectrometry imaging (MSI) (2, 3). The advantages of MALDI-MSI over more traditional analytical techniques, such as immunohistochemistry, is that MALDI-MSI is label-free, highly specific, and almost infinitely channeled, allowing researchers to distinguish between parent compounds and their metabolites and to detect thousands of analytes from a single experiment (3). However, analysis of NTs by MALDI-MSI can be difficult due to their poor ionization efficiency and their relative low abundance in brain tissue (3, 4). Thus, researchers have developed *in situ* chemical derivatization methods for easier detection and identification of NTs via MALDI-MSI (3).

In this study, a 2-fluoro-1-methyl pyridinium (FMP) cation was used as part of the structure of a reactive matrix that selectively target phenolic and primary amine groups that are present in many NTs. This reaction significantly increases their ionization efficiency. Using this novel compound and an optimized spraying protocol developed using the HTX TM-Sprayer, the distributions of several NTs in both healthy and diseased state rodent brain tissues were characterized via MALDI-MSI.

Experimental

Experimental Design

All animal experiments were conducted in agreement with the European Communities Council Direction of November 24, 1986 (86/609/EEC) on the ethical use of animals and were also approved by local ethics committee at the Karolinska Institute (N350/08 and N105/16). Male Sprague-Dawley rats (n = 8) were kept in separate, air-conditioned cages, under a 12-hour light/dark cycle with food and water provided ad libitum. Animals were injected with 6-hydroxydopamine (6-OHDA) under anesthesia to induce a loss of striatal dopamine (DA) to make them models for Parkinson's disease (PD), a neurodegenerative disease characterized by the loss of dopaminergic neurons. Two weeks after 6-OHDA administration, animals in the treatment group received intraperitoneal administration of 10 mg/kg L-DOPA and 7.5 mg/ kg benserazide once daily for 21 days. Animals were sacrificed by decapitation 30 minutes after the last intraperitoneal injec-tion treatment, which was benserazide (7.5 mg/kg) and deuterated L-DOPA-d_3 (10 mg/kg). Animals in the control group were sacrificed 4 weeks after the original 6-OHDA treatment. Two animals were un-treated. The brains of the animals were removed after decapitation and flash frozen and stored at -80°C.

Sample Preparation

Rat brain tissues were sectioned at 12 µm, using a Leica CM1900 microtome and thaw-mounted onto indium-tin-oxide-coated (ITO) coated glass slides. Slides were stored at -80°C until they were needed. Prior to internal standard and reactive matrix deposition, slides were placed under vacuum for 15 minutes to dry. To evaluate the quantitative response of the protocol for selected NTs, 0.2 µl of 10, 2.5, 0.63, 0.16, 0.039, and 0 ng/µl of homovanillic acid (HVA)-d_2 and $^{13}C_6$-DA were applied to the cortex region of the control rat brain tissue section. Before FMP-10 derivatization, internal standard solutions (DA-d4 33 µg/mL in 50% methanol and HVA-d5 33 µg/mL) were sprayed over the tissue sections with the parameters presented in Table 1. Then, the reactive matrix, FMP-10, available from Tag-ON AB (Uppsala, Sweden) was applied using the HTX TM-Sprayer with the parameters also displayed in **Table 1**.

Matrix	Internal Standards: DA-d_4 and HVA-d_5	FMP-10
Solvent	50:50 MeOH:H_2O	70:30 ACN:H_2O
Matrix Concentration (mg/mL)	0.033	0.18
Flow Rate (mL/min)	0.08	0.08
Spray Nozzle Velocity (mm/min)	1100	1100
Spray Nozzle Temperature (°C)	90	80 (90 for 10 µM imaging)
Track Spacing (mm)	2	2
Number of Passes	6	30
Nitrogen Pressure (psi)	6	6
Spray Pattern	HH	HH
Drying Time (sec)	0	0
Nozzle Height (mm)	40	40

Table 1. Spraying parameters for the deposition of internal standards and FMP-10 onto tissue sections.

Data Collection and Analysis

MALDI-MSI experiments were performed using either a MALDI-time-of-flight/time-of-flight (TOF/TOF) (rapifleX, Bruker Daltonik GmbH) or a MALDI Fourier transform ion cyclotron resonance (FTICR) (SolariX, 7T 2ω, Bruker Daltonik GmbH) mass spectrometer equipped with a Smartbeam II 2kHz laser or a Smartbeam 3D 10kHz laser (rapifleX) and operated in positive ion mode.

MS/MS analysis was performed on a MALDI FTICR instrument with a collision energy voltage of 30V in order to confirm the identification of analytes. Both instruments were externally calibrated using red phosphorous and FTICR-MS was also internally calibrated with the lock mass of the FMP-10 cluster ion (*m/z* 555.2231).

Data Collection and Analysis cont'd

Analyses were conducted in a random order, and flexImaging (Bruker Daltonik GmbH, v.4.1) was used to visualize the data. TOF/TOF spectra were normalized to total ion count (TIC), and FTICR spectra were normalized to the root mean square (RMS) of all data points. DataAnalysis (Bruker Daltonik GmbH, v.4.2) was used to examine the data, and msIQuant (v.2.0.1.15) was used for relative and absolute quantitation.

Results

Optimization of FMP-10 Derivatization

Derivatization of analytes prior to MALDI-MSI analysis aims to both increase the ionization/desorption yield of the compounds of interest and to reduce ionization bias by adding the same chemical group and permanent charge to each analyte. The goal of these experiments was to image precursor, signaling, and metabolized compounds of catecholaminergic and serotonergic NTs by targeting their phenolic hydroxyl, primary amine, and secondary amine chemical moieties (**Figure 1**).

The optimized deposition of the FMP-10 derivatization agent, using an automated sprayer (HTX TM-Sprayer) provided very homogenous crystallization with an average crystal size between 15-20 µm. The crystallize matrix layer was also found to be very reproducible between technical replicates. Using the optimized condition, FMP-10 derivatization enabled high sensitivity imaging of NTs e.g. DA, serotonin (5-HT), and gamma-aminobutyric acid (GABA) with no need for any further sample treatment such as heating or incubation (**Figure 1**).

High Lateral Resolution Imaging of NTs

The ability to control the temperature of the nozzle of the TM-Sprayer allowed for some tissues to be sprayed with FMP-10 at 90°C for high-resolution imaging at 10 µm. Unlike most of derivatization agents, FMP-10 does not need incubation and heating to complete the reaction. The latter advantage facilitates high lateral resolution imaging of tissue sections without delocalization of target compounds.

All major NTs e.g. DA and GABA were detected despite the smaller laser spot size, and the analytes localized to brain substructures with no significant sign of delocalization (**Figure 2**).

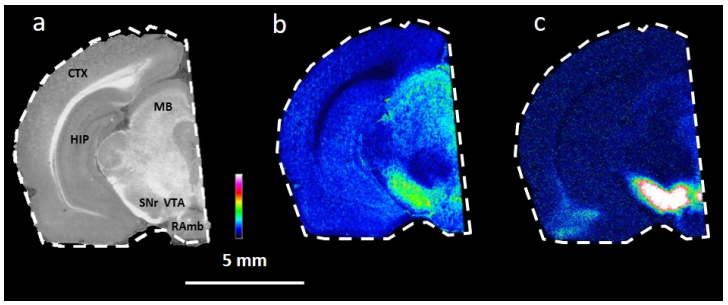
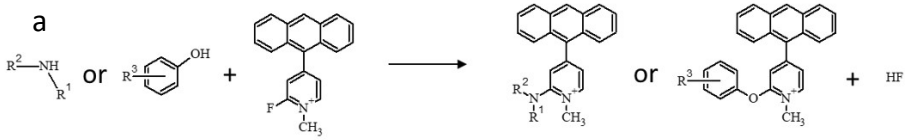


Figure 2. High lateral resolution MALDI-MSI analysis of rat brain tissue section. (a) Optical image of one hemisphere of a coronal rat brain tissue section. (b) Distribution of FMP-10 derivatized GABA, intensity scale 0-100%. (c) Distribution of FMP-10 derivatized DA, intensity scale 0-15%. Data were acquired by TOF-TOF MS (rapifleX, Bruker Daltonics) with lateral resolution of 10 µm. At each sampling position, 80 shots were used to acquire data for the *m/z* 300–900 range. Abbreviations: CTX, cerebral cortex; HIP, hippocampus; MB, midbrain; RAmb, midbrain raphe nuclei; SNr, Substantia nigra; VTA, ventral tegmental area.

Identification of NTs in Rat Brain Tissue

FTICR-MS allowed for the putative identification of dopaminergic, serotonergic NTs and metabolites from rat brain tissues based on ultra-high mass accuracy. In order to confirm these identifications, MS/MS analysis was performed on rat brain tissue for selected NTs e.g. DA and norepinephrine to verify the co-localization of the fragment ions to the parent NT in the rat brain images (**Figure 3**). Relative stability of the chemical structure of FMP-10 in collision induced dissociation process performs the fragmentation of derivatized compounds mainly from the target compound side (not the FMP-10 side). The latter property provides unique MS/MS fragmentation patterns for similar substances e.g. NTs and metabolites.



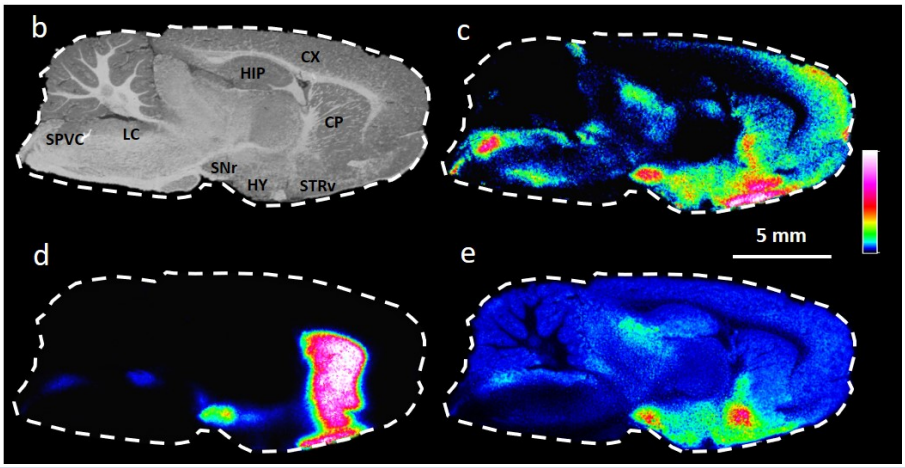


Figure 1. Improved detection of neurotransmitters and their metabolites facilitated by on tissue derivatization. (a) The reaction between the FMP-10 reactive matrix and amines and phenolic hydroxyls. R1, R2 and R3 indicate variable substituents. (b) Optical image of a sagittal tissue section from a rat brain. (c–e) MALDI–MSI analysis facilitated by FMP-10 reactive matrix maps the distribution of (c) 5-HT, (d) DA, and (e) GABA. Logarithmic color scaling for ion intensity from 0-60% was used for 5-HT and DA while a linear color scale ion intensity from 0-100% was used for GABA. Images were acquired by MALDI FTICR–MS with lateral resolution of 50 µm. At each sampling position, 100 shots were used to acquire data for the *m/z* 150-1500 range. CP, caudate putamen; CX, cerebral cortex; HIP, hippocampus; HY, hypothalamus; LC, locus coeruleus; SPVC, spinal vestibular nucleus; STRv, ventral striatum; SNr, substantia nigra pars reticulata.

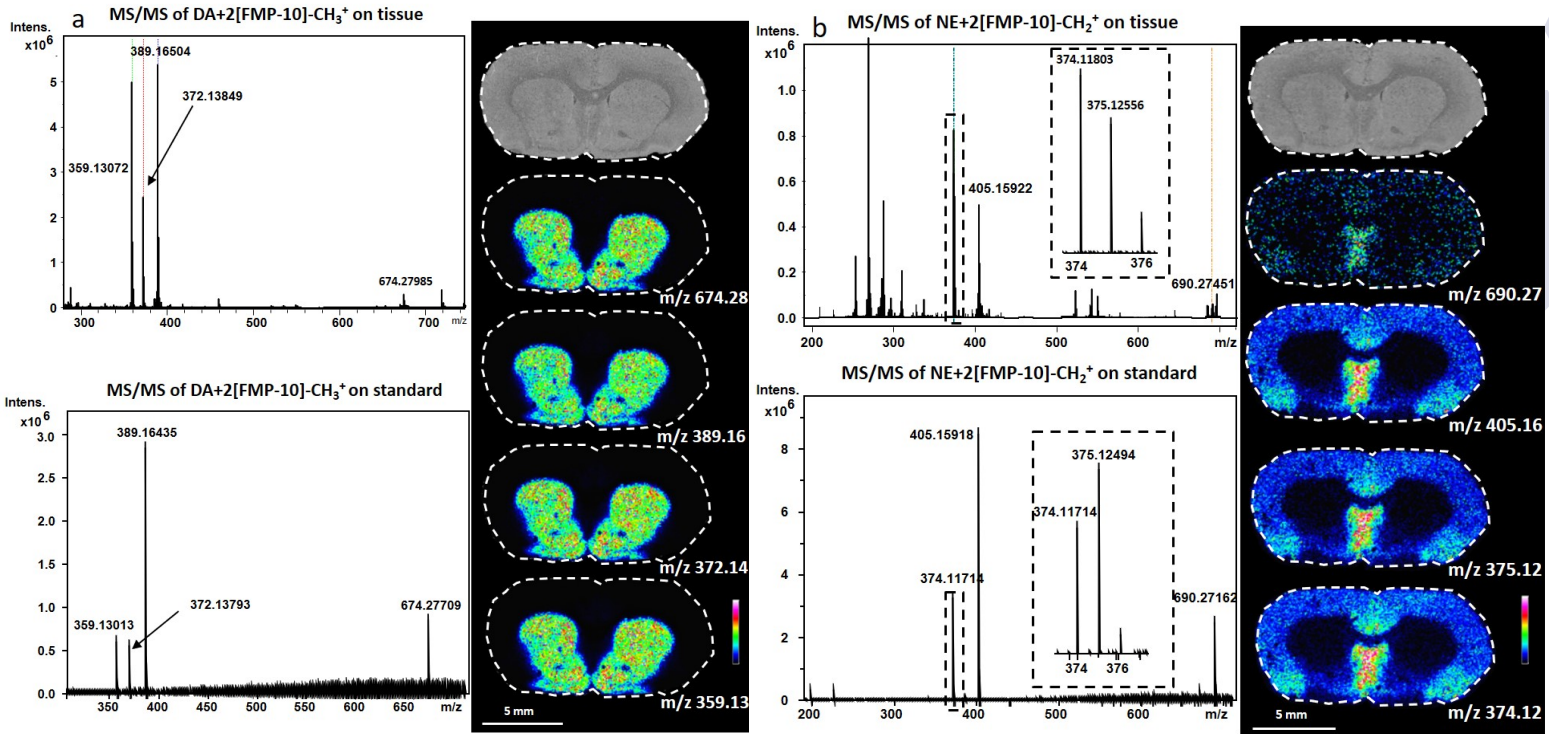


Figure 3. Identification of NTs and metabolites by MS/MS fragmentation of their FMP-10 derivatized species. MS/MS imaging experiments were conducted on rat brain tissue section. The MALDI MS/MS images show the distributions of product ions of FMP-10 derivatized (a) DA (double derivatized, *m/z* 674.28) and (b) NE (double derivatized, *m/z* 690.27). Upper panels represent average MS/MS spectra acquired on brain tissue sections, while lower panels show MS/MS spectra of standards spotted and derivatized on stainless steel plate. The ranges of the color intensity scale are 0-60% for all DA and NE precursors and fragments except for NE (*m/z* 690.27) where the intensity scale was set to 0-100% (a, b). MS/MS images were acquired using a MALDI FTICR-MS (solariX, Bruker Daltonics). At each sampling position, 100 shots were used to acquire data. The isolation window was set to 1 Da and the collision voltage was 30 V. Scale bar, 5 mm; lateral resolution = 80 µm.

MALDI-TOF-MSI of NTs in PD rat model

Even using low mass resolution MALDI-ToF instrument, several serotonergic, dopaminergic NTs and other phenol and/or amine containing endogenous metabolites could be visualized in 6-OHDA-lesioned rat model of PD with and without L-DOPA treatment. Administration of an L-DOPA-d_3 analog as the last dose enabled us to distinguish L-DOPA-d_3-derived metabolites from those derived from chronic L-DOPA administration and endogenously synthesized L-DOPA. As expected, the concentration of DA in the striatal regions of the diseased animals was significantly lower than those in the non-treated animals. Also in concordance with previous studies, GABA was increased in the striatal regions of the diseased animals compared to the control (**Figure 4**) (6).

L-DOPA, a precursor to DA, was administered to a group 6-OHDA-lesioned rats for 3 weeks prior to sacrifice in order to visualize the distribution of L-DOPA metabolites. While L-DOPA treatment was not correlated with any recovery of DA levels, treatment increased GABA in the striatum, but depleted NE in the cortex, in the lesioned side of the brain. Moreover, administration of the deuterated analogue of L-DOPA as the last dose facilitated imaging of NTs derived from the dosed drug (**Figure 4**).

Linear Response of Derivatized NTs and Quantitative MALDI-MSI

To evaluate the feasibility of the protocol toward absolute quantitation of NTs, different concentrations of isotope labeled DA and HVA were spotted on a control rat brain tissue section. In quantitative MSI uniform application of the reactive matrix as well as the yield of the reaction of the derivatization agent in different concentrations are the keys of success. Spraying FMP-10 using the HTX TM-Sprayer resulted calibration standard curves for DA and HVA with good linearity ($R^2 \geq 0.999$) not only for single derivatized DA and HVA but also for double derivatized DA (**Figure 5**).

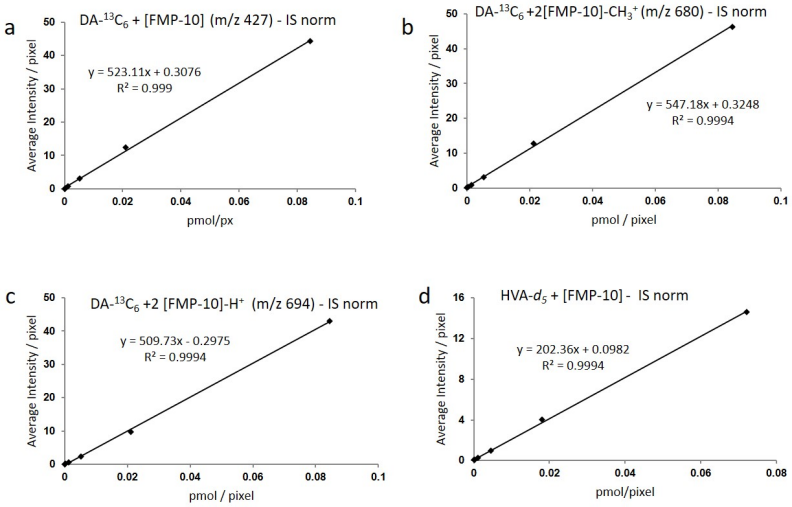


Figure 5. Quantitative analysis of FMP-10 derivatized standard solutions of NTs. Calibration standard curves for DA and HVA. Deuterated DA and HVA standard solutions were spotted on brain tissue sections. Quantitative data were acquired using MALDI FTICR-MS (solariX, Bruker Daltonics) for DA as (a) single derivatized, DA as double derivatized with loss of (b) methyl and (c) proton as well as (d) single derivatized HVA.

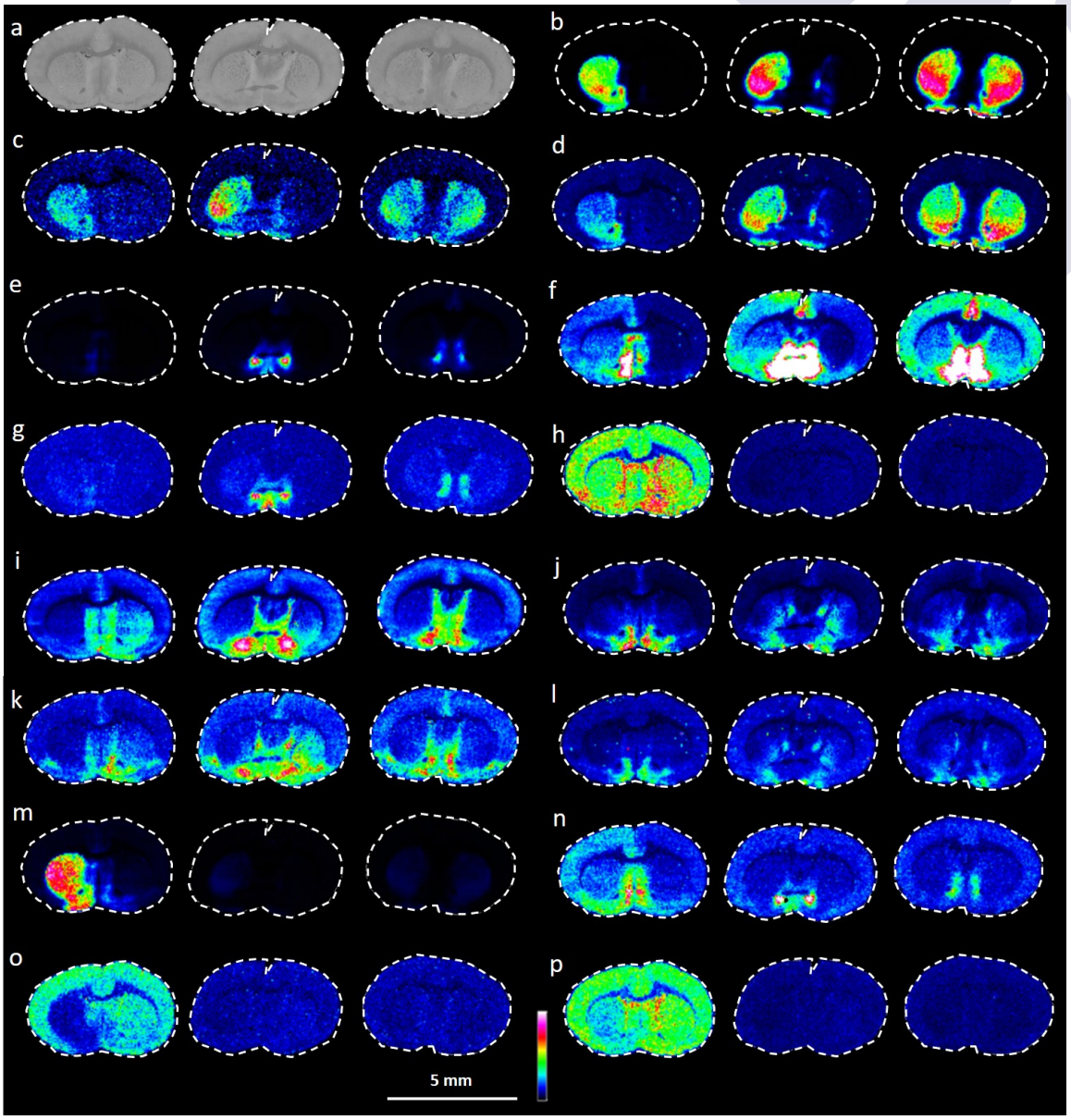


Figure 4. MALDI-MS images of NTs and metabolites acquired from control and PD disease model treated with L-DOPA. Imaging experiments were conducted on brain tissue sections from unilateral sham-lesioned (a, right panel), unilateral 6-OHDA-lesioned (a, middle panel), and unilateral 6-OHDA-lesioned animals that were treated with sub-chronic daily L-DOPA for 4 weeks, with a final dose of L-DOPA-d_3 (a, left panel). The subsequent MALDI-MSI images show the distributions of different NTs and metabolites in these three animals. The range of the color intensity scales and *m/z* values are presented in parenthesis. (b) DA (0-100%, 674.5), (c) 3-MT (0-100%, 702.5), (d) DOPAC (0-50%, 673.4), (e) NE (0-100%, 690.5), (f) NE (0-5%, 690.5), (g) EP (0-100%, 704.5), (h) 3-O-Methyldopa (0-100%, 479.3), (i) GABA (0-80%, 353.3), (j) 5-HT (0-100%, 444.4), (k) 5-HIAL (0-100%, 443.3), (l) 5-HTOL (0-100%, 445.3), (m) DA-d_3 (0-100%, 677.5), (n) NE-d_3 (0-60%, 693.5), (o) L-DOPA-d_3 (0-10%, 468.3) and (p) 3-O-Methyldopa-d_3 (0-100%, 482.3). At each sampling position, 1800 shots were used to acquire data for the *m/z* 300–900 range. Data were acquired by TOF-TOF MS (rapifleX, Bruker Daltonics) with lateral resolution of 150 µm.

Conclusions

NTs play essential roles in almost all aspects of biology and are known to dysfunction in a variety of diseases and disorders (1). The discovery of a new reactive MALDI matrix, FMP-10, makes MALDI-MSI analysis of NT distribution and abundance an appealing and robust method for further research on the role of NT in human health and disease. Importantly, this research has demonstrated that FMP-10 allows for the detection and quantitation of a variety of NT at high lateral-spatial-resolution in a wide variety of sample types.

The tissue images and MS data presented here were provided by Dr. Reza Shariatgorji, Dr. Anna Nilsson, and Dr. Per Andrén from the Uppsala University. This work was originally presented in: Shariatgorji M, Nilsson A, Fridjonsdottir E, Vallianatou T, Källback P, Katan L, Sävmarker J, Mantas I, Zhang X, Bezard E, Svenningsson P, Odell LR, Andrén PE. Comprehensive mapping of neurotransmitter networks by MALDI-MS imaging. Nat Methods. 2019 Oct;16(10):1021-1028. doi: 10.1038/s41592-019-0551-3.

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