Characterizing peptides in individual mammalian cells using mass spectrometry

Stanislav S Rubakhin & Jonathan V Sweedler

Department of Chemistry and the Beckman Institute, University of Illinois, Urbana, Illinois 61801, USA. Correspondence should be addressed to J.V.S. (jsweedle@uiuc.edu).

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Cell-to-cell chemical signaling plays multiple roles in coordinating the activity of the functional elements of an organism, with these elements ranging from a three-neuron reflex circuit to the entire animal. In recent years, single-cell mass spectrometry (MS) has enabled the discovery of cell-to-cell signaling molecules from the nervous system of a number of invertebrates. We describe a protocol for analyzing individual cells from rat pituitary using matrix-assisted laser desorption/ionization MS. Each step in the sample preparation process, including cell stabilization, isolation, sample preparation, signal acquisition and data interpretation, is detailed here. Although we employ this method to investigate peptides in individual pituitary cells, it can be adapted to other cell types and even subcellular sections from a range of animals. This protocol allows one to obtain 20–30 individual cell samples and acquire mass spectra from them in a single day.

INTRODUCTION

For more than a decade, one of our research goals has been the detection and characterization of cell-to-cell signaling peptides (SPs) using a variety of analytical methods. We describe a sample preparation protocol, optimized for direct single-cell measurements using MS, that is based on our prior investigation of invertebrate neurons and subcellular structures[1–5], as well as on single mammalian cell sampling methods recently reported during the 33rd annual meeting of the Society for Neuroscience and in *Analytical Chemistry* as well as highlighted in *Nature*[6–8].

Because biological structures contain numerous cells of differing biochemistry and function, SPs are often differentially expressed and processed. Cell-to-cell SPs perform a wide variety of functions in the body, and are involved in neuromodulation, neurotransmission, cell outgrowth and hormonal signaling between organs[9–14]. SPs have been reported to contribute to multiple aspects of behavior and play important roles in the physiological mechanisms of thirst (angiotensin)[15], feeding (neuropeptide Y and galanin)[16,17] and pain (enkephalins)[18–20]. Furthermore, a large body of research strongly suggests that an imbalance in chemical signaling mechanisms is associated with a number of neurological disorders[21–31].

Clearly, further research to understand the various intrinsic physiological processes in SPs is vital. This requires comprehensive analyses, relevant to the anatomical regions in which these peptides are found, such as the brain, gut or spleen. However, simultaneous detection, identification and quantification of the peptidome of complex multicellular structures create a number of analytical challenges, exacerbated by the inherent small quantities of SPs, their broad dynamic range and the diversity of their chemical and physical properties. As a consequence, the analytical measurement can be complicated, frequently yielding results that are difficult to interpret. One solution for resolving these challenges is to implement technologies that can simultaneously examine multiple analytes at the single-cell level.

Given this investigative dilemma, how does one approach the study of SPs? Prior single-cell research has relied heavily on immunohistochemistry[31,32]. Although the sensitivity and selectivity of immunohistochemical techniques can be quite high, these methods require analyte preselection, making it difficult to determine many compounds simultaneously and/or to identify unknown substances. Alternatively, one can consider biochemical methods of characterization of not only SPs but also classical transmitters. In fact, in the largest neurons, multiple neurotransmitters were identified more than 20 years ago[33] using single-cell biochemistry. Today, advanced analytical chemistry provides a suite of techniques to characterize SPs[34–40], the most noteworthy of which is MS.

Over the last decade, many novel neuropeptides have been determined with MS, making it a remarkably successful technology for the discovery of novel SPs. There are two primary mass spectrometric approaches to neuropeptide discovery. The first involves sample homogenization and separation (usually by a liquid chromatography system) followed by electrospray ionization. This combination has often been used for peptidomic studies to characterize hundreds of new SPs[41–46].

A second successful approach is matrix-assisted laser desorption/ionization (MALDI) MS, which was developed in 1988 (refs. 47–49). MALDI MS is a versatile technique for the detection and characterization of a broad range of biomolecules. Peptide profiling using MALDI provides a mass spectral fingerprint, characterized by the accurate molecular mass and relative intensity of the peptide signals comprising the sample. When using direct measurement methods, quantitative information can also be obtained, chiefly by implementing standards or by analysis of samples labeled with different stable isotopes[50]. Further, even a simple comparative analysis of MALDI MS-acquired peptide profiles from different samples produces a wealth of qualitative knowledge about their biochemical composition. Relatively pure samples, such as individual fractions resulting from multistage chromatographic separation, as well as complex mixtures (including enzymatic digests, homogenates of biological tissues or even structurally intact sections of organs and entire organisms) can be surveyed with MALDI MS. Although there are several ionization methods available, we highlight MALDI because of its compatibility with single-cell MS.

Peptide characterization and identification often requires tandem MS (MS/MS). State-of-the-art time-of-flight/time-of-flight (TOF/TOF) mass spectrometers have proven capable of *de novo*

peptide sequencing[51,52] using sample sizes of less than 100 fmol[7,53,54], typically providing a mass accuracy of 0.2–0.4 Da for fragment ions[7]. This combined sensitivity and mass accuracy is adequate for identifying many post-translational modifications and has a high probability of yielding at least partial sequence information for peptides, as well as identifying the prohormone precursors for many unknown SPs.

What about single neuron measurements? Single neuron analysis using small-sample MALDI MS has been one of the most successful approaches for detecting intercellular signaling molecules. In fact, direct single invertebrate cell and cell cluster MS studies have been used to discover hundreds of new invertebrate SPs over the last decade[1,55–59]. The ability of single-cell MALDI MS to enable discovery of new neuropeptides depends on ideally matching the analytical methodology to the properties of the sample under investigation. For example, the femtomole to zeptomole limits of detection intrinsic to MALDI MS are amenable to the detection of SPs, because they are generally expressed in relatively larger amounts than other cellular peptides. Indeed, in all invertebrate and vertebrate cells that we have studied using MS, most of the identifiable peaks in the appropriate mass range represent known or putative SPs. However, mass spectra obtained from non-peptidergic neurons tend not to show significant peaks (outside of a mass region containing an envelope of lipid-related peaks). This is partly because MALDI MS selectively detects peptides and proteins when MALDI matrices such as 2,5-dihydrobenzoic acid and α-cyano-4-hydroxy-cinnamic acid are used. Also, these MALDI matrices can be dissolved in water or water-containing solvents, producing an acidic environment (pH ~2) that inhibits the enzymatic activity of most proteases and peptidases, and promotes extraction of intact SPs[1,60].

Another important benefit of single-cell MS analysis is reduced sample complexity in the peptide mass region. The mass spectra from single cells typically have increased signal-to-noise ratios for SPs, which clarifies data interpretation. For instance, in single-cell MALDI MS, peptide signal identification can often be assigned by matching the molecular masses of the compounds predicted to be processed from prohormones. Because the majority of known SP prohormones are processed into multiple peptides, it is possible to use endogenous intracellular enzymatic digestion to verify prohormone expression and identify the final prohormone cleavage products. Additionally, the large amount of histological, immunohistochemical and genetic information available for many brain structures aids peptide identification.

Whereas the majority of initial single-cell MS studies have been performed on individual molluscan neurons that are several hundred micrometers in size, in several cases, smaller cells (10–30 μm) have been assayed[7,55,61,62]. As one example of micromanipulation techniques for handling even smaller volume samples for MS analysis, we demonstrated the MALDI MS analysis of multiple regions in processes of a cultured single neuron[4] and a single *Aplysia californica* atrial gland secretory organelle[3]. Pushing the sample size for this analytic technique down to the single-micron scale and the attoliter to femtoliter volume regime, more than ten peptides from at least four separate genes were detected within each vesicle. This study demonstrates that dramatic enhancements in mass sensitivity (low attomole) are achievable with commercial instrumentation using low-volume sampling methods, thus enabling the determination of a peptide profile from even a subcellular biological sample.

The experimental design of single mammalian cell MALDI MS projects

Here, we demonstrate that MALDI MS can be successfully employed for peptide profiling of individual mammalian cells. We use cells collected from the intermediate pituitary, containing a well-characterized peptidergic cell type, for development and optimization of a single mammalian cell MALDI MS protocol. As illustrated in **Figure 1**, the protocol contains four major sample preparation steps, followed by mass spectra acquisition, mass spectra analysis and analyte identification.

Even with the accuracy of peptide mass measurements attainable in a single MS experiment, as in a majority of biological investigations, meaningful results with single-cell MALDI MS are obtained only with an appropriate number of repeats. Typically, although the minimal number is three, the repeats required are determined by the type of study and questions posed[63,64]. Properly prepared single-cell samples will ensure that reproducible results are achieved. Over a period of several years, our group has observed similarities among peptide profiles of many identified neurons of *A. californica*, even though we have used different mass spectrometers and different investigators performed the sample preparation and analyses[1].

Another important consideration for potential users of this protocol is calibration of the mass spectrometer using peptide standards that have molecular masses close to the molecular masses of the analytes under investigation. Calibration of the mass spectrometer with appropriate standards deposited in close proximity to the single cell sample will ensure high measurement accuracy and, in some cases, will allow identification of detected analytes.

The described protocol has been validated for rat intermediate pituitary cells; however, it is also adaptable to many other cell types. We have actually used many elements of the protocol in our previous studies of single secretory vesicles[3], small segments of neurites[4] and neurons from mammalian brain[7]. Therefore, it is feasible that the protocol can be relatively easily adapted to any isolated, small individual biological structure. There are a variety of techniques that enable isolation of single cells and subcellular regions, such as synaptoneurosomes and organelles, from different tissues and organs. Enzymatic dissociation[65–67], homogenization followed by filtering[68] and laser capture microdissection[69] are

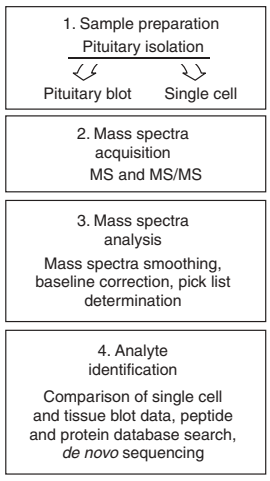

Figure 1 | Major steps in the investigation of peptide content in individual mammalian cells using single-cell MALDI MS.

among the available options. There are a number of cell types producing significant amounts of peptides that can be investigated with the described protocol. A few possible targets include hypocretin- and melanin-concentrating hormone-containing neurons from the lateral hypothalamic area[70,71], arginine vasopressin-producing magnocellular neurons in the supraoptic and paraventricular nuclei[72], dynorphin-, enkephalin- and substance P-synthesizing neurons of striatum[73] and insulin-containing pancreatic β-cells[74].

The advantages and disadvantages of single mammalian cell MALDI MS

Single-cell MALDI MS provides us with a unique tool to identify and characterize peptides from a single cell or even subcellular regions, as well as facilitating the detection of processing and post-translational modifications in both known and unknown SPs. The simplified mass spectra resulting from a single-cell experiment and the added information on cell-to-cell differences are key advantages of the methodology. There are, however, two drawbacks to the process. The first is sensitivity; peptides can be present below the detection level of the instrument. The second relates to the low throughput of the protocol and the large number of cells present even in a small region of the brain. Thus, this method does not allow measurement of even a fraction of the biochemically distinct cells contained in most targeted mammalian tissues. Efforts to address this low-throughput issue have already been initiated[75,76].

MATERIALS

REAGENTS

- 2,5-dihydrobenzoic acid (Sigma, cat. no. A3539)
- 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (Sigma, cat. no. H3375)
- Acetonitrile (Fisher Scientific, cat. no. A998-4) ! CAUTION Acetonitrile is toxic. When handling, wear gloves and use a pipetting aid.
- Calcium chloride (Sigma, cat. no. C7902)
- Glycerol (Sigma, cat. no. G7893)
- Magnesium chloride hexahydrate (Sigma, cat. no. M2393)
- Magnesium sulfate heptahydrate (Sigma, cat. no. M2773)
- 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) (Bruker Daltonics, part no. 222570)
- Potassium chloride (Sigma, cat. no. P5405)
- Potassium phosphate monobasic (Sigma, cat. no. P5655)
- Sodium bicarbonate (Sigma, cat. no. S5761)
- Sodium chloride (Sigma, cat. no. S5886)
- Sodium phosphate (Fisher Scientific, cat. no. S374-500)
- Trifluoroacetic acid (Sigma, cat. no. T6508) ! CAUTION Trifluoroacetic acid is highly corrosive. When handling, wear gloves, goggles, use a pipetting aid and operate in a ventilated chemical hood.

EQUIPMENT

- 1 ml insulin syringe with permanently attached 28G needle (Becton-Dickinson)
- 1-mm outer diameter, 0.75-mm inner diameter, thin-walled, single-barrel standard borosilicate glass tubing (World Precision Instruments, cat. no. TW100F-4)
- AxioCam MRc camera (Carl Zeiss)
- Axiovert 25 inverted microscope (Carl Zeiss)
- Diamond scriber (similar to the one that can be ordered at http://www.emsdiasum.com/, Electron Microscopy Sciences, cat. no. 62107-ST)
- FlexAnalysis software (Bruker Daltonics)
- FlexControl software (Bruker Daltonics)
- BioTools software (Bruker Daltonics)
- Mascot search engine (http://www.matrixscience.com/)
- Manual XYZ-manipulator (similar to M3301, World Precision Instruments, cat. no. M3301R)
- Mascot software (Matrix Science Ltd)
- MHW-3, three-axis water hydraulic fine micromanipulator (Narishige)
- MR Grab software package (Carl Zeiss)
- P-80 Flaming/Brown micropipette puller (Sutter Instrument Company), for fabrication of small-diameter glass micropipettes
- PP-83 vertical puller (Narishige), for creating large-diameter glass micropipettes
- ProbeOn Plus microscope slides (Fisher Scientific, cat. no. 15-188-52)
- Sharp Dumont #5 straight forceps (Fine Science Tools Inc., cat. no. 11253-20)
- Small animal guillotine (similar to a NEMI Model 701, NEMI Scientific Inc.)
- 4- to 6-inch scissors with pointed tips
- Leica MZ 7.5 high-performance stereomicroscope with 7.9:1 zoom (Leica Microsystems Inc.)
- Plastic 36-mm-diameter Petri dishes
- 1.5 ml plastic vials
- Gloves, goggles and paper such as Kimwipes absorbent wipes (Kimberly-Clark Inc., cat. no. 34155)

REAGENT SETUP

Animals Five-month-old male Sprague–Dawley rats were used (Harlan Sprague Dawley Inc.); however, a variety of cells and tissues from different animals can be studied following the guidelines presented here. Protocols for animal care and procedures have been approved by the UIUC Institutional Animal Care and Use Committee and are in full compliance with federal guidelines for the humane care and treatment of animals. ! CAUTION All experiments involving animals must be performed according to national and institutional regulations.

Stabilizing solution 33% (vol/vol) glycerol mixed with 67% modified Gey's balanced salt solution (mGBSS) containing (in mM) 1.5 $CaCl_2$, 4.9 KCl, 0.2 KH_2PO_4, 11 $MgCl_2$, 0.3 $MgSO_4$, 138 NaCl, 27.7 $NaHCO_3$, 0.8 Na_2HPO_4, 25 HEPES and 10 glucose, pH 7.2.

MALDI matrix solution 2,5-dihydrobenzoic acid saturated in a 1:1 mixture of acetonitrile:0.1% (vol/vol) aqueous trifluoroacetic acid. Many additional MALDI matrices are available for investigation of small metabolites, peptides, proteins, nucleotides and polysaccharides, and can be used in biochemical profiling of single cells.

EQUIPMENT SETUP

Setup for single mammalian cell sample preparation The setup consists of an Axiovert 25 inverted microscope with two micromanipulators mounted on the sample table (**Fig. 2**). Three objectives, ×10, ×20 and ×32, are used to observe isolated cell clusters, individual cells and final samples at different stages of sample preparation. Variable relief contrast technique, or VAREL, allows the highest flexibility for adjusting the contrast view of cells, including some intracellular structures.

MALDI TOF MS UltraFlexII MALDI TOF/TOF mass spectrometer (MALDI TOF/TOF) (Bruker Daltonics). A range of MALDI TOF instruments can be used to produce single-cell spectra; in addition to the Bruker instrument used here, a Voyager-DE STR BioSpectrometry Workstation (Applied Biosystems) also produces high-quality spectra from single-cell samples (for other details, see ref. 7). In what follows, we describe the instrument (**Table 1**) and software settings for a Bruker Ultraflex II TOF/TOF mass spectrometer, as the protocol has been validated using this system. Likely, similar procedures and settings will enable other mass spectrometers and software packages to collect and process similar data with minimal adaptation of these procedures.

WWW resources SWISS-PROT Protein Sequence Database, searchable through the SRS6 engine (http://srs6.ebi.ac.uk/) and maintained by the European Bioinformatics Institute; literature reports on rat peptides through http://pubmed.com/ and http://www.scopus.com/; SwePep (http://www.swepep.com/—a searchable database on peptides, including neuropeptides, present in nervous systems of different organisms; NeuroPred (http://neuroproteomics.scs.uiuc.edu/cgi-bin/neuropred.py)—a tool to predict cleavage sites in SPs and related prohormones; ProSight PTM (https://prosightptm.scs.uiuc.edu/)—a web portal allowing identification and characterization of intact proteins (and their post-translational modifications) using the top-down approach.

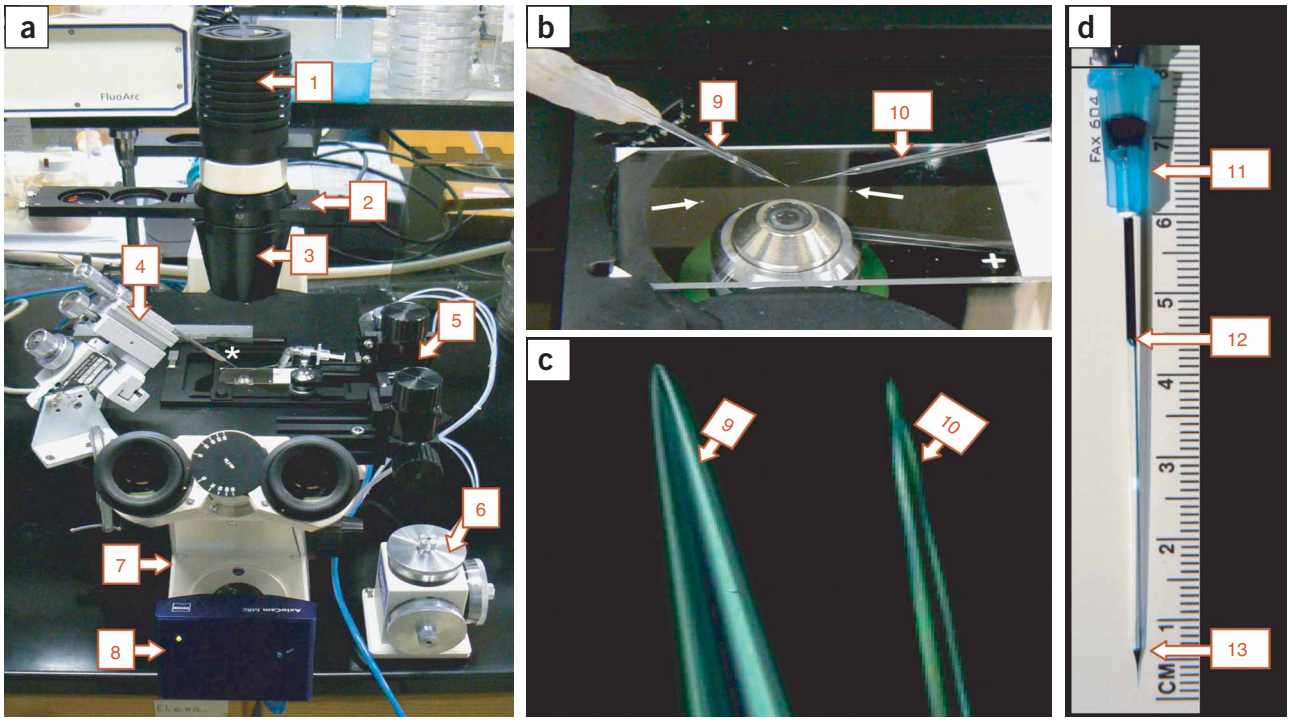

Figure 2 | Hardware setup used for single-cell preparation for MALDI MS investigation. (**a**) Front view of the setup consisting of a Zeiss Axiovert 25 inverted microscope with two micromanipulators mounted on its specimen stage. (1) Halogen light source; (2) Ph/H/Var slider with VAREL window placed in the light path; (3) 0.4 condenser; (4) MM-1 micromanipulator used to manage large-diameter glass micropipette filled with MALDI matrix solution; (5) MHW-3 micromanipulator's driving unit enabling fine movements as well as coarse manipulations of small-size micropipette; (6) MHW-3 micromanipulator's drum-type control unit hydraulically connected to the driving unit and used for fine control of the position of small-size micropipette; (7) Axiovert 25 microscope stand; (8) AxioCam MR color digital camera. (**b**) Enlarged view of the area marked in **a** by an asterisk. Arrows pointing to small white speckles are samples ready for MALDI MS investigation; (9) tip of large-diameter glass micropipette used for MALDI matrix application; (10) tip of small-diameter micropipette employed in cell transfer. (**c**) Microphotograph of large and small glass pipette tips used for MALDI matrix deposition and single-cell transfer (dark field image). Numbering is the same as in **b**. (**d**) The process of filling large-diameter glass micropipette with matrix solution. cThe 25G needle (11) attached to 1 ml plastic syringe is inserted into the barrel of the micropipette. The barrel is filled with the solution only to the level where the needle tip ends (12). Capillary and gravity forces take care of filling the tip of the micropipette (13). To improve visibility, the matrix solution is colored with Fast Green.

PROCEDURE

Preparation for pituitary isolation

1| Prepare stabilization solution as described in the REAGENT SETUP.

2| Clean and sterilize surgical/dissection instruments by ultrasonic treatment in SPECTRA-SONIC (or similar) solution (Spectrum Surgical Instruments Co.) at pH 7 for 5–10 min, followed by autoclaving according to the manufacturer's manual.

3| Collect gloves, goggles, vials and paper.

Rat pituitary isolation

4| Decapitate the animal using a sharp, clean guillotine. Decapitation should be done in proximity to the skull through the cervical vertebrae, preferably between the atlas and the axis (for graphical illustrations of anatomical features described in this protocol, see refs. 77 and 78). Decapitation too close to the skull may lead to dislocation of the bones composing the ventral floor of the cranium, causing a shift in the position of the pituitary, possibly damaging this structure. The main goal of Steps 5 and 6 is to gain access to the rat brain, allowing its careful removal from the skull. Although there are several approaches for successfully accomplishing this goal, only one is described here.

5| Using a sheet of firm paper, push the skin in a rostral direction, exposing the cranium.

TABLE 1 | The mass spectrometer operating parameters for the MS (positive polarity, reflector) and tandem MS (positive polarity, LIFT) modes.

Mode of operation	Pulsed ion extraction delay (ns)	Ion source voltage '1' (kV)	Ion source voltage '2' (kV)	Reflector voltage '1' (kV)	Reflector voltage '2' (kV)	Reflector detection voltage (kV)	Sample rate (Giga–sample s^{-1})
MS	20	25	21.7	26.3	13.8	1.867	0.5
Tandem MS		8	7.2	29.5	13.8	1.849	0.011

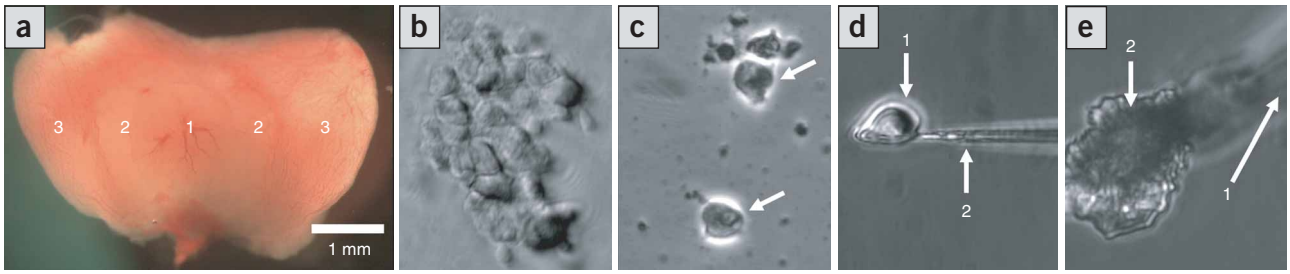

Figure 3 | Preparation of single pituitary intermediate lobe cell for MALDI MS investigation. (**a**) Rat pituitary preserved in 50 µl of 30% glycerol mGBSS solution. The main pituitary regions are numbered as follows: (1) posterior lobe (neurohypophysis); (2) intermediate lobe; (3) anterior lobe (adenohypophysis). Well-preserved elements of the circulatory system are visible as fine red threads. (**b**) Small group of cells isolated from intermediate lobe. (**c**) Dissociated individual cells (white arrows). (**d**) Single cell attached to small-diameter glass micropipette during transfer to clean location on a glass slide: (1) cell; (2) small-diameter glass pipette. (**e**) MALDI matrix application: (1) MALDI matrix-filled large-diameter glass pipette positioned in very close proximity to the cell; (2) patch of MALDI matrix forming around the isolated cell.

6| Insert the tip of a long scissors into the neural canal and cut the cranial bones in the frontal plane, which lies above the most dorsal part of the mandible, that is, the condyloid and coronoid processes. Possible damage to the brain induced by the scissors will not affect the integrity of the pituitary, and should be minimal when a trained investigator performs this operation.

7| Remove the previously cut dorsal part of the cranium, insert a fine forceps inside the brain and carefully lift it.
▲ **CRITICAL STEP** Sliding a spatula or another dissection tool underneath the brain for its removal should be avoided owing to possible damage to the pituitary. The rat pituitary is a several-millimeters-wide structure (**Fig. 3a**) located at the base of the brain. Typically, after brain removal, the pituitary remains on the bone, held in place by a sheath of connective tissue.

8| Remove the connective tissue, using a fine forceps to free the pituitary.

9| Place the pituitary into 300 µl of the described stabilization solution at room temperature (18–25 °C) for 10–15 min. Results of our previously reported capillary electrophoresis and MS investigations have demonstrated that glycerol treatment does not significantly change the biochemical profiles of cells[4,7,79].
■ **PAUSE POINT** Samples can be stored for a week in a refrigerator at ~4 °C in a small plastic or glass vial until use.

Single pituitary intermediate lobe cell isolation

10| Fabricate large- and small-diameter glass micropipettes (**Fig. 2c**) using specialized pullers such as the P-80 Flaming/Brown micropipette puller or the PP-83 vertical puller. Any of these pullers can be set appropriately and employed in preparing both types of micropipettes.

11| Fill a syringe with MALDI matrix solution. To do so, insert the syringe needle all the way into the barrel of a large-diameter micropipette and fill a portion of the syringe barrel; in our experiments, approximately one-fifth of the barrel is filled (**Fig. 2d**). Make sure that the solution has no undissolved crystals that might obstruct the free flow of solution inside the micropipette during the filling procedure, as well as outside the pipette during MALDI matrix deposition.
? TROUBLESHOOTING

12| Position the micropipettes vertically with the pipette tip down. Capillary and gravity forces will ensure complete filling of the tips.
▲ **CRITICAL STEP** Using premanufactured glass tubing with microfilaments is advantageous in achieving highly reproducible filling of micropipette tips with solution.

13| Place the pituitary in a Petri dish containing the stabilizing solution.

14| Under visual control with a stereomicroscope, isolate a small group of intermediate lobe cells (**Fig. 3b**) using two manually operated glass micropipettes as microscalpels. The intermediate lobe of the rat pituitary is an easily recognizable whitish structure localized between the more reddish anterior and posterior lobes (**Fig. 3a**).

15| Deposit the group of isolated cells on a glass slide mounted on the microscope's specimen guide. The presence of glycerol in the stabilizing solution covering these cells protects the sample from complete dehydration. Glass slides of varying thickness, size and/or chemical composition can be used; however, transparent glass slides with a conductive coating (e.g., Bruker Daltonics' glass slides for MALDI MS imaging, catalog no. 237001) aid in achieving better signal quality.

16| Intermediate pituitary cells are loosely attached to each other; drag the cell group on the glass slide surface using glass micropipettes to separate individual cells. Cells can be separated also by repetitive pipetting of this cell assembly through a glass pipette with a tip diameter slightly larger than the size of the isolated pituitary cell group. To make such a pipette, cut an appropriate length of any micropipette tip and fire-polish it. A piece of elastic tubing, plugged at one end and attached to the pipette at the other end, will allow positioning of this device with two fingers while simultaneously pipetting the liquid by squeezing and relaxing the tubing. This operation should be performed under visual control with the assistance of an inverted microscope to ensure the quality of single-cell isolation.

▲ CRITICAL STEP Owing to possible damage to some cells during the separation process, the extracellular solution should be sampled and analyzed using MS for the presence of bioanalytes. Collection of extracellular media and its subsequent analysis can be done in the same manner as described below for single-cell analysis—except that extracellular media will be collected with a fine glass pipette. Analysis of extracellular media at three random locations on the glass slide can be sufficient to verify the presence of analytes in the media. This analysis can be done at any time point of the experiment. However, this controlled probing performed at the start and end of an experiment can be very important when a different type of cell is to be studied with this protocol. We recommend repeating the entire cell isolation process should such contamination occur. If needed, an additional wash step for the sample or a single cell can also be introduced after isolation.

17| After isolating the desired number of separated individual cells (**Fig. 3c**), position the large-diameter micropipette filled with MALDI matrix solution and the small-diameter micropipette into holders attached to the appropriate micromanipulators. Holders can be replaced by a variety of sticky waxes (e.g., see the left micropipette mounting in **Fig. 2b**).

Single pituitary intermediate lobe cell collection and MALDI matrix application

18| Scrape and lift a single pituitary cell using a small-diameter glass micropipette attached to an MHW-3 micromanipulator (**Fig. 3d**). Use the fine micromanipulator controls for this operation.

19| By engaging the microscope's specimen guide, move the glass slide to the desired position where the isolated cell is to be placed and the MALDI matrix applied.

20| Lower the small-diameter glass micropipette and deposit the cell on the glass slide surface. A coordinated motion of the microscope's specimen guide and the micropipette may be required to dislodge the cell from the pipette tip.

21| Under visual control using an inverted microscope, slowly approach the deposited individual cell with the tip of the large glass pipette filled with MALDI matrix. After establishing contact, an increase in the volume of liquid surrounding the cell can be seen, with almost immediate MALDI matrix crystal formation (**Fig. 3e**). The amount of matrix to be applied is determined by visual observation and must be optimized for different samples and MALDI matrix solutions. In some cases, to facilitate MALDI matrix application, the very tip of the glass micropipette can be broken by gently pressing it against the glass slide surface at the spot where the individual cell is located.

▲ CRITICAL STEP To avoid cross-contamination, use a new application pipette for each single-cell sample prepared.

? TROUBLESHOOTING

22| Several samples that contain only the solution surrounding the cells (with no cells) should be prepared to verify that the analytes detected in single cells arise from the cellular contents and not from the extracellular solutions. Dipping of a small-diameter glass micropipette into the solution allows enough material to be collected for this control measurement. The same spot collection and spot deposition are helpful in increasing the sampled volume.

23| Additionally, samples containing peptide standards should be placed in close proximity to individual cell-containing preparations. Such placement will ensure optimal calibration of the mass spectra and eliminate mass errors induced by an uneven glass slide surface or misalignment after it is positioned into the instrument. To prepare such samples, peptide standards (e.g., peptide calibration standard mix II) can be added to the MALDI matrix solution and deposited onto the glass slide surface using micropipettes.

24| After preparation of the required number of single-cell samples, the spot containing multiple pituitary cells should be wiped relatively clean using Kimwipes absorbent wipers. Loading a glass slide containing excess liquids, such as water, into the MALDI mass spectrometer operating at high vacuum results in longer pumping times. Glycerol has a low vapor pressure and can be successfully loaded into the mass spectrometer. Experiments with metal matrices in glycerol, performed by Tanaka *et al.*[48], paved the way for the development of MALDI MS.

25| Locating the individual cell samples on a glass slide loaded into the mass spectrometer is difficult unless appropriate grid- or guidelines are drawn. We use a diamond scriber to manually draw arrows that point toward the sample location (**Fig. 4**). Although MS instruments are equipped with a video camera, the magnification as well as the resolution is not equivalent to that of a microscope, and so this step is important. Marking of the slide is most successfully performed using a stereomicroscope.

26| Either mount or load the glass slide with prepared single-cell samples on an appropriate sample holder. Bruker Daltonics supplies adapters specifically designed for standard glass slides (e.g., MTP Slide Adapter, catalog no. 235380) that can hold several slides. Glass slides can also be mounted using Scotch tape on top of other types of adapters.
▲ **CRITICAL STEP** Mounting of glass slides should be done in such a way that their surface will occupy a similar plane as the surface of regular MALDI sample plates. Positioning too low or too high may result in difficulties in obtaining a good signal, issues with calibration or, in the case of positioning it too high, breaking of the slide into pieces during the loading process, which in turn may lead to instrument malfunction.

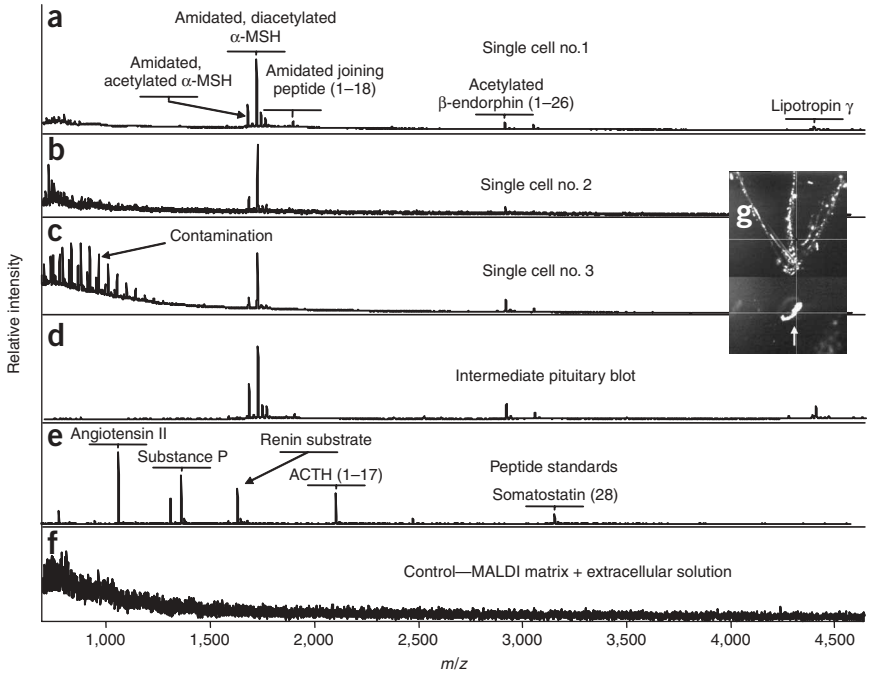

Figure 4 | Single-cell MALDI MS reveals expression of a number of POMC peptides in individual cells from the intermediate pituitary. (**a**–**c**) Representative mass spectra acquired from individual pituitary cells. (**d**) Mass spectrum demonstrating the cumulative peptide profile of intermediate pituitary blotted on a glass slide surface. (**e**) Mass spectrum of calibration standard peptides deposited in close proximity to single-cell sample. (**f**) Mass spectrum of control spot containing MALDI matrix and solution surrounding cells after their separation. (**g**) Microphotograph of glass slide loaded inside a Bruker Ultraflex II source. Guide arrow drawn on the glass surface is visible at the top half of the microphotograph. The arrow's tip is pointed toward the single-cell sample, also marked with a small white arrow. In **a**–**e**, a number of peaks, are not visible in this figure owing to their lower relative intensity compared to the most intense peptide signals, and therefore are not labeled.

MALDI MS analysis of single-cell samples

27| Load samples into the instrument and operate the mass spectrometer. The specific MS operation details are described in the EQUIPMENT SETUP section (**Table 1**). The first step is to find and analyze samples containing standard peptides. This allows calibration of the mass spectrometer and optimization of data acquisition parameters. There are many instrument settings that an investigator can select. Some options may allow better detection sensitivity or enhanced mass resolution—important parameters for the investigation of biochemically complex samples. Unfortunately, improvements in detection sensitivity often lead to a decrease in mass resolution, and vice versa. To work with single-cell samples, we operate the instrument in reflector mode, optimized for highest sensitivity (for details, see the EQUIPMENT SETUP section; **Table 1**). MALDI MS allows generation and detection of mostly monoprotonated (deprotonated in negative operation mode) ions.

28| Following the guide arrows, locate and measure single-cell samples or control samples containing extracellular solution. Analysis of single-cell samples should be started by determining the optimal level of laser intensity. Excess energy applied to a sample will deplete the limited analyte content too quickly. However, too little irradiation will produce poor mass spectrum quality. Therefore, we recommend that one first locate a sample and then begin analysis, with the laser power set below the standard threshold of laser intensity, when only digitization noise is obtained. Increasing the laser power then results in the detection of analyte signals. The appearance of only chemical noise and signals originating from the MALDI matrix may indicate that an inadequate amount of analyte was present in the sample, or that the target has to be moved to allow alignment of the correct region in the laser beam profile with the so-called 'hot spot' in the sample. This last adjustment highlights an intriguing feature of MALDI MS where even slight repositioning of visually homogeneous samples can lead to a drastic increase in signal intensity and overall quality. In our hands, samples containing a single mammalian cell can be probed twice while still obtaining high-quality mass spectra.

29| Process the mass spectra with the appropriate software. The main goal of this step is to generate a peak list, which will then be used in the SP identification stage. Depending on the quality of mass spectra obtained, additional processing such as recalibration, use of different filters and baseline corrections may be performed. Software packages provided by mass spectrometer manufacturers, or a variety of commercially available utilities designed to work with mass spectra, allow different types of mass spectral processing. For scientists who are involved in collaborative projects and who need a software tool for basic processing and analysis of single-cell mass spectra, we recommend a free demonstration version of MoverZ software (Genomic Solutions). This program is relatively compact, and works with spectra generated with a variety of instruments, including those generated by Ultraflex II workstations.

BOX 1 | APPROACHES TO IDENTIFY ANALYTES DETECTED IN SINGLE CELLS

Successful detection of peptides in single cells and determining their molecular mass is a first step toward analyte identification. There are several approaches that allow identification of SPs with a high degree of certainty. These include the following: (a) direct tandem mass spectrometric (MS/MS) sequencing of peptides in single-cell samples; (b) direct MS/MS peptide sequencing in the tissue where the cell under investigation originated; accurate mass information obtained in single-cell experiments guides selection of peptides for MS/MS sequencing; (c) MS/MS of analytes purified by liquid chromatography; again, the accurate mass information obtained in single-cell experiments guides selection of peptides for MS/MS sequencing; and (d) peptide identification using comparative analysis of all available information, including the accurate peptide mass detected in the single cell, and biochemical and genetic information about prohormone and/or peptide expression in the tissue or cells of interest. Obviously, if multiple peptides from a single prohormone are observed in a mass spectrum, then identification of other peptides from the same prohormones is easier. As an example, the observation of peaks that match ten different prohormone processing products in an individual pituitary cell is similar to detecting ten tryptic peptides from a particular protein and yields high confidence that the signals are from the prohormone. More information on MS peptide identification, including *de novo* sequencing, can be found in refs. 80–87. Owing to the low amount of SPs present in individual mammalian cells, a combination of approaches (b) and (d) is effective for analyte identification (described in Steps 30–36).

Identification of analytes detected in single cells

30| Using the peak list generated in Step 29, search—by accurate mass—the online databases (see the WWW resources section) to identify likely peptide candidates. SwePep is an easily searchable and comprehensive database for peptides and neuropeptides detected in mammalian nervous tissue (see **Box 1** for comments relating to the identification of analytes detected in single cells as well as refs. 80–87).

31| Dissect a small piece of intermediate pituitary: holding it by a fine forceps, blot (lightly touch) it on the surface of a metal MALDI sample plate.

32| Cover this blot with 0.5 µl of MALDI matrix solution, thus preparing a dried droplet sample.

33| Load a sample plate into the instrument and acquire spectra in reflector mode with the settings described above (**Table 1**). Determine the presence of analytes of interest in the sample, specifically searching for peptides predicted from the identification process in Step 30.

34| For the Ultraflex II, set the FlexControl settings as described in the EQUIPMENT SETUP section for MS/MS peptide sequencing (**Table 1**). Run the Ultraflex II in LIFT MS/MS mode and obtain MS/MS spectra of peptides of interest.

? TROUBLESHOOTING

35| Process MS/MS mass spectra with FlexAnalysis software. When the peak list for a particular mass spectrum is generated, open the BioTools software and proceed with the interpretation of MS/MS data. BioTools allows the user to identify a detected peptide by its fragmentation pattern using three different approaches: (i) by comparing the measured peptide fragmentation pattern with a predicted pattern from the putative peptide sequence (see Step 30) entered into the software tool; (ii) by exporting information about peptide fragmentation into the Mascot search engine, and comparing this information with *in silico*-produced fragmentation sets of proteins originating from different species; (iii) by employing specific algorithms for *de novo* peptide sequencing using the RapiDeNovo module (Bruker Daltonics).

36| For peptides detected in the single-cell samples that are positively identified in Step 35, collect information on their related prohormone sequence and post-translational processing (see WWW resources such as NeuroPred). Using this information, predict the presence of other products of prohormone processing in studied cells. Compare these predictions with the peak lists generated from the single-cell MS experiments and verify them using MS/MS analysis, as described in Steps 34 and 35.

● TIMING

Pituitary isolation and stabilization with glycerol-containing physiological solution takes 30 min. Single-cell sample preparation, including fabrication of glass micropipettes, ten single-cell isolations and matrix applications, can be done in 2 h. Approximately 1 h is necessary to acquire mass spectra from the single-cell samples, samples with standard peptides and control extracellular solution samples. Depending on the quality of the mass spectra, the data processing takes an additional 2–3 h. Preparation of tissue blots is relatively quick and requires just 20–30 min. Acquisition of mass spectra in MS and MS/MS modes can be done in 1–2 h if about five peptides are to be sequenced. The most time-consuming stage is the processing of MS/MS data and their comparison with data available in a variety of databases. This stage may take from 1 d to several weeks when *de novo* sequencing is attempted.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible cause	Solution
11	Visible MALDI matrix crystals form outside of the large-diameter micropipette before it contacts the glass slide surface	The inner diameter of the micropipette tip is too large or the tip is broken	Adjust micropipette-pulling parameters and investigate tip appearance under a microscope at high magnification
21	MALDI matrix does not exit the large-diameter micropipette	The MALDI matrix solution contains matrix crystals that clog the tip	Centrifuge or filter the solution before filling the large glass micropipettes (make sure that the filters will not be dissolved by MALDI matrix solution). MALDI matrix solutions with concentrations of matrices below their saturation point can be used to avoid crystal formation
34	Pituitary blots produce poor-quality signal	The optimal analyte to MALDI matrix ratio is not achieved	Examine samples under a stereomicroscope and add more MALDI matrix if 2,5-dihydrobenzoic acid crystals do not have the typical sharp, arrow-like appearance

ANTICIPATED RESULTS

Single-cell MALDI MS has been successful in characterizing new SPs. We and others have developed MALDI MS protocols to profile peptides in individual invertebrate neurons and cellular processes, including the first *de novo* sequencing of peptides in single cells[12,56,83,88–90]. Often, SP signals that are surprisingly intense, especially as compared to other work with multicellular samples, will be observed. This is true even with mammalian cells. Why? First, whether in an invertebrate or a vertebrate, the SP profiles in cells of the same tissue or organ are often different. As more and more cells are added to a sample, those peptides and proteins present in all the cells (for instance, structural proteins) produce increasingly intense signals; those that are different in each cell (such as cell-specific SPs) produce relatively smaller signals. Thus, prohormone processing tends to be easier to interpret for mass spectra collected from samples of ten or fewer cells, and SPs can be easily confirmed, as many peptides from each prohormone can be detected. With larger samples, the data become less clear. Of course, if the particular cell producing the peptide is known, and the cell's physiological function is also known, then the bioactivity of the peptide can be tested using directed experiments not possible without such information.

Here, we demonstrate the potential of single-cell MALDI MS for investigation of SPs in individual cells by isolating intermediate pituitary cells for MS analysis. **Figure 4** demonstrates the results of this investigation. On average, ten signals are observed in the peptide mass region and no peptide signals are detected in samples of extracellular media (**Fig. 4f**). Mass spectrometer calibrations using peptide standards deposited in close proximity to single-cell samples allow us to measure the accurate molecular mass for peptides detected in these single cells. Processing and analysis of these data, and their comparison with expected cleavages of suspected prohormones by proteolytic enzymes, allows us to identify these peptides as proopiomelanocortin

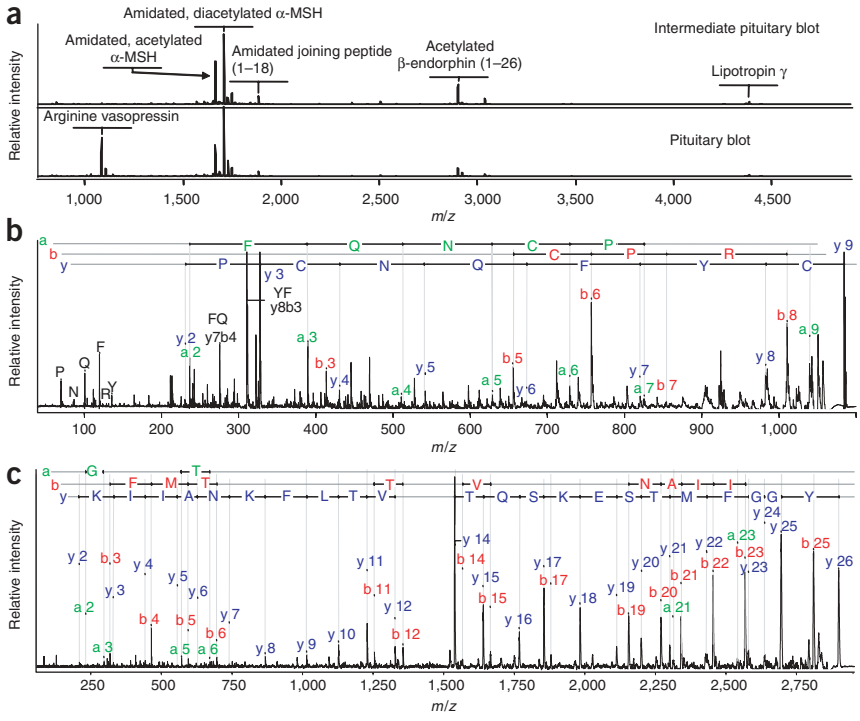

Figure 5 | MS/MS allows confirmation of signal identity assignments made using the precise molecular mass of peptides and a variety of data such as the peptide's known distribution in tissue, its prohormone expression and the presence of other peptides originating from the same prohormone. (**a**) Mass spectra acquired from a blot of intermediate pituitary lobe (top) and a tissue blot containing adjacent regions of intermediate and anterior pituitaries. (**b**) Mass spectrum acquired from a pituitary blot in MS/MS mode, confirming correct identification of Arg-vasopressin (CYFQNCPRG amide). (**c**) MS/MS confirmation of the identity of an acetylated β-endorphin fragment (1–26) (acetyl-YGGFMTSEKSQTPLVTLFKNAIIKNA) signal. Owing to space limitations, not all detected and identified fragments are labeled.

prohormone-derived molecules (with the detected post-translational modifications in parentheses): γ-melanocyte-stimulating hormone (MSH) (amidation), des-acetyl-α-MSH (amidation), α-MSH (amidation, acetylation), β-endorphin (13–27), diacetyl-α-MSH (amidation, diacetylation), joining peptide (1–18) (amidation), N-terminal peptide (52–74), β-endorphin, acetyl-β-endorphin (1–26) (acetylation) and lipotropin γ.

To validate these peptide identifications, we performed MS/MS experiments on selected peptides in blots of intermediate pituitary and larger areas containing intermediate and anterior pituitary (**Fig. 5a**). Arg-vasopressin and β-endorphin fragment (1–26) were successfully sequenced (**Fig. 5b,c**) using the MS/MS capabilities of the Ultraflex II operating in LIFT mode. Extensive fragmentation of molecular ions was achieved in both cases. Detection of multiple y and b ions confirms the primary sequences of both peptides.

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