Bioanalytical Instrumentation -- Mass Spectrometry

Lecture 16: MS and Hyphenated Techniques for Neuropeptide Research

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BME 595/CHEM 590

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Outline

- Introduction
- Direct tissue analysis (Profiling vs. imaging)
- Homogenization approaches
- Quantitation and differential display
- Neuropeptide release

Neuropeptides are involved in many functions

- feeding and body weight regulation
- energy expenditure
- drinking and fluid retention
- sleep / wake cycles
- memory
- pain
- reproduction / fertility
- stress
- anxiety
- depression
- emesis (vomiting) and nausea
- reward mechanisms (pleasure)

• Abnormal expression of signaling peptides might be related to disease

• Neuropeptides and their receptors are potential targets for drug discovery

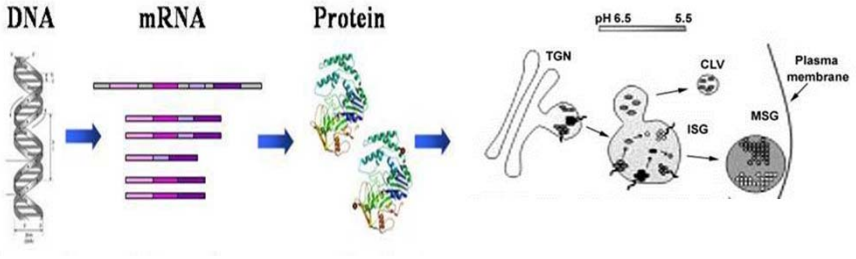
Potential Targets for Peptide-Based Therapeutics

Disease	Peptide
Pain	Opioid peptides Neurokinins
Depression	Corticotropin-releasing hormone Substance P
Epilepsy	Neuropeptide Y Dynorphins Somatostatin
Obesity	Leptin Ghrelin
Stroke	BDNF
Brain cancer	Various growth factors

Egleton & Davis, *NeuroRx 2*, 44-53 (2005).

The making of a neuropeptide

The biological effect of a specific neuropeptide depends on its exact chemical structure: dozens of enzymes are required process a prohormone to the final products



Neuropeptide processing defies prediction: make the measurement

Li and Sweedler, Annual Review of Analytical Chemistry, 2008, 1: 451.

How are neuropeptides discovered?

Two basic approaches:

- Find a "factor" that functions in cell-cell communication (using a bioassay).
  - Then, purify this factor and if a peptide, determine its sequence.

Function ⟶ **Peptide**

- Find a peptide.
  - Then find a function for this peptide in cell-cell communication.

Peptide ⟶ **Function**

Neuropeptide Analysis

- Edman Degradation
  - Chemical cleavage of single amino acids to identify sequence of peptide
- Immunocytochemistry
  - Antibody recognition of peptide
- Extraction, Purification, and Mass Spectrometry
  - Pooling of multiple organs/animals

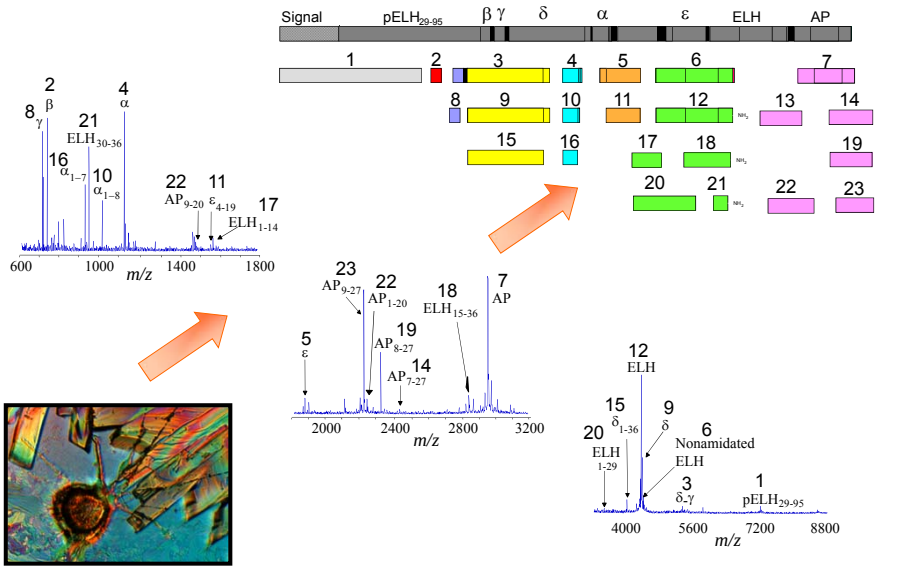
Analytical Challenges to Tissue Analysis by Mass Spectrometry

- High Salt and Lipid Content
- Low Concentration of Neuropeptides Compared to Surrounding Sample
- Wide Dynamic Range of Neuropeptides

Peptidomics *vs.* Proteomics

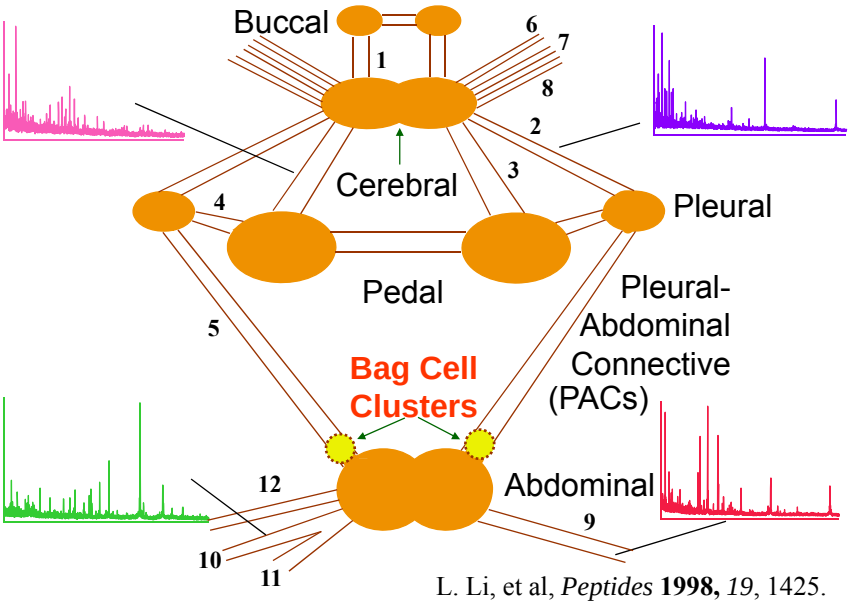
- Neuropeptides are produced by endogenous enzymatic cleavage from longer protein precursor molecules
- MS analysis of peptidomes is challenging due to the trace level of these signaling molecules
- 2D-gel methodology for proteomics is not suitable for looking at peptides
- Many of the isotopic labeling strategies do not work well for neuropeptides
- Database search is difficult or impossible for some of the peptidomic studies

MALDI MS Directly Reveals Prohormone Processing



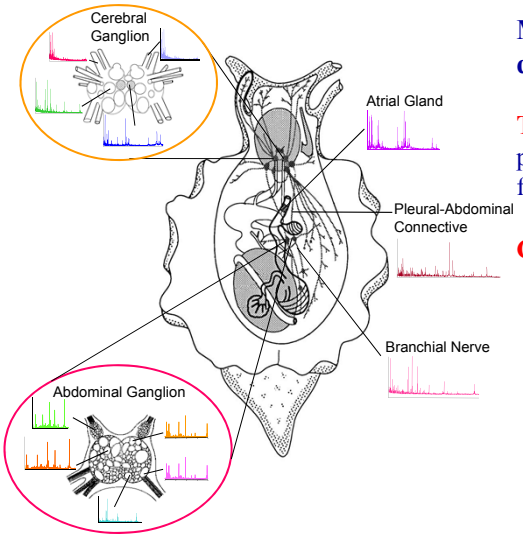
Garden et al., *PNAS* 95, 3972-3977 (1998) .
Li et al., *TIBTECH* 18, 151-160 (2000) .

Peptide Mapping in *Aplysia* Connectives



L. Li, et al, *Peptides* **1998,** *19*, 1425.

Strategies for Identifying New Bioactive Neuropeptides



MALDI Profiling of *Aplysia* CNS

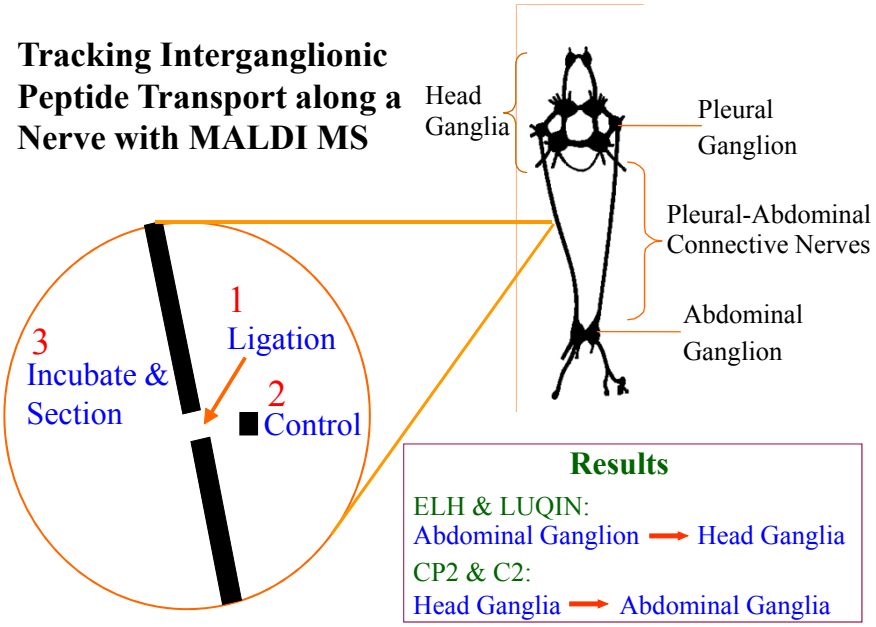
Multiple putative peptides are detected in individual neurons

The challenge: selection of the peptides most likely to be bioactive for further characterization

Criteria for preselection include:

- **Interganglionic transport (in nerves)**
- **Present in hemolymph (i.e. is released)**
- **Present in related species**
- **Posttranslational modifications**

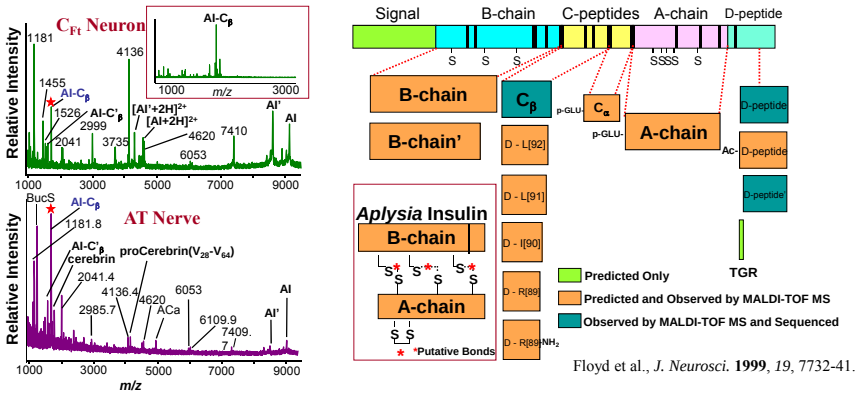
Li et al., *TIBTECH* 18, 151-160 (2000) .



L. Li, et al, *Peptides* **1998,** *19*, 1425.

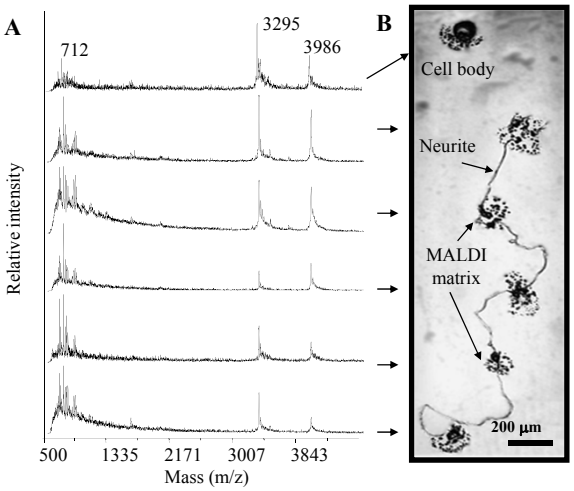
Peptide Transport Example: Discovery of *Aplysia* Insulin

- MALDI-MS of top-layer cerebral F cluster (C_{Ft}) neurons identified a peak at 1714 Da.
- The same peak was also observed in AT and UL nerves.
- 1714 Da peak was isolated using LC/MALDI and sequenced.
- Using PCR, the cDNA has been characterized, which encodes a 156 amino acid precursor and results in the first insulin prohormone to be reported in *Aplysia*.
- The expression of *Aplysia* insulin (AI) decreases when the animal is deprived of food, and injections of AI reduce hemolymph glucose levels.



Floyd et al., *J. Neurosci.* **1999**, *19*, 7732-41.

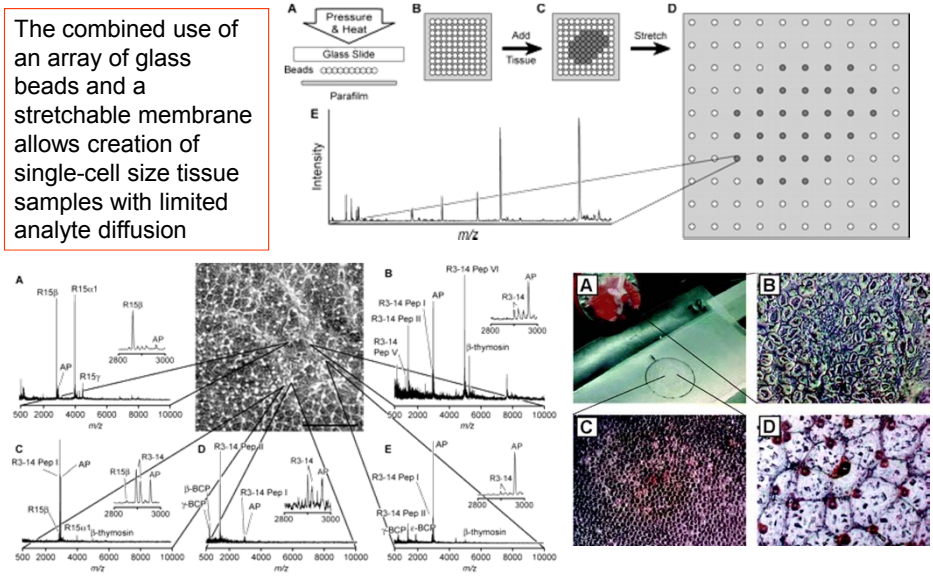
MALDI MS Profiling of Peptide Distribution between Soma and Dendrites of a Single Neuron



Rubakhin et al., Anal. Chem. 2003

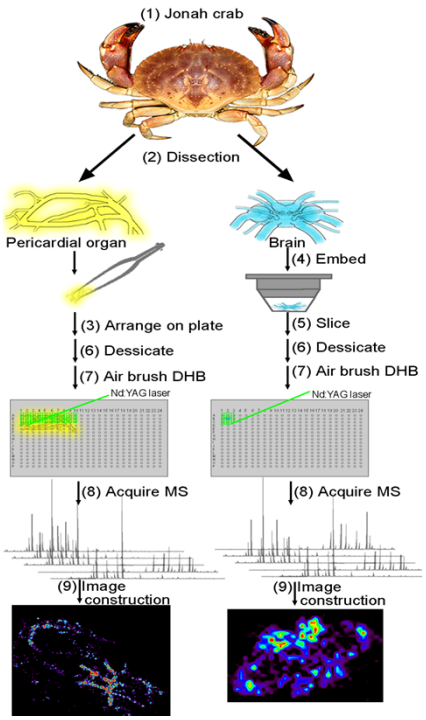
Massively Parallel Sample Preparation Method for MALDI MS Analysis of Tissues

The combined use of an array of glass beads and a stretchable membrane allows creation of single-cell size tissue samples with limited analyte diffusion



Monroe et al., *Anal. Chem. 78*, 6826-6832 (2006).

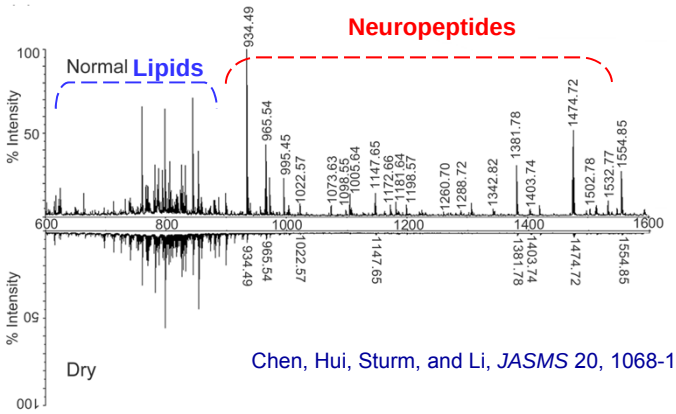
Mass Spectral Imaging of Neuropeptides in the Crustacean Nervous System by MALDI TOF/TOF



DeKeyser et al., *J. Proteome Res.* 6, 1782-1791 (2007).

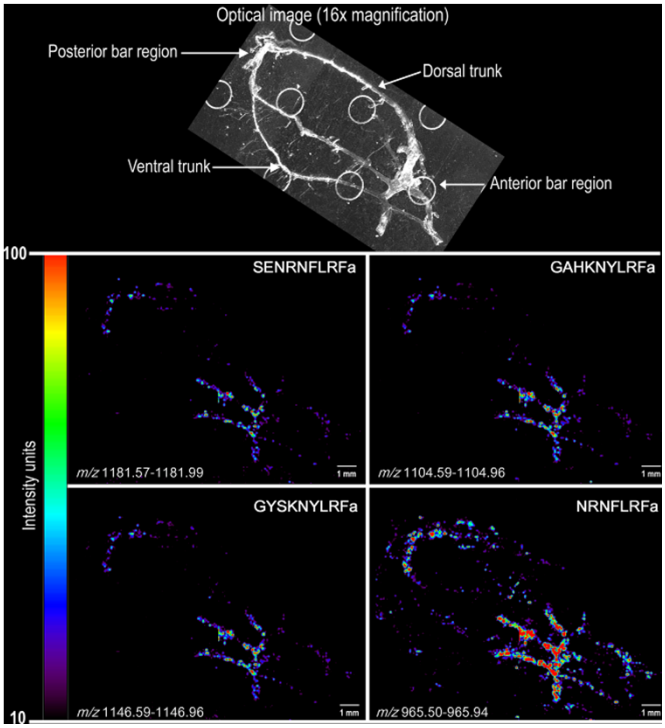
MALDI matrix application and its effects on tissue profiles

- Comparison of dry matrix spraying and regular matrix spraying for lipid and neuropeptide detection.
- Similar signal intensities and peak patterns for lipids below *m/z* 900
- Dry spraying yielded fewer peptides with lower signal intensities above *m/z* 900.



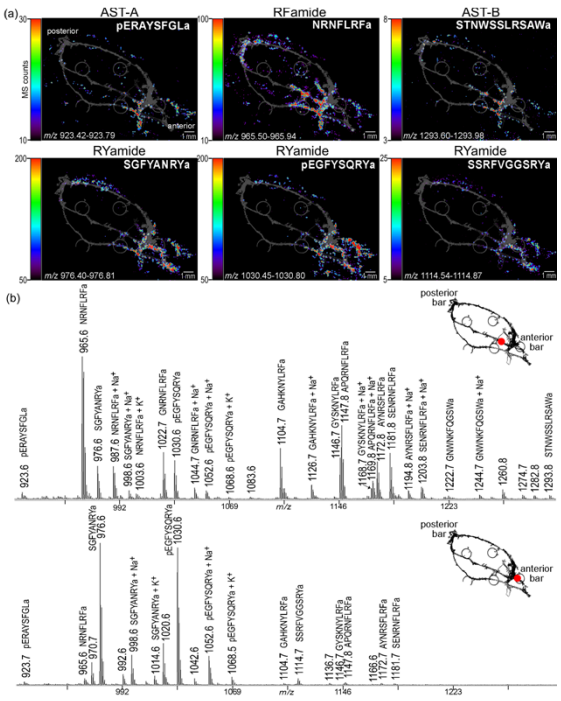
Chen, Hui, Sturm, and Li, *JASMS* 20, 1068-1077 (2009)

MALDI MS-Based Tissue Imaging of RFamide Neuropeptide Distribution in Neurosecretory Organ



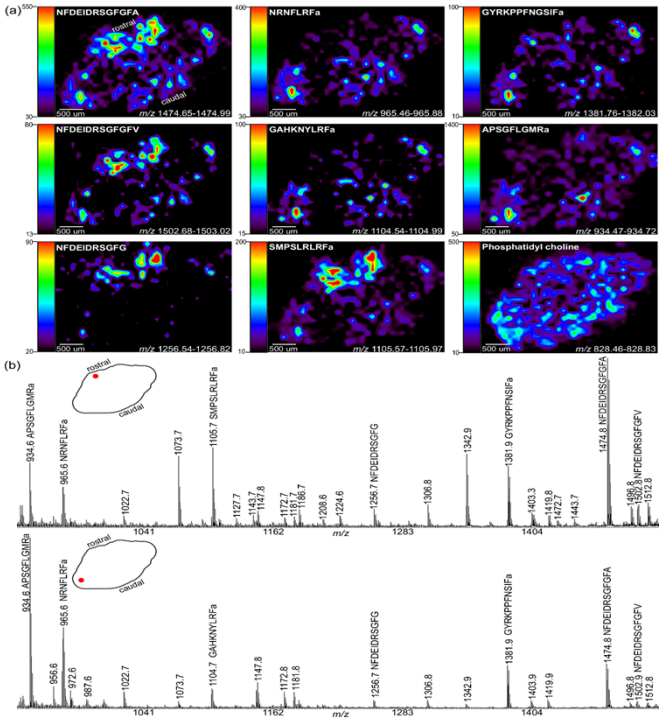
DeKeyser, Kutz-Naber, Schmidt, Barrett-Wilt, and Li. *J. Proteome Res. 6*, 1782-1791 (2007).

MALDI ion images and the resultant mass spectra from the pericardial organ of the crab showing differential distribution of peptides from multiple peptide families

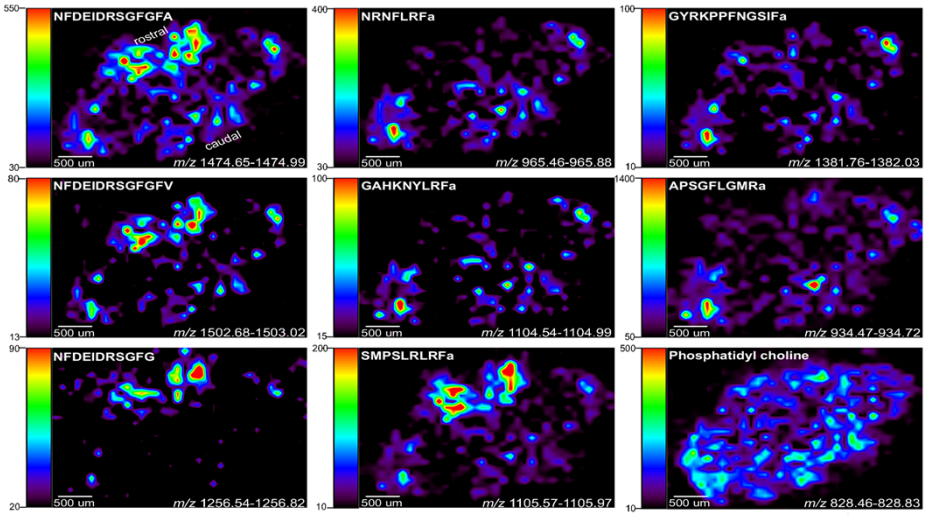


DeKeyser et al., *J. Proteome Res.* 6, 1782-1791 (2007).

MALDI-Based Neuropeptide Imaging of *C. borealis* Brain Tissue Section



MALDI-Based Neuropeptide Imaging of *C. borealis* Brain Tissue Section

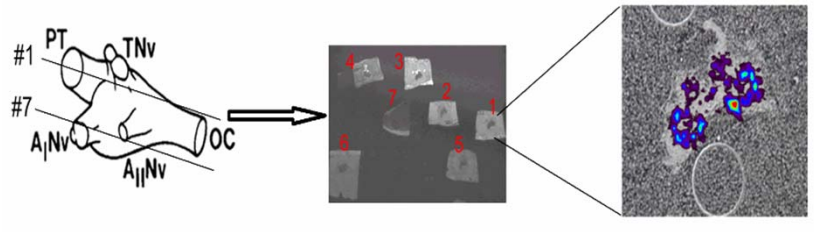


DeKeyser et al., *J. Proteome Res.* 6, 1782-1791 (2007).

DeKeyser, Kutz-Naber, Schmidt, Barrett-Wilt, and Li. *J. Proteome Res. 6*, 1782-1791 (2007).

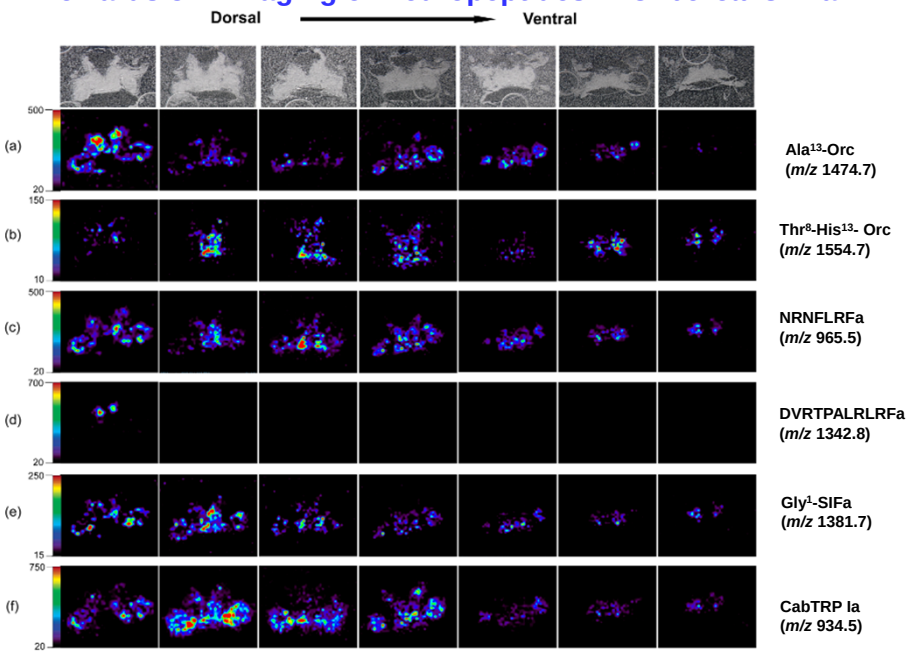
Three Dimensional Imaging Strategy

- **The schematic diagram of 3D imaging experiments showing serial sectioning along z-axis of the brain.**
- **Series of seven pieces of tissues were collected with 132 μm intervals in between**



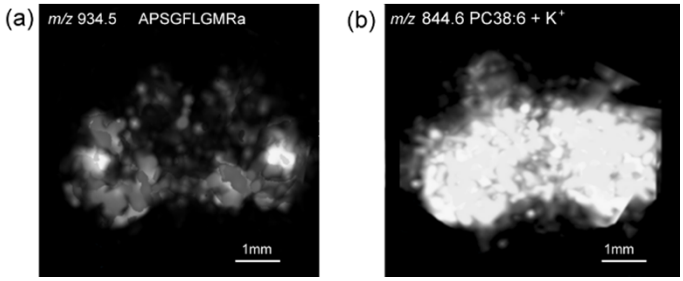
Chen, Hui, Sturm, and Li, *JASMS* 20, 1068-1077 (2009)

Towards 3-D Imaging of Neuropeptides in *C. borealis* Brain



Reconstructed 3D Images

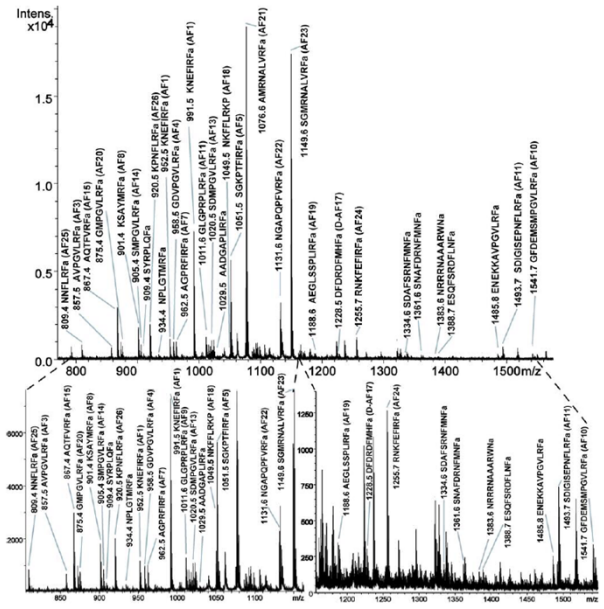
Neuropeptide **Lipid**



Peptide CabTRP 1a **Lipid PC 38:6**

- Lipids are distributed more evenly throughout the whole brain tissue
- Neuropeptide CabTRP 1a is concentrated in a few neuronal clusters

Chen, Hui, Sturm, and Li, *JASMS* 20, 1068-1077 (2009)



Yew et al., *J. Comp. Neurol.* 488, 396-413 (2005).

Fig 4. MALDI-TOF peptide profile from nerve ring. Peptide sequences have been assigned on the basis of calculated $[M+H]^+$. Sodium adducts, deduced by a m/z difference of 22 with another ion in the spectra (usually one of higher intensity) are not labeled. The bottom panel is an enlargement of the top panel.

Acetylated

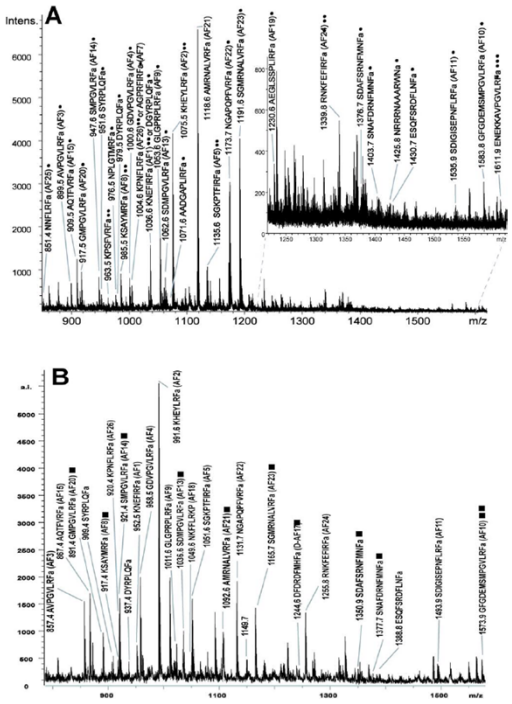
Applied 0.5 µl of 3:1 methanol/acetic anhydride solution on the tissue, evaporated, followed by the addition of matrix.

MALDI-TOF Profile of *A. suum* Nerve Ring

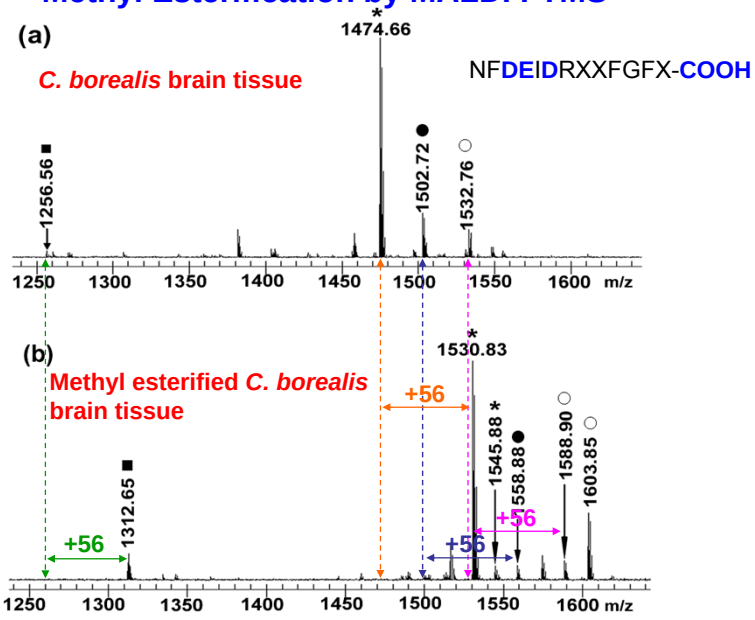
H_2O_2-treated

The tissue was covered with 0.5 µl of 0.1% H_2O_2 in 0.1% TFA. After 5 min incubation at RT, the H_2O_2 was removed followed by the addition of matrix.

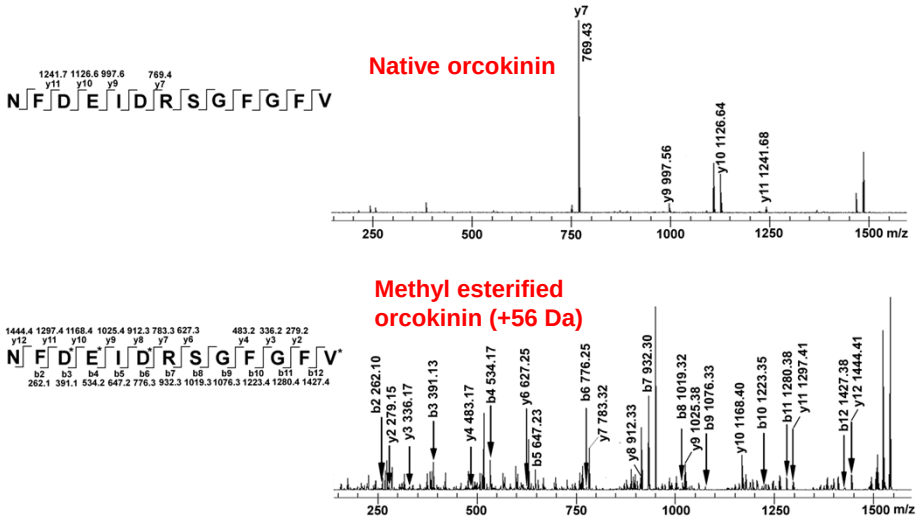
Yew et al., *J. Comp. Neurol.* 488, 396-413 (2005).



In Situ Analysis of Orcokinin Neuropeptides via Methyl Esterification by MALDI FTMS

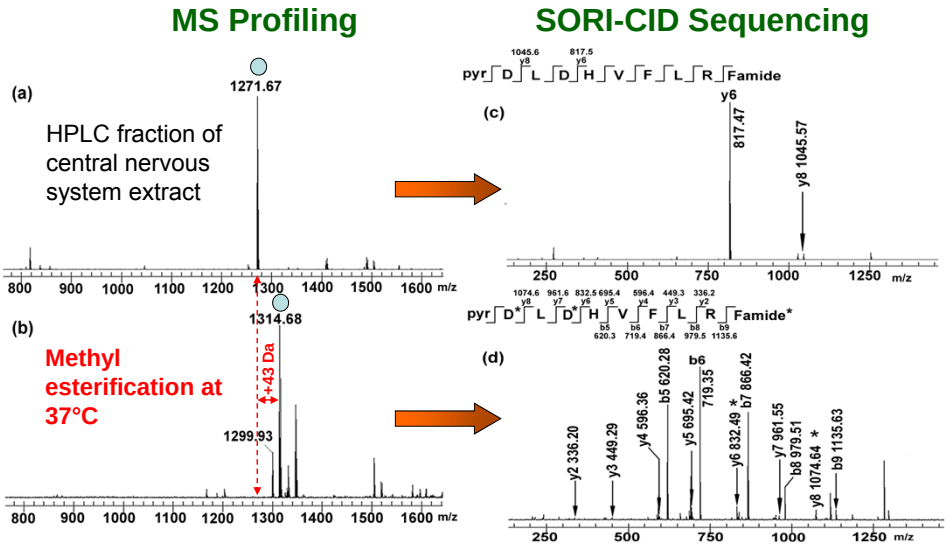


Methyl Esterification Improves MS/MS Fragmentation Efficiency of Orcokinin Neuropeptide

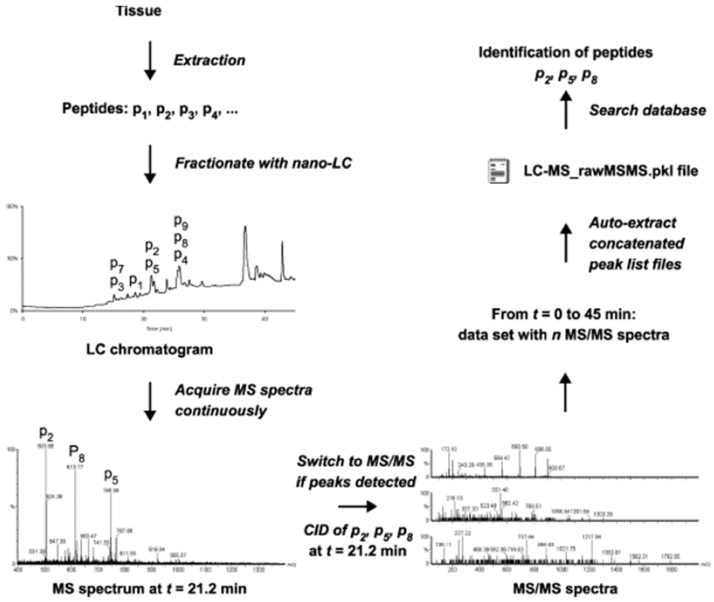


Ma et al., *Anal. Chem.* 79, 673-681 (2007).

Methyl Esterification Assisted MALDI FTMS Screening for C-Terminal Amidation and Identification of an RFamide in Lobster *H. americanus*

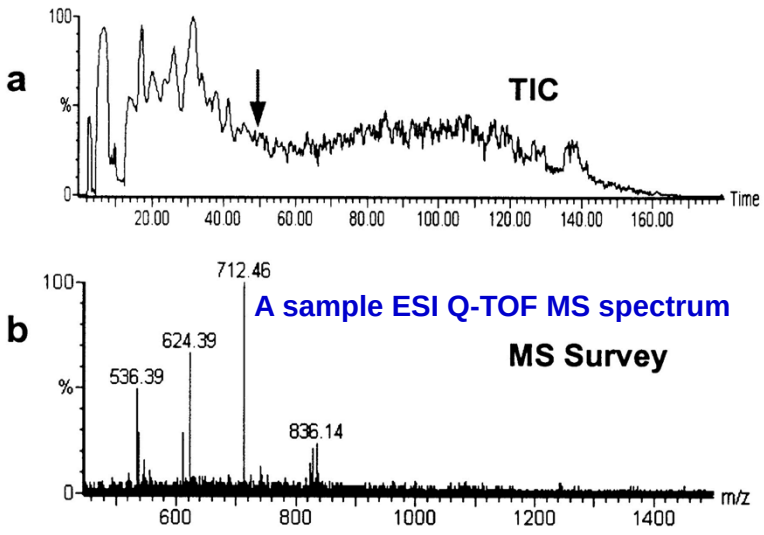


Experimental Setup for Peptidomic Analysis



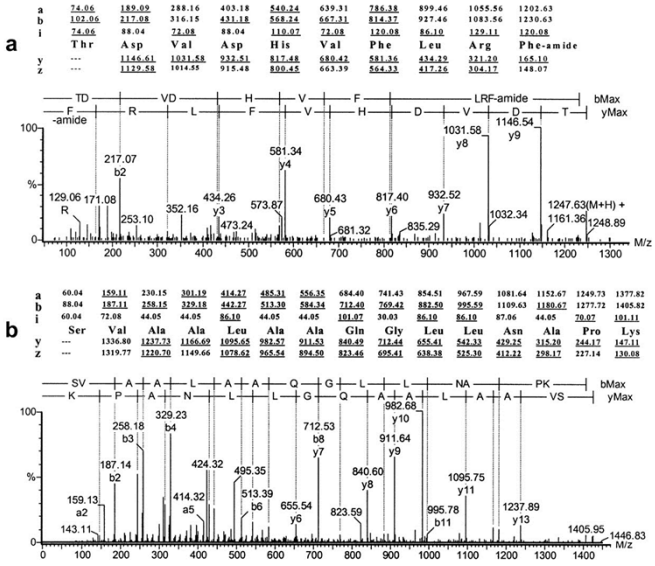
Baggerman et al., *J Chromatogr. B* 803, 3-16 (2004)

Total ion current chromatogram obtained by nanoLC-MS analysis of a methanolic extract of 50 larval Drosophila CNSs



Baggerman, G. et al. J. Biol. Chem. 2002;277:40368-40374

MS/MS fragmentation spectrum of the peptide at m/z 624.39(Dromyosuppressin) and m/z 712.47 (2+) that corresponds to an unannotated Drosophila gene



Baggerman, G. et al. J. Biol. Chem. 2002;277:40368-40374

The Challenges of *De Novo* Sequencing

1. **Lack of genomic sequence information**

   Homology with previously identified neuropeptides can be helpful.

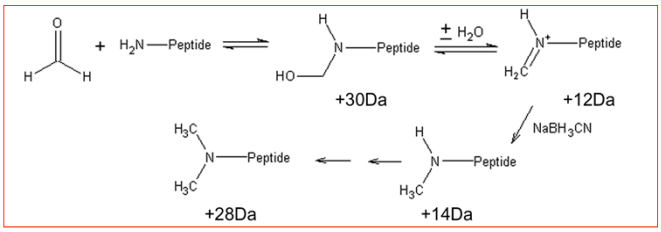
2. **Complexity of MS/MS spectra**
3. **Incomplete sequencing information in MS/MS**
4. **Isobaric amino acids**, such as:

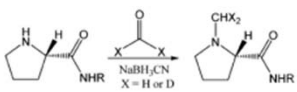
   L = I

   GG = N 87 ppb

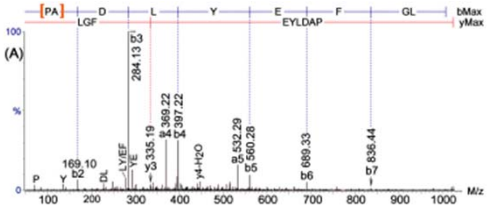
   GA = Q (K) 78 ppb

De Novo Sequencing of Neuropeptides via Formaldehyde Labeling

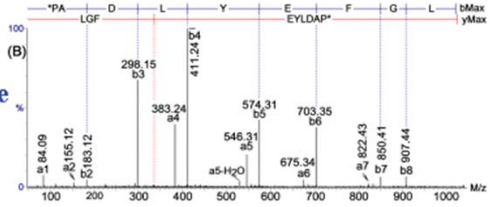




[PA]DLYEFGLamide
(1023.39⁺)



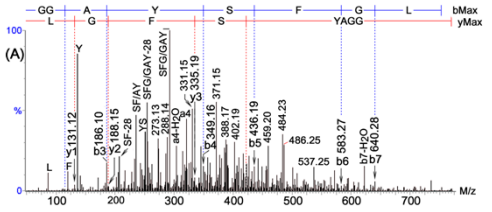
(CH_3)PADLYEFGLamide
(1037.28⁺)



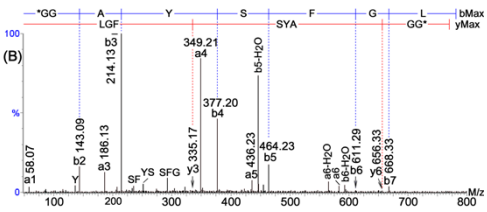
Fu and Li, *Anal. Chem.* 77, 7783-7795 (2005).

Reductive Methylation Simplifies MS/MS Fragmentation Pattern of Derivatized Peptides

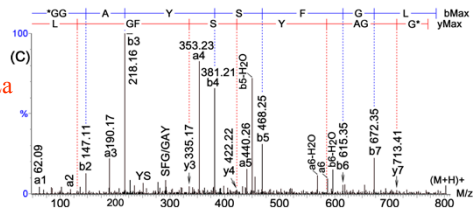
GGAYSFGLamide
(770.29⁺)



$(CH_3)_2$GGAYSFGLa mide (798.45⁺)

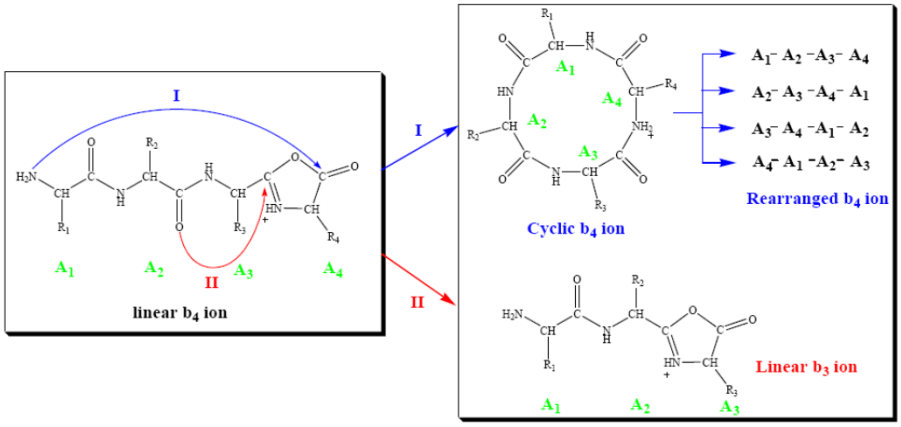


$(CHD_2)_2$GGAYSFGLa mide (802.51⁺)



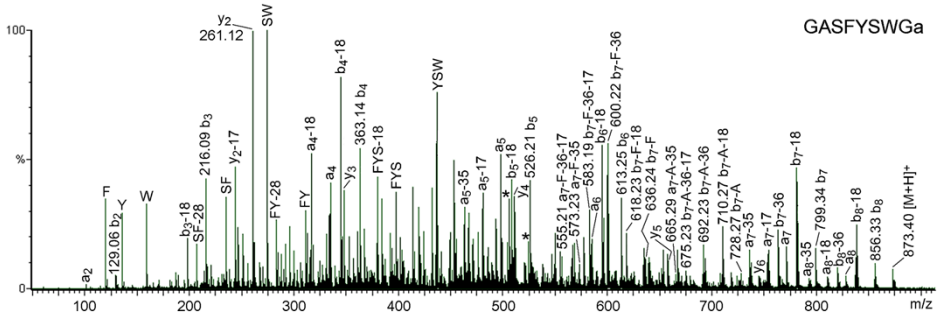
GGAYSFGLa A-type allatostatin from *C. borealis* PO
Sequencing result by PepSeq software
TPHGGGGST
TPHGGSGGT
TPAGGGGSAPa
TPPGGGASQa
TPAGGGPSQa
TPGAGGGSAPa
TPGPGGASQa
TPPGGGATNa
TPNGGASAPa
b_2 ion absent in (A)
GQYSFGLa 87ppb

The Formation of Cyclic b-ion Intermediate Could Result in Sequence Scrambling



Bela Paizs, ASMS 2006; Polfer et al., JACS 2005, 127, 17154; Jia et al., JASMS 2007, 18, 663.

Internal Losses of Amino Acids during Gas-Phase Fragmentation Complicates Spectral Assignment

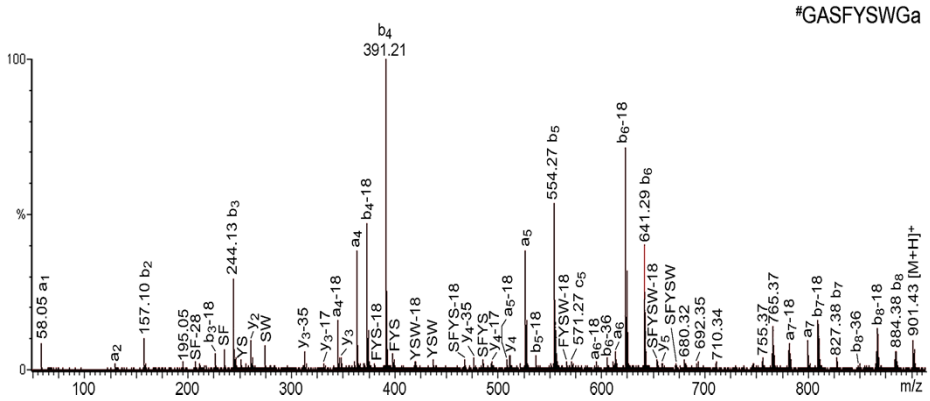


The internal residue loss from the b_7 ions are labeled.
The internal residue loss increase the MS/MS complexity.
The sequence 'scramble' makes *de novo* sequencing difficult.

PepSeq sequencing results (w/ C-terminal amide)

Sequence	#	Score	Joint Prob	Prob (%)	Calculated MW	Delta
QDPGGAGSEG	1	293	333	43.60	872.3624	0.17
YPHGAGSEG	2	361	333	31.57	872.3777	0.18
Q5PAAASGEG	3	385	332	15.43	872.3988	0.20
FSAGYSDAG	4	414	330	3.25	872.3665	0.17
TDGAGGPGDAG	5	544	330	2.29	872.3624	0.17

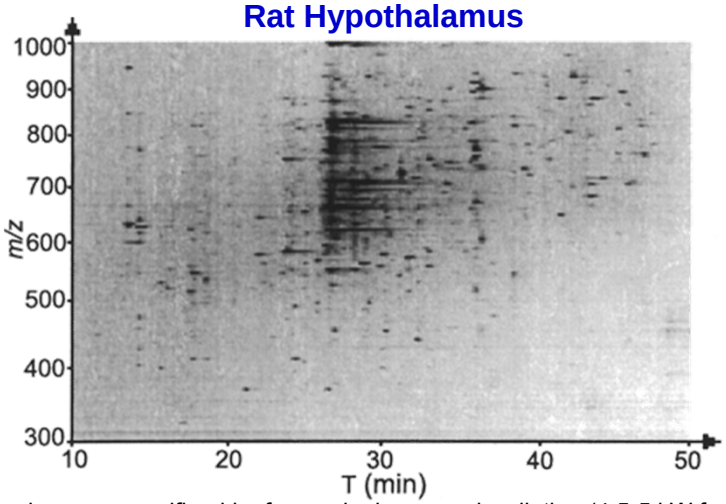
N-dimethylation Blocks the Cyclo-b Ion Formation



N-(di)methylation simplifies the MS/MS spectrum of singly charged peptides by

1. Increasing the b ion abundance and suppressing the y ions and internal fragment ions
2. Blocking the internal residue losses

2D Graph of the Neuropeptide Content from 1 mg of Rat Hypothalamus



- Rats or mice were sacrificed by focused microwave irradiation (4.5-5 kW for 1.4 s).
- Extraction with 0.25% acetic acid, and homogenized by microtip sonication
- The suspension centrifuged @ 20,000 xg for 30 min at 4°C.
- MWCO of 10,000 Da, centrifuged @ 14,000 xg for 45 min at 4°C.

Svensson et al., *J Proteome Res* 2, 213-219 (2003).

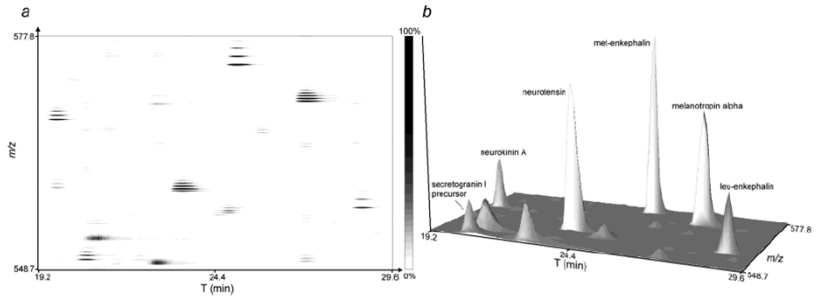


Figure 2. Selected region of a neuropeptide map of the rat hypothalamus displayed as two- and three-dimensional graphs. Both graphs present the nanoLC elution profile for 19.2−29.6 min and the *m/z* range 548.7−577.8. **a**, The distance between the coeluting parallel lines correspond to the charge states of the ions. Relative spot intensity in the two-dimensional graph is represented by color changes, black being the most intense reading and white the lowest. The seemingly unresolved spots at 20.9 min, *m/z* 552.2 $[M + 9H]^{9+}$ and 22.6 min, *m/z* 549.2 $[M + 8H]^{8+}$ are in fact resolved by further zooming. **b**, Three-dimensional graph showing the relative intensity of identified secretogranin I peptide (*m/z* 549.85 $[M + 2H]^{2+}$), neurokinin A (*m/z* 567.35 $[M + 2H]^{2+}$), neurotensin (*m/z* 558.31 $[M + 3H]^{3+}$), met-enkephalin (*m/z* 574.29 $[M + H]^{+}$), melanotropin alpha (*m/z* 569.66 $[M + 3H]^{3+}$), leu-enkephalin (*m/z* 556.35 $[M + H]^{+}$).

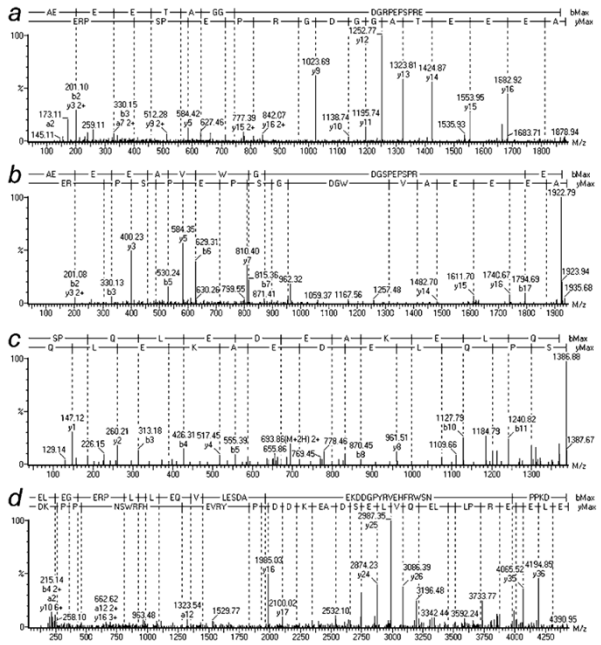
Svensson et al., *J Proteome Res* 2, 213-219 (2003).

POMC precursor-derived peptide

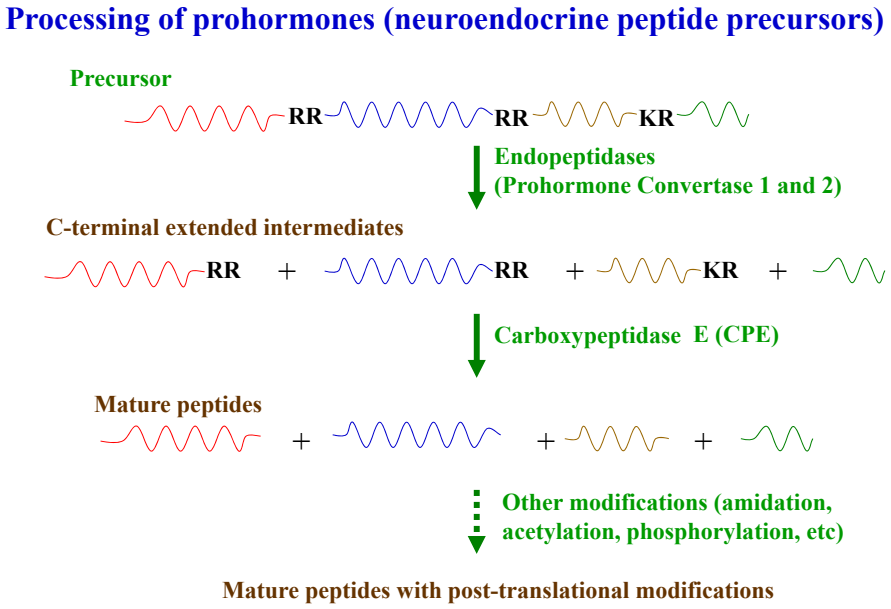
POMC precursor-derived peptide

Proenkephalin A precursor-derived peptide

Lipotropin gamma

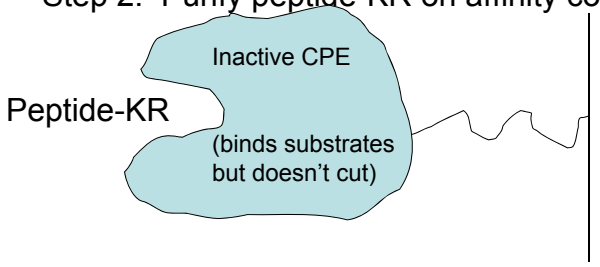


Svensson et al., *J Proteome Res* 2, 213-219 (2003).



Idea to identify neuropeptides

- Step 1: Block CPE – accumulate peptide-KR and -RR
- Step 2: Purify peptide-KR on affinity column



- Step 3: Elute peptides from column, sequence by Edman degradation

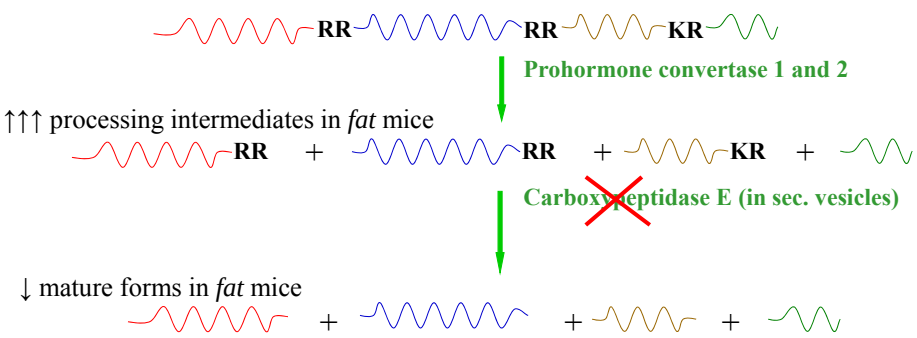
Cpe$^{fat/fat}$ mice

A natural mutation of CPE (Ser^{202}Pro)

CPE is inactive

Why are they fat?
Why are they alive???

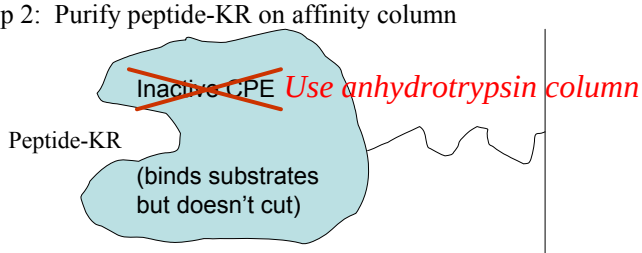
Left, wild type; right, *Cpe*$^{fat/fat}$



Idea to identify neuropeptides

Use fat/fat mice

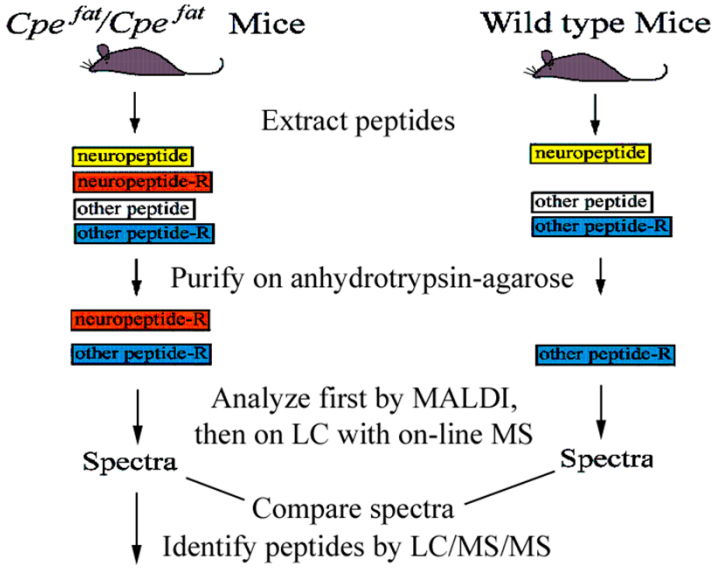
- Step 1: Block CPE accumulate peptide-KR and -RR
- Step 2: Purify peptide-KR on affinity column



- Step 3: Elute peptides from column, sequence by Edman degradation

Use mass spectrometry

Strategy for identifying neuropeptides in *Cpe* ^{fat}/*Cpe* ^{fat} mice

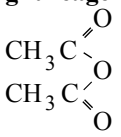
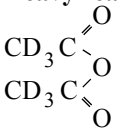
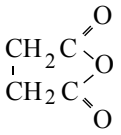
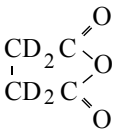
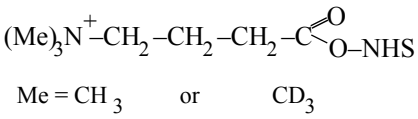


The ions that are *different* in the two spectra = CPE substrates (most are neuropeptides)

Using this technique…

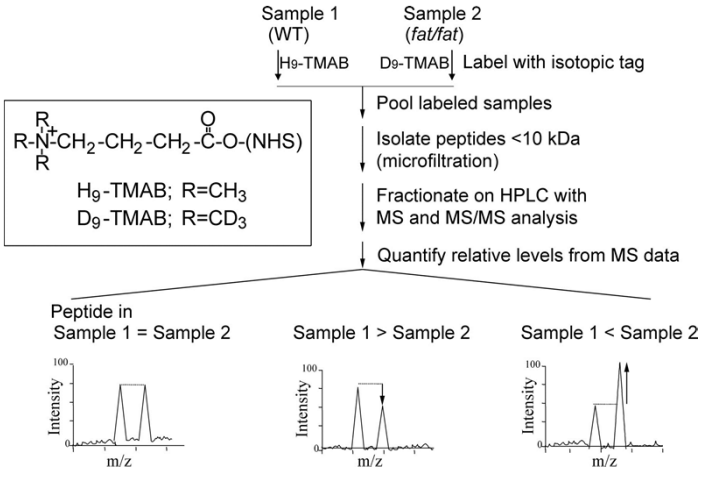
- Hundreds of peptides were detected in *Cpe*$^{fat/fat}$ mouse brain and pituitary that were not present in wild type mice
- Over 150 of these have been identified by MS/MS sequence
- These represent peptides from 20 different prohormones or other secretory pathway proteins
  - ProEnkephalin - ProGnRH
  - ProOpiomelanocortin - VGF
  - ProNeurotensin - ProTachykinin A and B
  - ProTRH - ProCholecystokinin
  - ProVasopressin - ProDynorphin
  - ProOxytocin - ProMelanin Concentrating Hormone
  - Chromogranin A and B - Secretogranin II
  - ProSAAS - Prohormone Convertase 1 and 2
  - ProPeptidyl-Amidating Monooxygenase

Differential isotopic tags for amine groups

	Light reagent	Heavy reagent	Δ Mass
Acetic anhydride			6 Da
Succinic anhydride			4 Da
Quaternary γ-amino butyric acid – N-hydroxysuccinimide ("TMAB")	 Me = CH_3 or CD_3		9 Da

Fricker et al., *Mass Spectrom Rev.* 25, 327-344 (2006).

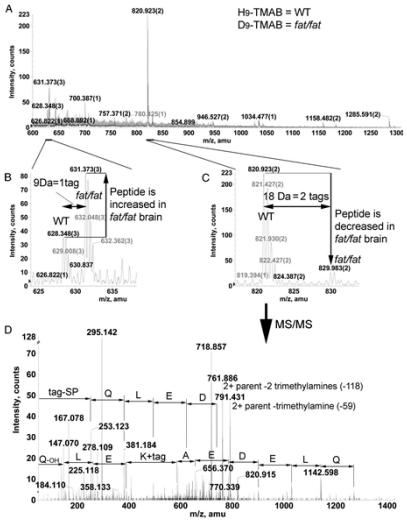
Outline of quantitative peptidomics approach using isotopic labels and mass spectrometry



Fricker, L. D. Endocrinology 2007;148:4185-4190

Endocrinology

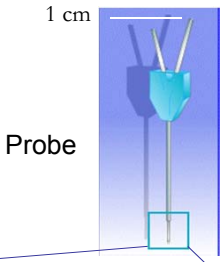
Selected liquid chromatography/mass spectrometry (LC/MS) and MS/MS data from an experiment in which the wild-type (WT) hypothalamic extract was labeled with H9-TMAB and the Cpefat/fat hypothalamic extract was labeled with D9-TMAB



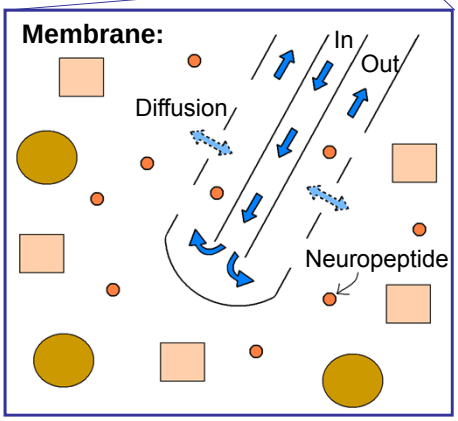
Fricker, L. D. Endocrinology 2007;148:4185-4190

Endocrinology

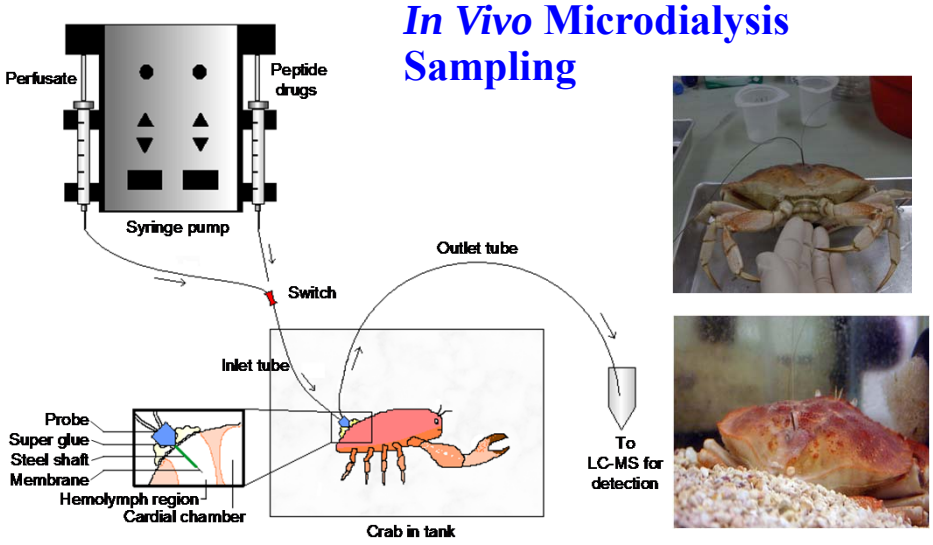
Microdialysis Sampling



- **Fluid is pumped through a probe, into the tissue, and out into collection vials**
- **On way out, fluid passes by a semi-permeable membrane**
  - Diffusion occurs between fluid and extracellular space
  - Only molecules smaller than the membrane pores can pass through and be collected
- **Benefits of microdialysis:**
  - Filters out larger, abundant molecules to produce cleaner samples
  - Can use on live, freely-moving animals
  - Minimizes effects of stress
  - Only samples neuropeptides that have been released from neurons (biologically significant)
  - Real-time monitoring
  - Can add drug, and observe effects

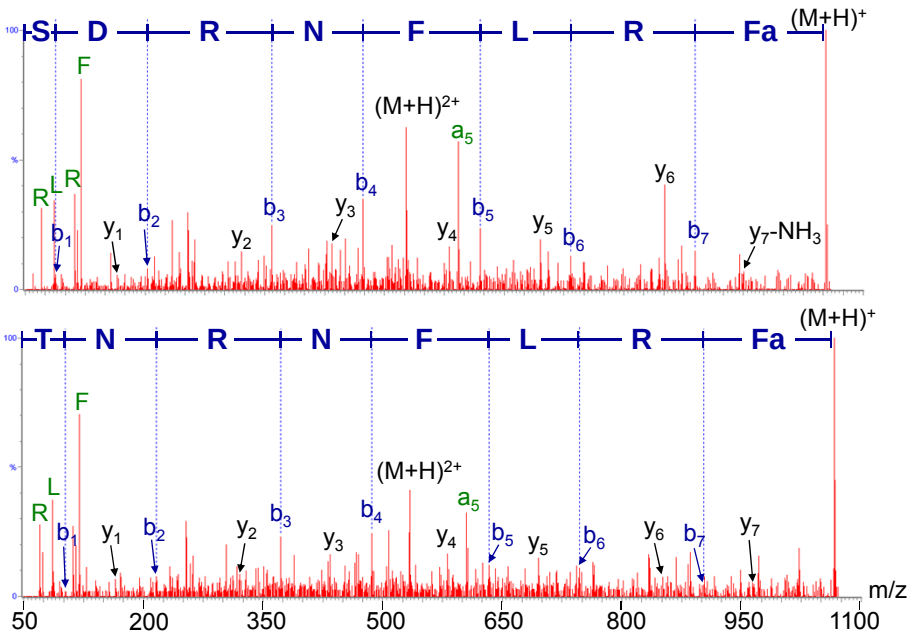


Experimental Setup for *In Vivo* Microdialysis Sampling

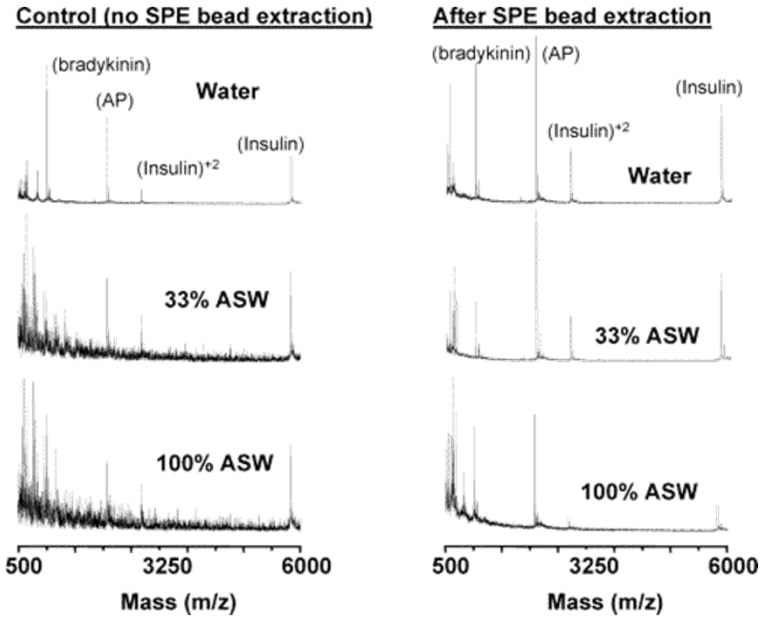


Behrens, Chen & Li, *Anal. Chem.* 80, 6949-6958 (2008).

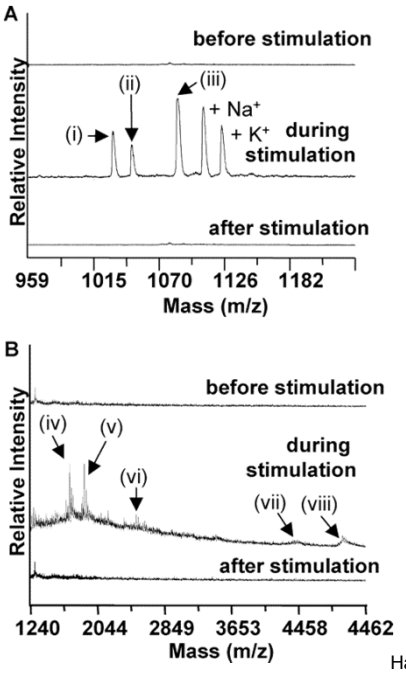
C. borealis Microdialysate



Ionization Suppression Due to Salts Can Be Minimized w/ Single-Bead SPE



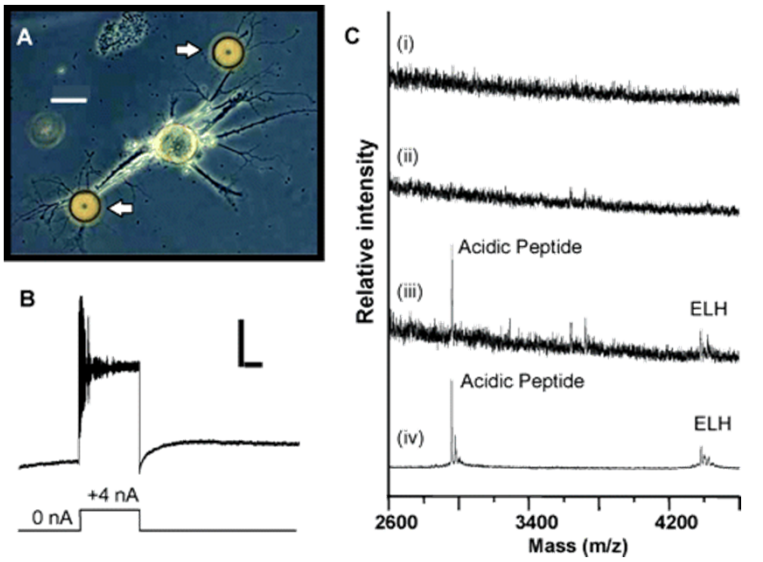
Hatcher et al., *Anal Chem* 77, 1580-1587 (2005).



Mass spectra of rat pituitary releasate collected with single SPE beads for 15-min intervals before, during, and after chemical stimulation with 50 mM KCl containing saline. A. The most prominent stimulation-dependent peaks are observed in the lower mass region, with masses corresponding to (i) α-MSH (6-13), (ii) vasopressin, (iii) arg-vasopressin, and Na+ and K+ salt adducts of arg-vasopressin. B. In relatively higher mass regions, peaks with masses matching (iv) diacetylated α-MSH, (v) joining peptide (1-18), (vi) CLIP (1-22), (vii) [His] lipotropin (1-38), and (viii) an unknown compound are readily observed in samples collected during stimulation.

Hatcher et al., *Anal Chem* 77, 1580-1587 (2005).

Single-Bead SPE Collection and MALDI MS Detection Confirmed Activity-Dependent Release of Peptides from Single Bag Cell Neurons



Hatcher et al., *Anal Chem* 77, 1580-1587 (2005).

Summary Points

- Mass spectrometry has become the method of choice for neuropeptide analysis.
- No single measurement platform can simultaneously provide chemical, spatial and temporal information content for probing neuropeptide transmission.
- Sample preparation is often key to obtain biologically relevant results.
- The development of direct tissue profiling and single cell MS has contributed significantly to neuropeptide research and show great promise in single-cell peptidomics.

Future Issues

- Dynamic measurements of signaling peptide release are among some of the most challenging analytical experiments. Future developments will address issues regarding sensitivity and temporal resolution.

- Future research in IMS will focus on the development of smaller sampling protocol and further improved spatial resolution to enable imaging of peptide distribution at the subcellular level.

- Several electron-based tandem MS fragmentation methods have shown great promise for sequencing large peptides.

- Continuous development of improved bioinformatics tools tailored to the unique features of signaling peptides will significantly accelerate our pace to neuropeptide discovery.