MEASUREMENT OF NEUROPEPTIDES: FROM PRESENCE TO RELEASE

BY

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THESIS

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ABSTRACT

Characterization of neuropeptides is still actively underway. Various resources, both animal and instrument, were utilized to best answer the questions about the presence of peptides. *Aplysia californica* cerebral neurons were used to verify predicted peptide processing of the putative prohormone AC2. However, the peptides were found to be absent in the forms given by the predictive software when analyzed with MALDI MS. Later studies used *Rattus norvegicus* brain tissue or microdialysate to look at dynamic peptide responses. The first compared pre- and post-stimulation profiles of rat brain slices that were exposed to a general stimulant. Though a stimulation affect was not seen, many peptides known to be present of the regions of interest, the suprachiasmatic nucleus and supraoptic nucleus, were observed. The final study involved creation of a peptide database for the hippocampus through high resolution mass spectrometry with which to reference microdialysate peptides. The rats were also exposed to various treatments including acute morphine, though the rats were most strongly distinguished by animal and not treatment via principal component analysis. A few peptides from the created hippocampus database were identified to also be in the microdialysate, while there was an interesting pair of fibrinogen protein fragments in the microdialysate that was not seen in the hippocampal extracts. The cumulative approach used in this work allows neuropeptide signaling to be characterized during behavior, enabling new insights into the functions of neuropeptides.

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION AND SUMMARY OF RESEARCH

Characterization of neuropeptides and hormones is still actively underway. The many aspects of their existence—chemical, spatial and temporal—can be examined separately or together with an equally diverse set of tools. The goals of the Sweedler research group are to develop instrumentation and sampling techniques to accommodate optimal conditions to answer the implied biological questions of identity, distribution and function of neurotransmitting molecules. This is accomplished mainly through various collection and separation methods coupled to mass spectrometry to provide accurate masses used for determining neuropeptide identities. Along with the correct tools, a fitting specimen is necessary to investigate the desired scope of interest. The work described here used two animal model systems of the many utilized by the group to best represent a measurable behavior, function, or neural system.

Chapter 2 describes the use of the invertebrate animal model, *Aplysia californica* that was chosen for the extensive background known about the neural system and the ease in studying their relatively large neurons and cluster anatomy. The well-mapped cells and connections naturally lead to the next step of attempting to understand their communication, which is where neuropeptide expression and processing information becomes an important building block. The search for a specific prohormone's peptide products in a discrete neural cluster tries to answer the question of cell contents. The expression of the products in the predicted cluster would connect a signaling molecule to a group of cells, which are in turn linked to cell connections and ultimately, to pathways and function.

The work described in Chapter 3 goes one step further in addressing tissue contents and their natural dynamics in living, responding tissue. Brain slices from the rat, *Rattus norvegicus*, were obtained, equilibrated in a bio-mimicking environment and allowed to respond to stimuli. Measurements were taken before and after the stimulus to be able to correlate the peptide changes to the stimulant. The stimulus of choice, KCl, provided a massive release event of the peptide reserve pool but other conditions could be used with this technique to probe increasingly specific release reactions. The implied future intention is to find the specific regulators of the two nuclei studied since they are known to be peptidergic regulators of body processes, or the "regulators of the regulators".

Chapter 4 is yet another step further; it incorporates peptide presence in a specific tissue, dynamic release, and the new aspect of a correlated observable behavior in a live subject. Sampling of the extracellular fluid was done via *in vivo* microdialysis while the rat was exposed to various conditions. Morphine was given as a stimulant condition followed by a measureable working memory task to assess an effect from the drug. The stimulation of the maze could also be treated as another stimulant condition itself. Discovering the peptide-condition relationship was also aided by supplementary sequencing information from the same hippocampal tissue. This brought identity information from the peptide level, reversed from the identity resource of the first *Aplysia* study. Though there is a common thread of peptide characterization throughout the studies, different tool, both animal and instrument, are needed to most efficiently answer the questions of interest.

CHAPTER 2

INVESTIGATION OF THE EXPRESSION AND PROCESSING OF *APLYSIA CALIFORNICA* PROHORMONE AC2

NOTES AND ACKNOWLEDGEMENTS

This work involves a collaboration between the Sweedler group and Sven Vilim and Klaudiusz Weiss from Mt. Sinai Medical School who supplied the gene sequence and *in situ* images. Dr. Elena Romanova is gratefully acknowledged for guidance and discussion regarding this material along with Dr. Fang Xie and Dr. Adriana Bora for further discussion and suggestions. Xiying Wang is also acknowledged for her dissection contributions and expertise. This material is based upon work that was supported by the National Institutes of Neurological Disease and Stroke through R01NS031609.

2.1 BACKGROUND

The cerebral ganglion of the *Aplysia californica* serves as processing center for various routes of receiving sensory information from other ganglia and the environment as well as sending motor commands. It consists of 15 discrete neuron clusters (7 bilateral pairs and 1 unpaired) with tens of visibly identifiable cells in each (JahanParwar 1976, Fredman 1975). The vast numbers of neurons forming connections in the cerebral ganglion enable the animal behaviors required for survival. These connections are either electrical or chemical, with the latter involving small molecule neurotransmitters and the peptide-based neuromodulators. Characterizing these signaling molecules aid our understanding of neuronal network function.

More than a decade ago, the Sweedler group was approached about finding peptide modulators within the cerebral ganglion neurons, and we turned to our other long term collaborators for help in determining these. Vilim and Weiss used a unique representation difference analysis approach to find peptide prohormone genes in this cluster (Jing 2010). One of these named AC2 (A-cluster clone 2), had in interesting structure suggesting it was a neuropeptide, and both western and *in situ* data were used to determine its localization. They confirmed it was expressed in A-cluster at high levels, with less but significant staining in the cerebral F-clusters. However, the enzymatic processing from RNA to peptide is complex because of the number of enzymes involved makes predicting final peptides problematic (Li 2008). Its processing and presence at the peptide level was investigated here through the use of direct MALDI MS of neurons and neuronal tissue chemical extractions.

2.2 EXPERIMENTAL

2.2.1 Animals. Adult *Aplysia californica* were obtained from Charles Hollahan (Santa Barbara Marine Bio., Santa Barbara, CA). Animal subjects were maintained in continuously circulating, aerated, and filtered seawater (Instant Ocean; Aquarium Systems, Mentor, OH) chilled to 14 °C.

2.2.2 Sample Preparation. Adult *Aplysia californica* weighing between 200 and 300 g were anesthetized by a ventral injection into the vascular cavity of 390 mM $MgCl_2$ in water equal to one-half to one-third the animal's body weight. Ganglia and connected nerve tissue were dissected out and placed in artificial seawater (ASW all in mM: 460 NaCl, 10 KCl, 10 $CaCl_2$, 22 $MgCl_2$, 26 $MgSO_4$, 2.5 $NaHCO_3$ and 10 HEPES, pH 7.7) supplemented with antibiotics (100 units/mL penicillin G, 100 μg/mL streptomycin, and 100 μg/mL gentamicin). The ganglia were

then incubated in 1% protease (Type IX, Roche Diagnostics) in ASW-antibiotic solution at 34°C for 30-60 min, depending on animal size, to enzymatically digest some of the surrounding sheath tissue for improved isolation specificity. After treatment, the ganglia were washed in ASW and then transferred to ASW-antibiotic solution. Under visual control with microscope assistance (Leica MZ 7.5 high performance stereomicroscope, Leica Microsystems, Bannockbum, IL), A-cluster cells are manually isolated from the cerebral ganglion with sharpened tungsten needles and scissors. A similar protocol is also described in Lapainis 2009.

2.2.3 Extraction. This protocol is also described in Bora 2008. Manually isolated cells were either used for direct cell analysis or extracted. When used for direct analysis, the cells/clusters were placed directly on a steel MALDI target with the sharpened tungsten needles. The cells were then ruptured and smeared onto the sample spot and then serially spread onto subsequent spots for a dilution affect followed by dried-droplet matrix application of 2,5-dihydroxybenzoic acid (DHB; 50 mg/mL in 50:50 acetone: deionized water). For extraction of the peptides, 10-15 cells were transferred into a microcentrifuge tube with 50 μL of acidified acetone (40:6:1, acetone/water/conc. HCl) with removal of ASW before addition of extraction solvent. Manual homogenization was then performed for approximately 1 min with a pellet pestle (Kimble Chase, Vineland, NJ) to enhance extraction. The samples were then centrifuged at 14,000 × *g* and 15 °C for 10 min. The supernatant was retained and concentrated to 7-10 μL.

2.2.4. Signal P 3.0 and NeuroPred. Online software was used to predict the peptide sequences of the AC2 prohormone. Signal P 3.0 predicted the length of the prohormone signal sequence that would be cleaved off before further peptide processing (Bendtsen 2004). Selected parameters

included eukaryote organism group and both the neural network and hidden Markov model methods. NeuroPred (UIUC) was then used to predict cleavage sites after indication of the signal sequence length (Southey 2006, Hummon 2003). Output sequences were manually confirmed by predicting cleavages after dibasic sites.

2.2.5 Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). MALDI-TOF MS data was acquired on an Ultraflex II TOF/TOF MS instrument (Bruker Daltonics, Bremen, Germany). Samples were spotted (either smeared cells or 0.5 μL extract spots) onto a steel MALDI target and covered with approximately 0.7 μL of DHB matrix. Samples were allowed to dry before insertion into the instrument. Mass spectra were obtained in reflectron mode within 100 ppm mass accuracy. The mass-to-charge (*m/z*) range was 450-5000 with variable accumulations of shots (200-1000). Bruker FlexAnalysis software was used for spectra visualization and picking of peaks signal-to-noise 3 or greater.

2.3 RESULTS AND DISCUSSION

The gene product AC2 is a putative prohormone based on sequence markers until other hallmarks of neuropeptides are confirmed. To help discover these products, *in situ* hybridization via a whole mount tissue image stained for AC2 mRNA transcripts (provided by Vilim with protocol described in Vilim 2001) is used as a map to guide cell selection (Figure 2.1). Then using the mass spectrometry tool MALDI-TOF MS, the final peptide products are measured by measuring the mass-to-charge values of the peptides present in the neurons. However, even with location information, the actual bioactive peptides are difficult to predict due to posttranslational processing like cleavage and chemical modifications (Xie 2010).

Preliminary predictions can be made using available online software that can calculate the most likely prohormone processing site cleavages using databases of known prohormone processing and cleavage predictions based on algorithms and a logistic regression model (Bendtsen 2004, Hummon 2003). The predicted products of the AC2 prohormone were four amino acid sequences separated by dibasic sites with one likely to also be amidated as shown in Table 2.1. This then provides a probable list of masses to look for within the direct tissue and tissue extracts.

Measurement of both types of cell extracts from the indicated A-cluster cells reveal profiles of detected peptides (Figure 2.2). The spectra show some conserved peaks throughout the samples as well as differences indicating variations from cell-to-cell. The first two spectra of Figure 2.2 show the advantage of serial smears; successive smears have less salt and signal is not competing with salt adducts. The spectra were searched specifically in the mass ranges of the predicted peptides. In three separate experiments, the four predicted peptides were not detected (*e.g.* the absence of the GSWN peptide in spectrum C of Figure 2.2). Their absence suggests that AC2 peptide products may not be expressed or detected in the predicted forms. This can be true for a number of biological and analytical reasons. Biologically, if the predicted sequences are correct, the peptides may not be present in those cells because they are dependent on the specific state of the animal (*e.g.* age, satiation/hunger, etc.) as in Floyd 1999 when insulin peptides were shown to decrease with food deprivation. It could be that the peptides are present but below the detection limit. The issue could also be in the mRNA staining. It could be detecting a similar but unrelated mRNA than the AC2 target (Manning 1998).

2.4 FUTURE DIRECTIONS

To further investigate the validity of AC2 as a peptide precursor that may be expressed in the cerebral ganglia A-clusters, other routes should be utilized. The pooled extracts of the clusters could be run on an instrument that offers fragmentation and sequencing capabilities (MS/MS). If any portion of the AC2 sequence was detected there, this would give strong evidence of expression of AC2 in some form. More predictive work may be done to consider alternative cleavages as may be also indicated by the MS/MS results. Consideration of post-translational modifications and even secondary structure (*i.e.* the possibility Cys-Cys bonds due to the high number of Cys residues) would provide more probable masses to verify in sample spectra.

In regards to *in situ* hybridization, the F-clusters could also be examined for the same peptides. If they reveal similar results then the source sequence of the hybridization could be reexamined for an alternative that could reconfirm the presence or absence of AC2 expression in the *Aplysia* cerebral ganglia.

2.5 TABLES AND FIGURES

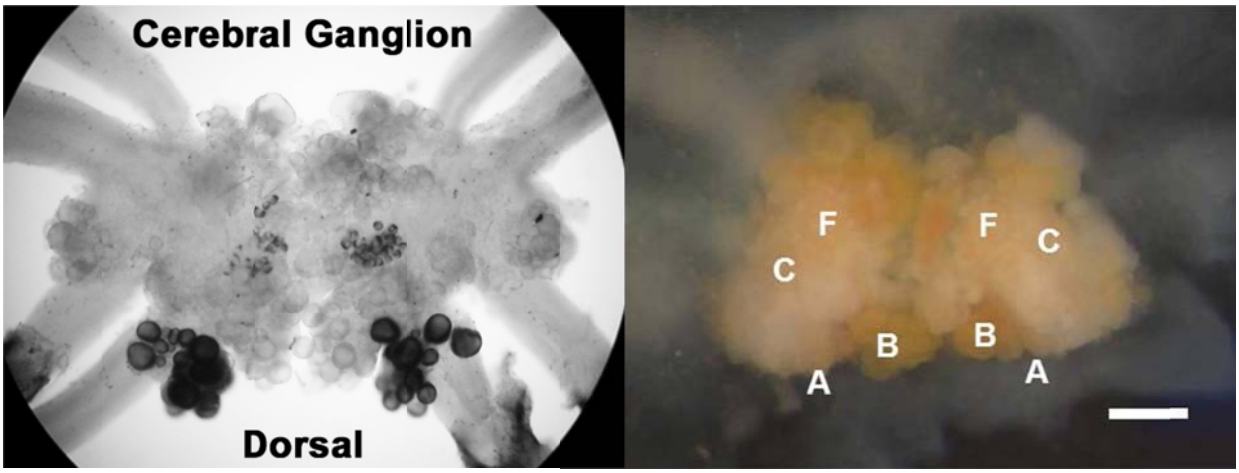

Figure 2.1 Whole mount *in situ* hybridization (ISH) of the AC2 precursor mRNA in the cerebral ganglia of *Aplysia* (left-ISH image provided by Sven Vilim). Strongest staining is found in the A and F-clusters. On the right is a micrograph of freshly dissected cerebral ganglia highlighting some distinct clusters and that the A-clusters have been removed for extraction and peptide analysis. (Scale bar is 1 mm).

Table 2.1 List of peptide sequences and corresponding calculated M+H from AC2 predicted with Signal P 3.0 and NeuroPred online software (Bendtsen 2004, Southey 2006, Hummon 2003).

Sequence	[M+H] calc.
GSWN	463.19
GPLDPCVFQCMRGVLKC	1865.89
TQAANTCNNNGHQHVPDFDDVVDLGQRFY	3275.46
EQSVVGLSQKVDCCQKYKMCYLVCRPDAESVPPCGGAK + amidation	4117.95

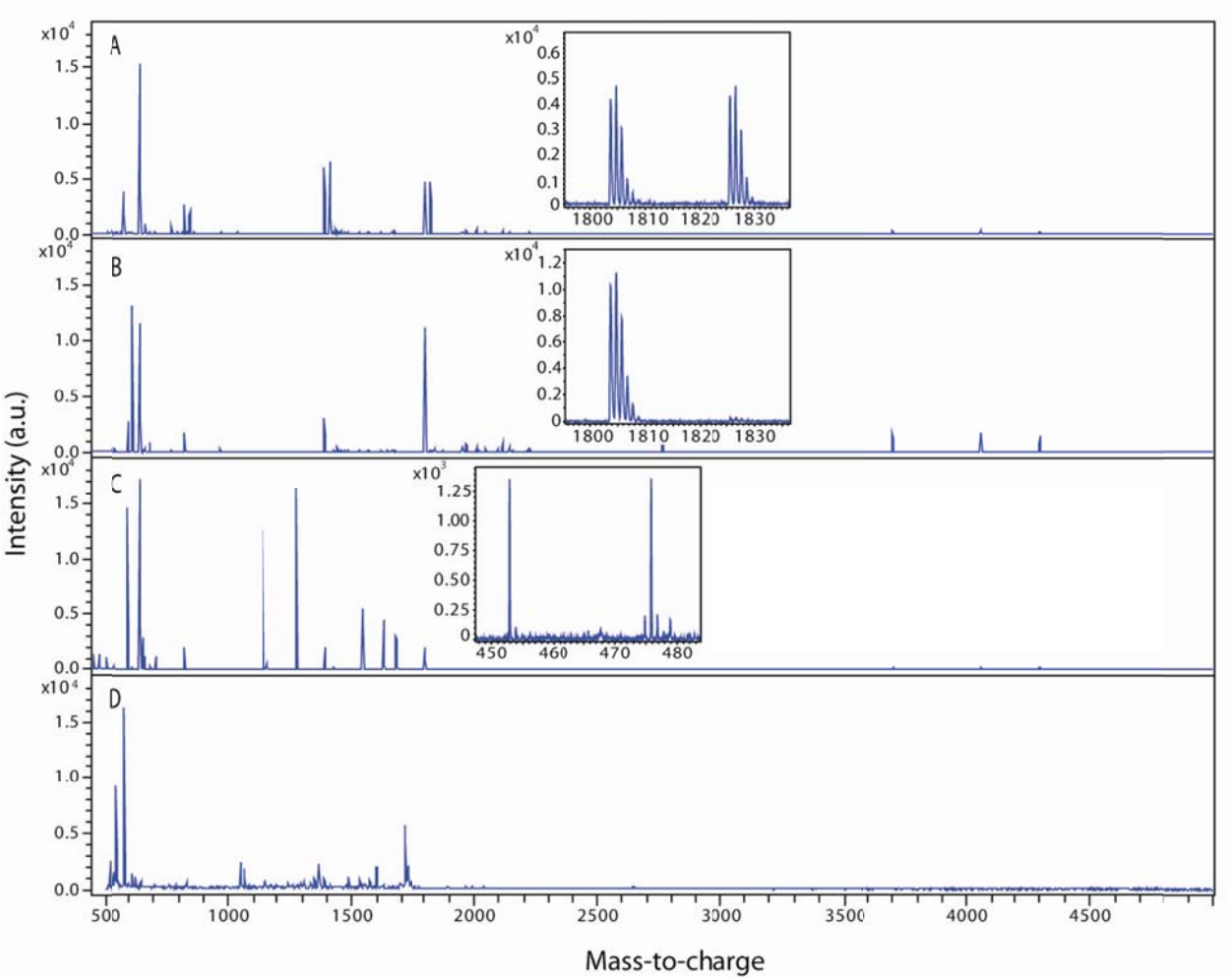

Figure 2.2 Mass spectra from three investigations into the presence of predicted AC2 peptides in the A-clusters of *Aplysia*. A and B are spectra from a serial cluster smear, C is a cluster smear from another animal and D is a spectrum of cell extract from the acidified acetone protocol. The inset of A shows a Na-adduct (*m/z* 1825.7) on the species with *m/z* 1803.7 which decreases in the serial smear of the same cluster (B inset). The inset of C shows the absence of predicted AC2 peptide GSWN at *m/z* 463.19.

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CHAPTER 3

EX VIVO COLLECTION AND DETECTION OF THE *RATTUS NORVEGICUS* SUPRACHIASMATIC NUCLEUS AND SUPRAOPTIC NUCLEUS PEPTIDE RELEASATE

NOTES AND ACKNOWLEDGEMENTS

This work involves a collaboration between the Sweedler and Martha Gillette research groups. Dr. Shifang Ren and Dr. Norman Atkins are acknowledged for their guidance in the SCN and SON release experiments. Dr. Stanislav Rubakhin and Agatha Luszpak are also acknowledged for their dissection contributions and expertise. This material is based upon work that was supported by the following agency and grants: National Institutes of Health NS031609 and DA018310.

3.1 BACKGROUND

The suprachiasmatic nucleus (SCN) and supraoptic nucleus (SON) are peptidergic brain regions that function in regulatory processes through the use of a variety of cell to cell signals (Lee 2010, Bora 2008). Release of neuropeptides and hormones is one form of their chemical signaling. The peptides are released from dense core vesicles into the extracellular space to diffuse to their targets (Bergquist 2009, Strand 1999). While it can be difficult to monitor peptide release in an intact organism, one can use brain slices maintained *in vitro* (the so called *ex vivo* brain slice preparation) to study mechanisms of release and the actual released species. This information gives a dynamic and functional picture of those particular tissues. Different tissues can release distinct sets of peptides with different stimulants, or one can use a generic stimulant, KCl, to study release from any neural tissue by depolarizing the membrane to produce a massive

release event of its vesicular pool. When working with a tissue slice maintained in a brain slice chamber, the released peptides do not reach their cellular targets but are released into the chamber where they could be collected. One can relate the specific "cocktails" of release peptides to specific tissues and stimuli. A connection between the release of peptides to even a general stimuli provides functional implications. Here my progress in using *ex vivo* brain slice preparation to monitor peptide release from the SCN and SON are highlighted. The research involves efficiently collecting the peptide release using solid phase extraction protocols and then characterizing the released peptides with mass spectrometry.

3.2 EXPERIMENTAL

3.2.1 Animals. Adult male Sprague-Dawley rats (Harlan Laboratories, Madison, WI) were caged individually with food and water available *ad libitum* and maintained on a 12 hr light-dark cycle (lights on at 0800). All procedures concerning the animals were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee and were in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

3.2.2 Sample Preparation. After decapitation the brain was removed and rinsed with approximately 1 mL ice cold Earle's balanced salt solution (EBSS, all in mM: 1.8 $CaCl_2 \cdot 2H_2O$; 0.81 $MgSO_4$; 5.4 KCl; 26 $NaHCO_3$; 116 NaCl; 1.0 NaH_2PO_4, 5.6 D-glucose). The tissue block (approximately 0.5 × 1.0 × 0.5, L × W × H in cm) was superglued (rostral side down with dorsal side facing blade) to the specimen tray of a Vibratome (Vibratome 3000 Series; Ted Pella, Redding, CA) and then submerged in ice cold EBSS bubbled with 95% O_2/5% CO_2. 400-µm

thick coronal slices were shaved off the tissue block until the SCN and SON were present in the same plane for several slices. These slices were transferred to an EBSS-filled chamber via large pasteur pipette.

3.2.3 Brain Slice Chamber Instrumentation. The brain tissue slices were sustained in a brain slice chamber (BSC1; Scientific Systems Designs Inc., Mississauga, ON) with circulating EBSS heated in a lower chamber to 41 °C. An elevated reservoir of EBSS was used to refill the brain slice chamber by a pressure difference while chamber EBSS is removed by vacuum. The tissue was placed on a net insert allowing the tissue to be submerged less than 1 mm below the EBSS surface. Two metal stands with fine controls stood on either side of the brain slice chamber to hold the sampling devices. Each suspended the tip of a C18-packed pipette (ZipTip; Millipore Corporation, Billerica, MA) within 1 mm of the tissue surface (while still below the EBSS surface) and was connected to a syringe pump under negative pressure (0.2 μL/min). Set-up and technique are also described in Hatcher 2008.

3.2.4 Sampling and Stimulation. The areas of interest (SCN and SON) were located visually and stimulated by the direct addition of 5 μL of 1 M KCl (5 μL per brain region). Prestimulation and stimulation collections were done over 20 min (each time with new a ZipTip). The stimulation collection was started immediately prior to the addition of the KCl.

3.2.5 Solid Phase Extraction. After collection the ZipTips were either immediately washed and eluted onto a pre-spotted MALDI target or temporarily stored until the end of the experiment in 20 μL of water in 0.6 mL microcentrifuge tubes (siliconized, low-retention; Fisher Scientific).

The protocol for ZipTip conditioning consisted of pipetting 20 uL of each of the following solution sequence: 50:50 ACN:water, water, EBSS. The washing consisted of rinsing the ZipTip with 20 uL of water. Finally the elution used 70:30 ACN:water. Elution was carried out directly from the ZipTip onto the analysis target through the use of a 100 µL Hamilton blunt syringe by introducing the elution solvent from the inner side of the ZipTip. Each sample was eluted as 5-6 0.2 µL spots and was allowed to air dry before analysis. Eluted samples were stored on the target in a case and storage bag at 4 °C before analysis.

3.2.6. Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). MALDI-TOF MS data was acquired on an Ultraflex II and Ultraflextreme TOF/TOF MS instruments (Bruker Daltonics, Bremen, Germany). Samples were eluted and spotted onto a Bruker/Eppendorf pre-spotted anchor chip target with alpha-cyan-4-hydroxycinnamic acid (CHCA) matrix along with pre-spotted calibrants on dedicated positions. Mass spectra were obtained in reflectron mode within 100 ppm mass accuracy. The mass-to-charge (*m/z*) range was 600-6000 with variable accumulations of shots (200-1000). Bruker FlexAnalysis software was used for spectra visualization and picking of peaks over signal-to-noise 6.

3.3 RESULTS AND DISCUSSION

Ex vivo stimulation and collection of peptides from regions of interest on brain tissue slices require bio-mimicking conditions to allow for release and to sustain the tissue throughout the sampling process. A temperature regulated and oxygen-supplemented system that constantly replenishes the slice with a fresh balanced salt environment allows for viability of the tissue to be

maintained. The brain slice chamber shown in Figure 3.1 was used to hold and maintain the brain tissue slice during sampling.

Collection of release has been carried out through various solid phase extraction (SPE) methods. Porous polymer monoliths (Ianncone 2009), SPE beads (Hatcher 2005) and functionalized surfaces in microfluidic devices (Jo 2007) have all been used to capture released peptides through the interaction of hydrophobic regions, which allows for separation from their native salt-rich environment that can hinder mass spectrometric detection. In this work C18-packed pipettes were used for the capture of peptide analytes, similar to work in Rubakhin 2001. Figure 3.1 shows two such suspended ZipTips to be used in peptide extraction from direct tissue. Released peptides are drawn to this conditioned SPE material through negative pressure and are allowed to bind over a sampling time period. The bound sample can then be desalted and carefully eluted by a small volume of high organic (Figure 3.5.2) onto a MALDI target with prespotted or manual addition of matrix. This method allows for the MALDI MS detection of peptides from live brain slices as shown in Figure 3.3. As evidenced in the inset, the use of a TOF mass analyzer allows for high resolution mass spectra to be collected with the baseline resolution of isotope peaks possible. The peaks also exhibit high mass accuracy (<100 ppm), allowing for the identification of the detected peptides using MS spectra alone. This can be accomplished by mass-matching to previous studies which utilized fragmentation (MS/MS) to characterize the same intact masses from tissue extraction studies of the same model system such as in Lee 2010 and Bora 2008. The use of this process allows for confident identifications even without the larger sample volumes required for quality MS/MS spectra, which cannot easily be attained with a sample limited brain slice experiment.

A comparison of peptide profiles of the baseline and stimulated release states can be made to suggest which peptides are released in a stimulation-dependent manner, and hence more likely to be bioactive, and perhaps even linked to a brain function. The desired result is to find a consistent change correlated with a specific stimulation, something that the Sweedler group had done with SCN release as a function of time of day (Hatcher 2008).

Here, unfortunately, my attempts to determine consistently released peptide profiles by comparing pre- and post-stimulation of the SCN and SON did not exhibit new patterns. Often the only difference was a change in signal intensity, but this was without a consistent trend as seen in Figure 3.4. Intensity and resolution were improved with the use of a higher performance instrument; however, sources of inconsistencies from the sampling process overshadowed the enhanced analysis. An inherent difficulty is the repositioning of the second ZipTip. The reproducibility of the positioning is currently only visually controlled and so the xyz locations of the pre- and post-stimulation sampling areas are independent despite being apparently similar to the eye. At the extremes, one of the two samplings would yield no detectable signal.

A distinct question involves the nature of the released compounds. To address this, identifications of released species can still be successfully made even without stimulation information. As previously mentioned , the quality of the spectral peaks provide an accurate mass that can be used to identify the species by comparison with identifications from MS/MS studies on tissue extracts of the same (or closely related) brain locations. Such previous studies were compiled; the sequences, monoisotopic neutral masses and source tissues were listed with all necessary species conversions made to the appropriate residues for *Rattus norvegicus* (Altelaar 2009, Boonen 2007, Bora 2008, Caprioli 1997, Che 2006, Decaillot 2006, Dowell 2006, Lee 2010, Parkin 2005, Zhang 2008). This list was referenced against consistently detected mass-to-

charge ratios not seen in ZipTips controls. Listed in Table 3.5.1 are the peptides that appeared at least once in the SCN or SON that have been found in the aforementioned peptide list. Peptide identification validity is strong due to matches to the same tissue type as well as related peptides being observed together (*e.g.* multiple neurotensin fragments).

3.4 FUTURE DIRECTIONS

Future release experiments should first incorporate an improved ZipTip conditioning protocol to allow more peptides to bind and be retained during desalting. Some recent attempts have shown improved results and should be further tested. In general, the organic gradient (50% ACN for conditioning followed by 70% ACN for desalting) may need to be reversed to sufficiently release all possible polymer contaminants from the ZipTips before the sample is introduced. The most recent trials have used 100% ACN instead of 50% ACN used in the initial conditioning protocol. An ion-pairing agent like TFA may also be incorporated to further enhance binding of desired peptide species.

A second improvement would be to provide a more localized stimulation to the region of interest in deter collection of the general mass release from the rest of the slice. This may be done by reduction of the slice size to eliminate tissue outside the region of interest. One possible study to supplement this effort would be to characterize the dynamics of the added stimulant in the brain slice chamber. This could be done by visualizing the diffusion of a colored stimulant replacement such as the addition of a dye (*e.g.* fast green). This way any contributing flow to the stimulant diffusion can be accounted for and controlled if needed. Lastly, the stimulations used here involved fairly universal and harsh stimulants such as KCl. Using electrical stimulation (such as via the retinohypothalamic tract) would eliminate issues with secretagogue application as this

would automatically involve stimulating the same neuronal structure. Thus, this likely will improve biological relevance and repeatability.

3.5 TABLES AND FIGURES

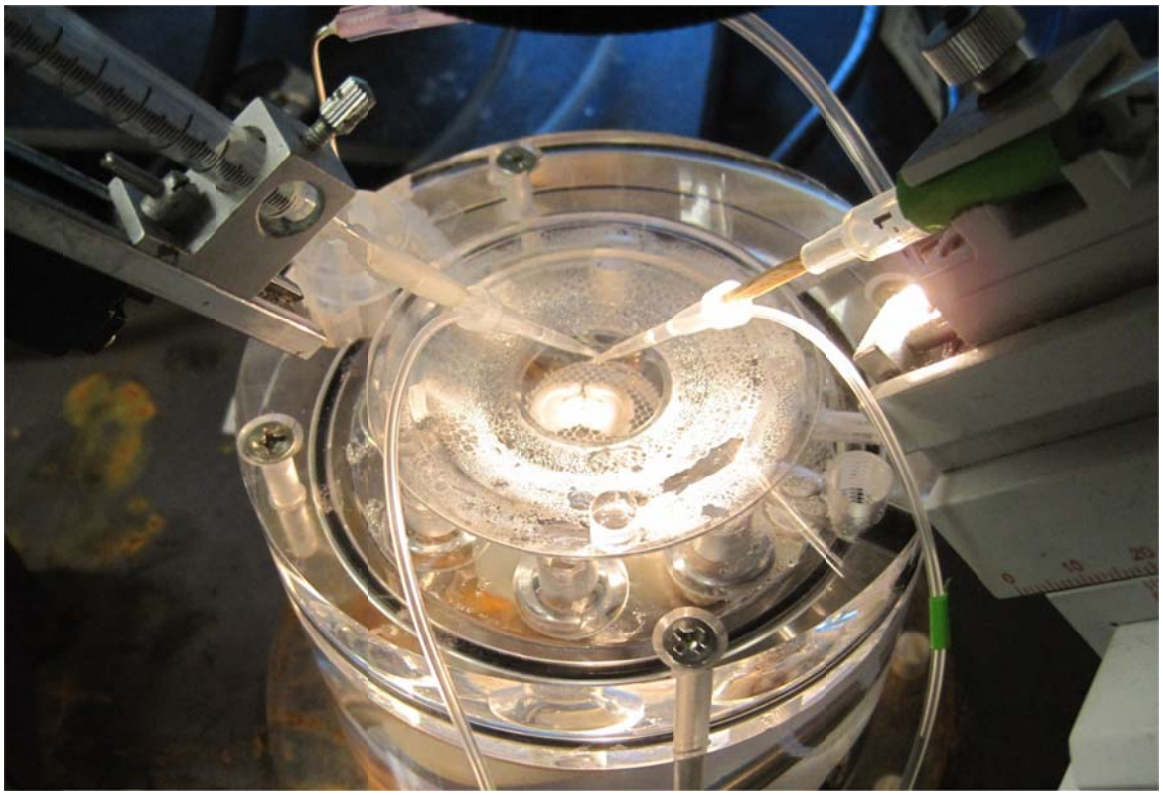

Figure 3.1 Image of the working brain slice chamber with a 400-µm rat brain tissue slice containing both the SCN and SON and positioned on the net insert. Also pictured are two conditioned ZipTips submerged away from the slice before positioning over the appropriate brain regions. [Image courtesy of Chris Dailey]

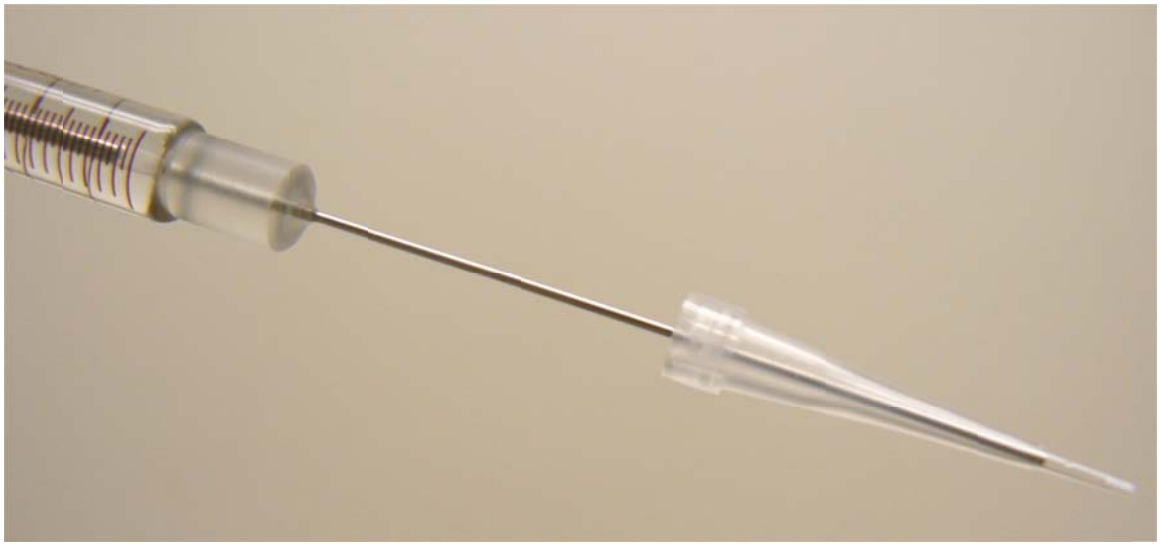

Figure 3.2 Image of elution method after peptide collection and desalting on a C18-packed pipette (ZipTip). Shown is a 0.6 µL ZipTip to be eluted with 70% ACN into 5-6 0.2 µL spots through the use of slow manual control of a 100 µL blunt tip syringe directly onto a MALDI target.

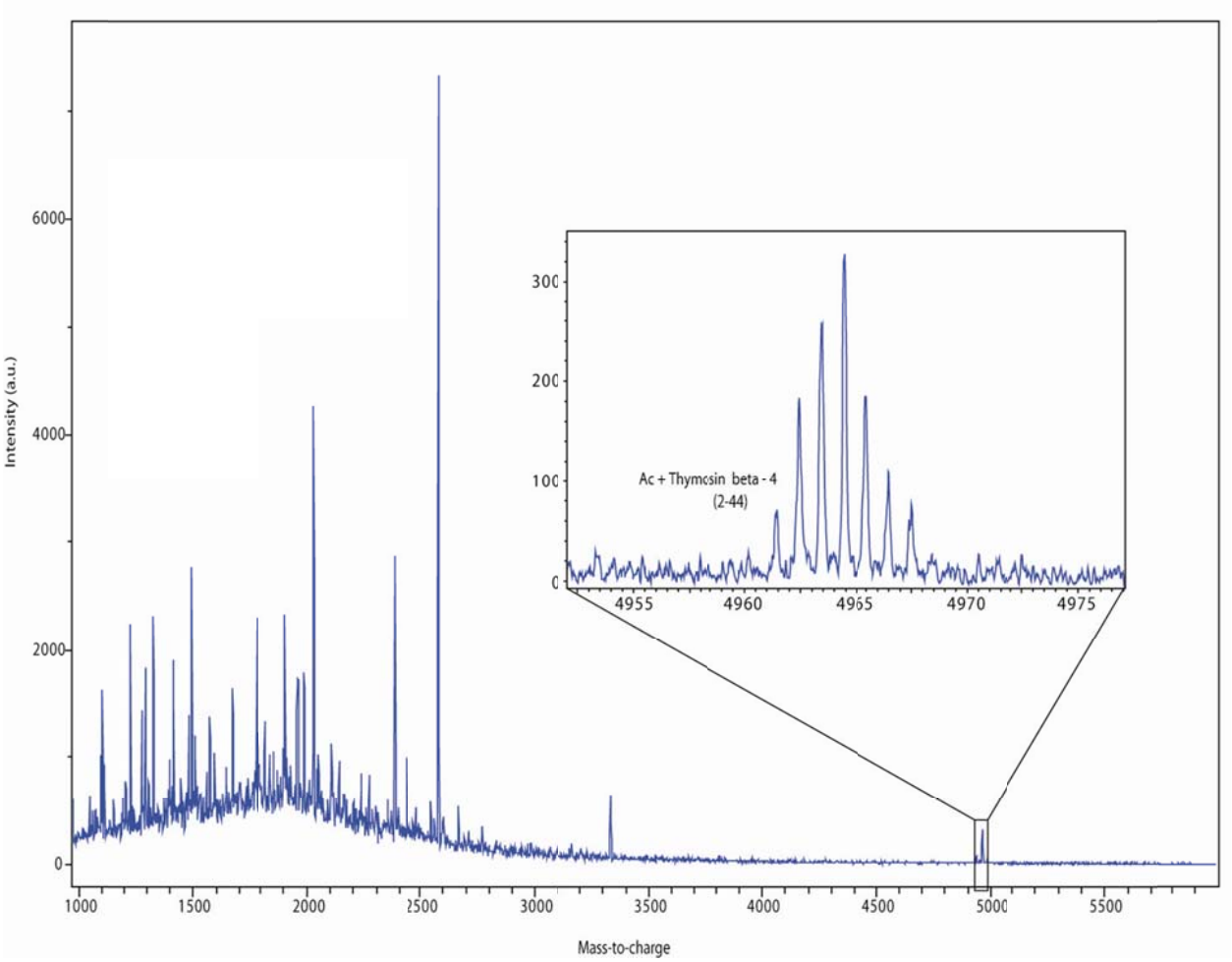

Figure 3.3 Baseline subtracted mass spectrum of SCN release from mass-to-charge 1000-6000 obtained on a Bruker Ultraflextreme TOF/TOF MS. Inset shows the isotopically resolved acetylated form of thymosin beta-4, a common small protein in rat brain slice release profiles, with a 14 ppm mass difference from its calculated mass.

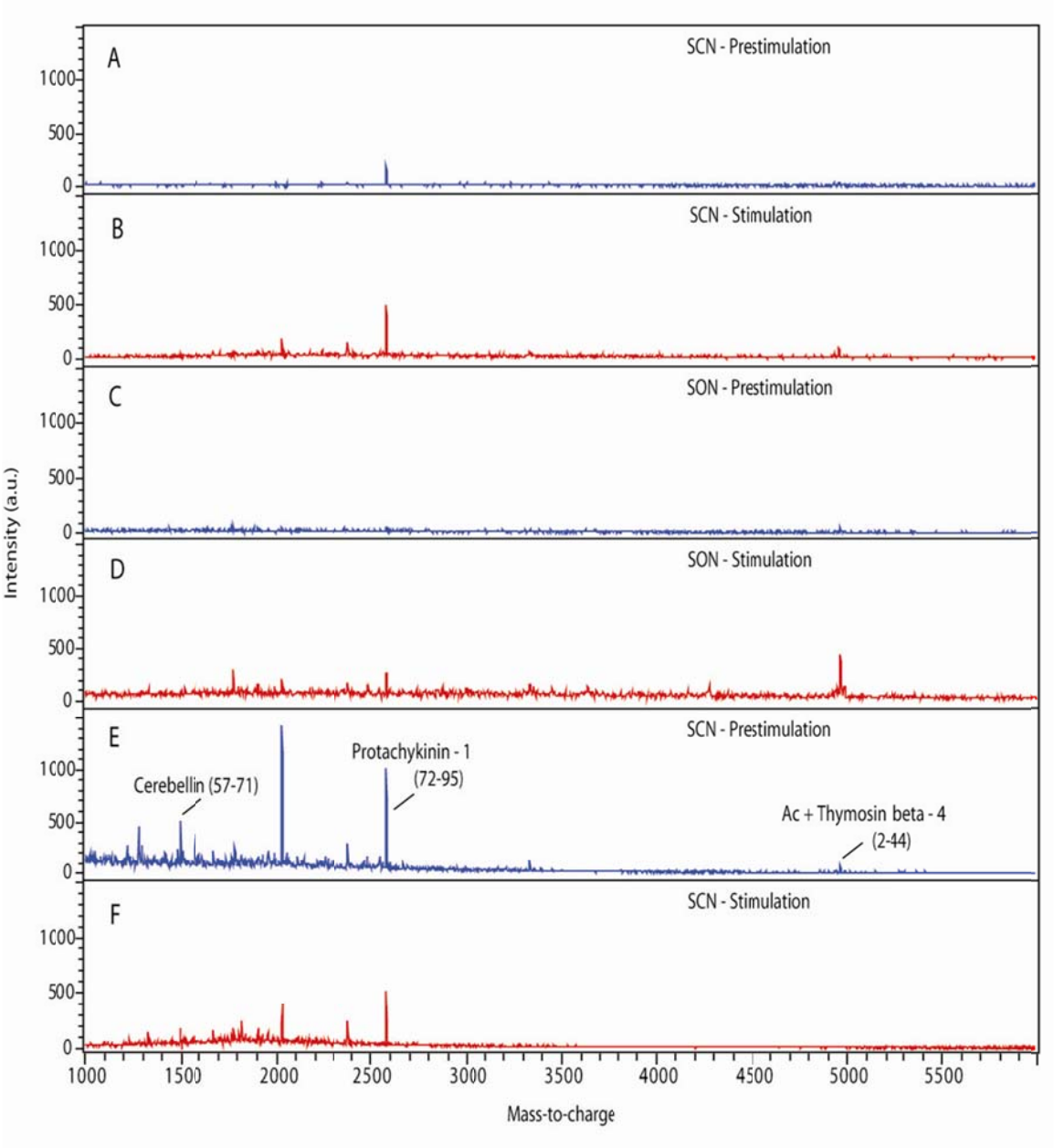

Figure 3.4 Baseline subtracted mass spectra of pre-stimulation and stimulation SCN and SON profiles from mass-to-charge 1000-6000 all scaled to intensity 1500 a.u. obtained on a Bruker Ultraflex II TOF/TOF MS (spectra A-D) and a Ultraflextreme TOF/TOF MS (spectra E-F). The first two spectra pairs show increased signal intensity after stimulation although the opposite has also been seen as in the last spectra pair. The last two also have improved resolution and signal intensity owing to the use of higher sampling rate and performance on a newer instrument model. All spectra show some common profile peaks and all three pairs were collected from different rats on different days.

Table 3.1 Table of identifications of SCN and SON release in *Rattus norvegicus* mass-matched to fragmentation studies in the same model system (Bora 2008, Dowell 2006, Lee 2010, Millet 2010). Other currently unidentified and commonly observed mass-to-charge ratios are also listed. Hi – hippocampus, Hy – hypothalamus, S - striatum.

Peptide Name	Theoretical m/z	Observed m/z	Mass Difference (ppm)	Previous ID tissue
		1295.68		
Cerebellin (57-71)	1495.78	1495.84	-35	SCN
Neurotensin + Pyroglu (150-162)	1672.92	1672.91	4	SON, S, Hy
		1776.78		
Neurotensin + Pyroglu (150-164)	1957.11	1957.06	26	SON
		2029.01		
		2380.20		
Protachykinin-1 (72-95)	2580.33	2580.22	46	SCN
Ac + Thymosin beta-10 (2-44)	4934.52	4934.98	-91	SON, Hi
Ac + Thymosin beta-4 (2-44)	4961.48	4961.43	14	SON, Hi

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CHAPTER 4

MEASURING PEPTIDE RELEASE FROM *IN VIVO* MICRODIALYSIS OF THE *RATTUS NORVEGICUS* HIPPOCAMPUS

NOTES AND ACKNOWLEDGEMENTS

This work involves collaboration between the Sweedler and Gold research groups. Ken Morris of the Gold group is acknowledged for his extensive collaboration on this material. Also making this work possible is Dr. Ji Eun Lee, Dr. Shifang Ren, Dr. Xian Chen and Agatha Luszpak. This material is based upon work that was supported by the following agency and grants: National Institutes of Health NS031609 and DA018310.

4.1 BACKGROUND

Chemical signaling among neurons is the foundation for communication and transfer of information in the brain. It involves a diverse group of chemicals. These include signaling peptides that contribute to various physiological processes and have multiple regulatory roles. Neuropeptides are neuron gene products that are packaged and released from large dense core vesicles into the extracellular space and are allowed to diffuse to their cellular targets (Strand 1999, Gordon 2000, Bergquist 2009). Specifically, neuropeptides are a category of interest among intercellular, chemical-signaling molecules because of their involvement in such processes as behavior, learning, and memory (Strand 1999).

One brain region intensively studied in neuroscience is the hippocampus, known for its roles in spatial memory and navigation (Burgess 2002). It is located in the medial temporal lobe and is shown in a coronal rat brain slice in Figure 4.5.1. Humans with damage or excision of the

hippocampal area exhibit deficits in declarative memory (Eichenbaum 2004). In rat hippocampal regions, there has been research on monitoring neurotransmitter levels (Zhang 2007), detecting specific peptides with labeled assays (Rocha 2003) and behavioral studies where single analytes are correlated to behavior that can be assessed with standard cognitive and behavioral performance tests (McNay 2006). It was in this particular study by our collaborator where it was shown that rats will have significant decreases in spontaneous alternation scores, a measure of working memory, in response to administration of systemic morphine. Since the hippocampus is known for memory roles, the results suggest that the morphine may be affecting response in that area and provide a good model for dynamic sampling of released peptides in response to a stimulus.

To target stimulated peptide release, a comparison of peptide profiles between two or more conditional states can be made. This can be done by coupling an *in vivo* microdialysis method that will allow collection of release in response to stimuli as well as observation of behavior with a non-selective discovery method like mass spectrometry to finally be compared to prior sequencing information for identification.

4.2 EXPERIMENTAL

4.2.1 Animals. Male Sprague-Dawley rats (Harlan Laboratories, Madison, WI) were caged individually with food and water available *ad libitum* for at least one week before surgery. The rats were maintained on a 12 hr light-dark cycle with lights on at 0800. Subjects were 3 months old and weighing between 315 and 350 g at the time of surgery. All procedures were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee and

were in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

4.2.2 Surgery. Subjects were anesthetized and bilaterally implanted with guide cannulae (CMA 12, CMA/Microdialysis, Stockholm) by Ken Morris (Gold group) using sterile stereotaxic procedures. The guides were implanted into the left and right ventral hippocampus (5.8 mm back and 5.0 mm lateral from Bregma, 2.3 mm deep from dura). Rats were allowed to recover from surgery for 1 week and then were handled daily for five consecutive days before the microdialysis procedure was conducted.

4.2.3 Microdialysis Instrumentation. All microdialysis instrumentation and accessories are products of CMA/Microdialysis, Stockholm. A CMA/100 microinjection pump infuses artificial cerebral spinal fluid (aCSF all in mM: 128 NaCl; 3.0 KCl; 1.3 $CaCl_2$; 1.0 $MgCl_2$; 21.0 $NaHCO_3$; 1.3 NaH_2PO_4; 1.0 D-glucose, pH 7.4) from two 1 mL microsyringes (corresponding to left and right sides of the rat brain) to two lengths of fluorinated ethylene propylene tubing (ID 0.12 mm, volume 12 μL/m) connected by tubing adaptors at all junctions. A mounted swivel was connected to the microsyringes by 0.5 m of tubing on the inlets and 2.5 meters of tubing connected the swivel outlets. The longer lengths of tubing are held together with Parafilm M and threaded through a pair of suspended steel rings approximately 1 m over the rat (Figure 4.5.2). The ends are then connected to the inlets on the left and right microdialysis probes. On the outlets, a pair of 0.5 m lengths of tubing were attached the perfusion output to a pair of Millipore 0.6 μL C18 ZipTips.

4.2.4 Microdialysis Procedure. On the day of testing, rats had two CMA-12 20 kDa microdialysis probes briefly inserted and removed from their implanted guide cannulae and were allowed to rest for 1 hour. After that time the rats were taken from their individual cages and placed in an opaque and open cylindrical holding chamber. The probes were reinserted and were perfused with aCSF at a rate of 2 μl/min. Perfusion continued for 1 hr 5 min before any samples were collected as shown in Figure 4.5.4. Samples were subsequently collected in 10 min intervals (20 μL perfusate) via solid phase extraction (SPE).

4.2.5 Stimulation. Rats were subjected to up to two subsequent stimulus conditions per microdialysis experiment. The types were morphine injection and maze training.

4.2.5.1 Morphine. The rat was removed from their cylindrical holding chamber and given a subcutaneous injection of morphine (5 mg/kg SC) or saline (0.9% NaCl) control into the dorsal surface of the animal. It was then replaced into their holding chamber where the subject was again free to move.

4.2.5.2 Maze Training and Spontaneous Alternation Task. The training apparatus was an elevated plus-shaped maze with black Plexiglass floor and walls. The four identical maze arms were distinguished as A, B, C, and D corresponding to the cardinal directions S, E, N, and W respectively. Maze training was performed in a closed, lit room with directional cues on the walls. Maze training, if performed, would follow an injection by 30 min, (120.5 min after start of microdialysis). For each maze training trial, the rat was place into arm B facing the center of the maze. The subject was then free to move about the maze for 20 minutes (4 samples taken, 2 left-

right sets). If the animal was stationary for 5 min in any one location, it was given a touch to the tail to restart movement. During the training time the animal's order of arm visitation was recorded. A spontaneous alternation score was given based on a +1 point system for every 4 novel arms visited in any 5 consecutive visited arms.

4.2.6 Solid Phase Extraction (SPE). The solid phase extraction was carried out by 0.6 µL C18 ZipTips (Millipore Corporation, Billerica, MA). Two protocols concerning their preparation and use were employed between the early and later microdialysis experiments. Both protocols involved passing over the C18 material with a conditioning solution, washing solution, and an equilibration solution before exposure to sample. The ZipTips were attached by tubing adapters to the simultaneous left and right microdialysis outlets for ten minutes. After removal the ZipTips were either immediately washed and eluted onto a pre-spotted MALDI target or temporarily stored until the end of the experiment in 20 µL of wash solution in 0.5 mL microcentrifuge tubes. The early protocol consisted of the following solutions (volume of each step was 2 x 10 µL): conditioning sol. – 50/50 ACN/water, washing sol. – water, equilibration sol. – aCSF, and elution sol. 70/30 ACN/water. The later protocol consisted of the following solutions (again volume of each step was 2 x 10 µL): conditioning sol. –ACN, washing sol. – water + 0.1% TFA, equilibration sol. – aCSF, and elution sol. 70/30 ACN/water. Elution was carried out directly from the ZipTip on the analysis target through the use of a 100 µL Hamilton blunt syringe introducing the elution solvent from the inner side of the ZipTip. One ZipTip was eluted out as 5-6 0.2 µL spots and were allowed to air dry before analysis. Eluted samples were stored on the target in a case and storage bag at 4 °C before analysis.

4.2.7 Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). MALDI-TOF MS data was acquired on an Ultraflex II and Ultraflextreme TOF/TOF MS instruments (Bruker Daltonics, Bremen, Germany). Samples were eluted and spotted onto a Bruker/Eppendorf pre-spotted anchor chip target with alpha-cyan-4-hydroxycinnamic acid (CHCA) matrix with along with pre-spotted calibrants on dedicated positions. Mass spectra were obtained in reflectron mode within 100 ppm mass accuracy. The mass-to-charge (m/z) range was initially 600-6000 with variable accumulations of shots (200-1000). Later experiments were narrowed and standardized to aid in statistical analysis and scanned over the m/z range of 960-4000 and summed 1000 shots. Bruker FlexAnalysis software was used for spectra visualization and peak picking.

4.2.8 Principal Component Analysis. Raw data files were uploaded in various folder structures to reflect the groups being compared with replicates kept in the same folders. Settings for spectra preparation included a resolution of 1000, baseline subtraction with Convex Hull algorithm, baseline flatness of 0.5, mass range of m/z 1000-4000 m/z, enabled Savitsky-Golay smoothing for 5 cycles and a width of m/z 1.0, null spectra exclusion and support of spectra grouping. Peak calculation settings included peak picking on a total average spectrum with a signal-to-noise of 6 and a 3% relative threshold base peak. Calculated peak statistics were used to determine significant peak differences between the groups.

4.2.9 Tissue Extraction. After decapitation both sides of the hippocampus were dissected out on ice within 7 min and were briefly rinsed with deionized water. 400-450 µL of preheated deionized water were added to the sample and continued to be heated for 10 min at 90°C, followed by

centrifugation at 14,000 × *g* for 10 min. The supernatant was saved and the tissue pellet was subjected to a second extraction stage. 400-450 µL of ice-cold acidified acetone (40:6:1, acetone/water/conc. HCl) was added to the sample before 1 min of sonication, 1 min of vortexing, and incubation on ice for 1 hr. The supernatant was saved again after another 1 min or vortexing and 20 min of centrifugation at 14,000 × *g* and 4 °C. A third extraction was performed on the tissue pellet with 400-450 µL of ice-cold 0.25% acetic acid and a 1 hr incubation on ice. The supernatant of this extraction was neutralized with 1 M NaOH and then dried to 25-35 µL with a SpeedVac to remove acetone. All three extracts were combined and filtered through a Microcon centrifugal filter (10 kDa MWCO). Lastly, the filtered extract was concentrated using a speed-vac.

4.2.10 Nano-Liquid Chromatography Fourier Transform Mass Spectrometry (LC-FTMS/MS). This protocol is similar to that described in Lee 2010. The tissue extracts were analyzed using a 12 Tesla LTQ-FT Ultra (Thermo Fisher Scientific) online with a 1D NanoLC pump (Eksigent Technologies, Dublin, CA). The 25-35 µL samples were loaded with helium bomb pressure (500 psi) to a trap column (75 µm ID, ?OD) packed with 10 µm C18 solid phase particles (YMC Co, Ltd., Allentown, PA) and fritted with 5 mm of LiChrosorb (EM Separations, Gibbstown, NJ). The analytical column used was a ProteoPrep II medium (C18, 300 Å, 5 µm; New Objective, Woburn, MA). The flow rates was 300 nL/min with the following gradient: 0–10 min, 0–10% B; 10–75 min, 10–30% B; 75–100 min, 30–45% B; 100–120 min, 45–60% B; 120–125 min, 60–65% B; 125–126 min, 65–5% B; 126–132 min, 5–85% B; and 132-150 min, 85-5% B. Data acquisition consisted of a full scan event (*m/z* 290–2000; resolving power, $m/\Delta m_{50\%}$ = 90,000 in which $\Delta m_{50\%}$ is the mass spectral peak full width at half-maximum peak height) and

data-dependent CID MS/MS scans (40,000 resolving power) of the five most abundant peaks from the previous full scan. MS/MS settings included: isolation width, *m*/*z* 5; minimum signal threshold, 1000 counts; normalized collision energy, 35%; activation Q, 0.4; and activation time, 50 ms. Dynamic exclusion was enabled with a repeat count of 4, an exclusion duration of 180 s, and a repeat duration of 30 s.

4.2.11 Data Analysis and Database Searching. This process is also described in Lee 2010. Raw data files were analyzed using ProsightPC 2.0 (Thermo Fisher Scientific, Boyne 2009). Its component algorithm cRAWler 2.0 converted the raw data files (.raw) into ProSight upload files (.puf) composed of resolved isotopic distributions through the use of the Xtract algorithm. The .puf file from a single nano-LC-MS/MS run contained hundreds of MS1 and corresponding tandem MS (MS2) experiments and their resulting mass lists (within user-defined tolerances such as m/z range, retention time and signal-to-noise ratio), which could be searched against a sequence databases. A search tree was constructed where the first level searched the experiments for absolute masses (within ±10 ppm intact mass tolerance) against a database of predicted rat neuropeptides processed *in silico* through the NeuroPred algorithm (Southey 2006). The second level of the tree processed the experiments without identifications in the first mass search through the use of a "biomarker" mode search against an intact rat database compiled from UniProt 15.0 and with parameter set to ±100 Da intact mass tolerance and ±10 ppm fragment mass tolerance. A Sequence Gazer tool was used for manually determining PTMs on the peptides.

4.3 RESULTS AND DISCUSSION

The goal of this work was to measure the peptides released from the hippocampus during activity and morphine exposure. Unfortunately, the amount of peptide released was small so the first part was to create a database of hippocampus peptides. This way released peptides could be matched to the list to provide identity information that can also hint at functional mechanisms.

Peptide sequencing information can be compiled to provide a database of hippocampus peptides present in the tissue without connection to any stimulus. This involves discovering the peptide complement in a whole tissue extract through analysis with a high mass confidence method capable of analyzing a range of peptides at once. The specific instrument used on the tissue extracts was a 12 tesla Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS) with chemical separation by nano-liquid chromatography. The peptides detected in the hippocampi of two animals are listed in Table 4.1. This technique allowed for high mass accuracy of both intact and fragment mass-to-charge ratios necessary for confident identifications when searched against large proteomic databases as evidenced in the E-values also in Table 4.1 (Boonen 2008, Mann 2008). Also, referencing previous studies with tandem mass spectrometry confirmed sequences in the same model system can contribute to the peptide lists as well (Altelaar 2009, Boonen 2007, Bora 2008, Caprioli 1997, Che 2006, Decaillot 2006, Dowell 2006, Lee 2010, Parkin 2005, Zhang 2008).

The database is expected to hold the subset of interest, peptides that are released and seen in microdialysis, as well as protein fragments that may not be found in a release. To analyze the samples obtained through microdialysis, another instrument was used: matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). It is non-selective, robust, salt-tolerant and can detect trace levels of analyte in complex samples (Boonen 2008). It

has the advantage over FT MS instrumentation in terms of sensitivity, ability to analyze limited volume samples, and salt tolerance. The spectra from the experiment involving only acute morphine exposure and controls (n=8) were evaluated manually and through principal component analysis. The peak lists were searched against the created and compiled databases for mass-matched identifications and putative matches were made but only two neuropeptide was seen in both the hippocampal extracts and the microdialysis samples: secretograinin-2 (571-573) and proenkephalin A (219-229).

At this point, a list of masses had been compiled from each animal, as well as a partial but growing list of identified peptides from the same brain region. Next, would be to identify which peptides/peaks correspond to the observed behaviors. To further examine the spectra and perhaps point out mass-to-charge ratios to put more effort into identifying, the spectra of the treatment groups were compared. The side-by-side comparison (Figure 4.4) did appear to show some differences between the groups but the spectra were too complex to meaningfully analyze manually.

Since the spectra are complex, a statistical approach was used to help simplify the data to the main differences between the groups. The tool to be used was principal component analysis software specific to MALDI MS data to enhance the eye in looking for differences between the treatment groups. Bruker ClinProTools software was used to reduce data complexity and distinguish major differences between conditional groups. Two comparisons were made to try to show the major contributions to variation in the data set. First, the data were organized by treatment group, which showed little clustering as seen in Figure 4.5. The second organization was by animal and this seemed to better define the variation and show that two sets of mass-to-charge ratios were responsible for most of the variation. Major contributors to PC1 included m/z

1121.5 and 1249.6, while 1209.5 and 1337.6 contributed mostly to PC2 (Table 4.2.). If presence of these m/z values was correlated to rats, there was a seemingly random separation among the rats. Four rats (2 morphine and 2 saline treated) display variable integration areas of PC1 components and constant areas of PC2 with the opposite true for the other four rats (again 2 morphine and 2 saline treated). A possible biological explanation for the separation shown by the first two principle components is the occurrence of a coding region single nucleotide polymorphism (SNP) resulting in a peptide that exists in two forms (Tost 2002). The other support for the peptides being related is that both pairs share the same difference of 128.1 Da, which may be the loss of an amino acid residue of lysine or glutamine.

4.4 FUTURE DIRECTIONS

The results show some variation in the data set but unfortunately it does not relate to the goal of finding differences due to stimulant conditions. As it is not described here, further analysis of the other treatment experiments (including the maze training) may reveal treatment specific changes that can be correlated to performance and essentially working memory. However, due to an instrument setting (cubic enhanced calibration vs. quadratic calibration), a recalibration will be required before being able to analyze this data through the same PCA software.

A new set of experiments under controlled conditions optimal for PCA analysis is also underway. The microdialysis samples are being acquired under automatic acquisition optimized to eliminate human bias towards saving spectra and to collect consistent shot counts from sample to sample. At the same time these same animals are being dissected immediately following their microdialysis testing to further correlate and identify peptides seen in microdialysis through tissue extraction and subsequent nano-LC FTMS analysis. Preliminary comparisons to the previous

acute morphine exposure experiments confirm the presence of the same mass-to-charge values as well as enhancing others that were more rarely seen under the previous acquisition conditions. PCA of these animals may give more pronounced differences especially using a so-called 'biomarker' training set option that will evaluate small differences between the treatment groups.

The consistent release and detection of protein fragments is interesting. Could they be an example of a non-classical type of neuropeptide or are they a blood contaminant from disturbing the tissue during probe insertion (Gomes 2010, Gelman 2010)? More work would be needed to confirm this. Infusion of the fibrinogen fragments by microdialysis or use as a tissue slice stimulant may give a response that can give clues to its function in the brain. If the addition of the protein fragments themselves caused a detectable change or release, their presence in the hippocampus may be the result of more than just the degradation of blood. A control for this could be performed by implanting probes in a different brain region to see if their presence is not ubiquitous.

In reference to using stimulants and better assessing their responses, it would be helpful to improve temporal resolution to the current microdialysis set-up. A future direction for the work is to integrate an oil plug system similar to that described in Wang 2008 to package peptide response that could be much easier correlated with the chemical or training stimulation.

Future experiments may also incorporate an evaluation of the microdialysis output not retained on the ZipTip. This can be analyzed via CE-MS for separation and detection of the smaller and important mass range that includes small molecule neurotransmitters known to play a role in memory.

4.5 TABLES AND FIGURES

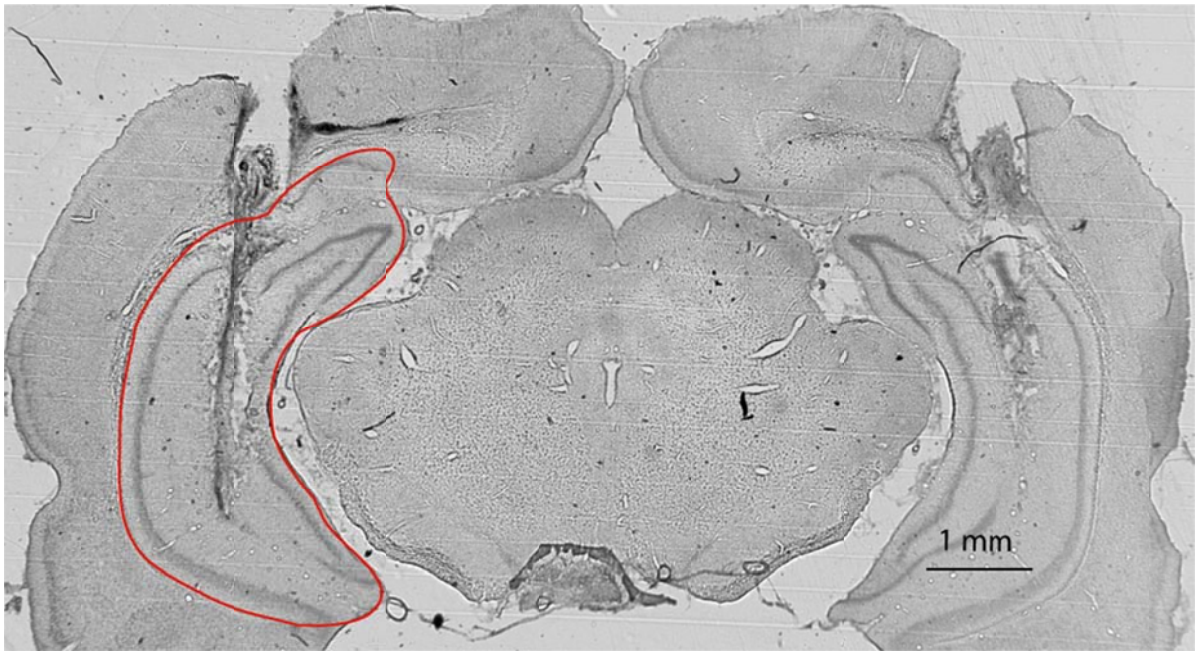


Figure 4.1 Micrograph of coronal rat brain slice stained with cresyl violet and microdialysis probe damage in one side of the ventral hippocampus with the hippocampus outlined in red. [Image courtesy of Ken Morris]

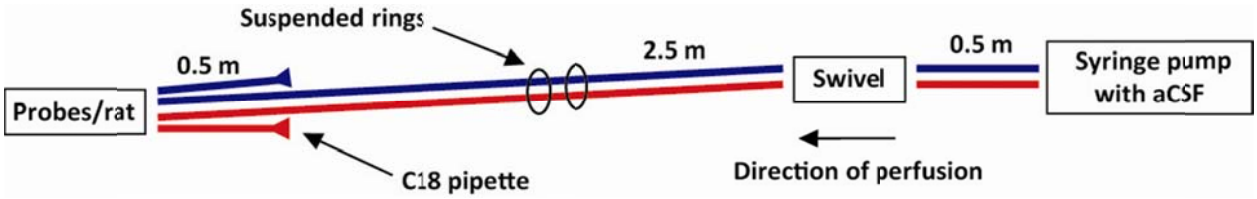


Figure 4.2 Microdialysis instrumentation set-up showing major components and tubing lengths.

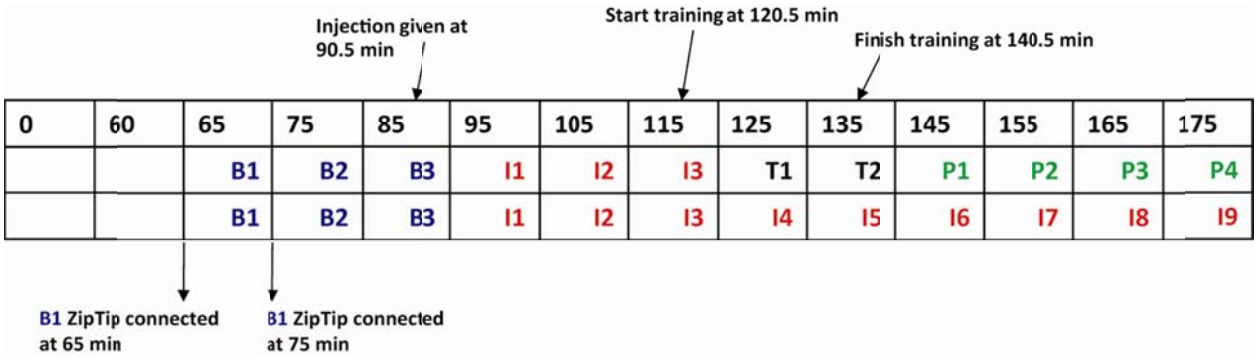


Figure 4.3 Timetable (in min) of equilibration, stimulation and sampling during microdialysis.

Table 4.1 Table of prohormone processed peptides identified from hippocampal tissue extracts from two rat subjects. Starred peptides were seen in both the microdialysis and hippocampus extract. E-value is a measure of confidence = P-score x database size with confident identification confirmed at E-value of 1E-4 or less.

	Prohormone Description	Peptide Name	Fragment	Sequence	Theoretical Mass	Mass Diff (ppm)	Mass Diff (Da)	E Value
1	Cerebellin-1	Cerebellin	57-72	SGSAKVAFSAIRSTNH	1631.84	0.88	0	1.29E-34
2	Cholecystokinin		36-44	AEEAPRRQL	1068.57	0.88	0	3.27E-08
3	Cholecystokinin		46-57	AVLRPDSEPRAR	1365.75	0.68	0	3.00E-09
4	Cholecystokinin		46-60	AVLRPDSEPRARLGA	1606.89	-0.1	0	4.42E-09
5	Cholecystokinin		72-94	APSGRMSVLKNLQGLDPSHRISD	2477.28	1.14	0	1.69E-36
6	Cholecystokinin		71-94	KAPSGRMSVLKNLQGLDPSHRISD	2605.38	1.82	0	1.87E-06
7	Neurogranin		59-75	GGPGGAGGARGGAGGGP	1208.57	0.19	0	1.05E-25
8	ProSAAS	Little SAAS	42-59	SLSAASAPLAETSTPLRL	1783.97	0.86	0	2.15E-34
9	Proenkephalin A		198-209	SPQLEDEAKELQ	1385.67	0.6	0	4.91E-33
10	Proenkephalin A*		219-229	VGRPEWWMDYQ	1465.64	1.18	0	1.53E-16
11	Proenkephalin A		239-260	FAESLPSDEEGESYSKEVPEME	2488.05	1.3	0	1.16E-35
12	Proenkephalin A		143-185	DADEGDTLANSSDLLKELLGTGDNRAKDS-HQQESTNNDEDSTS	4592.01	1.01	0	1.94E-20
13	Secretogranin-1		435-451	LLDEGHDPVHESPVDTA	1829.84	1.06	0	1.87E-34
14	Secretogranin-1		416-432	GRGREPGAYPALDSRQE	1857.91	0.5	0	5.34E-15
15	Secretogranin-1		513-532	LGALFNPYFDPLQWKNSDFE	2400.14	0.6	0	6.76E-40
16	Secretogranin-2*		571-583	IPAGSLKNEDTPN	1354.67	0.91	0	8.10E-28
17	Somatostatin		89-100	SANSNPAMAPRE	1243.56	0.75	0	1.01E-27

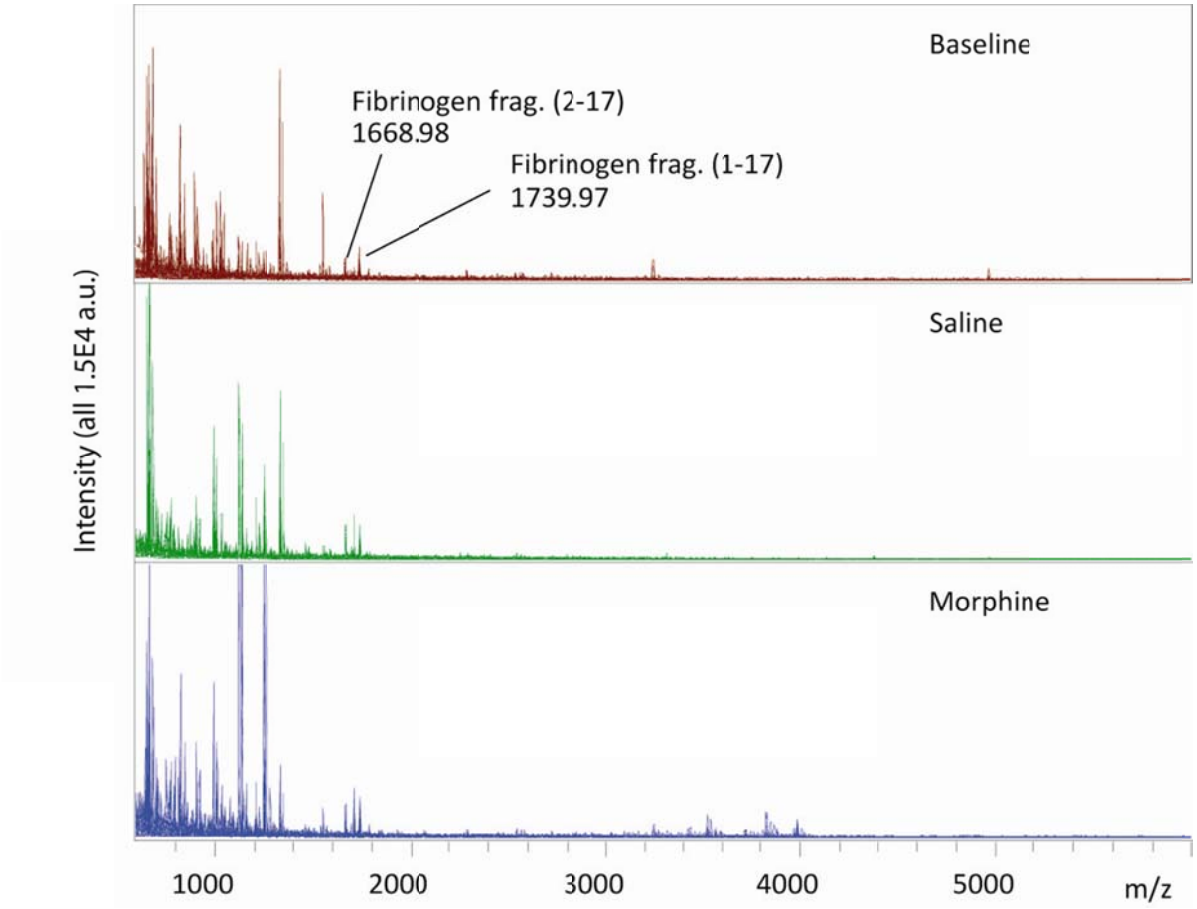


Figure 4.4 Summed spectra from 8 animals separated into 3 treatment groups. Labeled are two identified fragments that are prevalent in all treatment groups: Fibrinogen fragments 1-17 and 2-17 (Dowell 2006).

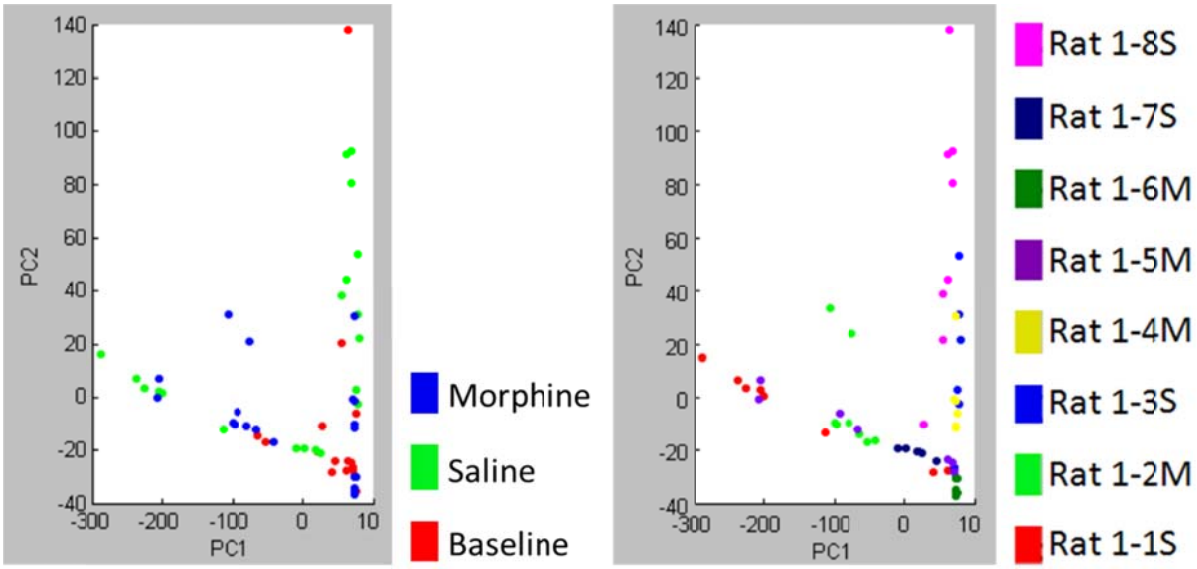
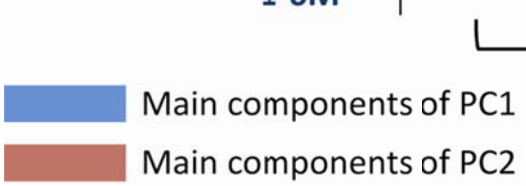
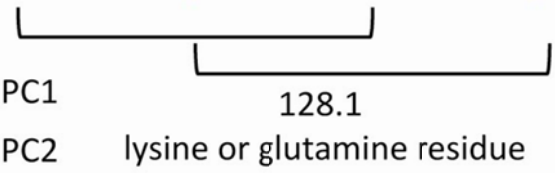


Figure 4.5 Plots of principal component 1 (PC1) vs. PC2 for two different organizations of the 8 animal data set. Points represent sample spectra. Clustering was most strongly defined by animal. No clear separations were seen between the morphine samples and the baseline or saline controls.

Table 4.2 Table of distributions of the major contributors to PC1 and PC2. The mass-to-charge ratios come in a pair with their oxidized species and the two pairs within the same PC are separated by the same mass shift. This mass shift mass be explained as a difference of one amino acid residue on the same base peptide.

	m/z			
Rat ID	1121.5 / 1137.5	1209.5 / 1225.5	1249.6 / 1265.6	1337.6 / 1353.6
1-1S	x		x	
1-7S	x		x	
1-2M	x	x*	x	x*
1-5M	x		x	
1-3S		x		x
1-8S		x		x
1-4M		x		x
1-6M		x		x





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