ABSTRACT

CALHOUN, SARAH ELIZABETH. Expanding Fast-Scan Cyclic Voltammetry to Enable In Situ Neuropeptide Quantitation (Under the direction of Dr. Leslie A. Sombers).

Deaths from opioid overdoses is increasing at an alarming rate and creating an epidemic, instating a cascade of negative repercussions affecting individuals, families and communities. Opioid peptides are heavily implicated in many behaviors including the perception of pleasure and pain, motivated behavior, and drug addiction. Unfortunately, little is known about how opioids function, largely due to the lack of scientific tools capable of monitoring and measuring opioids. The opioid system is complex, due in part, to the synthesis pathways of endogenous opioids\(^1\)\(^2\) and the promiscuity in which they bind to opioid receptors. Additionally, the inherent low abundance of endogenous opioids in the brain introduces analytical challenges which must be addressed before neurological questions about this system can be answered in detail. New analytical tools are needed to enable neuroscientist to gain a better understanding of the complex opioid system and help find solutions for society to combat the opiate epidemic.

The development of real-time electrochemical sensing technologies has significantly impacted our understanding of the dynamic molecular mechanisms underlying basic brain function, as well as a variety of neuropathologies and disease states. However, carbon-fiber microelectrodes (CFME) developed almost 30 years ago are still in use, and electrochemical waveforms have changed very little since fast-scan cyclic voltammetry (FSCV) was first introduced as a promising methodology for monitoring neurotransmission. The work presented here is to enhance and characterize the electrochemical detection of endogenous opioids measured on a time-scale comparable to the real-time dynamics of these molecules in the brain. A tool that directly monitors endogenous opioids will allow researchers to ask a host of new questions about these important molecules and obtain a mechanistic basis of the endogenous
opioid system which will inform new pharmacological studies with analytical data. The information gained from these studies will be of use for informing opioid therapies and enable the electrochemical study of many small peptides containing tyrosine or methionine.
Expanding Fast-Scan Cyclic Voltammetry to Enable *In Situ* Neuropeptide Quantitation

by
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DEDICATION

This work is dedicated to my family, specifically my parents, Kenny and Kim Calhoun who cultivated my curiosity, work ethic, and character as well as being a continuous source of encouragement. Also to Zachariah, Jennifer and Lucas Deyo for all the Sunday lunches filled with laughter and encouragement. To Aaron, Laura, Brenna and Malachi Berryman for taking care of everyone in the mountains and filling my heart with joy. Lastly, I dedicate this work to my little brother Mark Calhoun who made me a better human being and will never be forgotten.
BIOGRAPHY

It was the day after Christmas 19 hundred and 88 in Johnson City, Tennessee that Sarah Elizabeth Calhoun first saw the light of day, as the third daughter of Kenny and Kim Calhoun. Grass grew up the middle of the curvy dirt hollow where she grew up, in a house her uncles had built over 100 years earlier. Sarah had a storybook childhood in the Appalachian Mountains of North Carolina, spending her summers in the woods playing with her sisters Jennifer and Laura. Sarah often visited her neighbor great aunt Bertha Calhoun who would cook fried potatoes as a snack for her favorite great niece. After twelve long years of waiting she finally got the little brother, Mark, she had always asked for.

At 18 she left the hollow to return to Johnson City to earn her BS at East Tennessee State University in three years. A couple months after graduation she got the opportunity to work at Eastman Chemical where she found her passion for analytical chemistry. Sarah enjoyed her work from the regulatory controls lab to swing shifts in the quality control labs supporting manufacturing to temporarily moving to Texas to help startup a new plasticizer line. Sarah was fortunate to meet many friends and mentors within Eastman that helped mold her as a young scientist.

After four years at Eastman Chemical she finally pulled herself away from the Appalachian Mountains she loved so dearly to go back to school to further pursue her passion and matriculated at North Carolina State University for her PhD in analytical chemistry. It was there she met Dr. Leslie Sombers and joined her lab as an eager electrochemist falling under her guidance for the next step in her scientific career.

When not in the lab, Sarah is often found playing racquetball or cards with friends. Her favorite place to spend a Saturday morning is taking in the beauty of God’s creation while fly
fishing on the closest river. On Sundays you can find her at church with the Deyos of Hillsborough followed by the traditional family Sunday lunch.
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I would like to express my gratitude to my advisor and mentor, Dr. Leslie Sombers. Thank you for setting a high bar for me and seeing me through many challenges that appeared along the way. You have encouraged and challenged me to become a better scientist. A special thank you to all of my committee members for your guidance and support through the PhD process, Dr. Robert Kelly, Dr. Dave Muddiman, Dr. Gufeng Wang, Dr. Sabrina Robertson, and Dr. Ed Bowden. Your input has been key on my development as a scientist.

This work would not have been possible without funding from the Chemistry Department at North Carolina State University which has generously supported me as I have worked toward my PhD as well as the electrochemical community in the Research Triangle that I have had the opportunity to serve during my time as a graduate student.

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CHAPTER 1: Introduction to Neurotransmission, Endogenous Opioid Neuropeptides, and Techniques Used to Study Opioid Neuropeptides

1.1 Anatomy of a Neuron

Neurons, the building blocks of the central and peripheral nervous system (CNS and PNS), have three parts; the axon, a cell body, and dendrites. The cell body contains the nucleus, Golgi apparatus, and other organelles. Dendrites, which extend from the cell body, allow cells to receive information, usually via cation or chemical messengers that bind to a specific receptor. Receptors are proteins embedded in the cell membrane that elicit some physiological response, change in membrane potential or changes to the cellular activity, when bound by a ligand. If there is a change in the resting membrane potential above the cell’s threshold value, an action potential propagates an electrical signal from the cell body down the axon which eventually signals the neuron to release neurotransmitters. These neurochemicals are often released into a synapse where the dendrites or neighboring cells, typically 5-50 nm (Figure 1), can receive these chemical messages.

Figure 1.1: Neuronal Synapse. The synapse has a presynaptic axon releasing a chemical messenger and the postsynaptic cell’s dendrite can receive that chemical message with a receptor. 
1.2 Action Potentials

Action potentials occur as a result of electrical and chemical inputs in neuronal cells that have voltage-gated ion channels in their cell membrane. These ion-specific channels enable a rapid potential change across the cell membrane (Figure 1.2). Neurons at rest have high concentration gradients; specifically, sodium and chloride are much more concentrated outside the cell while potassium concentration is higher inside the neuron. This distribution of charged ions, leaky potassium channels, and ion pumps lead to a resting membrane potential in most neurons of ~70 mV. When the membrane potential increases above the threshold, in a typical cell above ~55 mV, the cell is depolarized. Na+ and K+ ion channels open allowing an influx of Na+ ions and an efflux of K+ ions which make up the rising phase of the action potential. Na+ channels close rapidly while K+ channels close slowly, allowing the cell to repolarize, and eventually hyperpolarize beyond the ~70 mV resting potential. This hyperpolarization is also known as the refractory period and the cell cannot be excited during this part of the action potential (Figure 1.2 Hyperpolarization). The action potential propagates as a wave down the axon through rapid membrane depolarization to the synaptic terminals. Action potentials facilitate an intracellular cascade leading to the vesicular release of neurotransmitters at the axon terminal.
Figure 1.2: Voltage from an Action Potential. Voltage versus time trace of an action potential as it would appear on an oscilloscope during a current clamp experiment.

1.3 Neurotransmission

Neurotransmitters, or chemical messengers, can modulate the activity of surrounding neurons through receptor binding. Intracellular cascades can result from binding at surface membrane receptors. This binding can initiate signal transduction or an intrinsic biochemical response in the neuron. The chemical messengers that can induce this mechanism can range from ions, small molecules like H$_2$O$_2$, monoamines and even large complex peptides. One highly studied group of molecules are monoamines, which are typically packaged in vesicles in the presynaptic neuron and released, via exocytosis, into the synapse. Released neurotransmitters can bind to surface proteins, be taken back up by transporter proteins on the cell’s membrane via reuptake, be broken down by enzymes in the synapse, or diffuse away from the synaptic terminal. Neurochemical dynamics occur on a sub-second time scale and are very complex in nature. Understanding the when specific ligands, such as endogenous opioids, are released into the extracellular space is essential in understanding the mechanistic details of normal function.
One example, if a researcher working with rats wanted to decrease the amount of catecholamine released during an experiment they could give the animal a cocktail of reserpine and alpha-methyl-p-tyrosine (α-MPT).\textsuperscript{9-10} Reserpine is known to inhibit the vesicular monoamine transporter (VMAT), key for transporting catecholamines into storage vesicles. The inability of the catecholamine to get into the vesicles enables their breakdown primarily by monoamine oxidase in the cytoplastm of the cell. This process decreases the amount of catecholamine found in vesicles that can be released. α-MPT reduces available catecholamine for release by blocking the synthesis pathway. Specifically this drug inhibits tyrosine hydroxylase, an essential enzyme in the synthesis of catecholamines.\textsuperscript{6, 10} These drugs can be given together as a cocktail to decrease the amount of catecholamine released from a cell. This kind of treatment is useful when catecholamines are the main ligand of interest.

1.4 Endogenous Opioid System

Opioid peptides are a subset of peptides, which are the largest and most varied group of signaling molecules in intercellular communication.\textsuperscript{11-12} Endogenous opioids are highly implicated in many functions necessary for survival including pain, stress response, gastrointestinal function, eating, learning, motivated behavior, and the biochemical response to multiple drugs of abuse.\textsuperscript{13-23} Unfortunately a full understanding of how many of the endogenous opioids function is unclear due to the lack of knowledge surrounding what happens to these types of molecules after released into the extracellular space. How long endogenous opioids are in the extracellular space and how the environment around them might degrade or activate these types of molecules is largely unknown.

Endogenous opioids can be broken down into three major families; the endorphins, enkephalins, and dynorphins.\textsuperscript{11} Opioid peptides are formed from larger precursor proteins that
undergo proteolytic cleavage (Figure 3). These peptides are heterogeneously distributed throughout the brain and peripheral nervous system, and implicated in a wide variety of physiological functions previously mentioned. Opioid peptides bind promiscuously to a variety of opioid G-protein coupled receptors including delta, mu, and kappa (δ, μ, and κ). These individual ligands will bind with various affinities to multiple families of opioid peptide receptors (described in Figure 4).

Preproenkephalin yields multiple forms of M-ENK after cleavage including four copies of the pentapeptide (tyrosine-glycine-glycine-phenylalanine-methionine; YGGFM), one copy of YGGFM-RF and one copy of YGGFM-RGL. These modifications to the X-terminal end could differentiate these molecules and make them bind differently to the different receptors.

Figure 1.3: Opioid Precursor Distribution in Rodent Brain. a) Three precursor proteins are enzymatically cleaved to non-specifically yield three different families of active neuropeptides: endorphins, enkephalins, and dynorphins. b) Distribution of relative abundance of precursor mRNA depicted with triangle size while different precursor proteins are color-coded in rodent brain.
Figure 1.4: Opioid Receptor Protein Distribution in Rodent Brain. A) The delta (δ), mu (μ), and kappa (κ) opioid receptors promiscuously bind to opioids from multiple families. b) Distribution of μ, δ and κ receptors through mRNA in rodent brain. Size of the diamond describes relative abundance while color depicts the receptor type.¹

1.5 Previous Work on Enkephalin Detection

Radioimmunoassay²¹, 3¹⁻³⁵ and ligand autoradiography³⁶ have defined distributions of the protein precursors and receptors in mammalian brains, which reveal a ubiquitous distribution. Also, these precursor proteins can yield multiple families with an opposite effect. For example, the precursor prodynorphin yields both leucine-enkephalin (L-ENK) and dynorphin peptides from proteolytic cleavage (Figure 1.3). L-ENK binds to δ and μ receptors associated with reinforcing behaviors while dynorphin peptides can bind to the aversive κ-receptor (Figure 1.4). The divergence in the molecular functions of the resulting peptides from a single precursor yields information gained from immunoassay inconclusive of the biological processes occurring in a given region. Due to the redundant synthesis and promiscuity of binding, anatomical characterization information is useful but insufficient for fully understanding the endogenous opioid system.
PET imaging of the displacement of radiolabeled agonist has also been a valuable tool showing activation of the opioid receptors during different behavioral manipulations. However, it is expensive, often requires human subjects and does not have the spatial resolution needed to look at many discrete brain regions. In vivo microdialysis sampling coupled to radioimmunoassay is the most established strategy to monitor changes in extracellular opioid peptide concentrations, despite several critical shortcomings. This technique utilizes a probe implanted in the tissue where artificial cerebral spinal fluid is perfused through the implanted probe allowing analytes to diffuse across an outer semi-permeable membrane. The analytes obtained in the perfusion solution are then coupled to a separation technique, commonly liquid chromatography, and then analyzed with a detector. The in vivo probes used in microdialysis are typically between 50 and 400 μm in diameter and up to a few mm in length. This size is greater than the size of most brain nuclei in rodents which leads to averaging a larger area spanning multiple anatomical nuclei and likely multiple chemical transmitter types and patterns.

Opioid peptides are also found at low abundance (1-100 pM), and the recovery rate in microdialysis for opioid peptides is also low. To overcome these issues analysis times must be increased (20-30 minutes per sample). Slow perfusion rates coupled to increased sampling time allows more analyte to diffuse through the membrane and accumulate in the sampling probe. The slower perfusion rate means samples must then wait for minutes to allow enough peptide to accumulate so that the detection system can obtain a quantifiable amount of peptide. This slow temporal resolution could also convolute results as peptides are known to undergo cleavage and radical oxidation within the synapse and oxidation of the peptide could occur during sample accumulation. A significant amount of tissue damage occurs during the implantation of a microdiasysis probe. This physical damage can lead to ischemia, gliosis, and
ultimately, cell death which can significantly alter the neuronal communication occurring in the recording environment\textsuperscript{57}. Therefore, this sampling technique lacks the spatial and temporal resolution needed to measure real-time peptide dynamics in an anatomically discreet location of the brain.

Given the complexity of the endogenous opioid system a new tool to directly monitor the complicated endogenous opioids is needed to inform the development of therapeutics. Opiates that bind to the opioid receptors are highly effective at relieving pain but have recently been abused leading to an epidemic level of deaths from opiate overdose.

\textbf{1.6 Electrochemical Techniques in Neuroscience}

One of the most promising analytical approaches for monitoring neuronal communication is electrochemistry coupled to ultra-microelectrodes. This analytical approach has enabled the direct measurement of chemical fluctuations on sub-second time scales for decades\textsuperscript{58-61}. Advantages of this approach include sensor size, renewable material, and temporal resolution. Most electrochemical studies that use FSCV use ultra-microelectrodes with a cylinder geometry (\textasciitilde7\mu m in diameter and \textasciitilde100 \mu m in length; Figure 1.5). The small footprint of this sensor makes it appropriate for targeting discrete anatomical locations in the brain while minimizing tissue damage.

There are several electrochemical advantages to using carbon-fiber ultra-microelectrodes. First high-speed measurements are possible because of a substantial decrease in ohmic drop as compared to traditional macro-electrodes\textsuperscript{62}. This decreased surface area also enables the use of only two electrodes to complete the electrochemical cell (working and reference), without the need for an auxiliary electrode\textsuperscript{62}. When looking for a ultra-microelectrode that can be used for in vivo studies, carbon is often chosen over platinum as it is chemically inert, has a renewable surface,
rapid electron transfer kinetics and a large potential window. Carbon is also widely available in a variety of sizes and maintains tensile strength even at micron diameters.  

![Figure 1.5: Glass Carbon-Fiber Ultra-Microelectrode.](image-url)

One of the first electrochemistry methods used in situ by neuroscientists was ultramicroelectrodes coupled to amperometry. Amperometry applies a constant potential to the working electrode relative to the reference electrode, typically Ag/AgCl. If the electrode is held at a potential above the oxidation potential of nearby analytes, these analytes will be oxidized, and the current generated from the oxidation of the analytes will be measured. This current is plotted against acquisition time in an amperogram. The signal obtained in the amperogram can be integrated to yield charge. Using Faraday's law, quantitation of charge is proportional to the number of moles of analyte (Equation 1).

\[ Q = nNF \]

**Equation 1: Faraday’s Law.** Where Q is charge obtained, n is the number of electrons transferred per molecule, N is the moles of analyte, and F is Faraday's constant (F=96,485.34 C/mole).
While the temporal resolution of amperometry is unparalleled, the main caveat to this approach is the lack of chemical selectivity. In a complex environment like the brain, there are multiple electroactive analytes present, making this a major issue. However, an electrochemical technique that can provide some selectivity is background-subtracted FSCV. Unlike the constant potential applied in amperometry, FSCV sweeps through a range of potentials cyclically with a forward and subsequent reverse scan, together making up the waveform. FSCV coupled to carbon fiber microelectrodes have been used for decades to selectively monitor real-time fluctuations of dopamine in discrete brain regions.\textsuperscript{72-80} Recently FSCV has been extended to other molecules such as adenosine,\textsuperscript{81-83} hydrogen peroxide,\textsuperscript{84-86} serotonin,\textsuperscript{75, 87-90} local pH changes,\textsuperscript{76, 91} norepinephrine\textsuperscript{92-93} and glucose.\textsuperscript{94} Our group was interested in extending FSCV to the opioid peptide methionine-enkephalin (M-ENK) because of its implied involvement in the pain and rewards circuits.\textsuperscript{95}

The most common waveform used in FSCV is the “Dopamine Waveform” which sweeps from -0.4 V to +1.3 V at 400 V s\textsuperscript{-1} on the forward scan and immediately sweeps back down to -0.4 V on the reverse scan. The differential nature of this technique makes it uniquely suited for measuring rapid changes, such as chemical signaling by exocytosis. A complete cycle of this waveform only takes 8.5 ms, and the application rate of this waveform is typically 10 Hz. When this waveform is applied in the presence of DA, the oxidation of DA occurs through a two-electron process converting it to dopamine-ortho-quinone on the forward scan. During the reverse scan, dopamine-ortho-quinone is reduced back to DA. Current is plotted as a cyclic voltammogram (CV), with the potential applied to the electrode on the x-axis and the resulting current on the y-axis (Figure 1.6 C). By plotting the CVs in this way, chemical selectivity can be achieved by evaluating the potential of the redox peaks. This method of peak analysis has been used in FSCV
to study multiple neurotransmitters including serotonin, adenosine, ascorbic acid, hydrogen peroxide, norepinephrine, epinephrine, dihydroxyphenylacetic acid, and levodopa.\textsuperscript{84, 96-97}

When applying the waveform at this frequency a relatively stable large background charging current is generated (Figure 1.6 A). Applying this waveform in the presence of DA yields peaks as the potential drives through DA’s oxidation and reductive potentials (Figure 1.6 B). Once the stable background is subtracted from this measurement the redox currents generated (DA oxidation; +0.5 V; reduction -0.3 V) can be observed (Figure 1.6 C). The amplitude of the peaks scales with concentration and through calibration can yield quantitative information. The potential where peak oxidation and/or reduction occurs can help identify the analyte.

**Figure 1.6: Background Subtracted Cyclic Voltammetry.** (A) Large background current (B) Background current plus faradaic current resultant from the oxidation of DA and reduction of dopamine-o-quinone overlaid on background current. (C) Background-subtracted voltammogram showing only redox current from DA.

Data collected with FSCV is usually collected in 20-90 second files at a frequency of 10 Hz corresponding to 200-900 voltammograms per file. To quickly interpret this amount of data, color plots are utilized, where cyclic voltammograms are unfolded at the switching potential and concatenated yielding time on the x-axis, data point (correlated to the potential) on the y-axis, and current on the z-axis depicted with false color (Figure 1.7 D and E). By grouping the data in this way information can also be visualized by extracting a horizontal line from the color-plot yielding
Much of the data presented in this document was collected \textit{in vitro} using a flow cell, which is an apparatus that allows electroactive species to be swept over the electrode in a continuous stream of buffer, mimicking vesicular release of neurotransmitters in the brain. A flow cell encompasses the working electrode in an electrochemical cell with a constant flow of buffer pumped across the electrode. A bolus of electroactive species is introduced to the electrode utilizing a six-port injection valve connected to a two-position pneumatic actuator. This allows a known volume and concentration of the analyte to pass the electrode enabling both characterization and quantitation of the electrochemical response to a given analyte.

\textbf{1.7 Previous Work on the Modified Sawhorse Waveform}

M-ENK is a five amino acid peptide with two electroactive amino acids, tyrosine and methionine. Unsuccessful attempts to detect M-ENK with the traditional triangular waveform were attributed to the previously described issue of tyrosine fouling the sensor surface (Figure 1.8 C).
When attempting to detect M-ENK with a triangular waveform the resulting colorplot includes multiple broad potential regions invoking current that continues after the analyte should have been removed from the vicinity of the electrode sensor. These streaks indicate that M-ENK is fouling the electrode surface (white asterisks), as predicted by the literature. Fouling of the sensor surface is a major analytical challenge as it can lead to irreproducible results and reduced sensitivity over multiple injections.\(^9\)

The Modified Sawhorse Waveform (MSW), was developed to overcome these analytical challenges and enable the detection of M-ENK with FSCV.\(^9\) The MSW utilizes multiple scan rates and a static potential hold in every application of the waveform to enable the reproducible detection of M-ENK. Specifically the first segment of the MSW starts at a potential of -0.2 V before ramping up to +0.6 V at a scan rate of 100 V s\(^{-1}\). By reducing the scan rate to 100 V s\(^{-1}\) rather than the typical 400 V s\(^{-1}\) in the first segment of this waveform, peak current is reduced. This is expected as described by the adsorption-controlled derivative of the Randles-Sevich equation (Equation 2; Figure 1.8 A,B). This is advantageous during the \textit{in vivo} application of this waveform as catecholamines, common interferences found in the vicinity of M-ENK, oxidize at ~0.5 V. Next the scan rate is increased to 400 V s\(^{-1}\) from +0.6 V to +1.2 V. Increasing the scan rate through this region also increases peak current from the oxidation of M-ENK (~1.0 V). At the top of the potential sweep a static potential of +1.2 V is held for 3 msec, which helps renew the sensor surface and negate the effects of tyrosine fouling (Figure 1.8 B).\(^6\) The MSW takes advantage of using multiple scan rates which is unprecedented in electrochemistry. This multi-
scan-rate approach is the technological advance needed to enable a sharp current response clear in both the colorplot and CV for M-ENK (Figure 1.8 B,D).

**Figure 1.8: Fast-Scan Cyclic Voltammetry of 2 µM M-ENK.** (A) Conventional triangle waveform applied to the electrode. (B) Modified sawhorse waveform (MSW). (C) Representative colorplot using the traditional waveform with a CV extracted from the time marked by black dashed arrow. (D) Representative colorplot obtained from using the MSW to detect a bolus injection of 2-µM M-ENK. Red bars indicate the time of sample introduction to the electrode.99

This MSW enabled reproducible peaks but also decreased sensitivity towards catecholamines, which are often found in the vicinity of M-ENK at concentrations that could mask M-ENK detection. The ability to simultaneously detect catecholamines and tyrosine containing peptides is largely due to our use of multiple scan rates. Equation 2 is the Randles-Sevcik equation, which defines the proportional relationship of peak current to scan rate.62
\[ i_p = (2.69 \times 10^5)n^{3/2}A^{1/2}C^*v^{1/2} \]

**Equation 2: The Randles-Sevcik Equation.** This equation describes the relationship between peak current and scan rate. \( i_p \) = peak current, \( n \) = number of electrons transferred in redox reaction, \( A \) = electrode surface area, \( C^* \) = bulk concentration, and \( v \) = scan rate.\(^5\)

By decreasing the scan rate in the range where catecholamines oxidize (\( \sim +0.6 \) V), less current is generated in oxidizing this ‘interferent.’ Further, this serves to shift the peak position to a lower potential, away from the critical potential for tyrosine oxidation (\( \sim 1.0 \) V). Next, to achieve additional peak current from M-ENK, the scan rate is increased to 400 V s\(^{-1}\) in the +0.6 V to +1.2 V range. Finally, tyrosine is known to foul the electrode.\(^1\) To mitigate this fouling, the potential is held at +1.2 V for 3 msec to weaken the adsorption of the oxidized molecule at potentials above +1.1 V.\(^1\) This has also been shown to strip an atomic layer of C from the electrode surface, providing a fresh surface with each successive scan.\(^6\)

To probe which amino acids correspond to the electrochemical response of M-ENK, the two terminating amino acids methionine and tyrosine were analyzed separately (Figure 1.9). These two amino acids were chosen for analysis because they are known to be electroactive within our potential window.\(^9\) Colorplots for tyrosine (left), M-ENK (middle), and methionine (right) in Figure 9A show the electrochemical response to these analytes. The voltammograms in Figure 9B show the two oxidation peaks obtained for M-ENK occur at roughly the same potentials as the oxidation of tyrosine (\( \sim 1.0 \) V) and methionine (\( \sim 1.2 \) V). The tyrosine peak was found to be more reproducible and was chosen as the peak for quantification of M-ENK. This waveform takes advantage of fundamental electrochemical properties to allow for the selective quantitation of M-ENK; a critical molecule that, was previously only quantifiable through physical sampling.\(^1\)
Figure 1.9: M-ENK Structure and Electrochemical Response. M-ENK is a five-amino-acid peptide with electroactive terminating tyrosine and methionine groups (A). Representative colorplots for 2 μM tyrosine (left), 1 μM M-ENK (middle), and 30 μM methionine (right). (B) To better visualize the current collected during the potential hold period, it is plotted versus waveform point number converted into potential. The displayed portion is indicated by the dashed box (top). The dagger and double-dagger indicate the first and second oxidation peaks of M-ENK, respectively.99

1.8 Principal Component Regression

One statistical treatment that is proven to work well with complex electrochemical signals from FSCV is principal component regression (PCR). PCR enables the deconvolution of individual electrochemical contributions from complex signals by reducing the dimensionality of the data.102 PCR uses principal component analysis (PCA) and least-squares regression to describe variability in the data and quantitatively predict concentrations from training set data. Training sets of a single redox contributor are built to define principal components (latent component vectors) that describe the variance of the single redox analyte and build predictors, with fewer variables, than initially present into factor space.63,64 The first PC describes the most variability in the data, for example, the peak current that occurs when running a standard of DA.103 The following PCs are ideally located orthogonal to one another and describe all other variability found in the data.103 Not all PCs describe significant variable components, but Malinowski’s F-test can be used to
statistically discriminate which components should be retained or discarded.\textsuperscript{104} PCR calculates the concentration of unknown samples as described in Equation 3.\textsuperscript{105}

\[
C_{unk} = F V_C^T A_{unk}
\]

\textbf{Equation 3: PCR prediction of unknown concentrations.} A is the voltammogram of the unknown to be predicted, F is the regression coefficient (assigned per analyte to relevant PCs), and V is a matrix of the relevant PCs (superscript T indicates matrix is transposed into factor space).

\section{1.9 Research Overview}

The goal of the research performed in this dissertation was to optimize the quantitative detection of opioid peptides using FSCV. The goal of this work was to further our current understanding of the electrochemical principles that affect the performance of this waveform with opioid peptides, as well as how this tool can detect fragments of M-ENK and analogs containing M-ENK. This work presents novel tool that can be used to detect tyrosine containing peptides.

\textbf{Chapter 2} systematically investigated how redox activity of M-ENK changes as specific parameters of the MSW were manipulated. This fundamental characterization led us to design a waveform which enabled the first FSCV measurements of M-ENK in the brain. Specifically, these results were recorded from the dorsal striatum of anesthetized and freely moving rats. Much of the work presented in this study focuses on the impact that waveform manipulation can have on the redox activity of M-ENK, but the data also demonstrate the variety of parameters that could be tailored to a specific analyte.

\textbf{Chapter 3} examines several analogs of M-ENK to determine how the type and number of amino acids present in a peptide affect the redox activity of tyrosine and methionine. Principal component regression (PCR) was used to assess how different peptide fragments can be
distinguished and quantified using this technique. Thus, this work will be of value to researchers interested in utilizing FSCV to further our current understanding of the electrochemical detection of opioid peptides. Results from these studies have given us a tool that can be used for the quantitative measurement of endogenous opioid peptides, which will aid in our understanding of the dynamic role these molecules play in both normal and maladaptive behaviors. The information gained from these projects will have broad implications for studying other peptides and any disorders that involve peptide signaling.
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CHAPTER 2: Characterization of a Multiple-Scan-Rate Voltammetric Waveform for Real-time Detection of Met-Enkephalin

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2.1 Abstract
Opioid peptides are critically involved in a variety of physiological functions necessary for adaptation and survival and, as such, understanding the precise actions of endogenous opioid peptides will aid in identification of potential therapeutic strategies to treat a variety of disorders. However, few analytical tools are currently available that offer both the sensitivity and spatial resolution required to monitor peptidergic concentration fluctuations in situ, on a time scale commensurate with that of neuronal communication. Our group has developed a multiple-scan-rate waveform to enable real-time voltammetric detection of tyrosine containing neuropeptides. Herein, we have evaluated the waveform parameters to increase sensitivity to methionine-enkephalin (M-ENK), an endogenous opioid neuropeptide implicated in pain, stress and reward circuits. M-ENK dynamics were monitored in adrenal gland tissue, as well as in the dorsal striatum of anesthetized and freely behaving animals. The data reveal co-fluctuations of catecholamine and M-ENK in both locations, and provide measurements of M-ENK dynamics in the brain with sub-second temporal resolution. Importantly, this work also demonstrates how voltammetric waveforms can be customized to enhance detection of specific target analytes, broadly speaking.

2.2 Introduction

Endogenous opioid peptides modulate a wide range of physiological functions including pain and reward processing, emotion, feeding behavior, and gastrointestinal activity by acting on the mu, delta, and kappa receptors (MOR, DOR, and KOR, respectively). These receptors are differentially expressed throughout the brain and peripheral nervous system. Mesolimbic opioid peptides are important mediators of hedonic and motivational aspects of reward processing, and aberrant opioid activity in the mesolimbic region is heavily implicated in drug addiction and drug-mediated reinforcing behaviors. However, the precise action of these opioid peptides and their interaction with mesolimbic dopamine (DA) remains ambiguous, despite nearly four decades of
research. This is largely due to the paucity of techniques for direct detection of opioid peptides in situ.

The complexity of the endogenous opioid system presents many hurdles when using existing approaches to dissect the action of specific opioid peptides at specific receptors (or receptor subtypes). Unlike receptors for many other small molecular transmitters, no endogenous opioid peptide family is associated exclusively with any one receptor type.\textsuperscript{15} Thus, even the complete and instantaneous blockade of a specific receptor type does not necessarily eliminate the action of a given opioid peptide. Similarly, quantification of mRNA expression and immunohistochemistry are often used to identify neurons that presumably contain the known opioid precursors: pre-proENK, pre-proopiomelanocortin, and pre-prodynorphin. However, cleavage of these larger molecules yields a variety of peptides that elicit a range of physiological responses.\textsuperscript{16} In fact, individual fragments from a given prohormone can generate opposing actions at postsynaptic cells in the brain.\textsuperscript{15, 17} As such, identification of neurons that synthesize precursor molecules provides no information on the contribution of specific opioid peptides to brain function, or whether release occurs in the somatodendritic region or at projection targets. Finally, it has even been shown that individual dopaminergic neurons in the mesolimbic circuitry can respond differentially to distinct DOR agonists.\textsuperscript{18} New analytical tools that are capable of directly monitoring rapid opioid peptide fluctuations in situ are needed to elucidate the contribution of individual opioid peptides to brain function.

Estimates of neuropeptide concentration are generally accomplished by coupling a sampling technique, such as microdialysis, to an ex situ analytical measurement of the collected fraction.\textsuperscript{19-27} This approach is widely used for monitoring small molecule neurotransmitters, but is difficult to apply to neuropeptides. These low abundance molecules are presumably released from
diffuse fibers, and the probe volume is large when compared to the volume of the nerve terminal, resulting in substantial dilution as steady-state concentrations are reached. Further, microdialysis recovery efficiencies are low, particularly for ‘sticky’ neuropeptides that readily adhere to polymeric materials (typically < 5% recovery). These issues are significant because the sensitivity of the detection method and the absolute recovery of the probe ultimately limit the temporal resolution. Recent studies have coupled microdialysis to liquid chromatography-mass spectrometry (LC-MS) to detect M-ENK in dialysate from the dorsal striatum, globus pallidus, and hippocampus of rodents. However, this approach necessitates sampling periods of at least tens of minutes. This exacerbates the analytical challenge, as peptides are known to rapidly undergo cleavage and oxidation during sample collection.

Background-subtracted, fast-scan cyclic voltammetry (FSCV) is commonly used to monitor DA fluctuations in the striatum during specific behavioral tasks related to reward seeking and consumption. We previously developed a modified-sawhorse waveform (MSW) for FSCV that incorporates three different scan rates in each sweep to address several challenges associated with the electrochemical detection of tyrosine-containing peptides, such as M-ENK. Herein, we systematically investigated the waveform parameters for the electrochemical detection of M-ENK in the presence of catecholamine (CA). Incorporation of the optimized parameters increased sensitivity to M-ENK by more than three-fold, and enabled simultaneous monitoring of M-ENK and CA at single, micron-scale recording sites in the rat dorsal striatum. The differential nature of this approach enables measurement of chemical fluctuations without interference from relatively stable or slowly changing electrochemical species that do not contribute to transient surges in chemical neurotransmitter. Thus, it provides the ability to reveal critical mechanistic details about rapid neuropeptide signaling, and promises to considerably advance understanding of peptidergic...
mechanisms implicated in normal physiological function and in maladaptive behaviors such as
drug addiction.

2.3 Results and Discussion

2.3.1 Introduction to the Modified Sawhorse Waveform. The classic triangular waveform
that is most frequently used in FSCV cannot be used to monitor opioid peptide fluctuations because
of a plethora of issues previously described by our group.\textsuperscript{40} We overcame these limitations by
designing a modified-sawhorse waveform (MSW), herein referred to as MSW 1.0 (Figure 1a). In
the first segment of the forward scan, the potential is swept at 100 V s\textsuperscript{-1} from an accumulation
potential of -0.2 V to a transition potential of +0.6 V. The scan rate is increased to 400 V s\textsuperscript{-1} in the
second segment of the forward sweep, which terminates at +1.2 V. The potential is held for 3 ms
of measurements at 1.2 V before returning to -0.2 V at 100 V s\textsuperscript{-1}. This waveform generates separate
peaks for tyrosine and methionine moieties (Figure 1b). Many peptides in the brain contain these
residues; however, most of these are present at relatively constant concentrations over the time
course of the measurement (tens of seconds). As such, they are subtracted with the background
signal. Only a peptide that contains both tyrosine and methionine, and that surges in concentration
over the seconds timescale, is putatively identified as M-ENK with this approach.

In order to optimize and explore the full potential of the MSW for detection of M-ENK,
the customizable waveform parameters were systematically evaluated. Application frequency,
accumulation potential, scan rate, amperometric hold potential, and the transition potential that
separates the first segment of the forward sweep from the second were varied to evaluate impact
on electrochemical performance (Figure 1a). Individual CVs typically display potential and
current on the abscissa and ordinate, respectively. As a result, current collected during the
amperometric hold portion of the MSW collapses into a vertical line (Figure 1b top). In this work,
data collected during the amperometric hold were plotted with respect to hold time, using a scaling factor. The modified cyclic voltammograms (mCVs; Figure 1b bottom) retain the conventional CV shape while also enabling visualization of currents generated during the amperometric hold period (shaded region). Chemical selectivity for M-ENK relies on the presence of distinct peaks generated in the oxidation of the tyrosine and methionine amino acid residues, which appear at ~0.95 V and in the amperometric hold, respectively. Plotting the data in the mCV format facilitates visualization of both peaks, even enabling M-ENK to be distinguished from the closely related pentapeptide, leu-enkephalin (L-ENK), which differs by a single amino acid (Figure 1b, bottom). Thus, the mCV convention is used throughout this work.
Figure 2.1: Introduction to the MSW. (a) A schematic of the MSW with the parameters of interest labeled. M-ENK, the target analyte, was used for waveform characterization. (b) Representative CVs (top) and mCVs (bottom) for M-ENK and L-ENK (normalized). Current collected during the amperometric period is plotted with respect to time (shaded region). M-ENK and L-ENK differ only at the C-terminus, with either a methionine or a leucine group, respectively. Both pentapeptides share the same first peak, but the presence of the second peak allows M-ENK to be visually distinguished.
2.3.2 Waveform Application Frequency. Adsorption plays a large role in the detection of many electroactive neurochemicals. For example, DA is positively charged at physiological pH, and it has been shown to concentrate at electrode surfaces during the period between voltammetric scans, when an electrode is negatively charged.\textsuperscript{41} Peptides contain many ionizable groups and other functionalities that influence electrochemical detection. To investigate this, the electrochemical response to 1 µM M-ENK was recorded using waveform application frequencies of 3, 5, 10, and 20 Hz, which correspond to inter-sweep accumulation times of 307, 174, 94, and 24 msec, respectively (Figure 2a, top). All other waveform parameters were held constant. As accumulation time increased, anodic current for the oxidation of M-ENK increased (Figure 2a,b). This suggests that M-ENK concentrates on the carbon-fiber microelectrode surface between scans, thereby amplifying the signal generated upon electrolysis. The 5 Hz waveform application frequency generated substantial current while maintaining sub-second temporal resolution. Thus, unless otherwise stated, 5 Hz was chosen as the waveform application frequency for subsequent experiments.

Figure 2.2: Waveform Application Frequency Impacts Sensitivity to M-ENK. (a) Top: Accumulation time as the MSW parameter under investigation. Bottom: Representative mCVs for bolus injections of 1 µM M-ENK. (b) Accumulation time (or application frequency) and current (tyrosine peak ∼0.95 V; dotted line) plotted on the abscissa and ordinate, respectively (n = 3 electrodes). Exponential line included as a guide for the eye.
2.3.3 Accumulation Potential and Scan Rate. The electrolysis of adsorption-controlled species is also impacted by accumulation potential. Thus, accumulation potential was systematically varied to investigate the impact on the voltammetric signal for M-ENK. Figure 3a (top) displays the waveforms used in this experiment. Accumulation potential was varied (0.0, -0.2, -0.4 and -0.6 V); all other parameters were held constant. Figure 3a (middle) depicts representative mCVs for 1 µM M-ENK. Figure 3a (bottom) presents a plot of the average peak current recorded in response to 1 µM M-ENK as a function of the accumulation potential (slope = -14.0 ± 0.9 nA V\(^{-1}\), \(R^2 = 0.93\)). Peak current clearly increased as the accumulation potential decreased (became more negative), consistent with adsorption-controlled electrolysis of M-ENK.

Figure 2.3: Characterization of the Voltammetric Signal for M-ENK When Varying Accumulation Potential (a) and Scan Rate (b). Top: Electrochemical waveforms investigated. The inset is an enlarged view of the region of interest. Middle: Representative mCVs collected in response to 1 µM M-ENK. Bottom: Peak anodic current generated in M-ENK oxidation (~0.95 V) increased as the accumulation potential decreased, and as the scan rate in the second segment of the forward scan increased. (n = 5 electrodes per parameter).
According to the Randles-Sevcik equation, current scales with scan rate. Thus, the scan rate in the potential window for tyrosine oxidation was systematically varied. Tyrosine oxidation occurs at ~+1 V in the second segment of the forward sweep. Figure 3b (top) highlights how changing the scan rate in this window alters the time it takes to reach the amperometric hold potential, as well as the total duration of the waveform. Representative mCVs for 1 µM M-ENK collected using scan rates from 200-1200 V s\(^{-1}\) in this segment of the waveform are shown in Figure 3b (middle). Figure 3b (bottom) depicts the relationship between scan rate and the peak current generated by oxidation of 1 µM M-ENK (slope = 0.0172 ± 0.0007 nA V\(^{-1}\), \(R^2 = 0.99\)). It is important to note that increasing the scan rate shifts the peak attributed to tyrosine oxidation (at ~+1 V), toward the amperometric hold. In fact, exceeding 800 V s\(^{-1}\) results in a complete loss of resolution, as the peaks that serve to identify the electroactive amino acids, tyrosine and methionine, completely overlap. Therefore, scan rates above 400 V s\(^{-1}\) should not be used to monitor M-ENK when using MSW 1.0.

2.3.4 Amperometric Potential and Transition Potential. In an attempt to recover the characteristic two-peak signature of M-ENK that was lost with incorporation of higher scan rates (Figure 3b middle), an amperometric potential of +1.3 V was investigated. In this experiment and in all subsequent analyses, unless otherwise stated, an accumulation potential of -0.4 V, transition potential of +0.7 V, and a scan rate of 600 V s\(^{-1}\) in the second segment of the forward scan were employed. Figure 4a (top) shows the waveforms, which incorporate an amperometric potential of either +1.2 or +1.3 V. Figure 4a (middle) depicts representative mCVs for 1 µM M-ENK collected using both waveforms. The data clearly show that extending the forward sweep to +1.3 V recovers peak resolution when using higher scan rates. Furthermore, Figure 4a (bottom)
demonstrates that this also improves sensitivity to M-ENK \((t(4)=3.803, p < 0.05; n = 5\) electrodes). Based on these results, all subsequent analyses utilized an amperometric potential of +1.3 V.

![Image of Figure 2.4: The Potentials Selected at Both Nodes of the Second Segment of the Forward Scan Influence the Voltammetric Response of M-ENK.](image)

There is significant evidence that neuropeptide and small molecule neurotransmitters are co-packaged within vesicles, and that release can occur simultaneously.\(^{43-44}\) Furthermore, estimates of CA concentrations in the extracellular space, for instance in striatum, are much higher than estimates of opioid peptide concentrations.\(^{21,45}\) Thus, CA molecules could easily interfere with the detection of low abundance peptides. The CA neurotransmitters oxidize in the +0.5 to
+0.7 V range. Ideally, CA oxidation would be completed prior to reaching the second segment of the forward scan, to facilitate accurate quantification of both analytes. Transition potentials of +0.6 V, +0.65, or +0.7 V (Figure 4b top) were investigated with other parameters held constant. Figure 4b (middle) shows representative mCVs for M-ENK collected using the different waveforms. Increasing the transition potential resulted in a decrease in the current generated for M-ENK oxidation, as shown in Figure 4b (bottom) (F(2, 8) = 95.44, p < 0.0001). However, this drawback was offset by a significant benefit.

Figure 5 displays representative mCVs for 1 µM M-ENK, 500 nM DA (physiologically relevant for work in rodent striatum), and a mixture of the two analytes (at the same concentrations) collected with transition potentials of +0.6 and +0.7 V. The total charge contribution to the signal collected in each segment of the forward scan was examined (excluding the amperometric hold period). The results indicate that in the mixed signal, DA contributes 28.5 ± 0.9% and 18.5 ± 0.7% of the total charge collected in the second segment of the forward scan with transition potentials of +0.6 V and +0.7 V, respectively (n=2 electrodes). Thus, increasing the voltage window of the initial segment (from +0.6 V to +0.7 V) allows for more complete electrolysis of DA. It should be noted that this issue is particularly important in adrenal tissue, where electrically stimulated CA release can substantially exceed 5 µM. Therefore, +0.7 V was selected as the transition potential for the subsequent studies. However, a tradeoff clearly exists between sensitivity and selectivity, and determination of the most appropriate transition potential is dependent on the presence or absence of interfering chemical signals.
Figure 2.5: The Transition Potential that Distinguishes the First Segment of the Forward Scan from the Second Can be Tailored to Maximize Sensitivity or Selectivity. Representative mCVs collected with transition potentials of +0.6 V (a) or +0.7 V (b) for detection of 1 µM M-ENK, 500 nM DA, and a mixture of both species containing the same concentrations.
2.3.5 MSW 1.0 vs MSW 2.0: A Direct Comparison. Due to the coexistence of CAs and M-ENK in tissues targeted in this study (adrenal medulla and striatum), subsequent recordings employed a +0.7 V transition potential. Given all of the above characterizations, a waveform referred to as MSW 2.0 was employed for tissue measurements using a 5 Hz application frequency. The potential was swept from -0.4 V to +0.7 V at 100 V s\(^{-1}\) before a faster sweep to +1.3 V at 600 V s\(^{-1}\). The potential was then held at +1.3 V for 3 msec before returning to -0.4 V at 100 V s\(^{-1}\) (Figure 6a). MSW 2.0 improves upon the previous waveform (MSW 1.0). It results in defined and distinct tyrosine and methionine peaks vital for chemical selectivity, as well as a greater than three-fold increase in sensitivity (Figure 6b,c, one-way ANCOVA, F (1, 4) = 304.9, p < 0.0001; n = 5 electrodes).

**Figure 2.6: Improved Detection of M-ENK with MSW 2.0.** (a) A graphic comparison of the two waveforms. (b) Representative mCVs collected for 1 µM M-ENK using MSW 1.0 and MSW 2.0. (c) A direct comparison of calibration plots for M-ENK using these waveforms. ***p < 0.001, n=5 electrodes.
2.3.6 Simultaneous Measurements of CA and M-ENK Fluctuations in Living Adrenal Tissue. Endogenous peptides are critically involved in numerous physiological functions that promote survival, including the response to stress.\(^6\), \(^50\) For example, the electrically excitable chromaffin cells that make up the adrenal medulla secrete several neuropeptides (including M-ENK) and relatively high concentrations of CAs (dopamine, norepinephrine and epinephrine) during fight-or-flight behavior.\(^6\), \(^51\), \(^52\) For measurements in the adrenal medulla, rats were pretreated with \(\alpha\)-methyl-DL-tyrosine methyl ester hydrochloride (\(\alpha\)-MPT) and reserpine to inhibit the synthesis and vesicular packaging of CAs, respectively.\(^53\)-\(^54\) This pretreatment effectively reduced CA content, in order to facilitate detection of M-ENK. Without it, the CA signal dominated the color plot, exceeding concentrations of \(\sim 5 \text{ M} \) (data not shown). Figure 7a displays representative color plots (top) and mCVs (bottom) for standards of 750 nM NE, 750 nM DA, and 500 nM M-ENK collected \textit{in vitro} using MSW 2.0. Figure 7b (top) displays a representative color plot of electrically evoked CA release and a second analyte, putatively identified as M-ENK, collected in an adrenal slice (data collected at 10 Hz). A voltammogram directly following the stimulation was extracted and compared with a voltammogram for a M-ENK standard (Figure 7b, bottom). The voltammetric signature for CA is clearly evident, and there is good agreement between the normalized mCVs in the potential region where M-ENK is detected (0.7 V – 1.3 V, \(R^2 = 0.83\), providing electrochemical evidence for the identification of M-ENK. These data suggest that CA and M-ENK are released on a similar timescale upon electrical stimulation of adrenal tissue, and establish the potential for MSW 2.0 in addressing a broad range of fundamental questions regarding endogenous opioid dynamics in live tissue.
Figure 2.7: Simultaneous Monitoring of M-ENK and CA Dynamics in an Adrenal Slice Preparation with MSW 2.0. (a) Representative data for standards of 750 nM DA and 500 nM M-ENK, and (b) M-ENK and CA released following electrical stimulation (administered at the time indicated by the red dashed line). Top: Color plots of raw voltammetric data. Bottom: mCVs extracted from the color plots at the time indicated by the white dashed line. There is good agreement between the normalized mCVs in the potential range where M-ENK is evident (to the right of the dashed line, 0.7 V – 1.3 V, \( R^2 = 0.83 \)).

2.3.7 Simultaneous Electrochemical Measurements of CA and M-ENK in the Dorsal Striatum. Enkephalins modulate motor output regions in the brain,\(^{31,55-57}\) nuclei involved in food intake,\(^{21,58}\) and they are involved in the integration of limbic information in the dorsal striatum.\(^{59}\) Approximately 90-95% of the cellular makeup of this region consists of medium spiny neurons, approximately half of which are known to express the DA D2 receptor, various opioid receptors,
and to contain enkephalin.\textsuperscript{60, 61} An infusion of 1 μM M-ENK was delivered to the local vicinity of the electrode in the striatum (within ~300-500 μm), to demonstrate the applicability of MSW 2.0 for voltammetric measurements of neuropeptide in brain tissue. Figure 8a demonstrates that MSW 2.0 can clearly detect the infused M-ENK. Interestingly, this signal was followed by an increase in extracellular CA, and the extracted mCV (inset) exhibits the defining characteristics of both CA and the M-ENK standard. Next, PBS was locally microinfused, with no observable neurochemical effects (Figure 8b). Finally, a cocktail of peptidase inhibitors was microinfused into the vicinity of the recording site (Figure 8c). This solution contained 20 μM bestatin added to a commercially available cocktail of 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.3 μM aprotinin, 116 μM bestatin, 14 μM E-64, 1 μM leupeptin, and 1 mM ethylenediaminetetraacetic acid. This manipulation should locally increase the concentration and extracellular lifetime of a variety of neuropeptides. The data show a voltammetric signal consistent with that recorded for infusion of exogenous M-ENK, as demonstrated by the Pearson’s correlation coefficient quantifying the covariance of the extracted mCVs (inset, $R = 0.88$).
Figure 2.8: M-ENK Recorded in the Dorsal Striatum of an Anesthetized Rat. Top: representative color plots collected during microinfusion of M-ENK (a), PBS (b), or a cocktail of enkephalinase inhibitors (c). Infusion is marked with the orange bar on the concentration vs time plots (bottom). Inset mCVs were extracted at the time point indicated by the corresponding white dashed lines. Microinfusion of PBS did not result in any observable neurochemical changes, but local infusion of the protease inhibitor cocktail resulted in voltammograms that correlated with those collected after infusion of exogenous M-ENK (R = 0.88).

When assessing a new strategy to detect endogenous molecules in the brain, an important step is to validate the approach by selectively modulating the signal using known pharmacology. However, to date, drugs to unambiguously and selectively manipulate the concentration of endogenous opioid peptides in the extracellular space do not exist. However, 6-OHDA lesioned animals exhibit increased pre-proENK mRNA expression. Furthermore, because the manipulation destroys the majority of the DA neurons in the substantia nigra that project to the dorsal striatum, there is also less interference from endogenous CA. Thus, putative M-ENK signals were monitored in the dorsal striatum of an intact and a 6-OHDA lesioned rat (both awake and freely moving).
Figure 9a (left) presents a representative color plot collected in the intact animal at rest. The voltammograms (inset) are indicative of spontaneous, dynamic DA fluctuations, and there are also signals that correlate well with the voltammogram for exogenous M-ENK infused into the striatum (Figure 8a, R =0.85). By contrast, the representative color plot collected in the 6-OHDA lesioned animal (Figure 9a, right) contains spontaneous signals consistent with voltammograms for a M-ENK standard (Figure 7, R = 0.84), with no evidence of a phasic DA signal.

Previous work has demonstrated a role for surges in ENK in the rat anterior dorsomedial striatum in the modulation of food-motivated behavior (albeit on the tens-of-minutes timescale).

Thus, voltammetric data were collected in anterior dorsomedial striatum of a male rat in response to unexpected palatable food reward (fruit loops, Figure 9b). Little electrochemical signal was recorded under baseline conditions, until the subject was presented with food reward. The subsequent bouts of food interaction and consumption were monitored by a trained observer while electrochemistry was recorded. The voltammograms correlate with those for the M-ENK standard (Figure 7, R = 0.80-0.88), consistent with a role for ENK surges in motivation to consume reward. Taken together, the data in Figure 9 suggest that endogenous M-ENK can be monitored in the striatum of awake animals; however, further investigation is needed to unambiguously identify M-ENK and to clarify the role that opioid peptides play in motivated behaviors.
Figure 2.9: Neurochemical Fluctuations Recorded in the Dorsomedial Striatum of Awake, Freely-Behaving Rats. Representative color plots are shown, with concentration vs time traces below. Inset CVs were extracted at the time point indicated by the white dashed lines. (a) Voltammograms that correlate with those collected after infusion of exogenous M-ENK into striatal tissue were evident in the intact striatum (left, R = 0.85), but no CA signal was evident in the 6-OHDA lesioned animal (right). These voltammograms correlate with the M-ENK standard (R = 0.84). (b) A voltammetric signal that correlates with M-ENK fluctuations was recorded in response to the presentation (left) and consumption (middle and right) of unexpected food reward (R = 0.80-0.88).
2.4 Conclusions

Direct, real-time measurements of opioid neuropeptides in live tissue and behaving animals will provide an improved understanding of their actions in both the peripheral and central nervous systems. This work takes a significant step toward that goal. The results demonstrate that the MSW allows for direct detection of tyrosine-containing peptides, such as M-ENK, in live tissue. Through a systematic characterization of the waveform parameters, we have enhanced selectivity and sensitivity for M-ENK, and demonstrated the simultaneous release of M-ENK and CA in both adrenal and striatal tissue. Importantly, this approach is not limited to endogenous opioids, and we have demonstrated how voltammetric waveforms can be customized to enhance detection of specific target analytes, broadly speaking. Overall, this work lays a foundation that can ultimately enable researchers to make direct, real-time measurements of tyrosine-containing endogenous peptides. It has the potential to enable investigation of questions concerning the specific conditions required for peptide release, peptidergic lifetime in the extracellular space, and how peptidergic flux is paired with specific behaviors. Further, the approach promises to provide valuable and unprecedented information to inform the development of therapeutic treatments of a myriad of physiological dysfunctions.

2.5 Methods

2.5.1 Chemicals: All chemicals were obtained with ≥95% purity (HPLC assay), and were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. The M-ENK acetate salt hydrate was obtained from LKT Laboratories (St. Paul, MN). Phosphate buffered saline (PBS; 10 mM Na₂HPO₄, 138 mM NaCl, and 2.7 mM KCl) was utilized for all in vitro experiments. Adrenal slice experiments were completed in bicarbonate buffered saline (BBS; 125 mM NaCl, 26 mM NaHCO₃, 2.5 mM KCl, 2.0 mM CaCl₂, 1.0 mM MgCl₂, 1.3 mM NaH₂PO₄, 10 mM HEPES, and
10 mM glucose) saturated with 95% O₂ and 5% CO₂. All buffers were made using ultrapure >18.2 MΩ water (Millipore, Billerica, MA) and were adjusted to pH 7.4 with 1 M NaOH and 1 M HCl.

2.5.2 Microelectrode Fabrication: In vitro and ex vivo electrochemical experiments were carried out with glass-insulated T-650 carbon-fiber microelectrodes (Cytec Industries, Woodland Park, NJ), fabricated as previously described.\textsuperscript{63} Briefly, a 7-μm diameter carbon fiber was aspirated into a glass capillary, and the glass was sealed around the carbon using a micropipette puller (Narishige, Tokyo, Japan). The fiber extending past the seal was cut to 100 μm. An electrical connection with the carbon fiber was established using a high ionic strength solution (4 M potassium acetate, 150 mM KCl) to backfill the capillary. A lead wire (Squires Electronics, Cornelius) was inserted to connect the electrode to custom instrumentation.

For experiments in animals, silica-insulated carbon-fiber microelectrodes were fabricated as described previously.\textsuperscript{64} Briefly, fused-silica tubing (90 μm outer diameter/ 20 μm inner diameter) with a polyimide coating (Polymicro Technologies, Phoenic Arizona) was cut to 1-1.5 cm in length and placed in a bath of 70% isopropyl alcohol. A T-650 polyacrylonitrile carbon fiber was inserted through the tubing and allowed to dry. An epoxy seal (McMaster Carr, Atlanta, GA) was created at one end, and an electrical connection was completed with conductive silver epoxy (MG Chemical, Thief River Falls, MN) and a gold pin (Newark Element 14, Palatine, IL). A second layer of insulation was established around the connection using liquid insulting tape (GC Electronics, Rockford, IL). Exposed carbon fibers were cut to 100–150 μm.

For the experiments in the anesthetized animal, an ‘injectrode’ device was fabricated, as described previously.\textsuperscript{65} The fused silica insulation was initially cut to 3 cm (164.7 OD and 98.6 μm ID), and all other aspects of silica-insulated microelectrode fabrication remained unchanged. The microelectrode was placed side by side with a guide cannula (26 GA, 11 mm from pedestal;
Plastics One, Roanoke, VA) and epoxy was used to secure them together as one device. Injection needles (33GA, extending 1 mm beyond the guide; Plastics One) were positioned in the guide cannula for at least 1 min prior to infusions, and remained in place for at least 1 min after infusions.

Reference electrodes were fabricated using a chloridized 0.25-mm diameter silver wire. A connection was made using a gold pin insulated with heat shrink. The silver wire and gold pin were positioned through a modified guide cannula stylet cap, and epoxy was used to secure it in place.

2.5.3 Electrochemical Data Acquisition In Vitro: All in vitro data were collected in a custom-built flow-injection apparatus housed within a Faraday cage. A syringe pump (New Era Pump Systems, Inc., Wantagh, NY) was utilized to enable a continuous buffer flow of 1 mL min\(^{-1}\) across the working and reference electrodes. A micromanipulator (World Precision Instruments, Inc., Sarasota, FL) allowed for precise positioning of the working microelectrode into the electrochemical cell. A Ag/AgCl pellet reference electrode (World Precision Instruments, Inc., Sarasota, FL) was used to complete the two-electrode cell. A six-port HPLC valve mounted on a two-position actuator controlled by a digital pneumatic solenoid valve (Valco Instruments, Houston, TX) enabled two-second bolus injections of analyte to be presented to the electrode.

Waveforms were applied at 3 - 20 Hz, and data were acquired at a sampling rate of 100 kHz using a custom-built instrument for potential application and current transduction (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility), or a WaveNeuro FSCV Potentiostat System (Pine Research Instrumentation, Durham, NC). High Definition Cyclic Voltammetry software (HDCV; University of North Carolina at Chapel Hill) was used in conjunction with data acquisition cards (National Instruments, Austin TX) to control waveform output, as well as to acquire and process resulting signals, including background subtraction.
Electrodes in all experiments were electrochemically conditioned at 25 Hz until stable, and then conditioned at the respective collection frequency needed per experiment for at least 10 additional min.

2.5.4 Animal Subjects and Care: Drug-naïve, adult male Sprague–Dawley rats (275–300 g, Charles River Laboratories, Raleigh, NC; n = 5) were allowed to acclimate to the facility for several days. One animal received a unilateral 6-hydroxydopamine (6-OHDA) lesion of the substantia nigra by the vendor prior to receipt. Animals were individually housed on a 12:12 hour light/dark cycle with free access to food and water. Animal care and use was in complete accordance with the North Carolina State University institutional guidelines (IACUC) and the NIH’s Guide for the Care and Use of Laboratory Animals.

2.5.5 Ex Vivo Adrenal Slice Preparation: Reserpine and α-MPT were prepared in 50% saline/50% dimethyl sulfoxide and in saline, respectively. Resperine (5.0 mg/kg, i.p.) was administered daily for 2 days prior to tissue removal, and again 90 min prior to tissue removal. The α-MPT (250 mg/kg, i.p.) was administered 30 min prior to tissue removal. On the day of the experiment, the animal (n =1) was deeply anesthetized with urethane (1.5 g/kg i.p.) and rapidly decapitated. The adrenal glands were removed and embedded in 3% agarose in BBS. The agarose gel blocks were placed in ice-cold BBS, and 400-μm thick slices were cut using a vibratome (World Precision Instruments, Sarasota, FL). Slices were allowed to rest in buffer for at least 1 h prior to placement in a recording chamber (Warner Instruments, Hamden, CT) that was superfused with BBS buffer maintained at 34 °C. They were maintained there for at least 30 min before FSCV recordings.

Glass-insulated, carbon-fiber microelectrodes were placed approximately 100 μm below the surface of each slice with the aid of a microscope (Nikon Instruments, Inc., Melville, NY), and
a Ag/AgCl pellet reference electrode (World Precision Instruments, Inc., Sarasota, FL) was placed in the tissue chamber to complete the electrochemical cell. A stimulating electrode comprised of two tungsten microelectrodes (FHC, Bowdoin, ME) was positioned 1 mm away from the working electrode. Electrical stimulations were carried out with a DS-4 Biphasic Stimulus Isolator (Digitimer Ltd., Welwyn Garden City, England) controlled by the HDCV software. Stimulation consisted of 165 biphasic, 500 µA pulses at a frequency of 165 Hz with a pulse-width of 1.5 msec. The MSW 2.0 waveform was applied at 10 Hz for this experiment.

2.5.6 In Vivo Experiments: Animals (n=4) were anesthetized with isoflurane (4% for induction and 1-3% for maintenance) and surgically prepared for electrode placement, as described. A heating pad (Harvard Apparatus, Holliston, MA) was used to maintain body temperature at 37 °C throughout the duration of the procedure. Briefly, holes for electrodes were drilled in the skull according to coordinates from the rat brain atlas of Paxinos and Watson. A guide cannula for a Ag/AgCl reference electrode was placed in the contralateral forebrain (BASi Instruments, West Lafayette, IN). The components were permanently affixed to screws in the skull with dental cement. The animals were allowed to recover for a minimum of 4 weeks prior to experiments, with daily handling. On the day of the experiment, a fresh Ag/AgCl reference electrode was inserted into the guide cannula.

For the anesthetized animal experiments, electrochemical data were collected in the dorsal striatum (+1.6 mm anteroposterior (AP); +2.0 mm mediolateral (ML) relative to bregma; -4.5 to -5.0 mm DV from skull) using the MSW 2.0 applied at 5 Hz. On the experiment day, the animal (n=1) was anesthetized with isoflurane (as described above), and a heating pad was used to maintain body temperature at 37 °C. The subject received intra-striatal microinfusions (0.75 µL over 1 minute) of PBS or a peptidase inhibitor cocktail (20 µM bestatin HCl added to a
commercially available cocktail (Sigma-Aldrich) that contained 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.3 μM aprotinin, 116 μM bestatin, 14 μM E-64, 1 μM leupeptin, and 1 mM ethylenediaminetetraacetic acid).

Animals used in the awake, freely-behaving experiments (n=3) were surgically prepared as described above, except silica-insulated electrodes were placed in the dorsal striatum (+1.2 mm AP, +2.0 mm ML, and -4.5 DV). The animals were allowed to recover for a minimum of 4 weeks and were handled daily. The animal used in the fruit loops experiment received fruit loops daily after surgical recovery, to prevent neophobia. On the day of the experiment, the animal was tethered and connected to a head-mounted voltammetric amplifier (current-to-voltage converter), commonly referred to as a headstage (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility). The headstage connects to the instrumentation via a swiveling commutator (SwivElectra; Crist Instument Co., Hagerstown, MD), to permit relatively unrestricted, free movement in the custom-built plexiglass chamber. Electrochemical data were collected using the MSW 2.0 applied at 5 Hz.

2.5.7 Statistics and Graphics: All data presented are shown as means ± SEMs, unless otherwise noted. Paired two-tailed student’s t tests, one-way repeated measures analysis of variance (ANOVA) with Bonferonni post-hoc tests or analysis of covariance (ANCOVA) tests were used where appropriate. Significance was designated at p < 0.05. Graphical depictions and statistical analyses were carried out using GraphPad Prism 6 or 7 (GraphPad Software, Inc., La Jolla, CA) and HDCV. MATLAB R2016a was used to convert traditional CVs to mCVs for visualizing the data, and Microsoft Excel 2013 was used to calculate correlation values.
2.6 Acknowledgements

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2.7 References


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CHAPTER 3: Electrochemical Quantification of Neuropeptides from Fragmented Amino Acid Sequences

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3.1 ABSTRACT

Opioid peptides function in many biological processes in organs ranging from the gastrointestinal system to the brain. However, a precise understanding of how endogenous opioid neuropeptides underlie pain and pleasure in the brain is currently lacking, largely due to the absence of tools capable of directly monitoring these endogenous species in vivo. Previously, we reported a methodology for detecting methionine enkephalin (M-ENK) using fast-scan cyclic voltammetry, enabling real-time monitoring of M-ENK dynamics in tissue. In the present study, several M-ENK related peptides were investigated to assess how amino acid composition can affect the local molecular environment and influence redox activity. The data reveal an increase in sensitivity as the number of amino acids in the M-ENK sequence increases, where intact M-ENK yields a much higher current response than singular amino acids. Principal component regression (PCR) was used to predict the concentration of M-ENK using training sets built from the electrochemical data of single amino acids or M-ENK fragments. The results demonstrate that PCR using training sets built from single amino acids were not capable of predicting M-ENK concentrations. However, training sets built from M-ENK-peptide fragments could predict M-ENK concentrations, likely due to the similar intermolecular forces of the peptide fragments to intact M-ENK. Furthermore, PCR using training sets from these peptide fragments was also effective for predicting the concentration of longer naturally occurring variants of M-ENK as well as synthetic peptides that contain two tyrosine or methionine amino acids. Thus, this work will be
of value to researchers investigating small peptides containing tyrosine and methionine in many contexts.

3.2 INTRODUCTION

Opiate compounds have been employed therapeutically in pain relief for millennia; however, pain remains a problem for many people. Recently, a variety of potent opiates have been developed to improve the pharmacological options for pain treatment. Unfortunately, these new drugs have also contributed to the dramatic increase in opiate overdoses, leading to an unprecedented number of fatalities, tragic psychosocial consequences, and billions of dollars in economic repercussions. The opiate epidemic is at least partly driven by the complexity of the endogenous opioid system these drugs impact. Targeting opiate receptors in the systemic treatment of pain also affects the wide range of biological functions modulated by endogenous opioid compounds, many of which are integral for adaptation and survival. For instance, endogenous opioids have been shown to play a role in the gastrointestinal tract, heart rate, respiration, renal function and immunological responses. Additionally, in the brain, these molecules are heavily implicated in motivated behavior and reward processing.

Despite being discovered over 50 years ago, many fundamental questions about opioid signaling remain unanswered, due largely to specific analytical challenges associated with the direct detection of endogenous opioid peptides in situ. A variety of approaches, including radioimmunoassay and ligand autoradiography, have been used to demonstrate that opioid peptide precursors and receptors are ubiquitously distributed in the peripheral and central nervous systems. Larger precursor proteins (prepro-enkephalin, prepro-opiomelanocortin, prepro-dynorphin) are synthesized and broken down through proteolytic cleavage to yield active peptides. These peptides bind non-exclusively to three principal classes of opioid receptors;
mu, delta, and kappa (MOR, DOR, and KOR).\textsuperscript{25-28} Thus, even the most accurate anatomical characterization of opioid precursors or receptors is inadequate for explaining the functional significance of a specific opioid peptide.

In an important study, Kennedy et al. coupled microdialysis to liquid chromatography-mass spectrometry (LC-MS) to detect met-enkephalin (M-ENK), a broadly expressed tyrosine-containing opioid pentapeptide,\textsuperscript{21-23} in dialysate extracted from rat striatum.\textsuperscript{10, 29-30} Weber et al. have also developed a robust and high-throughput method to quantify enkephalins and other neuropeptides in the rat hippocampus.\textsuperscript{31} These techniques offer the advantage of high selectivity because they are coupled with MS; however, the poor recovery rate inherent to microdialysis and the low basal concentrations of endogenous opioid peptides necessitate long sampling periods of up to an hour in length.\textsuperscript{31-34} These limitations are significant, as peptides are known to undergo cleavage and oxidation during sample collection.\textsuperscript{35-36} Furthermore, commonly used microdialysis probes are larger than some brain regions. Overall, this results in spatial and temporal averaging, compromising critical information needed to understand the mechanistic details that underlie opioid function. New analytical tools capable of monitoring real-time opioid peptide flux in discreet brain regions are needed to elucidate the exact mechanisms by which endogenous opioids modulate diverse biological processes.\textsuperscript{20}

Recently, fast-scan cyclic voltammetry (FSCV) has been coupled with carbon-fiber microelectrodes to selectively monitor real-time fluctuations of tyrosine-containing peptides, putatively identified as M-ENK, in the rat adrenal gland and striatum \textit{in situ}.\textsuperscript{37-38} A modified sawhorse waveform (MSW) was developed to enable these studies. This novel approach, which incorporates multiple scan rates in each voltammetric sweep, mitigates fouling issues and interference from endogenous dopamine. Broadly speaking, voltammetry can be used to resolve
multiple analytes in a complex mixture by evaluating the peak shape and position in cyclic voltammograms (CVs).\textsuperscript{39} Currents generated for samples containing multiple electroactive substances are typically additive, such that the sum of the CVs for each analyte yields a CV equivalent to one obtained from the direct detection of a mixed sample.\textsuperscript{40} However, detection of the single amino acids, tyrosine (Y) and methionine (M), yields voltammetric signatures that do not always align with the voltammogram for M-ENK.

The current study evaluated the redox activity of tyrosine and methionine singularly, and when integrated into various peptide sequences similar to M-ENK. The data were compared with voltammograms for intact M-ENK using principal component regression (PCR), a chemometric technique that is commonly used to evaluate voltammetric data.\textsuperscript{41-43} Predictions based on training sets of voltammograms for tyrosine and methionine were insufficient to reliably predict the redox activity of M-ENK (YGGFM). However, when training sets were constructed using four-amino-acid sequences that excluded the N-terminal tyrosine (-GGFM) or the C-terminal methionine (YGGF-), PCR was able to reliably and accurately predict known concentrations of M-ENK. This approach was also effective for the quantification of longer, M-ENK containing peptides (YGGFM-RGL and YGGFM-RF); however, the longer peptides could not be distinguished from the pentapeptide M-ENK. Finally, the data demonstrate that the PCR model can quantitatively predict synthetic amino acid sequences with more than one inherent tyrosine and/or methionine residue. This systematic exploration is one of the first FSCV studies done to explore how the local molecular environments of peptides effect redox activity. This study provides an improved method for the quantitative detection of M-ENK, and the findings can be used to inform the development of voltammetric strategies targeting other small peptides.
3.3 MATERIALS AND METHODS

3.3.1 Microelectrode Fabrication. T-650 carbon-fibers (Cytec Industries, Woodland Park, NJ) were used to fabricate microelectrodes as previously described. Briefly, a carbon-fiber was aspirated into a glass capillary, and sealed using a micropipette puller (Narishige, Tokyo, Japan). The fiber was cut to 100 μm away from the seal. A high ionic strength solution (4 M potassium acetate and 150 mM KCl) was injected into the unpulled end of the capillary to establish an electrical connection from the lead wire to the carbon-fiber. The other end of the lead wire was connect to the custom instrumentation. Electrodes in all experiments were electrochemically conditioned at 25 Hz until stabilized and allowed to cycle at 10 Hz for at least 10 min.

3.3.2 MSW. The MSW scanned from the accumulation potential of -0.2 to a transition potential of 0.6 V. The second segment of the forward sweep from 0.6 V to 1.2 V had a scan rate of 400 V s\(^{-1}\). Next, a 3 ms amperometric hold occurred at 1.2 V. After the hold, the reverse scan returned to -0.2 V at 100 V s\(^{-1}\). The total time to achieve one cyclic scan was 26.5 ms. This waveform was applied versus a Ag/AgCl reference electrode at an application frequency of 10 Hz.

3.3.3 Flow Injection. All data were collected in a custom-built flow injection apparatus encased within a Faraday cage. A syringe pump (New Era Pump Systems, Inc., Wantagh, NY) supplied a continuous buffer flow of 1.0 mL min\(^{-1}\) across the working and reference electrodes. A micromanipulator (World Precision Instruments, Inc., Sarasota, FL) allowed for precise positioning of the working microelectrode into the continuous stream of buffer flowing through the electrochemical cell. Bolus injections (5 s) were achieved using a six-port HPLC valve mounted on a two-position actuator. The valve was controlled by a digital pneumatic solenoid valve (Valco Instruments, Houston, TX) enabling precise 2-s bolus injections of analyte to be exposed to the electrode.
3.3.4 Data Acquisition. Potential application and current transduction were achieved using a custom instrument for potential application to the electrochemical cell and current transduction (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility). High-definition CV software (University of North Carolina at Chapel Hill) in conjunction with data acquisition cards (National Instruments, Austin TX) was used to control waveform output and acquire signals.

3.3.5 Chemicals. All chemicals were obtained at ≥95% purity (HPLC assay) from Sigma unless otherwise stated. Phosphate buffered saline (PBS; 10 mM Na₂HPO₄, 138 mM NaCl, and 2.7 mM KCl) prepared using ultrapure >18.2 MΩ water (Millipore, Billerica, MA) adjusted to pH 7.4 with 1 M NaOH and 1 M HCl was utilized for all experiments. All peptide fragments were obtained as trifluoroacetate salts from Biomatik (Ontario, Canada). A 5 mM stock solution of each analyte was prepared in PBS buffer and stored at -4 °C. The final working solutions were prepared daily by diluting the stock solution in PBS to the desired concentration.

3.3.6 Statistics and Graphics. All data presented are shown as the mean ± standard error of the mean, unless otherwise stated. Significance was assumed at p < 0.05. Graphical depictions and statistical analyses were carried out using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA) and HDCV. MATLAB R2016a was used to convert traditional CVs to modified CVs (mCVs) for visualizing the data. PCR data analysis was carried out in HDCV.

3.4 RESULTS AND DISCUSSION

3.4.1 Electrochemical Data Collected with the MSW. Previously, our group combined multiple-scan rates to overcome a myriad of electrochemical issues to enable the reproducible quantitation of tyrosine containing peptides using FSCV. This new approach enabled simultaneous monitoring of M-ENK and dopamine in rat adrenal medulla and dorsal striatum.
The presented work extends on this capability to quantitatively investigate the voltammetric detection of single amino acids and several-amino-acid sequences in vitro. More specifically, single amino acids and various amino acids integrated into peptide chains were evaluated to determine the redox activity of the two electroactive amino acids, tyrosine and methionine, of the endogenous pentapeptide M-ENK. (Figure 3.1 A).

All experiments in this study utilized an MSW that maintains a potential of -0.2 V between voltammetric sweeps. The first segment of the waveform scans up to +0.6 V at 100 V s\(^{-1}\). The scan rate is then increased to 400 V s\(^{-1}\) until reaching a potential of +1.2 V, where the potential is held for 3 ms, similar to amperometry. Finally, the reverse scan ramps down to -0.2 V at 100 V s\(^{-1}\) (Figure 3.1 B). Figure 3.1 C shows the issue and the existing solution for this dilemma, top and bottom respectively. In the top panel, CVs for tyrosine, methionine, and M-ENK are plotted in the traditional manner where multiple data points during the amperometric period are placed at the same potential. The solution to this issue is to plot all currents collected during the amperometric hold with respect to time (Figure 3.1 C gray box) using a scaling factor, as described previously.\(^{38}\) All data herein will be plotted as modified cyclic voltammograms (mCV) to enable visualization of currents recorded in the amperometric region at the highest potential, while retaining a conventional CV configuration.

3.4.2 Electrochemical Detection of Single Amino Acids. It is important to note the differences in the redox activity of the free tyrosine and methionine amino acids compared to the intact M-ENK peptide. Figure 3.1 C bottom overlays representative voltammograms where free tyrosine oxidations (red) can be observed at a slightly lower potential than tyrosine as a constituent of M-ENK (purple). Conversely, the oxidation of methionine within M-ENK occurs later in the amperometric hold than free methionine. Vast quantitative differences between the molar current
responses for tyrosine and methionine can be observed in Figure 3.1 D. The calibration plots for tyrosine and methionine are not on the same scale and therefore are plotted on separate axes. The concentrations of tyrosine use the top axis, while the bottom axis represents concentrations of methionine. The data shows that the sensitivity of tyrosine was 1.6 (± 0.1) nA µM$^{-1}$ and methionine was 0.069 (± 0.002) nA µM$^{-1}$. Importantly, the current response of M-ENK was higher for both constituents with current response of the tyrosine peak yielding a sensitivity of 4.0 ± 0.1 nA µM$^{-1}$ and the methionine peak yielding a sensitivity of 2.9 ± 0.1 nA µM$^{-1}$.

Electrochemically, it was expected that the voltammograms from the individual components of M-ENK could be mathematically combined with some form of multivariate analysis to predict the voltammogram for M-ENK, assuming there was no difference in the redox activity of free and bound amino acids. Given the vast differences in sensitivity and shifts in oxidation peak locations, this assumption was not likely valid, but testable with PCR.
Figure 3.1: Electroactive Amino Acids in M-ENK. M-ENK contains two electroactive amino acids that generate distinct electrochemical signatures. (A) Molecular structure of the pentatpeptide M-ENK with the electroactive amino acids tyrosine (red) and methionine (blue) highlighted. (B) MSW employed for the detection of tyrosine and methionine containing compounds with FSCV. The segments of the waveform that oxidize tyrosine and methionine are highlighted in red and blue, respectively. (C) Top: Conventional CVs for 4.3 µM tyrosine (Y, red), methionine 65 µM (M, blue), and 2 µM M-ENK (purple). Bottom: The CVs can be modified such that currents generated in the 1.2 V amperometric hold are plotted vs. time to enable visualization of the methionine peak (mCV). (D) Calibration plots for tyrosine (top x axis, red) and methionine (bottom x axis, blue). Sensitivity to tyrosine 1.6 ± 0.1 nA µM⁻¹ and methionine 0.069 ± 0.002 nA µM⁻¹ (n = 3 electrodes for each analyte).

3.4.3 Multivariate Analysis of the Redox Activity of M-ENK. PCR combines principal component analysis with inverse least-squares regression to quantitatively predict the concentration of mixed unknown samples. Voltammograms of M-ENK, like Figure 3.2 A, were used as the data sets to test a trained PCR model to determine the ability of the model to evaluate a known concentration. PCR reduces the dimensionality of the data in the training sets.
and effectively reduces noise by only keeping components that describe the highest correlated variability, like peaks in a voltammogram that change in intensity based on concentration. These training sets are then converted to principal components (PCs), or basis eigenvectors, that describe the variance in an abstract two-dimensional factor space in a score plot.\textsuperscript{34,37} Training sets in this experiment were constructed from five concentrations of tyrosine and methionine (Figure 3.2 C). To ensure the non-noise variance was adequately described, Malinowski’s F test was used to find the number of components to be retained in the PCR model.\textsuperscript{41, 48} The score plot in Figure 3.2 B displays the variance maintained in the PCR model as well as the inset mCVs which are abstract representations of the retained components retained from training sets (Figure 3.2 B insets of the k-Matrix for tyrosine (red) and methionine (blue)).

The representative color plot in Figure 3.2 D (left panel) shows typical voltammetric data collected for a 2-s, 1-µM bolus of M-ENK, with an inset mCV extracted from the time designated by the dashed line. The right panel in Figure 3.2 D displays the residual after fitting the representative data to the trained PCR model. The significant amount of residual current remaining in the colorplot and inset mCV, after application of the PCR model shows the deficiency of the training set’s ability to describe the redox activity of M-ENK. Evaluation of the PCR model, showed a $Q_a$ value (confidence threshold) which was less than the $Q_t$ value (squared and cumulative variance at a given time), signifying that there was a significant amount of current unaccounted for by these training sets.\textsuperscript{45}
**Figure 3.2: Predictive Power of Single Amino Acids.** PCR using training sets built from single amino acids is insufficient to reliably describe the electrochemical detection of M-ENK. (A) mCV for M-ENK with tyrosine and methionine peaks marked with red and blue vertical lines, respectively. (B) Score plot generated from tyrosine and methionine training sets displayed in (C). mCVs of the first two principal components are inset. (D) Left: Representative color plot for a 2-sec injection of 1-µM M-ENK. Right: Color plot describing the residual current that remains after subtraction of the principal components. Inset: mCVs taken at the time indicated by the vertical white line. (E) M-ENK concentration predicted using the PCR model versus the known sample concentrations. The diagonal-dashed line represents unity for a perfect prediction. The slope of the regression line based on the tyrosine peak is 2.0 ± 0.2 ($r^2= 0.99$) and based on the methionine peak the slope is 8 ± 2 ($r^2= 0.92$, n=3 electrodes per analyte). In both cases, M-ENK concentrations are overestimated.
Figure 3.2 E graphs the results of the PCR model’s ability to predict concentrations, by plotting the actual concentration of M-ENK injected into the system versus the PCR predicted concentration. The tyrosine or methionine peak can be used to predict the M-ENK concentration; the left panel displays the prediction based on the tyrosine component and the right panel shows the prediction based on the methionine component. Quantitation of M-ENK is typically based on the currents obtained from the tyrosine peak.37-38 The dashed line in both graphs represents a perfect prediction and it was observed that the tyrosine component was approximately twice the unified slope. Using the methionine component, the concentration of M-ENK was also overestimated by approximately 8-fold. These findings demonstrate that the electrochemical responses from singular amino acids are insufficient for predicting the voltammogram or concentration of M-ENK.

3.4.4 Detection of M-ENK Fragments Containing Tyrosine and Methionine Fragmented versions of M-ENK were assessed to gain a better understanding of how incorporation into different peptide chains affects the redox activity of tyrosine and methionine at carbon electrodes. Figure 3.3 A (left) shows the tyrosine-containing M-ENK fragments investigated, starting with tyrosine (Y; top), and systematically adding the amino acids until M-ENK (YGGFM) was formed (Figure 3.3 bottom). Voltammograms for the fragmented peptide show similar electrochemical signatures, with the exception that tyrosine alone presented a narrower and more ideal peak (Figure 3.3 A, pink). This aligns with previous research that modeled narrower oxidation peaks result from narrower peaks generated in the oxidation of single amino acids, as compared to the oxidation peaks of oligopeptides.50 This is also observed in mCVs found in figure 3.3 where peak shape changes depending on the presence of different amino acids. The redox signature is most different between the single amino acids and the intact M-ENK molecule. The shape of the redox
peaks shift closer to M-ENK the closer the peptide sequences gets to M-ENK. With the addition of glycine, the next amino acid in the M-ENK sequence, there is an observable increase in peak width. As additional amino acids are sequentially added, the CVs for the longer molecules show improved correlation with the voltammogram for the intact molecule (Figure 3.3 A bottom). Longer peptides oxidation peaks align better with the peptide of interest, M-ENK. If we use their voltammograms as inputs into PCA they should better describe the M-ENK redox signal than single amino acids using PCA.
Figure 3.3: Molecular Structures and Sensitivities of Tyrosine and Methionine-Containing Fragments. Sensitivity to tyrosine and methionine is enhanced by the presence of additional amino acids in the M-ENK sequence. Peptide fragments containing tyrosine are displayed on the left in the red box (A), while methionine-containing peptide fragments are displayed in the blue box (B). Top: Representative mCVs for the peptide fragments. Middle: Table listing the sensitivity to each fragment and M-ENK with this experimental approach. Bottom: Representative mCV for intact M-ENK with the tyrosine and methionine peaks marked with red and blue dashed lines, respectively. The CVs for tyrosine containing peptides have µM concentrations of Y-1.1, YG-1.7, YGG-3.1, YGGF-1.6. The CVs for methionine containing peptides have µM concentrations of M-50.5, FM-45.5, GFM-7.9, and GGFM-3.9.

M-ENK fragments were systematically evaluated from the singular methionine to the intact M-ENK peptide by the sequential additions of one amino acid to the C-terminus. The voltammograms of these molecules were overlaid in Figure 3.3 B (top) and the structures are displayed along the side. Interestingly, the decay profile observed in the different peptide fragments tested showed some variability in that they do not return to baseline with the same decay
rate. This could be due to a variety of reasons from mass transport to the concentration of analyte used. Conversely to the previously describe tyrosine containing fragments, the signal for singular methionine peptide does not return to baseline.

As more amino acids are added to the peptide sequence the decay profile approaches baseline. One possible contributing factor to the differences observed in the decay profiles is adsorption. As additional amino acids are added both size and electric charge change which influence the kinetics of this redox reaction. Also, both tyrosine and phenylalanine have benzene rings in their structure (Figure 3.3) which are likely the reason for this strong adsorptive interaction. Previously the Sombers group showed that the electro-oxidation is adsorption controlled. This strong adsorptive property would influence the way the molecule interacts with the surface of the electrode. Given the range of sensitivities and differences seen in the mCVs for these fragments, it is likely that the specific amino acids added to a peptide sequence could change a range of properties of the peptide including the electrostatic distribution of charge and hydrophobic properties the peptides display. These systems are quite complex in nature as we have observed changes in the redox activity even when the peptide had only a small non-electroactive amino acid was added.

The tables located in the middle of Figure 3.3 list analyte sensitivities for the tyrosine fragments of M-ENK on the left and those for the methionine fragments on the right. The current response of the methionine peak in intact M-ENK is over 40-fold higher than the current response observed for free methionine. This is likely due to the other amino acids bound to the methionine and how they are interacting with the electrode. This kind of interaction and how it changed the electrochemistry of cyclic voltammetry has previously been modeled. Clearly the number of
amino acid residues bound to the electroactive moiety altered the electrochemical response. This is likely due to changes such as mass transport and adsorption.

The next phenomenon investigated was the relationship of the position of the electroactive moiety relative to the N and C-terminus. To accomplish this, small peptides with either tyrosine or methionine were integrated into three amino acid sequences as a simple synthetic peptide model. Glycine was chosen to generate this three-amino-acid peptide chain as it has a single hydrogen as a side group and should have a minimal effect on redox activity when compared to more bulky amino acids. The model systems were YGG and GGY for tyrosine and GGM and MGG for methionine.

Figure 3.4 shows the resulting voltammograms for these peptide model systems. It was observed that the position of the tyrosine residue in the peptide did not significantly alter the detection sensitivity. The sensitivities for MGG and GGM are also equivalent. Also we can see that GGY has a narrower oxidation peak when compared to the intact M-ENK indicating that the molecules being detected in this peptide fragment are likely adsorbed to the surface of the electrode.

The data demonstrate that sensitivity for the methionine fragments MGG (0.031 ± 0.002 nA µM⁻¹) and GGM (0.025 ± 0.003 nA µM⁻¹) are roughly equivalent and closer to the reported sensitivity to singular methionine (0.069 ± 0.002 nA µM⁻¹) than to the tripeptide, GFM (0.60 ± 0.02 nA µM⁻¹). This highlights the importance of phenylalanine for increasing sensitivity and further supporting the hypothesis that this phenomenon is due to enhanced adsorption. Similarly, the data demonstrate that sensitivity for the tyrosine fragments YGG and GGY is roughly equal (within error). Overall, these data suggest that phenylalanine does play a role in the redox activity
of methionine and that the terminal position of electroactive amino acids does not affect redox activity.

![Table 3.4: Investigating the Effect of Terminal Location on Redox Activity.](image)

### Figure 3.4: Investigating the Effect of Terminal Location on Redox Activity.

Altering the position of tyrosine and methionine (C- versus -N terminus) does not significantly affect the electrochemical signature in a simple peptide model system. Information concerning peptide chains containing only glycine or tyrosine are found in panel (A), while the corresponding glycine- and methionine-containing amino acid chains are shown in (B). Top: Table listing sensitivity to the synthetic peptides with this experimental approach. Middle: Representative mCVs of synthetic model peptides. Bottom: Representative mCVs and molecular structures.

#### 3.4.5 PCR with Training Sets Built from Fragments of M-ENK.

Training sets built from mCVs of M-ENK fragments (YGGF- and -GGFM, **Figure 3.5 B**) are better at quantitatively predicting the concentrations of M-ENK than training sets built from mCVs for the individual amino acids. **Figure 3.5A** depicts overlaid mCVs for YGGF-, -GGFM, and M-ENK (YGGFM), with the tyrosine peak labeled with a red dashed line and the methionine peak labeled with a blue dashed line. A representative score plot built from the PCs that describe the variance in the -GGFM and YGGF- training sets is shown in **Figure 3.5 B**, with an mCV representation of the regression vector for each component inset. **Figure 3.5 D** (left) shows a representative color plot of raw data collected for a 2-s bolus injection of M-ENK (1 µM) at the time indicated by the red mark, as well as an mCV collected at the time designated by the dashed line is shown as an inset. After applying
PCR with the training sets of YGGF- and -GGFM, very little residual current remains (Figure 3.5 D right inset), demonstrating the peptide fragments can effectively describe the redox activity of M-ENK. Further statistical evaluation of this PCR model validates this observation as the remaining current is found to be insignificant.\textsuperscript{45} Figure 3.5 E describes the quantitative abilities of the PCR model to produce a linear regressed prediction of M-ENK that closely matches unity, especially when using the tyrosine peak. This result demonstrates that using these four-amino-acid peptide sequences in the training model more effectively described the voltammetric signature observed for M-ENK than the single amino acids.
Figure 3.5: Predictive Power of M-ENK Fragments. PCR using training sets generated from the electrochemical detection of four-amino-acid fragments of M-ENK improves predictive quantification of M-ENK. (A) mCVs for M-ENK, YGGF-, and -GGFM with tyrosine and methionine peaks marked with red and blue vertical lines, respectively. (B) Score plot comprised of mCVs generated from the YGGF- and -GGFM training sets displayed in C. (B) Inset: mCVs of principle components generated from training sets. (D) Left: Representative color plot of raw voltammetric data for a 2-sec injection of 1-µM M-ENK. Right: Color plot describing the residual after subtraction of principal components. Inset mCVs taken at the time indicated by the vertical white line. (E) M-ENK concentrations predicted using the PCR model plotted versus known sample concentrations. The diagonal line represents unity for a perfect prediction. The slope of the regression line based on the tyrosine peak is $1.05 \pm 0.04$ ($r^2 = 0.95$, $n = 3$). Methionine has a slope and regression line of $1.24 \pm 0.04$ ($r^2 = 0.95$, $n = 3$).
3.4.6 Physiologically Relevant Analogs of M-ENK. Given that this predictive model will likely be used for the quantification of data collected from *in vivo* models where the chemical composition and dynamics are not well known, we investigated peptides that are co-synthesized from the same precursor as M-ENK, preproenkephalin. During proteolytic cleavage, preproenkephalin can be divided into four pentapeptides of M-ENK (YGGFM), as well as one YGGFM-RF and one YGGFM-RGL. Given the inevitable presence of these analytes in biologically relevant preparations, these peptides were also tested to determine the ability of the model to predict known concentrations of these analytes with the PCR model trained with M-ENK fragments (YGGF- and -GGFM). Figure 3.6 A and B show representative calibration sets for these longer endogenous peptides on the left and the prediction plots on the right. The voltammograms for YGGFM-RGL and YGGRM-RF have the peaks similar to those previously observed in M-ENK attributed to the oxidation of tyrosine and methionine. The additional R and L amino acids are not electrochemically active within our potential window yielding the signature voltammogram for YGGFM-RGL very similar to M-ENK. Using the PCR model built from the four peptide fragments of M-ENK, analysis of the tyrosine peak yielded a near perfect prediction with the slope of the tyrosine prediction line roughly equal to unity. Conversely, the methionine peak is used to try to predict the concentration of YGGFM-RGL injected into our system, a twofold over-prediction occurs which we can see with the slope of the methionine prediction line being 1.96 µM of analyte predicted per 1 µM injected. These findings hold true for both YGGFMRGL and YGGFMRF. Evaluation of these PCR model data found the remaining current in the voltammogram to be insignificant, further validating that our peptide fragments can predict the current observed in these larger M-ENK analogs.45
Figure 3.6: Quantitative Prediction of Larger M-ENK Containing Peptides. Left: Representative mCVs. Right: Plots of PCR-predicted concentrations of M-ENK containing peptides vs. known concentrations. PCR training sets were built from CVs for YGGF- and -GGFM amino acid chains. Right: M-ENK concentrations predicted using the PCR model plotted versus known sample concentrations. The slope of the regression line based on the tyrosine peaks are plotted in red while the blue lines are regression lines based on the methionine peak. (A and B) YGGFM-RGL and YGGFM-RF are physiologically relevant peptides with a voltammetric signature similar to that of M-ENK. (C and D) MGG-YGGFM and YGGFM-GGY are synthetic peptides used to demonstrate the ability of the model to accurately predict the additional electroactive residues. All data are plotted as mean ± standard error (n = 3 electrodes per analyte).
3.4.7 Synthetically Derived Analogs of M-ENK. To extend the PCR model validation, artificially synthesized peptides were investigated that include additional electroactive amino acids, like MGG-YGGFM and YGGFM-GGY. These molecules were chosen to have a tyrosine and methionine amino acid residue ratio of 2:1 or 1:2, with terminating electroactive amino acids but it is important to note that these peptides do not have any physiological relevance. Figure 3.6 C shows a representative calibration set of mCVs for MGG-YGGFM where significantly more current can be observed during the amperometric hold where methionine oxidizes. Using either the tyrosine or methionine peaks, there was an over prediction in the model to determine the concentration of the non-physiological analyte. The slope of the prediction plot for the tyrosine peak is slightly higher than unity, meaning that, with the addition of MGG, more current is registered for the tyrosine peak. The methionine peak exhibits a little over two-fold higher sensitivity for MGG-YGGFM as we would expect for a molecule that contains two methionine amino acids.

For YGGFM-GGY (Figure 3.6 D), the tyrosine peak is appreciably larger than the methionine peak. Also the slope of the prediction plot for the tyrosine peak is approximately 2, as expected for a peptide that contains two tyrosine residues. The methionine peak prediction value is very close to that observed for native pentapeptide M-ENK. Thus, these results indicate that, by adding an additional tyrosine or methionine to the peptide sequence, we do not obtain a significant amount of current for the other amino acid. Evaluation of the residual current left after applying the PCR model built from the M-ENK fragments indicated that residual current was insignificant, further validating that our peptide fragments can predict the current observed in these synthetic analogs.45
3.5 CONCLUSIONS

These findings demonstrate that the redox activity of peptides is dependent on the local molecular environment, which is influenced by all amino acids in the peptide chain. Using the electrochemical responses of single amino acids did not adequately describe that of the full peptide. These findings show that peptide fragments that more closely resemble M-ENK can be used to build better predictive models. These findings are not surprising given the longer peptide fragments have are more similar to M-ENK in terms of electrostatic properties, and molecular size. All of these characteristics affect surface properties. The longer fragment peptides gave more similar electrochemical signals when compared to the intact M-ENK. This quantitative approach will be useful for the detection of M-ENK and other tyrosine or methionine-containing peptides in complex biological preparations, where the concentration dynamics of these peptides are unknown. This electrochemical approach will enable quantitative information to be obtained on how neuropeptides are synthesized, packaged, transmitted, and function in living tissue.

3.6 Acknowledgments

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CHAPTER 4: Summary and Outlook

4.1 Research Summary

The field of neuroscience has been advanced by numerous contributions made from the electroanalytical technique background-subtracted fast scan cyclic voltammetry (FSCV). This technique enables the quantitation of real-time fluctuation of neurotransmitters in the brain of anesthetized or behaving animals, tissue samples, and single cells. Common analytical approaches found in neuroscience often carry out measurements offline or in removed tissue like MALDI-MS, or immunoassay. Another advantage of voltammetry is the ability to yield qualitative information on the identity of the analyte detected, and quantitate the fluctuations of the analyte in behaving animals during an experiment that provokes a task or behavior. Similar studies focused on endogenous opioid neuropeptides will surely help inform the neuroscience community of the role these molecules might play in reward and addiction.

Chapter 2 in this work is a paper published in ACS Chemical Neuroscience in collaboration with Carl Meunier and Christie Lee. In this work, we optimized a multiple scan-rate waveform which allows increased sensitivity during the detection of M-ENK with FSCV. The multi-scan rate waveform was characterized so we could find which electrochemical variables influence the sensitivity of this system the most. This allowed us to develop a new, more sensitive waveform enabling the first FSCV measurements of opioid neuropeptide dynamics in the brain. The ability to monitor this family of molecules is highly significant to electrochemists, biologist, and medical researchers as peptides are the largest and most diverse family of cellular signaling molecules. The characterization of this waveform can guide scientists interested in detecting other peptides using FSCV. This new tool will facilitate many peptides, previously out of the reach of FSCV, to
be monitored and better understood providing a new arena of research and enabling the development of evidence based pharmaceuticals.

Broadly speaking, this work has enabled the direct monitoring of peptidergic signaling in the brain to enable us to start pairing the flux of opioid peptides to behaviors. Thus, it has advanced both analytical electrochemistry and neuroscience. Specifically, Chapter 2 optimizes a novel, multi-scan rate voltammetric approach, which enabled the first real-time electrochemical measurements of endogenous opioid peptide dynamics in the brain. Chapter 3 investigates how this technique can be used to detect several different peptide fragments and analogs of M-ENK. Also it highlights the quantitative differences observed with different peptide chains and how the number and type of amino acids making up the peptide chain affect redox activity. Together, this work provides researchers with significant characterization of an exciting new tool capable of the real-time detection of opioid peptides. This new tool will allow many crucial questions to be addressed that have been previously unreachable. For example, we now have a better understanding of how the electrochemical signature of peptides change when broken down into smaller peptide chains or singular amino acids. This information will be of great importance as we seek to better understand if these molecules are changed before reach their target sites. Our new tool will also enable us to monitor opioids flux during different rewarding behaviors and yield preliminary information of the normal function of endogenous opioids.

4.2 Outlook

Some of the limitations of FSCV include the inability to make measurements over tens of minutes to hours; however, our group is working on several drift removal techniques that could be used to enable long term measurements. These improvements could be applied directly to the research presented here and retroactively to any measurement made with FSCV to improve long
term quantitation. The sensitivity of our electrochemical approach to M-ENK was improved over three-fold. Future studies necessitating even more sensitive approaches could benefit from the implementation of new materials with advantageous electroanalytical properties like carbon nanotube yarns.\textsuperscript{8-9}

The ability to monitor opioid peptides in real-time could also have major implications on the research that can be conducted and how we approach solving the opioid epidemic. As the technology of our research tools improves, and the mechanisms that endogenous opioid modulate are better understood, our approach to alleviating pain and treating a wide range of disease will improve. The research outlined in this document describes steps taken to characterize the multi-scan-rate waveform our lab pioneered, taking this idea from a proof of concept to a more fully characterized technique. This characterization will make our electrochemical approach more appealing to practitioners of the FSCV community who could answer many mechanistic questions about endogenous opioids with this powerful new tool.
4.2 References


