ABSTRACT

SMITH, SAMANTHA KRISTINA. Advancing Microbiosensor Development for the Real-Time Electrochemical Detection of Multiple Analytes in Rat Brain Tissue. (Under the direction of Dr. Leslie A. Sombers)

Actions of opiates, alcohol, and major stimulants, such as cocaine, converge on the dopaminergic systems in the brain to modify synaptic activity and behavior. The neurotransmitter dopamine is critically involved in cognition, sensorimotor integration, and modulation of motivated behaviors. However, little is known about the energetic substrates that fuel these circuits, or how molecules such as glucose and lactate are altered upon neuronal activation and exposure to drugs of abuse. The brain utilizes glucose and lactate to fuel metabolic processes that control how we think, feel and behave. However, evidence is lacking to describe rapid changes in energy availability in discrete brain regions. The electrochemical approach, fast-scan cyclic voltammetry (FSCV), is well suited for monitoring neurochemical signaling because of the subsecond time resolution and selectivity. Recent developments in our research group have coupled FSCV to carbon-fiber microbiosensors to enable the measurement of glucose and lactate dynamics in brain tissue. This was a critical development as we can now monitor real-time fluctuations of energy availability in the extracellular space on the time scale of neuronal activation.

The work presented herein describes the development, characterization and utilization of microbiosensors for simultaneous detection of glucose and lactate in rat brain tissue. Investigations of enzyme attachment strategies for optimal subsecond detection of nonelectroactive chemicals were completed. A hydrogel entrapment strategy was found to be the most stable and reproducible method for enzyme placement on a microelectrode sensing surface. An optimized voltammetric waveform was developed for simultaneous detection of glucose and dopamine at a single recording
site in real-time. These unprecedented data revealed an increase in energy availability in response to metabolic demand after neuronal activation via electrical or pharmacological stimulation. Additionally, a microbiosensor was developed for sensitive, selective and stable detection of subsecond lactate dynamics. This novel microbiosensor was coupled with a glucose sensor to simultaneously quantify these species and dopamine at one location utilizing FSCV in the rat striatum. Data collected revealed diverse temporal dynamics of these molecules in response to neuronal activation.

Collectively, this dissertation describes the development, characterization, and application of important new tools to advance our fundamental understanding of brain energy availability, and to elucidate how key energetic substrates respond to neuronal activation. It has been used to demonstrate that glucose, lactate and dopamine are collectively implicated in brain function, and has the potential to describe modifications to chemical dynamics associated with dysfunction. As such, the microbiosensors described herein can potentially assist in the development of therapeutic strategies for treating drug addiction and other neurological disorders impacted by energy dysregulation.
Advancing Microbiosensor Development for the Real-Time Electrochemical Detection of Multiple Analytes in Rat Brain Tissue.

by
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DEDICATION

This work is dedicated to my closest friends and family, whom without their support and encouragement, I would not be where I am today. First, I would like to thank my better half, Alec Falzone, who fosters and strengthens my passion for science. Without you, I would have never accomplished this and the journey has been sweeter having my best friend along for the experience. Next, I would like to dedicate this work to my mother, Candice Smith. From a young age she has taught me the value of education and fought bravely to ensure I had access to necessary materials to thrive as a young female scientist. I would also like to thank my brother, Jacob Tenute. Albeit ten years younger, he has inspired me to work hard for my family through his courage and adaptability. Moreover, I dedicate this work to my tita, Mary Patterson, and maternal grandfather, Ronald Smith, for their calm demeanor and reassurance when I needed it the most.

Lastly, this dissertation is dedicated to all minorities. At some point in our lives we have been judged and mistreated for things we cannot control. We must overcome ignorant attitudes with grace and poise. Through hard work, determination, and grit, we can do anything.
BIOGRAPHY

Samantha Kristina Smith was born in Aurora, IL on August 12th, 1990. Since the age of 5, she wanted to be a professional scientist. Born into an underprivileged urban environment, science was not made readily available. This motivated her to explore various avenues in means of obtaining the most information she could get her hands on. Even as a young child, she remained goal oriented and results driven. Whether it was convincing her mother to take her to the library to immerse herself in scientific literature or asking for educational pricey books/kits for her birthday. The areas of science that fostered this hunger for knowledge ranged from biology, astrophysics to chemistry. As she began her journey towards her professional career, she still found diverse subjects intriguing and alluring. It’s that passion and commitment to collaboration, exploration, and discovery that has contributed to her interdisciplinary research and success.

Within her younger years, she lived and studied in various states ranging from Florida, Minnesota to Illinois. Settling in Wheaton, IL at the age of 11, she studied in DuPage County Public Schools until leaving for college at the age of 18. Along the way, Samantha participated in a wide variety of extracurricular activities including acting, student council, as well as chemistry club.

She left for college in 2008, attending Western Illinois University to attain a B.S. in Forensic Chemistry. There, she became heavily involved with student government, residence life and shortly thereafter, was the president of chemistry club for two years. Throughout these experiences, she learned about team dynamics and diverse cultures. These experiences would influence her professionalism and shape the women she turned out to be. Throughout her undergraduate career, she also participated in undergraduate research in three chemistry laboratories. Through her success, she was afforded an ungraduated research award as well as an
authorship on her first publication. These research experiences enlightened her to the intellectual challenge and joy from engaging in research. Through the encouragement of Alec Falzone, she applied to attend graduate school for a Ph.D. in chemistry. It was the best decision she ever made.

After graduating in 2013, Samantha Smith went to North Carolina State University to pursue her Ph.D. in chemistry. She joined Dr. Leslie Sombers’ multidisciplinary analytical chemistry research laboratory, falling under her mentorship and guidance for the remainder of her graduate career. Through Samantha’s love of science, she has discovered it’s a beautiful thing when passion meets purpose.
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1.1 Neuronal Communication

There are essentially two types of cells within the nervous system; glia and neurons. These cells are imperative to brain function and information processing. Neurons send messages in the form of chemicals at specialized junctions called synapses. Typically, a synapse contains a presynaptic terminal from the “sending” neuron and a postsynaptic terminal at the “receiving” neuron separated by a gap of 5–50 nm synaptic cleft. Neurotransmitters are chemicals that are released into this synaptic space where they can bind or interact with specific membrane proteins, called receptors, on the postsynaptic neuron allowing chemical communication (Figure 1.1).

Figure 1.1 Neurotransmission: Chemical Communication Between Two Neurons. The cell body of the neuron receives and processes incoming neurochemical signals. Once that the cell becomes depolarized, an electrical impulse called an action potential will propagate, down the axon to the terminals. Here, the electrical signal is translated into chemical communication, where vesicles of neurotransmitters fuse to the membrane and release their chemical contents into the synapse. Once in the extracellular space, the chemicals can bind to receptors where signal transduction can alter the receiving neuron’s cellular activity.
A neurotransmitter can bind to specific receptors causing a biochemical response in the postsynaptic neuron. In general, the endogenous neurotransmitters have a facilitatory, agonistic effect at specific receptor(s). The downstream effects of such agonism though can be varied. Some classical neurotransmitters include but are not limited to gamma-aminobutyric acid (GABA), glutamate, and dopamine (DA). GABA and glutamate play opposing roles in neuronal signaling by providing inhibitory or excitatory signals respectively, whereas dopamine modulates neuronal activity.

1.2 Dopamine

Within a dopaminergic neuron, tyrosine is enzymatically converted to L-Dihydroxyphenylalaine (L-DOPA) via tyrosine hydroxylase. Thereafter, L-DOPA can be converted to DA by dopa decarboxylase and the newly synthesized DA molecules are packaged into vesicles within the cytosol. When released into the extracellular space, DA can (1) bind to DA receptors, (2) be taken back up by a dopamine transporter (DAT) to be recycled and repackaged or (3) be enzymatically degraded by monoamine oxidase (MAO) or catechol-O-methyltransferase (COMT).

Dopamine has been heavily characterized since it’s discovery as a neurotransmitter by Arvid Carlsson in 1957. Since then, there have been several proposed roles for DA, which include an involvement in hedonic processing (pleasure), incentive salience (motivational drive or wanting), and in modulation of appropriate goal-directed behavior via associative learning. As such, DA plays an essential functional role in motivation through reward evaluation, seeking behavior for natural and drug rewards, and by facilitating reinforcement learning. DA also serves important motor and cognitive functions, thus techniques that afford real-time detection of
DA are critical to better understand DA function and dysfunction in Parkinson’s disease, Alzheimer’s Disease, and in substance abuse.

Cocaine, is a commonly abused drug that elicits a well-characterized and robust dopamine release in the striatum.\textsuperscript{7, 16} Although it is well known that cocaine induces patterns of neural, physiological and behavioral effects consistent with metabolic neural activation\textsuperscript{7, 10, 17-20}, direct attempts to evaluate metabolic effects of this drug have produced controversial results.\textsuperscript{21} Thus, there is a need to simultaneously detect DA (electroactive) and glucose (non-electroactive) molecules with one biosensor at discrete brain locations in order to elucidate the metabolic effects of drug induced neuronal activation from cocaine administration.

1.3 Neuronal Fuel Sources: Glucose and Lactate

Glucose metabolism provides the energy and precursors for biosynthesis of essential neurotransmitters such glutamate, aspartate and glycine.\textsuperscript{22-24} Glucose is able to access the brain by crossing the blood brain barrier (BBB) (Figure 1.2). Here, there is a wall of brain capillaries which limit the movement of substances into the extracellular fluid of the brain.\textsuperscript{25} These walls are composed of endothelial cells, pericytes, possible smooth muscle cells and a variety of basement membranes forming a continuous belt of tight junctions.\textsuperscript{26} Glucose transporters selectively allow glucose to cross the BBB and enter the extracellular fluid to fuel energetic demands upon neuronal activation.
Once glucose enters the brain, there are three main metabolic paths that it can undergo (Figure 1.3). First, glucose can be stored as glycogen to provide fuel to local regions upon functional activation or energy deficits. The second metabolic pathway is glycolysis, independent from the presence of oxygen, yielding a partial oxidation of glucose producing two molecules of adenosine triphosphate (ATP), two molecules of nicotinamide adenine dinucleotide (NADH) and 2 molecules of pyruvate. Thirdly, the pentose phosphate pathway (PPP), allows the complete oxidation of glucose resulting in more efficient glucose metabolism producing 30-36 ATPs.\textsuperscript{22}

Energy metabolism in the brain is a highly compartmentalized and convoluted process in which transcellular exchange of metabolites plays an essential role.\textsuperscript{28} Although glucose is the principle energy source of the brain, lactate has proven to be another critical source of energy.\textsuperscript{28-32} There has been considerable supporting evidence indicating that lactate is essential to maintain synaptic transmission, particularly during periods of intense activity. Moreover, lactate has been shown to demonstrate a potential neuroprotective role within certain conditions such as ischemia\textsuperscript{33-34}, epilepsy\textsuperscript{35}, and diabetes.\textsuperscript{36-38} However, access to this additional energy source is difficult to
determine precisely, as lactate is unable to cross the BBB. Nonetheless, within the brain lactate can be synthesized as it is a byproduct of glycolysis.

Nonetheless, within the brain lactate can be synthesized as it is a byproduct of glycolysis.  

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**Figure 1.3. Primary Mechanisms Involved in Glucose Metabolism.** (i) Glycolysis metabolism pathway. (ii) Pentose phosphate pathway (PPP). (iii) Glucose storage as glycogen.

A central role of astrocytes in coupling neuronal activity to energy metabolism. Astrocytes sense synaptic activity (glutamate release) which triggers a cascade of biological events that results in glucose uptake (Figure 1.4). This glucose can be processed via glycolysis, resulting in lactate production. Lactate is shuttled to the neuron where it is converted to pyruvate and provides fuel for the production of ATP by oxidative phosphorylation (28, 30, 32, 40-41) (see Figure 1.3 iv). However, demand for direct experimental proof of the lactate shuttling utilization is required.
to provide further evidence of this controversial theory. There have been discrepancies reported within literature due to various sample environments and methodologies utilized for lactate monitoring (for review see 24).

![Figure 1.4. Astrocyte-Neuron Lactate Shuttle](image)

**Figure 1.4. Astrocyte-Neuron Lactate Shuttle**. Real-time molecular detection of glucose and lactate dynamics is imperative to understand the complexities of brain energy availability in normal conditions as well as metabolic adaptations that occur within disease states.

### 1.4 Analytical Techniques to Measure Glucose Availability

Positron emission tomography (PET) is a neuroimaging technique that allows for non-invasive *in vivo* measurements of energy delivery and availability. PET involves an intravenous injection of short lived radioactive tracers attached to a biologically active carrier molecule, such as fluorodeoxyglucose (FDG) or [18F]-FLac analogs of glucose and lactate
respectively. These tracer molecules will be consumed by tissues and the scan reveals areas corresponding to regional analyte uptake.

Although this technique is noninvasive and ideal for visualizing regional indications of neuroenergetics, there are several limitations including high cost and an on-site chemical synthesis apparatus to create the radiopharmaceuticals.\textsuperscript{30, 32, 44} Furthermore, the technology does not allow measurements on the time scale of neuronal activation.

The traditional method for quantitatively measuring brain chemicals in animal research is microdialysis. The system includes a pump, catheter (probe) and a microvial. A probe with a diameter typically of 200-400 µm and 0.4 cm in length is placed in the brain of an intact animal (Figure 1.5).\textsuperscript{45} A thin dialysis tube with an inner diameter of 0.15-0.3 mm and a semipermeable membrane is at the tip of the probe.\textsuperscript{46} A perfusion fluid, typically artificial cerebral spinal fluid (aCSF), passes the membrane and is transported though the outlet tubing to be collected in the microvial (dialysate).\textsuperscript{46} During this process, molecules diffuse across the membrane down their concentration gradient and are collected over time in the dialysate. The dialysate is then taken for analyte identification with an offline technique such as high performance liquid chromatography (HPLC) to separate the molecules collected. This method offers high sensitivity, however, there are several disadvantages. For instance, the size of the probe is relatively large in comparison to the size of nerve terminals (nM) creating tissue damage upon implantation.\textsuperscript{47} The large size of the probe also results in spatial averaging across heterogeneous subregions of the brain. Because this is an equilibrium based technique, the typical time required to collect molecules results in one, static data point every 10 to 30 minutes.\textsuperscript{48}
1.5 Electrochemical Techniques Employed in Neuroscience

In contrast to offline or static analytical techniques, electrochemical measurements can be made in situ, providing a real-time approach for quantitative neurochemical monitoring. The advantages of these techniques stems from the micron dimensions of the electrodes utilized and high temporal resolution (sub-second measurements). The most commonly employed electrochemical techniques are amperometry and voltammetry.\textsuperscript{49} The main advantage within amperometry is the sub-millisecond temporal resolution. Within this technique, a constant potential is applied and current is measured vs. a reference electrode. Electrons are transferred at the electrode surface producing a measurable current, which is then plotted with respect to time in an amperogram. Information can be extracted from amperograms, such as charge. As shown in Equation 1.1, charge can be calculated by taking the integral of the current vs. time peak. This charge is directly proportional to the amount of species electrolyzed at the electrode surface utilizing Faraday’s law.
Equation 1.1. Faraday’s Law. Q is the total electric charge, n is the electrons transferred, N is the number of moles of analyte, and F is Farady’s constant (965000 C·mol⁻¹).

The primary disadvantage of amperometry is the lack of selectivity. All electroactive species oxidized or reduced at the applied static potential will add to the faradaic current measured. Thus, in most biological recording environments this technique is inherently non-selective and is therefore undesirable for measurements in complex media such as the mammalian brain. Alternative methods for *in situ* monitoring of molecules must be employed that enable real-time measurements with high spatiotemporal resolution, as well as selectivity.

Background subtracted fast-scan cyclic voltammetry (FSCV) has emerged as a primary alternative to microdialysis and amperometry for investigating electrochemically active species in the brain due to its combination of selectivity, sensitivity and temporal resolution. This approach provides a cyclic voltammogram where peaks at specific potentials of a voltammetric sweep can qualitatively identify electroactive species that oxidize and/or reduce within the potential window. With this standard approach, voltammograms are collected every 100 milliseconds providing the temporal resolution necessary to monitor changes on the time scale of neuronal signaling. FSCV also allows some analytes to preconcentrate to the electrode surface for enhanced sensitivity. With this approach, a triangular waveform is applied to the working electrode, resulting in measureable current. Importantly, this current collected is directly proportional to the concentration of species electrolyzed. For example, the DA waveform consists of a dynamic potential ramp in each scan, from -0.4 V to +1.3 V and back down to -0.4 V vs a Ag/AgCl reference electrode at a scan rate of 400 V/s (Figure 1.6). When DA is within the vicinity of the electrode during this application, DA is oxidized in a two electron redox reaction to form dopamine-ortho-quinone (DAQ) in the forward scan up to +1.3 V, and DAQ is reduced back to DA on the reverse scan (Figure 1.6).
Figure 1.6. Applied Triangular Waveform for DA Detection with FSCV. (A) Traditional triangular waveform employed for DA detection. (B) The waveform is applied for roughly 8.5 milliseconds and applied every 100 milliseconds at 400 V/s. (C) Reversible oxidation of DA to DAQ.

Due to the high scan rates employed in FSCV, a large non-faradic capacitive background current is generated from surface effects such as charging of the electrical double layer and adsorption and desorption processes at the electrode-solution interface. This large non-faradic current can obscure faradaic current, however, it is stable up to 60-90 seconds and can be subtracted out revealing the faradic current generated from the transfer of electrons at the electrode surface (Figure 1.7). Hence, this is a differential technique, ideal for monitoring sub-second dynamic changes in analyte concentration however it is not capable of quantifying absolute concentration of species in the brain.
Figure 1.7. Background-Subtraction for Cyclic Voltammogram Extraction. (A) Background current from electrode surface effects. (B) Background current plus faradaic current from analyte (DA). (C) Background-subtracted voltammogram solely from analyte (DA) oxidation and reduction reactions. The position and shape of the analyte’s anodic and/or cathodic peak allow for selectively identifying the analyte.

In order to visualize and interpret the large numbers of voltammograms collected (300 voltammograms for a single 30 second file), a three dimensional color plot is constructed. Each voltammogram is unfolded at the switching potential, and then lined up sequentially. The x-axis is collection time, the y-axis is potential applied to the electrode and the z-axis is current depicted in false-color (Figure 1.8). Color plots provide an easy way to interpret and visualize the data. Color plots allow the identification of analytes, the changes in concentration on a sub-second time resolution.
Figure 1.8. Data Visualization with Construction of a Color Plot. (A) Unfolding DA voltammogram at the switching potential. (B) Successively collected voltammograms that are unfolded at the switching potential to fabricate the color plot.

1.6 Microelectrodes

Microelectrodes offer various advantages for electrochemical measurements within complex biological environments due to the microscale dimensions of the electrodes. This generates a negligible ohmic drop across the electrolyte, allowing the use of a two electrode system (no auxiliary electrode required). The small size of the electrode allows for less tissue damage upon implantation and measurements in small discrete regions of the brain. This essentially eliminates the spatial averaging issue that is associated with measurements from larger probes typically utilized in microdialysis. Additionally, enhanced mass transport leads to higher
sensitivity. The lower amount of current detected allows for a faster scan rates to be employed, which can correspond to increase collected current for a given redox process.

Traditionally, electrochemists have used electrodes consisting of noble metals such as platinum as the sensing substrate. This provides an electocatalytic surface to facilitate electron transfer.\(^53\) However, there are several disadvantages associated with this sensing material for biological applications. For instance, the platinum surface is highly susceptible to biofouling when placed in a complex system. Biofouling can occur when proteins adsorb to the surface of the electrode causing less available sites for electron transfers to occur shifting the background signal. Thus, these probes are generally coated with polymeric membranes when used in tissue. This is disadvantageous because it can slow diffusion of analyte to the sensor surface.

Carbon materials have proven to be an ideal substrate for electrochemical measurements within biological environments.\(^54\)\(^-\)\(^56\) Carbon offers several advantages over traditional platinum microelectrodes for \textit{in vivo} applications (Figure 1.9). For instance, carbon itself is nontoxic, resists biofouling, is commercially available at a low cost, possesses a wide potential window and the electrochemistry is well characterized.\(^57\)\(^-\)\(^58\) Perhaps the most important feature of utilizing carbon as an electrode sensing substrate is the presence of oxygen containing functional groups on the surface to facilitate electron transfer.\(^58\)\(^-\)\(^60\)
Although a bare carbon fiber microelectrode is a powerful analytical tool for the detection of electroactive species, it is incapable of detecting molecules like glucose and lactate, essential energy sources of the brain, as they are nonelectroactive. However, biosensors can utilize clever strategies to enzymatically modify the carbon surface in order to detect non-electroactive species.\textsuperscript{5, 53-54, 61-62}

1.7 Biosensors

There have been several advances in the detection of both glucose and DA on a time scale that is commensurate of neuronal activation. Since the first discovery of the glucose electrode, biosensors have become one of the most active and clinically important areas of research.\textsuperscript{63} A biosensor is defined as a “self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative information using a biological recognition element retained in direct spatial contact with an electrochemical transduction element”.\textsuperscript{64-65} Several globally recognized laboratories focus on the detection of non-electroactive molecules such as glucose\textsuperscript{21, 60, 66-70}.
Since the glucose biosensor was developed by Clark and Lyons in 1962, the realm of biosensing has grown exponentially.\textsuperscript{71} This innovation entailed entrapping glucose oxidase over an oxygen electrode by a semipermeable dialysis membrane.\textsuperscript{71-72} The amount of non-electroactive glucose was determined by directly monitoring the enzyme-catalyzed reaction (Reaction Scheme 1.1).

\begin{align*}
\text{1A} & \quad \text{GOx}(\text{FAD}) + \text{glucose} \rightarrow \text{GOx}(\text{FADH}_2) + \text{gluconolactone} \\
\text{1B} & \quad \text{GOx}(\text{FADH}_2) + \text{O}_2 \rightarrow \text{GOx}(\text{FAD}) + \text{H}_2\text{O}_2 \\
\text{1C} & \quad \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}^+ + 2e^- 
\end{align*}

**Reaction Scheme 1.1.** Glucose Detection Reactions. Glucose with co-substrate molecular oxygen is synthesized to gluconic acid and hydrogen peroxide by means of glucose oxidase. (A) FAD works as the initial electron acceptor and is reduced to FADH\textsubscript{2}. (B) Then, FADH\textsubscript{2} is oxidized by the final electron acceptor, molecular oxygen (O\textsubscript{2}). O\textsubscript{2} will reduce to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) (C) Electrochemical detection occurs when a potential sufficient to oxidize H\textsubscript{2}O\textsubscript{2} is applied.

Inspired by Clark and Lyons, three generations of biosensors have developed in pursuit of the electrochemical detection of glucose. These are classified by the electron transfer pathways. The work presented herein focuses on first generation biosensing, dependent on the detection of the conjugate substrate or product of the biocatalytic reaction, in this case hydrogen peroxide.\textsuperscript{73} The following scheme describes the enzymatic generation of H\textsubscript{2}O\textsubscript{2} utilizing the glucose oxidase, though it should be noted this generation is applicable to other oxidase enzymes including lactate oxidase. Glucose oxidase is immobilized on the electrode surface and glucose reduces a flavin group (FAD) in the enzyme to FADH\textsubscript{2}. Then, reoxidation of the flavin group by molecular oxygen occurs to create the oxidized form of the enzyme GOx(FAD) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) (Reaction Scheme 1.2). Finally, the current from the oxidation of hydrogen peroxide is electrochemically detected. The concentration of H\textsubscript{2}O\textsubscript{2} is directly proportional to the concentration of glucose.
(Reaction Scheme 1.2), thus allowing glucose concentration to be determined by employing a calibration curve.

**Reaction Scheme 1.2.** Indirect Detection of Glucose via Detection of H$_2$O$_2$. Visual schematic of reaction scheme at the electrode/solution interface.

Second generation biosensors allow for electron transfer through mediators by artificial electron acceptors. These mediators shuttle electrons from redox center of enzyme to surface of the electrode. An advantage of second generation biosensors is the application of lower potential windows excluding undesirable faradaic current arising from interfering electroactive species. Disadvantages include instability, toxicity of the mediators employed, and competition with freely diffusing oxygen. In the case of third generation biosensors, electron transfer is directly from the redox center of the enzyme to the electrode surface. Theoretically this could be ideal, however the distance between the redox center and the electrode as well as electrode fouling, attenuates electron transfer. Furthermore, these biosensors are difficult to fabricate and utilize because of limitations arising from most enzymes.
1.8 Summary of Previous Research Conducted

Recently, our lab has fabricated a glucose oxidase-modified carbon-fiber microelectrodes (GOx EMEs) that enable detection of sub-second glucose fluctuations with unprecedented spatiotemporal resolution in discrete brain regions (Figure 1.10).\textsuperscript{75}

![Figure 1.10. Scanning Electron Micrograph of GOx EME.\textsuperscript{75}](image)

Typically, amperometry employs the use of several exclusion layers to inhibit additional signal from other interfering electroactive substances. This hinders diffusion of the targeted analyte increasing response time of the biosensor. These modifications do not allow identification or quantification of multiple species or interferents. Our novel biosensor is advantageous when compared to standard amperometric biosensors. The lack of exclusion membranes allows for faster response time. In conjunction with FSCV, we are able to detect and quantify multiple analytes and interferents within complex media, such as the brain. As show in Figure 1.11, the amperometric vs voltammetric response of this biosensor allows simultaneous identification and quantification of multiple analytes.
Figure 1.11. Comparison of FSCV and Amperometry. (A, B) Voltammetric data. Representative color plots (top panels) and respective analyte concentration vs time traces (middle panel) collected at a GOx EME in response to bolus injections (arrow) of (A) 1 mM glucose (black) or (B) a sample containing 1 mM glucose (black) in the presence of ascorbic acid (AA: 250 μM, blue). Analytes were distinguished and simultaneously quantified employing principal component regression, concentration vs time traces for both analytes were obtained. (C) Amperometric data. When the same solutions were interrogated with the microbiosensor held at a constant potential of +1.0 V, the two analytes could not be distinguished and the current from the oxidation of AA was evident in the signal (pink).  

This novel microbiosensor has been combined with FSCV to provide a completely new approach to biosensing. Figure 1.12 shows the voltammetric detection of glucose employing the GOx/chitosan modified carbon-fiber microelectrode (i.e. GOx EME). The waveform utilized ranges from +0.1 V to +1.4 V. These novel biosensors possess a sensitivity of 19.4 nA/mM and limit of detection of 13.1 ± 0.7 μM for glucose.
Figure 1.12. Voltammetric Detection of Glucose Employing the GOx/chitosan Modified Carbon-Fiber Microelectrodes. (A) Voltammetric waveform applied at a frequency of 10 Hz and a scan rate of 400 V/s, the voltammetric sweep was +0.1 V to +1.4 V ramped down to +0.1 V holding potential for glucose detection. (B) In vitro characterization. Background-subtracted voltammograms of glucose reporter molecule, H$_2$O$_2$. Inset, calibration plot showing physiological relevant glucose concentrations (n=5 electrodes). (C) Representative color plot of 15 seconds of successive voltammograms unfolded at the switching potential (+1.4V), where current is plotted in false color. (D) Chitosan does not significantly affect response time. Normalized response from a supra-physiological concentration of H$_2$O$_2$ on a bare carbon-fiber (black) and a GOx/chitosan modified carbon-fiber microelectrode (t(2)=0.67, p>0.05).

This dissertation explores the development and application of microbiosenors to optimize and enable quantification of multiple neurochemicals such as glucose, lactate and DA. Investigations presented herein assessed enzyme attachment strategies and the application of various triangular voltammetric waveforms for monitoring multiple neurochemicals, both electroactive and nonelectroactive. Moreover, this work examined the metabolic effects by monitoring glucose or lactate availability following neuronal activation elicited by cocaine administration or via electrical stimulation. Ultimately, these data will further our fundamental
knowledge of the chemical mechanisms that underlie neuroenergetics and advance the
development of therapies targeting disease states with energy regulation imbalances.
1.9 References


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CHAPTER 2 Quantitative Comparison of Enzyme Immobilization Strategies for Glucose Biosensing in Real-Time Using Fast-Scan Cyclic Voltammetry Coupled with Carbon-Fiber Microelectrodes


2.1 Introduction

Since the development of the glucose electrode in 1962 by Clark and Lyons,\textsuperscript{1} biosensors have been implemented in various fields of study including the food industry, environmental monitoring, and in clinical applications.\textsuperscript{2} Importantly, biosensors enable non-electroactive molecules, such as glucose, to be monitored with standard electrochemical methods. For example, glutamate,\textsuperscript{3-4} acetylcholine,\textsuperscript{5-6} lactate,\textsuperscript{7} and glucose,\textsuperscript{8-10} can be monitored via the generation of the electroactive mediator, hydrogen peroxide (H$_2$O$_2$). In the development of stable, sensitive, and reliable biosensors, the enzyme immobilization step is a crucial.\textsuperscript{11} As such, the biosensing field has continually explored enzyme immobilization conditions to optimize specificity, stability, and biosensor response time.\textsuperscript{12-13}

Amperometry is the electroanalytical detection scheme that is most commonly coupled with enzyme-based sensors for neurochemical monitoring.\textsuperscript{11} With this approach, a constant potential is applied to the electrode to drive redox processes that can be monitored with sub-millisecond temporal resolution. The measured current is directly proportional to the amount of species electrolyzed. However, all electroactive species that oxidize or reduce at the fixed potential add to the faradaic current, requiring additional strategies to limit the contribution from interfering analytes. These strategies often introduce limitations, such as slow response times due to the
incorporation of chemically selective coatings, or spatial averaging across recording sites. This has led to the investigation of other methods for in situ monitoring that enable real-time measurements with high spatiotemporal resolution, as well as selectivity.

Background-subtracted fast-scan cyclic voltammetry (FSCV) has recently emerged as an advantageous alternative for electroanalytical biosensing. This technique is most often coupled with carbon-fiber microelectrodes and used for selective quantification of fluctuating electroactive neurotransmitters, such as dopamine, in live brain tissue. It offers a combination of selectivity, sensitivity, and temporal resolution that has enabled unprecedented insight into biological processes that range from release and re-uptake kinetics to specific behavioral manifestations in awake subjects. Carbon-fiber microelectrodes are not as commonly used for biosensing as Pt electrodes; however, they offer several important advantages for electrochemical measurements in complex biological environments. The small size allows for minimal tissue damage and measurements in discrete regions of the brain. The carbon is nontoxic, resists biofouling, and is commercially available at a low cost. It boasts a wide potential window in aqueous solutions, and the presence of oxygen-containing functional groups on the carbon surface facilitates electron transfer. These carbon electrodes can be modified with glucose oxidase (GOx) to monitor glucose fluctuations in live brain tissue with sub-second temporal resolution. We have used this tool to simultaneously measure glucose and dopamine fluctuations at single recording sites in the rat striatum, directly demonstrating that glucose fluctuations are coupled with metabolic demand.

In the present work, we quantitatively compare three GOx immobilization strategies for the detection of glucose at carbon-fiber microelectrodes coupled with FSCV. Specifically, we assess the sensitivity and stability of glucose detection when the enzyme is physically adsorbed
onto the electrode surface and subsequently stabilized by a Nafion membrane, or when it is immobilized using entrapment in electrospun nanofibers or in a chitosan hydrogel, as previously established.\textsuperscript{8-9} The results demonstrate that entrapment of GOx in the chitosan hydrogel is most effective, in terms of sensitivity and stability, for monitoring physiological brain glucose concentrations in the rat brain. However, entrapment in electrospun nanofibers is advantageous for monitoring glucose at higher concentrations (> 3 mM). Importantly, the immobilization strategies characterized herein can be adapted to other enzymes, to expand the development of tools targeting a variety of non-electroactive target molecules.

\textbf{2.2 Results and Discussion}

\textbf{2.2.1 Three Methods of Enzyme Immobilization on a Carbon-Fiber Microelectrode.}

Three strategies for the immobilization of GOx on the surface of carbon-fiber microelectrodes were investigated; these are physical adsorption, hydrogel entrapment and entrapment in electrospun nanofibers. Figure 1 depicts a schematic overview of the fabrication procedure for each approach. Representative scanning electron micrographs (SEMs) of the finished GOx enzyme-modified electrodes are also included (scale bar 50 \(\mu m\)). Differences are easily observed at higher magnifications (inset images; scale bar 10 \(\mu m\)). For comparison, bare carbon-fiber microelectrodes exhibit clear and distinct vertical striations, as shown in Figure 2A. The white arrow highlights the transition from the glass insulation to the active carbon surface.
Figure 2.1. GOx immobilization strategies. (A) Immobilization via physical adsorption. Electrochemically pre-conditioned carbon-fiber microelectrodes are placed in GOx solution for 72 hours at 4°C. Bottom: representative scanning electron micrographs. (B) A chitosan hydrogel is created by applying a potential sufficient to reduce hydrogen in a 2% chitosan GOx solution. The representative SEM (bottom) depicts the membrane coating and a prolate spheroid electrode geometry. (c) Immobilization in electrospun nanofibers is achieved by dispensing a PVA GOx solution through a syringe (0.5 ml·hr⁻¹) with a 17.5 kV potential applied and manual rotation of the electrode to ensure an even coating.
Physical adsorption of GOx is fundamentally the most straightforward strategy evaluated. This approach leverages weak Van der Waal’s forces and electrostatic and/or hydrophobic interactions to immobilize the enzyme to the carbon sensing surface.\textsuperscript{2} This immobilization strategy has been widely utilized due to its simplicity, including at carbon-fiber microelectrodes.\textsuperscript{30} It is generally non-destructive to the enzyme, and boasts reduced production costs as compared to other immobilization methods.\textsuperscript{31-32} Carbon-fiber microelectrodes were electrochemically pre-conditioned by applying a triangular voltammetric waveform (−0.4 V to +1.4 V) for approximately 15 min at a frequency of 60 Hz, and then ~ 5 min at 10 Hz. Conditioned electrodes were soaked for 72 h in GOx solution at 4°C (Figure 1A; see Methods for details). Although this adsorption approach can theoretically work alone, our initial results were inconsistent (data not shown). Therefore, a Nafion film was adsorbed onto the enzyme-modified sensor to improve stability.\textsuperscript{33-34} Figure 1A depicts a scanning electron micrograph (SEM) of the finished electrode. The surface appears smooth, albeit with an uneven coating of material (inset).

The hydrogel entrapment strategy employs electrochemistry to control the solubility of chitosan, a low cost and nontoxic, natural polyaminosaccharide.\textsuperscript{35-36} Entrapment of GOx in a chitosan hydrogel immobilizes the enzyme at the pre-conditioned carbon surface without covalently binding the enzyme, as this has been shown to adversely impact enzyme activity.\textsuperscript{2} The immobilization strategy has been described previously,\textsuperscript{8-9} and is depicted in Figure 1B. Briefly, the conditioned electrode is submerged in mildly acidic chitosan solution (pH < 6.5) that contains GOx. Chitosan solubility is electrochemically controlled by the application of a negative potential sufficient to reduce hydrogen ions to hydrogen gas. In doing so, a steep pH gradient is generated immediately adjacent to the electrode surface. This initiates polymerization of the chitosan, affording entrapment of the GOx at the sensing surface. As demonstrated previously,\textsuperscript{8} the hydrogel
immobilization strategy creates a prolated spheroid or cotton swab-like electrode geometry (Figure 1B SEM).

Finally, electrospun poly(vinyl alcohol) (PVA) nanofibers were used to trap GOx at the carbon-fiber microelectrode surface. Electrospun nanofibers offer many advantages, including a high specific surface area\textsuperscript{13, 37} that enables effective mass transfer to the electrode surface, which is critical to achieve optimized biosensor response times.\textsuperscript{13, 38} PVA is a hydrophilic and biocompatible polymer that boasts excellent chemical and thermal stability.\textsuperscript{39-40} Enzyme immobilization is achieved by dispensing a PVA and GOx blend through a syringe (0.5 ml·hr\textsuperscript{-1}) with a 17.5 kV potential applied (Figure 1C). This produces ejection of a polymer solution from the syringe needle aimed at a collector plate. As the electric field increases, the droplet takes a conical shape known as a Taylor cone, and a liquid jet forms from the tip of the cone. The fluid is converted into a solid fiber as the electrified jet is continuously stretched, due to repulsions between surface charges and the evaporation of solvent.\textsuperscript{39, 41} The electrode is manually rotated in the electric field during electrospinning. Finally, hydroxyl groups on the PVA nanofibers (and the immobilized GOx itself) are crosslinked via glutaraldehyde vapor in an acidic environment.\textsuperscript{39} This renders the nanofibers insoluble in water, improves stability, and increases mechanical strength.\textsuperscript{42-43} Figure 1C includes a representative SEM of a completed, electrospun enzyme-modified carbon-fiber microelectrode, demonstrating randomly oriented, insoluble PVA nanofibers on the surface.

2.2.2 Fast-Scan Cyclic Voltammetry.

Background-subtracted fast-scan cyclic voltammetry (FSCV) can be employed to quantitatively characterize the performance of these microbiosensors. H\textsubscript{2}O\textsubscript{2} is readily detected with this approach using a bare carbon-fiber microelectrode,\textsuperscript{30} and this can be leveraged at microbiosensors to detect enzymatically-generated H\textsubscript{2}O\textsubscript{2} and to thus report on rapid changes in
glucose concentration. For the detection of H$_2$O$_2$, a triangular potential waveform (+0.1 V to +1.4 V) is applied to a carbon-fiber microelectrode at a frequency of 10 Hz, using a scan rate of 400 V·s$^{-1}$ (Figure 2A). During the application of potential, the current generated at the electrode surface is recorded. Representative recordings in the presence and absence of H$_2$O$_2$ are shown in Figure 2A (bottom left). To facilitate visualization of changes in the concentration of H$_2$O$_2$ at the electrode surface, a ‘background’ cyclic voltammogram collected prior to infusion of H$_2$O$_2$ is subtracted from subsequent voltammograms collected in the presence of H$_2$O$_2$ to generate background-subtracted voltammograms (Figure 2A, bottom right). These can be used to identify H$_2$O$_2$ by way of the single, well-defined oxidation peak near the switching potential (+1.4 V). Fluctuations in H$_2$O$_2$ concentration can be visualized over time using a color plot (Figure 2B, left), which provides an easy way to visualize and interpret the data. The x-axis contains information on collection time, the y-axis depicts the applied potential, and the z-axis (color) depicts the collected current. The magnitude of the H$_2$O$_2$ oxidation peak is correlated to concentration by way of standard calibration.
Figure 2.2. Fast-scan cyclic voltammetry for detection of $\text{H}_2\text{O}_2$. (A) A triangular waveform is applied with a scan rate of 400 V·s$^{-1}$ (top left). A large background current is generated (bottom left, gray). Additional faradaic current is generated at a bare carbon-fiber microelectrode (top right) in the presence of $\text{H}_2\text{O}_2$ (pink). Subtraction of these signals generates a background-subtracted cyclic voltammogram (CV), which serves to identify and quantify $\text{H}_2\text{O}_2$ (bottom right). (B) Sequentially collected CVs are plotted with time (sec) on the x-axis, potential (V) on the y-axis, and current (nA) in color.

Figure 3A contains representative voltammetric data collected for each of the electrode immobilization strategies in a benchtop flow-injection apparatus before and after a 2 sec bolus injection of glucose (1.6 mM). Note that differences in sensitivity to glucose necessitate the use of different current scales on these color plots. This is easily visualized in Figure 3B, which provides current (nA) vs time (sec) traces extracted from the corresponding color plots in Figure 3A. It is clear that microbiosensors fabricated using the physical adsorption method (blue) were less responsive to glucose than those fabricated using the chitosan hydrogel (red) or the electrospun nanofiber (green) entrapment strategies.
Figure 2.3. Glucose monitoring with FSCV at carbon microbiosensors created using physical adsorption of GOx (blue), hydrogel entrapment (red), and entrapment in electrospun nanofibers (green). (A) 30 sec of representative data demonstrating biosensor response to a 2 sec bolus of glucose (1.6 mM). Analyte identification is achieved by extracting voltammograms (inset) at the time indicated by the white dashed line. (B) Current vs time profiles extracted from the corresponding color plots shown in (A).

2.2.3 Sensitivity and Stability.

In order to quantitatively evaluate sensitivity to glucose, the sensors were calibrated in vitro using the flow-injection apparatus described above. After electrochemical conditioning (described in Methods), glucose calibration curves were constructed. Sensitivity to glucose is reported as mean ± standard error of the mean (SEM) (Figure 4 and Table 1). The sensitivity to glucose encompasses a large range (~0.4 – 15 nA·mM⁻¹ glucose). Analysis with one-way ANOVA reveals significant differences between the three enzyme immobilization strategies (F(2,8) =719, ****p<0.0001), with the highest sensitivity achieved using the chitosan hydrogel entrapment approach (Figure 4A,B). The extended calibration curve (Figure 4A) reveals that chitosan-immobilized enzyme activity was saturated at glucose concentrations greater than ~3 mM. However, these microbiosensors demonstrated a linear response to glucose over a physiological
Enzyme entrapment using electrospun nanofibers resulted in microbiosensors that were less sensitive to glucose when compared to the chitosan hydrogel probes; however, it is notable that this strategy is effective for the detection of an increased range of glucose concentrations (0.2 - 50 mM). Although not sufficiently sensitive for typical, physiological concentrations of brain glucose, this approach may be suited for detection of elevated brain glucose concentrations under pathophysiological conditions, in glucose monitoring applications in the periphery (blood), or in the food industry. Indeed, increased glucose concentrations have been reported in the peripheral nervous system (3.9 - 7.8 mM, adult rats).45

**Figure 2.4. Sensitivity to glucose.** (A) Calibration plots (0.2-50 mM), (B) Expanded view of more physiologically appropriate concentrations for glucose measurements in brain. (C) Sensitivities reported in terms of percent difference from the established hydrogel entrapment strategy for enzyme immobilization.
Microbiosensor stability was assessed over a 4 h period (Figure 5A), as this is approximately the length of a typical in vivo FSCV experiment. 2-sec bolus injections of glucose were delivered to the biosensor surface every 15 min (the linear-range median concentration for each immobilization strategy was selected). Normalized signals (mean ± SEM) were evaluated to assess biosensor stability across time. The color plots (Figure 5B) depict representative data collected with each of the three enzyme immobilization strategies investigated. Data are shown for the first (top) and last (bottom) glucose samples investigated over the recording session. Two-way ANOVA reveals significant differences between microbiosensors fabricated with the various immobilization strategies (F(2, 119) = 237.9, ***p<0.001). GOx immobilization by way of physical adsorption generates biosensors that are not stable, as ~42% of current is lost after the first injection (blue). Indeed, all responsivity to glucose is lost in the first 30 min of experimentation potentially due to the weak interactions between the enzyme and carbon surface. Microbiosensors fabricated with GOx immobilized in electrospun nanofibers are relatively stable, but they exhibit a ~75% decline in response relative to the first injection after 2.75 h of measurements (green). Finally, the chitosan hydrogel enzyme immobilization strategy generates a stable response across all time points (red), consistent with previously published results.8−9
2.2.4 Glucose Measurements in Live Brain Tissue.

Microbiosensors fabricated by entrapping GOx in electrospun PVA nanofibers (Figure 6), or in the chitosan hydrogel (Figure 7), were evaluated for performance in live brain tissue. Biosensors created by physical adsorption of the enzyme onto the carbon surface were not further evaluated, due to instability (Figure 5). Figure 6A presents scanning electron micrographs of an electrospun microbiosensor before (left) and after (right) placement in a rat brain slice containing the striatum (middle) for measurements of glucose dynamics in this *ex vivo* preparation. Placement in tissue did not appear to substantially displace the nanofibers; however, some swelling of the fibers is evident. The swelling of PVA fibers upon placement in an aqueous environment has been
documented in previous studies. Delivery of a single electrical pulse (450 µA) to the tissue elicited a measurable glucose signal, as well as dopamine release from local terminals in the striatum (Figure 6B). The color plot (left) depicts representative voltammetric data. Molecular dynamics are evident in the concentration vs time plot (right). These data demonstrate a dopamine release event (asterisk in color plot, blue trace) that coincides with the onset of a biphasic increase in extracellular glucose (triangle in color plot, red trace) that endures over at least 8 sec. Glucose delivery to the vicinity of the biosensor is not likely to be due to cerebral blood flow, due the nature of the brain slice preparation. However, astrocytes present in the brain slice contain glycogen and glucose transporter 1, providing a means by which glucose can be delivered to the extracellular space in response to an increase in metabolic demand. However, it is important to note that the amount of glucose monitored immediately after stimulation (~ 0.6 - 1 mM) is close to the theoretical limit of detection (LOD) for these microbiosensors (Figure 4).
Figure 2.6. Glucose measurements in a rat brain slice using an electrospun microbiosensor. (a) Scanning electron micrographs depicting the sensor surface before (left) and after (right) tissue implantation. The rat brain slice preparation is depicted in the middle panel. (b) Color plot of voltammetric data collected before and after delivery of a single electrical pulse (450 µA, red arrow). A voltammogram extracted at the time of the white vertical line is inset. Molecular concentration vs time profiles (right) extracted from these data (at the potentials indicated by the horizontal lines) provide dynamic information for both glucose and DA in the vicinity of the biosensor.

In a separate experiment, a microbiosensor fabricated by immobilizing GOx in a chitosan hydrogel was positioned in the dorsal striatum of an anesthetized, intact, male rat (experimental design depicted in Figure 7A). An intravenous infusion of saline (0.6 mL) was followed by electrical stimulation of midbrain neurons in the ventral tegmental area/substantia nigra region (lightning bolt with red arrow; 60 Hz, 120 pluses, 200 µA), and voltammetric data were recorded at the microbiosensor in the striatum. A representative color plot is presented in Figure 7B, left. Concentration vs time traces extracted from these data (white horizontal lines) reveal an increase in striatal glucose availability (triangle) immediately following dopamine release (asterisk),
consistent with previously published results. A voltammogram that serves to identify the analytes was extracted from the data at the time of the white, vertical dashed line (inset). The extracted concentration vs time traces are shown in the panel to the right. We speculate that dopamine release activated local cells in the striatum, increasing metabolic demand. This was accommodated by glucose influx to the region by some combination of an increase in cerebral blood flow and the release of glucose from glycogen reserves within astrocytes. A second (identical) electrical stimulation was delivered 15 min later, immediately following intravenous glucose administration (2.8 mM, Figure 7c). Again, the chitosan hydrogel microbiosensor afforded robust and reliable glucose monitoring. A larger increase in glucose concentration was evoked by this stimulation, suggesting that glucose availability was enhanced by intravenous administration of glucose. Importantly, the data clearly demonstrate that enzyme immobilization by entrapment in a chitosan hydrogel is an effective method for biosensor fabrication, measurements of brain glucose.
Figure 2.7. Glucose measurements collected in intact rat striatum using a chitosan hydrogel microbiosensor. A bolus of saline (B) or glucose (C) was administered intravenously. Extracellular glucose availability was monitored in response to electrical stimulation (60 Hz, 120 pulses, 200 µA, red arrow) of midbrain neurons projecting to the striatum. Triangle and asterisk indicate the voltammetric signal for glucose and dopamine, respectively. Voltammograms extracted from the data at the time of the vertical dashed line are provided in the inset. Molecular dynamics are plotted in the panels on the right.
2.3 Conclusion

This work quantitatively characterizes and compares three strategies of enzyme immobilization at the surface of a carbon-fiber microelectrode. Immobilization of GOx by physical adsorption generates a biosensor with poor sensitivity to glucose (Figure 4), and unstable performance (Figure 5). This is likely due to weak interactions between the enzyme and carbon surface. Thus, this approach is not suitable for extended glucose measurements. Entrapment of GOx in PVA nanofibers electrospun onto a carbon-fiber surface generates microbiosensors that are effective for glucose measurements over a large linear range (Figure 4), and these sensors exhibit stable performance over several hours (Figure 5). This approach may be particularly useful for measurements targeting glucose concentrations in excess of 3 mM, such as in the blood. Finally, entrapment of GOx in the chitosan hydrogel generates microbiosensors capable of sensitive glucose measurements over a 4 h period (Figure 4-5). Both the electrospun and chitosan hydrogel microbiosensors were capable of monitoring robust glucose signals in rat striatal tissue (Figure 6-7). However, the hydrogel immobilization strategy out-performed the other enzyme immobilization strategies, in terms of sensitivity and stability, for glucose measurements at physiologically relevant concentrations.

2.4 Experimental

2.4.1 Chemicals.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received, unless otherwise noted. *In vitro* electrochemical experiments were conducted in phosphate buffered saline solution (0.1 M PBS, containing 0.138 M NaCl and 0.0027 M KCl) at pH 7.4. Chitosan, β-D-Glucose, and GOx from *Aspergillus niger* were obtained from VWR International (West Chester, PA). The chitosan was created from shrimp shells and had an
approximate molecular weight of 190–375 kDa with a deacetylation percentage of ≥75% (practical grade). Glucose stock solution was prepared and underwent mutarotation for 24 h at room temperature. All aqueous solutions were made using doubly distilled water >18 MΩ·cm (Millipore Milli-Q, Billerica, MA).

2.4.2 Microelectrode fabrication.

Glass-insulated carbon-fiber microelectrodes were fabricated as described previously. Briefly, a single T-650 carbon fiber (7 μm diameter, Cytec Industries, West Patterson, NJ) was aspirated into a borosilicate glass capillary (0.6 × 0.4 mm or 1.0 × 0.5 mm, A-M Systems, Carlsburg, WA) and pulled using a micropipet puller (Narishige, Tokyo, Japan) to create two sealed microelectrodes. The exposed fiber was manually cut under a microscope to a desired length (∼100 μm). An electrical connection was established by inserting a stainless steel wire (Squires Electronics, Inc., Cornelius, OR) coated with conductive silver paint (GC Electronics, Rockford, IL) into the back of the microelectrode. Prior to deposition, carbon-fiber microelectrodes were electrochemically conditioned with a voltammetric waveform of −0.4 to +1.4 V at a scan rate of 400 V·sec⁻¹ for 15 min at 60 Hz, and then for 5 min at 10 Hz.

2.4.3 Physical adsorption.

Conditioned microelectrodes were submerged in an aqueous GOx solution (20 mg·mL⁻¹ GOx) for 72 h at 4°C. After a 15 min drying period at room temperature, GOx enzyme-modified electrodes were dip coated in a Nafion solution (D2020, Ion Power, DE) for 10 sec and allowed to dry for 30 sec. The Nafion dip coating process was repeated 3 times. The electrodes were stored at 4°C for 24 h prior to testing.
2.4.4 Hydrogel entrapment.

Conditioned electrodes were submerged in an aqueous solution containing 20 mg·mL⁻¹ GOx (specific activity: 100 U·mg⁻¹ at 37 °C) in 2% chitosan (pH ~ 5.3), as previously described.⁸⁻⁹ A potential of −3.0 V was applied for ~30 s using a DC power supply to electrodeposit a chitosan hydrogel encapsulating GOx at the electrode surface. GOx enzyme-modified electrodes were stored in PBS at 4 °C prior to use.

2.4.5 Electrospinning.

The electrospinning protocol was adapted from a previously published report.⁴³ Briefly, an aqueous 9.5 wt% PVA solution was prepared (Mowiol 40-88, average molecular weight 205 kg·mol⁻¹, 88% hydrolyzed) with 1X TRIS buffer and stored in the fridge 24 h prior to use. The PVA solution was combined with GOx (20 mg·mL⁻¹). The final PVA concentration in the blend was 7 wt.% loaded into a 10 mL syringe fitted with a stainless steel needle. A point-plate configuration was utilized to create the nanofibers with a flow rate of 0.5 mL·hr⁻¹, a 17.5 kV potential (Gamma High Voltage Research, D-ES-30PN/M692) and a collection distance of 15 cm between the tip of the needle and the ground collector plate. An electrochemically conditioned carbon-fiber microelectrode was placed in the line of nanofiber formation and manually rotated at 0.1 rotations per sec for 30 sec. The GOx/PVA nanofibers were chemically crosslinked by glutaraldehyde vapor. Electrodes were positioned for 15 min in a 100 mL container that held 11 mL of glutaraldehyde solution (50% wt) and 200 µL of 12.5 M hydrochloric acid,⁴³ then allowed to dry at room temperature for 15 min before they were stored at 4°C.

2.4.6 Electrochemical data acquisition.

All in vitro electrochemical data were collected in a custom flow-injection apparatus at room temperature. Microbiosensors were positioned in a custom electrochemical cell using a
micromanipulator (World Precision Instruments, Inc., Sarasota, FL), and supplied with a continuous flow of filtered PBS (0.5 mL·min⁻¹) using a syringe pump (New Era Pump Systems, Inc., Wantagh, NY). 2-sec bolus injections of analyte were introduced to the GOx microelectrode surface with a 6-port HPLC valve and pneumatic actuator (Valco Instruments Co., Inc., Houston, TX). The flow-injection apparatus was housed within a custom-built grounded Faraday cage.

Unless otherwise noted, a triangular voltammetric waveform ranging from + 0.1 V to + 1.4 V (vs Ag/AgCl) was applied at 400 V·s⁻¹ at a collection rate of 10 Hz using a custom instrument (Universal Electrochemistry Instrument, University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility). Each potential cycle had a duration of 6.5 ms, and the microelectrode was held at + 0.1 V between scans (Figure 2 A). TH-1 software (ESA, Chelmsford, MA) or HDCV software (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility) was used for waveform output with a DAC/ADC card (NI 6251 M). A second card (NI 6711) was used for triggering the DACs and ADCs and for synchronization of the electrochemical experiment with flow injection. Analog filtering was performed using a 4-pole Bessel filter, 2.5 KHz. Signal processing (background subtraction, signal averaging, and digital filtering) were software controlled.

2.4.7 Brain slice experiment.

All animal procedures followed the North Carolina State University & Institutional Animal Care and Use Committee (IACUC) guidelines. Male Sprague-Dawley rats (290 - 320 g, Charles River Laboratories, Raleigh, NC; n = 2) were deeply anesthetized with urethane (1.5 g·kg⁻¹, i.p.). Upon heavy sedation, the rats were decapitated and the brain was rapidly removed, mounted, and placed in cold artificial cerebral spinal fluid (aCSF). Utilizing a vibratome (World Precision Instruments, Sarasota, FL), 400 μm-thick coronal slices containing the striatum were obtained and
allowed to rest in buffer for at least 1 h. Brain slices were placed in a heated recording chamber (Warner Instruments, Hamden, CT) housed in a Faraday cage, and perfused with aCSF buffer maintained at 34 °C for at least 1 hour. Microbiosensor and stimulating electrodes were positioned about 100 μm below the surface of the slice with the aid of a microscope (Nikon Instruments, Inc., Melville, NY). To enable detection of dopamine and glucose in this preparation, data was collected using an ‘extended’ triangular waveform optimized for dopamine (-0.4 V - 1.4 V, 400 V·s⁻¹), applied at 10 Hz. Electrical stimulation of nerve terminals consisted of a single 450 μA biphasic pulse.

2.4.8 In vivo experiment.

Male Sprague-Dawley rats (290 - 320 g, Charles River Laboratories, Raleigh, NC; n =1) were anesthetized with urethane (1.5 g·mL⁻¹, i.p.) and body temperature was maintained at 37 °C with a heating pad. The animal was placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and a chitosan hydrogel microbiosensor was placed in the caudate putamen (CPu, +1.2 mm AP, 1.5 mm ML, and −5.0 to −7.5 mm DV, relative to bregma). A Ag/AgCl reference electrode was inserted in the contralateral forebrain. A bipolar stimulating electrode was placed in the area of the substantia nigra / ventral tegmental area in the midbrain (−5.8 mm AP, 1.0 mm ML, and −8.1 mm DV, relative to bregma). Infusion volume was 0.6 mL, manually injected. Data were collected with the triangular waveform (0.1V – 1.4V, 400·s⁻¹) applied at 10 Hz, before and after electrical stimulation consisting of 120 pulses (200 μA) applied at 60 Hz, using a pulse width of 2 ms.

2.4.9 Data Analysis and Statistics.

HDCV Analysis software (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility) was used for most aspects of data analysis. The limit of detection
(LOD) is defined as three times the standard deviation of the noise. All data are shown as the mean ± standard error of the mean (SEM). Statistical and graphical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). As appropriate, One-Way and Two-Way Analysis of Variance (ANOVA) were used to determine statistical differences. Significance was designated at 0.05.

2.5 Acknowledgements

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2.6 References


CHAPTER 3 Simultaneous Voltammetric Measurements of Glucose and Dopamine

Demonstrate the Coupling of Glucose Availability with Increased Metabolic Demand in the Rat Striatum


3.1 Introduction

Neuronal activation imposes a significant energetic demand that is fueled, at least in part, by glucose\textsuperscript{1-6}. Cerebral blood flow (CBF) delivers glucose to brain tissue in a process that is thought to be coupled to local metabolic rate\textsuperscript{7-10}. Over 100 years ago, Roy and Sherrington reported that the vascular supply of the brain responds to local variations in functional activity\textsuperscript{11}. Since then, many studies have described the functional recruitment of capillaries to accommodate an increased energetic demand during neuronal activation\textsuperscript{12-13}. However, little is known about how glucose availability changes on a second-by-second basis at discrete locations during neuronal activation \textit{in vivo}, largely due to a paucity of quantitative methods with sufficient spatial and temporal resolution to quantify this species on a timescale that is commensurate with that of neuronal firing. Identifying precisely how glucose availability correlates with metabolic demand is a critical piece to understanding brain function.

Actions of opiates, alcohol, and major stimulants, such as cocaine, converge on the dopaminergic systems in the brain to modify synaptic activity and, ultimately, behavior\textsuperscript{14}. The neurotransmitter dopamine (DA) is critically involved in cognition, sensorimotor integration, and modulation of motivated behaviors\textsuperscript{15-22}. Importantly, dopaminergic input to the striatum modulates basal ganglia output\textsuperscript{23}; however, little is known about the subsecond glucose dynamics that fuel these processes, or how they are changed upon exposure to drugs of abuse. Attempts to determine
how cocaine affects brain energy metabolism using positron emission tomography (PET), functional magnetic resonance imaging (fMRI), and high-field magnetic resonance spectroscopy have provided conflicting results\textsuperscript{24-28}. Broadly, imaging techniques selectively detect signals directly related to energy delivery and metabolism with a largely non-invasive method. Nonetheless, these approaches yield static information. Further, attempts to obtain quantitative information within discrete brain regions with these techniques truly emphasizes critical issues with using data without sufficient spatio-temporal resolution to infer chemical function\textsuperscript{29-31}. Certainly the response to cocaine is dynamic and regionally specific, confounding the issue.

Electrochemical approaches to molecular monitoring are ideal for the detection of neurochemical dynamics \textit{in situ} due to high spatial resolution and fast temporal response. Electrodes modified with glucose oxidase (GOx) are often coupled with amperometry for the detection of glucose \textit{in situ}\textsuperscript{32-33}. Although glucose is not electroactive, GOx on the electrode surface generates glucono-1,5-lactone and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) in the presence of glucose and molecular oxygen (as a cofactor). The H\textsubscript{2}O\textsubscript{2} serves as the electroactive reporter molecule for glucose. However, amperometry is a non-selective technique – any substances that can oxidize at an applied potential will contribute to the current detected. Thus, it is necessary to exclude electroactive species by incorporating chemically selective polymeric layers to the electrode surface\textsuperscript{34-35}. It is also common to incorporate separate ‘sentinel’ electrodes that are identical to the GOx recording site but lack the enzyme, thus responding to all interferents but not to glucose\textsuperscript{36-37}. With this approach, the difference between the two recording sites is used to report on glucose concentrations. These strategies effectively eliminate interferents, but introduce confounds associated with spatial averaging, as the chemical nature of one brain site cannot be assumed to be identical to that at another site\textsuperscript{38}. Additionally, and perhaps more importantly, they also preclude
the simultaneous detection of multiple analytes, as additional species are physically filtered from the recording site, or detected but subtracted from the signal.

An alternative strategy has recently been developed by our laboratory that couples GOx enzyme modified carbon-fiber microelectrodes (GOx EME) with background-subtracted fast-scan cyclic voltammetry (FSCV) for the detection of glucose with subsecond temporal resolution in live brain tissue\textsuperscript{39}. FSCV has been a transformative approach for quantitatively monitoring DA and other electroactive neurochemicals in brain tissue\textsuperscript{38, 40-44}, as cyclic voltammograms serve to identify individual components of complex samples based on their electrochemical properties without requiring strategies to exclude other analytes. By combining FSCV with GOx EME, the electrochemical selectivity inherent to voltammetry enables the simultaneous detection of glucose and electroactive analytes, provided that the voltammetric response for each species is unique.

The current work describes the systematic characterization and optimization of a voltammetric waveform for the reliable detection of glucose and DA at single recording sites using GOx EMEs. The optimized approach is used to simultaneously monitor both analytes in the dorsal striatum of anesthetized rats in response to electrical stimulation of the midbrain, or intravenous administration of cocaine and raclopride. The real-time measurements directly demonstrate the coupling of glucose availability with increased metabolic demand, and shed new light on the metabolic effects of augmented DA concentrations. Overall, this work provides a foundation that will enable detailed investigation of mechanisms that regulate the coupling of glucose influx with terminal activation under normal conditions, and in studies of drug abuse and addiction.
3.2 Results and Discussion

3.2.1 Immunohistochemistry Identifies Glucose Delivery Sites Proximal to Dopaminergic Axonal Fibers in the Striatum.

Immunohistochemistry was employed to evaluate the spatial distribution of vasculature, astrocytes, and dopaminergic axonal projections within striatal brain tissue. In Figure 1, dopaminergic axonal fibers (green; visualized with rabbit anti-tyrosine hydroxylase (TH) labeled with goat anti-rabbit Alexa Fluor 488) innervate the dorsal striatum. Here, the vasculature (red; visualized with biotinylated solanum tuberosum lectin labeled with Dylight 550 conjugated streptavidin protein) can carry CBF to deliver resources into the region and remove CO$_2$. Energetic support can also be provided by way of astrocytes (blue; visualized with mouse anti-glial fibrillary acidic protein (GFAP) labeled with goat anti-mouse Alexa Fluor 633) which encapsulate and surround the blood vessels. These supportive cells can act independently or via networks to shuttle glucose to neurons$^{45-46}$. Figure 1 visually describes the complexity of the recording microenvironment in striatal tissue. Both the vasculature (red; lectin label) and astrocytes (blue; GFAP) are positioned to deliver glucose and other nutrients to dopaminergic fibers (green; TH label, see sites at yellow and white arrows, respectively), or to the targets of striatal DA release (i.e., medium spiny neurons (MSNs), not shown).
Figure 3.1. Triple-Fluorescence labeling in the rat dorsal striatum. Confocal laser image (275 x 275 µm) taken from a 40 µm-thick tissue slice. Sites where dopaminergic axonal fibers (green; TH label) interact with blood vessels (red; lectin label) and/or astrocytes (blue; GFAP label) are indicated at the triangles (yellow and white, respectively). A scaled schematic of a GOx EME is superimposed. Scale bar is 50 µm.

3.2.2 An Optimized Voltammetric Waveform for the Simultaneous Detection of Glucose and Dopamine.

Recent developments in FSCV have enabled carbon-fiber microbiosensors to be used for the detection of rapid glucose fluctuations in the rodent brain with unprecedented temporal and spatial resolution\textsuperscript{39}. The previous work used a triangular waveform from +0.1 V to 1.4 V with a scan rate of 400 V/s to selectively monitor glucose concentration changes \textit{in vivo}, without requiring complicated spatial subtraction schemes or chemically selective coatings. Briefly, the oxidized
form of the GOx co-factor, flavin adenine dinucleotide (FAD), is reduced as glucose is oxidized to gluconolactone, generating \( \text{H}_2\text{O}_2 \). The electrochemical oxidation of \( \text{H}_2\text{O}_2 \) at +1.4 V generates \( \text{O}_2 \), 2 protons and 2 electrons, and is measured as a change in current\(^{47} \). The resulting voltammogram identifies the \( \text{H}_2\text{O}_2 \), even in the presence of interferents, enabling it to serve as a reporter molecule for the presence of glucose. However, this voltammetric approach was optimized for sensitivity to glucose, and did not allow for the simultaneous detection of physiologically relevant concentrations of DA (below limit of detection). Figure 2 depicts the voltammetric response to DA, \( \text{H}_2\text{O}_2 \), and glucose using a bare carbon-fiber microelectrode (CFME) and a GOx EME with the previously published waveform. Representative data are depicted in color plots with corresponding background-subtracted cyclic voltammograms (CVs) shown as insets. Color plots are a simplified display of many background-subtracted CVs that enable identification of the analytes present by demonstrating the current collected at all potentials. The applied potential is plotted on the y-axis (V), time on the x-axis (sec), and current is depicted in false color (nA). Here, each color plot contains 300 voltammograms. The CFME (shown schematically in Figure 2A, left) detects electroactive analytes (Figure 2A, middle), such as DA (1000 nM; oxidation at \(~+0.6\) V indicated by asterisk) combined with a supraphysiological concentration of \( \text{H}_2\text{O}_2 \) (50 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \); oxidation evident at +1.2 V on the reverse scan, indicated by triangle). However, no electrochemical signal was recorded upon delivery of 1.4 mM glucose to the CFME surface (Figure 2, right). In contrast, the GOx EME (shown schematically in Figure 2B left) was unable to detect the same concentrations of DA and \( \text{H}_2\text{O}_2 \) (Figure 2B, middle), but glucose was readily detected (Figure 2B, right). Thus, an approach to permit the simultaneous detection of physiological concentrations of both glucose and DA was sought.
Figure 3.2. Voltammetric response of carbon-fiber microsensors to Glucose, Dopamine and H$_2$O$_2$. (A) Left: Schematic representation of a bare CFME. Representative color plots depict current collected in response to the same solutions using a voltammetric waveform from +0.1 V to +1.4 V$^{39}$. A mixture of 1000 nM DA (asterisk) and 50 µM H$_2$O$_2$ (triangle) is detected (middle), but the detection of non-electroactive glucose (0.8 mM) is precluded (right). (B) Left: Schematic of the GOx EME. Right: Representative color plots demonstrate the detection of glucose (triangle, indicated by the oxidation of enzymatically generated H$_2$O$_2$). However, there is insufficient sensitivity for the detection of physiologically relevant concentrations of H$_2$O$_2$ and DA (middle panel).

A series of electrochemical waveforms was systematically characterized for the simultaneous detection of glucose and DA using the GOx EME. Triangular waveforms with holding potentials ranging from +0.1 V to -0.4 V were investigated (shown schematically in Figure 3A). GOx electrodes are most sensitive to glucose when a mildly positive holding potential is employed (+ 0.1 V). Increasingly negative potentials were explored in an attempt to pre-concentrate positively charged DA molecules at the electrode surface and thus boost sensitivity. The switching potential was held constant, as the oxidation of H$_2$O$_2$ occurs at ~+1.4 V (but is evident at ~1.2 V on the return scan as a result of filtering)$^{47-48}$. Calibration curves were constructed for physiologically relevant concentrations of glucose (Figure 3B; 0.2 - 2.0 mM) and DA (Figure
3C; 250 – 1000 nM) using each waveform. Values are reported as the average ± standard error of the mean (SEM; n=5 electrodes). The linear calibration curves demonstrate that sensitivity to both analytes is dependent on holding potential. One-way ANOVA revealed that using waveforms with negative holding potentials resulted in significant changes in sensitivity to glucose and DA when compared to the previously employed +0.1 V holding potential (glucose: $F(4,20)=37.28$, ***p<0.001 and DA: $F(4,20)=70.23$, ***p<0.001). Figure 3D plots the current collected in response to an injection of 2.0 mM glucose and 1000 nM DA using each of the waveforms, illustrating that sensitivity to DA improves as the holding potential decreases, but at the expense of sensitivity to glucose. Table 1 reports the sensitivities to both analytes and quantifies the percent difference from the previously characterized +0.1 V holding potential. Compared to original approach with the GOx EME, there was an increase in sensitivity to DA detection with negative holding potentials as positively charged DA can pre-concentrate at the sensing surface between voltammetric scans. We speculate a possible explanation for this decrease in glucose sensitivity lies with the net charge of GOx within physiological relevant pH buffer (7.4). The isoelectric point (IP) of GOx is 4.2, and when placed in a pH environment great than the IP, the GOx carries a net negative charge indicating conformational changes. These changes can lower GOx activity and thus decrease sensitivity with more negative holding potentials. However, as the data collected were reliably and reproducibly detected glucose, any compromise did not hinder the overall ability to monitor. Despite this inverse relationship, Figure 3D displays the -0.2 V holding potential affords optimal sensitivity for both glucose and DA within physiological concentrations and therefore was chosen as the holding potential for the optimal voltammetric waveform.
Figure 3.3. Systematic characterization of holding potential. (A) Investigation of a series of waveforms for the simultaneous voltammetric detection of glucose and DA with a GOx EME. (B) Glucose (0.2-2.0 mM) and (C) DA (250-1000 nM) calibration plots collected with the waveforms shown in (A). (D) The current collected in response to a sample containing 2.0 mM glucose and 1000 nM DA (note respective scales on left and right y-axes) is dependent on the holding potential applied between voltammetric scans. A -0.2 V holding potential provides a compromise that enables adequate detection of both analytes. One-way ANOVA analysis demonstrated all holding potentials were significantly different than the previously employed +0.1 V holding potential (glucose: $F(4,20)=37.28$, ***$p<0.001$ and DA: $F(4,20)=70.23$, ***$p<0.001$).
Table 1. Sensitivity to glucose and DA. Data were collected using a series of triangular waveforms coupled to a GOx EME, and are plotted in Figures 2B and C. Bonferroni post hoc comparisons revealed significant differences compared to a +0.1 V holding potential (*p<0.05, **p<0.01, ***p<0.001).

<table>
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<tr>
<th>Holding Potential</th>
<th>Glucose Sensitivity (nA/mM)</th>
<th>Glucose r²</th>
<th>Glucose % Difference vs. +0.1 V</th>
<th>DA Sensitivity (nA/μM)</th>
<th>DA r²</th>
<th>DA % Difference vs. +0.1 V</th>
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</thead>
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<td>+ 0.1 V</td>
<td>14.8 ± 0.4</td>
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<td>0</td>
<td>2.1 ± 0.2</td>
<td>0.98</td>
<td>0</td>
</tr>
<tr>
<td>-0.1 V</td>
<td>12.0 ± 0.3</td>
<td>0.99</td>
<td>-18.8 **</td>
<td>5.0 ± 0.4</td>
<td>0.98</td>
<td>138.1 *</td>
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<tr>
<td>-0.2 V</td>
<td>9.6 ± 0.5</td>
<td>0.99</td>
<td>-35.1 ***</td>
<td>5.8 ± 0.6</td>
<td>0.97</td>
<td>176.2 **</td>
</tr>
<tr>
<td>-0.3 V</td>
<td>7.3 ± 0.6</td>
<td>0.98</td>
<td>-50.7 ***</td>
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<td>0.96</td>
<td>242.9 ***</td>
</tr>
<tr>
<td>-0.4 V</td>
<td>4.7 ± 0.4</td>
<td>0.98</td>
<td>-68.2 ***</td>
<td>10.6 ± 0.5</td>
<td>0.99</td>
<td>404.8 ***</td>
</tr>
</tbody>
</table>

3.2.3 Stability.

The stability of a GOx EME coupled with this waveform was investigated over a four hour time window (Figure 4). 2 sec bolus injections of a solution containing glucose (0.8 mM) and DA (750 nM) were repeatedly introduced to the surface of the GOx EME every 15 min for four hours (typical time period of in vivo experiments), and stability to both analytes was assessed. Figure 4 demonstrates that the normalized current (mean ± SEM) collected in response to both analytes was stable across all time points relative to that collected in the first injection (n=4 electrodes, glucose: $F(16,48)=0.27$, p>0.05 and DA: $F(16,48)=0.60$, p>0.05). The color plots shown in Figure 4B provide representative data collected for the first and last samples analyzed at a single microbiosensor. The oxidation of DA can be identified at ~+0.6 V (asterisk), and the oxidation of H₂O₂ enzymatically generated in the detection of glucose is depicted with a triangle. CVs that identify these analytes (inset) were extracted at the time indicated by the dashed vertical line.
Figure 3.4. **Microbiosensor stability.** (A) Normalized current collected in response to repeated bolus injections of a solution containing glucose (0.8 mM) and DA (750 nM). The peak current remained stable for glucose \((F(16,48)=0.27, p>0.05)\) and DA \((F(16,48)=0.60, p>0.05)\) over 4 hours. (B) Representative color plots for the first (left) vs last (right) injection. Analyte identification (glucose: triangle, DA: asterisk) is achieved using CVs extracted from the color plot at the time indicated by the vertical dashed line (inset).

### 3.2.4 Local Glucose Availability Increases in Response to Metabolic Demand.

GOx EMEs were implanted in the striatum of adult, male, sprague-dawley anesthetized rats \((n=4)\) to simultaneously record endogenous DA and glucose fluctuations at discrete recording sites. Electrical stimulation of the midbrain \((60 \text{ Hz}, 100-125 \text{ pulses}, 200 \mu\text{A})\) elicited striatal DA release \((396 \pm 29 \text{ nM})\). Representative data are shown in Figure 5A (left, asterisk). Electrically-elicited striatal DA release has been shown to correlate with local neural activity in subsets of MSNs\(^{49-50}\). There is a significant energetic burden associated with the generation of action potentials, as well as in the regeneration of ionic gradients and resting potentials following synaptic events\(^{2-4}\). Accordingly, DA release was followed by an increase in the local glucose concentration.
(350 ± 23 μM, n=4 animals). Representative data are shown in Figure 5A (left, triangle). The voltammograms for these analytes agree with those collected in vitro (Figure 5A, right). Importantly, when a sample containing both glucose (triangle) and DA (asterisk) is detected in vitro, the electrochemical signal for glucose is generated before the DA signal (Figure 5B, right). H₂O₂ is enzymatically generated at the electrode surface; however, DA must diffuse through the chitosan hydrogel for detection. By contrast, (Figure 5B, left) after the electrical in the rat striatum stimulation (red arrow), the local glucose concentration (red) peaks 2.7 ± 0.2 sec after the DA release event (blue), demonstrating that glucose availability responds to meet metabolic demand. The energetic support likely fuels local MSN neuronal activation⁷⁻⁵¹, and also provides energy to other local targets including interneurons and glial cells. The combined electrochemical detection of glucose and DA at single recording sites is a powerful tool that can be used to quantify local mechanisms that regulate glucose influx in response to terminal activation. Indeed, identifying these processes is critical to understanding brain function under control conditions as well as in response to pharmacological challenge.
Figure 3.5. Comparison of *in vivo* vs *in vitro* chemical dynamics. Representative data in the form of (A) color and (B) concentration vs time plots provide a visual comparison of the dynamics of glucose and DA detection. Left: When recording in the striatum of the anesthetized rat, electrical stimulation of the midbrain (60 Hz, 100-125 pulses, 200 µA, indicated by lightning bolt and large red arrow) elicited DA release (asterisk) followed by an increase in extracellular glucose (triangle). Right: In comparison, glucose is detected prior to DA at this microbiosensor after 2 second injection bolus *in vitro*. These data demonstrate that *in vivo*, glucose availability responds to meet metabolic demand in the striatum following electrical stimulation of the midbrain.

3.2.5 Cocaine/ Raclopride Induced Fluctuations in Striatal Glucose.

The acute actions of opiates, alcohol, and major stimulants such as cocaine converge on the DA circuits to modify neurochemistry and behavior\(^{52-53}\). Certainly the administration of a drug like cocaine involves DA release in the striatum and activation of reward-associated circuits\(^{14}\), but the metabolic effects of cocaine administration have proven difficult to deconvolute. There are few direct measurements of fluctuations in extracellular glucose in the brain (but see\(^{33}\)), and the literature contains conflicting results\(^{24, 26-28, 54}\). Furthermore, imaging studies in human subjects are typically completed during a state of drug withdrawal or craving, as there are ethical concerns for studying an acute state, particularly with illicit substances such as cocaine. In order to directly
investigate how glucose availability correlates with augmented extracellular DA concentrations at discrete recording sites in the dorsal striatum, extracellular glucose and DA dynamics were simultaneously recorded using the optimized waveform. Measurements were made before and after an intravenous (i.v.) infusion of a cocktail containing cocaine (DA transporter inhibitor) and raclopride (D2 receptor antagonist), (COC/RAC), and upon electrical stimulation. This treatment has been shown to significantly increase striatal DA release in rat striatum\textsuperscript{55-56}.

GOx EMEs were placed in the dorsal striatum of anesthetized rats (n=4) and electrochemical data were recorded for 60 min after a systemic (i.v.) saline infusion. During this period, the midbrain was electrically stimulated after one min and every 15 min thereafter. The experimental procedure was repeated with an infusion of COC/RAC (2 mg/kg COC + 1 mg/kg RAC). At all time points, electrical stimulation evoked striatal DA release followed by local glucose influx. Representative data collected during the first electrical stimulation (lightning bolt) are shown in Figure 6, collected ~ 1 min after infusion of (A) saline or (B) COC/RAC. Spontaneous fluctuations in extracellular glucose concentration are evident, and glucose availability clearly increased in response to stimulation, as shown in the color plots (top panels) and in the corresponding concentration vs time traces (bottom panels). After saline administration, the amplitude of glucose influx was significantly increased by electrical stimulation, as compared to preceding spontaneous glucose signals (Figure 6C, light gray vs dark grey, two-way ANOVA: $F(3,24)=338.6$, ***p<0.001). This was also true after COC/RAC administration, Figure 6C, light red vs dark red, main effect of type, two-way ANOVA: $F(3,24)=7.4$, *p<0.05); however, statistical analysis failed to reveal any type X time interactions regardless of treatment. Glucose availability rapidly increased to meet metabolic demand in response to COC/RAC administration (Figure 6C). Both spontaneous (light gray vs light red, two-way ANOVA: $F(3,24)=36.5$ ***p<0.001), and
electrically evoked (dark gray vs dark red, two-way ANOVA: \(F(3,24)=5.6\) *p<0.05) glucose signals were significantly increased when compared to saline. COC/RAC-administration also elicited massive DA release in the dorsal striatum, consistent with prior reports of this treatment augmenting phasic DA release in the ventral striatum of anesthetized rats\(^{55}\). Electrically evoked DA release was significantly augmented at both 1 and 15 min after COC/RAC administration, as compared to saline (Figure 6D, two-way ANOVA, treatment X time: \(F(3,24)=10.1\), Bonferroni post hoc comparisons; ****p<0.0001 and **p<0.01 respectively). This drug-induced effect of intravenous cocaine is in accord with the involvement of the dorsal striatum in reward and addiction\(^{57}\). Importantly, striatal DA release has been shown to differentially activate subpopulations of striatal neurons\(^{49,58}\), and cocaine is well known to elicit neural activity in the region\(^{59-60}\). Overall, these data demonstrate that glucose influx follows electrically stimulated and pharmacologically evoked DA release, likely in response to metabolic demand within the dorsal striatum.
Figure 3.6. Glucose availability is coupled with metabolic demand. Top: Representative color plots collected after i.v. infusion of (A) saline or (B) COC/RAC. The time of electrical stimulation (60 Hz, 100-125 biphasic pulses, 200 µA) of the midbrain is indicated by the lightning bolt and red arrow. Bottom: Electrical stimulation evoked striatal DA release (blue) followed by local glucose influx (red), as evident in the concentration vs time profiles. (C) After saline infusion, electrical stimulation of the midbrain increase glucose influx to the recording site (dark gray v light gray), at all time points (**p<0.001). This trend held after treatment with COC/RAC (light red vs dark red, stats *p <0.05). COC/RAC treatment augmented spontaneous glucose influx to the sampling site (light gray vs light red, ***p<0.001). Electrical stimulation after COC/RAC further increased extracellular glucose concentrations, as compared to electrically evoked glucose influx after saline administration (dark gray vs dark red, *p<0.05). (D) COC/RAC treatment significantly increased electrically evoked DA release at the 1 and 15 min time points when compared to saline (Bonferroni post hoc ****p<0.001, **p<0.01 respectively).
Extracellular glucose fuels many critical aspects of brain function, including (but not limited to) the generation of ion gradients to establish postsynaptic potentials in response to synaptic activity, and the restoration of resting membrane potential (for review see 12). At any given moment, the extracellular glucose concentration is dependent on two opposing forces; glucose availability and metabolism. Increases in CBF and glucose consumption have both been correlated with an increase in metabolic demand upon neuronal activation in animal and human studies8, 10, 12, 61, and the physiological mechanisms that govern this have been reviewed elsewhere12. Wightman and colleagues have shown that electrical stimulation of the dopaminergic cell bodies in the midbrain elicits CBF in the striatum, as indicated by changes in extracellular O₂ and pH that are evident immediately following striatal DA release62. A combined electrochemistry/electrophysiology study has also shown that DA activates specific sub-populations of striatal targets49. Separate studies conducted in rats have shown that cocaine administration consistently increases CBF 63-65, and tissue oxygenation 65 in many brain regions, including the striatum. Furthermore, cocaine reward has been shown to increase firing in both the dorsal and ventral striatum of non-human primates60. The simultaneous measurements of glucose and DA at a single recording site reported herein are the first of their kind. They directly demonstrate that glucose availability increases following local DA release, whether evoked by electrical stimulation or COC/RAC administration (Figure 6), to meet energetic demand.

It should be noted the research conducted herein provides one perspective of the complex mechanisms that regulate neuroenergetics. We have focused this study was to provide a tool to monitor dynamic glucose and DA fluctuations that occur in response to increased metabolic demand. In such, this data serves to inform investigations of glucose’s role in the dopaminergic terminal region and broadly, the implicated molecular functions that require energy sources such
as glucose. However, it is important to note recent research has demonstrated lactate is a potential source of ATP to fuel cellular function and this certainly cannot be disregarded as a contributing energetic substrate under specific experimental conditions \(^1, 32, 46, 66-67\), despite considerable debate in the field\(^{66}\). Indeed, there are several aspects that regulate energy for essential brain function; astrocytes, CBF, lactate, oxygen, glucose, utilization, neuronal activation, etc. Ideally, future studies will be able to implement this data within a comprehensive framework of neuroenergetics including a variety of energy substrates and sources, to better understand the role of in situ, sub-second glucose fluctuations following neural activation.

3.3 Conclusion

To date, neurochemical studies have largely focused on monitoring single analytes at a time. However, the brain is a dynamic environment of many neurochemicals working together to generate complex physiological effects. Herein, we present the first simultaneous voltammetric detection of glucose and DA at a single recording site in the rat striatum. These distinct species – one electroactive, and one non-electroactive – both rapidly fluctuate in the dorsal striatum in response to electrical stimulation of the midbrain, or acute COC/RAC administration. Many of the acute reinforcing effects of abused drugs, including cocaine, have been predominantly attributed increases in extracellular levels of DA in the striatum. However, the effects of striatal DA release on local glucose availability, and the physiological effects of this modulation, remain unclear. The methodology introduced herein can be used in a wide range of future studies to evaluate how striatal function is correlated with glucose availability at spatially discrete recording sites in brain nuclei associated with addiction circuits, with subsecond temporal resolution. Such measurements can help clarify the fundamental physiological response that occurs with cocaine exposure, and thus ultimately inform improved therapeutic strategies to treat cocaine abuse.
3.4 Methods

3.4.1 Chemicals.

All chemicals were purchased and used as received from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. In vitro electrochemical experiments were carried out in phosphate buffered saline (0.1 M PBS, containing 0.138 M NaCl and 0.0027 M KCl) at physiological pH 7.4. β-D-glucose, chitosan, and GOx from Aspergillus niger were acquired from VWR International (West Chester, PA). The chitosan (from shrimp shells) had a deacetylation percentage of ≥75% and an approximate molecular weight of 190 000-375 000 Da (practical grade). Glucose stock solution was prepared and allowed to undergo mutarotation at room temperature for at least 24 hours. All aqueous solutions were made using doubly distilled water >18 MΩ·cm (Millipore Milli-Q, Billerica, MA).

3.4.2 Microelectrode fabrication.

Glass-insulated CFMEs were fabricated as described previously. Briefly, a single T-650 carbon fiber (7 μm diameter, Cytec Industries, West Patterson, NJ) was aspirated into a borosilicate glass capillary (0.6 × 0.4 mm or 1.0 x 0.5 mm, A-M Systems, Carlsburg, WA) and pulled using a micropipette puller (Narishige, Tokyo, Japan) to create two sealed microelectrodes. The exposed fiber was manually cut under a microscope to a desired length (~100 µm). An electrical connection was established by inserting a stainless steel wire (Squires Electronics, Inc., Cornelius, OR) coated with conductive silver paint (GC Electronics, Rockford, IL) into the back of the microelectrode.

3.4.3 Microbiosensor fabrication.

GOx EMEs were prepared as previously described. Briefly, electrodes were conditioned by the application of a voltammetric waveform of -0.4 V to +1.4 V for ~15 min at 60 Hz, and then
~5 min at 10 Hz. Thereafter, the electrodes were submersed in an aqueous solution containing 30 mg of GOx (6 mg/mL GOx in a 2% chitosan solution (pH~5.3); specific activity: 100 U/mg at 37°C). -3.0 V was applied using a DC power supply for ~30 sec to electrodeposit a chitosan hydrogel encapsulating GOx at the electrode surface. GOx EMEs were stored in PBS at 4°C prior to use.

3.4.4 In Vitro Experiments.

All in vitro data were collected at room temperature in a flow-injection apparatus using commercially available HDCV software (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility). A micromanipulator (World Precision Instruments Inc., Sarasota, FL) was used to position the GOx EME in a custom built electrochemical cell with a continuous flow of PBS (1 mL/min) supplied by a syringe pump (New Era Pump Systems, Inc., Wantagh, NY). Two-sec bolus injections of analyte were introduced to the GOx EME surface with a 6-port HPLC valve mounted on a two-position air actuator controlled by a digital pneumatic solenoid valve (Valco Instruments Co., Inc., Houston, TX). Triangular voltammetric waveforms were applied at 400 V/s and at a frequency of 10 Hz.

3.4.5 In Vivo Anesthetized Experiments.

All animal procedures were in accordance with the Institutional Animal Care and Use Committee (IACUC) at North Carolina State University and the National Institute for Health’s Guide for the Care and Use of Laboratory Animals. Adult, male sprague-dawley rats (290 - 320 g, Charles River Laboratories, Raleigh, NC; n =4) were housed on a 12:12 hr light- dark cycle with ad libitum access to food and water. Rats were anesthetized with urethane (1.5 g/mL, intraperitoneal (i.p.)) and body temperature was maintained at 37°C with a heating pad throughout the duration of the experiment.
First, animals received a catheter placement into the jugular vein for sterile saline or sterile drug infusions. Once fully anesthetized, the hair was shaved away from the region targeted by the in-house surgical procedure (right side of the chest, rostral back side, and top of the head) and the skin was disinfected with alternating swabs of 70% isopropyl alcohol and betadine (3 times each). For the jugular catheter placement, a ~20 mm incision was made on the back and the skin was separated from the muscle tissue using hemostats in a 4 cm$^2$ area to accommodate the exit port of the catheter. A small incision was made in the skin 5 mm anterior to the area where the jugular vein enters the rib cage. The jugular vein was separated from the surrounding tissue using hemostats. Next, a subcutaneous burrow as made between the chest/ back incisions and the silastic end of the catheter was inserted through the incision on the back and threaded through the burrow to the area of the jugular vein. Using microscissors, a small incision was made in the jugular vein and the silastic end of the catheter as inserted 33 mm into the vein, such that the catheter nodule was flush with the incision on the vein. Sterile silk surgical thread (Ethicon, 4-0) was tied around the vein and catheter on each side of the nodule in order to secure the catheter to the vein. Then the skin on the chest/ back was secured with sutures. The catheters were flushed with 0.1 ml 70 U/ml filtered sterile heparinized saline.

Thereafter, the stereotaxic surgical placement of electrodes was performed as described previously$^{39}$. A GOx modified microelectrode was placed in the dorsal striatum (+ 1.2 mm anteroposterior (AP), +1.5 mm mediolateral (ML), and − 5.0 mm dorsoventral (DV), relative to bregma), and a Ag/AgCl reference electrode was inserted in the contralateral forebrain. A bipolar stimulating electrode (Plastics One, Roanoke, VA) was placed in the ventral tegmental area/ substantia nigra area of the midbrain, −5.8 mm AP, +1.0 mm ML, and −8.0 mm DV. Electrical stimulations consisted of 200 µA biphasic pulses at 60 Hz, using a pulse width of 2 ms.
Electrodes were conditioned and data were collected using HDCV software (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility). A jugular vein catheter was used to administer systemic (i.v., 0.6 mL) infusions of saline or drug (cocaine 2 mg/kg + raclopride 1 mg/kg, 0.3 mL) followed by 0.3 mL of saline to flush the solution through the catheter. After ~1 min, a biphasic stimulation was induced (60 Hz, 100-125 pulses, 200 µA, 2 ms pulse width), and repeated in 15 min increments for one hour.

3.4.6 Immunohistochemistry.

Age-matched rats (n=2) were deeply anesthetized with urethane (1.5 g/mL) and transcardially perfused with 0.2 M potassium phosphate-buffered saline (KPBS), followed by 4% paraformaldehyde (PFA). All solutions were pH 7.4. Brains were removed, post-fixed for >24 hours in a 30% sucrose/ PFA solution, and cryoprotected in 30% sucrose in 0.2 M KPBS for >48 hours. Thereafter, tissue sections (40 μm) were cut with a freezing microtome (Leica) and collected in freshly prepared anti-freeze solution (30% glycerol, 30% ethylene glycol in KPBS).

Immunofluorescence procedures were adapted from elsewhere. Briefly, sections were washed 3 x 15 min in 0.2 M KPBS, then transferred to wells containing 3% goat serum in 0.2 M KPBS with 0.3% Triton X-100 (G-KPBS-T), which served as the blocking solution. Tissue was mildly agitated in this solution for 1 hour at room temperature. Thereafter, it was incubated for 72 hours at 4°C in a primary antibody cocktail prepared in G-KPBS-T, which contained antibodies directed against mouse glial fibrillary acidic protein (raised in mouse; 1:1000; G3893; Sigma) and tyrosine hydroxylase (raised in rabbit; 1:2500, AB5986P; Millipore). Next, the tissue was rinsed 3 x 15 min in 0.2 M KPBS. The secondary antibody cocktail was prepared in G-KPBS-T and contained AlexaFlour 633-conjugated goat-animal mouse immunoglobulin G (1:500; A-21052; Life Technologies) and Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (1:500; A-
Sections were incubated in the secondary cocktail for 90 min at room temperature with mild agitation. The tissue was thoroughly rinsed and placed into wells containing biotinylated solanum tuberosum lectin (2 µg/mL; B-1165; Vector Laboratories) in G-KPBS-T. The tissue was in this primary antibody solution at 4 °C for 24 hours. Tissue sections were then washed 3 x 15 min in 0.2 M KPBS and incubated for 90 min in a secondary solution of streptavidin-DyLight 550 (2 µg/mL; Cat. 84542; ThermoFisher) prepared in G-KPBS-T. Finally, sections were rinsed 3 x 15 min in 0.2 M KPBS, mounted on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA), coverslipped with glycerol-based mountant (50% glycerol in 4 M sodium bicarbonate), and stored at −80°C before visualization on a confocal microscope (Leica DM5000 scope). Images were acquired, edited, and exported via Leica Application Suite AF and made into a z-stack image using ImageJ software.

3.4.7 Data Analysis and Statistics.

HDCV Analysis software (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility) was used for data analysis with the multivariate statistical analysis method of principle component regression (PCR), which combines principal component analysis (PCA) with inverse least-squares regression. This approach is used to deconstruct data collected in vivo to reveal the relevant chemical contributors (DA, glucose, and pH) using training sets consisting of data collected in vitro. A chemical event was defined as an event with an amplitude that exceeded three times the standard deviation of the noise. All data are shown as the mean ± standard error of the mean (SEM). Paired t-Test, one-way and two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparisons post-hoc tests were used to determine statistical differences using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) when appropriate. Significance was designated as p<0.05.
3.5 Abbreviations

DA, dopamine; CFME, carbon-fiber microelectrode; GOx EME, glucose oxidase enzyme modified carbon-fiber microelectrode; COC/RAC, cocktail solution containing cocaine and raclopride.

3.6 Acknowledgments

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3.7 References


CHAPTER 4 Carbon-Fiber Microbiosensor for Monitoring Rapid Lactate Fluctuations in the Rat Striatum Using Fast-Scan Cyclic Voltammetry


4.1 Abstract

Considerable research has implicated glucose as the principal energy source of the brain. However, several recent studies have challenged this view with the demonstration that lactate is also an essential molecule for energy metabolism and memory formation. As such, real-time detection of lactate dynamics is imperative to understand brain energy availability and its involvement in neuronal function as well as dysfunction. To date, dynamic measurements of lactate concentrations in the brain have been limited. We have addressed this need by developing and characterizing a novel lactate oxidase-modified carbon-fiber microelectrode with a sensitivity and LOD for lactate of 22 ± 1 nA·mM⁻¹ and 7.0 ± 0.7 µM, respectively. The work presented herein enables detection of rapid lactate fluctuations with unprecedented spatiotemporal resolution as well as excellent stability, selectivity, and sensitivity; both in vitro and in vivo at discrete recording sites in the rat striatum. We provide evidence that striatal lactate availability increases in the extracellular space in response to electrical stimulation of dopamine release in the anesthetized rat. The lactate dynamics are distinct from those of concomitant glucose fluctuations that occur in response to the stimulation. The new tool described herein enables quantitative investigation of brain metabolism in both normal and disease states on a time scale that is commensurate with that of neuronal firing.

4.2 Introduction

The brain makes up roughly 2% of the body’s mass, but it accounts for ~ 25% of the body’s energy consumption. Investigation of brain energy metabolism, or neuroenergetics, has
traditionally focused on glucose as the principle fuel source of brain function, based on its role in
the formation of adenosine triphosphate (ATP). Glucose is transported into cells from the
extracellular space and, under resting conditions, aerobic cellular respiration converts glucose and
oxygen to pyruvate in the process known as glycolysis. Pyruvate and oxygen are appropriated by
mitochondria and, through the citric acid (TCA) cycle, generate a high yield of energy (~ 36 ATP).
Importantly, an anaerobic glycolytic pathway also exists, which yields only 2 ATP from the
formation of pyruvate. Additionally, fermentation of pyruvate can produce lactate\(^2\) which can later
be converted by lactate dehydrogenase back to pyruvate for use in the TCA cycle.\(^4\)

The conventional theory of neuroenergetics minimizes the role of lactate in energy
production, and describes it simply as a metabolic byproduct of glucose. However, there are
several studies challenging this notion that describe lactate as a key energy substrate in the brain.\(^5\)-\(^7\) For instance, when glutamate (the principal excitatory neurotransmitter) is released from a
neuron, it can be rapidly transported into local astrocytes. This creates an internal metabolic
demand within the astrocyte. In response, glucose is transported from the astrocytic end-feet
which ensheath the local vasculature that supplies nutrients from the blood. Then, glucose can be
converted to lactate via anaerobic glycolysis and shuttled from the astrocyte to the neuron via
monocarboxylate transporters (MCTs). This newly synthesized lactate can be used to create ATP
to meet energy demands.\(^8, 9\) Evidence in support of this “astrocyte to neuron lactate shuttle”
(ANLS) hypothesis includes theoretical modeling of brain lactate kinetics\(^10\), mathematical
stimulations.\(^11, 12\) Additionally, \textit{in vivo} experiments have demonstrated preferential lactate uptake
by neurons\(^7, 13\), as well as preferential glucose uptake by astrocytes\(^13\). Although several reports
challenge the ANLS hypothesis\(^14-16\), discrepancies in interpretation largely arise from the effects
of diverse sampling environments, as well as key differences in the methodologies and analytical
techniques utilized for lactate monitoring (for review see 17). Despite different perspectives, the preponderance of evidence indicates that lactate is an available and important energy substrate. The remaining debate focuses on which energy substrate, glucose or lactate, is preferentially available following neuronal activation.8, 9 Thus, there is a significant need for a simple, robust and powerful analytical tool with excellent spatiotemporal resolution that can enable selective, real-time detection of lactate in situ.

Several laboratories have made advancements in lactate monitoring utilizing first-generation biosensors coupled with electroanalytical methods.18-24 Amperometry is the electrochemical technique that is typically coupled with biosensors. With this detection method, a potential is applied to a sensor and any redox reactions that occur at that potential will produce current which, upon calibration, is indicative of analyte concentration. While amperometry is advantageous due to rapid temporal response, chemical selectivity becomes an issue when working in a complex chemical environment. All redox processes at the electrode surface may contribute to the current generated, including oxidation/reduction of interferents. Without complicated subtraction schemes or selective polymeric layers, deconvolution of the data proves difficult. While these and other methods are effective to improve selectivity, they increase response times and often result in spatial averaging across heterogenous brain regions concentrations. When working within a complex environment, such as the brain, selectivity, sensitivity, rapid response time and size are vital sensor components to consider.

The Sombers laboratory has advanced the biosensing field through the development of a glucose microbiosensor coupled with fast-scan cyclic voltammetry (FSCV). This glucose-enzyme modified microelectrode is capable of simultaneously monitoring glucose and dopamine (DA) in real time at the same micron-scale recording site.25, 26 We have optimized the methodology for
immobilization on carbon-fiber microelectrodes, and in this work the chitosan hydrogel protocol is used to encapsulate lactate oxidase (LaOx) on the electrode surface. The work presented herein describes the fabrication, characterization and implementation of this microbiosensor for lactate monitoring in real-time. To validate the sensor performance in biological tissue, dynamic concentrations of lactate are recorded in the extracellular space of the striatum. Furthermore, we systematically vary electrical stimulation of the dopaminergic midbrain and monitor striatal lactate and glucose availability, simultaneously. This tool will provide the means to further examine the precise role of lactate in the scope of neuroenergetics, and thus it will advance the development of therapies targeting disease states that involve dysfunctional energy regulation.

4.3 Experimental Section

4.3.1 Chemicals

All chemicals all were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. All in vitro experiments were carried out in 0.01 M phosphate buffered saline (PBS) at physiological pH 7.4. Lactate oxidase (LaOx; specific activity: 100 U/mg at 37°C) from Aerococcus viridans was combined with chitosan solution, for microbiosensor fabrication. The chitosan (from shrimp shells) had a deacetylation percentage of ≥ 75% and an approximate molecular weight of 190,000-375,000 Da. All aqueous solutions were prepared using >18 MΩ·cm distilled water (Millipore Milli-Q, Billerica, MA).

4.3.2 Microelectrode Fabrication

Glass insulated carbon-fiber microelectrodes were prepared as described. Briefly, a single T-650 carbon fiber (7 μm diameter, Cytec Industries, West Patterson, NJ) was aspirated through a glass capillary (0.6 x 0.4 mm or 1.0 x 0.5 mm, A-M Systems, Carlsburg, WA). The glass capillary was then placed in a micropipette puller (Narishige, Tokyo, Japan) to create two glass tapered
ends. The exposed carbon fiber was then cut under a microscope at a length of approximately 100 μm (±10 μm). Once cut, a wire lead (Squires Electronics, Inc., Cornelius, OR) coated with a thin layer of silver conductive paint (GC Electronics, Rockford, IL) was inserted into the back, open-end of the capillary to establish an electrical connection.

### 4.3.3 Microbiosensor Fabrication

A 2% chitosan solution was prepared in 87 mM acetic acid as described\textsuperscript{26, 27} 30 mg LaOx was dissolved in 200 μL of deionized water. Then, 800 μL of the 2% chitosan solution was added to the LaOx solution. The mixture was slowly stirred until homogenous at 37\textdegree C, and was stored for at least 24 hrs before use. Microelectrodes were electrochemically conditioned with a triangular voltammetric waveform ramping from -0.4 V to +1.4 V and back down to -0.4 V with a scan rate of 400 V/s at a frequency of 60 Hz for 15 mins, then at 10 Hz for 5 mins. Immediately after conditioning, the electrode was immersed in the enzyme-chitosan solution and -3.0 V was applied to the electrode for 30 sec before it was slowly removed. Each LaOx enzyme-modified electrode was stored in PBS (pH 7.4) at 4\textdegree C prior to use.

### 4.3.4 Microbiosensor Characterization

All \textit{in vitro} data were collected in a flow-injection apparatus, at room temperature, in a custom-built grounded Faraday cage, using freely available High Definition Cyclic Voltammetry (HDCV) software (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility). A micromanipulator (World Precision Instruments Inc., Sarasota, FL) was used to the position the biosensor in a custom electrochemical cell supplied with a continuous flow of PBS (0.5 mL/min) via a syringe pump (New Era Pump Systems, Inc., Wantagh, NY). A 6-port HPLC valve was mounted on a two-position air actuator that was controlled by a digital pneumatic solenoid valve (Valco Instruments Co., Inc., Houston, TX). This allowed for two-second bolus
injections of analyte to be reliability introduced to the LaOx microbiosensor surface. A triangular voltammetric waveform was applied from +0.1 V to + 1.4 V and back \(^{25-27}\) at a frequency of 10 Hz using a scan rate of 400 V/s.

4.3.5 Anesthetized Experiment to Monitor Dynamic Concentrations of Lactate In Vivo

Drug-naïve, male Sprague–Dawley rats (n=2; 275–300 g, Charles River Laboratories, Raleigh, NC) were allowed to acclimate to the facility for several days before experiments commenced. Animals were individually housed on a 12:12 h light/dark cycle with free access to food and water. Animal care and use was in complete accordance with the NC State University institutional guidelines (IACUC) and the NIH’s *Guide for the Care and Use of Laboratory Animals*. Rats were anesthetized with 4% isoflurane (Vetequip; Pleasanton, CA) and positioned in a stereotaxic frame (Kopf Instrumentation; Tujunga, CA) where isoflurane was maintained at ~1.5-2.0% for the remaining duration of the experiment. A heating pad (Harvard Apparatus, Holliston, MA) was used to maintain body temperature at ~37°C. Electrodes were positioned according to coordinates from the Paxinos and Watson\(^{29}\) rat brain atlas. The Ag/AgCl reference electrode was placed in the contralateral forebrain, and working electrodes were placed in the dorsal striatum (anteroposterior (AP): +1.2 mm and mediolateral (ML): +2.0 mm, relative to bregma; dorsoventral (DV): -5.0 mm, relative to skull). A bipolar stimulating electrode was placed in the ventral tegmental area/ substantia nigra complex (AP: -5.0 mm, ML: +2.0 mm, DV: -8.0 mm). The stimulating and reference electrodes were permanently affixed with screws and dental cement. Electrical stimulations were delivered to the VTA/SN complex (15-60 Hz, 20-240 pulses with 2ms width, 300 µA) and voltammetric data were recorded with a WaveNeuro Potentiostat (Pine Instrument Company; Grove City, PA).
4.3.6 Data Analysis and Statistics

HDCV Analysis software (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility) was used for data analysis. Principal component regression (PCR) was used to determine specific chemical contributors to \textit{in vitro} data. Training sets for DA, lactate, and glucose were constructed using data collected \textit{in vitro}. All plots depict data from three consecutive replicates of a single stimulation parameter, all within a single animal subject and are presented with the mean ± standard error of mean (SEM). GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) was used to determine statistical differences. Two-tailed Student’s t-test and one-way or two-way analysis of variance (ANOVA) with a Bonferroni’s post-hoc test were used as appropriate. Statistical significance was identified as p < 0.05.

4.4 Results and Discussion

4.4.1 Background-Subtracted Fast-scan Cyclic Voltammetry

FSCV is a power electroanalytical technique coupled with carbon-fiber microelectrodes, to monitor rapid changes in molecules with precise spatiotemporal resolution. Nonelectroactive molecules that are essential to brain function, such as glucose and lactate, cannot be directly monitored with this robust technique. However, several distinguished laboratories have extended the power of electrochemistry with adapted sensor fabrication and detection schemes. These labs have incorporated enzyme-modified sensors to monitor essential nonelectroactive molecules such as glucose, acetylcholine, and glutamate. The Sombers lab has contributed to this expansion through the development and characterization of glucose enzyme-modified carbon-fiber microelectrodes coupled to FSCV capable of selective glucose monitoring in spatially discrete regions of brain tissue. In this study, we have adapted this technology to quantitatively monitor lactate dynamics. LaOx-modified carbon-fiber microelectrodes were
coupled with FSCV to allow the selective detection of rapid lactate fluctuations. As shown in Figure 4.1a, a triangular waveform from +0.1 V to +1.4 V was applied at 400 V/s. In the presence of lactate and molecular oxygen, pyruvate and hydrogen peroxide (H$_2$O$_2$) are produced by the activity of LaOx. This enzymatically generated H$_2$O$_2$ is voltammetrically detected, serving as a reporter molecule for lactate.$^{39, 40}$ The high scan rates employed with our technique generates a large non-faradaic background current (Figure 4.1b and Figure 4.1c) that is stable over 10s of seconds and can be subtracted from the overall current collected. This reveals the faradaic contribution to the signal and results in a background-subtracted cyclic voltammogram indicative of H$_2$O$_2$ $^{40}$ (Figure 4.1d). This voltammogram can serve to identify lactate even in the presence of additional electroactive analytes or interfering chemical species.
Figure 4.1 Voltammetric detection of lactate via enzymatically generated H$_2$O$_2$. (a) Left, a triangular voltammetric waveform is applied to the microbiosensor shown in the representative scanning electron micrograph (scale bar is 100 µm). The waveform dynamically ramps the applied potential, with a scan rate of 400 V/s, from 0.1 V to 1.4 V and back to 0.1 V and this occurs at a frequency of 10 Hz. Right, mechanism of lactate and molecular O$_2$ interaction with LaOx immobilized to the electrode surface. Here, H$_2$O$_2$ is enzymatically produced then oxidized at 1.4 V (red star). (b) Cyclic voltammogram of large non-faradaic background current monitored in black. (c) Cyclic voltammogram of background current plus faradaic current, from H$_2$O$_2$ oxidation in blue, which is masked by the background current. (d) Background-subtracted cyclic voltammogram of current detected from the oxidation of enzymatically produced H$_2$O$_2$.

4.4.2 Electrochemical Lactate Monitoring

To determine sensitivity, LaOx microbiosensors were placed in a flow injection apparatus and in vitro voltammetric data were recorded following 2 sec bolus injections that introduced lactate to the surface of the electrode. Concentrations ranging from 75 µM to 2000 µM were detected (Figure 4.2a). A linear calibration for physiological brain concentrations (0 to 1000 µM) was evident with a sensitivity of 22 ± 1 nA/mM (Figure 4.b, r$^2$ = 0.98; n=15). A representative color plot demonstrates the electrochemical detection of enzymatically generated H$_2$O$_2$, the reporter molecule for lactate (Figure 4.2c). Color plots provide a method to simultaneously view
data collected across time for the entire window of applied potentials. Individual cyclic voltammograms can be extracted to provide qualitative identification (Figure 4.2c inset), and the dynamic molecular profile can be extracted as well (Figure 4.2c, current vs time trace, blue). Figure 4.2d provides a series of voltammograms indicative of \( \text{H}_2\text{O}_2 \) oxidation across the linear range. The limit of detection was \( 7.0 \pm 0.7 \, \mu\text{M} \), defined as three times the standard deviation of the noise. Response time was defined as the time span for a signal to rise from 10% to 90% of its maximum value and was determined to be \( 1.6 \pm 0.1 \, \text{sec} \) (n = 3).

![Image](image_url)

**Figure 4.2** Electrochemically characterizing lactate current response *in vitro*. (a) Calibration plot demonstrates enzyme saturation past 1000 µM (n = 15 electrodes). (b) From data plotted in a, linear range of lactate calibration. (c) Representative color plot depicting electrochemical detection of 250 µM lactate dynamics utilizing FSCV. Analyte identification extracted at white dashed line (inset). Current vs time trace extracted at 1.2 V on back scan (red line) of the color plot. (d) A series of representative cyclic voltammograms collected from bolus injections (75 µM to 1000 µM) of lactate.
4.4.3 Microbiosensor Stability

Typical *in vivo* data collection involves monitoring chemical dynamics for up to 4 hrs and requires a sensor to respond predictably for precise quantification over the course of the entire experiment. To validate sensor stability, 500 µM lactate was detected every 15 min over 4 hrs (n = 4). For a given electrode, the current responses were normalized to the maximal response recorded during the first injection. There was no significant main effect for current responses across time (F(3,51)= 0.82, p=0.66), indicating the current response on the LaOx microbiosensor was stable after repeat injections of lactate over a 4 hr period (Figure 4.a). Figure 4.b displays representative color plots collected for the first (left) and last (right) injections of lactate.

![Figure 4.3 Lactate microbiosensor stability.](image)

(a) Normalized current collected in response to repeated bolus injections of lactate (500 µM) across 4 hours. (b) Representative color plots for the first (left) and the last (right) voltammetric data collected on the same electrode. The CV for enzymatically generated H\(_2\)O\(_2\), via lactate interacting with LaOx, was extracted at the vertical dashed line for analyte identification (inset CV).
4.4.4 Selectivity

In a complex environment such as the brain, selective detection of the molecule of interest is a primary concern. In order to assess selectivity, null electrodes were fabricated in a manner identical to fabrication of the LaOx-modified electrodes, but the chitosan mixture was void of LaOx. Figure 4.4a-f depicts representative color plots collected on null electrodes (left) and LaOx microbiosensors (right) in response to lactate (lac, 500 µM), a supraphysiological concentration of H$_2$O$_2$ (50 µM), and dopamine (DA; 1000 µM). Voltammograms (insets) were extracted for analyte identification at white dashed vertical lines. The complete data set is quantitatively compared in Figure 4.4g. Two-way ANOVA reveals significant main effects of current response on null electrodes (black) and the LaOx microbiosensors (blue) (F(1, 33)=78.72, p< 0.0001); and across the analytes examined (F(4,33)=59.88, p<0.001). Moreover, there was a significant interaction of electrode type by analyte examined (F(4, 33)=65.37, p<0.0001). Post-hoc comparisons indicate a significant difference in the current response to lactate between the electrode types (p<0.0001), though there was no significance difference in the current generated in response to the other analytes investigated (p > 0.05). The sensitivity to lactate was considerably greater than that to all of the other chemical species examined, including a supraphysiological concentration of H$_2$O$_2$. This verifies the reliable performance of this novel microbiosensor, and demonstrates selective detection of lactate dynamics using FSCV, without the requirement for additional subtraction schemes or selective exclusion coatings.
Figure 4.4 Electrochemical evaluation of current response to potential interfering chemical species. (a-f) Representative color plots of bolus injections containing either lactate, supraphysiological H$_2$O$_2$, or DA introduced to null electrodes (left column) or LaOx microbiosensors (right column). Analyte identification (see inset CVs) was extracted at the white dashed lines, respectively. (g) Bar graph of current response to lactate (lac), supraphysiological H$_2$O$_2$, glucose, DA and ascorbic acid (AA) on null electrodes (black) and LaOx microbiosensors (red) (n=3 electrodes; ****p<0.0001).
4.4.5 Lactate Dynamics in the Rat Striatum

Lactate’s perceived role in the brain is slowly changing from that of a mere metabolic waste product, to that of an important supplemental fuel source, signaling molecule, and an essential precursor for synthesis of other energetic substrates. Nonetheless, many critical details regarding the specific role that lactate plays in a given biological situation, and the temporal aspects of lactate’s modulatory influence in fueling neurotransmission, remains obscure. To begin to answer some of these questions, an individual LaOx microbiosensor was paired with a null electrode and positioned as a unit in rat striatum to monitor lactate dynamics in vivo. Figure 4.5a demonstrates the experimental design for in vivo voltammetric data collection in anesthetized rats. A bipolar stimulating electrode was placed in the VTA/SN complex to drive the nigrostriatal projection to the striatum, and elicit neurotransmitter release from these neurons in a controlled manner. Release of neurotransmitter from dopaminergic terminals in the striatum elicits neural activity in local subsets of medium spiny neurons (MSNs), and this activity is implicated in both motor control and habit formation. Voltammetric data were recorded in response to electrical stimulation of the midbrain (60 Hz, 120 pulses, 300 µA). Representative color plots and current versus time traces are presented in Figure 4.5b. The null electrode (left, black) recorded the release of DA in response to electrical stimulation (red arrow and lightning bolt). In contrast, the lactate microbiosensor (right, red) recorded the release of DA and a subsequent increase in extracellular lactate. These data demonstrate that lactate availability increases ~3 sec after DA release, presumably to provide fuel upon activation of MSNs and local astrocytes.
Figure 4.5 Electrochemical monitoring striatal lactate and DA dynamics in response to electrical stimulation of the midbrain. (A) A bipolar stimulating electrode is implanted in the midbrain. The microbiosensors are implanted in the dorsal striatum of an anesthetized rat. Voltammetric data are simultaneously collected at a null and a lactate microbiosensor. Representative color plots and current vs. time traces demonstrate (B) DA release and (C) increased lactate availability in response to electrical stimulation (arrow and lightning bolt; 60 Hz, 120 pulses, 300 µA). Individual voltammograms (inset) were extracted at the white dashed lines.
Next, stimulation frequency and duration (pulses) were systematically varied. Figure 4.6a depicts normalized lactate dynamics recorded in response to 120 stimulation pulses applied over a range of frequencies (15, 30 and 60 Hz). It has been clearly shown that driving these cells with high frequency stimulations in this range increases the amount of dopamine released.\textsuperscript{44} Thus, we hypothesized that more intense stimulations (higher frequencies or increased pulse numbers) would result in increased metabolic demand and an increased lactate response. At 60 Hz, a biphasic lactate response was evident, with an initial increase in lactate availability that rose after the DA event, followed by a longer, and more gradual increase in local lactate concentration. The initial lactate response is clearly evident in the 20 sec view of the data (Figure 4.6a, below). Striatal lactate measurements after the 15 and 30 Hz midbrain stimulations lacked the rapid lactate response and demonstrated only the delayed, broad concentration increase. Next, a series of stimulations was applied at 60 Hz, and the number of stimulation pulses was varied (Figure 4.6b). The magnitude of the lactate response corresponds with the number of pulses applied (20, 60, and 120 pulses). A biphasic lactate concentration profile was evident at 60 Hz and 120 pulses. The shortest stimulation duration (20 pulses, ~0.3 sec) only elicited a slow, broad increase in lactate. Table 4.1 provides a summary of the quantitative data (Appendix B). Consistent with previous reports\textsuperscript{44}, one-way ANOVA revealed main effects of stimulation pulse number (F(4, 8)=153.5, p<0.0001) and frequency (F(4, 8)=331.7, p<0.0001) on elicited DA concentration. Interestingly, there were also significant main effects of both pulse number (F(4, 10)=32.24, p<0.0001) and frequency (F(2,8)=13.75, p<0.01) on extracellular lactate concentration. With a 60 Hz stimulation, post-hoc tests revealed that application of 60 Hz and 120 pulses evoked increased extracellular lactate concentrations as compared 20 pulses (p<0.05 and p<0.0001, respectively). 120 stimulation pulses applied at frequencies of 30 Hz (p<0.05) and 60 Hz (p<0.01) elicited
significantly greater increases in striatal lactate than 120 pulses applied at 15 Hz. The time to reach maximum lactate concentration increased with increased pulse number (F(4,14)=268.8, p<0.0001). This was expected, because longer stimulation trains require more time for pulse delivery. However, there was no significant difference in the time to reach maximum lactate concentrations when various stimulation frequencies were compared (F(2,8)=0.4129, p>0.05). Importantly, striatal lactate availability ([Lac]_{max}) is positively correlated with the amount of DA detected when varying either frequency (Figure 4.6c, r^2 = 0.92) or pulse number (Figure 4.6d, r^2 = 0.99).

![Figure 4.6 Extracellular lactate availability corresponds with stimulation intensity and DA release.](image)

Normalized lactate dynamics evoked with (A) varied stimulation frequencies (120 pulses; 15, 30, 60 Hz), or (B) stimulation durations (60 Hz; 20, 60, 120 pulses). (C,D) Extracellular lactate concentrations directly correspond with evoked DA concentrations.
When recording in intact brain tissue, the type and duration of the anesthesia can significantly impact the data, due to effects on the vascular response of blood flow or metabolism.\textsuperscript{45, 46} Furthermore, extensive neuronal activation over long durations can result in a hypoglycemic state, due to long periods without food consumption or supplemental nutrient delivery.\textsuperscript{47, 48} Interestingly, after multiple (~20) stimulation trains were delivered over the course of several hours, the lactate dynamics significantly changed. A representative trace recorded in response to a 60 Hz stimulation is shown in Figure 4.7. Upon application of 20 pulses, the slow, broad lactate signal that was evident earlier in the experiment (Figure 4.6) was largely attenuated (Figure 4.7a). Furthermore, only the initial, phasic response was evident in response to longer stimulations (120 and 240 pulses; Figure 4.7.a top and emphasized in the bottom enlarged view, all normalized). Only subtle changes in lactate dynamics were evident in response to 60 Hz stimulations of shorter duration. Indeed, the response to 20 pulses delivered at 60 Hz was essentially no different from that recorded upon delivery of this stimulation early in the recording session. A biphasic increase in lactate availability was recorded in response to delivery of 60 stimulation pulses late in the recording session (Figure 4.7.a top), which was not evident in earlier stimulations of this type (Figure 4.6). Although the dynamics of lactate availability clearly shifted over the duration of the recording, Students t-test indicated no significant difference in maximum striatal lactate concentrations evoked by electrical stimulation of the midbrain (60 Hz with 120 pulses) when data collected early in the experiment (normal, i.e. norm; striped bar) were compared to data collected 3-4 hours into the recording session (hypoglycemic, i.e. hypo; solid bar) ($t(4)=0.5118$, $p > 0.05$; Figure 4.7d).

The lactate biosensors respond to DA, albeit with low sensitivity (Figure XX). Nonetheless, DA dynamics were also evident in these stimulation files. Figure 4.7 compares the
amplitude of striatal lactate ([Lac]_{max}, red) and DA (black) concentrations evoked by 120 stimulation pulses delivered with various frequencies (15, 30, and 60 Hz; Figure 4.7.b), or in response to varying the number of pulses (20, 60, 120, and 240 pulses; Figure 4.7.c) delivered at 60 Hz. One-way ANOVA revealed main effects of stimulation frequency (F(2,8)=32.88, p<0.001) and pulse numbers (F(3,11)=76.09, p<0.0001) on evoked lactate concentrations. When delivering 120 pulses, lactate availability in the striatum was elevated in response to 30 and 60 Hz stimulations, as compared to 15 Hz (p<0.01 and p<0.001, respectively). However there was no significant difference found for [Lac]_{max} when comparing 30 vs. 60 Hz. Striatal lactate concentrations also increased as the number of pulses delivered at 60 Hz increased (p<0.01-0.0001). Striatal DA release was also dependent on stimulation duration and intensity at this point in the experiment. One-way ANOVA demonstrated main effects of frequency (F(2,8)=464.9, p<0.0001) and pulse number (F(3,11)=249.0, p<0.0001) on the DA concentrations evoked. Striatal DA concentrations ([DA]_{max}) increased with increased pulse numbers delivered (p<0.01-0.0001). However, the effects of stimulation frequency were somewhat less apparent. No DA was detected in response to a 15 Hz stimulation, and evoked DA concentrations did not significantly differ between 30 and 60 Hz stimulation frequencies (p>0.05). This is somewhat surprising, because striatal DA release has been shown to be dependent on the frequency of midbrain stimulation.\textsuperscript{44} However, these data are inconclusive at this time, due to the low sensitivity of the LaOx microbiosensor to DA (insert 2.1 ± 0.2 nA/µM; data not shown) and the long duration of the overall recording period. The data presented in Figure 4.7e demonstrate that the concentration of striatal DA ([DA]_{max}) evoked by midbrain stimulation was reduced after prolonged anesthesia (t(4)=21.43, p < 0.0001). Future studies will combine these measurements with an assessment of peripheral blood glucose concentrations, to confirm hypoglycemia.
Figure 4.7 Electrically evoked lactate fluctuations shift after 3-4 hours of recording. (A) Normalized lactate dynamics evoked at the end of the recording session in response to various stimulation durations (60 Hz; 20, 60, 120, 240 pulses). (B) A summary of striatal lactate ([Lac]$_{\text{max}}$, red) and DA ([DA]$_{\text{max}}$, black) concentrations evoked in response to stimulations of varying (B) frequency and (C) duration. (D) Lactate and (E) dopamine concentrations measured in response to a 60 Hz, 120 pulse, 300 µA stimulation early (stripes; data from Figure 4.6 and Table T.1) vs late (solid) in the experiment, when the animal is presumably hypoglycemic.

In a separate animal subject, the dynamic profile of glucose and lactate was compared to elucidate how they respond to induced metabolic demand. The data were collected using a tri-microelectrode device (TRIMED) that consisted of lactate, null and glucose microbiosensors aligned in parallel and affixed into a single unit, with the sensor tips separated by ~1.0 mm. The experimental design was essentially identical to that shown in Figure 4.5a, with the inclusion of the glucose microbiosensor. Figure 4.8 shows normalized lactate and glucose traces recorded simultaneously in response to midbrain electrical stimulation (120 pulses applied at 15, 30, and 60 Hz). Across all three stimulation frequencies, both glucose (black) and lactate (red) exhibit a biphasic concentration profile. Immediately following stimulation, extracellular glucose availability dominates the signal (Figure 4.8 a,b,c; see bottom panel for an enlarged view).
Figure 4.8 Differential temporal response of neuroenergetic substrates in the striatum to various midbrain stimulation frequencies. (A) 15 Hz, (B) 30 Hz, and (C) 60 Hz midbrain stimulation (120 pulses, 300 µA).

A similar trend is evident in response to increasing the stimulation duration (number of pulses) applied at 60 Hz (Figure 4.9). A dominant glucose signal precedes sustained elevations in lactate availability. These signals are quantitatively compared in Figures 8 and 9 and summarized in Table 4.2 (Appendix B). Immediately following stimulation (first 15 sec), one-way ANOVA reveals significant main effects for stimulation frequency (lactate, F(2,8)=288.8, p<0.0001; glucose, F(2,8)=90.78, p<0.0001) and duration (lactate, F(3,11)=181.6, p<0.0001; glucose, F(3,11)=392.5, p<0.0001) on lactate and glucose concentrations (Figure 4.10.ab).
Figure 4.9 Dynamic temporal response of striatal glucose and lactate following various electrical stimulation durations (by pulse number) delivered to the midbrain. (A) 60 pulses (1 sec) (B) 120 pulses (2 sec) and (C) 240 pulses (4 sec).
Figure 4.10 Simultaneous extracellular $[\text{Lac}]_{\text{max}}$ (red) and $[\text{Gluc}]_{\text{max}}$ (black) availability in the striatum immediately following (within the first 15 seconds) midbrain electrical stimulations. (A) Representative full 90 sec collection file from a 60 Hz 120 pulse stimulation. Maximum concentrations of glucose and lactate response in (B) the first 15 sec and (C) 90 sec of midbrain electrical stimulation. Varied stimulation frequencies (120 pulses; 15, 30, and 60 Hz) or pulse numbers (60 Hz; 20, 60, 120, or 240 pulses)

Overall, these data are consistent with the ANLS model of energy delivery. The initial, rapid increase in glucose concentrations could be attributed to cerebral blood flow responding to the metabolic demand. The subsequent, slower and broader increase in glucose
and lactate availability could result from increases could be sourced from astrocytes from glycogen storage. Indeed, local increases in neuronal activity are coupled with increases in cerebral blood flow, presumably because there is a lack of sufficient glycogen storage in astrocytes to fuel the demand. Astrocytic end feet are well positioned to receive glucose influx from increased blood flow that can be converted to lactate to meet metabolic demand.\textsuperscript{49, 50, 52} Additionally, neurons and glia can transport lactate via MCTs, which can subsequently be converted to pyruvate for energy utilization to fuel metabolic demand.\textsuperscript{1} These data are compatible with that of reported in literature\textsuperscript{8, 53} that local increases in neuronal activity are accompanied by anaerobic glycolysis. Nonetheless, many questions remain unanswered, and the specific cellular and molecular mechanisms that govern the apparent extracellular availability of these molecules in the striatum requires warrants further investigation.

4.5 Conclusions

We have adapted our previously employed methodology to create a lactate microbiosensor to enable selective detection of lactate dynamics in the rat dorsal striatum. The work characterizes the lactate biosensor performance to enable reliable, sensitive, and selective measurements of lactate at a single sensing substrate without additional exclusion layers. Interestingly, we have shown an increase in lactate availability within the extracellular space in the striatum upon electrical stimulation of the VTA/SN complex. We have demonstrated a direct correlation to the \([\text{Lac}]_{\text{max}}\) and \([\text{DA}]_{\text{max}}\) monitored and their dependence on stimulation frequency as well as stimulation duration. Moreover, we have shown duration of anesthesia with isoflurane altered electrically evoked \([\text{DA}]_{\text{max}}\) and a shifted the temporal response of the striatal \([\text{Lax}]_{\text{max}}\). Our data corroborates recent evidence\textsuperscript{7, 13, 54} indicating lactate is certainly a player in neuroenergetics (Figure 4.5.1). The cellular origin and specific role of striatal lactate in fueling neurotransmission
source remains to be determined. Future studies are necessary and now possible through the utilization of the novel LaOx and the established glucose-enzyme microbiosensors\textsuperscript{25-27} coupled to FSCV to simultaneously monitor these neuronal fuel sources and DA in real-time.

Figure 4.11 Energy availability coupled to neurotransmitter release. (A) Excitation of dopamine neurons evoked DA release and subsequently increase activity of MSNs\textsuperscript{55} and ANLS theory of lactate energy delivery.\textsuperscript{1,9} (B) Summary of the data collected: Glucose, lactate and DA dynamics in response to neuronal activation. Striatal lactate availability is positively correlated with electrically evoked DA release. (C) Schematic depicting the overall hypothesis whereby multiple pathways of energy delivery can occur after striatal DA release.
4.6 References


CHAPTER 5 Summary and Outlook

5.1 Research Summary

Background subtracted fast-scan cyclic voltammetry (FSCV) is clearly a powerful electrochemical technique for investigating chemical dynamics in the living brain, due to its combination of selectivity, sensitivity and temporal resolution. The advancement and application of this analytical technique has enabled enormous contributions to the field of neuroscience. It has shed light upon subsecond chemical mechanisms that underlie behavior, particularly for dopamine (DA), under normal conditions and disease states. To date, neurochemical studies have largely focused on monitoring only one analyte at a time in a single recording session. However, the environment in the brain is chemically complex and dynamic, with many neurochemicals collectively generating physiological effects. The work presented herein demonstrates that FSCV is much more than a technology for sensing DA. It can monitor multiple analytes simultaneously, even when non-electroactive species are targeted.

Decades of DA research have proven that DA plays an important role in motivation, particularly with respect to drug abuse and addiction, and it is also important for motor and cognitive function. In contrast, little is known about subsecond energy availability in brain tissue. This dissertation begins to elucidate the dynamic profile of glucose and lactate, key molecules that fuel metabolic processes in the brain. Considerable research has implicated these metabolic fuel sources in cognition and neuronal activation globally, but a lack of molecular monitoring techniques for non-electroactive species with sufficient spatiotemporal has hindered research in vivo. The advancement and implementation of electroanalytical techniques that can simultaneously monitor fluctuations of glucose, lactate and small molecule neurotransmitters in real time is critical to better understand neuroenergetics in a living organism. The work presented
describes important first steps toward this goal - the development and characterization of novel microbiosensors for monitoring subsecond dynamics of glucose, lactate and dopamine in rat brain tissue.

Chapter 2 is a paper published in ChemPhysChem\textsuperscript{7} in collaboration with Saad Khan from the Department of Chemical and Biomolecular Engineering laboratory at North Carolina State University. This work quantitatively characterizes and compares three strategies for immobilization of enzyme at the surface of a carbon-fiber microelectrode.\textsuperscript{7} The three immobilization strategies explored were: physical adsorption, immobilization in electrospun poly(vinyl alcohol)(PVA) nanofibers, and entrapment in a chitosan hydrogel. Immobilization of glucose oxidase (GOx) by physical adsorption generated a biosensor with poor sensitivity and unstable performance. Data collected on the electrospun microbiosenors demonstrated stable glucose measurements over a large linear range appropriate for peripheral measurements (~ 3 mM). Finally, entrapment of GOx in the chitosan hydrogel generated microbiosensors capable of sensitive glucose measurements over a 4 hour period. Both the electrospun and chitosan hydrogel microbiosensors were capable of monitoring robust glucose signals in rat striatal tissue. However, the hydrogel immobilization strategy out-performed the others in terms of sensitivity and stability. These findings confirmed an optimal enzyme immobilization strategy capable of selectively monitoring subsecond fluctuations of glucose within discrete regions of the brain. Most importantly, this work contributes to the field of biosensing as a whole, due to the potential adaptability of the optimal enzyme immobilization strategy presented. This strategy can be used to create sensors for other essential analytes of interest through the utilization of various oxidase enzymes.
Chapter 3 is an article recently published in *ACS Chemical Neuroscience* which demonstrates the first simultaneous detection of glucose and DA at a single recording site in the rat striatum. The work investigated multiple triangular voltammetric waveforms to optimize the simultaneous electrochemical detection of glucose and DA. Simultaneous detection of an electroactive and non-electroactive species at a single recording site is unprecedented. The data revealed that glucose in the dorsal striatum rapidly responds to increased metabolic demand induced by electrically evoked dopamine release, or by pharmacological augmentation of DA dynamics through acute cocaine/raclopride administration. This important study provides a demonstrated research tool that can be used to help clarify fundamental questions in neuroscience regarding normal brain function as well as responses that occur with cocaine exposure. Ultimately the application of this tool should inform improved therapeutic strategies to treat cocaine abuse and opens the door to monitoring other combinations of electroactive and nonelectroactive species. This biosensor application with FSCV will pave the way for future investigations of glucose function in states with altered neuroenergetics, such as after food consumption and in neurodegenerative states such as Parkinson’s disease and Alzheimer’s disease.

Chapter 4 presents a microbiosensor capable of selective detection of real-time lactate dynamics in the rat dorsal striatum. The methodology for optimal enzyme entrapment strategy describe in Chapter 2 was utilized to create this microbiosensor. The stability of lactate oxidase-modified carbon fiber microelectrodes was characterized, and the experiments demonstrate that the microbiosensors are capable of selectively monitoring lactate up to four hours without inclusion of additional exclusion layers to impart chemical selectivity. Interestingly, data collected *in vivo* showed extracellular lactate availability was augmented in the striatum after electrical stimulation of dopaminergic neurons. This microbiosensor was combined with the previously
established glucose sensor and null electrodes for the simultaneous detection of glucose, lactate and DA with subsecond temporal resolution at a single striatal recording site. These data provide a direct look at availability of two neuroenergetics substrates, simultaneously, after activation of DA release within intact brain tissue. The data revealed a positive correlation between lactate and DA concentrations in the extracellular space, as well as a dependence on stimulation frequency and duration. Moreover, that data demonstrated key differences in lactate dynamics under initial (baseline) conditions as compared to late in the recording session (hypoglycemic conditions). Together, these sensors can now be used to investigate the role of lactate and glucose as a neuronal fuel sources in the striatum as well as other brain nuclei. The controversial topic of preferential fuel sources and their role in neuronal activation can now be examined. Through these investigations, the origin of fuel sources and how their mechanisms impact DA release implicated in drug addiction behaviors as well as neurological disease states can finally be observed.

Many of the acute reinforcing effects of abused drugs, including cocaine, have been predominantly attributed to increased extracellular levels of DA in the striatum. However, the effects of glucose and lactate availability on striatal DA release, and the physiological effects of this modulation, remain unclear. Glucose and lactate are ubiquitous molecules in the brain, however, how their availability is elicited is likely complex and crucial for specific physiological conditions. Drugs of abuse have primary actions in the brain’s DA systems that are involved in motor control, habit formation, and motivational behavior. Interestingly enough, psychostimulants, hallucinogens, and opioids all have different mechanisms of action that promote DA release. These drugs of abuse alter cellular activation differently, suggesting the response of energy availability upon metabolic demand may be different in a variety of ways. Moreover, Parkinson’s disease (PD) is a debilitating disorder whereby a large proportion of midbrain DA
neurons die and reduce movement initiation and motor control. There is compelling evidence of altered neuroenergetics in PD and overactive glucose utilization by oxidative phosphorylation in cells may contribute to abnormal oxidative load via excess intracellular reactive oxygen species. Conversely, studies have shown prolonged movement with exercise can elevate brain lactate levels and the technology outlined in this work could enable the simultaneous measurements of lactate and glucose. These data can evaluate the phenomena associated with ANLS function with increase physical activity. Such experiments would positively impact studies regarding dopamine-associated brain circuits and depression, as symptoms such as psychomotor slowing (also evident in PD) and lack of pleasure could very well involve altered metabolic function. Thus, the described biosensors and voltammetric applications could be used in the future to investigate how metabolic changes (glucose and lactate) contribute to the initiation and progression of PD, addictive pathologies, and normal behaviors like exercise. Therefore, the research described will broadly impact a future research investigating how, what, where, and why glucose and lactate concentrations dynamically change in the living brain. Future studies are necessary and now possible through the utilization of these microbiosensors coupled to FSCV to simultaneously monitor these neuronal fuel sources and DA in real-time.

Overall, the work presented described that development, characterization and optimization of voltammetric techniques and microbiosensors to selectively monitor multiple neurochemicals vital to cellular function and ultimately, behavior. Collectively, these data provide a fundamental understanding on the chemical dynamics of brain energy availability and how these chemicals change upon neuronal activation. This foundational work promises to inform how glucose, lactate and DA collectively are implicated in normal brain function/dysfunction. In doing so, this
information can potentially assist the development of therapeutic strategies for treating drug
dependency and other neurological disorders impacted by energy dysregulation.
5.2 References


APPENDIX A Unmasking the Effects of L-DOPA on Rapid Dopamine Signaling with an Improved Approach for Nafion Coating Carbon-Fiber Microelectrodes

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Author’s Note: My dissertation has primarily focused on the development and characterization of enzyme-modified electrodes for real-time electrochemical measurements in brain tissue. However, throughout my tenure at North Carolina State University, I have collaborated with various colleagues on diverse projects. The work presented herein describes one of those collaborations that resulted in a publication. I contributed to the fabrication and characterization of a size exclusion membrane on carbon-fiber microelectrodes for selective detection of dopamine dynamics in intact brain tissue.

A.1 Introduction

Parkinson’s disease (PD) is a neurodegenerative disorder that affects more than a million people in the United States 1. It is characterized by motor deficits including bradykinesia, rigidity, and resting tremor, resulting from a progressive loss of nigrostriatal dopamine (DA) neurons 2-3. DA does not readily cross the blood-brain barrier, thus its use for treating the symptoms of PD is precluded 4. 3, 4-dihydroxyphenyl-L-alanine (L-DOPA), the metabolic precursor to DA, has routinely been used for symptomatic treatment of PD since the late 1960s 3, 5-6. However, the efficacy of prolonged L-DOPA treatment wanes over time and patients develop serious motor complications 3, 7-8. Despite common use in the therapeutic management of PD, remarkably little is known about how L-DOPA replacement therapy alters the dynamics of pulsatile DA fluctuations that occur on a fast (seconds) timescale.

The vast majority of studies that have investigated these questions have analyzed dialysate collected in the striatum 9-12. Microdialysis is a diffusion-based sampling method that is well suited to examine steady state or slowly changing levels of analytes in the extracellular fluid 13. Thus, these studies have significantly advanced our knowledge on how L-DOPA serves to gradually increase striatal DA levels 9-12. However, quantification of the effects of L-DOPA on rapid DA
dynamics is also important, because patients with Parkinson’s disease are impaired in cognitive tasks that require learning from positive (and negative) feedback\textsuperscript{14-15}, and rapid DA signaling in both the sensorimotor and limbic corticostriatal circuits has been shown to play a key role in reinforcement learning processes\textsuperscript{16-20}. Over the past twenty years, electrochemical techniques have proven to be particularly useful for monitoring rapid changes in DA concentration resulting from discrete neurochemical events, and voltammetric studies have provided fundamental information to describe how rapid DA fluctuations underlie discrete aspects of motivated behavior in animal subjects. For example, phasic dopamine release in the nucleus accumbens core has been shown to correlate with the presentation of Pavlovian reward-predictive cues and to precede the initiation of reward-seeking actions\textsuperscript{19}. However, few electrochemical studies have investigated the effects of L-DOPA administration on phasic DA fluctuations in the dorsal striatum (but see Phillips et al.\textsuperscript{21}), and measurements of the effects of L-DOPA on electrically evoked DA release have reported conflicting results with significant variability\textsuperscript{22-25}. Clarifying this question is critical to developing improved therapies for the treatment of PD.

We hypothesize that much of the uncertainty in the electrochemical data is due to complications associated with the chemical nature of L-DOPA and DA. Both species self-polymerize in a mechanism that likely involves oxidation of the catechol to a quinone\textsuperscript{26}. Near neutral pH, the amine group inherent to dopamine-o-quinone (o-DQ) can serve as a nucleophile, initiating an intramolecular cyclization to generate leucodopachrome (LDC)\textsuperscript{27}. Further oxidation generates dopachrome (DC), which may polymerize to melanin by a free radical polymerization\textsuperscript{28} (Scheme A1). The oxidative cross-linking generates robust water resistant bonds that enable the formation of thin, surface-adherent films on a wide range of materials, including the surface of
electrodes. This film reduces the active surface area of the sensor and attenuates sensitivity.

Scheme A1. DA polymerization pathway.

Nafion, a perfluorinated ion-exchange polymer, has been extensively used on the surface of sensors to repel interfering and adsorbing species. There are numerous protocols for creating a Nafion membrane at the electrode surface; however, a reliable means for consistent generation of a simple Nafion membrane on cylindrical carbon-fiber microelectrodes has not yet been achieved (but see Heien et al. for description of a robust Nafion/polyethylenedioxythiophene composite membrane). Electrochemical data collected using Nafion-coated electrodes can be highly variable, presumably due to inconsistent adhesion of the Nafion (both hydrophobic and hydrophilic regions) with the electrode surface. Reliable generation of a membrane is confounded by the fact that the morphology of Nafion is very sensitive.
to the nature of the solvent in which it is dissolved. The problem is exacerbated at carbon-fiber microelectrodes, as electrochemical conditioning changes the chemical functionalities inherent to the carbon surface, even etching the surface when electrodes are rapidly scanned to anodic limits of ~1.3 V. By refining the parameters by which a Nafion coating is applied to the electrode, we have advanced a technique for generating a carbon fiber microelectrode that is less sensitive to fouling by polymerization of L-DOPA or dopamine at the surface. We use fast-scan cyclic voltammetry (FSCV) to demonstrate that generation of a Nafion membrane after electrochemical conditioning reliably produces a uniform film on the sensor surface that preserves a rapid response to DA while preventing fouling. We then use this approach to assess the effects of various concentrations of systemic L-DOPA administration on DA dynamics measured in rat striatum with sub-second temporal resolution. In addition to providing chemical information that is highly relevant to the most common therapy used in the treatment of PD, this work serves to benefit a wide variety of studies plagued by electrode fouling because Nafion is so commonly used in electroanalytical chemistry.

A.2 Experimental Section

A.2.1 Chemicals.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received, unless otherwise noted. Solutions were prepared using doubly distilled deionized water (Milli-Q Millipore, Billerica, MA). In vitro characterization of electrode response to DA and ascorbic acid (AA) was conducted in phosphate buffered saline (PBS: 140 mM NaCl, 3 mM KCl, 10 mM NaH2PO4) at pH 7.40. Calibration of electrodes was accomplished in Tris buffer (15 mM Trisma HCl, 3.25 mM KCl, 1.2 mM CaCl2, 1.2 MgCl2, 2.0 mM Na2SO4, 1.25 mM NaH2PO4, and 145 mM NaCl) at pH 7.40.
A.2.2 Carbon-fiber Microelectrode Fabrication.

Carbon-fiber microelectrodes were fabricated as described previously \(^{49}\). Briefly, a single T-650 carbon fiber (7µm diameter, Cytec Industries, West Patterson, NJ) was aspirated into a glass capillary tube (0.60 mm external diameter and 0.40 mm internal diameter, A-M Systems, Carlsburg, WA) and heat pulled with a micropipette puller (Narishige, Tokyo, Japan) to taper the glass to form two sealed microelectrodes. The carbon fiber extending beyond the glass seal was cut to approximately 100 µm under an optical microscope. A stainless steel lead with conductive silver paint (GC Electronics, Rockford, IL) was inserted into the capillary for electrical contact.

A.2.3 Data Acquisition.

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) supplied a continuous buffer flow of 1 mL/min across both the working and reference electrode. Two-second bolus injections of analyte were accomplished using a six-port HPLC valve and air actuator controlled by a digital valve interface (Valco Instruments Co., Inc., Houston, TX). All electrodes were conditioned prior to data collection by applying a triangular cyclic waveform (-0.4 V to +1.4 V versus Ag/AgCl) with a resting potential of -0.4 V at a scan rate of 400 V·s\(^{-1}\) and at a frequency of 60 Hz for ~15 minutes, followed by another 15 minutes of conditioning at 10 Hz. In some instances, this conditioning occurred after Nafion electrodeposition. Electrochemical measurements used the same waveform applied at a frequency of 10 Hz, as described previously \(^{50}\). Commercially available TH-1 software (ESA, Chelmsford, MA) was used with a DAC/ADC card (6251, National Instruments, Austin, TX), for waveform generation and data collection. A second National Instruments card (6711) was used for synchronization of waveform, data
collection, and electrical stimulation. Signal processing (background subtraction, signal averaging, and digital filtering) was software-controlled.

A.2.4 Nafion-Coating Protocols.

Prior to use, all bare carbon-fiber microelectrodes were soaked in filtered isopropyl alcohol purified with Norit A® activated carbon (MP Biomedicals, LLC, Solon, OH) for at least 30 min to remove surface impurities. Two different protocols were used to create the Nafion membrane: dip coating and electrodeposition. For the dip-coating procedure, the tip of the microelectrode was immersed in the Nafion solution (DE520, Ion Power, DE) for 5 min, allowed to air dry for 10-15 s, and then dried in an oven for 10 min at 70°C. For electrodeposition, the carbon-fiber microelectrode tip was lowered into the Nafion solution and a continuous potential (+0.5, +1.0, or +1.5 V vs. Ag/AgCl) was applied for 30, 60, or 90 s with a DC power supply (3B Scientific, Tucker, GA) to generate the Nafion layer. The electrodes were then allowed to air dry for 10-15 s, and subsequently dried in an oven for 10 min at 70°C. All electrodes were stored at room temperature. Prior to Nafion coating, some carbon-fiber microelectrodes were electrochemically conditioned with a triangular waveform (-0.4 V to 1.4 V vs. Ag/AgCl, 400 V/s) applied at a frequency of 60 Hz for 10 min, and subsequently at a frequency of 10 Hz for an additional 5 min.

A.2.5 Surgery.

Surgical procedures were performed as described previously. Briefly, adult male Sprague-Dawley rats weighing 250-300 g were purchased from Charles River Laboratories (Wilmington, MA). Rats were deeply anaesthetized with an intraperitoneal (i.p.) injection of sodium urethane (3 g/kg) and positioned in a stereotaxic frame (Kopf Instrumentation; Tujunga, CA). A heating pad (Harvard Apparatus, Holliston, MA) was used to maintain body temperature at 37 °C. Holes for electrodes were drilled in the skull according to coordinates from the brain atlas of Paxinos and
Watson. Working electrodes were placed in caudate-putamen (CPu, +1.2 mm anterior-posterior and + 2.0 mm medial-lateral relative to bregma, -4.5 mm dorsal-ventral from the skull surface). The Ag/AgCl reference electrode was placed contralateral to the working electrode. The bipolar stimulating electrode (Plastics One, Roanoke, VA) was placed above the medial forebrain bundle (MFB, -4.6 mm anterior-posterior and + 1.3 mm medial-lateral to bregma, - 8.5 mm dorsal-ventral from the skull surface). Biphasic stimulation pulses (60 Hz, 24 pulses, 150 µA, 2 ms per phase) were delivered to the MFB every 5 min to evoke DA release in the terminal region of the CPu. The working electrode was cycled as it was lowered into tissue, and the positions of the working and stimulating electrodes were optimized to maximize electrically evoked DA signaling. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the North Carolina State University.

A.2.6 In-Vivo Experimental Design.

Following electrode implantation, carbon-fiber microelectrodes were cycled at 10 Hz for 15 min to stabilize the background current. L-DOPA methyl-ester and benzerazide-hydrochloride, a peripheral DOPA decarboxylase inhibitor, were dissolved together in physiological saline. After baseline data collection, each animal received an acute treatment with L-DOPA methyl-ester/benzerazide cocktail at a clinically relevant dose (6 mg/kg + 10 mg/kg benzerazide, i.p.)25,55, followed by a higher dose (250 mg/kg + 400 mg/kg benzerazide, i.p.). These doses of L-DOPA methyl-ester are equivalent to 5 mg/kg and 200 mg/kg of L-DOPA, respectively 25. Data was collected for one hour after each drug administration.

A.2.7 Statistics.

All data are presented as the mean ± standard error of the mean (SEM), unless otherwise noted. Statistical and graphical analyses were carried out using GraphPad Prism 5 (GraphPad
Software, Inc., La Jolla, CA) or SPSS (IBM SPSS Statistics Software v. 17.0, Armonk, NY). The Student’s t-test was used to compare two groups. One-way analysis of variance (ANOVA) with paired samples t-test was used for post hoc determination of statistical differences between three or more groups across a single independent variable. When two classes of variables were compared, two-way ANOVA was applied with independent t-tests. Significance was designated at p<0.05.

A.3 Results and Discussion

A.3.1 The Effects of L-DOPA on Electrically Evoked Dopamine Release.

DA is normally synthesized from tyrosine in a two-step process. First, a hydroxyl group is attached to tyrosine by the enzyme tyrosine hydroxylase (TH), using oxygen, tetrahydrobiopterin, and Fe^{2+} as cofactors to produce L-DOPA^{56}. L-DOPA is then efficiently converted to DA by the enzyme L-amino acid decarboxylase (AADC), using pyridoxal phosphate as a cofactor. There are several physiological mechanisms by which exogenous L-DOPA can affect presynaptic DA release (for review see^{57}). Studies using single cells have demonstrated that L-DOPA is effectively loaded into the neuronal cytosol by the L-amino acid transporter^{58}. In primary cultures of murine substantia nigra neurons, L-DOPA treatment increases DA levels in the cytosol by > 100-fold^{59-60}. The DA is loaded into vesicles by the vesicular monoamine transporter (VMAT2). Amperometric recordings from these cells have demonstrated that the number of DA molecules released in single synaptic vesicle fusion events (termed quantal size) is increased from ~3,000 to ~10,000 DA molecules (~300%) in only 30 minutes^{61-62}. A similar increase in quantal size occurs in PC12 cells, and electron micrographs have shown that L-DOPA treatment also serves to increase the volume of individual dense core vesicles in these cells, in order to accommodate the additional DA^{63-64}. However, in healthy brain tissue L-DOPA treatment
could decrease evoked dopamine release by D2-mediated autoinhibition of DA release \(^{65}\). Additionally, the principal enzymes involved in DA synthesis, TH and AADC, are both regulated by DA autoreceptor-mediated second-messenger systems, such that DA synthesis is decreased when extracellular DA concentrations are increased \(^{66-68}\).

Initial experiments assessed the effects of L-DOPA administration on real-time striatal DA dynamics using bare carbon-fiber microelectrodes in intact Sprague-Dawley rats. Representative color plots, each containing 150 background-subtracted voltammograms, are shown in Figure 1A. These plots provide a representation of all changes in current collected across the entire potential window, enabling discrimination of specific electroactive species as they fluctuate over time \(^{69}\).

The left column shows DA dynamics elicited in response to electrical stimulation of the MFB (arrow) prior to any pharmacological manipulation. A current versus time trace extracted at the oxidation potential of DA (~0.6 V), and converted to concentration following post-calibration of the electrode, is shown in the middle of each panel. A cyclic voltammogram that serves to identify DA is also shown (lower panel). In this representative example, electrical stimulation elicited the release of 595 nM DA in the vicinity of the carbon-fiber recording electrode positioned in the dorsal striatum. Electrically-evoked DA release appeared to decrease to 482 nM after administration of L-DOPA (5 mg/kg, i.p. middle), and to decrease even further (to 292 nM) after a higher dose (200 mg/kg i.p. right) was administered. By contrast, administration of an identical volume of saline (vehicle control) did not have any significant effect on the evoked DA signal (Figure S1). Figure A.1B summarizes the entire data set (n = 5 animals) by plotting the concentration of electrically evoked DA release measured after administration of L-DOPA as a percentage of the baseline DA concentration. Twenty-five minutes after L-DOPA (5 mg/kg, orange arrow), electrically evoked DA release was significantly decreased from 529 ± 19 nM to
428 ± 24 nM (* p<0.05). This DA signal was further decreased to 362 ± 54 nM after 50 min. Subsequent administration of L-DOPA at a higher dose (200 mg/kg, red arrow) further decreased the amplitude of electrically evoked DA to ~45% of baseline (207 ± 33 nM, *** p< 0.001). However, it is important to note that large increases in extracellular L-DOPA are observed in the brain after systemic L-DOPA administration using microdialysis 70-72, and polymerization of L-DOPA could foul the electrode, confounding interpretation of the results.
Figure A.1 L-DOPA administration affects electrically evoked DA release recorded at bare carbon-fiber microelectrodes. (A) Representative data collected at a bare carbon-fiber microelectrode before L-DOPA administration (left column), 20 min after administration of L-DOPA (5 mg/kg, i.p., middle), and 50 min after administration of a higher dose (200 mg/kg, i.p., right). Top: Color plots depicting all changes in current (false color) collected over a 15-s window (x-axis) across all potentials (y-axis). Time of electrical stimulation is indicated by the black arrow. Middle: Current vs. time traces extracted at the oxidation potential of DA (+0.6V). Bottom: Cyclic voltammograms identifying DA. (B) Across the entire data set, there is a significant decrease in electrically evoked DA release upon acute L-DOPA treatment (n = 5, F(23,92) = 9.05, ****p<0.0001, one-way repeated measures ANOVA). Post-hoc analysis with a paired samples t-test demonstrated a significant decrease in dopamine release at specific time points (n = 5, *p < 0.05, **p<0.01, ***p<0.001).
A.3.2 Microelectrode Fouling by L-DOPA.

Background subtracted FSCV is a differential measurement that measures rapid changes in electroactive analytes at the electrode surface. As such, basal levels of DA and L-DOPA in brain tissue are not detected, even if high concentrations of these analytes are present. However, the catechol moiety inherent to both DA and L-DOPA is easily oxidized to the reactive o-quinone by the potentials used in this study \(^{13,73-74}\), and both molecules can also be enzymatically oxidized in the brain. As described above, the oxidized form can then immediately undergo a series of complex chemical polymerization reactions to produce melanin\(^{75-79}\). To investigate if the decrease in electrically evoked DA release measured at the bare carbon-fiber electrodes following L-DOPA administration (Figure A.1) could be attributed to fouling, electrode sensitivity to 1 μM DA was assessed \textit{in vitro}. Sensitivity was not compromised following exposure to 1 μM L-DOPA (Figure A.2A); however, exposure to 50 μM L-DOPA significantly attenuated sensitivity to DA (Figure A.2B, \(n = 6, *** p < 0.001\)). Indeed, Hefti et al. have reported a peak value of \(~100\ \mu M\) L-DOPA (20 μg/g) in the rat striatum after administration of a very high dose of L-DOPA (500 mg/kg) \(^{80}\). Thus, the decrease in evoked DA detected at bare carbon-fiber microelectrodes after a 200 mg/kg L-DOPA treatment suggests fouling of the electrode surface. If not taken into account, this significantly skews interpretation and quantification of \textit{in vivo} data.
Figure A.2. L-DOPA fouls bare carbon-fiber microelectrodes \textit{in vitro}. Normalized electrode response to 1 μM DA in the absence (black) and presence (grey) of (A) 1 μM or (B) 50 μM L-DOPA in the running buffer of the flow-injection apparatus. Sensitivity was significantly attenuated in the presence of 50 μM L-DOPA (n = 6, paired t-test, $$***$$ p<0.001).

A.3.3 Systematic Characterization of Nafion-Coating Procedure.

Nafion is a cation-exchange polymer. Thus, when coated on the carbon-fiber electrode surface it serves to increase sensitivity to positively charged species, such as DA at physiological pH 38-41, 43-44. It also decreases sensitivity to several negatively-charged species, including AA, which is ubiquitous in the brain 38. L-DOPA is a zwitterion at physiological pH. It is electrochemically active, with a voltammogram that looks identical to that for DA. Nafion is not effective at excluding L-DOPA when relatively low concentrations (1 μM) are investigated. However, Nafion is quite effective at excluding higher concentrations of L-DOPA (50 μM). These trends are demonstrated by the representative cyclic voltammograms presented in the upper panel of Figure A.3, which were collected \textit{in vitro} using bare and Nafion-coated carbon-fiber microelectrodes (Nafion electrodeposited at 1.0 V for 90 s onto electrochemically pretreated electrodes). The entire data set is summarized in the Table.
Figure A.3. The presence of a Nafion membrane significantly affects sensitivity to L-DOPA, DA, and AA. Upper panel shows representative cyclic voltammograms for bare (black) and Nafion-coated (gray) electrodes for L-DOPA (1 µM), DA (500 nM), and AA (200 µM). Lower panel: A summary of the entire data set. The values are averages ± SEM. (n = 3-9 electrodes per group, unpaired t-test, **p <0.01, *p <0.05).

The presence of a Nafion layer can be confirmed by reporting a sensitivity ratio for DA (the quotient of the sensitivities measured before and after coating). A large deviation from unity indicates the presence of a robust membrane. Figure A.4 presents normalized sensitivity ratios for DA. Electrodes were prepared using a variety of Nafion deposition procedures. The most straightforward protocol is dip coating. With this approach, the electrode surface is covered with Nafion by dipping the electrode in the polymer solution and evaporating the solvent. Alternatively, Nafion can be electrodeposited onto the carbon surface by application of a potential sufficient to generate an anodic current. The data demonstrate that coating cylindrical carbon-fiber electrodes by way of electrodeposition is more effective than a simple dip coating procedure. Thus, all subsequent studies presented herein utilized electrodeposition to generate a Nafion membrane.
Figure A.4. Systematic characterization of Nafion coating procedures. (A) Dip coating versus electrodeposition (F(2,40)=4.76, * p<0.05), (B) Electrochemical pretreatment before (blue) vs. after (gray) Nafion electrodeposition (F(2,36)=12.20, **p<0.01, ***p<0.001), (C) Electrodeposition potentials (F(3,33)=7.78, **p<0.01), (D) Electrodeposition times (F(3,51)=8.30, *p<0.05, **p<0.01, ***p<0.001), (E) Stability of coated electrodes. In panels A-D, data are presented as normalized DA sensitivity ratio (n = 6–17 electrodes per protocol, one way ANOVA with Tukey’s post-hoc test, * p<0.05; ** p< 0.01; *** p<0.001).

It is well established that electrochemical pretreatment of the carbon fiber surface can enhance electron transfer kinetics and significantly improve electrochemical performance by shifting the surface chemistry of the sensor to that which is present during use. Thus, carbon-fiber microelectrodes are commonly conditioned immediately prior to data collection.
has also more recently been established, using various spectroscopic techniques including X-ray photoelectron spectroscopy, \textsuperscript{88} thermal desorption mass spectrometry, \textsuperscript{89} enzyme-immobilized fluorescence microscopy, \textsuperscript{90-91} optical spectroscopy, \textsuperscript{92} and Raman spectroscopy, \textsuperscript{47} that application of potentials greater than \(\sim 1.3\) V can chemically alter, or even etch, the electrode surface. \textsuperscript{47-48, 87}

Thus, we hypothesized that electrochemical conditioning with a commonly used waveform (triangular, ranging from -0.4 V to 1.4 V vs. Ag/AgCl) before membrane deposition would improve adhesion of Nafion to the electrode surface. We quantitatively compared the performance of carbon-fiber microelectrodes that were conditioned before and after Nafion coating with microelectrodes that were electrochemically pre-treated and left bare. The data unequivocally demonstrate that Nafion electrodeposition (by application of 1.0 V for 30 sec) was most effective when electrodes were conditioned prior to the deposition procedure (Figure A.4B). Based on this, all subsequent protocols included electrochemical pretreatment of bare carbon-fiber microelectrodes prior to electrodeposition of Nafion.

Next, the potentials employed to electrodeposit the Nafion membrane were systematically investigated. Three potentials were selected: +0.5, +1.0 and +1.5 V vs. Ag/AgCl, as these have all been previously reported in the literature. \textsuperscript{41, 44, 93-94} The results indicate that +1.0 and +1.5 V were more effective than +0.5 V in generating a reliable membrane (Figure A.4C). However, as described above, the application of positive potentials can modify the surface of the carbon fiber, making it highly adsorptive and potentially slowing electrode response time, convoluting electrochemical performance. \textsuperscript{85, 87} Thus, 1.0 V (vs. Ag/AgCl) was selected for the electrodeposition of Nafion.

It is important to note that the Nafion membrane itself presents a diffusion barrier that can reduce electrode response time. To maintain the rapid temporal response required for detection of
neurotransmitter fluctuations *in vivo*, a thin Nafion layer is required. Indeed, previous studies have demonstrated that Nafion membranes can be generated such that electrode response times are not significantly different from uncoated microelectrodes. Figure A.4 shows that with our approach, an electrodeposition time of 90 s produced the best membrane performance in terms of sensitivity (Figure A.4D) and stability (Figure A.4E). With this approach, electrode performance was stable for at least two hours, presumably because the structural integrity of the Nafion coating (a perfluorosulfonated polymer) is derived from strong interactions between Nafion chains, rather than interactions between the Nafion and the carbon surface. This membrane did not significantly affect the sub-second response time of the electrode to a step change in the concentration of DA in a flow injection system (Figure A.5A), or to the stimulated secretion of DA in an intact brain (Figure A.5B). Finally, Nafion-coated electrodes prepared using different electrodeposition times were tested *in vivo*. Figure A.5C shows representative data collected in the striatum after administration of 5 mg/kg (orange arrow) and 200 mg/kg (red arrow) L-DOPA. These data suggest that the Nafion-coated electrodes prepared using electrodeposition times of 30 s (blue) and 60 s (green) did not resist L-DOPA induced fouling as efficiently as the Nafion-coated electrode prepared using an electrodeposition time of 90 s (brown), which was able to detect a robust increase in electrically-evoked DA release following L-DOPA treatment.
Figure A.5. DA concentrations recorded at Nafion coated electrodes. The optimized Nafion membrane (90 s electrodeposition time) did not affect the sub-second response time of the electrode when it was used (A) to record bolus injections of 500 nM DA in a flow cell, or (B) to record electrically evoked DA release in rat striatum. In both (A) and (B), the mean current (solid) ± SEM (dashed) is plotted for n = 6 electrodes. (C) Electrical stimulation was used to evoke striatal DA release every 5 minutes after L-DOPA administration. The x-axis shows the 2-h window of data collection. The y-axis represents the normalized amplitude of electrically evoked DA release collected using electrodes that were electrodeposited with Nafion for 30 (blue), 60 (green), or 90 (brown) seconds. Arrows indicate the time at which L-DOPA was administrated (orange: 5 mg/kg, red: 200 mg/kg).

A.3.4 The Effects of L-DOPA on Striatal DA Dynamics.

The optimized Nafion deposition procedure (electrodeposition using 1.0 V for 90 s) was used to investigate the effects of L-DOPA administration on electrically evoked striatal DA release. Representative color plots with corresponding DA concentration traces and cyclic voltammograms are shown in Figure A.6. These data demonstrate that DA release increased
across the entire data set (****p<0.0001, one-way repeated measures ANOVA). Post-hoc analysis with a paired samples t-test demonstrated that a clinically relevant dose of 5 mg/kg had no significant effect on electrically-evoked DA release as compared to baseline; however, L-DOPA administered at dose of 200 mg/kg significantly increased DA release (~200%), 25 min after drug administration (*p < 0.05). These data suggest that the regulatory mechanisms of intact animals are capable of controlling extracellular DA very efficiently in response to acute administration of a clinically relevant dose of L-DOPA (5 mg/kg). However, administration of a higher dose of L-DOPA (200 mg/kg) enhances DA overflow. This finding is consistent with previous studies using microdialysis, which report that striatal DA was increased by ~200% in intact animals following a 200 mg/kg or higher dose of L-DOPA \textsuperscript{10, 24, 95}. Importantly, the data collected using the Nafion-coated electrodes significantly contrast with the data collected using bare electrodes (Figure A.1 and re-plotted here in light orange and red). The differences between these data sets (gray bar, ++++p = 0.0001-0.05, repeated measures two-way ANOVA) confirm that a robust Nafion membrane is necessary to quantitatively report the effects of L-DOPA treatment on DA dynamics when using carbon-fiber microelectrodes and high concentrations of L-DOPA.
Figure A.6. The optimized Nafion membrane reveals that L-DOPA administration increases electrically evoked DA release. (A) Representative data collected at a Nafion-coated carbon-fiber microelectrode in an intact animal before L-DOPA administration (left column), 20 min after administration of L-DOPA (5mg/kg i.p., middle), and 20 min after administration of a higher dose of L-DOPA (200 mg/kg i.p., right). (B) When using Nafion-coated electrodes for the measurements, L-DOPA administration increased the amplitude of electrically evoked DA release overall (n = 4, bright orange and red trace, F(23,69) = 2.880, ****p < 0.00001, one-way repeated measures ANOVA). Post-hoc analysis with a paired samples t-test demonstrated a significant increase 25 minutes after administration of 200 mg/kg L-DOPA (*p < 0.05). Data collected using bare electrodes are also shown (n = 5, light orange and red trace) to enable direct statistical comparison of electrode performance in vivo. The differences between the two data sets are significant at several time points (gray boxes, repeated measures two-way ANOVA, all main and interaction effects, F(1-23, 7-161) = 2.196 – 54.432, +++p = 0.00001- 0.05).
A.4 Conclusions

Overall, the data indicate that when systemically treating with high doses of L-DOPA, a carbon-fiber microelectrode implanted in the brain is easily fouled, resulting in decreased sensitivity to DA. This is likely due to the polymerization of catecholamines on the electrode surface. By refining the parameters by which a Nafion coating is applied to the electrode, we have advanced a technique for generating a carbon fiber microelectrode that maintains a rapid electrode response time and is less sensitive to fouling. The improved performance of these electrodes allows a better measure of L-DOPA augmented DA release in vivo when using FSCV. With this approach, an acute dose of 5 mg/kg L-DOPA had little effect on DA release in healthy striatal tissue, consistent with several reports in the literature that have used microdialysis measurements or mathematical modeling to demonstrate that DA terminals play a crucial role in the clearance of extracellular DA formed from exogenous L-DOPA in healthy striatum. In contrast, administration of 200 mg/kg L-DOPA was capable of significantly increasing evoked DA release.

A.5 Acknowledgements

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A.6 Associated Content

Supplemental Figure A.6.1.
Figure A.7 Saline administration does not affect the amplitude of evoked dopamine release. Bare (black, n = 1) or Nafion coated (gray, n = 1) electrodes were used to monitor DA release in rat striatum evoked by electrical stimulation of midbrain DA neurons every 5 minutes after an i.p. infusion of saline. The x-axis shows the 60 min. window of data collection. The y-axis represents the normalized amplitude of DA release.
A.7 References


30. Azari, S.; Zou, L. D., Using zwitterionic amino acid L-DOPA to modify the surface of thin film composite polyamide reverse osmosis membranes to increase their fouling resistance. *Journal of Membrane Science* 2012, **401**, 68-75.


APPENDIX B Supplemental Information to Chapter 4

T.1 Supporting Information

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**Table T1.** Quantitative measurements of [DA]_{max}, [Lac]_{max} and time to reach [Lac]_{max} at each stimulation condition investigated.

T.2 Supplemental information

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**Table T2.** Quantitative measurements of [Lac]_{max}, [Gluc]_{max}, and time to reach each throughout all stimulation conditions investigated.