ABSTRACT

WILSON, LESLIE RAE. Studying Oxidative Stress: Real-Time Detection of Hydrogen Peroxide and Dopamine Dynamics in the Brain. (Under the direction of Dr. Leslie A. Sombers).

Parkinson’s disease (PD) is a neurodegenerative disease characterized by the slow degeneration of dopaminergic neurons found in a region of the midbrain called the substantia nigra. Dopamine (DA) plays a key role in regulating motor function. Thus, the destruction of these neurons and the consequential decrease in DA concentrations in the striatum leads to the deterioration of motor control. The drug Levodopa has been used to treat PD by helping to increase the concentration of DA in the brain. This drug has been proven to alleviate the motor symptoms of PD; however, after a short period of time, L-DOPA-induced dyskinesias (L.I.D.s), or abnormal involuntary movements, can develop. It is thought that oxidative stress is a principal contributor to the destruction of dopaminergic neurons, and possibly to the development of dyskinesias, in PD and its treatment. Hydrogen peroxide (H₂O₂) can serve as an indicator of the presence of more aggressive reactive oxygen species in brain tissue; but few analytical techniques provide adequate temporal and spatial resolution to monitor dynamic fluctuations of these molecules. Recent studies in the Sombers’ lab have described the real-time in vivo detection of this analyte using fast-scan cyclic voltammetry at carbon-fiber microelectrodes.

The work presented in this dissertation furthers our understanding of H₂O₂ and DA dynamics in the striatum, as well as their role in L.I.D.s. First, a new analytical sensor was developed and characterized to aid in the selective detection of H₂O₂. This sensor allows a reliable signal for H₂O₂ while excluding larger molecular endogenous as well as pharmacological interferents. Next, investigations into the neurochemical contributors to
L.I.D.s revealed a role of oxidative stress in modulating nigrostriatal DA signaling throughout prolonged daily administration of L-DOPA to hemiparkinsonian rats. After 1-week, excessive swings in DA and H$_2$O$_2$ were recorded in hemiparkinsonian rats in the hours after L-DOPA administration. These fluctuations were no longer apparent after 3 weeks of L-DOPA treatment. Additionally, it was determined that with the onset of involuntary rotational bouts of lesioned rats, the signal for H$_2$O$_2$ increases while the DA signal decreases after 3 weeks of L-DOPA treatment. To further investigate the neurochemical sources underlying these signals, pharmacological manipulations of the serotonergic and mitochondrial pathways were performed. Blockade of serotonergic transporters in hemiparkinsonian rats resulted in attenuation of L.I.Ds as well as excessive concentrations of H$_2$O$_2$ and DA recorded during hours after L-DOPA administration. Pharmacological manipulation of mitochondrial-derived H$_2$O$_2$ attenuated the H$_2$O$_2$ signal in the lesioned striatum while increasing the total number of $360^\circ$ rotations in hemiparkinsonian rats administered L-DOPA. Overall, results show a dynamic interplay of DA and H$_2$O$_2$ dynamics, which are time-locked with specific dyskinetic behaviors. Results from this dissertation hope to guide future research regarding the manifestation of dyskinetic behaviors in PD, as well as improve the development of therapeutic strategies to reduce oxidative stress and DA dysfunction.
Studying Oxidative Stress: Real-Time Detection of Hydrogen Peroxide and Dopamine Dynamics in the Brain.

by
Leslie Rae Wilson

A dissertation submitted to the Graduate Faculty of
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APPROVED BY:

____________________
Dr. Leslie Sombers
Committee Chair

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Dr. Edmond Bowden

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Dr. Gufeng Wang

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Dr. John Meitzen
DEDICATION

Dedicated to Blanche Adele Horne, my grandmother who suffered from a debilitating neurodegenerative disease. You had an amazing way of standing up for what you believed, even if meant standing alone.
BIOGRAPHY

Leslie Rae Wilson was born in Salt Lake City, Utah, on November 2, 1986. As a child, she was reserved and quiet, but she was also very observant of ongoing events around her. Her ability to observe events with an introspective view, while holding off of making quick judgements, is an attribute that was with her as a child and still defines her character to this day. Leslie is currently in Dr. Leslie Sombers’ research laboratory where she is expected to receive her Ph.D. in Analytical Chemistry (Fall 2018) from North Carolina State University, Raleigh, NC. Her research focus is on the measurement of oxidative stress within the brain using electroanalytical approaches to monitor real-time neuro-chemical dynamics for neurodegenerative diseases. Her Master’s in Chemistry, from Western Carolina University (2011), concentrated on the quantification of arsenic and antimony isotopes utilizing an ICP-MS to trace sources of pollution in the Rio Loa River Basin, northern Chile. Leslie earned her B.S degree in Environmental Chemistry (2009) at the University of Mary Washington, located in Fredericksburg, Virginia. Her work as an undergrad included an extensive research project quantifying trace metals and organic pollutants on aquatic wildlife due to mining operations within the piedmont region of central Virginia.

Even as a young adult, Leslie was concerned with making sure there were facts to backup events that needed explanations. She prided herself with being careful about judgment, even it meant gathering more information before she came to a conclusion. Her research reflects this trait, and as a result, she has been asked to present her findings to numerous conferences and meetings. The Pittsburg Conference, the Society for Neuroscience, and the American Chemical Society, are just to name a few of the many conferences she has attended as a presenter. She has also been an invited speaker for numerous student and research
symposiums, the Triangle Society for Neuroscience, The Indiana University Animal Behavior Conference, and Brain Awareness Night at the NC Museum of Natural Sciences. Some of her research work while with Dr. Sombers has been published in Analytical Chemistry. Leslie has obtained awards for her findings and presentations, which she is happy to accept only with the acknowledgement of those that helped her along the way. Some of these include the Sigma Xi Award for Excellence in Graduate Research, The NCSU Graduate Research Symposium Poster Award, the Comparative Medicine Institute Award for Chemistry at the Interface of Medicine, the Triangle Society for Neuroscience Poster Award, and the Western Carolina University Dean’s Outstanding Scholar Award.

In addition to performing quality science, Leslie has a gift for helping others, especially through teaching. During the years while pursuing her education, she has had many opportunities to pass on her knowledge and enthusiasm for science. Her love for teaching and her adherence to detail when it comes to science, makes her a natural mentor and teacher. She has received tremendous satisfaction while sharing her love of science with others and mentoring younger scientists as they make their start in the field. Leslie has been recognized with teaching awards for her contribution towards making a difference in education. This recognition, and the consequent awards, include the first recipient of the Early Mentoring Award from the Undergraduate Research Student Advisory Council, the Miles F. Anderson Award for Excellence in Teaching, the University Graduate Student Association Award for Excellence in Laboratory Teaching, a Certificate of Accomplishment in Teaching (CoAT), a Foundations in Teaching (FIT) Certification, and the Graduate Assistant Teaching Experience (GATE) Certification.
As a person that intends to make a difference in the world of science and research, Leslie is an active member of several societies, including the Triangle Society for Neuroscience (SfN), Triangle Electrochemical Society (ECS) Student Chapter, W.M. Kick Center for Behavioral Biology, Chemistry Graduate Student Association (CGSA), and the North Carolina American Chemical Society. She holds several officer positions within these organizations, as well as having been a judge at the NC State Science Fair, and a field trip volunteer for Science, Technology, Engineering, and Mathematics (STEM). Her support and leadership in these organizations helps maintain awareness in the sciences, which allows students and upcoming scientists to develop a relationship with industry and their community.
ACKNOWLEDGMENTS

This work is dedicated to those that helped me through the many research hours I spent collecting and analyzing data; those that helped me with the initial writing; and those that have mentored me through preparing this dissertation. A special thanks goes to Catherine Mason and Sambit Panda, two outstanding undergraduate students who assisted with the data analysis and preparing electrodes, while also giving me the strength to continue through many difficult times. Catherine and Sambit came to me as freshman, and because of their passion for and dedication to neuroscience, they are developing into great scientists. I was encouraged by their engagement with the science, which was rewarded by watching them win awards and attending local and international conferences. It was a pleasure to help them to further their own careers, and mentoring them had the added bonus of their friendship. In addition, my colleagues and friends, Christie Randall, Karen Butler, Lingjiao Qi, and Xiaohu “Tiger” Xie, have taught me research skills, helped me become a better writer, and kept me motivated throughout the long hours in the lab. I also want to thank my parents, Dr. Mark and Glenda Wilson, who instilled a love for science in me at a young age and brought the gift of curiosity into my life.

I would like to acknowledge the Chemistry Department at North Carolina State University for awarding me with a Chemistry Scholars Graduate Research Assistantship, which gave me a stipend and aided in my research funding. I’d also like to thank the Keck Center for Behavioral Biology for providing me with a travel award, which allowed me to present this research at the Monitoring Molecules in Neuroscience International Conference, held in Gothenburg, Sweden, during the summer of 2016. Additionally, I’d like to thank the collaborators in this dissertation; Drs. David Muddiman from NC State and Peter Wipf from University of Pittsburgh who have contributed their knowledge, instrumentation, and expertise.
to advance the findings of this work. Also, thank you to my committee members, Drs. John Meitzen, Ed Bowden, and Gufang Wang. Your guidance and mentorship was instrumental in my development, and provided me with not only technical knowledge but also the much needed wisdom and perspective necessary for a successful scientific career.

As a final note, I’d like to thank my advisor, Dr. Leslie Sombers. The accomplishments in this dissertation, and at NC State, would not have been possible without her guidance and encouragement. Her continued attention to my research and its progress was an invaluable asset. The knowledge she shared while designing experiments and performing analysis methods, contributed greatly to the completion of this work. I will always be grateful for her support throughout my time in her lab. She believed in me, gave me the necessary tools to succeed in research, and launched me into an exciting career as a scientist.
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1.1 Anatomy of a Neuron

Neurons are specialized cells that are electrochemically excitable and can transmit information to other cells through molecules called neurotransmitters\(^1\). Different neuronal cells in the central nervous system (CNS) release different types of neurotransmitters. Some neurons that are commonly studied are: (1) gamma aminobutyric acid (GABA), (2) cholinergic, (3) glutamatergic, (4) dopaminergic, and (5) serotonergic neurons. A typical neuron consists of three basic parts; the cell body, axon, and synaptic terminal (Figure 1.1). The cell body is where the nucleus, mitochondria and other cell organelles are located. Cell bodies can have long extensions which extend (axon) and branch (dendrite), communicating with other neurons in the CNS through contacts with other neurons, called synapses. In the CNS, the cell body transmits a signal by propagating the action potential, a quick change in the neuronal membrane potential, through its axon and ultimately releasing its neurotransmitters.

Figure 1.1: Anatomy of A Neuron. Typical neuron showing cell body, axon, and synaptic terminal. At the synaptic terminals, neuron’s communicate with each other by translating electrical signals from action potentials into chemical signals called neurotransmitters.
1.2 Action Potentials

Action potentials are electrochemical events where the potential difference across a neuronal cell membrane can rapidly rise and fall (Figure 1.2). Maintaining the appropriate voltage gradients across the membrane of the neuron is necessary to facilitate action potentials. In general, the resting potential of the neuron is maintained by the metabolically driven Na+-K+ pumps. These pumps bring extracellular K+ ions into the cell while taking Na+ ions out of the cell. Charged ions in the cytosol can cause the resting potential to shift to a more positive threshold value which causes the cell to depolarize. Depolarization causes an electrochemical impulse that is generated across the membrane where voltage-gated Na+ channels rapidly open, and there is a rapid influx of Na+ ions. At the same time, voltage gated K+ channels open, allowing an efflux of K+ from the cell. This is the rising phase of the action potential. To return to the resting potential the Na+ channels rapidly close, while the K+ channels slowly close, causing the cell to repolarize and even hyperpolarize, to a point below the resting membrane potential. This stage is called the refractory period and it keeps the neuron from firing another action potential too quickly. The action potential propagates down the axon through rapid membrane depolarization to synaptic terminals. At the terminal, action potentials facilitate the release of neurotransmitters into the extracellular space, typically across the synaptic cleft.
Figure 1.2: Action Potential as it would Appear on an Oscilloscope. The shape is due to rapid depolarization and repolarization from ion channels opening and closing.

1.3 Neurotransmission

A synapse is the space between neurons typically about 10-20 nm wide, which allows neurons to communicate with one another through brain chemicals (Figure 1.3). Neurotransmitters can range from small amine molecules to large peptides. Generally, these chemicals are stored inside the neuron within vesicles, and upon a depolarization event, the vesicles fuse with the terminal membrane thereby releasing into the synaptic space, and bind to selective protein receptors located on the postsynaptic neuron. When a neurotransmitter binds to its receptor it can elicit a physiological response in the postsynaptic neuron’s membrane, thus initiating the transduction of information between two neurons. Some neurotransmitters are excitatory, and elicit action potentials from the postsynaptic neuron, while some are inhibitory and decrease the probability of an action potential happening. It should be noted however, not all neurotransmitters proceed to bind to receptors. The released molecules may diffuse away from the synapse, where they can be taken back up into the presynaptic terminal through transporters and essentially recycled to be re-packaged into vesicles. Moreover, some are broken down by enzymes located in the synaptic space. In addition to
neurotransmitters, there are neuromodulators in the cytosol that regulate the release and actions of neurotransmitters. One example of a type of neuromodulator is H$_2$O$_2$, which is believed to regulate DA release$^{2-10}$.

There are many ways to pharmacologically manipulate neurotransmitter release extracellular concentrations, and receptor responses. Manipulation of these biological processes is necessary for neurochemical research investigating neurodegeneration and drug addiction. Researchers can administer compounds with a variety of receptor-mediated mechanisms into a brain region to either facilitate and/or inhibit the cell’s physiological response. Certain drugs can act on receptors of interest. Specifically, receptor agonists bind to a selective type of receptor and can facilitate a physiological response while receptor antagonists act to block that particular response. Pharmacological agents that prevent a process related to neurotransmission are inhibitors. For example, the action of endogenous vesicular monoamine transporter (VMAT), which is necessary for catecholamine storage, can be inhibited by a pharmacological agent reserpine. Additionally, specific inhibitors can bind to enzymes or mitochondrial complexes of the electron transport chain and block their activity. Both of these particular targets, DA neurotransmission and mitochondrial function have been implicated as potential contributors to oxidative stress in Parkinson’s disease (PD).
Figure 1.3: Chemical Synapse Showing Pre-synaptic Terminal and Postsynaptic Neuron. Neurotransmitters are synthesized and the neuron releases the neurotransmitters into the extracellular space. Here, the neurotransmitters can bind to protein receptors on the postsynaptic neuron, which can cause a cascade of signaling processes. Neurotransmitters that do not bind to receptors are removed from the synapse either from reuptake by the pre-synaptic neuron or degrading enzymes.

1.4. Basal Ganglia and Parkinson’s disease

From the cortex of the brain, an organism can initiate body movements. Mid-brain and cortex neurons project to different brain regions, termed the basal ganglia, which make up the motor loop in the brain. Ultimately interactions of signals along the pathways of these neurons either initiate movement through a direct pathway, or inhibit motor output through an indirect pathway. The basal ganglia are mainly comprised of five brain structures; (1) substantia nigra pars compacta (SNpc), (2) dorsal striatum, (3) globus pallidus, (4) thalamus and (5) subthalamic nucleus. In rats, the dorsal striatum is made up of a fused caudate nucleus and putamen nuclei (Figure 1.4), although these areas are separated in humans. Excitatory, inhibitory, and modulatory synapses through these five brain regions make up the motor loop.
Figure 1.4: The Basal Ganglia Located Within the CNS. The excitatory, inhibitory, and modulatory synapses send signals that ultimately go to the spinal cord and initiate or inhibit motor function.

Regulation of motor function through the basal ganglia is complex and prone to complications and disorders. PD is a neurodegenerative disease that which affects approximately 1 million people in the United States\textsuperscript{12}. This neurological disorder leads to significant, debilitating motor and cognitive symptoms. PD is marked by dopaminergic cell death at the SN\textsubscript{pc} which is the dopaminergic source innervating the dorsal striatum (nigrostriatal pathway). The nigrostriatal pathway is involved in maintaining normal functions including sensorimotor integration, habit learning, and motor functions. PD patients are typically diagnosed when they exhibit the cardinal hypokinetic symptoms and resting tremor; regrettably at a point of no return as \sim 80\% of the DA innervation to the striatum has already been lost. Due to overall reductions in nigrostriatal DA, administration of levodopa (L-3,4-dihydroxyphenylalanine; i.e. L-DOPA), the metabolic precursor to DA, is used to treat PD as it can be enzymatically converted to DA. Despite the initial alleviation of Parkinsonian
symptoms, after 4-6 years about 40% of patients on L-DOPA will develop periodic abnormal movements\textsuperscript{13}, or L-DOPA induced dyskinesias (L.I.D.s).

Eventually, nearly all patients on L-DOPA acquire these involuntary motor complications, which can be as debilitating as PD itself, thereby limiting the long-term therapeutic benefit of this drug. Many studies have demonstrated that slow variations (minutes) in striatal DA release contribute to locomotion\textsuperscript{14}. However, the role of phasic DA in motor control has not been well defined, but these fluctuations could play a major role in regulating this circuit\textsuperscript{15}. Moreover, indirect evidence suggests that the effects of L-DOPA treatment could be due, in part, to oxidative stress resulting from the strain put on the remaining DA neurons in metabolically converting excess L-DOPA to DA\textsuperscript{16-18}.

1.5 Oxidative Stress

Reactive oxygen species (ROS), such as the oxygen radical (O\textsubscript{2}\textsuperscript{-}), nitric oxide (NO\textsuperscript{-}), and the hydroxide radical (\cdot OH), are byproducts of cellular metabolism under normal physiological conditions\textsuperscript{19}. Increased ROS concentrations can cause neuronal cell death and, over time, contribute to neurodegenerative disorders such as PD\textsuperscript{20-22}. Unfortunately, normal mitochondrial function can be disrupted by either idiopathic or genetic factors\textsuperscript{23}, which increases ROS formation in the cell, causing oxidative stress. Direct measurement of many ROS is complicated due to the instability and relatively low concentrations of these species. Although measurement of H\textsubscript{2}O\textsubscript{2} can serve as a measurement of oxidative stress, as O\textsubscript{2}\textsuperscript{-} is converted to H\textsubscript{2}O\textsubscript{2} by the enzyme superoxide dismutase (Figure 1.5). Quantification of H\textsubscript{2}O\textsubscript{2} is promising because it can accumulate to relatively high concentrations\textsuperscript{5,24}, is more stable than O\textsubscript{2}\textsuperscript{-}, and is electroactive.
Oxidative stress is implicated at various time-points during the progression of PD, including the initiation and maintenance of this disease state\textsuperscript{19,25}. Major research emphasis has been focused on the nigrostriatal DA system because it is particularly prone to oxidative stress for several reasons:

1. In DA neurons, H\textsubscript{2}O\textsubscript{2} is formed in mitochondrial metabolism, but additional H\textsubscript{2}O\textsubscript{2} may arise from byproducts of DA oxidation or deamination by monoamine oxidase (MAO) activity\textsuperscript{26}. Further, MAO concentrations are high in the SN and numbers of the MAO isoform increase with age\textsuperscript{26}.

2. Multiple studies have shown the accumulation of iron in the SNpc as well as striatum of PD patients. Moreover, iron, in combination with DA, can cause neurotoxic forms of DA through redox reactions\textsuperscript{27}. This is typically accelerated by the Fenton reaction. The Fenton reaction, the decomposition of H\textsubscript{2}O\textsubscript{2} through iron-salt dependence, can play a role in generating the destructive ROS, \textbullet OH, when H\textsubscript{2}O\textsubscript{2} comes into contact with ferrous iron (Fe\textsuperscript{2+})\textsuperscript{28,29} (Reaction 1).

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-
\]

Reaction 1.1: The Fenton reaction.

3. Patients with PD have decreased concentrations of the antioxidant glutathione, and the severity of this depletion increases with the disease severity\textsuperscript{30-33}. This contributes to a vicious oxidative cycle which targets these specific DA neurons in the nigrostriatal pathway.

4. New evidence suggests that metabolically driven L-type calcium channels can produce oxidative stress due to the relatively high firing rate of DA neurons\textsuperscript{18,34}. 

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-
\]
Currently, no direct evidence pinpoints to one of these theories as the root cause of PD. Although H$_2$O$_2$ serves an indicator of oxidative stress in the CNS, endogenous H$_2$O$_2$ is also important for normal physiological function. Evidence suggests that H$_2$O$_2$ can modulate vesicular release of neurotransmitters, specifically DA in the nigrostriatal DA system which has been demonstrated to be through adenosine triphosphate (ATP) sensitive potassium channels. To date, little is known about the real-time dynamics of both H$_2$O$_2$ and DA at a single location due to previously employed methodologies lacking temporal, spatial, and chemical selectivity. Millisecond temporal resolution is especially important when correlating specific behavioral abnormalities, such as motor movements, with quantitative measurements of neurotransmitter release. Additionally, it is unknown whether L-DOPA treatment for PD could further the oxidative stress induced neurodegeneration of the already impaired DA neurons. To help answer these questions, a detection method is needed that operates on a level commensurate with the real-time biology. The technique employed must be able to:

1. Provide chemical selectivity for the simultaneous quantification of both H$_2$O$_2$ and DA.
2. Offer spatial resolution sufficient to target specific brain regions such as the SN or dorsal striatum.
3. Provide temporal resolution on the order of milliseconds to measure transient H$_2$O$_2$ and DA fluctuations and relate them to behavioral observations.
Figure 1.5 Mitochondria and Oxidative Stress. Under normal physiological conditions, mitochondria produce O$_2^\cdot$ radicals, which are quickly broken down superoxide dismutase into H$_2$O$_2$. The H$_2$O$_2$ is further broken down by either catalase or glutathione peroxidase into water and molecular oxygen. Oxidative stress can occur due to mitochondrial dysfunction that increases the formation of O$_2^\cdot$ radicals, herein increasing H$_2$O$_2$ after the dismutation of O$_2$.

1.6 Common Methods to Measure Oxidative Stress

Few analytical methods have been developed for dynamic measurements of ROS in vivo. Fluorescence imaging offers low limits of detection, but lacks chemical selectivity across all ROS species and has limited dynamic range$^{19,35,36}$. Additionally, this method lacks a useful way to calibrate the fluorescent dyes, excluding their use for quantitative analysis$^{37}$. A second analytical method to measure ROS or DA concentrations is microdialysis. A microdialysis probe is coupled with spin trap agents to collect ROS molecules, which are then separated offline by high-performance liquid chromatography (HPLC) and specific analyte concentrations are determined using an electrochemical detector$^{38}$. Microdialysis, coupled with chemical separation and detection instrumentation, allows for good chemical selectivity for many types of neurotransmitters$^{39}$, but it is a diffusion-based (steady-state) technique which suffers from slow temporal resolution, typically in the range of minutes to seconds at best. Additionally, the microdialysis probe is on the order of 0.5-1mm in diameter and 3-4mm in
length, offering extremely low spatial resolution because it can extend across multiple brain regions and cause damage in tissue of the sampling region.

Electrochemical methods are perhaps the most promising analytical tools available to measure ROS and DA dynamics in the brain. These methods offer excellent temporal resolution and the sensors can be made small, on the μm scale\textsuperscript{40}, allowing specific brain regions to be targeted. Electrochemical methods that employ ultramicroelectrodes (μm scale) have a small ohmic drop, obliterating the need for an auxiliary electrode, where a two electrode set-up containing a reference electrode, typically Ag/AgCl, and a working microelectrode is employed. Redox reactions of analytes occur at the working ultra-microelectrode, which creates a current that is measurable using specialized electronic instrumentation. One of the most common electrochemical methods utilized in neuroscience is amperometry\textsuperscript{40}. In this method, the working microelectrode is held either above the oxidation potential or below the reduction potential for the species of interest. When an analyte comes into contact with the electrode surface, a redox reaction occurs and the current is measured. An amperogram, which is a graph of current vs. time, is obtained. By using Faraday’s law, quantification is possible by integrating of the current with respect to time. This law states that the flow of electrons is directly proportional to the amount of charge generated (Equation 1)\textsuperscript{40}.

\[ Q = nNF \]

**Equation 1: Faraday’s Law.** The letter Q is the charge in Coulombs, n is the number of electrons transferred per analyte, N is number of moles of analyte reacted, and F is Faraday’s constant (96,485.34 C/mole).

Although temporal resolution is excellent, one major drawback of using amperometry is the sampling environment of the brain is extremely chemically complex, which contains
multiple electroactive species. While using amperometry, it is difficult to distinguish specific electroactive contributors to the signal; and thus, a lack of chemical selectivity as a result.

1.7 Fast-Scan Cyclic Voltammetry

Fast-scan cyclic voltammetry (FSCV) is an analytical technique frequently used with carbon-fiber microelectrodes for molecular monitoring in brain tissue. This method offers both quantitative and qualitative analysis on a millisecond time scale. Typical FSCV scan rates exceed 100 V/s. These fast scan rates produce a large background current that can be 100 times larger than current collected from processes involving the faradaic electron transfer from neurochemical analytes. The background current is relatively stable, and can be subtracted out, making FSCV a differential technique ideal for detecting the phasic release of neurotransmitters. As such, only concentration changes are quantified, absolute concentrations are not measured. Carbon fibers are used with FSCV due to their fast electrochemical properties and resistance to biofouling. Typically, an ultra-microelectrode used in FSCV consists of one cylindrical carbon-fiber that is 7µm in diameter cut to ~100µm in length. FSCV can be used to study many neurochemicals such as DA, norepinephrine, epinephrine, homovanillic acid, 3,4-dihydroxyphenylacetic acid (DOPAC), L-DOPA, serotonin, adenosine, ascorbic acid, uric acid, and \( \text{H}_2\text{O}_2 \). Through utilization of FSCV, many of these neurochemicals can be quantified simultaneously.

The goal of this work is to quantify oxidative stress and the influence it has on DA dynamics at a single location in vivo with FSCV. An electrode can be placed into the SNpc or the dorsal striatum to simultaneously detect the release of both DA and \( \text{H}_2\text{O}_2 \). A triangular waveform consisting of a forward scan from -0.4 V to +1.4 V, and a reverse scan from +1.4 V to -0.4 V is applied at a frequency of 10 Hz every 100 msecs at 400 V/s. During the forward
scan, if both DA and H$_2$O$_2$ are within the vicinity of the microelectrode surface, DA will oxidize to dopamine-ortho-quinone at +0.6 V and H$_2$O$_2$ is oxidized to O$_2$ and two protons at +1.4 V. A voltammogram is a plot of the current generated vs. the applied potential. Inherent differences in the formal potentials (E$^\circ$) of various analytes are observed on the voltammogram as separate peaks, allowing qualitative identification of electroactive analytes. No cathodic current is observed for the reduction of O$_2$ because the reverse scan does not reach a potential sufficiently negative for reduction to occur. Therefore, in these experimental conditions the oxidation of H$_2$O$_2$ is an irreversible process. A resulting voltammogram is obtained from these redox processes which can both identify and quantify DA and H$_2$O$_2$ concentrations (Figure 1.6). One way of observing all of these voltammograms collected over time is with a three dimensional color plot which unfolds and concatenates the voltammograms. Time is plotted on the X-axis, potential on the Y-axis, and current in false color on the Z-axis (Figure 1.7). Dynamic information can be obtained from the color plot by extracting a horizontal line through the data at a desired potential. Qualitative information can be obtained by extracting a vertical line through the data at a desired time point.
Figure 1.6: DA and H$_2$O$_2$ simultaneous detection using FSCV. A potential is applied to a carbon-fiber microelectrode from -0.4 V to +1.4 V. When faradaic analytes are present within the vicinity of the electrode surface, they will undergo redox reactions and generate a voltammograms, where the x-axis is the potential applied to the electrode and the y-axis is current collected from faradaic analytes near the electrode surface.

Principal component regression (PCR) is a statistical approach that can be utilized to reduce the dimensionality of the data when multiple sources are contributing to an electrochemical current$^{43}$, enabling individual chemical contributions to be extracted from the complicated signal. PCR can be thought of as a combination of both principal component analysis (PCA) and inverse least-squares regression. PCA accounts for variability in the data and assigns principal components (PC), which describes the data in fewer variables than originally present. The first PC is the direction through the data which describes the most variability, for example the peak current when running a standard of DA. The following PCs are orthogonal to one another and describe the amount of variability remaining in the data. Ultimately, only PCs that describe relevant information (as detected by an F-test) are retained and given scores. Unknown concentration values can be calculated through regression analysis (Equation 2)$^{44}$ where $A$ is the voltammogram of the unknown to be predicted, $F$ is the
regression coefficient which relates unknown concentration values of each analyte to the relevant PCs, and V is a matrix of the relevant PCs (superscript T means matrix is transposed). PCR works well as an analytical chemometric tool when the voltammograms of target analytes are distinctly different, such as DA, pH-shifts, and H₂O₂. However, this approach can be complicated when voltammograms of separate analytes have similar oxidation peaks.

**Figure 1.7: Generation of a three-dimensional color plot.** Cyclic voltammograms are unfolded at the switching potential and aligned chronologically. They are then concatenated into a color plot where time is on the x-axis, potential is on the y-axis, and current is plotted in false color.

\[
C_{unk} = FV^T_A_{unk}
\]

**Equation 2:** Equation used for PCR prediction of neurochemical concentrations.

1.8 Electrode Fabrication and Insulation Techniques

Chronic electrodes (fused-silica insulated carbon-fiber microelectrodes) are another tool that has advanced the use of FSCV for *in vivo* measurements. Long term measurements of DA and H₂O₂ have proven difficult using glass-insulated carbon-fiber microelectrodes because this type of electrode is fragile and it must be removed after every acute experimental
session. Recent developments have enabled the use of fused-silica insulation. This method is particularly advantageous because silica-insulated carbon-fiber electrodes can be permanently implanted in the brain to allow stable detection of analytes across time (weeks to months) at one spatially discrete recording site. The carbon fiber used to make glass and chronic microelectrodes is the same, which results in comparable performance in H$_2$O$_2$ detection (Figure 1.8; $t_0=0.02427$, $P = 0.98$; $n=6$ electrodes). Chronic electrodes are also sufficiently robust enough to be fabricated together to allow for recording on two separate channels less than 200 µm apart. We have termed these devices “dual-microelectrode devices” (DMEDs). The fabrication with silica insulation also allows the electrode to be easily attached to a steel microinjection cannula positioned less than 100 µm away, which will allow neuroscientists to microinject various drugs right next to the recording electrode(s).

![Figure 1.8](image)

**Figure 1.8: Chronic carbon-fiber microelectrodes.** (A) Scanning electron microscopy (SEM) image of chronic microelectrodes. (B) Chronic electrodes have comparable sensitivity to H$_2$O$_2$ as glass insulated electrodes (silica= 0.18 ± 0.01 nA/µM, glass= 0.227 ± 0.007 nA/µM).
1.9 Research Overview

The goal of the research performed in this dissertation was to utilize FSCV to further our current understanding of oxidative stress in the CNS, how it modulates DA in the striatum, and how it is implicated in the onset and progression of L-DOPA induced dyskinesias (L.I.D.s). First, a sensor was designed for the selective detection of H$_2$O$_2$ to accurately and reliably quantify H$_2$O$_2$ when the signal is complicated by endogenous or pharmacological interferents that share a similar oxidation potential. Next, hemiparkinsonian rat models were used to investigate the effects of L-DOPA treatment on DA and H$_2$O$_2$ fluctuations as well as L.I.D.s over 3 weeks. The neurochemicals were correlated to L.I.D.s during weeks 1 and 3 of L-DOPA treatment on both the long-term timescale (mins-hours) as well as sub-second timescale. Lastly, the DA and H$_2$O$_2$ signals were pharmacologically manipulated in an effort to elucidate the various sources of these analytes in the striatum and how they correlate to L.I.D.s. Results from these studies will aid in understanding more of the dynamic molecular mechanisms that are underway during L-DOPA treatment for PD, and will have broad implications for studying other neurodegenerative disorders that involve oxidative stress.
2.0 References


CHAPTER 2: Selective and Mechanically Robust Sensors for Electrochemical Measurements of Real-Time Hydrogen Peroxide Dynamics In Vivo

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2.1 Abstract

Hydrogen peroxide (H₂O₂) is an endogenous molecule that plays several important roles in brain function: it is generated in cellular respiration, serves as a modulator of dopaminergic signaling, and its presence can indicate the upstream production of more aggressive reactive oxygen species. H₂O₂ has been implicated in several neurodegenerative diseases, including Parkinson’s disease (PD), creating a critical need to identify mechanisms by which H₂O₂ modulates cellular processes in general, and how it affects the dopaminergic nigrostriatal pathway, in particular. Furthermore, there is broad interest in selective electrochemical quantification of H₂O₂ because it is often enzymatically-generated at biosensors as a reporter for the presence of non-electroactive target molecules. H₂O₂ fluctuations can be monitored in real time using fast-scan cyclic voltammetry coupled with carbon-fiber microelectrodes. However, selective identification is a critical issue when working in the presence of other molecules that generate similar voltammograms, such as adenosine and histamine. We have addressed this problem by fabricating a robust, H₂O₂-selective electrode. 1,3-phenylenediamine was electrodeposited on a carbon-fiber microelectrode to create a size-exclusion membrane, rendering the electrode sensitive to H₂O₂ fluctuations and pH shifts, but not other commonly studied neurochemicals. The electrodes are described and characterized herein. The data demonstrate that this technology can be used to ensure the selective detection of H₂O₂, enabling confident characterization of the role this...
molecule plays in normal physiological function, as well as in the progression of PD and other neuropathies involving oxidative stress.

2.2 Introduction

Reactive oxygen species (ROS) such as the superoxide radical (O$_2^-$), nitric oxide (NO·), and the hydroxide radical (·OH), play important physiological roles in brain function. For instance, they serve as important byproducts of cellular metabolism under normal conditions, can serve as neuromodulators, and can become dysregulated in pathological conditions.$^1$–$^5$ Indeed, oxidative stress has been implicated in the initiation and progression of several neurodegenerative disorders, including the slow destruction of dopamine (DA) neurons in Parkinson’s disease (PD).$^5$–$^{13}$ Direct measurements of ROS in brain tissue can significantly advance our understanding of the role that these molecules play in modulating brain function and dysfunction; however, the measurements are challenging due to the instability and relatively low concentration of many of these short-lived species. Hydrogen peroxide (H$_2$O$_2$) is more stable than other ROS, it can accumulate to relatively high concentrations, and can readily diffuse through biological membranes. It can serve as a measure of oxidative stress, because more reactive species (i.e. O$_2^-$) are readily converted to H$_2$O$_2$ by enzymes such as superoxide dismutase. Furthermore, there is broad interest in quantitative detection of this molecule, because it is often enzymatically-generated at biosensors to serve as a reporter for the presence of non-electroactive species.$^{14}$–$^{19}$

There are few analytical methods available for dynamic measurements of H$_2$O$_2$ and other ROS in vivo. Florescence imaging probes offer low limits of detection, but these generally suffer from poor chemical selectivity and irreversible activation.$^{20,21}$ Additionally, quantitative analysis with this method can be problematic, due to the challenges of calibrating
fluorescent dye intensity. Second-generation dyes for ROS are promising due to improved chemical selectivity; though, they are also limited by kinetics and temporal resolution.\textsuperscript{22,23} ROS in intact brain tissue can be measured using microdialysis sampling, by including spin trap reagents in the dialysate.\textsuperscript{24} These are then separated offline by high-performance liquid chromatography and subsequently quantified. However, microdialysis is a diffusion-based (steady-state) sampling technique that typically operates in the range of minutes, preventing a second-by-second assessment of molecular dynamics. Furthermore, the microdialysis probe is generally on the order of 0.5-1 mm in diameter and 3-4 mm in length. These are dimensions that can span multiple brain regions and cause significant damage to tissue in the sampling region, confounding results.
Figure 2.1: Multiple neurochemicals found endogenously oxidize at ~1.4V, the peak oxidation potential for H$_2$O$_2$. Voltammograms are presented for (A) H$_2$O$_2$ (40 μM), (B) adenosine (2.0 μM), and (C) histamine (7.5 μM).

Electrochemical methods are perhaps the most promising analytical tools available to measure rapid chemical dynamics in the brain. These methods offer excellent temporal resolution and electrodes can be made on the micrometer scale$^{25}$, allowing specific brain regions to be targeted. Fast-scan cyclic voltammetry (FSCV) is a differential technique
frequently used with carbon-fiber microelectrodes to quantitatively monitor rapid neurotransmitter fluctuations in brain tissue.\textsuperscript{25,26} This method also offers qualitative information, because characteristics such as peak shape and position can be used to identify a given class of molecules. Real-time detection of H$_2$O$_2$ with FSCV has recently been accomplished in brain tissue, generating voltammograms with a single oxidation peak at $\sim$1.4 V.\textsuperscript{27,28} However, care must be exercised when using voltammetry to detect H$_2$O$_2$ in live brain tissue, because other endogenous molecules such as adenosine\textsuperscript{29,30} and histamine\textsuperscript{31,32} also oxidize near 1.4 V, confounding the direct measurement of H$_2$O$_2$ when using this approach (Figure 2.1). Each of these molecules has an inherent and requisite function in the brain and, as such, selective measurements of each are essential. Many times, pharmacology can be used to verify analyte identity – administration of known pharmacology should alter the signal in a predictable way.\textsuperscript{33} However, this approach to signal validation is precluded when distinguishing H$_2$O$_2$ from adenosine or histamine, as the dynamics of these molecules are presumably interlinked.\textsuperscript{3,34} Any molecule that increases cellular activity in the brain drives energy demand, which drives cellular pathways of energy metabolism and mitochondrial respiration to produce adenosine triphosphate and ROS.

In order to address this issue, we have developed a reproducible technique for the electrodeposition of 1,3-phenylenediamine (mPD) onto the carbon-fiber sensing surface. Electrodeposition of this polymer creates a size-exclusion membrane to reject larger molecular interferents that could be falsely identified as H$_2$O$_2$. The mPD membrane has been used extensively as a coating in both microdialysis sampling\textsuperscript{35-37} and electrochemical sensors\textsuperscript{38-40}, typically coupled with amperometric methods. The goal of this project is to fully characterize
mPD-coated carbon-fiber microelectrodes for voltammetric measurements, and to then apply this tool in vivo to selectively measure real-time H$_2$O$_2$ fluctuations in the rat dorsal striatum.

2.3 Experimental Section

2.3.1 Chemicals.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received, unless otherwise specified. In vitro electrochemical experiments were carried out in 0.1 M phosphate buffered saline (PBS) at a physiological pH of 7.4 and at room temperature. mPD solutions were prepared on the day of electrodeposition. Brain slice experiments used artificial cerebral spinal fluid (aCSF) saturated with 95% (O$_2$ and 5% CO$_2$, at pH 7.4. aCSF consisted of 124 mM NaCl, 26 mM NaHCO$_3$, 3.7 mM KCl, 2.4 mM CaCl$_2$, 1.3 mM MgCl$_2$, 1.3 mM NaH$_2$PO$_4$, and 10 mM glucose. All aqueous solutions were made from double deionized water >18 MΩ·cm (Millipore, Billerica, MA).

2.3.2 Microelectrode Fabrication.

Fused silica tubing (164.7 μm outer diameter/98.6 μm inner diameter) with a polyimide coating (Polymicro Technologies, Phoenix Arizona) was cut to 2 cm in length and placed in a bath of 70% isopropyl alcohol. A T-650/35 polyacrylonitrile carbon fiber was inserted though the tubing under a stereoscopic microscope. After insertion, the carbon fiber and silica were allowed to dry for 24 h. A seal was created using fast-hardening epoxy (McMaster Carr, Atlanta, GA) at one end of the silica tubing. An electrical connection with the carbon fiber was made using highly conductive silver epoxy (MG Chemical, Thief River Falls, MN) and a gold pin (Newark Element 14, Palatine, IL). This was allowed to dry for at least 24 h. The connection was insulated using fast-hardening epoxy and electrodes were subsequently placed in a 100º C oven to cure for 20 min. After drying, a second layer of insulation around the
connection was made using liquid insulting tape (GC Electronics, Rockford, IL) and allowed to dry. Exposed carbon fibers were then cut to 100-150 µm under a stereoscopic microscope. Dual-microelectrode devices (DMEDs) were created by placing two microelectrodes less than 200 µm apart under a stereoscopic microscope and cementing them together using fast-hardening epoxy. For brain-slice experiments, the fused silica insulation was cut to 4 cm. All other aspects of the microelectrode fabrication protocol remained unchanged. “Injectrodes” were made under a stereoscopic microscope by positioning a 26 gauge, 10 mm long, microinjector cannula (Plastics One, Roanoke, VA) 0.5 mm above the exposed carbon fibers in a DMED and cementing with fast-hardening epoxy. After positioning, injectrodes were allowed to dry for at least 24 h before use.

2.3.3 Flow-Injection Apparatus.

In vitro calibration was performed using a flow-injection apparatus. Individual microelectrodes were lowered into a custom electrochemical cell (North Carolina State University, College of Science Machine Shop) using a micromanipulator (World Precision Instruments, Inc., Sarasota, FL). A syringe pump (New Era Pump Systems, Inc., Wantagh, NY) supplied a continuous flow (1 mL/min) of PBS across both the working and reference (Ag/AgCl) electrodes. Three-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).

2.3.4 Electrochemical Data Collection.

A waveform optimized for the electrochemical detection of H\textsubscript{2}O\textsubscript{2} was applied to the carbon-fiber microelectrode. The potential window ranged from -0.4 V to +1.4 V, applied at 10 Hz using a scan rate of 400 V/s. The electrodes were conditioned with this waveform for
30 min prior to data collection. Potential application and current transduction were performed using a Universal Electrochemistry Instrument (UEI, University of North Carolina - Chapel Hill, Department of Chemistry Electronics Facility) for brain slice experiments or a WaveNeuro (Pine Research Instrumentation, Durham, NC) for in vitro and anesthetized experiments. These instruments were operated using HDCV software (University of North Carolina - Chapel Hill, Department of Chemistry Electronics Facility) to control waveform input and output. A 6363 PCIe card (National Instruments Corp., Austin, TX) was used to interface to a computer. Signal processing (background subtraction, signal averaging and digital filtering (2-pole Sallen-Key Filter, 2 KHz)) were software controlled. All electrochemical data collection was performed within a custom-built Faraday cage.

2.3.5 Animal Subjects and Care.

Drug-naïve, male Sprague–Dawley rats (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 Guide for the Care and Use of Laboratory Animals.

2.3.6 Brain Slice Preparation.

Rats were deeply anesthetized with urethane (1.5 g/kg, intraperitoneal administration(i.p.)), decapitated, and the brain was rapidly removed (< 2 min). The brain was mounted and placed in a bath of cold aCSF saturated with 95% O₂ / 5% CO₂ gas. Tissue was sliced (400 μm) on a vibratome (World Precision Instruments, Sarasota, FL) and coronal slices containing the striatum were allowed to rest in the aCSF for at least 1 h before the start of an
experiment. Brain slices were subsequently placed in a recording chamber (Warner Instruments, Hamden, CT), and superfused with continuously oxygenated ACSF buffer maintained at 34°C for at least another hour. DMEDs and bipolar tungsten stimulating microelectrodes (FHC, Neural micro Targeting Worldwide, Bowdoin, ME) were positioned about 100 µm below the surface of the slice with the aid of a microscope (Nikon Instruments, Inc., Melville, NY) and micromanipulators (Scientifica ltd., United Kingdom). Biphasic electrical stimulation consisted of five 500 µA pulses at 60 Hz with a pulse width of 4 msec, generated with a DS-4 Biphasic Stimulus Isolator (Digitimer Ltd., Welwyn Garden City, England). Local drug application (microinfusion) was achieved using a 33 gauge microinjector needle (Plastics One, Roanoke, VA) positioned 100-200 µm away from the working microelectrode(s) using a syringe pump (KD Scientific, Holliston, MA) with a flow rate of 26.5 µL/min over 3 sec.

2.3.7 Anesthetized Animal Surgery.

Rats were deeply anesthetized with urethane (1.5 g/kg, i.p.), 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 rat brain atlas of Paxinos and Watson, relative to bregma. The DMED was placed in the dorsal striatum (anterior-posterior: +1.5 mm, medial-lateral: + 2.5 mm, dorso-ventral: -5.0 mm from the skull surface). The Ag/AgCl reference electrode was placed contralateral to the working electrode. Electrodes were cemented to the skull using acrylic dental cement (Lang Dental Manufacturing Company, Wheeling, IL). All microinfusions (saline and mercaptosuccinic acid, MCS) were performed as described above, but at a flow rate of 0.5 µL/min for 1 min.
2.3.8 Data Analysis and Statistics.

All data are shown as the mean ± standard error of the mean (SEM). To determine calibration factors, injections of four concentrations spanning a physiological range were performed in triplicate and peak oxidative currents were averaged. Differences between slopes were assessed using an analysis of covariance (ANCOVA) with Tukey’s post-hoc test. Principal component regression (PCR) is a multivariate statistical method that was performed using HDCV Analysis software for quantitative determination of individual chemical contributors to the voltammetric data. Training sets consisted of four clean cyclic voltammograms collected for 2-sec bolus injections of DA (250-1000nM), H$_2$O$_2$ (20-80 µM), and acidic shifts in pH (0.05-0.20 pH units), collected in vitro in the flow-injection apparatus. Paired t-tests were used to compare data collected before and after drug administration. One-way analysis of variance (ANOVA) with a Bonferroni’s multiple-comparison post-hoc test was used to assess electrodeposition time. All statistical and graphical analyses were carried out using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). In all cases, significance was designated at p < 0.05.

2.4 Results and Discussion

2.4.1 Electrodeposition of mPD.

Microelectrodes were individually placed into a solution of 5 mM mPD in PBS, and a triangular waveform (0.0 to +1.0 V versus Ag/AgCl, 5 V/s) was applied at 1 Hz to electrodeposit the mPD membrane on the carbon-fiber surface (Figure 2.2 A and B). Ideally, the mPD coating should exclude molecules found in vivo that are larger than H$_2$O$_2$, such as adenosine. To optimize electrodeposition time (1-60 secs), bolus injections of 80 µM H$_2$O$_2$ and 2 µM adenosine were run in a flow cell using a triangular waveform. The potential was
scanned from -0.4 V to +1.4 V at 400 V/s and a frequency of 10 Hz, and the ratio of the peak oxidation currents was calculated. The data demonstrate that a 5 sec electrodeposition time was most effective at facilitating the preferential detection of \( \text{H}_2\text{O}_2 \) (Figure 2.2 C). Thus, this deposition time was used for the remainder of the study. At shorter electrodeposition times, adenosine wasn’t effectively excluded from the electrode surface. With deposition times longer than 5 sec, adenosine was completely attenuated but the current generated in the oxidation of \( \text{H}_2\text{O}_2 \) was also substantially decreased. This was most likely due to increased polymer thicknesses that hindered diffusion of \( \text{H}_2\text{O}_2 \) to the electrode surface. Scanning electron micrographs of the uncoated and mPD-coated microelectrodes provide visual verification that a thin coating of the mPD polymer was electrodeposited onto the carbon surface with this approach (Figure 2.3 A and B).

![Optimization of mPD electrodeposition](image)

**Figure 2.2: Optimization of mPD electrodeposition.** (A) Electrodeposition waveform. (B) Representative current vs. time trace collected at +1.0 V during a 5 sec electrodeposition. (C) Ratio of peak oxidation currents collected for \( \text{H}_2\text{O}_2 \) and adenosine with electrodes fabricated using various electrodeposition times. The electrodes created using the 5 sec electrodeposition performed significantly better than those created using other electrodeposition durations (One way ANOVA, Bonferroni's Multiple Comparison Test, \( F(7,81)= 14.06, ****p<0.0001, ***p<0.001, *p<0.05, n=4 \)).
2.4.2 Characterization of mPD membrane.

A DMED allows two parallel electrodes to simultaneously experience the same solution (Figure 2.3 C), permitting direct comparison of electrode performance. Physiological concentrations of adenosine\textsuperscript{44}, DA\textsuperscript{45}, histamine\textsuperscript{46,47} and H\textsubscript{2}O\textsubscript{2}\textsuperscript{27} were sampled \textit{in vitro}, along with a pharmacologically relevant concentration of mercaptosuccinic acid (MCS, Figure 2.4 D-F). MCS is an irreversible inhibitor of glutathione peroxidase, an enzyme that protects the brain from oxidative damage by reducing free H\textsubscript{2}O\textsubscript{2} to water. It is redox active and oxidizes at a potential close to that of H\textsubscript{2}O\textsubscript{2} oxidation. The mPD coating effectively excludes adenosine, histamine, DA, and MCS from the electrode surface, but allows H\textsubscript{2}O\textsubscript{2} to permeate the polymer for detection. Notably, sensitivity to H\textsubscript{2}O\textsubscript{2} at the coated electrode is decreased by about 30\% as compared to detection at the uncoated electrode, likely due to the loss of active sites on the microelectrode surface and restricted diffusion through the polymer.

![Figure 2.3. A dual-microelectrode device (DMED).](image)

(A) Scanning electron micrograph of an uncoated carbon fiber, and (B) a fiber after electrodeposition of the mPD polymer. (C) Graphical representation of a DMED.
Voltammograms collected using the mPD-coated electrode exhibit a concentration dependent, acidic pH shift in response to MCS (Figure 2.4 E), but the majority of the current due to inherent redox activity is eliminated. The mPD membrane cannot exclude a shift in pH, since both the hydronium (H$_3$O$^+$) and hydroxide (OH$^-$) ions are sufficiently small to diffuse through pores in the membrane and affect charging current generated at the electrode surface. A small acidic pH-shift is also evident in the voltammogram for H$_2$O$_2$ when using an mPD-coated microelectrode (Figure 2.4 N). Protons are generated in the oxidation of H$_2$O$_2$ (Reaction 1), and their diffusion from the electrode surface appears to be slowed by the membrane.

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (1)$$

Fortunately, this pH signal does not interfere with H$_2$O$_2$ detection, because it can be quantified and subtracted using PCR (Figure 2.5). A training set consisting of voltammograms for acidic pH shifts can be used to remove the pH signal from the raw data (black trace), leaving the residual signal corresponding to H$_2$O$_2$ (red trace). Overall, these results demonstrate that the mPD membrane acts as a size-exclusion polymer, allowing smaller species such as H$_2$O$_2$, H$_3$O$^+$, and OH$^-$ to diffuse to the electrode surface while effectively excluding larger molecules.
Figure 2.4. The mPD membrane ensures selective detection of H$_2$O$_2$. Representative color plots with cyclic voltammograms inset (white) for various species detected on uncoated (left) and mPD-coated (middle) microelectrodes bundled in a DMED. mPD-coated microelectrodes effectively exclude all of these analytes except for H$_2$O$_2$ (M-O), as quantified with the calibration curves (right). The slopes (coated vs. uncoated) were significantly different for all panels (ANCOVA with Tukey’s post hoc test, C: adenosine (ADO), F(1,36)=306.07, I: DA, F(1,35)=418.75, F: MCS, F(1,36)=332.28, L: histamine (HIST), F(1,26)=325.33, O: H$_2$O$_2$, F(1,36)=44.79, ****p<0.0001, n=3-4).
Figure 2.5: Removing pH contributions using PCR. The mPD-coated microelectrodes record an acidic shift in pH when monitoring H$_2$O$_2$ (black). A training set for acidic pH shifts was created (inset) and PCR was used to remove the pH contribution from the voltammogram, leaving the signal from H$_2$O$_2$ intact (red).

2.4.3 Membrane Stability.

The stability of the mPD membrane was evaluated in vitro over the course of 4 h with a flow-injection apparatus using uncoated and mPD-coated microelectrodes bundled in a DMED. The peak current obtained in the oxidation of a bolus injection of 2 µM adenosine was plotted every 20 min (Figure 2.6). The currents collected across the 4 h recording session did not significantly deviate for either electrode type, demonstrating stable electrode performance. Color plots of raw data collected at the end of the experiment are shown (Figure 2.6 B,C). These demonstrate both the integrity of adenosine detection on the uncoated electrode after extended use, and the ability of the mPD-coated electrode to continuously exclude this species (Figure 2.6 B,C). The uncoated microelectrode maintained a mean current response of 38.2 ± 0.3 nA over the 4 h period; whereas the mPD-coated electrode recorded
significantly less current in response to adenosine, with a mean of 1.3 ± 0.1 nA (Unpaired two-tailed t-test, ****p<0.0001, n=4).

Figure 2.6 mPD membrane stability. (A) Maximum currents recorded simultaneously at bare and mPD-coated electrodes for the oxidation of 2 µM adenosine, plotted every 20 min over a 4 h recording session. (B) Adenosine detection at the uncoated electrode was stable, and (C) the mPD-coated electrode consistently excluded adenosine. An unpaired two-tailed t-test was used to compare data collected at the last time point, (****p<0.0001, n=4 DMEDs).

2.4.4 H$_2$O$_2$ Measurements in Live Tissue.

The capacity of the mPD coating to ensure selective measurements of H$_2$O$_2$ in tissue was evaluated by using a DMED to investigate the extracellular environment in a rat brain slice encompassing the striatum. A mild electrical stimulation was used to induce striatal DA release and to increase local cellular activity, which should generate H$_2$O$_2$ by way of cellular respiration. As anticipated, the uncoated microelectrode detected a rapid increase in DA concentration as a result of vesicular release, as well as a small signal that was putatively assigned to H$_2$O$_2$ (Figure 2.7 A). The mPD-coated microelectrode detected a small increase in H$_2$O$_2$ in response to the stimulation, but a DA signal was not observed (because DA is effectively excluded by the membrane, Figure 2.7 C).
To pharmacologically validate the voltammetric signal attributed to H$_2$O$_2$, 5 mM MCS was microinfused in the vicinity of the working electrodes (DMED). An electrical stimulation delivered 15 min later elicited significantly more current at the peak oxidation potential for H$_2$O$_2$ (~1.4V) at both microelectrodes, as compared to the stimulation before MCS, validating H$_2$O$_2$ identification (uncoated: paired two-tailed t-test, ***p<0.001; n=5; mPD-coated: paired two-tailed t-test, ***p<0.001; n=5). DA release was recorded only with the bare electrode. The mean DA concentrations recorded before and after application of MCS were not significantly different (data not shown, paired two-tailed t-test, p>0.05; n=5). Previous studies have reported a decrease in evoked DA release when H$_2$O$_2$ levels are amplified by MCS.$^{28,48-51}$ However, those were completed in other preparations using different MCS concentrations and electrical stimulation parameters over a range of timescales.

A representative H$_2$O$_2$ concentration versus time trace collected at an mPD-coated microelectrode is shown in Figure 2.7 E, and an overall analysis of amplitude, area, duration, and decay time (tau) for the H$_2$O$_2$ events recorded on both electrode types is shown (Figure 2.7 F-I). Microinfusion of MCS, a glutathione peroxidase inhibitor, significantly increased event area. Analysis of event duration and tau demonstrates that this treatment increased the lifetime of H$_2$O$_2$ in the extracellular space. Overall, these data confirm that electrical stimulation locally elicits the generation of H$_2$O$_2$, and that the mPD membrane ensures its selective detection by excluding larger species also elicited by the stimulation, including DA.
2.4.5 H$_2$O$_2$ Measurements in the Intact Animal.

A DMED injectrode was used to selectively detect H$_2$O$_2$ and DA dynamics in the dorsal striatum of an intact rat. Sterile saline was microinfused into the recording environment and voltammograms were simultaneously collected at both bare and mPD-coated electrodes for 30 min. Next, 200 mM MCS was microinfused for 1 min at the same rate, and data were collected for another 30 min. H$_2$O$_2$ generation was recorded at both electrodes, with significant increases
in the amplitude and area of H₂O₂ events clearly evident in the first 5 min after MCS administration (Figure 2.8 A-C). Importantly, much of the current that was generated at the oxidation potential for H₂O₂ on the uncoated electrode is likely due to the oxidation of MCS itself. MCS generates substantial current at ~1.4 V (Figure 2.4 D), it was directly introduced to the vicinity of the electrode, and attempts to distinguish the H₂O₂ contribution to the signal from that of MCS using PCR⁵² which failed because the residual error tolerance threshold, Q_α, was greatly exceeded (Figure 2.8 D). These data directly demonstrate the utility of the mPD coating when using pharmacological agents, such as MCS, which can directly interfere with the measurement.

**Figure 2.8. Quantification of H₂O₂ events in intact brain tissue.** Representative color plots simultaneously recorded on (A) bare and (B) mPD-coated microelectrodes upon local microinfusion of MCS (green arrow). Note the different scale bars for current (color). (C) Representative H₂O₂ concentration vs time traces extracted from (B) to demonstrate the effects of local microinfusion of saline (black) and MCS (red). MCS significantly increased the area and amplitude of the currents generated at ~1.4 V on the mPD coated electrode (see Table 2.1 for statistics). (D) At the bare electrode, the residual error tolerance threshold, Q_α, was greatly exceeded (note logarithmic scale).
Table 2.1: Local microinfusion of MCS increased the amplitude and area of \( \text{H}_2\text{O}_2 \) events recorded on the mPD-coated microelectrode, pharmacologically validating the measurement.

<table>
<thead>
<tr>
<th></th>
<th>Amplitude (µM)</th>
<th>Area (µM*s)</th>
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<tbody>
<tr>
<td>Saline (control)</td>
<td>4.9 ± 0.4</td>
<td>357 ± 111</td>
</tr>
<tr>
<td>MCS</td>
<td>48 ± 3</td>
<td>8608 ± 1606</td>
</tr>
<tr>
<td>t-statistic</td>
<td>t(3)=20.00</td>
<td>t(3)=4.820</td>
</tr>
<tr>
<td>p-value</td>
<td>***p&lt;0.001</td>
<td>*p&lt;0.05</td>
</tr>
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2.5 Conclusion

The results presented herein clearly demonstrate that caution should be executed when analyzing data collected in the complex environment of the brain. FSCV allows for the direct quantification of endogenous \( \text{H}_2\text{O}_2 \) dynamics, and it is also useful for detecting enzymatically generated \( \text{H}_2\text{O}_2 \) using enzyme-modified carbon-fiber microelectrodes.\(^{14-17}\) There are certainly many instances in which PCR analysis is sufficient to distinguish \( \text{H}_2\text{O}_2 \) from interfering species evident in the collected data; however, selectivity is always a primary concern when using electrochemistry. Additional measures must be taken to ensure selectivity when there is not a clear distinction between electroactive species potentially contributing to the signal (i.e., when PCR fails). In this work, we have described a straightforward approach to modifying the electrode surface with a size-selective, mPD membrane to provide optimized sensitivity to \( \text{H}_2\text{O}_2 \) while excluding larger molecular interferents. We have determined that the mPD membrane is stable on the surface of the fiber for at least 4 h, and we have demonstrated its utility \textit{in vivo}. This advance is important, because it will enable confident measurements describing the role that \( \text{H}_2\text{O}_2 \) plays in normal physiological function, as well as in the progression of neuropathies involving oxidative stress.
2.6 Acknowledgements

Funding for this work was provided by the U.S. National Institutes of Neurological Disorders and Stroke (1R01NS076772–01 to L.A.S.), the Goodnight Scholars Program (support of S.P.), the NCSU Office of Undergraduate Research (support of S.P.), and the NCSU Keck Center for Behavioral Biology (partial support of L.W.). We would like to thank Nicholas Williams and Catherine Mason for their help in the fabrication of the microelectrodes that were used in this study, Xiaohu Xie for his assistance with statistics, and James Roberts for assistance in manuscript preparation.
2.7 References


CHAPTER 3 Real-Time Striatal Measurements of Oxidative Stress and Dopamine in Hemiparkinsonian Rats Expressing L-DOPA Induced Dyskinesias

Leslie R. Wilson, Catherine F. Mason, Christie A. Lee, Karen E. Butler, Sitora Khodjaniyazova, David C. Muddiman, and Leslie A. Sombers

3.1 Abstract

Parkinson’s disease is a neurodegenerative disorder commonly treated with levodopa (L-DOPA), which eventually induces abnormal involuntary movements (A.I.M.s). The neurochemical contributors to these dyskinesias are unknown; however, evidence indicates interplay of dopamine (DA) and oxidative stress. Here, we simultaneously monitored real-time DA and hydrogen peroxide (H$_2$O$_2$) fluctuations with fast-scan cyclic voltammetry. This was done bilaterally in the dorsal striatum of control and hemiparkinsonian rats after L-DOPA. Infrared matrix-assisted laser desorption electrospray ionization mass spectrometry imaging validated the lesions. In hemiparkinsonian rats, DA and H$_2$O$_2$ tone increased during A.I.M.s after one week of treatment. By the third week, this was abolished. However, rapid chemical fluctuations were precisely correlated with involuntary bouts of rotation induced by L-DOPA administration. H$_2$O$_2$ increased and DA concentrations recorded at the same location simultaneously decreased with rotation onset. These results help clarify how oxidative stress can modulate nigrostriatal DA signaling, and the behavioral consequences of this interaction.

3.2 Introduction

Worldwide, there are ~10 million people diagnosed with Parkinson’s disease (PD)\(^1\), a neurodegenerative disorder that results in muscle rigidity, slowed movement, and resting tremor. Pathologically, PD is marked by the extensive loss of nigrostriatal dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) which innervate the dorsal striatum, a region involved in action selection, locomotion, and habit formation\(^2-4\). There is no specific
known cause for idiopathic PD; however, multiple lines of evidence implicate oxidative stress as an underlying factor in both the initiation and progression of the disease\textsuperscript{5}. For instance, decreased mitochondrial complex I activity is evident in postmortem PD tissue\textsuperscript{6}, and is a common feature of many of the neurotoxin-induced experimental models of PD\textsuperscript{7}. This causes the enhanced generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and hydroxyl radical\textsuperscript{8}. H\textsubscript{2}O\textsubscript{2} is particularly interesting because it is relatively stable and can accumulate to significant concentrations, allowing it to serve distinct biological roles as a key player in both signaling and oxidative stress\textsuperscript{9}. Dopaminergic replacement therapy with L-DOPA (levodopa; L-3,4-dihydroxyphenylalanine), the metabolic precursor to DA, can initially alleviate hypokinetic symptoms of the disease. Unfortunately, about 40\% of patients undergoing this therapy develop hyperkinetic, involuntary dyskinesias after about 4-6 years of treatment\textsuperscript{10}. Eventually, nearly all patients on L-DOPA acquire these involuntary motor complications\textsuperscript{11}, which can be as debilitating as PD itself, limiting the long-term therapeutic benefit of L-DOPA.

Remarkably little is known about how the dynamics of DA release events change to preserve motor control in PD, or how these dynamics are modified during L-DOPA replacement therapy. One prevailing theory is that surviving DA neurons functionally compensate for the progressive loss of nigrostriatal DA neurons by up-regulating DA release, which is further augmented by L-DOPA treatment\textsuperscript{12}. This likely contributes to a highly heterogeneous and pulsatile DA concentration profile in the dorsal striatum. Striatal DA levels are increased in dialysate collected from the 6-hydroxydopamine (6-OHDA) rat model of PD while the animals are exhibiting L-DOPA-induced A.I.M.s and rotations\textsuperscript{13,14}. Excess DA can act at supersensitized DA D1 receptors, which are involved in dyskinetic movements\textsuperscript{15}. It can
also contribute to the generation of ROS. For instance, monoamine oxidase catalyzes the deamination of DA through a two-electron reduction of O$_2$ to H$_2$O$_2$. The auto-oxidation of L-DOPA or DA generates toxic metabolites and ROS that can be rapidly converted to H$_2$O$_2$. However, neither rapid fluctuations in brain DA concentrations (“DA transients”), nor the dynamics of H$_2$O$_2$ have been examined in L-DOPA-treated dyskinetic rats. Thus, many unanswered questions remain regarding the role of these species in the development and expression of dyskinesias.

Fast-scan cyclic voltammetry (FSCV) is an electroanalytical technique that enables molecules in the brain to be monitored in situ with chemical selectivity and temporal resolution on the millisecond timescale. It has been widely used to characterize the role of striatal DA dynamics in modulation of specific behaviors, such as interaction with conspecifics, reward learning, and action selection. Recently, FSCV has been used to simultaneously monitor H$_2$O$_2$ and DA in intact striatal tissue of anesthetized rats. In this work, bilateral neurochemical measurements of striatal H$_2$O$_2$ and DA were time-locked to dyskinetic behaviors exhibited by unilaterally 6-OHDA-lesioned rats treated chronically with L-DOPA. The data indicate that the expression of A.I.M.s and contralateral rotations coincides with an overall increase in striatal H$_2$O$_2$ and DA tone after one week of daily L-DOPA treatment. This was not evident after three weeks. Examination of rapid neurochemical dynamics revealed a novel relationship that progressively emerged across time in both the ipsilateral and contralateral striata. After 3 weeks of L-DOPA treatment, striatal H$_2$O$_2$ concentrations rapidly increased precisely with the onset of rotation, as DA concentrations recorded at the same site simultaneously decreased. Finally, infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) imaging source coupled to a high resolution accurate mass
(HRAM) MS platform was used to quantify the extent of DA loss. The unanticipated and novel correlation of these chemical dynamics with the expression of motor complications associated with PD will prove paramount to our understanding of the neuromodulatory role played by H$_2$O$_2$ in the brain. These results promise to inform the development of new therapeutic interventions, neuroprotective strategies, and promising antiparkinsonian drugs based on redox biology.

3.3 Results

3.3.1 Evaluation of Global A.I.M.s

Animals were treated daily for three weeks with saline or L-DOPA (6 mg/kg, i.p.). After 1 week of treatment, increased A.I.M.s were evident in the 6-OHDA -lesioned + L-DOPA group (vs. all control conditions; lesioned + saline, intact + L-DOPA, intact + saline. Figure 3.1 B; main effect of treatment, $F_{3,12} = 4.59$, $P = 0.0232$; treatment x time interaction, $F_{3,12} = 2.21$, $P = 0.0022$). This effect peaked 40-100 min after L-DOPA administration (Figure 3.1 B; 40 min: $P = 0.0165$; 60 min: $P = 0.0165$; 80 min: $P = 0.0004$; 100 min: $P = 0.0020$). Dyskinetic behaviors were not significantly different from controls for the remainder of the evaluation period (Figure 3.1 B; 120-180 min). Table 3.1 (top) displays a quantitative summary of dyskinetic behaviors assessed at week 1 (day 7) and week 3 (day 21). It should be noted that dyskinetic behaviors were not observed in control groups. These animals mostly rested during the recording sessions, with little ambulation or exploration.

After 3 weeks of daily L-DOPA treatment, the lesioned + L-DOPA group exhibited increased A.I.M.s 40 min ($P < 0.0001$) after L-DOPA administration (Figure 3.1 C; main effect of treatment, $F_{3,10} = 14.6$, $P = 0.0003$; main effect of time, $F_{27,90} = 3.63$, $P = 0.0005$; treatment x time interaction, $F_{27,90} = 4.92$, $P < 0.0001$). This reached a maximum at 60-100 min ($P <
0.0001), and began to decrease at 120 min ($P = 0.0476$). The remaining time points were not significantly different from controls (140-180 min). Overall, the lesioned + L-DOPA animals exhibited a longer period of dyskinesia during the week 3 test session, from 40-120 min after L-DOPA administration, vs. 40-100 min in week 1. Moreover, the average amplitude/severity of the dyskinetic behaviors also increased ~100% from week 1 to week 3 during the peak time period (40-100 min; two-tailed paired t-test, $t_3 = 30.0$, $P < 0.0001$).

### 3.3.2 DA and H$_2$O$_2$ Tone during Dyskinetic Behavior

As a first metric to quantify chemical signaling in the dorsal striatum, DA and H$_2$O$_2$ tone were simultaneously measured with concentration vs. time traces from bilateral recordings ipsilateral and contralateral to the lesion. The chemical signal was averaged over 300-sec bins of data by calculating the area under the respective concentration vs. time trace (see representative data in Figure 3.1 A), and normalized to the corresponding baseline for each rat. A complete summary of these data is provided in Table 3.1. The DA (blue) and H$_2$O$_2$ (red) tone in the ipsilateral (Figure 3.1 D-G) and contralateral (Figure 3.1 H-K) striata are graphically presented in Figure 3.1.

In week 1, the DA tone in the ipsilateral striatum varied based on treatment condition (Figure 3.1 D; main effect of treatment, $F_{3,10} = 5.6$, $P = 0.0162$); However, this was generally not observed in the contralateral striatum (Figure 3.1 H). In hemiparkinsonian animals, ipsilateral DA tone increased to values ~900-1200% greater than those recorded at baseline 40 min ($P = 0.0473$), 60 min ($P = 0.0436$), 80 min ($P = 0.0456$), and 100 min ($P = 0.0067$) after L-DOPA administration (Figure 3.1 D, green). Ipsilateral H$_2$O$_2$ tone (Figure 3.1 F, green) was simultaneously elevated ~300-500% above baseline measurements from 60 – 140 min ($P = 0.0120-0.0416$) after L-DOPA administration (main effect of treatment, $F_{3,10} = 4.9$, $P =$
0.0239), except for the 120 min time point. Similarly, in the contralateral striatum (Figure 3.1 J, green), $\text{H}_2\text{O}_2$ tone concurrently increased to values ~300-400 % greater than baseline from 60 - 180 min ($P = 0.0025$-$0.0478$) after L-DOPA administration (main effect of treatment, $F_{3,10} = 8.27$, $P = 0.0046$), except for the 120 min time point. Interestingly, the changes in chemical tone elicited by L-DOPA administration were largely attenuated after 3 weeks of treatment (Figure 3.1 E,G,I,K, green; Table 3.1).
Figure 3.1: Correlation of chemical tone in the striatum with dyskinetic behavior. A, Representative color plot collected in the ipsilateral striatum of a 6-OHDA-lesioned animal 160-165 min after L-DOPA administration on the 21st day (week 3) of treatment. A mixed H₂O₂ and DA signal is evident. The CV (inset) was extracted at the time of the vertical dashed line. H₂O₂ (red) and DA (blue) tone was approximated by integrating the area under the respective concentration vs time traces. B, After 1 week of L-DOPA (6 mg/kg) treatment, lesioned rats exhibited dyskinetic movements 40-100 min after drug administration. C, After 3 weeks of L-DOPA, the amplitude and duration of the dyskinetic movements increased. D, F, After 1 week of L-DOPA treatment, ipsilateral striatal recordings demonstrated elevated DA and H₂O₂ tone in the lesioned animals (green), this effect was not evident after 3 weeks of daily L-DOPA administration (E, G). H, I, DA tone in the contralateral hemisphere did not significantly vary between treatment groups at any time point examined. However, contralateral H₂O₂ tone was elevated in lesioned animals (green) after 1 week of L-DOPA treatment (J), but this effect was abolished by week 3 (K). Sidak post-hoc comparisons between treatment groups, #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$; ####, $P < 0.00001$ (See Table 3.1).
Table 3.1: Statistical analysis of A.I.M.s and chemical tone (for data presented graphically in Figure 3.1) using a two-way repeated measures ANOVA. Significant effects are shown in bold.

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<th>Treatment</th>
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<tr>
<td></td>
<td>F₃,₁₂ = 4.59</td>
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<tr>
<td>Week 3</td>
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<tr>
<td></td>
<td>F₃,₁₂ = 14.6</td>
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<td>F₂₇,₉₉ = 4.92</td>
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<td>H₂O₂</td>
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3.3.3 A Closer Look at H₂O₂ and DA Dynamics Following L-DOPA Administration

A principal advantage of FSCV is that it enables detection of neurochemical dynamics on the millisecond timescale. Neither DA nor H₂O₂ fluctuations robustly correlated with the expression of individual axial, orolingual or limb A.I.M.s in lesioned rats treated with L-DOPA (Figure 3.3.1 A-C). However, after 3 weeks of daily administration, both DA and H₂O₂ fluctuations were precisely time-locked with the onset of rotational behavior. Figure 3.2 shows 300 sec of representative data collected in the ipsilateral striatum of a unilaterally lesioned rat 3 weeks into L-DOPA treatment and ~90 min after drug administration (Figure 3.2 A). The corresponding concentration vs. time traces for DA (blue) and H₂O₂ (red) are presented in panel B. The 15 periods shaded in grey indicate when the rat was engaged in rotational
behavior for at least 1.7 sec, without breaks in excess of 1.7 secs. Interestingly, DA and H$_2$O$_2$ dynamics were time-locked with the onset of each bout of rotational behavior. To facilitate visualization and quantification of this result, 4-sec long “snippets” of data were extracted from the data files, centered around the onset of each period of rotation. In the representative snippet shown in Figure 3.2 E, DA and H$_2$O$_2$ are inversely correlated with one another – i.e., H$_2$O$_2$ increased with the onset of rotation, as DA simultaneously decreased at the same recording site (Spearman’s Rho = -0.661, $P = 4.03 \times 10^{-8}$). Color plots for this representative snippet are also shown (Figure 3.2 C,D). Background subtraction conventionally occurs at the time of the lowest current recorded at each species’ peak oxidation potential. As such, the background subtraction is optimized to facilitate visualization of H$_2$O$_2$ in panel C, and DA in panel D. Voltammograms extracted from these data are also provided (insets). All 15 snippets collected in this recording were averaged and are presented in panel F (Spearman’s Rho= -0.921, $P = 0.00$).
Figure 3.2: H₂O₂ and DA fluctuations in the striatum correlate with the onset of rotational behavior evoked by L-DOPA. A, Representative data in the form of a color plot, collected in the lesioned striatum after 3 weeks of L-DOPA treatment. B, H₂O₂ (red) and DA (blue) traces extracted from these data using PCR. Periods where the rat was engaged in robust rotational behavior (≥ 1.7 sec) are shaded in grey. Data collected around the start of one representative bout of rotational behavior (± 2 sec, vertical black box) are optimized for H₂O₂ (C) and DA (D). E, The respective concentration vs. time traces extracted from this “snippet” of data. F, Concentration traces from all snippets are averaged for the entire 300 sec evaluation period.

After 1 week of L-DOPA treatment, the frequency of 360° rotations recorded in hemiparkinsonian rats increased (Figure 3.3 A, green; interaction of treatment x time, F_{27,108} = 1.88, P = 0.0120). This is consistent with the time course of the increase in A.I.M.s (axial, orolingual, and limb) evoked by L-DOPA treatment (Figure 3.1 B, green), and prior reports. Lesioned animals treated with L-DOPA completed more rotations than the control groups at 20 min (5 ± 3 rotations/min, P = 0.0068), 40 min (5 ± 2 rotations/min, P = 0.0113), and 60 min (5 ± 2 rotations/min, P = 0.0041). From 80 min through the end of the recording session few rotations were noted, and their frequency was not different from that exhibited by the control rats (Figure 3.3 A). After 3 weeks of L-DOPA treatment, the lesioned animals (Figure 3.3 H, green) continued to exhibit an increased frequency of 360° rotations compared to controls 20 min (7 ± 3 rotations/min, P = 0.0001) and 40 min after L-DOPA administration (6 ± 3 rotations/min, P = 0.0004. Treatment x time interaction, F_{27,90} = 2.04, P = 0.0068). Overall, the
duration of the rotational behavior was shorter than that recorded in week 1, which is consistent with other reports\textsuperscript{25,26}.

Neurochemical dynamics were correlated with rotations at three time points in each recording. These were: shortly after rotations began (~35-40 min after L-DOPA administration; Figure 3.3 A,H, green), in the middle of the rotational period (~55-60 min after L-DOPA; yellow), and when the rotations were subsiding (~85-90 min after L-DOPA; gray). After 1 week of treatment, a small but reliable increase in DA was evident in both striata with the start of robust rotation. 35 – 40 min after L-DOPA administration, ipsilateral DA concentrations increased 0.6 ± 0.1 nM (Figure 3.3 B, blue; \( P < 0.0001 \)) with rotation onset (main effect of analyte, \( F_{1,8} = 81.4, P < 0.0001 \); main effect of behavior, \( F_{1,8} = 77.3, P < 0.0001 \); analyte x behavior interaction, \( F_{1,8} = 46.7, P < 0.0001 \)). Similarly, contralateral DA concentrations increased 1.0 ± 0.1 nM (Figure 3.3 C, blue; \( P < 0.0001 \)) with the start of rotation (main effect of analyte, \( F_{1,8} = 70.0, P < 0.0001 \); main effect of behavior, \( F_{1,8} = 101, P < 0.0001 \); analyte x behavior interaction, \( F_{1,8} = 64.4, P < 0.0001 \)). 55-60 min after L-DOPA administration, DA concentrations in the ipsilateral striatum did not significantly correlate with initiation of rotational behavior (Figure 3.3 D, blue; \( P = 0.320 \)); however, DA recorded contralateral to the lesion did increase 0.7 ± 0.1 nM (Figure 3.3 E, blue; \( P < 0.0001 \)) as the animals transitioned to rotation (main effect of analyte, \( F_{1,8} = 15.2, P = 0.0046 \); main effect of behavior, \( F_{1,8} = 121, P < 0.0001 \); analyte x behavior interaction, \( F_{1,8} = 96.8, P < 0.0001 \)). Finally, ~85-90 min after L-DOPA administration, a small but significant increase of 0.4 ± 0.1 nM DA (Figure 3.3 F, blue; \( P = 0.0126 \)) was recorded in ipsilateral striatum (main effect of analyte, \( F_{1,8} = 39.7, P = 0.0002 \); main effect of behavior, \( F_{1,8} = 23.4, P = 0.003 \)), and 0.3 ± 0.1 nM DA (Figure 3.3 G, blue; \( P = 0.0456 \)) was recorded in contralateral striatum with rotation.
onset (main effect of behavior, $F_{1,8} = 6.88, P = 0.0305$; interaction of analyte x behavior, $F_{1,8} = 5.6, P = 0.0455$). The H$_2$O$_2$ story is different. After 1 week of L-DOPA treatment, striatal H$_2$O$_2$ concentrations (red) were not highly correlated with rotation onset in either hemisphere. The one exception was noted 55-60 min after L-DOPA administration. At this time point, striatal H$_2$O$_2$ concentrations ipsilateral to the lesion significantly increased $0.34 \pm 0.03$ µM (Figure 3.3 D, red; $P = 0.0106$) with rotation onset (main effect of analyte, $F_{1,8} = 81.5, P < 0.0001$; main effect of behavior, $F_{1,8} = 22.8, P = 0.0014$). Interestingly, this was also the only time period during week 1 where no DA increase was correlated with the onset of rotational behavior.
Figure 3.3: Striatal DA and H₂O₂ dynamics precisely correlate with rotation. A. After 1 week of L-DOPA treatment, lesioned animals (green trace) exhibited more frequent 360° contralateral rotations than all control groups. A detailed analysis of rotational behavior was performed at three time points (green, yellow, and gray vertical bars) during the 185 min recording session. 35-40 min into the experiment, the data reveal an increase in DA that precisely correlates with the onset of rotational behavior in both the ipsilateral (B, blue) and contralateral (C, blue) striata. 55-60 min after L-DOPA administration, the DA concentrations in the contralateral striatum continued to increase with rotation onset (E, blue); however, this trend was not observed in the ipsilateral striatum, where an increase in H₂O₂ concentrations was noted to coincide with the start of a rotational bout (D, red). When rotations were subsiding (~85-90 min after L-DOPA administration), DA concentrations increased with the onset of rotational behavior in both ipsilateral (F, blue) and contralateral (G, blue) striata. Different trends were noted 3 weeks into L-DOPA treatment. Lesioned animals exhibited robust 360° rotations 20-40 min after L-DOPA treatment (H, green trace). I, 35-40 min after L-DOPA administration, both DA (blue) and H₂O₂ (red) concentrations in the ipsilateral striatum increased with rotation onset. However, in the contralateral striatum (J), an inverse relationship was observed between DA (decreasing) and H₂O₂ (increasing) concentrations centered about the onset of rotation. This trend was evident in both striatal hemispheres at all later time points analyzed across all animals (K-N). Specific time points of significance were identified through Sidak post-hoc comparisons. Between treatment groups: #, P < 0.05; ####, P < 0.00001. Within groups: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. For each hemisphere and respective time point, n = 3 rats with the exception of week 3 at the 85-90 min time point for the contralateral striatum, where n = 2 rats.
Table 3.2: Statistical analysis of rotations and neurochemical fluctuations (for data presented graphically in Figure 3.3) using a two-way repeated measures ANOVA. Significant effects are shown in bold.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Treatment</th>
<th>p-value</th>
<th>Time</th>
<th>p-value</th>
<th>Interaction</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>(F_{3,12} = 2.60)</td>
<td>(P = 0.101)</td>
<td>(F_{9,108} = 1.39)</td>
<td>(P = 0.202)</td>
<td>(F_{27,108} = 1.88)</td>
<td>(P &lt; 0.0120)</td>
</tr>
<tr>
<td>Week 3</td>
<td>(F_{3,9} = 2.5)</td>
<td>(P = 0.12)</td>
<td>(F_{9,81} = 2.0)</td>
<td>(P = 0.057)</td>
<td>(F_{27,81} = 2.3)</td>
<td>(P &lt; 0.01)</td>
</tr>
</tbody>
</table>

| Week 1   | Ipsilateral | \(F_{1,8} = 81.4\) | \(P < 0.0001\) | \(F_{1,8} = 77.3\) | \(P < 0.0001\) | \(F_{1,8} = 46.7\) | \(P < 0.0001\) |
|          | Contralateral | \(F_{1,8} = 70.0\) | \(P < 0.0001\) | \(F_{1,8} = 101\) | \(P < 0.0001\) | \(F_{1,8} = 64.4\) | \(P < 0.0001\) |
| ~35-40   | Ipsilateral | \(F_{1,8} = 81.5\) | \(P < 0.0001\) | \(F_{1,8} = 22.8\) | \(P = 0.0014\) | \(F_{1,8} = 2.94\) | \(P = 0.125\) |
|          | Contralateral | \(F_{1,8} = 15.2\) | \(P = 0.0046\) | \(F_{1,8} = 121\) | \(P < 0.0001\) | \(F_{1,8} = 96.8\) | \(P < 0.0001\) |
| ~55-60   | Ipsilateral | \(F_{1,8} = 39.7\) | \(P = 0.0002\) | \(F_{1,8} = 23.4\) | \(P = 0.003\) | \(F_{1,8} = 2.15\) | \(P = 0.181\) |
|          | Contralateral | \(F_{1,8} = 3.02\) | \(P = 0.120\) | \(F_{1,8} = 6.88\) | \(P = 0.0305\) | \(F_{1,8} = 5.60\) | \(P = 0.0455\) |
| ~85-90   | Ipsilateral | \(F_{1,8} = 142\) | \(P < 0.0001\) | \(F_{1,8} = 66.8\) | \(P < 0.0001\) | \(F_{1,8} = 4.35\) | \(P = 0.0704\) |
|          | Contralateral | \(F_{1,8} = 414\) | \(P = 0.538\) | \(F_{1,8} = 32.9\) | \(P = 0.0004\) | \(F_{1,8} = 257\) | \(P < 0.0001\) |
|          | Ipsilateral | \(F_{1,8} = 0.127\) | \(P = 0.730\) | \(F_{1,8} = 17.6\) | \(P = 0.0030\) | \(F_{1,8} = 107\) | \(P < 0.0001\) |
|          | Contralateral | \(F_{1,8} = 168\) | \(P < 0.0001\) | \(F_{1,8} = 46.6\) | \(P = 0.0001\) | \(F_{1,8} = 175\) | \(P < 0.0001\) |
|          | Ipsilateral | \(F_{1,8} = 19.1\) | \(P = 0.0024\) | \(F_{1,8} = 5.83\) | \(P = 0.467\) | \(F_{1,8} = 178\) | \(P < 0.0001\) |
|          | Contralateral | \(F_{1,8} = 156\) | \(P = 0.0002\) | \(F_{1,8} = 0.227\) | \(P = 0.626\) | \(F_{1,8} = 209\) | \(P = 0.0001\) |

These neurochemical dynamics were substantially different after 3 weeks of L-DOPA treatment. Shortly after rotations began (~35-40 min after L-DOPA), both DA (blue, 0.3 ± 0.1 nM, \(P = 0.0155\)) and H\textsubscript{2}O\textsubscript{2} concentrations (red, 0.43 ± 0.03 µM, \(P = 0.0005\)) in the ipsilateral striatum increased with the onset of rotation (Figure 3.3 I; main effect of analyte, \(F_{1,8} = 142, P < 0.0001\); main effect of behavior, \(F_{1,8} = 66.8, P < 0.0001\)). However, in the contralateral striatum, DA concentrations decreased 0.4 ± 0.1 nM (blue, \(P = 0.0005\)) as H\textsubscript{2}O\textsubscript{2} concentrations
increased $0.87 \pm 0.06$ µM (red, $P < 0.0001$) with the onset of rotation (Figure 3.3 J; main effect of behavior, $F_{1,8} = 32.9, P = 0.0004$; analyte x behavior interaction, $F_{1,8} = 257, P < 0.0001$). In both hemispheres, a decrease in DA and an increase in $H_2O_2$ concentrations consistently correlated with the onset of all bouts of rotation at all further time points.

In the middle of the rotational period (~55-60 min after L-DOPA), DA concentrations in the ipsilateral striatum decreased $0.20 \pm 0.1$ nM (blue, $P = 0.0150$) with a simultaneous $0.47 \pm 0.02$ µM increase in $H_2O_2$ concentrations recorded at the same site (red, $P < 0.0001$), specifically at the onset of rotation (Figure 3.3 K; main effect of behavior, $F_{1,8} = 17.6, P = 0.0030$; analyte x behavior interaction, $F_{1,8} = 107, P < 0.0001$). Similarly, in the contralateral striatum, DA concentrations decreased $0.32 \pm 0.048$ nM (blue, $P = 0.0114$) as $H_2O_2$ concentrations simultaneously increased $1.01 \pm 0.09$ µM (red, $P < 0.0001$) with the start of rotational behavior (Figure 3.3 L; main effect of analyte, $F_{1,8} = 168, P < 0.0001$; main effect of behavior; $F_{1,8} = 46.6, P = 0.0001$; analyte x behavior interaction, $F_{1,8} = 175, P < 0.0001$).

When robust rotations had almost subsided (~85-90 min after L-DOPA), DA concentrations in the ipsilateral striatum continued to decrease (blue, $0.43 \pm 0.06$ nM, $P = 0.001$) while the $H_2O_2$ concentrations simultaneously increased (red, $0.48 \pm 0.04$ µM, $P < 0.0001$) when rotational behavior began (Figure 3.3 M; main effect of analyte, $F_{1,8} = 19.1, P < 0.0024$; analyte x behavior interaction, $F_{1,8} = 178, P < 0.0001$). A similar trend was observed in the contralateral striatum (Figure 3.3 N; main effect of analyte, $F_{1,4} = 156, P = 0.0002$; analyte x behavior interaction, $F_{1,4} = 209, P = 0.0001$), where DA concentrations decreased $0.8 \pm 0.1$ nM (blue, $P = 0.0036$) as $H_2O_2$ concentrations increased $0.85 \pm 0.08$ µM (red, $P = 0.0027$) with rotation onset. Table 3.2 provides a complete summary of the statistical results that are graphically presented in Figure 3.3.
3.3.4 IR-MALDESI MSI Reveals the Extent of the Dopaminergic Lesion

The relative abundance of DA in the dorsal striata was assessed using mass spectrometry imaging (IR-MALDESI MSI). Figure 3.4 shows representative coronal slices of both unilaterally 6-OHDA-lesioned (A, C) and intact (B, D) animals, with the relative DA abundance quantified for each treatment group. DA abundance significantly differed across hemispheres in lesioned animals (Figure 3.4 E, \( P < 0.0001 \)), validating the animal model. In L-DOPA-treated hemiparkinsonian animals, DA abundance quantified in the lesioned striatum was only \( 18 \pm 3 \% \) of the total DA measured across both striata (\( n = 3 \)). The lesioned striatum of saline-treated rats contained only \( 24 \pm 4 \% \) of the abundance attributable to DA (\( n = 4 \)). By contrast, intact rats exhibited a more equal distribution of striatal DA (Figure 3.4 C; L-DOPA treated: \( 48 \pm 5 \% / 52 \pm 5 \% \) across striata, \( P = 0.936 \); Saline treated: \( 53 \pm 4 \% / 47 \pm 4 \% \), \( P = 0.890 \)). Importantly, the overall analysis suggests that L-DOPA administration did not alter the amount of remaining DA terminals compared to saline treatment conditions (Figure 3.4 C; main effect of hemisphere, \( F_{1,15} = 84.7, P < 0.0001 \); main effect of treatment, \( F_{3,15} = 0.00204, P = 0.999 \); hemisphere x treatment interaction, \( F_{3,15} = 28.5, P < 0.0001 \)). The extent of the lesion did not correlate with either the sum of the A.I.M.s scores (axial, orolingual, limb) or the number of 360° rotations recorded in week 1 (Figure 3.S.2, blue; A.I.M.s: \( R^2 = 0.132 \); Rotations: \( R^2 = 0.035 \)). However, after 3 weeks of L-DOPA treatment, there was a moderate correlation between the extent of the lesion and the A.I.M.s scores (Figure 3.S.2 A, pink; \( R^2 = 0.825 \)).
Figure 3.4: IR-MALDESI MSI was used to quantify relative DA abundances in the dorsal striatum. Representative images (coronal brain slices) show the relative DA abundance in each hemisphere of a unilaterally 6-OHDA-lesioned (A, C) and intact (B, D) rat. The relative abundance of DA was plotted for all treatment groups (E). 50% relative abundance is indicated by the dashed line. Significantly less DA was measured in the striatum ipsilateral to the lesion in all lesioned animals. Asterisks indicate significant differences in comparing contralateral vs ipsilateral striata for each group (**** $P < 0.0001$).

3.4 Discussion

In this study, we evaluated A.I.M.s behavior in unilaterally 6-OHDA-lesioned rats while simultaneously monitoring striatal DA and H$_2$O$_2$ fluctuations bilaterally over several weeks of L-DOPA treatment. The extent of dopaminergic denervation in the striatum was
assessed using IR-MALDESI MSI\textsuperscript{23} (Figure 3.4). DA depletion was extensive ipsilateral to the lesion, similar to dopaminergic neurodegeneration evident in advanced stages of PD\textsuperscript{27}. Consistent with previous reports\textsuperscript{28}, the 6-OHDA-lesioned rats exhibited contralateral rotations ~20-40 minutes after L-DOPA administration (Figure 3.3 A,H), and severe A.I.M.s were evident ~40-100 minutes after L-DOPA (Figure 3.1 B,C). Concomitant neurochemical dynamics recorded in both striatal hemispheres correlated with dyskinetic behaviors. Compared to controls, ipsilateral striatal DA tone and bilateral striatal H\textsubscript{2}O\textsubscript{2} tone increased in the lesioned animals after 1 week of L-DOPA treatment (Figure 3.1 D,F,J). This subsided by the 3\textsuperscript{rd} week of treatment (Figure 3.1 E,G,I,K). At this time, a novel and inverse correlation developed between DA and H\textsubscript{2}O\textsubscript{2} fluctuations simultaneously recorded at single sites in both striata that was time-locked to the onset of rotational behavior (Figure 3.3 J-N).

FSCV has been used to monitor DA dynamics in the dorsal striatum of anesthetized 6-OHDA-lesioned rats\textsuperscript{29,30}, intact rats engaged in operant discriminative tasks\textsuperscript{4}, as well as after L-DOPA treatment in intact animals with escalating cocaine self-administration\textsuperscript{31}. However, the role of rapid DA dynamics in locomotion remains unclear, DA transients have not been measured with FSCV during the development and expression of dyskinetic movements, and rapid H\textsubscript{2}O\textsubscript{2} dynamics have not been measured with precise temporal and spatial resolution, to date, in any freely-moving animals. In this study, FSCV was used to correlate DA and H\textsubscript{2}O\textsubscript{2} chemical tone with the progression of A.I.M.s recorded in hemiparkinsonian rats treated chronically with L-DOPA. Concentrations of these species were estimated from 300 sec voltammetric recordings (Figure 3.1 A). After 1 week of L-DOPA treatment, DA and H\textsubscript{2}O\textsubscript{2} tone in the ipsilateral striatum was elevated while the lesioned animals were expressing A.I.M.s behavior (Figure 3.1 D,F). Excessive increases in DA concentration have been reported in the
ipsilateral striatum of dyskinetic rats in studies that used a variety of measurement approaches including microdialysis sampling, positron-emission tomography (PET) imaging, and chronoamperometry, as well as in human patients as determined using PET imaging shortly after L-DOPA administration. Periodic, pulsatile swings in ipsilateral DA concentration can result from altered monoamine transporter function, ectopic DA release from serotonergic neurons, or non-neuronal cells. These cells have the ability to synthesize DA from L-DOPA, but lack the associated mechanisms to regulate its release. Excess DA in the extracellular space can be auto-oxidized, non-enzymatically oxidized by O₂, or de-aminated by MAOs; each process generates H₂O₂. Furthermore, H₂O₂ production could result from other DA metabolites or a glial neuro-inflammatory response. As such, pulsatile chemical signals were anticipated.

By the 3rd week of treatment, the observed A.I.M.s escalated in magnitude and overall duration, consistent with other reports. However, the neurochemical tone normalized in both striata of hemiparkinsonian animals treated with L-DOPA, and was not different from controls (Figure 3.1 E,G,I,K). DA denervation can be compensated by increased DA synthesis and release from remaining DA cells, as well as increased DA D2 receptor expression in indirect pathway neurons ipsilateral to the lesion. However, there is also evidence that L-DOPA can increase cystolic DA in cultured SNpc neurons, resulting in neurotoxicity. Multiple in vitro studies have shown that L-DOPA can auto-oxidize or metabolize to form toxic species that produce ROS and eventual cell death. Additional dopaminergic cell death would theoretically decrease extracellular DA tone, consistent with our data. However, it has also been shown that L-DOPA treatment results in partial recovery of tyrosine hydroxylase and DA transporter expression, as well as reversal of increased D2 receptor expression in the
denervated striatum after moderate nigrostriatal lesions\textsuperscript{45}. Here, after 3 weeks of daily L-
DOPA treatment, the extent of the DA depletion was not different from that in lesioned rats
treated daily with saline, consistent with other studies\textsuperscript{13}. No bilateral differences in DA
abundance were noted in the intact animals treated with either L-DOPA or saline. Thus, despite
a clear elevation in ipsilateral DA and bilateral H\textsubscript{2}O\textsubscript{2} tone in the striata of the lesioned animals
after 1 week of L-DOPA treatment, chronic administration of L-DOPA does not appear to
exacerbate striatal oxidative stress or DA concentrations after 3 weeks.

Hemiparkinsonian animals treated with saline exhibited no changes in DA or H\textsubscript{2}O\textsubscript{2}
concentrations in the contralateral striatum (beyond intact controls) after 1 or 3 weeks (Figure
3.1 H-K, gray). Nonetheless, there are many reports of compensatory mechanisms at work in
the contralateral striatum of unilaterally lesioned animal models. For example, altered levels
of peptide precursor mRNA have been shown in hemiparkinsonian rat striatum that are not
evident with bilateral lesions\textsuperscript{46}. Considering that ~1-3\% of dopaminergic fibers cross the
midline\textsuperscript{47}, Wightman and colleagues have used FSCV to uncover D2-like adaptations in cross-
hemispheric, contralateral DA projections after unilateral DA depletion\textsuperscript{30}. In addition,
unilaterally reducing activity in the SNpc in anesthetized cats has been reported to increase
DA concentrations recorded in the contralateral striatum\textsuperscript{48}. Such studies directly demonstrate
that adaptive, compensatory changes occur contralaterally. These are likely specific to the
experimental and analytical recording conditions. However, they do occur and, as such, the
contralateral striatum may not provide an optimal, unaffected internal control.

Our contralateral striatal measurements in the L-DOPA-treated, dyskinetic
hemiparkinsonian rats indicated no differences in DA tone, as compared to the control groups
at either test week (Figure 3.1 H-K), consistent with microdialysis studies\textsuperscript{49,50}. Interestingly,
contralateral H$_2$O$_2$ tone was elevated in this treatment group only after 1 week of daily L-DOPA administration. After three weeks of treatment, this signal was no longer elevated. Overall, these data suggest that despite some indicators of oxidative stress at early stages of treatment, compensatory mechanisms are in place to normalize excessive chemical swings over time.

Hemiparkinsonian rats exhibited robust $360^\circ$ contralateral rotations that began ~20 minutes after L-DOPA treatment. Similar to other reports$^{25,51}$, the number of rotations increased but the duration of these rotational bouts decreased from the 1$^{\text{st}}$ to the 3$^{\text{rd}}$ week of treatment (Figure 3.3 A,H). This suggests a sensitized behavioral response that occurs simultaneously with a “wearing-off” phenomenon, similar to that reported in human PD studies$^{52}$. These contralateral rotations are not a measure of dyskinesia per se, but they provide a quantifiable behavioral measure of antiparkinsonian efficacy. The contralateral locomotion after L-DOPA treatment$^{53,54}$ is reported to result from asymmetry in dopaminergic circuits$^{55}$, and DA receptor supersensitivity across striata$^{56}$.

Dogma dictates that slow (minutes) changes in striatal DA regulate motor control. Indeed, many studies have demonstrated that slow variations in SNpc cell firing and striatal DA release contribute to locomotion$^{57}$. However, phasic DA fluctuations also play a major role in regulating this circuit. There is substantial evidence correlating phasic DA fluctuations, particularly in the ventral striatum, with specific reward-related behaviors$^{19-21}$. However, there is little direct evidence defining the role of phasic DA release in locomotion. Here, the concentration traces reveal frequent fluctuations of both DA and H$_2$O$_2$ across the recording sessions. After 1 week of L-DOPA treatment, a noticeable increase in striatal DA was recorded in both hemispheres precisely at the onset of rotational behavior (Figure 3.3 B-G). This is
consistent with a previously described role for phasic, nigrostriatal DA activity in initiating bouts of locomotion. The one exception was in the ipsilateral recordings ~55-60 minutes after L-DOPA administration (Figure 3.3 D). At this time point, DA dynamics did not increase with the onset of rotation; rather, a rapid increase in H₂O₂ correlated with rotation onset. H₂O₂ generation could result from a variety of processes including auto-oxidation of extracellular DA, enzymatic DA metabolism, or the continued activation of the MSNs themselves (cellular respiration). Importantly, it has been shown in striatal brain slices that DA release can be suppressed by endogenously generated, membrane-permeable H₂O₂ by way of ATP-sensitive K⁺ (K<sub>ATP</sub>) channels on DA axons. Notably, it is equally likely that H₂O₂ can diffuse to act at K<sub>ATP</sub> channels located on serotonergic terminals. This would suppress the ectopic release of L-DOPA-derived DA in the denervated striatum, as these channels are expressed on a multitude of cellular subtypes. Thus, it is entirely possible that at peak dyskinetic time points (i.e. ~55-60 min, Figure 3.3 D), elevated H₂O₂ concentrations could act to downregulate DA release.

The trend in H₂O₂ concentrations observed only during the peak dyskinetic period in the first week of treatment (~55-60 min, Figure 3.3 D) was reliably noted at all time points investigated after 3 weeks of chronic L-DOPA administration. Specifically, increased H₂O₂ concentrations correlated with the onset of each bout of contralateral rotation. Interestingly, DA concentrations simultaneously decreased (Figure 3.3 J-N), with the exception of the first time point analyzed on the ipsilateral side (Figure 3.3 I). DA burst firing is sufficient to produce striatal DA release, which influences MSNs to generate a cascade of signaling through the motor loop that ultimately results in locomotion. Some nigrostriatal DA cells and MSNs increase activity before action initiation. In vivo photometry in genetically modified mice has shown transient increases in neural activity in direct and indirect pathway MSNs that precede
and predict (within 500 ms) the start of contraversive movements, but not ipsilateral orientations. It has been shown that AMPA receptor activation of MSNs results in H$_2$O$_2$ generation through mitochondrial respiration. D1 receptor activation of these cells is sufficient to induce dyskinetic behavior, and could also lead to generation of H$_2$O$_2$ that can negatively modulate subsequent DA release. Inversely correlated DA and H$_2$O$_2$ signals time-locked with the onset of contralateral rotational behaviors were only apparent after 3 weeks of L-DOPA treatment, suggesting that this is an adaptive, progressive neurochemical response evident in both striata.

The role of nigrostriatal DA in motor control is slowly becoming more clear. Phasic activity of SNpc neurons has long been implicated in reward prediction error, and several more recent lines of evidence have implicated rapid DA signalling in the dorsal striatum in action initiation, voluntary action selection, or the salience of sensory stimuli. Here, we have shown that DA dynamics also precisely correlate with the onset of involuntary bouts of rotation induced by L-DOPA administration in hemiparkinsonian rats. The simultaneous voltammetric measurements of H$_2$O$_2$ and DA lend credence to the argument that dramatically fluctuating DA concentrations in the dorsal striatum may contribute to the development of L-DOPA-induced behavioral abnormalities, as suggested previously. Future studies that simultaneously evaluate oxidative stress with other principal neuromodulators, their receptors, and downstream signaling targets implicated in dyskinesia will continue to clarify the mechanisms that underlie these debilitating side effects. Such studies will ultimately aid in the development of improved therapeutic strategies in the treatment of PD.
3.5 Materials and Methods

3.5.1 Chemicals.

All chemicals were purchased from MilliporeSigma (St. Louis, MO) and used as received, unless otherwise specified. In vitro electrochemical experiments were carried out in 0.01 M phosphate buffered saline (PBS) at physiological pH 7.4. All aqueous solutions were made from double deionized water >18 MΩ-cm. L-3,4-dihydroxyphenylalanine methyl ester (L-DOPA) and benserazide hydrochloride were dissolved in sterile saline (0.9% NaCl; Hospira, Lake Forest, IL).

3.5.2 Electrode Fabrication.

Fused-silica tubing (75-µm outer diameter/18-µm inner diameter) with a polyimide coating (Molex, Lisle, IL) was cut to 10 mm in length and placed in a bath of 70% isopropyl alcohol. A T-650/35 polyacrylonitrile carbon fiber (7-µm diameter, Cytec Industries, West Patterson, NJ) was inserted into the tubing under a stereoscopic microscope. The carbon fiber and silica were allowed to dry for 24 hours. A seal was created at one end of the silica tubing using fast hardening 5 min epoxy (McMaster Carr, Atlanta, GA). An electrical connection with the carbon fiber was made using conductive silver epoxy (MG Chemical, Thief River Falls, MN) and a gold pin (Newark Element 14, Palatine, IL). This was allowed to dry for at least 24 hours. The connection was insulated using fast curing epoxy and the electrodes were subsequently placed in a 105º C oven for 20 min to allow the epoxy to completely cure. The connection was insulated a second time with GC Electronics Insulating Coating (GC Electronics, Rockford, IL). The exposed carbon fibers were cut to ~100 µm under a stereoscopic microscope. Thereafter, carbon-fiber microelectrodes were electrochemically conditioned in vitro and the shape of the background signal was inspected prior to implantation.
Ag/AgCl reference electrodes were made using 0.25 mm silver wire with >99.99% trace metals basis. A connection was made to the silver wire using a gold pin and heat shrink was used to insulate. The silver wire and gold pin were positioned through a hollowed MD 2250 guide cannula stylet cap (BASi Instruments, West Lafayette, IN), and 5 min epoxy was used to secure it in place. The exposed wire was chloridized in 0.1 M HCl with the aid of a 9V battery just prior to implantation on the day of each experiment.

3.5.3 Flow Injection Apparatus.

*In vitro* calibration of carbon-fiber microelectrodes was performed with a custom flow-injection apparatus in a home-built Faraday cage. The working electrodes were lowered into a custom electrochemical cell using a micromanipulator (World Precision Instruments, Inc., Sarasota, FL). A syringe pump (New Era Pump Systems, Inc., Wantagh, NY) supplied a continuous flow (1 mL/min) of PBS across both the working and reference (Ag/AgCl) electrodes. Three-second bolus injections of analyte were introduced to the electrode surface using a six-port HPLC valve and air actuator controlled by a digital valve interface (Valco Instruments Co., Inc., Houston, TX). The triangular voltammetric waveform was applied from -0.4 V to 1.4 V at 400 V/s. Electrochemical conditioning was completed at 60 Hz for a minimum of 15 min until the electrochemical background current stabilized, and data was subsequently collected at a frequency of 10 Hz. Calibrations of dopamine (DA), hydrogen peroxide (H$_2$O$_2$), and an acidic pH shift were performed using 5 standards for physiological concentrations of DA (250-1000nM), H$_2$O$_2$ (20-80 μM), and acidic shifts in pH (0.05-0.20 pH units). Bolus injections of each concentration were performed in triplicate, and the peak oxidative current was averaged. Linear regression was used to determine the slope (calibration factor) using GraphPad Prism 7.04.
3.5.4 Animal Subjects and Care.

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*. Animals were individually housed on a 12:12 hr light/dark cycle with free access to food and water. Drug-naïve, male Sprague–Dawley rats (275-300g, Charles River Laboratories, Raleigh, NC) were allowed to acclimate to the facility for several days prior to the start of experiments. Some animals received a unilateral 6-OHDA lesion of the left SNpc, completed by the vendor, prior to being received.

3.5.5 Stereotaxic Surgery.

Rats were initially anesthetized with 4% isoflurane (Henry Schein, Dublin, Ohio), and isoflurane was maintained at 1.5-2.0% during surgery. Rats were 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 rat brain atlas of Paxinos and Watson. The working electrode(s) 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 guide cannula (BASi Instruments, West Lafayette, IN) for the removable Ag/AgCl reference electrode and a tether pedestal were placed in sites posterior to the chronic working electrode(s). The animals were allowed to recover for a minimum of 2 weeks before experiments commenced. On days when electrochemical data was collected, an acute, removable Ag/AgCl reference electrode was placed into the guide cannula.
3.5.6 Experimental Design.

After two weeks of recovery, saline (0.6 mL/kg intraperitoneal (i.p.)) or L-DOPA (6 mg/kg L-DOPA + 12 mg/kg benserazide hydrochloride, i.p.) was administered daily for 21 days. Electrochemical data were recorded and behavior was simultaneously monitored every 7 days during the 21-day drug treatment. Recordings consisted of a 10 min baseline period prior to drug administration, after which the recording continued for an additional 185 min.

3.5.7 Electrochemical and Behavioral Recording.

Awake, freely moving, rats were placed into a custom-built (North Carolina State University, Chemistry Dept. Machine Shop, Raleigh, NC) Lexan acrylic (Piedmont Plastics, Raleigh, NC) behavioral chamber with a flat floor. The center of the behavioral chamber was a square with an area of 412.9 cm$^2$. The sides of the box were 57.6 cm high. The bottom 10.8 cm of each side was sloped at a 45° angle. The chamber was enclosed within a custom-built Faraday cage. A neuroelectrochemical headstage kit (24” cable) for current amplification (Pine research instrumentation, Durham, NC) was connected to a SwivElectra™ commutator (Crist instrument company, Hagerstown, MD) to allow unrestricted motion in the chamber. This was connected to a multi-channel, custom-built instrument for potential application and current transduction (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility). A 6363 PCIe bus card (National Instruments Corp., Austin, TX) was used for waveform output. HDCV software (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility) controlled the waveform input and output. Signal processing (background subtraction, signal averaging and digital filtering (2-pole Sallen-Key Filter, 2 KHz)) was software controlled.
Prior to each electrochemical recording, the electrodes were conditioned with the detection waveform applied at 60 Hz for 10 min, and then at 10 Hz until stable over a 300 sec timeframe. Experimental data were then collected at this frequency. Four web cameras were controlled using iSpy Connect software version 6.0 to record behavior using TechSmith Camstasia software version 8.5. A BOB-4 video character generator (Decade Engineering, Turner, OR) was synchronized to the HDCV software to timestamp the electrochemical recording for precise correlation with the behavioral recording.

3.5.8 Quantification of Dyskinetic Behavior.

The A.I.M.s rating score outlined by Lindgren and Lane was used to quantify dyskinetic movements. Video-recorded behavior was scored by two researchers blind to treatment condition. The global A.I.M.s value for axial, orolingual, and limb dyskinesias was calculated by multiplying the amplitude score by the frequency score in 1 min time bins every 20th min during the 185 min recording session in week 1 (day 7) and week 3 (day 21). Rotations were quantified by counting the total number of full (360°) rotations every 21st min of these sessions.

3.5.9 Brain Slice Imaging using Mass-Spectrometry.

IR-MALDESI coupled to a Q Exactive Plus (Thermo Scientific, Bremen, Germany) mass spectrometer was used to measure the spatial distributions of DA in rat brain slices. The details of home-built ambient ionization source have been described elsewhere. Briefly, an infrared laser (2940 nm) was used to ablate neutral species from brain slices, followed by post-ionization in an orthogonally oriented electrospray plume.

Rats were deeply anesthetized with 4% isoflurane. After decapitation, brains were rapidly removed (< 2 min) and quickly frozen with either liquid nitrogen (ARC3 Gases,
Raleigh, NC, USA) or a 2-methylbutane/dry ice bath (Millipore Sigma, St. Louis, MO, USA). Biological samples were stored at -80°C until time of analysis. A thin layer of optimal cutting temperature (OCT) mounting medium (Scigen Scientific, Gardena, CA) was applied to a cryostat specimen disk. Samples were placed onto the OCT-coated specimen holder and thermally equilibrated for 10 min at -20°C. The brains were sectioned using a Leica CM1950 cryomicrotome (Buffalo Grove, IL) and thaw-mounted onto a pre-cleaned microscope slide. High-profile coated microtome blades were purchased from VWR (Batavia, IL). Tissue thickness varied from 25-100 µm, depending on tissue freshness. Fresh samples were easily cut at 25 µm, while older samples were sliced at 100 µm to ensure uniform slicing. Sample sectioning is the key step in MALDESI MSI, because every voxel (volumetric element corresponding to one image pixel) on a slice has to be positioned the same distance from the laser to ensure uniform sampling.

Slides were placed onto an XY translation stage and housed inside an acrylic enclosure with relative humidity lowered to ~10% by purging with nitrogen gas. The stage temperature was lowered to -9°C and held constant for 10 min to allow the tissue to reach thermal equilibrium, at which time the enclosure door was opened to increase humidity and to form a thin layer of ice on the tissue. The enclosure door was again closed and relative humidity lowered to ~10% to prevent further ice formation during imaging. When humidity stabilized, a 2940-nm laser (JGM Associates, INC., Burlington, MA) was used to ablate material from the sample with two mid-IR laser pulses. Spot-to-spot spacing varied: 200×200 µm, 250×200 µm, and 150×150 µm. ESI flow rate also varied from 1-2 µL/min.

A solution of 0.2% formic acid in 50:50 (v/v) methanol/water was used as the ESI solvent. In positive ESI mode, DA readily showed up as an [M+H⁺]⁺ ion. Since IR-MALDESI
is a pulsed ionization source, the automatic gain control function was disabled and the injection
time was set to 75 ms. All spectra were generated at a resolving power of 140,000\textsubscript{fwhm} at \( m/z \) 200 within an \( m/z \) range of 100-400. To achieve a low mass measurement accuracy, peaks of
carboxylic anhydride (\( m/z \) 149.0233 [M+H\(^{+}\)]\(^{+}\)), polysiloxane (\( m/z \) 371.1012 [M+H\(^{+}\)]\(^{+}\)), and
diisooctyl phthalate (\( m/z \) 391.2843 [M+H\(^{+}\)]\(^{+}\) and 413.2662 [M+Na\(^{+}\)]\(^{+}\)) were used as lock-
masses for internal calibration. At the end of MSI analysis, each slice was stained using
Histogene\textsuperscript{TM} staining solution (Thermo Fisher Scientific, Carlsbad, CA). Optical images were
acquired using a 10× objective on an LMD7000 (Leica, Buffalo Grove, IL, USA).

The .RAW files generated by the Q Exactive Plus were processed in Xcalibur software
(version 2.2, Thermo Fisher Scientific, San Jose, CA) and then converted into .mzML format
using the opensource MSConvertGUI tool from ProteoWizard. The .mzML files were then
converted to .imzML files using the imzML converter tool. The .imzML files were finally
loaded into MSiReader\textsuperscript{69}, an open-source MSI data analysis software developed at NC State,
to produce ion abundance heatmaps.

3.5.10 Data Analysis and Statistics.

DA and H\textsubscript{2}O\textsubscript{2} tone were analyzed in 300 sec bins collected every 20 min. Principal
component regression (PCR), a multivariate statistical method, was performed using HDCV
Analysis software for quantitative determination of individual chemical contributors to the
voltammetric data\textsuperscript{70}. Training sets consisted of representative cyclic voltammograms collected
\textit{in vitro}. Concentration vs. time traces were extracted from the experimental data and the
magnitude of the signal was determined by integrating the area under the curve using GraphPad
Prism 7.04 (GraphPad Software, Inc., La Jolla, CA). Signals that exceeded 3 times the standard
deviation of the noise were termed “tone” of the respective analyte. The minimum peak width to be considered analyte tone was set to 50 data points (5 secs).

Analysis of rapid (less than 5 secs) chemical transients was performed by investigating small bits of data, termed “snippets”, that were extracted from the concentration vs time traces and centered around the onset of contralateral rotational behavior (± 2 secs; 4 sec window). Changes in H$_2$O$_2$ and DA concentrations in each individual snippet were assessed by averaging the data collected 1-2 secs before the onset of rotation and comparing it with averaged data collected 1-2 secs after rotation onset.

All plots are presented as mean ± S.E.M. Plots and statistics were achieved using GraphPad Prism 7.04 for Windows. Animal behavior and chemical tone were analyzed using a two-way repeated measures ANOVA. The sources of variance were treatment group and time. Snippet statistical analysis used a two-way with non-repeated measures ANOVA with analyte (DA and H$_2$O$_2$) and behavior (non-rotating and rotating) as factors. Spearman’s correlation analyses were used to quantify the correlation between the two analytes. IR-MALDESI MSI images of lesioned vs intact striata were quantitatively assessed using a two-way non-repeated measures ANOVA with striatal hemisphere and treatment condition as factors. All significant ANOVA results were compared using Sidak post-hoc comparisons. Linear regression was used to relate the extent of the lesion to abnormal behaviors. For all statistical tests, significance was set to $P < 0.05$. 
3.6 Acknowledgments

We would like to thank Dr. Xiaohu Xie for his help in snippet data analysis. We would also like to thank Sambit Panda and Nicolas Williams for their help with making electrodes, data collection, and drug preparation and administration.

3.7 Author Contributions

L.R.W., C.F.M., C.A.L., and L.A.S. contributed to experimental design, data collection, data analysis and interpretation, and manuscript preparation. K.E.B. contributed to data collection and analysis. S.K. and D.C.M. contributed to data collection, analysis, and methods preparation for IR-MALDESI MSI.
Figure 3.S.1: Representative neurochemical dynamics recorded in ipsilateral striatum of a lesioned rat after 3 weeks of daily L-DOPA treatment. In all panels, shaded regions depict the time over which specific behaviors were observed. H$_2$O$_2$ and DA fluctuations were not specifically correlated with limb (A), orolingual (B), or axial (C) A.I.M.s. However, precise fluctuations of these neurochemicals were correlated with the onset of each rotation (D). E, A complete map of all L-DOPA-induced dyskinesias (L.I.D.s) observed during this representative 120 sec recording.
Figure 3.S.2: A linear regression relating the extent of the lesion to dyskinetic movements. A, The total A.I.M.s score calculated for the week 1 recording session (blue) does not significantly correlate with the extent of the lesion, as quantified using IR-MALDESI MSI; however, that for the week 3 recording (pink) does correlate with DA depletion. B, The total number of 360° rotations did not correlate with the extent of the lesion in either week.
3.9 References


CHAPTER 4: Hydrogen Peroxide, Dopamine, and Serotonin: Overlapping Chemical Systems Contribute to the Control of Dyskinetic Movements in the Rat During Chronic L-DOPA Treatment for Parkinson’s Disease

Leslie R. Wilson, Karen E. Butler, Christie A. Lee and Leslie A. Sombers

4.1 Introduction

For patients with Parkinson’s disease (PD), DA replacement therapy with 3,4-dihydroxyphenylalanine (L-DOPA) to ameliorate their symptoms is a ticking time-clock. It has been estimated that 90% of PD patients taking L-DOPA experience involuntary abnormal movements (A.I.M.s), or L-DOPA induced dyskinesias (L.I.D.s), after ten years of L-DOPA use. While the exact mechanism underlying the development of dyskinesia is unclear, most animal model research involving L.I.D.s indicates that plastic changes of the post-synaptic neurons located in the striatum occur. These changes primarily involve dopamine (DA) receptors, which point to excessive swings in extracellular DA as being critical to the involvement of these abnormal behaviors. However, these swings in DA could accelerate the progression of PD through generation of oxidative stress, which has been known to come from a variety of sources including the breakdown of DA through monoamine oxidase (MAO) or the auto-oxidation of L-DOPA and DA which form toxic metabolites. Overall, to date, neurochemical mechanisms underlying these L.I.D.s remains largely unclear, but evidence suggests that the serotonergic terminals along with modulation from the generated hydrogen peroxide ($H_2O_2$; Chapter 3) are important contributors.

The serotonergic terminals projecting from the dorsal raphae nucleus into the caudate putamen may be an important contributor L.I.D.s. The terminals are able to take up L-DOPA via serotonin (5-HT) transporters (SERT), convert it to dopamine using L-amino acid decarboxylase, and co-release it into the synapse with 5-HT. While this dopamine is able to be enzymatically degraded in the extracellular space, serotonergic terminals from the dorsal...
raphae nucleus lack regulatory mechanisms, such as D₂ dopamine auto-receptors, to regulate extracellular dopamine concentrations\textsuperscript{11}. This leads to an increase in synaptic dopamine following the administration of L-DOPA. Additionally, these afferents have been shown to have increased in density on the lesioned hemisphere in animals that have received a unilateral 6-OHDA injection to the substantia nigra\textsuperscript{12}, and have elevated expression of the 5-HT transporter\textsuperscript{13}.

To date, little is known about the role oxidative stress plays in L.I.D.s due to the paucity of analytical techniques to measure reactive oxygen species (ROS) in real-time. Therefore, much of what is known about abnormal movements and oxidative stress has been limited to cell cultures, brain slices, or post-mortem studies\textsuperscript{9}. Previous work has demonstrated that H\textsubscript{2}O\textsubscript{2}, a downstream indicator of oxidative stress\textsuperscript{14}, is an important neurochemical modulator of the DA signal after the onset of rotational behaviors observed after L-DOPA treatment (Chapter 3). XJB-5-131, a novel synthetic antioxidant, has been demonstrated to reduce oxidative stress and relieve cells from damage by adhering to the mitochondrial membrane through the use of a gramicidin-like backbone\textsuperscript{15}. This construct brings the other end of the molecule, an antioxidant radical trap, in close proximity to the mitochondria, and traps ROS that are by-products of the electron transport chain (Figure 4.1). This molecule, synthesized by the lab of Dr. Peter Wipf at the University of Pittsburgh, has prevented motor decline and other physiological characteristics in a long-term study using a mouse model of Huntington’s disease\textsuperscript{16-18}. The accumulation of ROS is disrupted with XJB-5-131 treatment; therefore it could be used to elucidate information on sources of oxidative stress that develops in response to L-DOPA administration or possibly even reverse the effects of this oxidative stress\textsuperscript{19}. Yet,
it is still unknown how successful XJB-5-131 is at combatting mitochondrial oxidative stress on a sub-second timescale in other disease states such as PD.

![Figure 4.1: Structure of XJB-5-131. The gramicidin-like backbone (grey) adheres to the mitochondrial membrane to bring the tempo radical trap (blue) within close proximity to the mitochondria. This construct will trap ROS molecules that are generated by the mitochondria.](image)

The goal of this work is to further understand the complex neurochemical systems underling A.I.M.s and rotational behavior by pharmacological manipulation of both mitochondrial oxidative stress, as well as blockade of SERT. A portion of this study focuses on correlating chemical dynamic information with behavioral changes induced by the administration of L-DOPA following pretreatment with citalopram, a clinically-relevant selective 5-HT reuptake inhibitor (SSRI), while another portion works to determine the role of mitochondrial respiration in relationship to the onset of L.I.D.s through pretreatment with XJB-5-131. This project will use fast-scan cyclic voltammetry (FSCV), an electroanalytical technique that proves to be optimal for monitoring H₂O₂ and DA with high temporal and spatial resolution¹⁴,²⁰. These analytes were simultaneously detected, before and after pharmacological manipulation, during continuous behavioral monitoring to correlate the neurochemical activity.
dynamics with observed L.I.D.s. Findings from this study indicate a complex mechanism involving many chemical messengers, including H$_2$O$_2$, DA, and 5-HT. Results from this study will further elucidate therapeutic targets to improve patients who are coping with neurodegenerative diseases such as PD.

4.2 Additional Materials and Methods

**Author note:** Please see Chapter 3 for materials and methods relating to electrode fabrication, flow-injection apparatus, animal subjects and care, stereotaxic surgery, electrochemical and behavioral recording, and quantification of dyskinetic behavior.

4.2.1 Chemicals

All chemicals were purchased from MilliporeSigma (St. Louis, MO) and used as received, unless otherwise specified. *In vitro* electrochemical experiments were carried out in 0.1 M phosphate buffered saline (PBS) at physiological pH 7.4. All aqueous solutions were made from double deionized water $>$18 MΩ·cm. A 50/50 racemic mix of R,S-citalopram hydrobromide (LKT LABS, St. Paul, MN) was dissolved in sterile saline. XJB-5-131 was a gift from the laboratory of Peter Wipf (University of Pittsburg, Pittsburgh, PA). The vehicle for XJB-5-131 administration consisted of anhydrous 2-propanol, poly(ethylene glycol)-300 (PEG-300) (Fisher Scientific, Pittsburgh, PA), liquid polysorbate-80 (TWEEN-80), and triple deionized water. The ratios of these constituents were 6.8/36/37.2/20 (v/v) respectively. XJB-5-131 was prepared by vortexing 10 mg of with 2-propanol until all of the XJB-5-131 had dissolved and a yellow solution was generated. PEG-300 and TWEEN-80, were added to the solution, which was then vortexed for an additional 2 mins. Water was added to the solution, vortexed for 1 min until the resulting suspension was sufficiently mixed, then heated in a water bath at 50°C for 10 mins. The solution was then removed from the hot water bath and vortexed again for 1 min to create a homogeneous, slightly yellow solution. All drugs (L-DOPA,
Citalopram, and XJB-5-131) were prepared approximately 10 minutes prior to administration via intraperitoneal (i.p.) injection.

4.2.2 Experimental Design

During the first week of the experiment, rats were administered a control vehicle injection (1.0 mL/kg saline, or the 1.0 mg/kg vehicle for XJB-5-131, i.p.) 30 min prior to administration of L-DOPA (10 mg/kg L-DOPA + 20 mg/kg benserazide HCl, i.p.) for 3 days. After a four day washout period, rats were administered a drug pre-treatment (10 mg/kg citalopram or XJB-5-131, i.p.) 30 min prior to administration of L-DOPA (10 mg/kg L-DOPA + 20 mg/kg benserazide HCl, i.p.) daily for 3 days. Behavioral evaluation with simultaneous electrochemical recording was performed on the first and third days each week. Rotarod testing was performed on all three days of the experiment prior to any drug administration (baseline), and >3 hours after the L-DOPA injection. An experimental design for this Chapter is outlined in Figure 4.2.

Figure 4.2: Experimental design for Chapter 4. Rats were pre-treated with either saline or vehicle (1.0 mL/kg, i.p.) 30 min before L-DOPA administration. After 4 days of washout, rats were pre-treated for 3 days with either citalopram or XJB-5-131 (10 mg/kg, i.p.) 30 min before L-DOPA administration. Electrochemical and behavioral recording was completed on the first and third day. Rotarod testing was performed each week on all 3 days of the experiment and 2 days before test days to establish stable baseline performance.
4.2.3 Rotarod-Testing

Rotarod testing was used to assess motor coordination and balance alterations in the subjects. Testing was performed on a Roto-Rod Series 8 (IITC Life Science, Inc., Woodland Hills, CA) which was accelerated from 0 to 20 RPM over 60 seconds and then continued at 20 RPM for another 60 seconds. Each test session consisted of three trials that concluded either when the rat fell off the rotarod or after the full 120 seconds had transpired. Prior to the evaluation of drug treatments, the rats were trained for 3 days to perform the test and a baseline was established\textsuperscript{21}. After training, rats were tested in the morning before any drug treatment to acquire a value for the L-DOPA off-time recording. The rats were then tested >3 hours after the L-DOPA treatment to acquire values for L-DOPA on-time assessment. For each session, the mean of three trials was taken. Time, RPM and distance values were recorded. As RPM reaches a maximum value after 60 secs, this parameter was not analyzed. Time and distance were observed to follow similar trends; therefore only distance values were reported herein.

4.2.4 Data Analysis and Statistics

To determine if XJB-5-131 or citalopram pre-treatments changed the mean frequency or amplitude of the dyskinetic episodes (both A.I.M.s and rotations) observed after administration of L-DOPA, a two-tailed paired t-test was performed to compare the vehicle control pre-treatment (vehicle or saline) with drug-pre-treatment sessions, a two-way repeated measures ANOVA was performed. Variance was analyzed between the different pre-treatments (control vs drug), the analyte, and the interaction of pre-treatment x analyte. Statistical analysis on the snippet traces was performed using ordinary two-way ANOVA with the sources of interaction set to be between behavior (non-rotating vs rotation) and treatment (control vs drug), as well as the interaction between behavior x treatment. Spearman’s
correlation analyses were used to quantify the correlation between the two analytes. To determine if vehicle, XJB-5-131, or citalopram pre-treatments varied the motor performance (distance traveled) of the rat during the L-DOPA on or off-times, one-way repeated measures ANOVA with Sidak post-hoc tests were performed on the rotarod data.

4.3 Results and Discussion

4.3.1 Correlating H₂O₂ and DA Tone with Dyskinetic Episodes after Pharmacological Manipulation of Mitochondrial-Derived H₂O₂

Mitochondrial-derived H₂O₂ was pharmacologically manipulated through pre-treatment with XJB-5-131 (10 mg/kg i.p.) 30 minutes before treatment with L-DOPA (10 mg/kg + 20 mg/kg benserazide i.p.) in a 6-OHDA lesioned rat for 3 consecutive days. Figure 4.3 shows the global A.I.M.s scores (pink) and a count of the 360⁰ rotations (green) observed in this rat over 185 mins after L-DOPA treatment (arrow at time 0) following either vehicle (A) or XJB-5-131 (B) pretreatment. Under both pretreatment conditions, an increase in A.I.M.s and 360⁰ rotations was observed after the administration of L-DOPA. The mean ± SEMs of the A.I.M.s scores and rotations across all time points post L-DOPA administration for both pretreatments was plotted (Figure 4.3 C). There were no significant differences in the mean A.I.M.s scores after XJB-5-131 treatment (18 ± 3) compared to vehicle (20 ± 4; t₈ = 0.486, P > 0.05; pink bars; Figure 4.3 C). However, XJB-5-131 pretreatment did appear to increase the mean total number of 360⁰ rotations (5 ± 1 rotations) vs. vehicle pretreatment (2 ± 1 rotations; t₈ = 3.78, P < 0.01; green bars; Figure 4.3 C).

The neurochemical dynamics of H₂O₂ and DA were simultaneously recorded in both hemispheres of this animal subject. Figure 4.3 shows the H₂O₂ (red) and DA (blue) tone (see Chapter 3; Figure 3.1 A) recorded in both the ipsilateral and contralateral striatum after pretreatment with vehicle (Figure 4.3 D) and after pharmacological manipulation with XJB-5-
131 (Figure 4.3 E). Similar to a previous study analyzing the DA and H$_2$O$_2$ tone after chronic L-DOPA administration (Chapter 3), pretreatment with only the vehicle before administration of L-DOPA elevated the DA and H$_2$O$_2$ tone in the ipsilateral striatum (top Figure 4.3 D; ipsilateral). Specifically, the H$_2$O$_2$ peaked at 40 min (228 % of baseline; red) and the DA tone peaked at 20 min after L-DOPA (251 % of baseline; blue) above baseline after vehicle pretreatment and L-DOPA administration (Figure 4.3 D, F; ipsilateral). However, these excessive swings in DA and H$_2$O$_2$ in the ipsilateral striatum were not evident after L-DOPA administration when the rat was pretreated with XJB-5-131 (Figure 4.3 E; ipsilateral). Analysis was performed to compare the vehicle pretreatment to the XJB-5-131 condition. In the ipsilateral striatum, both H$_2$O$_2$ (41 ± 11 % of vehicle; red, p < 0.05) and DA tone (34 ± 11 % of vehicle; blue, p < 0.01) were attenuated with XJB-5-131 pre-treatment (top Figure 4.3 E; ipsilateral; main effect of pre-treatment, F$_{1,34} = 11.6$, $P = 0.0017$). In the contralateral striatum, H$_2$O$_2$ and DA tone were not attenuated with XJB-5-131 pretreatment and L-DOPA administration as compared to measurements taken after vehicle pretreatment and L-DOPA administration (Figure 4.3 F; contralateral; no main effect of pretreatment: F$_{1,32} = 0.37$, p > 0.05). Therefore, there was no evidence that the pretreatment strategy altered the concentrations of these analytes in the contralateral striatum, as H$_2$O$_2$ and DA tone both remained elevated after administration of L-DOPA with either pretreatment (Figure 4.3 D-F; contralateral).

It is hypothesized that the tone of the H$_2$O$_2$ is attenuated after pre-treatment with XJB-5-131 because this molecule is trapping ROS coming from mitochondrial-derived sources$^{16,18}$. The attenuated DA tone after L-DOPA administration in the ipsilateral striatum with XJB-5-131 pretreatment was unexpected, as no prior research has been conducted to investigate
potential effects of this drug on analytes other than ROS. One explanation for the observed result could be that treatment with XJB-5-131 reduced the degree of oxidative stress in this brain region, allowing the remaining DA terminals to undergo arborization to effectively regulate DA release. Interestingly, there also seemed to be excessive swings in the extracellular DA fluctuations in the contralateral striatum for this animal (note change of DA scale; Figure 4.3 D, E), which is typically not observed in this hemisphere following L-DOPA treatment due to the presence of more intact regulatory mechanisms (for discussion see Chapter 3). However, it is possible for changes in the contralateral striatum to be observed following L-DOPA treatment due to some hemispheric cross-over of axons from the SNpc to the contralateral striatum. Moreover, here, a comparison is made to determine changes in the neurochemical fluctuations based solely on the two pretreatment conditions (vehicle vs XJB-5-131) of the same rat.

Overall, pretreatment with XJB-5-131 before L-DOPA administration did not alter the total A.I.M.s score in this rat compared to the vehicle pretreatment and L-DOPA administration performed the week prior. Although, the total 360° rotations performed by this rat increased with XJB-5-131 pretreatment when compared to the rotations observed in this rat after vehicle pretreatment and L-DOPA administration. These increased rotations are atypical, as previous research has shown that with chronic L-DOPA treatment, a decrease in the total rotations is typically observed over time. Here, however, we observed an opposite trend with XJB-5-131 pretreatment. It is possible that the increased rotations observed after XJB-5-131 pretreatment may be from less regulated DA release, since H\textsubscript{2}O\textsubscript{2} has been shown to negatively modulate DA release by way of K\textsuperscript{+}\textsubscript{ATP} channels. As shown and discussed in Chapter 3, the onset of rotational behavior in 6-OHDA lesioned rats has been correlated with sub-second
increases in extracellular DA, which acts on D1 receptors located on the medium spiny neurons (MSNs) in the striatum to drive locomotive behavior. In Chapter 3, DA dynamics were not time-locked with any specific type of A.I.M. (axial/orolingual/or limb). However, around the onset of rotational behavior, DA concentrations decreased and H\textsubscript{2}O\textsubscript{2} concentrations increased after 3 weeks of chronic L-DOPA treatment. Therefore, after pretreatment with XJB-5-131, an increase in the locomotive behavior along with no change in the A.I.Ms score is an interesting find. This elucidates that mitochondrial H\textsubscript{2}O\textsubscript{2} may play a pivotal role in down regulating involuntary rotational behavior. These preliminary findings were investigated further through detailed snippet analysis in an attempt to investigate the sub-second neurochemical dynamics underlying these rotational behaviors.

**Figure 4.3: Pharmacological manipulation of mitochondrial-derived H\textsubscript{2}O\textsubscript{2} through pretreatment with XJB-5-131.** A.I.M.s (pink) and total 360° rotations (green) of a 6-OHDA lesioned rat were scored for both (A) vehicle and (B) XJB-5-131 pre-treatments (first dotted lines; time point -30 min). These pretreatments were administered 30 minutes before L-DOPA administration (second dotted line; time point 0 min). There was in increase in the total number of 360° rotations after pretreatment with XJB-5-131 and L-DOPA administration. Additionally, H\textsubscript{2}O\textsubscript{2} and DA dynamics in both striata were simultaneously recorded after both (D) vehicle and (E) XJB-5-131 pretreatments. F, In the ipsilateral striatum there was a decrease in both H\textsubscript{2}O\textsubscript{2} and DA tone overall after pretreatment with XJB-5-131 and L-DOPA administration, this was not recorded in the contralateral striatum. Asterisks indicate significance: * $P < 0.05$, ** $P < 0.01$. n=1 rat.
4.3.2 Pre-Treatment with XJB-5-131 Changes the Neurochemical Dynamics Centered Around Rotational Behavior

To compare the effect of the different pretreatment conditions (vehicle or XJB-5-131) on rapid chemical dynamics associated with L-DOPA induced rotations, snippet analysis (See Chapter 3- Figure 3.2) was performed at 3 time points after L-DOPA administration. The colored bars on the total rotations plots (Figure 4.4 A-B) denote the 300 sec time bins where detailed snippet analysis was performed. These bins were selected because they occur during peak period of rotational behavior for this rat. Specifically, the time bins selected for snippet analysis were 20-25 min (left green panel), 40-45 min (middle red panel), and 60-65 min (right yellow panel) after L-DOPA administration. Quantification of data from both the contralateral (Figure 4.4 C-F) and ipsilateral (Figure 4.4 G-I) striata are shown for pretreatments of both vehicle and XJB-5-131. The DA signal recorded in the ipsilateral striatum was excluded from the analysis, as these data contained significant electrochemical drift and a low signal-to-noise at the oxidation potential for DA.

Overall, pretreatment with XJB-5-131 seemed to disrupt the correlation of H$_2$O$_2$ and DA dynamics (with the onset of rotational behavior) that is typically observed in 6-OHDA lesioned rats treated chronically with L-DOPA (Chapter 3). Similar to what was observed in Chapter 3, analysis of the snippet data of this rat revealed that H$_2$O$_2$ increased (both striata) and DA decreased (contralateral striatum) after the onset of rotation in the contralateral striatum when the rat was pretreated with the vehicle (Figure 4.4 C,G). Calculation of a correlation coefficient from the snippet data revealed an inverse correlation between the H$_2$O$_2$ and DA signal was apparent around the onset of rotational behavior in the contralateral striatum with vehicle pretreatment (Figure 4.4 C; 20-25 min: Spearman’s rho = -0.64, $P < 0.0001$; 40-45 min: rho = - 0.70, $P < 0.0001$; 60-65 min: rho = -0.65, $P < 0.0001$). However, when this rat
was pretreated with XJB-5-131, these dynamics were disrupted and there was no longer an increase in the H$_2$O$_2$ (both striata) and decrease in the DA signal (contralateral striatum) with the onset of rotational behavior (Figure 4.4 D, H). Additionally, an inverse correlation between these two analytes was not recorded in the contralateral striatum during the last two time points analyzed after XJB-5-131 pretreatment and L-DOPA administration (Figure 4.4 D; 40-45 min: Spearman’s rho = 0.34, $P = 0.012$; 60-65 min: rho = 0.42, $P = 0.0014$). However, snippets analyzed 20-25 min after XJB-5-131 pretreatment and L-DOPA administration did reveal an inverse correlation between the H$_2$O$_2$ and DA signals (Figure 4.4 D: Spearman’s rho = -0.72, $P < 0.0001$). Although, the DA signal seemed to be increasing with the onset of rotational behavior, which is atypical of what is observed after chronic L-DOPA administration (Chapter 3). An increase in the DA signal is typically observed during voluntary, locomotive behavior$^{35}$, or only after 1 week of L-DOPA treatment (Chapter 3, Figure 3.3 B-G). This result furthers investigations into how XJB-5-131 might be altering the neuronal adaptations which seem to occur after chronic treatment of L-DOPA.

The dynamics of H$_2$O$_2$ (both striata) and DA (contralateral striatum) before and after the onset of rotational behavior were quantified for both pretreatments (Figure 4.4 E,F,I). In the contralateral striatum, H$_2$O$_2$ dynamics increased after rotational behavior when the rat was pretreated with the vehicle but not after XJB-5-131 pretreatment across all time bins analyzed (Figure 4.4 E; main effect of behavior, 20-25 min: $F_{1,72} = 6.75$, $P < 0.05$; 40-45 min: $F_{1,68} = 45.2$, $P < 0.001$; 60-65 min: $F_{1,56} = 7.59$, $P < 0.01$; main effect of treatment, 40-45 min: $F_{1,68} = 29.6$, $P < 0.0001$; interaction of treatment x behavior, 40-45 min: $F_{1,68} = 11.3$, $P < 0.01$). Specifically, after vehicle pretreatment and L-DOPA administration, H$_2$O$_2$ increased after the onset of rotational behavior at time points 20-25 min from $1 \pm 0.2 \mu$M to $3 \pm 0.4 \mu$M ($P <$
0.001), from 0.6 ± 0.2 µM to 3 ± 0.3 µM at 40-45 min (P < 0.0001), and from 0.50 ± 0.09 µM to 1.2 ± 0.2 µM (P < 0.05) at 60-65 min (Figure 4.4 E; striped bars). However, these increases in H$_2$O$_2$ concentrations correlating with the start of rotation did not occur with XJB-5-131 pretreatment and L-DOPA administration (Figure 4.4 E, solid bars).

Even though a reliable DA signal was not recorded in the ipsilateral striatum, the H$_2$O$_2$ dynamics recorded followed a trend similar to those in the contralateral striatum. When pretreated with vehicle and administered L-DOPA, H$_2$O$_2$ increased after the onset of rotation behavior, but this was disrupted when XJB-5-131 pretreatment was administered (Figure 4.4 H; main effect of behavior, 20-25: F$_{1,72}$ = 10.8, P < 0.01; 40-45 min: F$_{1,68}$ = 7.25, P < 0.01); main effect for treatment, 40-45 min: F$_{1,68}$ = 12.4, P < 0.001). Specifically, after vehicle pretreatment, the H$_2$O$_2$ concentrations increased from 0.4 ± 0.1 µM to 1.4 ± 0.2 µM (P < 0.001) in the 20-25 min bin and increased from 0.627 ± 0.003 µM to 1.1 ± 0.1 µM (P < 0.05) during the 40-45 min time point (Figure 4.4 I; striped bars). The last time bin analyzed, 60-65 mins, did not show an increase in the H$_2$O$_2$ concentrations with rotation onset with vehicle pretreatment and L-DOPA administration. This is possibly because a wearing-off effect of the L-DOPA treatment during this later time point, lowering the total number of rotations that the rat performed, therefore a low number of snippets (n=7) were analyzed. After pretreatment with XJB-5-131 and L-DOPA administration, there was no longer an increase in the H$_2$O$_2$ dynamics observed after the onset of rotations at any time point analyzed (p>0.05 for all time bins; Figure 4.4 I; solid bars).

The dynamics of the DA concentrations centered around the onset of rotational behavior was also disrupted after pretreatment with XJB-5-131 (Figure 4.4 F; main effect of behavioral, 40-45 min: F$_{1,60}$ = 9.33, P < 0.01; main effect of treatment, 20-25 min: F$_{1,72}$ = 4.37,
$P < 0.05$; 60-65 min: $F_{1,56} = 16, P < 0.01$; main effect for the interaction between behavior x treatment, 20-25 min: $F_{1,72} = 5.71, P > 0.05$; 40-45 min: $F_{1,60} = 16.2, P < 0.001$; 60-65 min: $F_{1,56} = 9.73, P < 0.001$). Specifically, with vehicle pretreatment, the DA concentrations decreased from $3 \pm 0.5$ nM to $1.1 \pm 0.3$ nM ($P < 0.001$) in the snippets analyzed from 20-25 mins, $2.3 \pm 0.2$ nM to $0.7 \pm 0.1$ nM ($P < 0.0001$) from 40-45 mins, and $1.7 \pm 0.2$ nM to $1.1 \pm 0.1$ nM ($P < 0.05$) from 60-65 mins (Figure 4.4 F; stripped bars). However, after XJB-5-131 pretreatment and L-DOPA administration there were no longer any DA dynamics centered around the onset of rotations ($P > 0.05$; all time bins; Figure 4.4 F; solid bars). Even though a reliable DA signal was not recorded in the ipsilateral striatum, the $H_2O_2$ dynamics recorded followed a trend similar to those in the contralateral striatum after both pretreatments. A complete summary of the two-way repeated measures ANOVA results can be found in Table 4.1.

Here, a modulatory relationship of $H_2O_2$ with DA concentrations, centered around rotational behavior, was observed when the rat was pretreated with the vehicle and administered L-DOPA. This relationship was previously determined and discussed in Chapter 3, after rats had been treated chronically with L-DOPA for 3 weeks. However, the data indicate that pretreatment with XJB-5-131 disrupts the modulatory role that $H_2O_2$ has on the DA signal with rotation onset. XJB-5-131 is a ROS trap that attaches to the mitochondrial membrane; therefore these results implicate that the signal for $H_2O_2$ typically seen increasing after rotational behavior is derived from mitochondrial activation of the medium spiny neurons\textsuperscript{36}. When the rat was pretreated with only the vehicle and administered L-DOPA, the DA signal was attenuated by $H_2O_2$ after rotational behavior, presumably by way of $K^{+}_{ATP}$ channels\textsuperscript{28}. However, with XJB-5-131 pretreatment to L-DOPA administration, there were no DA
dynamics centered around the onset of rotational behavior. This is most likely because mitochondrial H$_2$O$_2$ is not available to attenuate the DA signal, therefore no decrease in the signal was observed.
Figure 4.4: Pretreatment with XJB-5-131 disrupts neurochemical dynamics typically observed with rotational behavior. Three time bins of data were analyzed during peak rotational behaviors after L-DOPA treatment for both vehicle (A) and XJB-5-131(B) pretreatments to a lesioned rat. In both striata, snippet analysis was performed during 3 time bins after vehicle (C,G) and XJB-5-131 (D,H) pretreatments to L-DOPA administration. After treatment with XJB-5-131, the inverse correlation between H$_2$O$_2$ and DA with the onset of rotational behavior was no longer apparent for any time point. Quantitative comparisons of the two pretreatments show how the dynamics of both DA (F) and H$_2$O$_2$ (F,I) no longer correlate with the behavior after XJB-5-131 pretreatment. Asterisks indicate significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; n=1 rat.
Table 4.1: Complete summary of the results from a two-way non-repeated measures ANOVA performed on data in Figure 4.4. Areas of significance are shown in bold.

<table>
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<th>Analyte</th>
<th>Time point (min)</th>
<th>Behavior</th>
<th>p-value</th>
<th>Treatment</th>
<th>p-value</th>
<th>Interaction</th>
<th>p-value</th>
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</thead>
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<td>H₂O₂</td>
<td>20-25</td>
<td>F(1,72)=10.8</td>
<td>**p&lt;0.01</td>
<td>F(1,72)=3.9</td>
<td>p=0.052</td>
<td>F(1,72)=2.7</td>
<td>p=0.110</td>
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<tr>
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<td>-40-45</td>
<td>F(1,68)=7.3</td>
<td>**p&lt;0.01</td>
<td>F(1,68)=12.0</td>
<td>***p&lt;0.001</td>
<td>F(1,68)=2.6</td>
<td>p=0.110</td>
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<tr>
<td></td>
<td>-60-65</td>
<td>F(1,56)=0.0028</td>
<td>p=0.96</td>
<td>F(1,56)=2.5</td>
<td>p=0.12</td>
<td>F(1,56)=1.1</td>
<td>p=0.290</td>
</tr>
</tbody>
</table>

4.3.3 Correlating H₂O₂ and DA Tone with Dyskinetic Episodes after Pharmacological Blockade of the 5-HT Transporter

Injections of saline (1.0 mL/kg i.p.) or citalopram (10 mg/kg i.p.) were performed for 3 consecutive days 30 minutes before L-DOPA administration (10 mg/kg +20 mg/kg benzerazide). Figure 4.5 shows the global A.I.M.s scores and number of rotations on the third day of observations for saline pretreatment and L-DOPA administration (Figure 4.5 A) along with citalopram pretreatment and L-DOPA administration (Figure 4.5 B). The mean A.I.M.s score and total 360° rotations performed by this rat for all time points analyzed after administration of L-DOPA are shown in the bar graph (Figure 4.5 C). With saline pretreatment and L-DOPA administration, the hemiparkinsonian rat exhibited A.I.M.s and 360° rotations (Figure 4.5 A; mean A.I.M.s: 50 ± 8; mean rotations/min: 5 ± 2). However, pretreatment with citalopram significantly decreased A.I.M.s (5 ± 2; t₁₆ = 5.6, P < 0.0001) and 360° rotations/min (Figure 4.5 C; 0.3 ± 0.1 rotations/min; t₁₆ = 2.5, P < 0.05).
The neurochemical dynamics of H$_2$O$_2$ and DA were recorded in both hemispheres during the behavior observations. When pretreated with only saline, a large increase in DA tone (blue) was observed after L-DOPA administration in both the ipsilateral (top) and contralateral (bottom) striata (Figure 4.5 D), consistent with previous experiments (Chapter 3). Specifically, the DA and H$_2$O$_2$ tone in the ipsilateral striatum peaked 40 min after L-DOPA administration (DA: 580 % of baseline; H$_2$O$_2$: 150 % of baseline). In the contralateral striatum, DA tone peaked 20 min after L-DOPA (340 % of baseline), whereas the H$_2$O$_2$ tone did not show any excessive swings in concentrations after L-DOPA for this rat. Interestingly, the swings in H$_2$O$_2$ (red) and DA (blue) tone were not evident with citalopram pretreatment in either hemisphere (Figure 4.5 E). Next, analysis was performed to quantify the overall effects of citalopram on the total H$_2$O$_2$ and DA tone (Figure 4.5 F). A significant main effect of pretreatment type was observed ($F_{1,32} = 4.44$, $P < 0.01$), however this was not the case for either analyte ($F_{1,32} = 0.72$, $P > 0.05$), or the interaction term ($F_{1,32} = 0.72$, $P > 0.05$; ipsilateral Figure 4.5 F top). In the ipsilateral striatum (top), citalopram pretreatment (prior to L-DOPA) significantly decreased DA tone overall (18 ± 8 % of saline; $P = 0.024$). In the contralateral striatum, the total DA tone in the recordings after L-DOPA administration did not differ based on pretreatment condition (30 ± 11 % of saline; no main effect of pretreatment: $F_{1,32} = 0.99$, $P > 0.05$; no main effect of analyte: $F_{1,32} = 3.01$, $P > 0.05$; no interaction effect: $F_{1,32} = 0.99$, $P > 0.05$). The tone recorded for H$_2$O$_2$ remained unchanged when comparing saline pretreatment and L-DOPA administration to pretreatment with citalopram and L-DOPA administration in either striata (ipsilateral: 65 ± 12 % of saline; contralateral: 80 ± 24 %).

Large, transient increases in DA caused by L-DOPA administration have been causally linked to motor difficulties$^{37,38}$, and excessive swings in DA in the striatum demonstrated by
microdialysis\textsuperscript{39,40} and PET imaging in humans\textsuperscript{6}. The cause of these excessive swings has been studied through double-lesions of both dopaminergic and serotonergic afferents into the striatum. These lesions have been shown to lower synaptic dopamine following administration of L-DOPA in animal models\textsuperscript{41}. Similarly, the dampening of 5-HT neuron activity by use of 5-HT\textsubscript{1A} and 5-HT\textsubscript{1B} agonists has been shown to decrease L.I.D.s in 6-OHDA lesioned rats with both partial and full lesions\textsuperscript{11}, however, administration of 5-HT\textsubscript{1A} agonists has also been shown to cause 5-HT syndrome in rats\textsuperscript{42}. Studies have shown increased 5-HT transporter to dopamine transporter ratios are correlated with increased A.I.M.s, indicating a functional shift caused by the L-DOPA treatment\textsuperscript{43}. Additionally, pretreatment with SSRIs, 15-30 mins before L-DOPA administration, has attenuated the A.I.M.s score of 6-OHDA lesioned rats\textsuperscript{42-45}, without causing 5-HT syndrome side-effect\textsuperscript{42}.

In this experiment, citalopram pretreatment 30 mins before L-DOPA administration further verified that the excessive swings in DA after L-DOPA are due to co-release of L-DOPA derived DA and 5-HT from these dorsal raphae nuclei terminals. Citalopram pretreatment attenuated both the excessive swings in DA concentrations and dyskinetic behavior observed in this rat. Yet, reducing these excessive swings in DA after citalopram pretreatment and L-DOPA administration did not seem to reduce oxidative stress in the striatum of this individual rat. In order to draw appropriate conclusions, the experiment will need to be repeated with sufficient replicates.
Figure 4.5: Pharmacological blockade of 5-HT transporter through administration of citalopram. A.I.M.s (pink) and total 360° rotations (green) of a lesioned rat were scored for vehicle (A) and citalopram (B) pretreatments (first dotted line; time point -30 min) which were administered 30 minutes before L-DOPA treatment (second dotted line; time point 0). There was a decrease in the A.I.M.s score and number of 360° rotations after pretreatment with citalopram to L-DOPA administration (A.I.M.s: t₈=5.98, ***P < 0.001; Rotations: t₈ = 2.62, *P < 0.05). H₂O₂ and DA dynamics in both the ipsilateral and contralateral striata were simultaneously recorded to correlate with the behavior after both saline (D) and citalopram (E) pretreatments. (F) In the ipsilateral striatum, citalopram reduced DA tone evoked by L-DOPA treatment. Two-way non-repeated measures ANOVA with Sidak post-hoc; **P < 0.01. n=1 rat.

4.3.4 Assessment of Motor Function after Pharmacological Manipulation of DA and H₂O₂ Pathways

A Rotarod performance test provides a measure of the motor activity and coordination of an animal, typically a rodent. The animal is forced to walk on a spinning rod that is slowly increasing in speed until they are unable to maintain their pace with the spinning rod and eventually fall off. The motor function of a single rat was assessed via rotarod during the time course of the L-DOPA treatment after either vehicle, XJB-5-131, or citalopram pretreatments. The distance that the rat traveled on the rotarod before any daily drug manipulation (off-time) and 3 hours after the L-DOPA treatment (on-time) with various pretreatments was recorded for 3 days (Figure 4.6). One-way ANOVA between the pre-treatment groups revealed the
animal traveled a longer distance when tested before the daily L-DOPA administration had been performed (during the L-DOPA off-time) throughout the 3 days of pretreatment with XJB-5-131 (4 ± 1 m) as compared to either vehicle (1 ± 0.2 m) or citalopram (2 ± 0.3 m) pretreatments (Figure 4.6 A; One way ANOVA: $F_{2,25} = 3.85, P < 0.05$). When the motor function was tested during the L-DOPA on-time, analysis determined that pretreatment with XJB-5-131 did not increase the distance that the rat traveled on the rotarod (3.0 ± 0.6 m) compared to vehicle pre-treatment (2 ± 0.4). However, the rat traveled a significantly further distance when pretreated with citalopram over 3 days (5 ± 0.5 m) during the L-DOPA on-time compared to either XJB-5-131 or vehicle pre-treatments (Figure 4.6 B; One-way ANOVA: $F_{2,26} = 12, P < 0.001$).

Previous studies assessing chronic treatment with XJB-5-131 have shown improved grip strength and rotarod performance in Huntington’s disease modeled rats$^{16,18}$. XJB-5-131 also decreased the frequency of ‘foot dragging’$^{16}$. Here, XJB-5-131 improved the rotarod performance in hemiparkinsonian rats, but only during L-DOPA off-time. The motor performance during the L-DOPA on-time was impaired because the rat was still experiencing rotational behaviors during the rotarod testing period. The data show that pretreatment with citalopram improved the distance traveled when compared to saline pre-treatment during L-DOPA on-time. This indicates that, for these dosages, citalopram facilitates the efficacy of L-DOPA in improving motor function, likely by decreasing the excessive swings of extracellular DA caused by L-DOPA being converted to DA by serotonergic terminals. The effect of SSRIs on motor performance after L-DOPA treatment has previously been studied using hemiparkinsonian rats$^{42,43}$, as well as in human PD patients$^{46}$. Studies in hemiparkinsonian animal models have shown results similar to those discovered here, with several finding a dose-
dependent attenuation of L.I.D.s and improved motor performance, as assessed by forepaw adjusted steps test\textsuperscript{43,47}. Although a different motor performance test was used in this work, the data reported herein is consistent with previous studies showing attenuation of L.I.D.s, as well as improvement of motor performance.

![Figure 4.6: Motor function assessed through rotarod performance during L-DOPA on and off times.](image)

4.4 Conclusion

The data from this series of experiments is but a promising beginning to further investigations into the mechanisms behind L.I.D.s. Administration of XJB-5-131, a molecule that is capable of specifically trapping mitochondrial ROS, was able to attenuate both DA and H\textsubscript{2}O\textsubscript{2} signals in the lesioned hemisphere in 6-OHDA lesioned rats. Additionally, contralateral rotations were increased after administration indicating improved locomotive function during L-DOPA on-time\textsuperscript{48}. However, there was no apparent impact on the A.I.M.s behaviors. Citalopram, a clinically-relevant SSRI, was able to attenuate fluctuations in DA on the lesioned side, in addition to attenuation of L.I.D.s. Overall, these data indicate that L-DOPA treatment causes a complex problem that involves the overlap of several neurotransmitters and
neuromodulators, indicating a need for a multi-phase treatment strategy for PD. Further experimentation will need to be performed in order to improve the statistical power of these results. Ultimately, results from this study will guide future therapeutic strategies as they seek to improve the quality of life for patients with this neurodegenerative disorder.
4.5 References


CHAPTER 5 Summary, Clinical Relevance, and Outlook

5.1 Research Summary

Oxidative stress has been implicated in the initiation and progression of multiple neurodegenerative diseases such as PD. The nigrostriatal DA system has been a major research focus for PD as it has a high susceptibility to oxidative stress. Dopaminergic replacement therapy with L-DOPA is the gold-standard, however at the inevitable price of eventually developing abnormal involuntary movements (A.I.M.s; associated with L-DOPA-induced dyskinesias (L.I.D.s) behavior). The discrete mechanisms underlying L-DOPA’s function to increase extracellular DA in the denervated striatum are becoming more clear\(^1\)\(^-\)\(^3\), yet how it contributes to A.I.M.s and oxidative stress is not well understood. Research has implicated L-DOPA treatment in furthering neurodegeneration via oxidative stress, however some studies indicate it may be neuroprotective\(^4\). The understanding of the in situ effects of L-DOPA treatment and L.I.D.s is limited to date, as few analytical techniques able to provide adequate temporal and spatial resolution to monitor dynamic concentrations of oxidative stress and DA. The work presented here addressed this with the development of a new analytical sensor and with in vivo studies investigating striatal DA and H\(_2\)O\(_2\) dynamics during A.I.M.s behavior in rats following L-DOPA treatment and anti-dyskinetic agents to examine the mechanisms that govern L.I.D.s.

Chapter 2 is an article published in Analytical Chemistry\(^5\) characterizing a coated sensor for the real-time quantification of H\(_2\)O\(_2\) in vivo with background subtracted FSCV\(^6\),\(^7\). With FSCV, rapid fluctuations of multiple neurotransmitters can be identified and quantified based on the oxidative peak shape and peak position. However, the selective detection of H\(_2\)O\(_2\) in live brain tissue is complicated as other endogenous molecules and pharmacological agents
also oxidize at or near +1.4 V, the oxidation potential for H₂O₂. The electrodeposition of 1,3-phenylenediamine (mPD) onto the carbon-fiber sensing surface, a size-exclusion polymer, allows for the exclusion of larger molecular interferents such that only small molecules, such as H₂O₂, were electrochemically detected. Characterization was performed through optimization of the electrodeposition time of the mPD membrane to the electrode surface, determining the stability of the mPD membrane over time, and determination of sensor selectivity through the use of a dual-microelectrode device (DMED). Further investigation on the utility of this sensor was performed through measurements with a DMED both in live tissue as well as in the intact brain after pharmacological manipulation. The mPD coated electrode selectively detected H₂O₂ after application of mercaptosuccinic acid, an irreversible inhibitor of the enzyme glutathione peroxidase. This enzyme protects the brain from oxidative damage by reducing free H₂O₂ to water. This mPD coated voltammetric sensor will enable researchers to confidently measure H₂O₂ in the complex environment of the brain to investigate the role of H₂O₂ in normal as well as pathophysiological states that are associated with increased oxidative stress. However, the sensor was not characterized for its stability beyond 4 hours and therefore was not used in other investigations within this dissertation that required sensors to be implanted 3+ weeks.

Chapter 3 is an article in preparation to Nature Communications where FSCV was used for the real-time, simultaneous measurements of striatal DA and of H₂O₂ dynamics in non-lesioned and hemi-parkinsonian rats undergoing daily, chronic L-DOPA treatment. Rapid neurochemical measurements were time-locked to dyskinetic behaviors in an effort to reveal their precise role in the expression of A.I.M.s. After 1 week of daily L-DOPA treatment, lesioned rats exhibited A.I.M.s and contralateral rotations that coincided with increased
ipsilateral DA and bilateral H₂O₂ tone in the hours following L-DOPA administration. Interestingly, specific examination of real-time DA and H₂O₂ dynamics revealed a novel relationship with rotational bouts that progressively emerged across time. After 3 weeks of L-DOPA treatment, H₂O₂ concentrations increased at the onset of rotational behavior, as DA concentrations simultaneously decreased in the striata of lesioned animals. In collaboration with Dr. David Muddiman from the Chemistry Department at North Carolina State University, IR-MALDESI MSI was used to verify the considerable loss of the DA terminals in the striata of the hemi-parkinsonian rats. The findings of this studied prompted further investigation of the various sources of H₂O₂ in the dorsal striatum which might simultaneously contribute to A.I.M.s and rotations.

In Chapter 4, the neurochemical systems underlying A.I.M.s and rotational behavior were further investigated by pharmacologically manipulating sources of H₂O₂ and DA. We sought to attenuate ROS, and thus the generation of H₂O₂, derived from mitochondrial sources with the synthetic bifunctional antioxidant XJB-5-131⁹. This work was done in collaboration with Dr. Peter Wipf in the Chemistry Department at the University of Pittsburgh. XJB-5-131 contains a gramicidin-like backbone with a mitochondrial targeting end of the molecule along with a radical scavenger on the other. Administration of XJB-5-131 prior to L-DOPA reduced elevations in H₂O₂ and DA in the lesioned striatum and improved locomotive function; however, A.I.M.s were not attenuated. In depth, snippet analysis of these data revealed that the inverse relationship between H₂O₂ and DA centered at the onset of rotational behavior previously observed with chronic L-DOPA treatment was disrupted after administration of XJB-5-131. As increased DA in the denervated striatum following L-DOPA administration is largely thought to arise from ectopic release from 5-HT terminals, an additional animal was
pre-treated with citalopram, a serotonin reuptake inhibitor (SSRI), prior to daily L-DOPA. Citalopram pre-treatment attenuated the fluctuations of DA in the lesioned striatum after L-DOPA treatment. Additionally, A.I.M.s and rotation were significantly reduced and voluntary motor function was improved. The data provided in this study show promising results which will ultimately guide future investigations regarding the sources of striatal DA and H$_2$O$_2$ in the development and progression of L.I.D.s.

5.2 Clinical Relevance and Outlook

Overall, excessive swings in DA and H$_2$O$_2$ tones were observed in the rat striatum in the hours after L-DOPA treatment at 1 week with a dose of 6 mg/kg (Chapter 3), and after 3 days of a high dose of L-DOPA (10 mg/kg) (Chapter 4). These swings were positively correlated with A.I.M.s scores and rotational behaviors. The relationship between L.I.D.s and ROS concentrations in the <4 hours post L-DOPA has not been directly investigated in humans. However, a post-mortem study of the cerebral spinal fluid of patients treated with L-DOPA (450 mg, 8 weeks) reported higher levels of 3-nitrotyrosine, a marker for oxidative stress, compared to untreated patients$^{10}$. Additionally, blood samples looking at the concentration of homocysteine from PD patients on L-DOPA indicated increases in systemic markers of neurodegeneration, such as amyloid-β and α-synuclein$^{11}$. Most research has been focused on DA related effects in PD patients after L-DOPA treatment. Excessive swings in DA have been reported 1 hour after L-DOPA administration using $^{11}$C-raclopride PET imaging, which detects synaptic DA fluxes as changes in D$_2$ receptor availability. These results also correlate with increased PD symptoms after 1 hour, but not 4 hours post L-DOPA administration$^{12}$. Moreover, Politis et al. also demonstrated, also through PET imaging, that dyskinetic patients had increased concentrations of extracellular DA when compared to non-
dyskinetic controls. These results performed in PD patients beg the question of how these excessive swings in DA are generated, particularly when a majority of the DA terminals gone.

Depression is a major non-motor symptom of PD patients\(^{13}\). Therefore, the impact of combination therapy of SSRIs with L-DOPA on motor performance is particularly interesting. There is amassing evidence supporting the role of serotonin neurons in the uptake of L-DOPA, converting it to DA, and releasing it into the extracellular space with few regulatory mechanisms available to prevent excessive concentrations. In Chapter 4 of this dissertation, citalopram pre-treatment reduced L.I.D.s along with DA concentrations in the lesioned striatum in the 3 hours after L-DOPA treatment. Human clinical data has conflicting reports on the utility of SSRIs to attenuate dyskinetic behavior without negating the efficacy of L-DOPA. Much of the discrepancy in the data is due to the range of L.I.D. severity in PD patients before starting the SSRI pretreatment. In a study using 5 PD patients who had been on L-DOPA from 4-15 years, pretreatment with buspirone, a serotonin 1A agonist, reduced L.I.D.s in all patients but increased the frequency of “off” periods in 3 of the patients\(^{14}\). Additionally, using \(^{11}\)C-DASB, a radioligand marker for serotonergic function in positron-emission tomography imaging, Politis et al determined that pretreatment with buspirone improved L.I.D.s in PD patients who exhibited moderate L.I.D.s. Furthermore, using \(^{11}\)C-raclopride, the excessive swings of extracellular DA concentrations in this subset of patients were attenuated with the buspirone pretreatment to those of non-dyskinetic patients\(^{15}\). However, in this study, the pretreatment had no neurochemical or behavioral effects on patients who previsously displayed severe L.I.D.s. Moreover, Lee et al. have shown that the SERT to DAT binding ratio has been shown to be increased in dyskinetic compared to non-dyskinetic patients\(^{16}\). The Citalopram is perhaps one of the more promising SSRIs, as it has no apparent deleterious effect
on motor performance in PD patients, and may show improvement in motor function with long-term use\textsuperscript{17}. Altogether, these data indicate that future studies should consider targeting the serotonin pathway as a contributor to the progression of L.I.D.s in PD patients.

Overall, the work presented in this dissertation furthers the understanding of oxidative stress in the striatum and its role in the chronic administration of L-DOPA treatment. Collectively, this dissertation has shown that H\textsubscript{2}O\textsubscript{2} plays a neuromodulatory role in the brain, is elevated after 1 week of L-DOPA treatment in hemiparkinsonian rats, and is generated from mitochondrial as well as enzymatically derived sources, such as glutathione peroxidase. We have also shown that DA is elevated in hemi-parkinsonian rats after the onset of rotations with 1 week of L-DOPA treatment, is modulated by H\textsubscript{2}O\textsubscript{2} after rotational behaviors at 3 weeks of L-DOPA treatment, and is dysregulated, causing L.I.D.s, in the hours after L-DOPA treatment due to the interplay of serotonergic neurons. These results will inform the development of improved therapeutic strategies, which will ultimately alleviate the disabling dyskinetic episodes that are side-effects of the L-DOPA treatment.
5.3 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: 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 Nafion-coated, ion-exchange electrodes for the enhanced sensitivity to DA.

A.1 Introduction

Parkinson’s disease (PD) is a neurodegenerative disorder that affects more than a million people in the United States. It is characterized by motor deficits including bradykinesia, rigidity, and resting tremor, resulting from a progressive loss of nigrostriatal dopamine (DA) neurons. DA does not readily cross the blood-brain barrier, thus its use for treating the symptoms of PD is precluded. 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. However, the efficacy of prolonged L-DOPA treatment wanes over time and patients develop serious motor complications. 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. 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. Thus, these studies have significantly advanced our knowledge on how L-DOPA serves to gradually increase striatal DA levels. 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 \(^{29-31}\). This film reduces the active surface area of the sensor and attenuates sensitivity \(^{32-34}\).

![Scheme A1. DA polymerization pathway.](image)

Nafion, a perfluorinated ion-exchange polymer, has been extensively used on the surface of sensors to repel interfering and adsorbing species \(^{35-37}\). There are numerous protocols for creating a Nafion membrane at the electrode surface \(^{35,38-46}\); 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. \(^{46}\) 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 \(^{45}\). 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 mM MgCl₂, 2.0 mM Na₂SO₄, 1.25 mM NaH₂PO₄, and 145 nM NaCl) at pH 7.40.

A.2.2 Carbon-fiber Microelectrode Fabrication.

Carbon-fiber microelectrodes were fabricated as described previously⁴⁹. 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 previously⁵⁰. 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 benserazide-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/benserazide cocktail at a clinically relevant dose (6 mg/kg + 10 mg/kg benserazide, i.p.)25,55, followed by a higher dose (250 mg/kg + 400 mg/kg benserazide,
i.p.). These doses of L-DOPA methyl-ester are equivalent to 5 mg/kg and 200 mg/kg of L-DOPA, respectively. 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. 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 ). Studies using single cells have demonstrated that L-DOPA is effectively loaded into the neuronal cytosol by the L-amino acid transporter. In primary cultures of murine substantia nigra neurons, L-DOPA treatment increases DA levels in the cytosol by > 100-fold. 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. 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.

However, in healthy brain tissue L-DOPA treatment could decrease evoked dopamine release by D2-mediated autoinhibition of DA release. 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.

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 A.1. 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. 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 A.S.1). Figure A.1 B 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 microdialysis70-72, 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\textsuperscript{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\textsuperscript{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.2 A); however, exposure to 50 μM L-DOPA significantly attenuated sensitivity to DA (Figure A.2 B, n = 6, *** p < 0.001).

Indeed, Hefti et al. have reported a peak value of ~100 μM L-DOPA (20 μg/g) in the rat striatum after administration of a very high dose of L-DOPA (500 mg/kg)\textsuperscript{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.
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\textsuperscript{38-41,43-44}. It also decreases sensitivity to several negatively-charged species, including AA, which is ubiquitous in the brain\textsuperscript{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. It 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 \textasciitilde 1.3V 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.4 B). 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.4 C). 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\textsuperscript{35, 37}. 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.4 D) and stability (Figure A.4 E). 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.5 A), or to the stimulated secretion of DA in an intact brain (Figure A.5 B). Finally, Nafion-coated electrodes prepared using different electrodeposition times were tested in vivo. Figure A.5 C 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. 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 \textit{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\textsuperscript{10, 96} or mathematical modeling\textsuperscript{57} 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.S.1 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


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