REVIEW OF FAST SCAN CYCLIC VOLTAMMETRY AND MULTIPLEXING

NEUROCHEMICAL DETECTION WITH CARBON FIBER MULTIELECTRODE ARRAYS

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REVIEW OF FAST SCAN CYCLIC VOLTAMMATERY AND EXPERIMENTAL CHARACTERIZATION OF THE NOVEL MULTI ARRAY IN NEUROCHEMICAL DETECTION

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ABSTRACT

Fast scan cyclic voltammetry (FSCV) is an analytical technique that was first developed over 30 years ago. Since then, it has been extensively used to detect dopamine using carbon fiber microelectrodes (CFMEs). More recently, electrode modifications and waveform refinement have enabled the detection of a wider variety of neurochemicals including nucleosides such as adenosine and guanosine, neurotransmitter metabolites of dopamine, and neuropeptides such as enkephalin. These alterations have facilitated the selectivity of certain biomolecules over others to enhance the measurement of the analyte of interest while excluding interferants. In this thesis, we detail these modifications and how specializing CFME sensors allows neuro-analytical researchers to develop tools to understand the neurochemistry of the brain in disease states and provide groundwork for translational work in clinical settings.

Multiplexing neurochemical measurements in multiple brain regions simultaneously have long been in demand in the greater neuroscience community. Metal microelectrode arrays have been vastly used for neural recordings but cannot measure biomolecules in the brain. Conversely, carbon fiber microelectrodes (CFMEs) have been known to measure neurotransmitters with FSCV due to their ability to adsorb cationic monoamine neurotransmitters on the surface through electrostatics or $\pi-\pi$ stacking. Although FSCV in tandem with CFMEs have high temporal and spatial resolution, only a single channel potentiostat and electrodes have been utilized. In this
work, we configured the carbon fiber multielectrode array (MEA), to a commercially available 4-channel potentiostat for multiplexing neurochemical measurements. The MEA’s relative performance was compared to single channel CFMEs. Dopamine detection was found to be adsorption controlled on the surface of the multielectrode array. Multiple waveforms were applied to each fiber of the multi array simultaneously to detect four different analytes on each electrode of the MEA. A proof of concept ex-vivo experiment showed that the MEA could record redox activity in the mouse caudate putamen and detect dopamine in a 3mm² area. To our knowledge, this is the first use of the MEA paired with a commercial multichannel potentiostat for multi-waveform application and neurotransmitter codetection. This novel array may aide in future studies to better understand complex brain heterogeneity, the dynamic neurochemical environment, and how disease states or drugs affect separate areas concurrently.
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TABLE OF CONTENTS

ABSTRACT......................................................................................................................... ii
ACKNOWLEDGMENTS ........................................................................................................ iv
LIST OF TABLES .................................................................................................................. vi
LIST OF ILLUSTRATIONS ............................................................................................... vii
LIST OF ABBREVIATIONS ............................................................................................... viii

CHAPTER 1  INTRODUCTION ......................................................................................... 1

CHAPTER 2  RECENT ADVANCES IN FSCV DETECTION OF NEUROCHEMICALS VIA WAVEFORM AND CARBON MICROELECTRODE MODIFICATION ................................................. 3

CHAPTER 3  MULTI ARRAY ............................................................................................ 26

CHAPTER 4  EXPERIMENTAL METHODS AND MATERIALS ........................................ 31

CHAPTER 5  RESULTS .................................................................................................... 36

CHAPTER 6  DISCUSSION AND CONCLUSION ............................................................ 47

CHAPTER 7  FUTURE DIRECTIONS ............................................................................. 49

APPENDIX A  SUPPLEMENTAL FIGURES .................................................................... 51

REFERENCES ................................................................................................................... 55
LIST OF TABLES

Table 1. Neurotransmitters detected by FSCV ................................................................. 24
LIST OF ILLUSTRATIONS

Figure 1 ......................................................................................................................... 8
Figure 2 ......................................................................................................................... 12
Figure 3 ......................................................................................................................... 16
Figure 4 ......................................................................................................................... 18
Figure 5 ......................................................................................................................... 20
Figure 6 ......................................................................................................................... 35
Figure 7 ......................................................................................................................... 37
Figure 8 ......................................................................................................................... 38
Figure 9 ......................................................................................................................... 39
Figure 10 ....................................................................................................................... 42
Figure 11 ....................................................................................................................... 43
Figure 12 ....................................................................................................................... 44
Supplemental Figure 1 ................................................................................................. 51
Supplemental Figure 2 ................................................................................................. 52
Supplemental Figure 3 ................................................................................................. 52
Supplemental Figure 4 ................................................................................................. 53
Supplemental Figure 6 ................................................................................................. 54
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSCV</td>
<td>Fast Scan Cyclic Voltammetry</td>
</tr>
<tr>
<td>CFME</td>
<td>Carbon Fiber Microelectrode</td>
</tr>
<tr>
<td>NTs</td>
<td>Neurotransmitters</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine Transporter</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine Oxidase</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NET</td>
<td>Norepinephrine Transporter</td>
</tr>
<tr>
<td>Adn</td>
<td>Adenosine</td>
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<tr>
<td>Gn</td>
<td>Guanosine</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen Peroxide</td>
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<tr>
<td>ENK</td>
<td>Enkephalin</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

Overview of the Project

The aim of this research project was to detail a review of recent advancements in the analytical chemistry technique, fast scan cyclic voltammetry (FSCV) and carbon fiber microelectrodes (CFMEs). This served as the basis of learning the theory behind FSCV and its application in detection of neurotransmitters.

Next, we studied and worked with a novel multi array, to determine the advantages it had over the traditional CFMEs. This array was used to detect multiple analytes, apply several waveforms simultaneously, and to test in mouse brain tissue in dopamine rich areas. In electrochemistry, the waveform is a function of voltage over a time course and are generally described by their shape.

Research Questions and Hypotheses

Aim 1: Review FSCV and recent advances in surface modifications and waveform development

Aim 2: Experiments conducting using the Multi array

- Explaining what a “Multi array” is
- Dopamine Experiments
- Comparing to traditional CFMEs
- Co-detection simultaneous
- Ex-vivo mouse brain experiments
- Relevance
CHAPTER 2
RECENT ADVANCES IN FSCV DETECTION OF NEUROCHEMICALS VIA WAVEFORM AND CARBON MICROELECTRODE MODIFICATION

Introduction

Many human diseases and disorders can be traced back to an imbalance of biomolecules and neurotransmitters (NT). Although we can identify which are implicated for certain disease states, detection, differentiation, and quantification is still a challenge for analytical chemists and neuroscientists alike. Electrochemical techniques have been used to resolve this issue, but not all methods are equal in successfully detecting neurochemicals.

Amperometry involves the application of a constant potential to monitor the electron transfer of an analyte\(^1\). Larger molecules, such as cholesterol have been detected using amperometry with enzymatic surface modification \(^{1,2}\). Additionally, neurotransmitters like dopamine\(^3\) have also been detected using amperometry and because the potential is held constant during the experiment, no charging currents are generated. However, although amperometry provides high sensitivity for a single molecule, it has relatively low selectivity compared to other electrochemical techniques such as voltammetry \(^3\).

Other electrochemical detection methods include electrochemical impedance spectroscopy or EIS. EIS measures the resistive and capacitive properties of an analyte by applying a periodic voltage signal. Impedimetric detection has been used to observe immunological binding between an antibody and an antigen where minute changes in impedance are proportional to concentration of the antigen \(^4\). Additionally, this detection method has been utilized as DNA, protein, and cell apoptosis sensors \(^5\). The EIS working electrode can be easily modified for high specificity in detection, however, the identical conditions and surface
regeneration are not easily reproducible. False positive results must be analyzed with precaution, especially with the low signal to noise ratio.

In addition to these electrochemical techniques, quantifying neurotransmitters has been accomplished using sampling techniques such as microdialysis. Used in tandem with liquid chromatography and mass spectroscopy (LC-MS), this technique allows for multiplexed detection of neurotransmitters. Since many analytes, such as dopamine, are not CNS permeant or unable to cross the blood brain barrier into the central nervous system, microdialysis requires in situ measurements. A cannula is implanted into a specific area of the brain with a buffer solution, such as Ringers, perfusing through a microdialysis probe. The probe membrane has a semi-permeable sheath to allow for the passage of small molecules, rather than larger macromolecules. Samples are collected and quantified using high performance liquid chromatography through columns that are selective for the analyte of choice. This method allows for freely behaving animal studies and pairing locomotor activity with dopamine measurements. However, it does not have fast sub second temporal resolution at the rate of neuronal firing. The size of the probe also makes targeting specific brain areas difficult leading to poor temporospatial resolution in addition to an immune response and inducing tissue damage. Although these and other methods exist, they lack spatiotemporal resolution that voltammetric sensors such as CFMEs have.

In this review we detail recent advances made in the analytical technique, fast scan cyclic voltammetry (FSCV), for detection of neurotransmitters with the use of carbon fiber microelectrodes. Rapid detection is necessary to understand human diseases such as schizophrenia and Parkinson’s, the second most common neurodegenerative disease by
measuring transient dopamine release in vivo. Both diseases involve an imbalance of the neurotransmitter dopamine.

Dopamine (DA) is a catecholamine and a chemical messenger in the central nervous system and is found in brain regions such as the substantia nigra and basal ganglia. DA is synthesized by dopaminergic neurons, stored in the neuron’s vesicles, and released to influence the reward-motivation behavior. Dopamine, and serotonin, an indolamine NT, are also implicated in major depressive disorder (MDD), which affects nearly 4.5% of all adults in the United States. In MDD, the mechanism of action of the serotonin (5-HT) is not fully understood. It is also debatable if only the depletion of 5-HT is the sole cause for clinical depression. These are just two of the many biologically relevant molecules where enhanced detection and quantification can aide in furthering our understanding of the roles of these NTs in diseases.

FSCV and carbon fiber microelectrodes (CFMEs) match the time scale of neurotransmitter release with their combined high temporal and spatial resolution. Utilizing rapid scan rates produces an output of current versus voltage or a cyclic voltammogram (CV). The CV acts as a “fingerprint” where the shape and the position of the voltammogram peaks are specific for each neurochemical. A waveform is applied onto the surface of electrode which encompasses the reduction and oxidation potentials of the molecule of interest. The frequency and scan rate can be adjusted to optimize spatial and temporal resolution, respectively.

Although FSCV has been used in the past, recent advances have led to substantial progress in detecting many biologically relevant molecules beyond DA and 5-HT. We begin by detailing recent advances made in waveform optimization for analyte detection in addition to how CFME
surface modification can enhance detection by improving selectivity and decreasing electrode fouling.

**Dopamine and Carbon Electrodes**

CFMEs are individually made by aspirating a ~7-micron carbon fiber into a 1.2 mm glass capillary tube. Commonly used fibers include Goodfellow USA and Cytec Thornel. Using a pipette puller, the glass capillary is stretched to a tapered point and is sealed using oven-cured epoxy resin. The protocol has been used numerous times in the fabrication of CFMEs which adds to consistency and reproducibility between numerous research labs \(^{24}\). Through electrochemical pretreatment, the surface area and roughness of the electrode increases the amount of carbonyl and hydroxyl functional groups on the fiber’s surface and is constantly renewed by an oxidative etching process \(^{25}\). A silver-silver chloride (Ag/AgCl) disk or pellet is used as a reference electrode that is submerged in the same buffer solution washing over the CFME. The CFMEs, or the working electrode’s potential is observed with respect to the fixed Ag/AgCl reference potential of 0.197 V \(^{26}\). Typical buffers used are phosphate buffered saline (PBS), Tris buffer, or artificial cerebrospinal fluid (aCSF). Although they are inherently salt solutions to mimic physiological pH, differences in ionic strength can impact the resulting sensitivity signal \(^{27}\).

To detect dopamine, the common triangle waveform is applied onto CFMEs. The triangle waveform scans from the holding potential (lower limit) of –0.4V to a switching potential (upper limit) 1.3 V at 400 V/sec \(^{25}\) (Figure 1.A). Utilizing a flow cell (Pine Instruments, Durham, NC), the CFME can be lowered into a well or opening where a constant stream of buffer flows past the exposed carbon fiber. The flow cell has tubing junctions which allow for injections of analytes. When the triangle waveform is applied, using commercially available software, such as HDCV (UNC, Chapel Hill, NC), WCCV (Knowmad Technologies, Tucson, AZ), and Demon (Wake
Forest Baptist Medical Center, Winston-Salem, NC) among others, a CV is produced (Figure 1.B). The fast scan rate is necessary to ensure DA kinetics can be observed where DA is oxidized at approximately +0.7 V on the forward scan to dopamine-o-quinone and reduced back to DA at approximately -0.2 V on the backward scan. A consequence of scanning at such high rates is the creation of a large non-faradaic background charging current which increase proportionally to the scan rate. However, because charging currents are similar in each experimental trial per electrode, they can be subtracted out to divulge a faradaic current, also known as a background subtracted CV (Figure 1.C). The oxidation and reduction of an analyte can also be seen in false color plots (Figure 1.E), while taking time into account. The waveform voltage is shown on the y-axis, and time is displayed on the x-axis, while color indicates current. The yellow to navy blue hues represent negative reduction current, whereas the green hues represent positive, oxidative currents. The color plot can also be reproduced as a 3D plot which shows the change in potential and current at each time point (Figure 1D). Higher switching potentials can oxidize the surface of the electrode and enhance sensitivity by breaking carbon-carbon bonds to increase surface area and roughness, which is a key property for faster DA adsorption/desorption. Furthermore, it can functionalize the carbon electrode with negatively charged oxide groups, such as carboxyl groups, to electrostatically attract cationic neurotransmitters such as dopamine and serotonin.
Figure 1: FSCV of dopamine. (A) Applied potential waveform using −0.4 V holding potential, +1.3 V switching potential, 400 V/s scan rate, and 10 Hz repetition rate. (B) Example CVs with background: blank (PBS pH 7.4) (black) and buffer with 1 μM dopamine (red). Dashed boxes emphasize the difference between them. (C) Background-subtracted CV of 1 μM dopamine. (D) Three-dimensional current–potential–time plot and (E) conventional false color plot with anodic peak current–time trace of 5 s bolus injection of 1 μM dopamine.

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In addition to in vitro detection, DA has been successfully measured at a fast, sub second timescale in animal studies of addiction. In a rodent model, a CFME was inserted into the rat nucleus accumbens (NAc), a dopamine rich area of the brain. Freely moving rats were allowed to self-administer cocaine, via a catheter implanted into the jugular, every time they pressed a lever. It was found that extracellular DA levels rose just before the lever press and immediately after, showing the changes in DA levels in real time using FSCV with CFMEs. A similar response was also observed with cannabinoid activation of another dopaminergic area, the
ventral tegmental area (VTA) which projects to the NAc. Increased firing of DA neurons in short bursts have been related to sensory processing related to reward and lead to increased concentration of DA. Cannabinoid receptor agonists also increased DA levels in these areas. These small bursts of DA signal were measured and CFMEs provided the high temporal resolution needed for in vivo kinetics of dopamine. Understanding the dynamics of DA in addiction and receptor binding is imperative to visualizing pathology of addiction diseases.

The triangle waveform parameters and resulting peak positions are specific to DA while using a conventional CFME. The same waveform may not be ideal for every ionizable analyte or for simultaneous co-detection. Waveform development has helped create assays for enhancing the detection of neurotransmitters. By altering the scan rate, analytes with slower electron transfer kinetics can be detected and differentiated from other molecules. Moreover, specific neurotransmitters are measured by specialized waveforms to enhance detection including providing a renewable surface for the detection of dopamine and anti-fouling waveforms for serotonin measurement. Other waveforms include upper limits of 1.4 V or greater in order to detect molecules with higher oxidation potentials such as hydrogen peroxide, adenosine, guanosine and others. Additionally, the surface of the carbon fiber can be modified with polymers for enhanced sensitivity while repealing interferants. By altering the waveform and modifying the surface of CFMEs, scientists have been able to detect and differentiate many neurochemicals apart from dopamine that has led to many exciting applications for in vivo and ex vivo measurements.

Carbon Electrode Modifications for Enhanced Neurochemical Measurements

In recent years, numerous assays have been developed to enhance the detection of neurotransmitters with CFMEs. Carbon nanotubes (CNTs), for example, have been utilized due
to their high conductivity, aspect (surface area: volume) ratio, which are optimal for neurotransmitter detection. Discovered by Iijima via arc discharge synthesis, CNTs dip coated or electrodeposited onto CFMEs have shown enhanced co-detection of dopamine and serotonin with respect to unmodified electrodes. CNTs functionalized with negatively charged carboxylic acid groups further enhanced detection through an electrostatic interaction, while vertically aligned CNT forests with iron chloride also greatly enhanced sensitivity by over 90%. Fibers and yarns solely made from CNTs also further enhanced neurotransmitter detection. CNT fiber microelectrodes wet spun from polyethyleneimine (PEI) were found to be fouling resistant to serotonin, while CNT yarn microelectrodes were shown to trap dopamine at the surface. They have a sensitivity independent of the wave application frequency for high temporal measurements of dopamine and serotonin. Moreover, carbonaceous material such as CNTs and carbon nanospikes were grown on metal wires for the development of enhanced neurotransmitter sensors. Additionally, there are two subtypes of CNTs with single wall- and multi walled CNTS. SW-CNTs are made up of single sheets of graphene rolled into tubes while MW-CNTs are multiple layers of graphene cylinders. Orientation and deposition methods of these CNTs can also impact their sensitivity to neurochemicals.

In addition to carbon nanotubes, CFME have been modified with polymers. Nafion has been utilized by many researchers due to its ability to form cation conducting networks which are able to attract the positively charged DA. The sulfonate group on this polymer repels negatively charged metabolites and interference such as ascorbic acid (AA), which all share similar oxidation peaks. Although, Nafion alone was not stable for reliable measurements, as it did not strongly bond with the carbon fiber surface, PEDOT was utilized. Electrodeposition of PEDOT:Nafion was accomplished by applying a triangle wave, from +1.5
V to ~0.8 V, on a CFME surface submerged in the polymer solution. Nafion was also found to repel 5-HIAA, a metabolite of serotonin, to enhance 5-HT detection. PEDOT:Nafion was also shown to increase electron transfer and enhance DA detection, in vivo, and in ex vivo zebrafish retina. However, the two-fold selectivity came at a cost where temporal resolution suffered by 400 ms. Polyethyleneimine (PEI) coatings were also applied to enhance the detection of anionic neurotransmitters such as DOPAC, while PEDOT:PEI coatings helped detect and differentiate other dopaminergic metabolites such as DOPAL and 3-methoxytyramine (3-MT). The sensitivity of these different analytes can be characterized by calculating the limit of detection (LOD) measurements. Having a lower LOD indicates the analyte can be detected even when present at nanomolar concentrations. These measurements of common analytes can be found in Table 1.

Additionally, surfactants have been used with PEDOT:Nafion electrodes, such as sodium dodecyl sulfate (SDS) and sodium dodecyl benzene sulfonate (SDBS). Scanning electron microscope (SEM) images of these polymers on CFMEs can be seen in Figure 2. SDS was used as it increased the sensitivity with aiding in Nafion assembly and enhancing conductivity of the PEDOT due to the sulfate group. It was also less sensitive to the anionic compounds such as ascorbic acid. SDBS acted similarly except the improvement was due to the sulfonate group which competed, rather than being incorporated, with Nafion. Both, however, showed greater sensitivity to DA by up to a five-fold increase.

Coating for enhanced detection is not limited to polymers, but can include noble metal-nano particles, specifically gold (Au). Other coatings such as gold nanoparticles, have also enhanced the sensitivity and temporal resolution by increasing the conductivity and electroactive surface area of the carbon electrode substrate. As gold microelectrodes have been used to
detect many NTs and are comparable to CFMEs, electrodeposition of Au nanoparticles (NP) was considered. Electrodepositing AuNPs can increase the surface area available for NT adsorption as they can be purified and fixed onto the carbon fiber. The electrodeposition was accomplished by electro-reduction of $\text{Au}^{3+}$ to $\text{Au}^0$ and deposition onto the carbon fiber as NPs. Direct comparisons between bare and AuNP CFMEs showed higher sensitivity for dopamine detection. Energy-dispersive X-ray spectroscopy (EDS/EDX) measurements detailed a heterogeneous carbon-gold surface that increased sensitivity and provided for faster electron transfer kinetics.

Figure 2: SEM images carbon fiber 7 μm x 100 μm for Bare carbon (a), PEDOT:Nafion (b), PEDOT:Nafion-SDS (c), PEDOT:Nafion-SDBS (d). The PEDOT:Nafion coatings were deposited from a solution containing 200 μM EDOT.

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Dopamine Metabolites

Other catecholamines that have been detected using FSCV are metabolites of DA. DOPAL (3,4-Dihydroxyphenylacetaldehyde) is metabolized by monoamine oxidase or MAO and is an aldehyde product of DA. It has been postulated to play an important role in Parkinson’s disease, contributes to reduction of dopamine, and increases toxicity to dopaminergic neurons in the substantia nigra and striatum. Due to its potential to act as a biomarker for PD, it is necessary to differentiate between DA and DOPAL. Using unmodified CFMEs with the triangle waveform, DOAPL’s oxidation peak was observed at 0.7 V. When DA and DOPAL were measured together, only a single peak was observed. To differentiate DOPAL’s peak from dopamine, the electrode surface was modified by the electrodeposition of the polymers polyethyleneimine (PEI) and Poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS). The PEI coated CMFEs had a higher sensitivity for DOPAL detection due to the electrodeposited polymer forming a thin, positively charged layer on the electrode’s surface, which attracted the negatively charged DOPAL. Although this enhanced DOPAL detection by repelling the DA molecules, it was not sufficient in creating two separate peaks for co-detection.

Another pre-synaptic metabolite of DA is DOPAC (3,4-Dihydroxyphenylacetic acid) and is synthesized via MAO. The utility in discerning DOPAC from dopamine lies with its significant role in mitochondrial dysfunction, in tandem with nitric oxide (NO), where oxidative stress and dysfunction can lead to degeneration of dopaminergic neurons. This catecholamine oxidizes in a quasi-reversible process in a two-electron transfer where only some of the oxidized product is reduced back to its original form. The carboxyl group, which differentiates DOPAC from DA, is deprotonated and gives it an overall negative charge at a physiological pH. Although both metabolites are detected via the same triangle waveform, DOPAC is also
indistinguishable from the DA’s signal without a form of electrode modification. To further increase the selectivity of DOPAC over DA, a novel waveform was used which scanned from 0 V to 1.3 V at 400 V/sec. By shifting the holding potential from a negative to neutral voltage, the CFME became more sensitive towards DOPAC due to the diminished electrostatic repulsion of the anion. In combination with that waveform, PEI and Nafion coated CFMEs were used. The PEI coating provided a positive charge to electrostatically attract DOPAC. However, for the co-detection of the two molecules, PEI-CFMEs were used with the DA triangle waveform which produced two distinctive peaks corresponding to DA and DOPAC at approximate 0.6 V and 0.7 V, respectively.

Norepinephrine

Norepinephrine (NE) plays a major role in the central nervous system in arousal, and a mediator of the reward pathway \(^{74}\). NE’s role in stress response has been implicated in multiple disorders related to stress such as addiction and post-traumatic stress disorder (PTSD) \(^{75,76}\). Clinical studies also indicate NE may play a role in depression \(^{77}\). In animal tissue, NE can be found in the ventral bed nucleus of the stria terminalis (vBNST) via stimulation of the VTA as noradrenergic bundles are seen in this pathway. Dopaminergic neurons do not innervate the fusiform nucleus of this area, making it ideal for detecting NE independently. However, when measured within the BNST, DA and NE yield very similar redox peaks \(^{78,79}\). This is not surprising however, as they have similar structures, are both synthesized from tyrosine, and are only distinguished by a single hydroxyl group \(^{80}\). For this reason, NE and DA peak differentiation still requires further waveform development.
**Hydrogen Peroxide**

Hydrogen peroxide (H$_2$O$_2$) is another analyte that is involved in stress and cause cellular level oxidative stress. Also known as an oxygen reactive species (ROS), H$_2$O$_2$ can form free radicals that are toxic to surrounding cells, which causes oxidative damage to DNA, carcinogen activation, and tumor promotion $^{81,82}$. Although lethal effects of DNA repair suppression exist, H$_2$O$_2$ also plays a role in redox signaling and cascades in normal functions and neuromodulation $^{83}$. Enzymatically, it is used as a reporter due to its electroactive properties to indirectly reveal presence of non-electroactive species $^{84}$. The enzymes (such as glutamate oxidase) convert molecules into reporter molecules which can be oxidized and detected via FSCV, with peak oxidative current corresponding to their relative concentration. This method has been used for other biomolecules such as glucose, lactate, acetylcholine, GABA, and glutamate where ionization is not a possibility $^{85-87}$. For detection, a modified triangle waveform was used with a –0.4 V holding potential, similar to the DA waveform, but with a slightly higher switching potential at 1.4 V. At a scan rate of 400 V/sec, a 100 µM bolus of H$_2$O$_2$ was detected with this waveform $^{40}$. As the reaction is irreversible, only one peak was seen in the forward scan: an oxidation peak at 1.2 V $^{40,88}$.

**Serotonin**

Serotonin (5-HT) plays many roles in the brain such as in depression, mood, emotion. Microdialysis with HPLC has been used for 5-HT detection with a response time as quick as under a minute $^{56}$. However, to achieve a higher temporal resolution, FSCV with waveform modification has been utilized to isolate and detect 5-HT.

To better visualize 5-HT alone, FSCV assays utilize waveforms that use a high scan rate to avoid the adsorption of unwanted products with a piecewise function, known as the Jackson
waveform (JWF)\textsuperscript{39}. Since its conception, it has been modified with two additional waveforms to detect 5-HT at both 1000 V/sec and 400 V/sec. Serotonin fouls on the surface of the electrode at higher concentrations and timescales and diminishes sensitivity. An extended serotonin waveform (ESW) was developed to prevent fouling at the electrode surface. Similar to the JWF, this waveform used a piecewise function from 0.2 V to 1.3 V then −0.1 V to 0.2 V, at 1000 V/sec. As higher switching potentials were used to renew the CFME surface and remove impurities\textsuperscript{25}, this higher scan rate was paired with a modified piecewise function. ESW yielded an oxidation peak at 0.9 V, similar to JWF but more resolved, and a reduction peak at 0.0 V\textsuperscript{89}.

![Waveform Diagram](image)

Figure 3: Waveforms tested. A. Traditional serotonin “Jackson” waveform with a 1.0 V switching potential and 1000 V s\textsuperscript{−1} scan rate. B. Traditional dopamine waveform with a −0.4 V holding potential, extended 1.3 V switching potential, and 400 V s\textsuperscript{−1} scan rate. C. Extended serotonin waveform (ESW) with 1.3 V switching potential and 1000 V s\textsuperscript{−1} scan rate. D. Extended hold serotonin waveform (EHSW) with a 1 ms hold at 1.3 V and 400 V s\textsuperscript{−1} scan rate. All waveforms were repeated at 10 Hz.

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Additionally, another waveform was established that made use of a lower scan rate. The extended hold serotonin waveform (EHSW) was comparable to the ESW except the switching potential of 1.3 V was held for 1 ms, and at a scan rate of 400 V/sec. The “sawhorse” shape aided in 5-HT detection as holding at the switching potential allowed extra time to oxidize the surface of the electrode. The EHSW yielded similar results to the DA triangle waveform for 5-HT with redox peaks at 0.0 V and 0.6 V, respectively. The different waveform shapes are shown in Figure 3. In comparing the 5-HT waveforms, it was found that the JWF fouled the most, minimal fouling occurred with both 5-HT waveforms, and no fouling occurred with the DA waveform. This was not surprising as the DA waveform is the most anti-fouling due to its ability to renew the electrode surface. The fouling was attributed to a downstream metabolite, 5-hydroxyindoleacetic acid (5-HIAA), which produced a free radical that fouled the surface. This radical intermediate dimerized and electropolymerized and, thus, fouled the CFME surface.

In addition to waveform development, electrode modification was also performed with Nafion coating to increase the selectivity of the CFME to 5-HT over 5-HIAA in vivo. The combination of the above experiments validated the detection of 5-HT in vivo for the first time with the Nafion-CFME and the JWF. Although multiple waveforms were presented, they all were able to detect 5-HT indicating that there are several ways to identify the same analyte with FSCV.

**Adenosine**

Adenosine is a neuromodulator found in the brain that can have neuroprotective effects in response to hypoxia and neurodegenerative diseases. Adenosine is difficult to differentiate from biomolecules that share similar oxidation peaks, such as H₂O₂, ATP, and histamine. Specifically, with ATP and H₂O₂, oxidation peaks were nearly identical, at 1.45 V, when
detected with a triangle waveform that scanned from –0.4 to 1.45 V at 400 V/sec \(^91\). To enhance selectivity, a modified sawhorse waveform was developed which scanned from –0.4 V to 1.35 V, held for 1.0 ms, then back to –0.4 V, at 400 V/sec. The hold at the switching potential enabled more adenosine molecules to oxidize on the electrode’s surface, similar to 5-HT. With the sawhorse waveform, a primary peak for adenosine was observed at 1.45 V and a smaller secondary peak, especially at higher concentrations, at 1.0 V. This secondary peak was attributed to a change in background current in response to adenosine adsorption on the electrode’s surface. Additionally, the oxidation of adenine, the nucleobase of adenosine, also had a secondary peak but with a lower current. Due to this adsorption peak being present for adenosine, adenine, and ATP, but not \(\text{H}_2\text{O}_2\), it may be a result of the product of the nucleobase \(^91\). The modified sawhorse waveform enabled discrimination between adenosine and similar oxidative peak biomolecules, however, it was not possible to discriminate a mixture of ATP and adenosine.

![Graph showing Adenosine, ATP, and Hydrogen Peroxide](image)

**Figure 4:** A schematic showing background subtracted, unfolded CVs of adenosine, ATP, and hydrogen peroxide using the modified sawhorse waveform \(^91\).

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**Guanosine**

Guanosine (Gn) is another important purine neuromodulator that not only impacts adenosine levels but is structurally similar to adenosine. The signaling mechanism for Gn is not well understood but both nucleosides are found in astrocytes and are the main extracellular...
source of purines. Additionally, both can control glutamate transmission, implicating a joint role in Alzheimer’s Disease (AD)\textsuperscript{92}. This guanine-based purine is also known to increase in concentration in response to brain injury. To pick out Gn from adenosine, the initial guanosine waveform scanned from $-0.4$ V to $1.3$ V, at a rate of $400$ V/s and produced only two oxidation peaks. The primary oxidation peak was observed at the switching potential, $1.3$ V, on the backward scan. If the switching potential was increased to $1.45$ V, the primary peak shifted to the forward scan, indicating slow kinetics of guanosine. A secondary peak was observed at $0.8$ V on the forward scan. The presence of two peaks indicated the oxidation reaction involved a two-electron transfer on the guanine moiety. First, a radical formed and dimerized prior to detection. The second, although reversible, was highly favored. As the original waveform did not exceed $1.3$ V, an adenosine signal was successfully omitted.

To optimize a waveform that would detect both guanosine and adenosine, a modified scalene waveform was used with a slower rise to the switching potential from $-0.4$ V to $1.45$ V, at $100$ V/sec, then faster at $400$ V/sec on the backward scan down to $-0.4$ V. With the scalene waveform, guanosine’s primary peak was observed at $1.2$ V and the secondary peak was observed at $0.66$ V. Adenosine’s primary peak appeared at $1.29$ V, with the secondary peak at $0.88$ V. The slower waveform enhanced the separation between the primary and secondary peaks of both purines. The distance between the peaks also indicated that adenosine’s electron transfer was slower than that of guanosine. Also, the primary oxidation steps for both were irreversible and resulted in peak potential shifts as a function of scan rate. This novel scalene waveform yielded a nanomolar limit of detection (Table 1) and resolved all four peaks within an unfolded single CV. When utilizing the guanosine triangle waveform, on the other hand, there was minimal separation of peaks and secondary peaks for both purines were absent\textsuperscript{93}.  

19
Analyte multiplexing was also performed with the addition of DA (Figure 5). All three analytes had distinct peaks with DA oxidation seen at 0.46 V, however, secondary oxidation peaks for either adenosine or guanosine were not present. Although co-detection for all three analytes has yet to be validated in vivo, Gn and adenosine primary oxidation peaks have been confirmed in rat caudate putamen slices.

Figure 5: Triple detection of guanosine, adenosine, and dopamine using the scalene waveform. (A) Example false-color plot showing 5 μM (each) dopamine (DA), guanosine (GN), and adenosine (AD). Dopamine oxidation occurs at 0.46 V when the modified waveform is used. (B) Opened cyclic voltammogram for the purine–dopamine mix. The Δt between dopamine and guanosine, its nearest neighbor, is 6.96 ms. 93


Melatonin

In addition to nucleosides, recently, the hormone melatonin has also been measured with CFMEs and FSCV. Melatonin regulates circadian rhythms, body temperature, oxidative stress, and mitochondrial homeostasis. Early research limited melatonin production to the pineal gland, but newer literature shows melatonin receptors and synthesis in the retina, lymphocytes, and gastrointestinal tract. Immune related functions also include modulation of inflammation where melatonin may suppress inflammation via COX-2 enzymes. With such critical roles in
the nervous system, a specific waveform was developed to detect and enhance sensitivity of melatonin with CFMEs.

The oxidation scheme of melatonin involves a single electron abstraction which generates a radical cation. This is further oxidized into a quinoneimine by electron and proton loss. This oxidation is irreversible and is adsorption controlled. Because the quinoneimine is highly reactive, it electropolymerizes in solution and leads to unwanted adsorption products, causing it to foul the surface of the electrode. To circumvent this, a modified waveform was developed, which scanned from 0.2 V to 1.3 V at 600 V/sec and the higher scan rate eliminated the radical peak. To ensure selectivity, this waveform was tested with structurally similar serotonin, dopamine, and their respective metabolites; 5-HIAA, 6-hydroxymelatonin (6-HMA), and N-acetyl serotonin (NA-5HT). As the modified waveform lacked a negative holding potential, DA was not measured with this assay. 5-HT had a higher sensitivity, but a different oxidation peak potential than melatonin, which was observed at 1.1 V, allowing for peak distinction. Melatonin and 5-HT were then co-detected and serotonin’s oxidation peak was observed at .7 V. However, it was more challenging to differentiate from serotonin’s downstream metabolite, NA-5HT and melatonin’s metabolite, 6-HMA.

Neuropeptides

Using CFMEs with FSCV for detecting biomolecules is not limited to small molecules. Recent assays include the detection neuropeptides such as enkephalin as well. Originally, this was challenging as neuropeptides are made up of multiple amino acid chains with limited oxidizable residues. The larger size may prevent adsorption onto the electrode’s surface, and the ionized amino acid may be difficult to discern on the CV when multiple oxidizing amino acids are present. However, certain smaller peptides have been detected with voltammetry through the
oxidation of the ionizable tyrosine or methionine $^{101,102}$. Moreover, other peptides such as insulin $^{103,104}$ and glucagon $^{105}$ have been measured with amperometry. The ability to detect neuropeptides with FSCV has expanded the potential applications where a carbon fiber is able to detect large and complex molecules as opposed to solely small molecules.

Enkephalin, or ENK, is an opioid neuropeptide involved in a wide variety of functions, including analgesic effects, cell proliferation activation, and can act on various, nonspecific opioid receptors $^{106}$. There are two different types of ENK that arise from proteolytic cleaving: methionine-enkephalin (M-ENK) and leucine-enkephalin (L-ENK) $^{107,108}$. The detection of ENKs, however, becomes difficult due to the presence of tyrosine which requires higher oxidation potentials and fouls the electrode surface. To overcome these challenges, the modified sawhorse waveform (MSW) was designed and used to detect M-ENK $^{109}$. The MSW utilized two distinct scan rates in each anodic sweep with a holding potential at $-0.2$ V that ramped up to $0.6$ V at 100 V/sec. The potential was increased to $1.2$ V at 400 V/sec and held for 3 ms before returning to $-0.2$ V, also at 100 V/sec. The faster scan rate was used around the oxidation peak to capture tyrosine’s oxidation, while the slower scan rate at the beginning and end of the MSW reduced faradaic signals from interfering analytes. The resulting CV had two distinct peaks for M-ENK: at $1.0$ V corresponding to tyrosine and at $1.2$ V for methionine. The other amino acids in the neuropeptide were not electroactive. The data suggested the MSW successfully detected M-ENK and may facilitate the detection of other tyrosine containing opioid neuropeptides $^{109}$.

**Conclusion**

FSCV is not the only technique used to detect biomolecules, but the high spatial and temporal resolution, easy equipment setup, and ability to manually produce and modify CFMEs have made this technique a standard in analytical chemistry and neuroscience research. Over the
years, this method has been utilized for the detection of dopamine, which is important for understanding the basis of many neurological diseases, behaviors, learning and memory, and drug abuse among others. For other neurotransmitters, our understanding of their physiological roles in vivo has previously been limited by our ability to detect and distinguish them from one another. However, more recently, assays have been developed to not only isolate their signal, but detect additional biomolecules such as neurohormones, neuropeptides, and DNA bases. With the help of surface polymer coatings of CFMEs and waveform modifications, it has become more facile to identify multiple analytes in vitro, ex vivo, and in vivo. With novel sensors, the possibility of clinical relevance and human patient work may also be a possibility.

Enhancing the detection of neurochemicals will further help in understand their complex roles in vivo. Formulating better sensors with high spatiotemporal resolution is a critical step in understanding the chemical processes and neuroanatomy of specific brain regions.
Table 1. Neurotransmitters detected by FSCV

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Acronym</th>
<th>Sensor</th>
<th>LOD (µM)</th>
<th>Buffer</th>
<th>Waveform</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>DA</td>
<td>PEDOT:Nafion-SDBS CFME</td>
<td>9 nM</td>
<td>Tris Buffer</td>
<td>Triangle</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>3,4-Dihydroxyphenylacetaldehyde</td>
<td>DOPAL</td>
<td>PEDOT-PEI CFME</td>
<td>100 nM</td>
<td>aCSF</td>
<td>Modified Triangle</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>3,4 dihydroxyphenyl acetic acid</td>
<td>DOPAC</td>
<td>PEDOT:Nafion-SDS CFME</td>
<td>19 nM</td>
<td>Tris Buffer</td>
<td>Modified Triangle</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>NE</td>
<td>CFME</td>
<td>4 µM</td>
<td>Tris Buffer</td>
<td>Triangle</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>Hydrogen Peroxide</td>
<td>H2O2</td>
<td>CFME</td>
<td>50 µM</td>
<td>Tris Buffer</td>
<td>Modified Triangle</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>Serotonin</td>
<td>5-HT</td>
<td>CFME</td>
<td>.6 nM</td>
<td>PBS</td>
<td>Piecewise</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>Histamine</td>
<td>His</td>
<td>CFME</td>
<td>1 µM</td>
<td>Tris Buffer</td>
<td>HSW</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>Adenosine</td>
<td>Adn</td>
<td>Nafion-CNT CFME</td>
<td>7 nM</td>
<td>Tris Buffer</td>
<td>Modified Sawhorse</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>Analyte</td>
<td>Acronym</td>
<td>CFME</td>
<td>LOD</td>
<td>Buffer</td>
<td>Waveform</td>
<td></td>
</tr>
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<td>-------------------</td>
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<td></td>
</tr>
<tr>
<td>Guanosine</td>
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<td>CFME</td>
<td>50 nM</td>
<td>Tris Buffer</td>
<td>Scalene</td>
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<tr>
<td>Melatonin</td>
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<td>CFME</td>
<td>24 nM</td>
<td>Tris Buffer</td>
<td>Triangle</td>
<td></td>
</tr>
<tr>
<td>Enkephalin</td>
<td>ENK</td>
<td>CFME</td>
<td>.5 µM</td>
<td>PBS</td>
<td>MSW</td>
<td></td>
</tr>
</tbody>
</table>

*Note:* Discussed analytes and their acronym, the type of CFME used for detection, the limit of detection (LOD) at that surface modification, type of buffer solution used, and corresponding waveform used for detection. It is critical to note other differences can affect the LOD such as scan frequency rate, length of carbon fiber, and assembly of polymer coating. This in turn can cause peak shift in resulting CVs. All structures taken from Cayman Chemicals Product Catalog and open domain, non-copyrighted images.
CHAPTER 3
MULTIELECTRODE ARRAY

The brain is the most complex, heterogeneous organ responsible for a variety of processes. Different neurotransmitters serve as messengers for relaying electrochemical signals into physical actions. The complexity of studying these neurochemicals arises not only from their size, but the extremely short time scale at which they are metabolized, released, or taken up into the pre-synaptic neuron. One of the major neurotransmitters (NTs) is the catecholamine, dopamine (DA), responsible for functions such as motivation, reward learning, and the contributes to “wanting” a reward \(^{115}\). Dopaminergic neurons are found in brain regions such as the substantia nigra and make up nearly 5% of striatal neurons. The greater majority of these cells are located in the ventral mesencephalon or midbrain, an area that further divides into dopamine rich subregions \(^{116}\). Death of dopaminergic cells in the substantia nigra leads to Parkinson’s disease while presence of excess DA is implicated in schizophrenia. Serotonin (5-HT) is an indoleamine NT derived from tryptophan and is a precursor of melatonin \(^{117}\). It is involved in mood regulation, susceptible to depressive symptoms and various roles in cognition and in the gastrointestinal tract \(^{118}\). While 5-HT is usually found in the dorsal raphe nuclei (DRN), it is not limited to the CNS where majority of serotonergic neurons are in the body’s periphery \(^{119,120}\). Norepinephrine (NE), another catecholamine with similar structure to DA, is found in another brain nucleus called the Locus Coeruleus (LC). NE plays roles in sleep-wake cycles, mood disorders, and implications in psychostimulants. The noradrenergic neurons from the LC extensively branch and extend into various regions of the cerebral cortex and the VTA or ventral tegmental area, also a source of DA \(^{121}\). Additionally, the hippocampus is another area where the three previously mentioned NTs can be found concurrently via fibers that project onto hippocampal neurons \(^{122,123}\). As these NTs do not exist in isolation, but rather act on similar brain
regions, it is necessary for studies of neurological disorders to differentiate between their signals
to further understand their impact in dysregulation and diseases. Therefore, it is important to
have a sensor array that can target these regions simultaneously for making neurochemical
measurements.

To detect brain biomolecules, the carbon fiber microelectrodes, or CFMEs have been
used in neurochemical research. These electrodes are relatively small in size with a <10 µm
diameter and have low impedance and low signal to noise ratio\(^{124}\). They are highly
biocompatible with well characterized electrochemical properties that make them ideal for
biological applications\(^{52,125}\). CFMEs can be easily batch constructed in any lab setting and can
make rapid measurements of neurochemicals when used in tandem with the analytical technique
fast scan cyclic voltammetry. FSCV was first introduced by Millar in the late 1970s and
expanded by Wightman et al. with the use of microelectrodes\(^{126}\). FSCV allows for application of
high sampling rates onto CFMEs to measure kinetics of neurotransmitters such as the DA, 5-HT,
and NE\(^{125}\). Moreover, carbon electrodes are biocompatible and highly efficacious for making
measurements \textit{ex vivo} in brain slices or tissue \textit{in vivo} in anesthetized animals. CFMEs do not
elicit an immune response when chronically implanted and their small size mitigates tissue
damage\(^{127}\). With fast, sub-second temporal and spatial resolution, CFMEs with FSCV are used
to understand the dynamics of neurotransmitter release and uptake by factoring in the voltage at
which they are oxidized or reduced\(^{128}\). When a voltage pattern or waveform is applied onto the
CFME, redox reaction of an analyte can be detected and a cyclic voltammogram (CV) is
produced. The shape and current peak position of the CV act as a fingerprint for biomolecule
detection and facilitate discrimination of analytes. However, to understand the complex
environment of the brain, a sensor with multiple electrodes is needed for simultaneous targeting of several brain subregions.

To target multiple areas simultaneously, metal arrays have been used to study neuronal firing and electrical signals in addition to performing electrical stimulation. Metal arrays, often used in electrophysiology, record neuronal activity down to a singular cell scale. These penetrating microelectrodes reveal information about cortical activity, but they are limited to this form of electrical detection\textsuperscript{129}. Additionally, high impedance, variable signal to noise ratios, inflammation in response to implantation, and unreliable performance make them unideal for use outside the laboratory\textsuperscript{130}. Moreover, metal arrays have limited applicability as neurotransmitter sensors as they have been known to elicit an immune response and have lower overpotentials for water oxidation. To counter some of these drawbacks and enhance functionality as biosensors, metal electrodes have also been electrodeposited with conductive polymer carbon-based coating to increase biocompatibility and longevity of recording capabilities\textsuperscript{131}. The polymer Poly(3,4-ethylenedioxythiophene), or PEDOT, is known for its electrochemical stability and electrolyte compatibility\textsuperscript{132,133}. Probes with this polymer were seen to have decreased impedance and astrocytic, or immune cell, response\textsuperscript{134}. Polymers such as PEDOT, CNTs, and Nafion, an anionic polymer, have been electrodeposited onto the surface for increased selectivity\textsuperscript{24,69}. Furthermore, carbon nanotubes, both single walled (CW) and multi walled (MW), can also be used with PEDOT coatings for structural enhancement. An analysis of MWCNT-doped PEDOT coated onto platinum/iridium electrodes showed encapsulation by inflammatory tissue and then a sharp increase in impedance due to the inflamed tissue\textsuperscript{130}. More recently, metal microelectrodes that were modified with carbon nanotubes\textsuperscript{56} and carbon nanospikes\textsuperscript{135} have also been used as neurotransmitter sensors with FSCV\textsuperscript{136}. Although these coated metal arrays show promise in in-
vivo, extensive method development is needed for this new type of sensor, limiting its use to primarily electrical measurements. However, the literature indicates to better understand the neurochemical environment of the brain, a carbonaceous surface is required on the exterior of the electrode surface to enable neurotransmitter adsorption and subsequent oxidation.

To address the limitations of a single channel CFME, a novel CFME array (CFMEA) has been utilized. This CFMEA or multi array is custom developed with four separate fibers protruding from a custom printed, polyimide circuit board. The carbon fibers are reinforced with a layer of Parylene C and soldered to a head stage \(^{137}\). With the use of four fibers but acting as a single electrode setup, the multi array is able to make simultaneous measurements spatially resolved and with the option to apply four separate waveforms to each fiber. In the current study, we coupled and interfaced this novel multi array with a commercially available WaveNeuro FSCV multichannel potentiostat with the use of an Omnetics connector. We compared the multi array’s selectivity and sensitivity to single CFMEs and measured various biologically relevant NTs. Optical characterization showed a highly carbonaceous surface and comparable performance to a single channel CFME. Dopamine was adsorption controlled to the surface of the electrode as shown by concentration, stability, and scan rate experiments. Additionally, the simultaneous use of multiple waveforms was applied to the four different fibers to multiplex NT detection and target multiple brain subregions simultaneously. Upon applying multiple waveforms and potentials to each electrode of the multi array, we also performed proof of principle testing in brain slices where we measured the KCl-stimulated release of extracellular dopamine in approximately a 3mm\(^2\) area of the mouse caudate putamen (CPu). The development of carbon fiber multielectrode arrays coupled with multichannel potentiostat provides for novel developments in multiplexing fast neurochemical measurements in multiple brain regions.
simultaneously. This technology may help understand complex brain neurochemistry by measuring changes in NT concentration concurrently in different brain regions in response to disease and pharmacological states in addition to behavioral changes.
CFME Fabrication

Carbon fiber microelectrodes (CFMEs) were produced by aspirating a single carbon fiber (GoodFellow, Huntingdon, England) into a 1.2 mm diameter glass capillary tube (1.2-0.68 mm, A-M Systems, Inc., Carlsberg, WA) via vacuum (Gast). They were pulled to a fine taper using a pipette puller (PC-100, Narishige Puller, Narishige Group, Japan). The tips were epoxied with Epon Resin 828 resin (Miller-Stephenson, Morton Grove, IL) and diethylenetriamine hardener (Fisher Scientific, Waltham, MA) at a ratio of 3:1 to form a seal and cured in an oven at 125 °C for approximately 4 hours. The protruding fiber was trimmed under a compound microscope to approximately 100 µm in length.

To conduct measurements, the electrode was backfilled with a 0.1 M KCl and placed with an electrode holder with a silver wire (Universal Pipette Holder, Dagan, Minneapolis, MN) that was interfaced with the Malli Adapter from Pine Research. The electrode was placed into the flow cell setup. A 200-ampere head stage connected to the holder and then interfaced to a Wave Neuro potentiostat (Pine Research Instrumentation, Durham, North Carolina). A manual micromanipulator (WPI, Sarasota, FL) attached to the flow cell was used to lower the CFME to make consistent contact with a buffer solution flowing over the electrodes tip. Artificial cerebrospinal fluid (aCSF) buffer was used as the buffer solution and made from the following: 145 mM NaCl, 2.68 mM KCl, 1.40 mM CaCl₂·2H₂O, 1.01 mM MgSO₄·7H₂O, 15.5 mM Na₂HPO₄, and 0.45 mM NaH₂PO₄·H₂O. The salts were sequentially dissolved into deionized water (Millipore, Billerica, MA) and the pH adjusted to 7.4. Similarly, phosphate buffered saline (PBS) was used as an alternative buffer consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM
Na$_2$HPO$_4$ and 1.8 mM KH$_2$PO$_4$. The aCSF or PBS solution was applied over the electrode tip using a 50 mL syringe (BD, Franklin Lakes, NJ) and the NE-1000 programmable syringe pump (New Era Pump Systems Inc. Farmingdale, NY) with solutions and injected analytes flowing through the FSCV Flow Cell (Pine Research, Durham, NC) at a rate of 1 mL/min.

Neurochemicals such DA, 5-HT, and their metabolites were purchased from Fisher Scientific and Sigma-Aldrich (St. Louis, MO). They were prepared by diluting a 10 mM perchloric acid stock solution of NTs to make a 10 mM solution of NTs. During the day of an experiment, the 10 mM stock solution of NT was diluted to a desired concentration, ranging from .1 µM to 100 µM.

**Multi array**

Carbon fiber multielectrode array (also called CFMEAs or multi arrays) were obtained from the University of Michigan and used in the flow cell in a similar fashion to the CMFE. In brief, the multi array is made up of 4 carbon fibers (T-650/35: 3K, Cytec Thornel, Woodland Park, NJ) arranged in a row, but can be custom made to fit a variety of different arrangements. Each fiber is spaced approximately 400 to 600- µm apart, lengthwise, and are cut to a length of approximately 200 µm. These protruding fibers may be trimmed shorter or left at longer lengths. The distance between the fibers can also be customized. With this iteration of the multi array, the horizontal distance between all four fibers was asymmetrical. The fibers were attached to a custom circuit board using silver epoxy and the electronics covered in a protective, insulating epoxy. The exposed fibers were also coated with Parylene C for support and 45 µm were re-exposed using 532 nm Karl Suss green laser to uncover the electroactive carbon fiber microelectrode.
Statistics

Statistical data analysis was completed using Graph Pad Prism 9 and significance testing performed using students t-test.

Tissue Extraction

Tissue samples of the striatum were collected from C57BL/6J in accordance with IACUC and animal facility protocols at American University. Mice were housed in 12-hour light and dark cycles and provided food and water ad libidum. Mice were removed from their home cage one at a time and placed in a chamber for anesthetization via isoflurane. Once reflexes were assessed via toe and tail pinch, euthanasia was completed by cervical dislocation. The head was decapitated, and tissue cut away using surgical scissors to expose the skull. Surgical rongeurs were used to quickly peel away the skull bones to expose and remove the brain. For striatum tissue sample extraction 138, on a petri dish over ice, the extracted brain was cut in half via a slice down the sagittal suture, using a razor blade. The striatum was visible after removing the hippocampus and its surrounding structures. Bilateral excision of the striatum was performed, and tissue samples were placed into ice cold aCSF until use. For slice preparation, a similar protocol as followed except a coronal cut as made at approximately bregma +1.0 to uncover the caudate putamen. Coordinates and anatomical landmarks were located using Allen Mouse Brain Atlas (2004) and Paxinos Atlas 139,140.

After extraction, the striatum was placed in a petri dish with cold aCSF until the setup was prepared. Set up procedure was adapted from Papouin and Haydon 141 where a 600 mL plastic beaker was filled with ice cold aCSF solution. Within the beaker, an empty 50 mL conical tube, with the bottom removed, was superglued to the side. An open bottom petri dish had nylon hosiery glued to it and also superglued to the beaker 141. To oxygenate the buffer and
brain tissue, an aquarium air stone was used to bubble oxygen into the beaker using tubing and an air pump (Tetra, Blacksburg, VA)\textsuperscript{142}. Airline tubing is conventionally connected to an oxygen tank, however; the bubbler was sufficient for the short duration and small volume size in this experiment, as done similarly in previous literature\textsuperscript{143}. The beaker was then placed in an ice bath to maintain a cooler the temperature of the aCSF solution.

**Multiple Array Setup**

From the potentiostat, an adapter was used to connect the 4-channel WaveNeuro to a head stage cable, and then to a head stage coupler. This directly connected to another adapter which linked the Omnetics head stage on the multi array to the head stage coupler. All 4-channel accessories were also purchased from Pine Research with the exception of the array adapter which linked the Omentics head stage on the multi array to the head stage coupler. Images of the setup can be seen in Figure 6D and Supplemental Figure 1.

To hold the multi array in place, a three-prong extension clamp was inserted into the micromanipulator arm and the clamp held the multi array in place. Using the adjustments knobs on the micromanipulator, the multi array was lowered into the brain slice until the oscilloscope showed the applied waveform on the electrode and was allowed to equilibrate from 30 min. Afterwards, 1 \( \mu \)L of 0.1 M KCl\textsuperscript{144} was pipetted (Eppendorf, Enfield, CT) onto the surface of the tissue to detect NT release. The same software was used to record the CV data. Trials were repeated until degradation of the signal.

**Scanning Electron Microscopy**

SEM images were obtained using a JEOL JSM-IT100 (JEOL, Tokyo, Japan). Gold sputtering of the CFMEA was not required as the protruding carbon and paraylene were clearly
visible. The entire array was placed on an SEM metal puck and secured with carbon tape. The puck was placed into the SEM stage and the working distance det to 10mm and adjusted as needed for focus and resolution. The accelerating voltage was set to 5.0 kV.

Figure 6: A) the multi array B) a view of the carbon fibers of the multi array under a light microscope. C) SEM image of a single fiber of the multi array. D) Connection schematic of multi array showcasing the separate pieces of hardware that is required for adapting it to a commercial potentiostat. See supplemental figure 1 for setup photos.
CHAPTER 5

RESULTS

Setup and Characterization

As shown in Figure 6 and in the supporting information, the multi array has to be coupled with an Omnetics adapter in order to connect to the WaveNeuro FSCV multichannel potentiostat. As shown in Figures 6A and 6B, a custom-made carbon fiber multielectrode array (CFMEA) displays four protruding carbon fibers from the array surface that are approximately 200 µm in length. Figure 6C shows, a high magnification scanning electron microscope image (SEM) of the electrode surface. The parylene coating is clearly visible in the SEM image as well as the protruding carbon fiber surface, which was exposed after laser treatment. The dimensions of the carbon fiber were approximately 45 µm in length and ~7 µm in width. Figure 6D provides a schematic of how the CFMEA was connected and interfaced to the WaveNeuro FSCV multichannel potentiostat. An illustration of that schematic is provided in Supplemental Figure 1. Colored wires were soldered with silver epoxy to create an electrical connection to connect the electrodes to adapter that are interfaced with potentiostat for measurements to be made.

In-Vitro

We first compared the conventional single channel CFMEs to the multi array for dopamine detection with fast scan cyclic voltammetry. We compared the multielectrode array performance to single channel CFMEs that were trimmed to approximately the same protruding length, 45 µm. A triangle a waveform that scans from -0.4 to 1.3 V at 400 V/sec was applied onto the electrodes to test the neurotransmitter dopamine. Sensitivity to DA was measured by detecting 100 nM concentration of the analyte. For the multi array, the DA triangle waveform
needed to be applied onto each fiber and one experiment produced four separate CVs, corresponding to channels 1 – 4. The waveform applied onto CFMEs oxidized DA to dopamine-ortho-quinone (DOQ) and yielded an oxidation peak at approximately 0.7 V. DOQ is then reduced back to DA with a reduction peak at approximately -0.2 V on the back scan. In trials lasting 30-seconds, DA was injected at the 5 second mark and a CV was recorded. Following background subtraction, both electrodes showed CVs with redox peaks at the appropriate voltages as seen in the literature \(^{145}\). The single channel CFMEs and multi array electrodes, yielded similar peak oxidative currents with no significant differences, thus showing similar performance between the array and single channel electrode.

![Figure 7: Cyclic voltammograms of the A) CFME compared to the B) 4 channels of the multi array overlayed on one another. The CVs indicated no differences in the peak oxidative current of the electrodes. (N = 3 for each electrode type). Only the DA triangle waveform was applied onto thee each channel and electrode type.](image)

To ensure the electron transfer kinetics of DA were also equivalent on both arrays, scan rate experiments were performed. If an analyte is adsorption controlled onto the surface of the CFME, the peak oxidative current will have a linear relationship with increasing scan rate \(^{146}\). 10 \(\mu\text{M}\) of DA, a concentration large enough that allows for peak visualization at lower scan rates,
was tested on both electrodes at several scan rates ranging from 50 V/sec to 1000 V/sec (Figures 8 A and B). Both electrodes yielded a linear relationship between peak oxidative current and scan rate, and thus, were found to be adsorption controlled to the surface of the carbon fiber, as shown in previous literature \^{145}. The signal response of both electrodes over time was also confirmed with stability tests (Figure 8C and D). Again, using 10 µM DA, electrodes were applied with the triangle waveform for four consecutive hours, and DA was injected every hour. The time limit of the stability experiments was set to four hours as that is the typical duration of an electrochemical in vivo experiment. Moreover, a stable response also showed the analyte was not polymerizing or fouling the electrode surface, which would decrease sensitivity over time. Similarly to before, the multi array behaved comparably to the CFME and did not have a significant difference in normalized oxidative current (nA) over time (Figure 8).

![Figure 8: Comparison of A) CFME versus B) multi array linear relationship of normalized current versus increasing scan rate from 50 – 1000 V/sec. Peak oxidative current was found to be linearly dependent and proportional to scan rate. Stability of C) CMFE and D) multi array over the course of four hours showed no significant difference in peak oxidative current over time. Error bars are shown as standard error of the mean. (N = 3 for each electrode type).](image)
Finally, *in vitro* calibration experiments were performed where a range of .1 µM to 100 µM of DA were tested on both electrodes (Figure 9). The CFME and multi array both had clear plateaus above 10 µM and strong linear relationships when testing the lower concentrations. This indicates that dopamine becomes saturated at the surface of the electrode at higher concentrations, hence the asymptotic curve. The calibration curves are especially important at lower concentrations as it allows for electrode pre-calibration, and in turn, to extrapolate *in-vivo* concentrations of a known analytes. The calibration curve serves as a reference for extrapolating
*in-vivo* neurotransmitter concentration using the non-normalized current after pre- and post-calibrations.

In addition to DA, several other neurochemicals were detected using the CFMEA to confirm resulting CV peaks agreed with the literature (Supplemental Figures 2 and 3). Norepinephrine (NE) was detected using the DA triangle waveform and a clear peak was seen at a similar voltage as DA. Hydrogen peroxide (H$_2$O$_2$) was also detected using a triangle waveform that scanned from -0.4 to +1.4 V $^{40}$. Due to H$_2$O$_2$ production as a by-product of synaptic transmission as well as acting as a secondary measurement, it was also tested to ensure the multi array could detect the reactive oxygen species $^{147}$.

**Codetection**

After ensuring that the multi array had equivalent electrochemical properties in detecting DA, the co-detection of dopamine with other analytes was performed. With a single channel CFME, a potentiostat is used to apply a waveform an analyte of choice is injected into the flow cell, and the redox peaks are measured. Aside from DA, other analytes, such as 5-HT $^{39,89}$ and Melatonin $^{100}$ for example, needed waveform modification to differentiate the analyte from other neurochemicals. Adjusting the waveform leads to enhanced detection by allowing the analyte to sufficiently adsorb onto the CFME surface and decrease fouling intermediates in the redox scheme. However, a major limitation in the method development of waveform modification is that only a single waveform can be tested at once. In other instances, a single waveform has been developed to distinctly identify two biomolecules concurrently, such as the nucleosides adenosine and guanosine $^{42,93}$. An advantageous feature of the multi array is that multiple
waveforms can be applied to each the four different channels to aid in co-detection and test multiple waveforms without signs of electrode crosstalk.

Figure 10 shows oscilloscopes of successful simultaneous waveform application onto each channel for the multi array. Channel 1 had the triangle dopamine waveform applied. Channel 2 was applied with the 5-HT waveform proposed by Jackson et al. \(^{39}\) to reduce \textit{in vivo} fouling. The Jackson waveform (JWF) scanned from a holding potential of 0.2 V to the switching potentials of 1.0, to -0.1, and back to 0.2 V at 1,000 V/sec. The two modified waveforms for 5-HT were the extended WF, where the switching potential was increased to 1.3 V instead of 1.0 V (applied to Channel 3, Fig. 5A), and the extended hold WF (EHWF) which scanned from 0.2 to 1.3 V (applied to Channel 4, Fig. 5A). It was held at that voltage for 1 msec then went back to then -0.1 to 0.2 V. In Figure 10 A, we see the oscilloscopes from the waveforms applied. The triangle, Jackson, Extended, and Hold waveforms are shown in Figure 10 B. While in Figure 10 C, we observed the aforementioned four waveforms applied and superimposed with the oscilloscope response from each electrode.

All three 5-HT waveforms, in addition to the DA waveform, were applied to the four fibers to detect 1 µM 5-HT (Figure 11). 5-HT was successfully detected with redox peaks comparable to CMFE peaks, with 5-HT peaks approximately at 0.6 V for channels 1 and 4, and 0.9 V for channels 2 and 3. The different waveforms applied changed the shape and position of the respective cyclic voltammograms by manipulating electron transfer at the surface of the electrode. This data indicates that waveforms of different functions, such as triangle versus piecewise or sawhorse shape, could be applied onto the multi array simultaneously and still produce comparable peak positions to single CFMEs.
Figure 10: Multiple waveform application onto the multi array. A) Oscilloscope of waveforms applied onto each multi array and B) the waveform shapes: Dopamine, Jackson, Extended, and Hold. C) Overlay of waveforms applied onto the multi array and the oscilloscope of channels 1 – 4. Channel 1 corresponds to the dopamine triangle waveform, channel 2 is applied with the serotonin Jackson waveform, channel 3 shows the serotonin extended waveform, and channel 4 displays the serotonin extended hold waveform, as seen in the legend on the right-hand side of C.
We then applied the adenosine triangle waveform instead of the extended hold waveform in channel 4 to continue measuring multiple compounds. As adenosine cannot be detected with upper limit switching potentials below 1.4 V, applying the adenosine triangle waveform allowed us to determine how various switching potentials affected the overall signal and oxidative peak positions. The adenosine (AD) triangle waveform, which scanned up to 1.45 V was applied to channel 4. A mixture of 10 µM each of DA, 5-HT, and adenosine were injected into the flow cell for co-detection (Figure 12A). Normally, AD has 2 oxidation peaks at 1.4 V and 1.0 V and lacks a reduction peak. The first oxidation peak was clearly observed but the second was slightly shifted, with a third at 0.5 V (Figure 12E). When DA is co-detected with adenosine, the carbon fibers are more sensitive to DA. The third peak is then suggested to be DA, as seen in 12B and 7E, where the change in oxidative current may be ascribed to the higher switching potential. This phenomenon is explained by when increasing the switching potential, the CFME is overoxidized, which causes the increase of sensitivity. The oxidation peaks for the 5-HT CVs
(Figure 12C and 7D) were similar to when they were detected in the flow cell and not in a mixture. Although, clear evidence of one fiber occluding the signal of another was not present. However, the slight shifts present in the CVs may arise from the change in electron kinetics and adsorption due to presence of multiple analytes with waveforms that are optimized for their detection.

![Figure 12: Codetection of a 10 µM solution of DA, 5-HT with 2 waveforms, and Adenosine on a single multi array with application of 4 separate types of waveforms. A) overlayed CVs of each channel, which corresponded to a separate waveform. The colored arrows correspond to the channel’s analyte, such that; black is dopamine, green is 5-HT with the JWF, red is 5-HT with the EWF, and blue is adenosine. B) Extracted CV of channel 1 alone where the dopamine waveform was applied. The peaks present were of adenosine primary and secondary peaks, and of dopamine. C) Channel 2 CV with the JWF applied to detect 5-HT, with an oxidation peak approximately at 9.0 V. D) Channel 3 CV with the EWF to detect 5-HT, where the oxidative peak is normally seen at 0.9 V. Additional peak is suggested to be for DA. E) CV with the adenosine triable waveform applied. Peaks for DA and adenosine were apparent.

Additionally, a mixture of 5 analytes was also co-detected. Including the previous neurochemicals (DA, 5-HT, and AD) another 10 µM mixture was made with the addition of melatonin and guanosine. Channel 2 was changed to a modified scalene waveform which scanned from -0.4 to 1.45 V at 150 V/sec on the forward scan and 400 V/sec on the backward scan to co-detect Gn and AD with a single waveform. Channel 3 was changed to the 5-HT
EHWF. Channel 4 was changed to the melatonin waveform, which is similar to the DA triangle waveform. The original waveforms scans at rate of 600 V/sec, but due to errors in the CV, it was changed back to 400 V/sec (Supplemental figure 4). The resulting CVs indicated a DA peak on channel 1 at ~.7 V. Primary peaks for guanosine (Gn) and AD were seen on channel 2 near the switching potential. Channel 3 indicated a 5-HT peak a small shoulder was present that may be attributed to one of the many analytes present. Chanel 4 showed 2 melatonin peaks with a larger secondary peak due to fouling intermediates. Again, although the fibers did not interfere with one another, this experiment, being the first of its kind to our knowledge, showed multi analyte co-detection with multi-waveform application.

**Mouse striatum**

Finally, the multi array was used to perform proof of concept measurements and assess signal strength and durability in animal tissue. After extracting a mouse brain, it was sliced at approximately bregma +1 to expose the striatum (Figure 13B). As previously mentioned, the striatum is innervated by neuron fibers that release various neurotransmitters, such as dopamine, 5-HT and norepinephrine, in addition to hydrogen peroxide as a byproduct of cellular activity. Therefore, a subregion, the caudate putamen (CPu) was targeted. The CPu is approximately 1.5 mm in length from the corpus callosum and the targeted area is indicated by a yellow circle (Figure 13B). Once the CPu region was exposed, it was placed into the aCSF and the multi array lowered by 100 µm. After a 30-minute equilibrating period, neurochemical release was elicited by pipetting on 1 µL of .1M KCl near the multi array via a micropipette. The resulting CV (Figure 13A) indicated an oxidation peak at approximately 0.7 V which is thought to be the oxidation peak of DA with other neurochemicals present that may have caused the peak to broaden and shift in tissue.
Figure 13: A). The cyclic voltammograms of dopamine release stimulated by the application of KCl in vivo. B). The multi array targeted the caudate putamen or dorsal striatum, as indicated by the yellow circle, which was previously performed by Zang et al. The mouse brain atlas was adapted from Allen Mouse Brain Atlas (2004) and the Paxinos Atlas. Neurotransmitter release was elicited by “puffing” on 1µL of .1M KCl. C) Shows a color plot of the resulting CV and a clear oxidative current for DA. The extended time the analyte remained adsorbed onto the surface was due to tissue detection taking place in a beaker rather than a flow cell. The “puff” of KCl was administered at the 5 second mark.
CHAPTER 6
DISCUSSION AND CONCLUSION

CFMEs have been extensively used in a multitude of applications from \textit{in vitro} measurements of neurotransmitters to insertion into tissue to detect physiological concentrations of biomolecules. More recently, a novel electrode has been developed that has 4 carbon fibers. In this work, the novel multi array was characterized and compared to the CFME. For electrodes with similar protruding lengths and electroactive surface areas, the multi array had a comparable sensitivity to single channel CFMEs. The kinetics of DA were similar on both types of electrodes as observed in the stability and scan rate experiments.

The most prominent feature of the multi array was the ability to simultaneously apply multiple waveforms to the different carbon fiber electrodes with minimal crosstalk between the exposed carbon fibers. Although there were slight variations in exact peak positions for co-detected analytes, they were still within the bounds as seen in literature. This novel array also serves as an improved method for waveform development where multiple waveforms can be assessed at the same time to determine, which waveform yields the highest sensitivity for a particular analyte.

Additionally, measurements in brain tissue \textit{ex vivo} showed that more than one sub region within the caudate putamen could be targeted. CFMEAs could not only target different brain regions simultaneously, but also the same region simultaneously if the electrodes were closely positioned and spaced together. Eliciting neurotransmitter release via KCl produced non-specific neurotransmitter release, especially for the electrode targeting the lateral ventricle and nucleus accumbens, which had a shifted position. Interestingly, when the multi array was inserted into a region where dopaminergic neurons are not typically found, a DA signal was not easily detected.
Also, as this array can be customized with a great number of electrodes, spacings, and configurations, it has the potential to target areas that are also further apart. This would be useful for targeting neurotransmitter rich areas simultaneously and to determine how those concentrations might change in response to certain disease states and drugs.

Conclusion

In conclusion, we interfaced a carbon fiber multielectrode array with a commercially available multichannel FSCV potentiostat. This work will expand the use of FSCV to better understand the brain while using a singular device. The ability to simultaneously measure neurotransmission in several areas within the brain will aide in understanding how the environment changes as a whole instead of looking at one brain region at a time with only one electrode. To our knowledge, this was the first coupling of a carbon fiber multielectrode array to a commercially available multichannel potentiostat, which will make this technology accessible to laboratories and researchers around the world. Future work includes modifying existing metal-based multielectrode arrays with conductive carbonaceous coatings to enhance their ability to monitor neurotransmitters in multiple brain regions simultaneously.
CHAPTER 7

FUTURE DIRECTIONS

Taking what I have learned from these experiments as well as a basic understanding of FSCV, future plans include expanding more into neurobiological research. First, we plan to repeat ex-vivo experiments using electrical stimulation to have specific areas targeted. Then to repeat these experiments in-vivo to confirm the electrode functions within mouse brain tissue. These will be used to support the findings with the CFMEA. The next set of experiments will be looking at pairing a mouse model of addiction to serotonin and dopamine concentrations.

Accepted Proposal for Summer Work

In the proposed study we aim to correlate the types of gut bacteria present to concentrations of serotonin in a mouse model of heroin addiction. Using male and female C57BL/6J mice, animals will be allowed intravenous access to heroin to form opioid dependence. Briefly, subjects will be implanted with a catheter for IVSA. Following a 1-week recovery period, mice will be allowed to self-administer in 1-hour sessions, daily for 5 days. After this acquisition phase, mice will be divided into 1-hour or 6-hour subgroups and allowed to self-administer at higher doses during the allotted time. This will continue for 10 sessions with 1-2 day breaks in between. A control group will undergo the same surgery but only receive saline intravenously. Mice will then be sacrificed to extract the brain and portions of the small intestines. Opioid use disorder is a major health issue as over 11 million people, as young as 12 years old, have abused these drugs. The proposed experiments will provide a distinct approach to understanding the biological basis of addiction by assessing gut bacteria, concentrations of 5-HT, and sex differences present in this model of addiction.
Supplemental Figure 1: A) WaveNeuro Four Multichannel Fast Scan Cyclic Voltammetry (FSCV) Potentiostat, from Pine Research. Attached to the top of the potentiostat are interface cables 0 and 1, which connect to the data acquisition interface board within computer. On the lower left of the potentiostat, the four channel headstage adapter, in purple, is inserted and tightened with screws to hold it in place. To this adapter, the B) black, four-channel headstage cable which connects to multiple tiers of adapter to fit the multi array. The blue, four channel FSCV headstage amplifier (5 MΩ, 200 nA/V) attaches to the black and red four channel headstage-to-microelectrode coupler wire assembly. C) At the end of the coupler are gold pins that connect to the multi array’s D) Omnetics connector via another adapter. The white wire connects to a reference wire with a soldered Ag/AgCl pellet at the end. The other 4 colors correspond to channels 1-4 with red as channel 1, green as channel 2, blue for channel 3, and yellow for channel 4.
Supplemental Figure 2: Multi array CV 10 µM of norepinephrine (NE) using the DA triangle waveform

Supplemental Figure 3: Multi array CV of 10 µM of hydrogen peroxide
Supplemental Figure 4: A) Multi array CV of co-detection of a 10 µM solution of the following analytes: dopamine (B), adenosine and guanosine (C), serotonin (D), and melatonin (E).

Supplemental Figure 5: Corresponding color plots to the multi array channels as seen in supplemental figure 4
Supplemental Figure 6: photographs of the mouse tissue experiments taken using a Samsung camera. A. Implantation of Flex carbon fiber multielectrode array in brain tissue. B. Zoomed-in high magnification of multielectrode array in brain tissue. C. Approximate brain region targeted by Multi array. Note, the tissue is submerged under water but the waterline is not visible in the image.
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