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The Background Signal as an In Situ Predictor of Dopamine Oxidation Potential: Improving Interpretation of Fast-Scan Cyclic Voltammetry Data

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Abstract

Background-subtracted fast-scan cyclic voltammetry (FSCV) has emerged as a powerful analytical technique for monitoring sub-second molecular fluctuations in live brain tissue. Despite increasing utilization of FSCV, efforts to improve the accuracy of quantification have been limited due to the complexity of the technique and the dynamic recording environment. It is clear that variable electrode performance renders calibration necessary for accurate quantification; however, the nature of *in vivo* measurements can make conventional post-calibration difficult, or even impossible. Analyte-specific voltammograms and scaling factors that are critical for quantification can shift or fluctuate *in vivo*. This is largely due to impedance changes, and the effects of impedance on these measurements have not been characterized. We have previously reported that the background current can be used to predict electrode-specific scaling factors *in situ*. In this work, we employ model circuits to investigate the impact of impedance on FSCV measurements. Additionally, we take another step toward *in situ* electrode calibration by using the oxidation potential of quinones on the electrode surface to accurately predict the oxidation potential for dopamine at any point in an electrochemical experiment, as both are dependent on impedance. The model, validated both in adrenal slice and live brain tissue, enables information encoded in the shape of the background voltammogram to determine electrochemical parameters that are critical for accurate quantification. This improves data interpretation and provides a significant next step toward more automated methods for *in vivo* data analysis.

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AUTHOR CONTRIBUTIONS

L.A.S., G.S.M., and J.G.R. conceived of the work, all of the authors collectively designed experiments and prepared the manuscript, and C.J.M. collected and analyzed data.

Keywords

carbon-fiber microelectrode; electrochemical impedance spectroscopy; electrochemistry; calibration; in vivo

1. Introduction

Background-subtracted fast-scan cyclic voltammetry employing carbon-fiber microelectrodes (CFME) is a powerful tool for making real-time, sub-second measurements of electroactive molecules in discrete brain regions.^{1,2} This approach originated with the work of Millar and colleagues,^{3,4} and has seen increasing use in the last decade (Figure 1) due to thorough characterization and technological improvements made largely by Wightman and colleagues.^{5–10} When combined with behavioral and pharmacological paradigms, it has provided remarkable information about the molecular mechanisms underlying specific aspects of goal-directed behavior and associative learning, significantly advancing this field.^{11–19} Despite this, broad acceptance by the neuroscience community has been hindered by remaining difficulties associated with its practice, such as reliable electrode calibration.

Individual CFME performance is variable and dependent on physical characteristics inherent to the handmade electrodes and the recording microenvironment. As a result, electrode-specific calibration is required to accurately relate the observed current to analyte concentration. Traditionally, calibration is done in a flow-injection system following an experiment, as the electrode surface changes in response to tissue exposure and this alters electrode sensitivity.^{20,21} However, post-calibration assumes that the homogeneous buffered electrolyte solution used *in vitro* replicates the unknown, complex, and chemically dynamic environment of brain tissue. Traditional calibration schemes do not account for the involvement of spectator species, such as proteins, which can affect both working and reference electrodes *in vivo*. Also, traditional calibration assumes that electrode sensitivity is constant over the course of the experiment. These assumptions contradict fundamentals of analytical chemistry, which state that the calibration medium should resemble (as closely as possible) the environment in which the measurement was made,²² or calibration may result in significant misinterpretation of data. Additionally, univariate calibration fails when the signals from multiple analytes overlap, which is often the case for *in vivo* electrochemical data. Principal component analysis combined with inverse least-squares regression, also

called principal component regression (PCR),²³ is a multivariate calibration technique. When used with FSCV, training sets are composed of voltammograms for individual electroactive analytes to resolve chemical contributions to the signal, as well as calibration factors to estimate sensitivity and enable quantification.^{24,25,26} A more detailed description of the use of PCR with FSCV can be found elsewhere.²⁷

PCR has been effectively employed in analysis of *in vivo* data for acutely implanted CFMEs.^{24,28} The relatively brief exposure to tissue minimizes the neuroimmunological response,²⁹ and acutely implanted electrodes can be easily removed for post-calibration to obtain *in vitro* calibration factors as estimates of sensitivity *in vivo*. However, chronically implanted electrodes introduce a significant procedural constraint with respect to calibration, due to the duration of the implant (weeks to months),³⁰ and difficulties in safely recovering the electrode for post-calibration. These constraints have led to use of 'standard' or 'universal' training sets, obtained from other electrodes, recording sessions, or subjects, to analyze *in vivo* data.^{31–33} Even when training sets are constructed from data collected *in vitro* using the electrode that was implanted *in vivo*, so called 'matrix effects' can lead to inconsistencies between the *in vivo* data and the data that comprise the training set (i.e. changes in the shape and position of peaks in the voltammogram).³⁴ For these reasons, analytical chemistry textbooks conclude that standards used for calibration should be run in the same matrix as the unknown.^{22,35} Thus, when monitoring molecules in brain tissue a reliable and straightforward method for *in situ* electrode calibration is essential.

Recent work in our group has moved toward this goal by demonstrating that the total background current can be used as a predictor of electrode sensitivity to multiple analytes, *in situ*.²¹ Herein, we expand upon this work by exploring changes in electrochemical performance resulting from variable impedance. Electrode fouling is mimicked by the addition of impedance to the electrochemical system. The resulting shifts in sensitivity, and in the oxidation potentials of both quinone-like moieties on the carbon surface ($E_{p,QH}$) and of dopamine ($E_{p,DA}$), are characterized. Finally, an *in vitro* model that predicts $E_{p,DA}$ based on $E_{p,QH}$ is described and validated. This powerful tool will facilitate *in situ* determination of appropriate electrochemical parameters for accurate quantification. Additionally, the information within will be fundamental in the development of more automated data analysis methods.

2. Results and Discussion

Combining Fast-Scan Cyclic Voltammetry and Principal Component Regression

The detection of catecholamines using FSCV is commonly performed with a triangular waveform ramping from a -0.4 V holding potential to $+1.3$ V and back.^{1,2} Herein, this waveform (Figure 2A) is applied to a CFME with a scan rate of 400 V s^{-1} , an application frequency of 10 Hz , and a sampling frequency of 200 kHz (except for data present in Figure 3 which were sampled at 100 kHz). When applied to a CFME, it generates a large but stable background signal composed predominately of nonfaradaic capacitive (charging) current,³⁶ with smaller faradaic contributions from electroactive species that are statically present in the extracellular space¹ and redox groups inherent to the electrode surface.³⁷ When the concentration of an electroactive analyte fluctuates in the immediate vicinity of the

electrode, the background current (Figure 2B, black dashed line) can be subtracted to reveal the faradaic current generated in response to the concentration change. The resulting voltammogram serves to identify the analyte and can also be used for quantification (Figure 2C). This effectively removes contributions to the current that result from processes inherent to the electrode, and from species that exist at relatively constant concentrations. The vertical dashed lines delineate the oxidation potentials of quinone-like surface functionalities and surface confined quinone species, ($E_{p,QH}$, Figure 2B),^{37–39} and the oxidation potential of dopamine ($E_{p,DA}$, Figure 2C). The current at $E_{p,DA}$ (ordinate) can be plotted versus analyte concentration (abscissa) and the slope of a fitted regression line determines electrode sensitivity (Figure 2D).^{1,2}

A comprehensive training set consists of voltammograms for all analytes expected to be present in the experimental data, with concentrations spanning the physiological range.^{27,34} Voltammetric signals collected *in vivo* are highly variable in terms of peak width, position, and relative intensity. Additional variation results from instrumentation, inherent differences in carbon-fiber preparations and surface structure,^{38,40} reference electrode performance,⁴¹ and differences in recording microenvironments. In fact, oxidation potentials can dynamically shift *during* an *in vivo* electrochemical experiment. When oxidation potentials are intentionally offset from those used in the training set, systematic misestimation of analyte concentrations and/or failures in residual analysis for model validation arise.^{27,34} Thus, training a model for PCR is not straightforward, and the selection of an inappropriate training set could result in unreliable assignment of analyte concentrations.^{27,34} Realization of this unresolved issue initiated investigation into how electrode surface states and recording microenvironments influence oxidation potentials.

The Carbon-Fiber Microelectrode Surface Influences Oxidation Potential

Conditioning of the CFME changes the chemical nature of the carbon surface,⁴² and as such is expected to influence analyte oxidation potentials. To verify this, three surface conditions were investigated using a flow-injection system. Dopamine was detected in a TRIS buffered saline 1) before implantation in the striatum of an anesthetized rat for 30 min, 2) after removal from the striatum, and 3) following post-implantation cleaning in isopropyl alcohol (IPA) overnight. Representative CVs are displayed in Figure 3A–B. Potential is plotted on the abscissa and normalized current (I/I_p) on the ordinate. The dashed lines indicate nominal values for the oxidation potentials, $E_{p,QH}$ and $E_{p,DA}$. The difference between the two potentials ($E_{p,DA-QH}$) is displayed in Figure 3C. Data are presented as the mean $\pm$ standard deviation ($n=12$ electrodes). One-way ANOVA with Tukey's post-hoc test was employed for multiple comparisons of $E_{p,QH}$, $E_{p,DA}$ and $E_{p,DA-QH}$ resulting from different electrode states. There was a significant effect of the surface state on $E_{p,QH}$ and $E_{p,DA}$: $F(2,33)=6.895$, $**p=0.0031$; and $F(2,33)=16.83$, $****p<0.0001$, respectively. A Tukey's post-hoc test revealed that $E_{p,QH}$ and $E_{p,DA}$ are shifted to more positive potentials following implantation in striatal tissue. This is attributed to fouling of the electrode surface by adsorbed biological material. Cleaning the electrode in IPA reverses the effects of fouling, returning the oxidation potentials to approximately their original values, suggesting removal of adsorbed material. Perhaps the most interesting result is that $E_{p,DA-QH}$ is conserved for each electrode condition; $F(2,33)=0.8282$, $p>0.05$. Thus, the oxidation potentials are similarly

affected by changes to the electrode surface, suggesting that the position of $E_{p,QH}$ could serve as a predictor for the position of $E_{p,DA}$. To investigate this, a series of circuits of varying impedance was used to controllably shift oxidation potential positions *in vitro*.

Model Circuits to Simulate Impedance Contributions Due to Electrode Fouling

The non-specific adsorption of material to the electrode surface perturbs the electrochemical system. This can be treated as a change in impedance and modeled using equivalent circuits.³⁶ Generally, an electrode-electrolyte interface can be modeled with a Randles equivalent circuit consisting of solution resistance (R_s) in series with both double-layer capacitance (C_{DL}) and charge-transfer resistance (R_{CT}); such that C_{DL} is in parallel with R_{CT} (Figure 4).^{36,43–45} Warburg impedance is a constant phase element that describes impedance due to diffusing redox species. It is observed at low frequencies (<100 Hz) not relevant to this work, so it is not included.⁴⁶ The simplified circuit model represents a two-electrode electrochemical system in tissue. It consists of a potentiostat connected to two Randles equivalent circuits representing the working and reference electrodes (R_E is the resistance inherent to the electrode). A variable impedance element is included to represent impedance contributions due to fouling (of both working and reference electrodes), and the system is completed by including solution resistance (R_s). Work characterizing the impedance properties of single carbon-fiber microelectrodes *in vivo* is minimal. However, fouling contributions to metal and silicon microelectrode impedance have been studied extensively using electrochemical impedance spectroscopy (EIS).^{47–51} These studies rely on the assumption that the electrode state does not change significantly over the duration of the experiment. However, impedance is expected to vary due to the dynamic nature of the tissue and slower changes in the microenvironment (inflammation, gliosis, etc.) that occur over the course of an extended electrode implant. The data presented in Figure 3C define the approximate range of $E_{p,QH}$ and $E_{p,DA}$ that must be achievable by controlling impedance.

Figure 5A (inset) displays a schematic of the Randles circuit used to mimic shifts in impedance due to biological fouling, and a table of the values for resistance (R) and capacitance (C) used. These values represent a myriad of possible contributing factors such as bulk tissue resistance and tissue response to the implanted electrodes by way of matrix proteins, microglia, and astrocytes (the response is dynamic across the duration of the implant).^{47,49,52} Changes in capacitance can be attributed to microglia/astrocyte cell membrane capacitances as well as the adsorption of proteins (which affects surface area), and changes in extracellular ionic composition.^{47,49,50} A representative Nyquist plot resulting from an EIS simulation of a model Randles circuit is also shown (Figure 5A), in which the real and imaginary components of impedance are plotted on the abscissa and ordinate, respectively. Each point making up the Nyquist plot represents impedance due to different alternating current frequencies, decreasing from left to right. A standard voltammetric sweep is completed in ~8.5 msec when using common waveform parameters in FSCV (–0.4 V to 1.3 V applied at 400 V/sec). If this triangle were constantly repeated, the time of the applied triangular waveform (8.5 msec) could be thought of as the ‘period’ of the waveform, approximated by that of a sine wave of ~118 Hz (dotted line, Figure 5A inset). Thus, this frequency was sampled for a series of 27 circuits, including one with no added impedance, to study how impedance modulates the position of $E_{p,QH}$ and $E_{p,DA}$.

Figure 5B displays a contour plot describing the relationship between capacitance (x-axis), resistance (y-axis), and total impedance (z-axis) of the circuits at 118 Hz. Total impedance is also depicted in color. It is evident that impedance increases as resistance increases, and it decreases as capacitance increases. The effects of capacitance are much more evident at high versus low resistance. However, the plot clearly indicates that these relationships are not linear. Rather, there is a complex relationship that spans a range of impedance values.

Employing Model Circuits to Investigate How Impedance Shifts Redox Potential

Following characterization, the circuits were coupled with CFMEs in either phosphate-buffered saline (PBS) or TRIS-buffered saline (TRIS). Both buffers are commonly used in electrochemical experiments at physiological pH; however, TRIS contains amines (present in many biomolecules) that competitively bind to the electrode,⁵³ creating a situation that is thought to more closely resemble conditions in the brain. The model circuits were implemented between the potentiostat and the working electrode; the reference electrode was unaltered. Impedance is additive across the electrochemical cell. As such, altering the impedance of one electrode should serve to shift impedance of the entire circuit. Background CVs and background-subtracted dopamine CVs were used to determine $E_{p,QH}$ and $E_{p,DA}$ values. Triplicate standards were averaged across five electrodes for each impedance preparation in PBS and TRIS.

Figure 6A displays representative background CVs (left) and background-subtracted dopamine CVs (1 μ M, right) for impedance preparations in which the total resistance was systematically increased (60, 110, 160, 210, 310 k Ω) while the capacitance was held constant (3.19 nF) in PBS (top) or TRIS (bottom) buffer. The black traces were collected with no added impedance (control), and simulated impedance values are described in the legend. The dashed lines indicate the mean potentials (n=5 electrodes) corresponding to $E_{p,QH}$ and $E_{p,DA}$ in the control circuit, with no impedance added. The data clearly demonstrate that adding impedance impacts the nature of the electrochemical system, as both $E_{p,QH}$ and $E_{p,DA}$ shift to more positive potentials as impedance is added. A positive shift in these oxidation potentials is also observed in the presence of TRIS, an amine that competitively binds to the electrode surface, as compared to PBS solution (with no impedance added: $E_{p,QH}$: t(4)=5.74, **p=0.0023 and $E_{p,DA}$: t(4)=3.675, *p=0.0107, one-tailed t-test, data not shown). Increased impedance also results in decreased current amplitude (Figure 6B, PBS: F(5,24)=15.05, ****p<0.0001; TRIS: F(5,24)=21.07, ****p<0.0001), as well as decreased electrode sensitivity (Figure 6C, F(2,30)=56.52, ****p<0.0001). Working in TRIS buffer also reduces the amplitude of the signal, but this is largely due to the presence of the amines that occupy dopamine adsorption sites on the sensor surface.⁵³ Taken together, these data underscore the importance of improving calibration strategies, and the need for an accurate *in situ* approach.²¹

Figure 7A plots the position of $E_{p,QH}$ (blue) and $E_{p,DA}$ (red) as a function of added impedance in both PBS (left) and TRIS (right) buffers. At 118 Hz, it is apparent that this relationship is linear (R^2 0.87, n=5). Figure 7B compares the slopes of the regression lines fit to these data. Slope (mV/k Ω ***) is plotted on the ordinate, with the various peak potentials identified on the abscissa; columns are color coordinated according to buffer. A

two-way ANOVA determined that the source of variation in the slopes is due to the oxidation potential monitored, $F(1,100)=9.034$, $**p=0.0033$, and not due to the buffer, $F(1,100)=0.2363$, $p>0.05$. Thus, $E_{p,QH}$ is modulated differently (per unit impedance) than $E_{p,DA}$. These results also demonstrate that the effects of impedance are consistent across these buffers. Figure 7C displays the relationship between $E_{p,QH}$ (abscissa) and $E_{p,DA}$ (ordinate) in both PBS (black) and TRIS (gray) buffers. Each point represents the mean ($n=5$) value for a given impedance preparation, with the lowest values (bottom left) collected with no added impedance and the highest values (top right) with 310 k Ω of added impedance. $E_{p,DA,PBS}$ values are plotted on the left ordinate and $E_{p,DA,TRIS}$ values on the right ordinate. The linear relationship demonstrates that $E_{p,QH}$ can be used as a reliable predictor of $E_{p,DA}$. This is important, because it shows that this model can be used to identify training set parameters that are most likely to be appropriate for PCR and data analysis, and to thus improve interpretation of *in vivo* data. ANCOVA was used to compare regression models in both PBS and TRIS, and no differences were found ($F(1,50)=0.154$, $p>0.05$). The more physiologically relevant TRIS model was employed for further *ex vivo* validation.

Validation of the Impedance Model

Electrically evoked catecholamine (CA) release was detected in CA-depleted adrenal tissue slices as well as in the nucleus accumbens (NAc) of a freely moving animal to validate a predictive model for $E_{p,DA}$ using $E_{p,QH}$. The adrenal medulla contains significant quantities of epinephrine and norepinephrine, and lower levels of dopamine, even when the animal has been treated with reserpine and α -MPT to decrease CA levels.^{54,55} The oxidation potentials of these CAs are indistinguishable from one another using the waveform employed and the resulting CVs are quite similar.^{40,56} Adrenal tissue provides an effective model for validation because it is significantly impeding, as evident by the fact that catecholamine CVs have $E_{p,QH}$ and $E_{p,DA}$ values higher than in the NAc (Figure 8A). This may be due to high levels of CA fouling the electrode via electropolymerization.⁵⁷ Electrodes ($n=5$) were positioned in the medulla and allowed to cycle for 30–60 min prior to electrical stimulation every 5 min for 30 min. To further evaluate the model, electrically evoked catecholamine release was also detected at a CFME chronically implanted in the NAc over the course of three weeks. Background CVs and background-subtracted catecholamine CVs were used to determine $E_{p,QH}$ and $E_{p,DA}$, respectively.

Figure 8A displays representative color plots and CVs from the two tissue preparations. Figure 8B displays the relationship between the critical oxidation potentials for individual electrical stimulation events (black symbols) as they relate to the TRIS regression model (gray). Measured values for $E_{p,QH}$ and $E_{p,DA}$ are plotted on the abscissa and ordinate, respectively (black). It is apparent that the relationship between $E_{p,QH}$ and $E_{p,DA}$ approximates the relationship established *in vitro*, confirming that the range of impedances used to create the model was appropriate. The individual data points should be normally distributed about the regression model; however, the tissue data is offset from the regression line. In tissue, ongoing background redox processes are present that are not accounted for in the flow-cell. To investigate the role of background redox processes on the $E_{p,QH}$: $E_{p,DA}$ relationship, physiological concentrations of electroactive species present in the NAc were

added to the buffer. Ascorbic acid (AA, 50 μ M) and 3,4-dihydroxyphenylacetic acid (DOPAC, 5 μ M) were used to create a TRIS-AA-DOPAC buffer (Figure 8B, blue),^{58,59} and a TRIS-DA buffer (Figure 8B, red) contained 5 μ M dopamine. ANCOVA determined no significant difference in the slopes of TRIS, TRIS-AA-DOPAC, TRIS-DA and of a regression line fit to the live tissue data ($F(3,119)=0.84$, $p=0.4766$). ANCOVA did reveal that the y-intercepts were significantly different ($F(3,122)=13.2$, **** $p<0.0001$). Tukey's post-hoc test determined that TRIS-AA-DOPAC, TRIS-DA and the live tissue regression lines had intercepts that were not different ($p=0.37$), but that were all different from the TRIS model (**** $p<0.0001$). Therefore, the presence of background redox processes is critical when predicting $E_{p,DA}$ in tissue. Figure 8C plots the relationship between the measured $E_{p,DA}$ (abscissa) and $E_{p,DA}$ predicted (ordinate) using the linear model for the TRIS (gray), TRIS-AA-DOPAC (blue), and TRIS-DA (red) buffers. If the models reliably predict $E_{p,DA}$, then all the resulting points should fall on, or be normally distributed about, the dashed unity line, as in the models that incorporate background redox processes.

3. Conclusion

Precise and reliable quantification of neurochemical concentration is critical in order to accurately interpret the molecular mechanisms underlying any aspect of brain function. Electrode calibration is necessary for accurate quantification; however, the nature of *in vivo* measurements can make conventional post-calibration difficult, or even impossible. Even when calibration is feasible, the analyte-specific voltammograms and scaling factors that lie at the core of PCR can shift or fluctuate *in vivo*, largely due to changes in impedance brought about by long-term biological processes. Thus, an *in situ* method for accurate electrode calibration is imperative. We provide a step toward this goal by describing a model that uses the oxidation potential of quinone-like moieties in the background signal as an accurate predictor for the oxidation potential of dopamine at any time point in an electrochemical experiment. Importantly, this model does not assume that current-potential relationships are fixed across the entirety of an experiment. One example of its utility would be when the experimental signal to noise ratio is insufficient to resolve peak oxidation potential. The nature of the background signal could bring attention to inconsistencies between the training set and the experimental data, so that the training set could be adjusted as required. Overall, this work characterizes the effects of impedance on FSCV measurements, and provides a tool for researchers to make informed decisions when constructing appropriate PCR models. It promises to simplify data collection protocols for *in vivo* applications, and to facilitate widespread use of FSCV by allowing more freedom in experimental design, particularly when combined with a previously described approach to predict electrode sensitivity *in situ*²¹. Thus, it represents a significant step toward more automated analysis of *in vivo* data.

4. Materials and Methods

Chemicals

All chemicals (95%, HPLC assay) were purchased from Sigma-Aldrich Co. (St. Louis, MO), unless otherwise stated, and used without additional processing. *In vitro*

electrochemical experiments were performed in either a phosphate-buffered saline (10 mM Na₂HPO₄, 138 mM NaCl, and 2.7 mM KCl) or TRIS buffered saline (15 mM 2-amino-2-hydroxymethyl-propane-1,3-diol, 3.25 mM KCl, 1.20 mM CaCl₂, 1.2 mM MgCl₂, 2 mM Na₂SO₄, 1.25 mM NaH₂PO₄, and 145 mM NaCl). Adrenal slice experiments used bicarbonate-buffered saline (125 mM NaCl, 26 mM NaHCO₃, 2.5 mM KCl, 2.0 mM CaCl₂, 1.0 mM MgCl₂, 1.3 mM NaH₂PO₄, 10 mM HEPES, and 10 mM glucose) saturated with 95% O₂ and 5% CO₂. All buffers were adjusted to pH 7.4 with 1 M NaOH and 1M HCl. Reserpine (10 mg mL⁻¹) and α -methyl-DL-tyrosine methyl ester hydrochloride (α -MPT, 250 mg mL⁻¹) were prepared in 50% saline:50% dimethyl sulfoxide and in saline, respectively. All aqueous solutions were made using ultrapure 18.2 M Ω water.

Microelectrode Fabrication

Cylindrical carbon-fiber microelectrodes were fabricated using T-650 carbon fibers (Cytec Industries, Inc., Woodland Park, NJ) as previously described.¹ In short, a single carbon fiber was aspirated into a glass capillary, a glass seal was created using a micropipette puller (Narishige, Tokyo, Japan), and the fiber extending past the seal was cut to 100 μ m in length. Electrical connection to the carbon-fiber was established via a conductive silver epoxy. A Ag/AgCl pellet reference electrode (World Precision Instruments, Inc., Sarasota, FL) was used to complete the two-electrode electrochemical cell.

Model Circuit Fabrication and Characterization

Resistance and capacitance values for circuit components were confirmed with a voltmeter. Five circuits with constant resistance (60, 110, 160, 210, and 310 k Ω) were produced in a manner that allowed capacitors (0, 3.93, 7.43, 10.19, 13.82 nF) to be interchanged. Additionally, a circuit containing four 10 k Ω resistors in series was fabricated to add data points between 0 and 40 k Ω . This resulted in 29 different circuits. EIS Spectrum Analyzer⁶⁰ software (available online at <http://www.abc.chemistry.bsu.by/vi/analyser/>) was used to simulate electrochemical impedance spectroscopic values for each circuit.

Flow-Injection System

Working electrodes were positioned in a custom electrochemical flow-cell using a micromanipulator (World Precision Instruments, Sarasota, FL). A syringe pump (New Era Pump Systems, Wantagh, NY) supplied a continuous buffer stream of 0.5 mL min⁻¹ (1.0 mL min⁻¹ for data in Figure 3) across the working and reference electrodes. Two-second bolus injections of analyte to the microelectrode surface were accomplished with a six-port HPLC valve mounted on a two-position actuator controlled by a digital pneumatic solenoid valve (Valco Instruments, Houston, TX). The entire apparatus was enclosed in a custom-built grounded Faraday cage.

Data Acquisition

A triangular waveform from -0.4 to +1.3 V with a -0.4 V holding potential was applied at a scan rate of 400 V s⁻¹, an application frequency of 10 Hz, and a sampling frequency of 200 kHz (100 kHz for data in Figure 3) using a custom built instrument for potential application and current transduction (University of North Carolina at Chapel Hill, Department of

Chemistry, Electronics Facility). High Definition Cyclic Voltammetry software (University of North Carolina at Chapel Hill) was used for waveform output, data analysis and signal processing in conjunction with data acquisition cards (National Instruments, Austin TX) used for measuring current and synchronizing the electrochemical cell with the flow-injection system. Analog filtering was accomplished with a 2-pole Sallen-Key, low-pass filter at 2 kHz. There was no additional analog or digital filtering of the data.

Animal Experiments

Male Sprague-Dawley rats (250–300 g, Charles River Laboratories, Raleigh, NC) were used in all animal experiments. Animal care and use was in accordance with North Carolina State University Institutional Animal Care and Use Committee (IACUC) guidelines. One animal was used as an acute electrode preparation. The animal was urethane anesthetized (4 g kg^{-1} intraperitoneally), placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and holes for electrodes were drilled in the skull using stereotaxic coordinates, as described previously.¹ The working electrode was placed in the striatum (1.2 mm AP, 3.0 mm ML, -5.0 mm DV relative to bregma), and the Ag/AgCl reference electrode was placed in the contralateral forebrain. The animal's body temperature was maintained at 37°C by a heating pad.

One animal was treated with reserpine (5 mg/kg) and α -MPT (250 mg/kg) daily for three days. The animal was urethane anesthetized (4 g kg^{-1} intraperitoneally), decapitated, and adrenal glands were quickly removed, trimmed of fat tissue, and embedded in 3% agarose gel in bicarbonate-buffered saline (BBS). The gel blocks were placed in cold BBS and $400 \mu\text{m}$ thick slices were cut with a vibratome (World Precision Instruments, Sarasota, FL). The slices were allowed to rest in BBS for one hour, then transferred to a recording chamber (Warner instruments, Hamden, CT) and superfused with BBS maintained at 34°C for at least 30 min prior to electrical stimulation. Carbon-fiber microelectrodes were positioned in tissue near twin tungsten stimulating electrodes. Electrode placements were made with the aid of a microscope (Nikon Instruments, Inc., Melville, NY) and micromanipulators (Scientifica, Ltd., United Kingdom). Electrical stimulations consisted of trains of 16 biphasic, $500 \mu\text{A}$ pulses at a frequency of 60 Hz with a pulse width of 2 ms.

A chronic electrode preparation was investigated in one animal. Surgery was performed as previously described.³⁰ Briefly, the animal was anesthetized with 2.5%–4% isoflurane, placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and holes for electrodes and screws were drilled in the skull using stereotaxic coordinates. The working electrode was placed in the NAc ($+1.2 \text{ mm AP}$, $+1.4 \text{ mm ML}$, -7.0 mm DV relative to bregma), the stimulation electrode in the ventral tegmental area (-5.2 mm AP , $+1.0 \text{ mm ML}$, -8.2 mm DV relative to bregma), and a Ag/AgCl reference electrode was placed in the contralateral forebrain. The components were permanently affixed with dental cement. The animal's body temperature was maintained at 37°C using a heating pad. The animal was allowed to recover for two weeks prior to administering electrical stimulation. Electrical stimulations consisted of trains of 12 biphasic, $175 \mu\text{A}$ pulses at a frequency of 60 Hz with a pulse width of 2 ms, performed every other day.

Statistics

Data are presented as the mean $\pm$ standard deviation, unless otherwise noted. Paired t-tests were employed to compare two groups of data. One-way ANOVA with a Tukey's post-hoc test and two-way ANOVA were used to compare significance between multiple groups. ANCOVA (with Tukey's post-hoc test) was used to compare linear regression models. Significance was determined at $p < 0.05$. Statistical analyses and graphical depictions of the data were carried out using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA) and MATLAB R2012B (Mathworks, Natick, MA).

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Abbreviations

FSCV	fast-scan cyclic voltammetry
PCR	principal component regression
CV	cyclic voltammogram
E_{p,DA}	oxidation potential of dopamine
E_{p,QH}	oxidation potential of quinone-like moieties
CFME	carbon-fiber microelectrode
EIS	electrochemical impedance spectroscopy
PBS	phosphate-buffered saline
TRIS	Tris-buffered saline
CA	catecholamine
α-MPT	α -methyl-DL-tyrosine methyl ester hydrochloride
AA	ascorbic acid
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid

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Figure 1.
The number of references in PubMed using ‘fast-scan cyclic voltammetry’ per year, since 1984.

Figure 2.

Overview of fast-scan cyclic voltammetry. A) A triangular waveform from -0.4 V to $+1.3$ V is applied at 400 V/s and repeated at 10 Hz. B) Representative background (black) and dopamine (red) CVs. C) A representative background-subtracted CV for 1 μ M dopamine. In both B) and C), potential and current are plotted as the abscissa and ordinate, with dashed lines indicating positions of $E_{p,QH}$ and $E_{p,DA}$, respectively. D) A calibration curve for dopamine using a single CFME. Dopamine concentration and current at $E_{p,DA}$ are plotted on the abscissa and ordinate, respectively. Data are presented as mean $\pm$ standard deviation ($n=3$ replicate injections).

Figure 3.

The electrode surface influences oxidation potential. Normalized A) background CVs and B) background-subtracted dopamine CVs collected on an electrode: 1) before tissue implantation (black), 2) post-implantation (red) and 3) post-implantation and cleaning in IPA (blue). Potential and normalized current are plotted as the abscissa and ordinate, respectively. Dashed lines indicate nominal values of the oxidation potentials. C) Summary of $E_{p,QH}$ (top), $E_{p,DA}$ (middle), and $E_{p,DA-QH}$ (bottom) as a function of the electrode state. Data represent mean $\pm$ standard deviation ($n=12$ electrodes). Significance was determined via a one-way ANOVA with Tukey's post-test.

Figure 4.

Equivalent circuit diagram depicting an *in vivo* electrochemical system. Both the working and reference electrodes are represented by Randles equivalent circuits with an impedance contribution due to adsorbed material (fouling) that is simulated by adding impedance (Z) between the potentiostat and working electrode.

Figure 5.

Impedance characterization of model Randles circuits. A) Representative Nyquist plot for a Randles circuit (top right), as well as a table of the resistance (R) and capacitance (C) values employed. Triangular and sine waveforms are overlaid (bottom right) to depict the period used in determining an appropriate impedance frequency (118 Hz). B) Total impedance at 118 Hz as a function of resistance and capacitance. Capacitance, resistance, and impedance are plotted on the x, y, and z axes, respectively; color also denotes impedance. Simulated data points are indicated by the black dots.

Figure 6.

Impedance impacts the nature of the electrochemical system. A) Representative background CVs (left) and background-subtracted dopamine CVs (1 μ M, right) resulting from systematic increases in impedance using the model circuits. Values in the legend are simulated impedance at 118 Hz for each circuit. In each case the capacitance was held constant (3.93 nF) and the resistance varied (60, 110, 160, 210, 310 k Ω). Data was collected in PBS (top) and TRIS (bottom). Mean values of $E_{p,QH}$ and $E_{p,DA}$ (n=5) for the preparation with no added impedance are indicated with dashed lines. B) Added impedance significantly decreases peak current (at $E_{p,DA}$) for dopamine oxidation when working in either PBS (left)

or TRIS buffer (right). A one-way ANOVA with Tukey's post-hoc test was used to compare each preparation with added impedance to that with no added impedance, for both PBS and TRIS (n=5). C) Sensitivity to dopamine is significantly decreased with additional impedance. Data was collected in PBS with no added impedance (black), 60 k Ω (red), and 310 k Ω (blue) of added impedance (n=3). A summary of the sensitivities is provided in the table. Slopes were compared via ANCOVA with Tukey post-hoc test. All data are presented as mean $\pm$ standard deviation.

Figure 7.

Establishing a relationship between $E_{p,QH}$ and $E_{p,DA}$. A) $E_{p,QH}$ (blue) and $E_{p,DA}$ (red) as a function of added impedance (abscissa) in both PBS (left) and TRIS (right). B) Comparison of the slopes (mV/k Ω) of the regression model for both $E_{p,QH}$ and $E_{p,DA}$ as a function of added impedance in PBS (black) and TRIS (gray). Significance was determined via a two-way ANOVA. C) Plot of $E_{p,QH}$ (abscissa) versus $E_{p,DA}$ (ordinate) resulting from all impedance preparations in PBS (black, left ordinate) and TRIS (gray, right ordinate). ANCOVA was used to compare the two regression models. In all cases error bars represent the mean $\pm$ standard deviation (n=5).

Figure 8.

Model validation in live tissue. A) Representative color plots (top), background CVs (middle), and background-subtracted catecholamine CVs (bottom) collected in adrenal and brain (NAc) tissue. In the color plots, time is the ordinate, potential is the abscissa, and current is plotted in false color with the scale bar below the color plot. Time of electrical stimulation is denoted by red arrows. B) Measured values for $E_{p,QH}$ and $E_{p,DA}$ from individual electrical stimulations are plotted on the abscissa and ordinate, respectively. One group of data points was obtained from five CFMEs acutely implanted in adrenal tissue. The other group of data points was obtained over three weeks using a chronically implanted CFME. The four lines display the linear regressions for the TRIS (gray), TRIS-DA (red, $n=3$ electrodes), and TRIS-AA-DOPAC (blue, $n=3$ electrodes) models, and for the live tissue data (black). ANCOVA with Tukey's post-hoc test was used to compare the slopes and intercepts of the regression models. A table summarizing the regression statistics is provided below. C) Measured $E_{p,DA}$ (abscissa) and predicted $E_{p,DA}$ (ordinate) obtained using $E_{p,QH}$ in the TRIS (gray), TRIS-DA (red), and TRIS-AA-DOPAC (blue) models. The dashed line has a slope equal to unity.