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An In Situ Electrode Calibration Strategy for Voltammetric Measurements In Vivo

James G. Roberts, **J. Vincent Toups**, **Eyob Eyualem**, **Gregory S. McCarty**, and **Leslie A. Sombers**
North Carolina State University, Raleigh, North Carolina, 27695, United States.

Abstract

Technological advances have allowed background-subtracted fast-scan cyclic voltammetry to emerge as a powerful tool for monitoring molecular fluctuations in living brain tissue; however, there has been little progress to date in advancing electrode calibration procedures. Variability in the performance of these handmade electrodes renders calibration necessary for accurate quantification; however, experimental protocol makes standard post-calibration difficult, or in some cases impossible. We have developed a model that utilizes information contained in the background charging current to predict electrode sensitivity to dopamine, ascorbic acid, hydrogen peroxide, and pH shifts at any point in an electrochemical experiment. Analysis determined a high correlation between predicted sensitivity and values obtained using the traditional post-calibration method, across all analytes. To validate this approach in vivo, calibration factors obtained with this model at electrodes in brain tissue were compared to values obtained at these electrodes using a traditional ex vivo calibration. Both demonstrated equal powers of predictability for dopamine concentrations. This advance enables in situ electrode calibration, allowing researchers to track changes in electrode sensitivity over time and eliminating the need to generalize calibration factors between electrodes or across multiple days in an experiment.

Keywords

Dopamine; fast scan cyclic voltammetry; carbon fiber microelectrodes; electrochemistry; neurotransmission

INTRODUCTION

Background-subtracted fast-scan cyclic voltammetry (FSCV) is a powerful and emerging tool for monitoring chemical fluctuations in living tissue.[1] It combines sensitivity and electrochemical selectivity for making real-time measurements of electroactive molecules in discrete brain regions. This approach originated with the work of Millar and colleagues,[2-3] and has been popularized through characterization and significant technological improvements made largely by Wightman and colleagues.[4-9] When coupled with specific behavioral challenges, it has provided unprecedented information about the molecular mechanisms underlying specific aspects of goal-directed behaviors, significantly advancing these studies.[10-16] However, broad acceptance by the neuroscience community has been hindered by remaining difficulties associated with its practice. One technical aspect that needs attention is that of electrode calibration.

*Corresponding Author leslie_sombers@ncsu.edu; Phone: 919-515-0320. .

Carbon-fiber microelectrodes are individually fabricated by hand, thus each sensor is distinct in terms of area and surface chemistry. Voltammetric current is directly dependent on electrode surface area, as described by the Randles-Secvik equation.[17] Furthermore, the surface of the electrode chemically changes during electrochemical conditioning, and this significantly affects sensitivity.[18-20] As a result, individual electrode calibration is required to accurately relate the current to analyte concentration. This is commonly done at the end of an experiment, because sterilization is often required prior to implantation in tissue, and tissue exposure alters electrode sensitivity.[21] Typically, the sensor is carefully removed from tissue and post-calibrated in a flow-injection system. Importantly, this calibration scheme assumes that the homogenous buffered electrolyte solution used ex vivo replicates the unknown environment of living tissue, where analyte sources and sinks are highly localized. It does not take into account the involvement of spectator species, such as proteins, which can affect both the reference and working electrodes in vivo (fouling). Finally, it also assumes that electrode sensitivity is constant over the course of the experiment, and that the electrode surface was not altered (or broken) when removed from tissue. These assumptions may significantly skew interpretation of the data.

Electrode-to-electrode variability deems calibration necessary, but ex vivo calibration is often impractical and in some cases impossible. Data interpretation is dependent on the complex nature of the brain nucleus in which it was collected; however, the electrode tip is too small to leave a visible track in brain tissue. Thus, the recording electrode is frequently used to lesion the surrounding tissue, so that the recording site can be verified using post-mortem histological methods. This process requires the passage of high currents, destroying the electrode and preventing post-calibration. Another example is inherent to the use of chronically implanted carbon-fiber microelectrodes. These permanent electrodes provide an important tool that enables the study of longitudinal neurochemical changes over a longer time course (days to months), commensurate with the time course of learning processes or disease progression in animal models.[16,22-23] However, chronically implanted electrodes are difficult to remove for calibration. Thus, calibration factors are often generalized, assuming all electrodes are identical.[24] Finally, FSCV has recently been implemented in human studies, in an important step forward.[25-26] However, medical protocol requires sterilization of the electrode prior to implantation, and post-calibration is abandoned because the electrode is considered medical waste. These examples clearly demonstrate the need for a method to eliminate standard calibration procedures, while preserving the accurate quantitation of chemical fluctuations measured in an in vivo environment.

Herein, we present and validate a simple method that uses the characteristics of the largely non-faradaic background charging current as an accurate predictor of electrode sensitivity to several biologically important analytes including dopamine, ascorbic acid, hydrogen peroxide (H_2O_2), and shifts in pH, eliminating the need for traditional in vitro calibration. This novel and facile mathematical approach for predicting the sensitivity of carbon-fiber microelectrodes to a number of analytes detected using FSCV does not assume that all electrodes are the same, or that the sensitivity of a given electrode remains constant. Rather, it individually assesses the sensitivity of each sensor in real time, while it is in the recording environment. It was verified and applied to empirical data representing phasic dopamine signaling collected in the ventral striatum of a freely-moving rat, and is demonstrated to beat least as effective at predicting analyte concentration as a traditional ex vivo calibration. This powerful tool for quantitative electrochemical measurements can have an immediate and significant impact on a broad range of in vivo studies, eliminating the requirement for ex vivo calibration or the use of generalized calibration factors.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich Co., (St. Louis, MO) unless otherwise specified and used without additional processing. All electrochemical experiments were done in physiological pH 7.4 buffered solution (15 mM Tris, 3.25 mM KCl, 1.20 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2 mM Na_2SO_4, 1.25 mM NaH_2PO_4, and 145 mM NaCl). All aqueous solutions were made using double-deionized water (Millipore, Billerica, MA).

Electrode Fabrication

Cylindrical carbon-fiber microelectrodes were fabricated using T-650 carbon fibers (Cytec Industries, Inc., Woodland Park, NJ) as previously described.[1] In brief, a single carbon fiber was inserted into a glass capillary tube (A-M Systems, Sequim, WA), sealed with a micropipette puller (Narishige, Tokyo, Japan), and cut to 100 μm in length. A Ag/AgCl pellet reference electrode was employed (World Precision Instruments, Inc., Sarasota, FL) to complete the two electrode electrochemical cell.

Flow-Injection System

The working electrode was positioned in a custom electrochemical cell using a micromanipulator (World Precision Instruments, Sarasota, FL). A syringe pump (New Era Pump Systems, Wantagh, NY) supplied a continuous buffer flow of 1 $mL \cdot min^{-1}$ 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 air actuator controlled by a digital pneumatic solenoid valve (Valco Instruments, Houston TX). The entire apparatus was housed within a custom-built grounded Faraday cage. All experiments were performed at room temperature.

Data Acquisition

All in vitro data was collected in a custom flow-injection system. Unless otherwise noted, a triangular waveform ranging from −0.4 to +1.3 V with a holding potential of - 0.4 V versus Ag/AgCl was applied at a scan rate of 400 $V \cdot s^{-1}$ and at a frequency of 10 Hz using a custom built instrument for potential application to the electrochemical cell and for current transduction (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility). When multiple waveforms were used at a single electrode, the wavelimits were sequentially increased. TH-1 software (ESA, Chelmsford, MA) was used for waveform output and data analysis, along with data acquisition cards (6251 and 6711, National Instruments, Austin, TX) used for measuring currents and synchronization of the electrochemical experiment with the flow-injection apparatus. Signal processing (background subtraction and signal averaging) was software controlled. Analog filtering was accomplished with a 2-pole Sallen-Key, low-pass filter at 2 kHz. There was no digital filtering of the data.

Traditional Electrode Calibration

Pre-calibration was performed before the electrode was implanted within brain tissue; post-calibration was performed after it was removed from tissue, rinsed in deionized water, and soaked in isopropyl alcohol overnight. A minimum of three concentrations of analyte within the electrode's linear range were used as follows: dopamine (0.50, 0.75, 1.0 μM), hydrogen peroxide (250, 500, 1000 μM), ascorbic acid (50, 100, 200 μM), and pH shift (+0.1, +0.2, +0.3, relative to pH 7.4). Peak current was plotted versus concentration and the fitted slope was used as a calibration factor for predicting in vivo analyte concentration.

In Vivo Experiments in Anesthetized Rats

Male Sprague-Dawley rats (n = 3, 250 - 300 g, Charles River Laboratories, Raleigh, NC) were urethane anesthetized (4 $g \cdot kg^{-1}$ 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.

In Vivo Experiments in Awake Rat

A male Sprague-Dawley rat with a jugular vein catheter was anesthetized with ketamine hydrochloride (100 mg/kg i.p.) and xylazine hydrochloride (20 mg/kg i.p.), and stereotaxic surgery was performed as described previously.[1] The Ag/AgCl reference electrode was placed in the forebrain, and a guide cannula (Bioanalytical Systems, West Lafayette, IN) was positioned above the contralateral NAc (1.7 mm AP, 0.8 mm ML, −2.5 mm DV relative to bregma). A combination bipolar stimulating electrode/steel guide cannula (26 gauge; Plastics One, Roanoke, VA) was implanted unilaterally into the VTA at a 6° angle toward the midline to avoid the midline sinus (5.4 mm posterior, 1.2 mm lateral, 7.8 mm ventral relative to bregma). The components were permanently affixed with dental cement. The animal recovered for 3 days.

Measurements were performed as described previously.[1] Briefly, the animal was in an operant chamber containing an infusion pump for drug delivery through the jugular catheter. The electrodes were connected to a head-mounted amplifier, connected through a swivel to the remaining electronics. The rat was acclimated to the chamber for ~1 hr before recording began. A 6 sec infusion of cocaine (3 mg/kg) was administered to elicit dopamine transients. All animals were individually housed on a 12:12 hour light cycle with *ad libitum* access to food and water. All procedures were performed in accordance with the North Carolina State University Animal Care and Use Committee.

Statistics

Data are presented as the mean ± standard deviation. One-way ANOVA with Tukey-Kramer post-test was used to compare significance between multiple groups. Statistical and graphical analyses were carried out using MATLAB R2008a (The MathWorks, Natick, MA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

RESULTS AND DISCUSSION

Electrochemical Properties and Sensitivity

A voltammetric waveform that spans from −0.4 to +1.3 V is most often used for complex measurements in live brain tissue.[1,27] However, these wavelimits are routinely adjusted based on the goals of the experiment. In order to develop and validate a broadly applicable method to eliminate standard electrode calibration procedures, a set of electrochemical descriptors was evaluated for five waveforms spanning commonly-used wavelimits (Figure 1A). All of the applied waveforms utilized a holding potential of −0.4 V and ramped at 400 $V \cdot s^{-1}$ to the switching potential, then returned to −0.4 V at the same scan rate. All waveforms were applied at a frequency of 10 Hz, so collection of a voltammogram containing 1000 data points was completed every 100 msec and took ≤ 9 msec to record.

In FSCV, the high scan rate generates a large capacitive current that is dependent on surface area. This background current is significantly greater than the faradaic currents that result from redox processes at the microelectrode surface. Representative background current

voltammograms are shown in Figure 1B. When a species of interest is present, this largely non-faradaic background current is subtracted from the overall signal to reveal the faradaic response to the electroactive analyte.[1,27] This strategy effectively removes all contributions to the measured current that result from double-layer capacitance and redox processes inherent to the electrode surface. For instance, Figure 1C-F shows representative background-subtracted voltammograms for single concentrations of dopamine, ascorbic acid, H_2O_2, and a basic pH shift, collected using each of the waveforms. The characteristic shape of each voltammogram serves to identify the analyte,[9,28-29] and the current amplitude is used in quantification. The background current is generally not given much attention; however, it contains a significant amount of valuable information. As the switching potential increases, the magnitude of the background current also increases across all potentials (Figure 1B). When the sum of the absolute value of all currents in the background voltammogram is plotted with respect to the switching potential, a non-linear relationship is evident (Figure 2). This graph illustrates that the total background current changes in a predictable manner, enabling it to be used as a reliable electrochemical descriptor. Additionally, Figure 1C-F reveals that the amplitude of the faradaic current also increases as the switching potential of the applied waveform increases. This is likely a factor of surface chemistry, as previous research has shown that increasing the anodic limit of the applied waveform generates more oxygen-containing functional groups on the graphitic-carbon surface, effectively increasing electrode sensitivity.[19,29]

Traditional calibration is performed by exposing known concentrations of analyte in a physiological buffer to the electrochemical sensor, and extracting peak current from the background-subtracted voltammograms.[1,27] This current is then plotted versus analyte concentration and the slope of a fitted regression line determines electrode sensitivity to the chosen analyte. This value can then be used to predict analyte concentration from voltammograms of unknown analyte concentration, collected in vivo. Figure 3 presents linear calibration data collected using all five waveforms for dopamine, ascorbic acid, H_2O_2, and basic pH shifts. It should be noted that for most analytes there were only slight differences in electrode sensitivity between the +1.0V and +1.1V wavelimits, but as the wavelimit was further increased there were marked increases in sensitivity. As such, plotting the traditionally calculated sensitivity versus the switching potential of the applied waveform resulted in a nonlinear relationship (Figure 4), reminiscent of data shown in Figure 2.

Since both sensitivity and total background current increased as the range of the applied waveform was increased, electrode sensitivity was directly plotted versus total background current. The data shown in Figure 5 were collected at ten electrodes using five different waveforms, resulting in 50 individual data points, and a linear relationship was evident for all analytes. Thus, a model can be developed that uses the total background current as a reliable descriptor of electrode sensitivity.

A Novel Approach to Calculating Electrode Sensitivity

It is known that background charging current is in part a function of electrode capacitance, which is dependent on surface area.[17] We have shown that electrode sensitivity increases in a reproducible and predictable manner as the wavelimit is increased (Figure 4). Since faradaic current is dependent on electrode surface area and analyte concentration,[17] we sought to use the background charging current collected during a typical experiment to define the sensitivity of the electrode to a particular analyte. Data collected at a set of 10 electrodes were fit to the following model:

$$\text{Sensitivity} = \alpha C + \beta S + \gamma S^2 + \delta \quad (1)$$

$$\Sigma_i^{\text{electrode}}\left(\left(\alpha C_i+\beta S_i+\gamma S_i^2+\delta_i\right)-\text{Sensitivity}_i\right)^2=0 \quad (2)$$

where electrode sensitivity is predicted for a given analyte as a function of both total background current (C) and the switching potential (S). Multiple linear regression was used to solve for electrode sensitivity, by determining the coefficients α, β, γ, and δ, minimized with Equation 2. These values are presented in Table 1. The predicted sensitivity to all four analytes was then plotted versus the sensitivity determined by the traditional in vitro calibration method (Figure 6). The slope of the regression line is reported within each panel, where a slope of 1.0 demonstrates a perfect match between our model and the traditional method of electrode calibration. None of the slopes deviates significantly from unity, demonstrating the power of the model to predict electrode sensitivity utilizing information contained in the background current. Importantly, there are wide variations in electrode sensitivity for all analytes investigated. This clearly demonstrates the critical need for a reliable calibration method, and reinforces the importance of exercising caution when assuming an average electrode sensitivity to quantify analyte concentration in live tissue. It is also important to note that many factors underlie the generation of the background current, including scan rate, the differential capacitance of the double layer at the electrode surface, and solution resistance.[17] Thus each laboratory should generate a unique set of regression coefficients using their own instrumentation, electrodes, and experimental protocols.

Validating the Model

To evaluate the utility of this model, a set of predicted calibration factors was directly compared to values obtained using a traditional ex vivo calibration completed before (pre-calibration) and after (post-calibration) implantation in brain tissue. Calibrations were carried out using dopamine concentrations ranging from 250 to 1000 nM, and a cyclic waveform that ranged from −0.4 V to +1.3 V at a scan rate of 400 $V \cdot s^{-1}$. Electrodes were pre-calibrated in a flow-injection system and then immediately lowered into the dorsal striatum, a brain region rich in dopaminergic terminals. After the electrode stabilized, the characteristics of the background current collected in vivo were used with Equation 1 and the coefficients of α, β, γ, and δ determined for electrodes fabricated in our lab (Table 1) to predict electrode sensitivity to dopamine. The electrode was then removed from the animal, rinsed and stored in isopropyl alcohol overnight, following traditional protocol.[1,27] The following day, post-calibration of the electrode was performed in a manner identical to the pre-calibration procedure. Figure 7 shows that the pre-calibration sensitivity (13.74 ± 1.07 $nA \cdot \mu M^{-1}$) was significantly greater ($p = 0.011$) than either post-calibration sensitivity (9.66 ± 1.29 $nA \cdot \mu M^{-1}$) or that predicted using our model (9.25 ± 0.77 $nA \cdot \mu M^{-1}$). Consistent with previous findings, electrode sensitivity decreases after tissue exposure.[21] More importantly, there was no significant difference between the sensitivity calculated using the traditional ex vivo post-calibration method and the model presented herein, verifying the efficacy of this approach.

A direct comparison of the mathematical approach to estimating electrode sensitivity and a traditional calibration is presented in Figure 8. Dopamine transients collected in the ventral striatum of an awake rat were elicited by cocaine administration (3.0 mg/kg, i.v.). The current was extracted at the peak oxidation potential for dopamine (Figure 8A, white dashed line) and divided by electrode sensitivity to generate a concentration profile (Figure 8B). Electrode sensitivity to dopamine determined by in situ calibration was 7.97 $nA \cdot \mu M^{-1}$ (red line), and using traditional methodology was 9.00 $nA \cdot \mu M^{-1}$ (black line). The disparity between the resulting concentration traces is less than 10% of the overall signal (plotted in Figure 8C). The sign of the differential indicates whether the in situ calibration predicted a dopamine concentration that was greater than (positive value) or less than (negative value)

that predicted by standard procedures. These data explicitly demonstrate the practicality of this novel method to quantify in vivo electrochemical data collected using FSCV.

CONCLUSIONS

Precise quantification of neurotransmitter concentration is necessary in order to accurately interpret the molecular mechanisms underlying any aspect of brain function. The concentration of an analyte in the extracellular space determines which receptor subtypes are activated, and thus functionally alters the postsynaptic response. We describe a model that uses the information encoded within the background current to accurately predict electrode sensitivity, eliminating the need for traditional post-calibration procedures. Importantly, this model is not simply a means to obtain a generalized calibration factor after an experiment. Using a single calibration factor assumes an average sensitivity over multiple electrodes and across the course of an experiment. However, individual electrodes are variable and experiments may last several hours, or even several days or weeks. Electrode sensitivity can slowly improve over time with application of the electrochemical waveform (electrochemical conditioning). Alternately, an electrode may lose sensitivity during the course of an in vivo experiment (fouling). The true power of this model lies in the fact that sensitivity to a given analyte can be assessed at any point during an experiment using a set of regression coefficients that accurately describes the background charging current generated at the electrode surface. This tool will be particularly valuable in cases where calibration is difficult, such as when lesioning is required to histologically verify the recording site, in human studies, and in research that implements chronically-implanted electrodes that cannot be removed. This method has the potential to simplify electrochemical data collection protocols for in vivo applications, and to facilitate more widespread utilization of FSCV by allowing researchers more freedom in experimental design.

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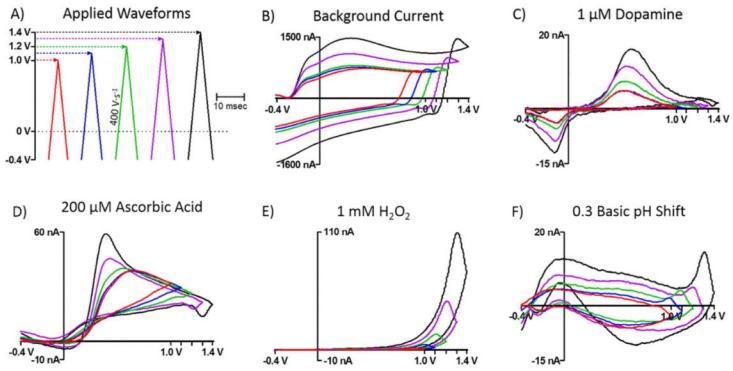

Figure 1.

Background-subtracted fast-scan cyclic voltammetry. (A) Five different waveforms were applied to each electrode. (B) Representative background voltammograms resulting from waveform application to a carbon-fiber microelectrode. (C-F) Representative background-subtracted voltammograms, collected at multiple waveforms for (C) 1 µM dopamine, (D) 200 µM ascorbic acid, (E) 1 mM H_2O_2, and (F) a basic pH shift to 7.7.

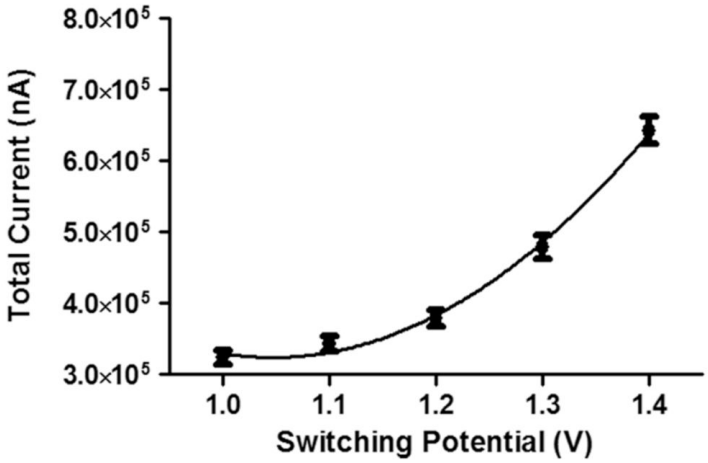

Figure 2.

The magnitude of the background current increases as the switching potential of the applied waveform is increased. Total current is determined by summation of the absolute value of the 1000 data points comprising the voltammogram. It exhibits a non-linear relationship with the switching potential (n = 10 electrodes).

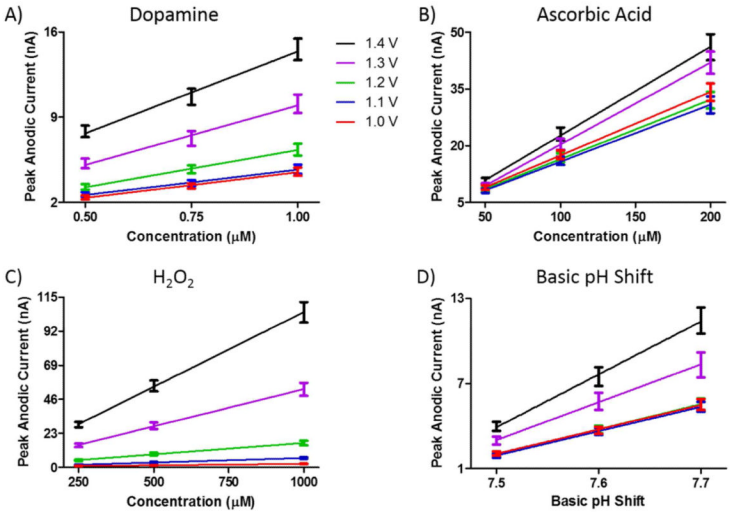

Figure 3.

Traditional calibration curves determining electrode sensitivity. Peak current was plotted versus analyte concentration, for all applied waveforms. A linear relationship was found for (A) dopamine, (B) ascorbic acid, (C) H_2O_2, and (D) basic pH shifts, where the slope of the fitted line represents electrode sensitivity (n = 10 electrodes).

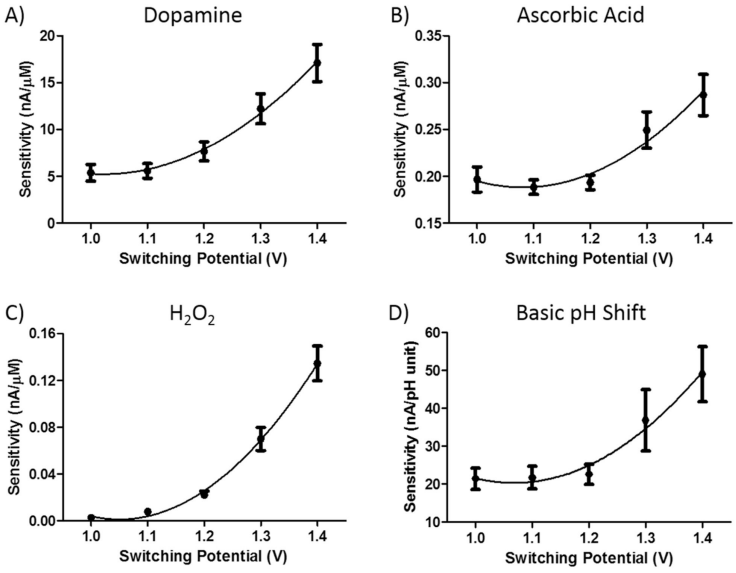

Figure 4.

Extended wavelimits increase electrode sensitivity. Sensitivity was determined using a traditional in vitro calibration method. It exhibited a non-linear relationship when plotted versus the switching potential used in detecting (A) dopamine, (B) ascorbic acid, (C) H_2O_2, and (D) basic pH shifts. (n = 10 electrodes)

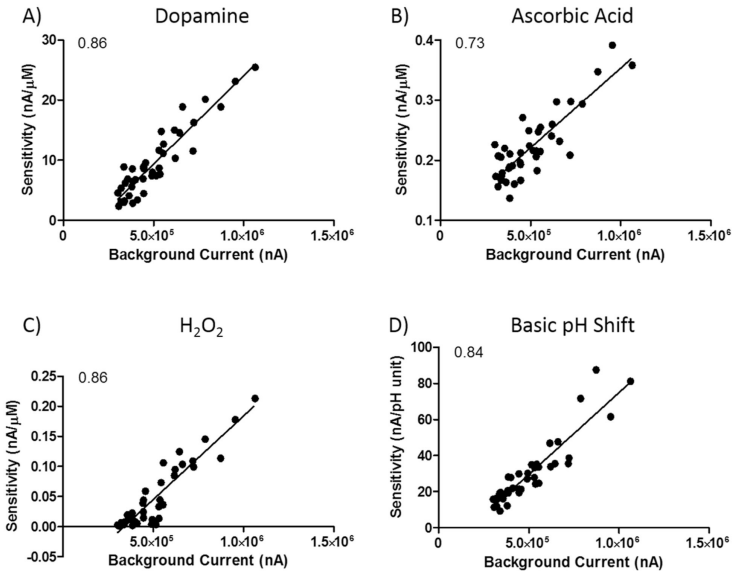

Figure 5.

The background current is a reliable descriptor of electrode sensitivity. Sensitivity was determined by a traditional in vitro calibration method and it exhibited a linear relationship with background current in the detection of (A) dopamine, (B) ascorbic acid, (C) H_2O_2, and (D) basic pH shifts. Correlation coefficients for each fitted line are inset within the respective panel (n = 10 electrodes).

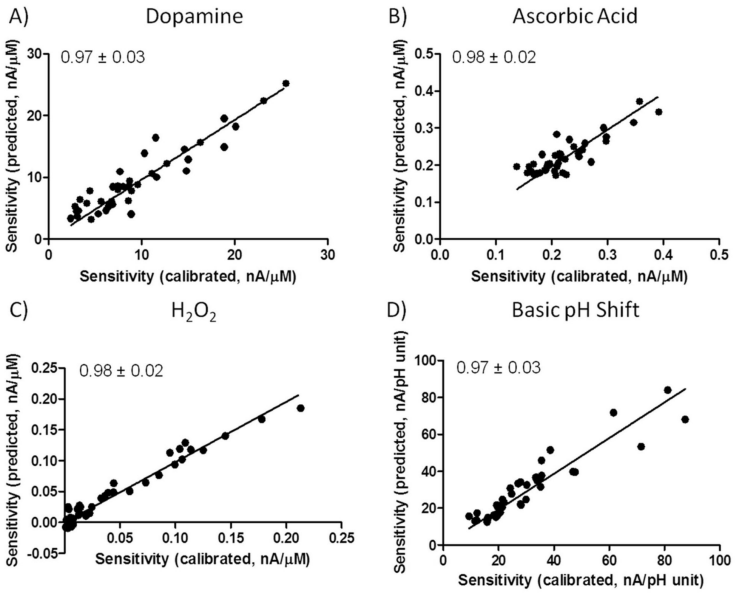

Figure 6.

Characteristics of the background charging current accurately predict electrode sensitivity. Predicted sensitivity was calculated and compared to values for sensitivity determined using the traditional calibration method. Fitting a line through the data points gave a slope at unity for (A) dopamine, (B) ascorbic acid, (C) H_2O_2, and (D) basic pH shifts, demonstrating the validity of the model. Actual slopes for each fitted line are inset within the respective panel (n = 10 electrodes).

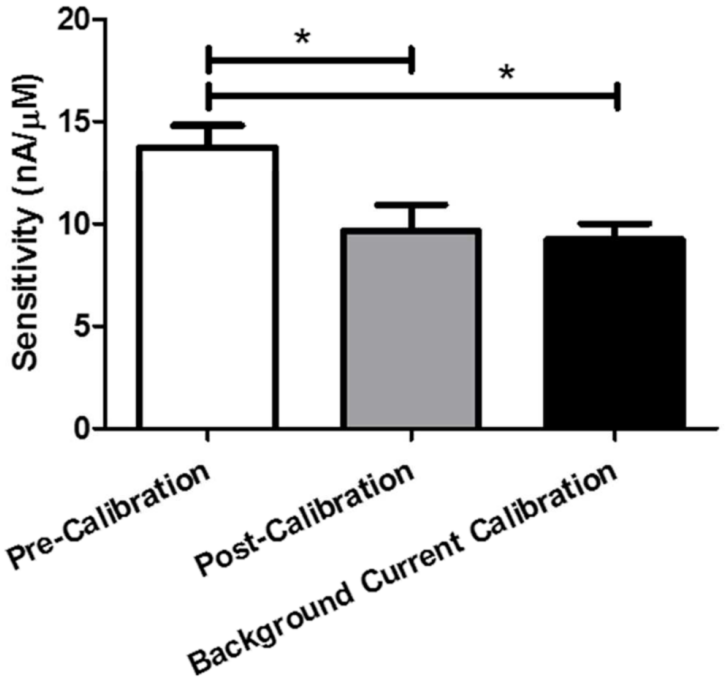

Figure 7.

Comparison of electrode calibration methods. Sensitivity was determined by the traditional calibration method before implantation (pre-calibration), after implantation (post-calibration), and using background voltammograms collecting in tissue along with Equation 1 (background current calibration). There was no significant difference between the sensitivity calculated using the traditional ex vivo post-calibration method and the model presented herein, verifying the efficacy of this approach. (*, $p = 0.011$, one-way ANOVA, Tukey-Kramer test, $n = 8$ electrodes).

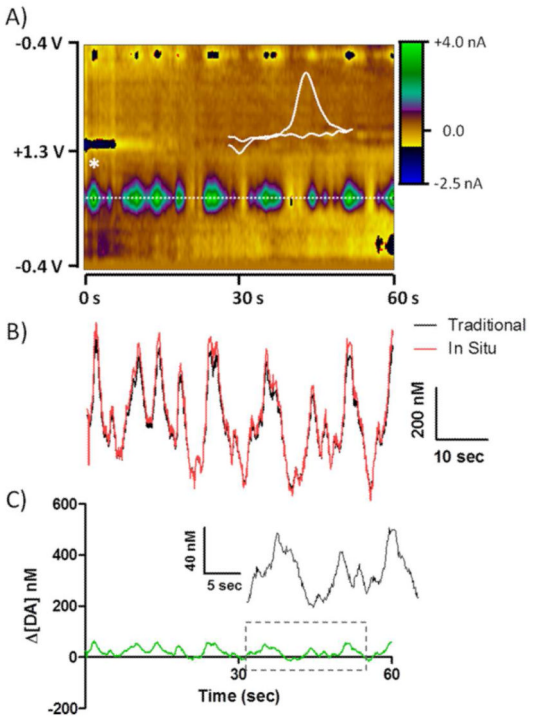

Figure 8.

Direct comparison of the mathematical approach to estimating electrode sensitivity and a traditional calibration. (A) Representative voltammetric data depicting dopamine transients collected in the rat ventral striatum upon i.v. cocaine administration. The color plot contains 600 background-subtracted voltammograms. The ordinate is potential applied to the carbon-fiber electrode, the abscissa is time, and the current is depicted in false color. A dashed line marks the peak oxidation potential for dopamine, and a voltammogram corresponding to the starred event is shown as an inset. (B) Concentration profiles extracted from the data using either traditional calibration (black line) or the in situ mathematical approach (red line). (C) Differential between the concentration traces plotted in (B), (in situ - traditional). A segment of these data (boxed) is enlarged and presented as an inset.

Table 1

Coefficients determined by multiple linear regression on Equation 1 for the training set (n = 10 electrodes).

Coefficients	Dopamine	Ascorbic Acid	H_2O_2	pH
α	2.55×10^{-5}	2.55×10^{-7}	1.62×10^{-7}	1.10×10^{-4}
β	−12.2	−0.589	−1.26	128.0
γ	7.79	0.248	0.598	−66.7
δ	−0.083	0.438	0.604	−82.5