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Correspondence/Rebuttal

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Response to Comment on "Improved Calibration of Voltammetric Sensors for Studying Pharmacological Effects on Dopamine Transporter Kinetics in Vivo"

Fast-scan cyclic voltammetry is a powerful tool for the in vivo study of electroactive neurotransmitters and neuromodulators (e.g., dopamine and serotonin).[1] Prior to 2002,[2] studying release and reuptake of these molecules was mostly limited to examining electrically evoked signals because the methodology did not have the capability to measure rapid endogenous concentration changes. Electrical stimulations result in large changes in dopamine concentration (often in excess of 1 μM). Naturally occurring transient dopamine events are much smaller in amplitude (~50 nM) and reflect endogenous processes.[3,4] Common methods that were in use ca. 2002 had limits-of-detection of ~50 nM, limiting practical quantification to ~200 nM. To overcome this limitation, overoxidation of carbon-fiber microelectrodes is used to enhance dopamine adsorption and thus increases sensitivity.[5] Overoxidarion lowers the limits-of-detection to less than 5 nM, allowing endogenous signals to be measured.[3,4]

The increased sensitivity is caused by adsorption to the carbon-fiber surface.[3,6,7] The amplitude of the signal is larger than that expected for a purely diffusion-controlled process, because dopamine accumulates on the electrode between voltammetric scans. This preconcentration lowers detection limits; however, it slows the response time of the electrode because of the increased time to reach steady-state.[8] Slower temporal responses have been previously reported with electrochemically pretreated carbon electrodes.[9] When making measurements with sensitive probes using fast-scan cyclic voltammetry, the required time for sensors to reach equilibrium is often longer than the duration of neurotransmitter release and clearance. Phasic dopamine release occurs on the millisecond time scale and is cleared in normal conditions in less than one second. Due to this short time scale, when using highly sensitive (i.e., overoxidized) probes, there is not enough time for the voltammetric signal measured to reach steady-state. In such a situation, the stronger the analyte adsorption, the greater this systematic error. Electrodes with weak adsorption have minimal error, and this is shown in our original article.[10] Thus, researchers that use "overoxidized electrodes" that are capable of measuring endogenous signals are more susceptible to the challenges introduced by a slower electrode response time caused by adsorptive electrodes. The referenced paper[10] adapts and modifies the recently developed technique of fast scan controlled adsorption voltammetry (FSCAV). The technique is applied to experimental data and a computational model based on a mass-transport limited diffusion. This eliminates the need for flowing solutions in calibration to more closely mimic the endogenous environment in the brain.

RESISTANCE TO MASS TRANSFER

The observation that fast-scan cyclic voltammetry has a slower response time is not a new one. Figure 1 shows two traces obtained in a brain slice using amperometry and fast-scan cyclic voltammetry.[11] Amperometry at bare carbon surfaces does not rely upon adsorption processes, and thus has a fast response time (microseconds), that is limited primarily by electron transfer kinetics. This graph shows that voltammetry introduces an error (the traces are not identical) even though the calibration procedures used were identical, directly contradicting the point made in the rebuttal. Wightman and coworkers used deconvolution to correct for this error prior to fitting kinetic parameters. The most accurate data is directly obtained from experiments that do not introduce errors in the first place; that is, if the goal of the experiment is to measure kinetic parameters, amperometry is the gold standard as presented by Venton et al.[7] However, amperometric measurements are not always possible, nor are they as sensitive; when examining endogenous dopamine signaling, voltammetry provides critical information on the chemical identity of a species.

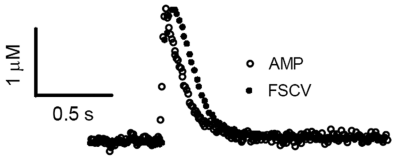

Figure 1. Stimulated dopamine release monitored with fast-scan cyclic voltammetry repeated at 60 Hz and amperometry. Both measurements were in the presence of 500 μM ascorbate. A time delay due to adsorption can be seen in the fast-scan cyclic voltammetric response indicated by a longer time to reach a maximum and a slower decay to baseline. The adsorptive delay with cyclic voltammetry was accounted for using the convolute-and-compare method to obtain rate constants. Reprinted with permission from ref 11. Copyrignt 2012 American Chemical Society.

Because adsorption is used to preconcentrate dopamine, the electrode effectively removes some dopamine from the extracellular space. When dopamine is adsorbed to the electrode surface, the adsorbed dopamine cannot interact with receptors, reuptake transporters, or other proteins. As a sample back-of-the envelope calculation, a typical 6 μm diameter carbon fiber with a 50 μm length has a geometric area of approximately 10^{-5} cm^2. An adsorption strength ("b" in ref 10) of 2×10^{-3} cm (values for dopamine in FSCV papers range from 5×10^{-4} to 2×10^{-2} cm) would mean that if the concentration in solution is 1 μM, the surface coverage on the electrode would be given by, $\Gamma_{DA} = b[DA] = 2$ pmol/cm^2. For the electrode area above, that is 2×10^{-17} moles of material, or approximately 12 million molecules. If each small synaptic vesicle contains 10^4 molecules,[12] this corresponds to 1200 dopaminergic vesicles (released into the extracellular space near the electrode) that are on the electrode surface, where they cannot interact with receptors and transporters. The volume

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near the electrode (extending out six radii, 50 μm length, with an extracellular volume fraction of 0.2) contains about 6×10^6 molecules. This means that, under equilibrium conditions, the equivalent of approximately twice the number of dopamine molecules in the diffusion layer of the electrode is removed from the solution and put on the electrode surface for us to measure. Clearly, this can be a significant effect. The stronger the adsorption (termed "*b*" in[10]), the more material must be removed from solution, thus slowing the electrode response time.

This is not a new idea: in 1957, Delahay Trachtenberg[13] said that "adsorption with diffusion control is a slow process." They further explain "The qualitative interpretation is simple: the gradient of concentration of adsorbate decreases continuously as adsorption proceeds, and the rate of diffusion drops accordingly." The studies in question use cylindrical microelectrodes, which reach a quasi-steady-state. But as the dopamine accumulates on the electrode, the resulting concentration gradient is made smaller, slowing the rate of mass transport (i.e., a smaller dC/dx leads to a smaller flux). The closer the system gets to equilibrium, the less the concentration gradient, resulting in a lower flux.

The two factors affecting the response time are the strength of adsorption (how much material is removed from the solution and adsorbed to the electrode) and the rate of mass transport (how fast material can be brought to the electrode). Flow cells are used for calibrating electrodes with fast-scan cyclic voltammetry because they create a step-change in concentration and the technique is a background-subtracted. The use of flow cells is clever as a way to rapidly introduce a change in dopamine concentration at the electrode so that a background-subtracted measurement can be made. However, this introduces convective flow, which is dissimilar from the comparatively quiescent endogenous environments. Figure 2 shows the response time of sensors in still solutions and for solutions with flow present (convection typical in a flow cell calibration) as monitored by fast scan controlled adsorption voltammetry (FSCAV). Note that with both the standard FSCAV method (Figure 2a) and the 10 Hz variation (Figure 2b) the response in the stirred solution is faster (this is identical to the experimental method in Figure 3 of ref 10). Although there is a stationary layer near the electrode, convection increases the rate of mass transport of new material to the volume surrounding the electrode. The 10 Hz variation is not affected as much because it is less adsorptive than FSCAV (smaller *b*). This can be rationalized by the fact that the response of the electrode in the 10 Hz variation is faster because the adsorption strength is smaller, which results in less material accumulating on the electrode surface. Yet, the response is even faster in a convective environment. The letter writer is correct to point out that there are differences between FSCAV and FSCV: as you have less adsorption, you have less material to remove from solution, resulting in a faster response time, which is evident in the original paper and Figure 2 here.

The letter writer states, "If the electrode is immune to the flow, then no calibration error occurs." This would mean that if the electrode is not immune to the flow (i.e., the diffusion layer is larger than the stationary boundary layer), then an error is present, but does not address the time scale involved. The letter states that "Venton and coworkers[14] showed that FSCV electrodes are immune to the flow... ," but this result is being overstated by the letter writer; Venton et al. show that "the change in flow rate does not affect the amount of dopamine

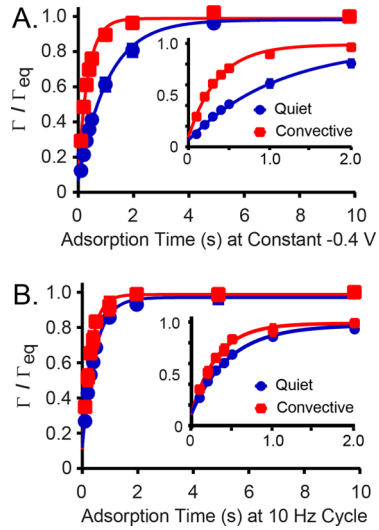

Figure 2. Differences in mass-transport limited adsorption kinetics are observed under different controlled-adsorption conditions. In each experiment, the FSCAV technique was employed to minimize adsorbed dopamine at the electrode prior to the adsorption time. (a) In the standard FSCAV methodology, the working electrode potential is held at a constant −0.4 V during the entire controlled adsorption time. The adsorption rate constant for quiet solution is 0.908 ± 0.015 s^{-1} (R^2 = 0.9931), whereas a convective (stirred) solution exhibits a rate constant of 2.761 ± 0.208 s^{-1} (R^2 = 0.9848). The inset graphic shows the importance of convective mass transport on short time scales (<2.0 s) (b) A modified FSCAV methodology (similar to Figure 3 in ref 10) is used to study the adsorption dynamics under typical FSCV conditions by applying a standard triangle waveform at 10 Hz during the adsorption period. The quiet solution exhibited an association rate constant of 2.146 ± 0.139 s^{-1} (R^2 = 0.9902), and the convective solution exhibited a rate constant of 3.189 ± 0.305 s^{-1} (R^2 = 0.9740). All adsorption data were fit with a first order exponential association.

adsorbed." The steady-state amount present on the electrode surface is not changed by flow, but we demonstrate that the rate at which it is attained is affected by mass transport.[10] They state that the "rate of adsorption is mass-transport-limited"; additionally, the "2.5 μm" figure is calculated specifically for their model over a 90 ms time frame. Voltammetric measurements have different time scales, and most measurements are longer than 90 ms, with typical signals lasting several seconds.

In our work, we perform a simulation to model diffusion in three dimensions. Prior work was limited to using phenomenological parameters to model the data. To investigate the effect of flow on the diffusion later, we performed a Simulation in Comsol Multiphysics (Figure 3, methods in ref 10). Amperometric simulations are presented, where the concentration at the electrode surface is set to zero. This is done to model the extent of diffusion that the electrode draws material from in solution (the electrodes "sphere of influence"). The graph on the left shows the concentration of dopamine after 2 s in a purely diffusive system. On the right, convection is introduced (0.16 cm/s). There is still a stationary layer immediately adjacent to the electrode, but the diffusion later is compacted. This increases the rate of mass transport. As an aside, when flow cell calibrations are used, the linear flow velocity is rarely reported in literature; volumetric flow rates are reported, and without the physical dimensions of the flow cell, it is not possible to know what the linear velocity is.

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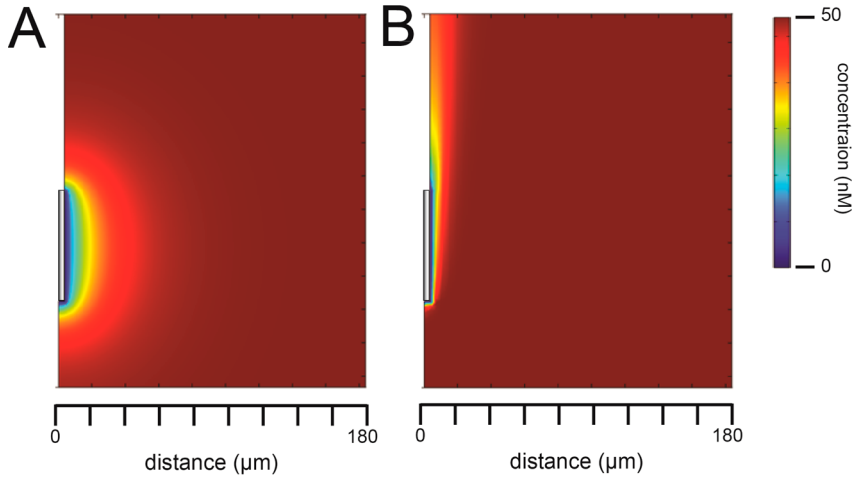

Figure 3. Modeled diffusion layer for cylindrical microelectrodes. Comsol Multiphysics was used; methods in ref 10. The concentration profiles at time = 2.0 s are shown. The electrodes are located on the left, and have a radius of 3 μm. (A) A purely diffusive system with $D = 5 \times 10^{-6}$ cm^2/s. The concentration at the electrode surface is fixed as 0. (B) Convective flow (0.16 cm/s) is introduced to the system, changing the concentration profile. This results in increased flux to the electrode surface.

THE CALIBRATION PROCEDURE

Carbon-fiber microelectrodes have a strength of adsorption and FSCV masks that true value, because once the steady state is reached due to repetitive waveform application, maximum adsorption (equilibrium coverage) cannot occur. FSCAV makes use of this to measure the absolute concentrations of dopamine. In Figure 3 in the original article,[10] we present how the calibration factor is obtained for the work presented. In Figure 3, FSCV was used with 100 ms between scans for a fixed interval (instead of a fixed holding potential) and then the amount adsorbed on the electrode was measured by FSCAV. In effect, we employ a variation of FSCAV where instead of applying a holding potential (which would allow the electrode to reach equilibrium), we apply a 10 Hz waveform. The experiment is outlined in the original article, but briefly we apply a 100 Hz waveform to minimize adsorption, then we apply a waveform at 10 Hz (identical to most FSCV measurements) which allows the system to reach steady-state and then we return to applying the waveform at 100 Hz. This allows us to calibrate with the same waveform used in FSCV measurements; the steady state value for the dopamine adsorbed is not different. The experimental procedure described in Figure 3 in the original work contains a clever attempt to determine the steady-state adsorption constant (b) during FSCV by modifying FSCAV to incorporate an FSCV waveform during the controlled adsorption period. This allows us to determine the strength of adsorption and time response in the absence of flow.

The two assumptions we rely upon for this calibration are

(1) that the steady-state adsorption constant (b) during FSCV scanning does not change between in vivo and in vitro conditions (this is the same assumption that is made for traditional flow-injection calibrations);
(2) the diffusion coefficient of dopamine in vivo is $\sim 2.0 \times 10^{-6}$.

Assumptions 1 and 2 allow us to calculate a time response for the electrode that is appropriate for in vivo calibrations by using the model described in Figure 2 of the original work, and then use deconvolution. The time response is faster than it would be when compared to FSCAV where more adsorption is present. This exponential function (eq 3 in the original paper) invokes two parameters: A_0 and the response time. We define A_0 using a logical argument based upon the assumption that, at equilibrium, the response function condition is satisfied. Thus, our deconvolution function is defined by two terms: b, which is an **equilibrium constant that is determined empirically using our novel modification of FSCAV to incorporate FSCV waveforms**, and the response time which is determined by modeling diffusive mass transport to the electrode (Figure 2 in the original paper).

MODELING REUPTAKE PARAMETERS

We are not the first to use convolution theory prior to modeling voltammetric data, and this mathematical technique has been an important part of the development of in vivo electrochemical methods across four decades.[14−16] Convolution theory has been commonly used to remove the response due to a film of Nafion being used on the electrode. With adsorptive electrodes, a similar phenomenon occurs. The letter writer correctly points out that the model by Wu et al. does not produce negative excursions.[17] Indeed, no negative excursions are illustrated in our original paper. The figure in question has no "y" axis, but simply a scale bar. This is because fast-scan cyclic voltammetry involves a background-subtraction to measure concentration changes; the "zero point" is not directly known. To model this data, the baseline was assigned to be "0" and the falling part of the curve was modeled until it reached the baseline ("0") value again. The traces are not shown for simplicity, but the fit parameters are directly in the figure in a bar graph; they can be used to reproduce the fit of the data. The fits are not shown as the authors chose instead to show the standard error of the mean on the original and deconvolved data. This is present so that the propagated error in the process can be directly seen on the raw data. The "superior fit" was validated in that before the convolution procedure, a well-known reuptake inhibitor did not change K_M as predicted (the changes were masked by the adsorption process at the electrode), whereas after the deconvolution procedure K_M increased as predicted. The fitting procedure used here does not involve arbitrary parameters, but rather empirically

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determined constants or variables, which have been described in detail in the biochemical literature.

THE NEED FOR NEW CALIBRATION METHODS

Determining the concentration of neurotransmitters in vivo is critical for understanding the brain. In an ideal experimental setup, researchers would perform an in vivo calibration, but despite continued improvements in calibration strategy,[18] a purely in vivo calibration has not yet been realized. The letter writer asserts that "Validation of the deconvolution result is not possible," and also that "calibration in a flow cell does not cause any error." However, neither calibration method cannot be presently validated in vivo, meaning that the assertion that "calibration in a flow cell does not cause any error" cannot be validated. If the time scale of the measurement is on the same time scale of the response time of the sensor, it follows that an error is introduced. Our paper directly addresses this logical argument and models the result. This model presents a hypothesis: one that can be tested once we can perform purely in vivo calibrations. Additionally, the statement that "calibration in a flow cell does not cause any error" does not ring true in the context of FSCV-related literature: issues with an in vitro calibration continue to persist. Using buffers containing calcium and magnesium are important when calibrating electrodes; the presence of calcium and magnesium in the buffer solution caused a 2−3-fold decrease in signal.[19] This is presumably due to changes in the adsorption of dopamine at the electrode surface when these ions are present. Differences in calibrations performed before an electrode is implanted in vivo ("precalibration") and calibrations performed after an electrode is implanted in vivo ("postcalibration") are common.[20,21] Roberts et al. introduces a novel calibration method based on the background to quantify changes in sensitivity at an electrode and uses it to quantify signals. Also, for similar electrodes and scan parameters, calibration factors ranging from ∼10 to ∼60 nA/μM are reported.[18,22] Indeed, the above papers confirm that researchers using fast-scan cyclic voltammetry are concerned with quantification and recognize calibration must be carefully considered to convert in vivo signals to concentrations. This may result in a more robust way of giving a value to the amount of dopamine release in response to various stimuli. Progress toward improved calibrations does not invalidate previous experiments; in fact, it will only increase their significance as in vivo electrochemical measurement becomes a mature technique in which many valuable options in design of experiments, choice of calibration method, and data analysis exist for the experimentalist probing the complexity of the brain.

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Notes

The author declares no competing financial interest.

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