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

In vivo fast scan cyclic voltammetry (FSCV) has been used extensively since its introduction in the 1980s to measure extracellular dopamine (DA) in the brain. A recent study[1] claims that the ubiquitous practice of calibrating carbon fiber FSCV microelectrodes in a flow cell leads to a substantial calibration error. Reference 1 also reports an alternate calibration procedure, called kinetic calibration, which is based on deconvolution. The deconvolution dramatically alters both the amplitude and temporal profile of electrically evoked DA responses measured in vivo. Thus, ref 1 claims that the calibration error has caused inaccurate analyses of DA release and clearance, hidden from view a negative excursion of DA concentrations just after the stimulus, and prevented the detection of certain spontaneous DA transients.

Reference 1 suggests that that all prior FSCV conclusions must be questioned and that all future FSCV studies should avoid the flow cell calibration error by adopting instead kinetic calibration.

On the other hand, ample counter evidence lends confidence that calibration in a flow cell does not cause any error, that alternative explanations exist for the calibration results reported in ref 1, and that kinetic calibration produces deconvolution artifacts. The goal of this commentary is to assist in clarifying these potentially confusing issues.

THE PITFALLS OF CONVOLUTION AND DECONVOLUTION

The kinetic calibration employs deconvolution. It is appropriate, therefore, to offer some discussion of convolution and deconvolution and some potential pitfalls that accompany their use. In vivo responses as measured by FSCV can be described as a convolution of DA's intrinsic extracellular concentration and an instrument transfer function, eq 1 of ref 1:

$$g(t)*h(t) = S(t) \quad (1)$$

where $g(t)$ is the transfer function, $h(t)$ is the intrinsic function, $S(t)$ is the measured function, and the asterisk means convolution.

Equation 1 serves as the basis for two procedures for determining the intrinsic DA function. The first uses a kinetic model to calculate trial intrinsic functions, which are convoluted and compared to the measured function. The process is repeated iteratively until agreement with the measured function is obtained. The trial intrinsic function producing the best possible fit is the estimate for the actual intrinsic function. This convolution procedure is employed in ref 1 for the kinetic analysis of DA clearance kinetics.

The second procedure is deconvolution of the measured signal and the transfer function. This single-step procedure is simpler, but it amplifies noise and suffers from so-called deconvolution artifacts, as explained below. This deconvolution procedure is employed in ref 1 for kinetic calibration.

It is important to emphasize that FSCV provides no direct measure of the actual in vivo intrinsic function. This creates two pitfalls. First, it is impossible to verify the accuracy of the convolution procedure. Second, it is impossible to verify the accuracy of the deconvolution procedure.

KINETIC CALIBRATION: AN ATTEMPT TO MEASURE A TRANSFER FUNCTION

Reference 1 can be viewed as an attempt to determine the transfer function for the temporal effects of DA adsorption to FSCV electrodes. Conceptually, this is very appealing: direct knowledge of both the measured FSCV signal and the transfer function would enhance confidence in the validity of the estimated intrinsic function.

THE EFFECTS OF DA ADSORPTION: THE GOOD AND THE BAD

The issues at hand revolve around the mechanism underlying the electrochemical detection of DA with carbon fiber FSCV electrodes. DA molecules adsorb to these electrodes.[2,3] So, the measured electrochemical current arises from the oxidation of DA diffusing to the electrode and from the oxidation of DA adsorbed on to its surface.

DA adsorption carries with it two key benefits. First, it promotes the sensitivity of the DA measurements because oxidation of adsorbed DA is an additional source of electrochemical current over and above that from diffusion alone. Second, it promotes the selectivity of the DA measurements because other electroactive substances in the brain, acid metabolites of the biogenic amine neurotransmitters, ascorbate, urate, and so forth, do not adsorb to the electrode (additional factors contribute to DA selectivity but are beyond the scope of this discussion).

The benefits of DA adsorption come at a price: since adsorption takes time, it slows the electrode's temporal response.

The use of a flow cell apparatus for FSCV calibration has become ubiquitous because it provides investigators with a convenient way to quantify the sensitivity, selectivity, and temporal response of their electrodes.

THE PERCEIVED CALIBRATION ERROR

Reference 1 claims that the flow cell causes calibration error due to convection forces that accelerate the electrode's response. This is claimed to cause an error because such convective forces do not exist in stationary solutions, including brain extracellular fluid. Reference 1 states: "Flow-injection analysis for FSCV introduces convective flow, which causes electrodes to reach a steady state within one second."

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However, the claim that convective forces should accelerate the response violates basic principles of hydrodynamic electrochemistry[4,5] and the fundamentals of electrochemical detection with microelectroes.[5,6] Flowing solutions form stationary boundary layers at surfaces, including electrode surfaces. Microelectrodes form very small, quasi-steady state diffusion layers. When the diffusion layer is smaller than the stationary boundary layer, the electrode's behavior is immune to the flow.

Venton and co-workers[3] showed that the FSCV electrodes are immune to the flow for the reasons just explained. Those authors state "The diffusion layer extends only 2.5 μm from the electrode surface, and since it remains very close to the electrode, it is undisturbed by the flow."[3] If the electrode is immune to the flow, then no calibration error occurs.

■ ADSORPTION EQUILIBRIUM VS STEADY-STATE

The introduction of ref 1 states that "to accurately determine the concentration of analyte in the brain, the electrode surface must be at equilibrium with its surrounding environment." This statement must be closely examined.

Prior studies[2,3] clearly establish that DA absorbs to the electrode during the interval between each FSCV scan when the electrode potential is too low to cause oxidation. During each FSCV scan, however, the electrode potential is made positive: this causes oxidation of DA to its quinone, which rapidly desorbs from the electrode. So, the balance between adsorption of DA and desorption of quinone determines the net amount of adsorbed DA. During FSCV, therefore, the steady state amount of adsorbed DA is less than the equilibrium amount due to the repetitive desorption of quinone.

What this means in practice is that the DA sensitivity is a function of the FSCV protocol (scan rate, potential limits, scan frequency, etc.). For example, increasing the time between the FSCV scans increases sensitivity because there is more time for DA adsorption and less time for quinone desorption. It is imperative, therefore, that calibration and measurements follow identical FSCV protocols: otherwise, one is comparing apples to oranges.

As long as the calibration and the measurement adhere to identical protocols, accurate determination of the analyte concentration does not require the electrode surface to be at equilibrium with its surroundings.

■ FSCV VERSUS FSCAV: APPLES VERSUS ORANGES

Figure 1 of ref 1 is presented as evidence that the flow cell accelerates the speed of the response. The figure compares electrode responses measured in a flow cell by FSCV and in stationary solutions by fast scan controlled adsorption voltammetry (FSCAV[1,7]). FSCV and FSCAV follow different electrochemical protocols, so comparing their results is a case of comparing apples to oranges.

As explained above, during FSCV, the steady state amount of absorbed DA will be less than the full equilibrium amount. For this reason, the time needed to reach steady state is less than the time needed to reach full equilibrium coverage. FSCAV requires full equilibrium coverage, which takes longer to achieve. The response time difference reported in ref 1, therefore, is not due to convective forces but rather the use of FSCAV.

The FSCV response in Figure 1 of ref 1 is plotted incorrectly because the FSCV prevents the electrode from reaching full equilibrium coverage (so Γ/Γ_{eq} can not rise up to 1). Had the response been plotted correctly, it would be readily apparent that the response of FSCV is faster because the FSCV does not wait for full equilibrium coverage.

Figure 1 of ref 1 includes an in vitro FSCAV response measured under hindered diffusion conditions. The relevance of this very slow response to in vivo DA measurements is not established. In vivo FSCV is used routinely to monitor evoked DA release during stimulus trains as short as 200 ms.[8,9] In the case of fast sites in the rat dorsal striatum, when the 200 ms stimulus ends, the FSCV response begins its descent on the first FSCV measurement taken after the stimulus ends. So, there is ample counter evidence to the claim that FSCV overestimates the duration of release events.

Figure 1 of ref 1 includes an in vivo response that exhibits a pronounced initial lag at the onset of the stimulus. The implication is that these features arise from distortion caused by adsorption. But the in vivo response in Figure 1 is a slow-type DA response. The temporal characteristics of such responses are highly sensitive to raclopride, a D2 autoreceptor antagonist, to quinpirole, a D2 autoreceptor agonist, and to nomifensine, a DA uptake inhibitor.[9,10] The suggestion that the dynamics of the in vivo response are determined by the mass transport control of adsorption does not account for any of these published observations.

Figure 1 of ref 1 also plots the in vivo FSCV responses incorrectly: no justification is provided for reporting the in vivo response as Γ/Γ_{eq}.

It was mentioned above that the different response times of FSCV and FSCAV are due to the difference in electrochemical protocol. Figure 3 of ref 1 shows this to be the case. Figure 3 compares two FSCAV responses, one obtained with the electrode potential held steady at −0.4 V during the controlled adsorption interval and one obtained while scanning the potential FSCV-style during the adsorption interval. With the FSCV-style waveform, the response comes to its lower steady state faster. This shows that FSCV is faster than FSCAV even in stationary solution: the difference in response time has absolutely nothing to do with forced convection.

■ THE KINETIC CALIBRATION ERROR

The kinetic calibration procedure reported in ref 1 applies the response time measured by FSCAV to DA responses measured by FSCV: this is an error. Reference 1 states that "From this novel use of FSCAV, τ for the average Nafion-coated T-650 was determined to be 1.5 ± 0.1 s (±SEM, n = 3 electrodes)." The τ value appears in the argument of the exponential transfer function (eq 2 of ref 1): estimating that it takes about 3τ for the exponential to decay, this imparts a 4.5 s response time to the electrode, which is the time required for the "−0.4 V" FSCAV data in Figure 3B to reach equilibrium. But, that is more than 4-fold longer than the time needed by FSCV ("Flow-injection analysis for FSCV introduces convective flow, which causes electrodes to reach a steady state within one second."[1])

Deconvolution of in vivo responses with the overestimated τ value produces 100% increases in the amplitude of evoked DA transients and negative excursions of the DA concentration after the stimulus (Figures 4 and 5, ref 1). These are artifacts that arise because deconvolution with the overestimated τ value leads to differentiation of the measured response. The negative excursions of the deconvoluted responses arise because the derivative of the measured response is negative. FSCV does not measure the derivative of the DA concentration.

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Reference 1 reports that GBR 12909, a DAT inhibitor, decreases the amplitude of the negative excursion of the deconvoluted responses. Reference 1 correctly points out that this shows the role of the dopamine transporter (DAT) in the negative excursion but misidentifies that role. GBR 12909 decreases the negative excursion because it decreases the negative slope of the descending phase of the response.

Deconvolution artifacts relate to the pitfalls associated with convolution and deconvolution, explained earlier. Validation of the deconvolution result is not possible, so it is hard to know that the result is accurate but just as hard to know that it is inaccurate.

STUDYING PHARMACOLOGICAL EFFECTS OF DOPAMINE TRANSPORTER KINETICS IN VIVO

A focus of many prior FSCV studies has been the kinetic analysis of DA release and clearance. The pairing of FSCV with electrical stimulation procedures has proven especially powerful for kinetic analysis: the exact knowledge of the stimulus frequency and duration renders the evoked responses ideal targets for numerical analysis. One focus of much attention has been the kinetic analysis of uptake inhibitors, given their high significance in the context of substance abuse. Given that fact, ref 1's claim that "Pharmacologically Inhibited Dopamine Reuptake Kinetics Are Observable Only When Calibration by Deconvolution Is Used" ignores a substantial block of published literature. FSCV reports of pharmacologically inhibited DA reuptake kinetics have appeared regularly in the literature, from 1988[11] to the present day.[9] None of those prior reports have used calibration by deconvolution.

Reference 1 claims that kinetic calibration produces a more accurate analysis of DA clearance kinetics: "By separating the response of the electrode from the measured release event using the kinetic calibration, a more accurate determination of DAT kinetics following the previous model described by Wu et al. is performed"[1] (here, the Wu citation is ref 12). Determination of DAT kinetics follows the convolution procedures, explained earlier. However, the model described by Wu et al.[12] is incapable of generating trial intrinsic functions with any negative excursions:[13] this makes it difficult to understand the claim that the kinetic calibration improved the accuracy of the kinetic analysis. Such a claim requires evidence that the model provides a superior quality fit to the responses obtained by kinetic calibration compared to those obtained by standard calibration. This is easily done: it is usual practice to superimpose the model on the data, to report sums-of-squares of residuals and correlation coefficients, and to evaluate the statistical significance of the correlations. Especially given the title of the paper, the absence of any such documentation of the accuracy of the analysis is a glaring omission.

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Notes

The author declares no competing financial interest.

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