Supporting Information

Construction of Training Sets for Valid Calibration of *In Vivo* Cyclic Voltammetric Data by Principal Component Analysis

Nathan T. Rodeberg,[a] Justin A. Johnson[a], Courtney M. Cameron,[b] Michael P. Saddoris,[d]

Regina M. Carelli,[b,c] R. Mark Wightman[a,c]

[a]Department of Chemistry, [b]Department of Psychology & Neuroscience, [c]Neuroscience Center, University of North Carolina at Chapel Hill, Chapel Hill, Chapel Hill, NC, [d]Department of Psychology and Neuroscience, University of Colorado, Boulder, CO.

This supporting information provides more information on the methods employed in this study, key terms and concepts within PCA-ILS, and supporting data not included in the main manuscript.

Supporting Experimental Methods

Surgery

Rats in the multiple schedule reinforcement and cue discrimination task were surgically implanted with jugular vein catheters [1]. For subjects participating in multiple schedule reinforcement (n=8) and Pavlovian conditioning (n=4), rats were anesthetized with a mixture of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg i.m.) and a guide cannula (Bioanalytical Systems, West Lafayette, IN) for the working electrode was implanted above the nucleus accumbens (NAc) core (+1.3 mm anterior, +1.3 mm lateral, all measurements from bregma). A Ag/AgCl reference electrode was implanted in the contralateral hemisphere. A bipolar stimulating electrode (Plastics One, Roanoke, VA) was positioned above the ventral tegmental area (VTA) (-5.2 mm posterior, +1.0 mm lateral, -7 mm ventral from brain surface). The stimulating electrode was lowered in 0.2 mm increments until electrical stimulation resulted in diminished physical response, suggesting proximity to the desired stimulation site. Stainless steel screws and dental cement were used to secure all items to the skull surface.

Rats for intracranial self-stimulation (ICSS, n=5) underwent similar surgery, with minor differences. They were anesthetized with isoflurane (1.5-4%). The guide cannula (Bioanalytical Systems, West Lafayette, IN) was implanted above the NAc shell (+1.7 mm anterior, +0.8 mm lateral). Another guide cannula was implanted in the contralateral hemisphere for experiment-day implantation of the reference electrode. The bipolar stimulating electrode (Plastics One, Roanoke, VA) was implanted 8.4-8.6 mm ventral from skull surface.

Behavior

Three separate behavioral paradigms were investigated in this study. All training and experiments were conducted in plexiglass operant chambers housed in sound- and noise-attenuated cubicles (Med Associates Inc., St. Albans, VT. USA).

Multiple Schedule Reinforcement

The multiple schedule reinforcement paradigm was described previously [1]. Prior to surgery, rats (n=8) were trained to press a lever for sucrose (45 mg pellet; TestDiet, St. Louis, MO, USA) on a fixed-ratio 1 (FR1) schedule. A cue light above the lever was illuminated with lever extension. Each lever press was followed by the onset of a tone (65 dB, 2900 Hz, 20 s) and a timeout (20 s). Rats were trained until

stable responding of at least 50 presses per behavioral session. Rats were subsequently trained to lever press for cocaine (0.33 mg/infusion, approximately 1 mg/kg/infusion, 6s) at a separate lever; each lever press was followed by a different tone (65 dB, 800 Hz, 20 s) and a timeout (20 s).

Following behavioral training, rats underwent voltammetric surgery. After recovery, rats were retrained for two consecutive days in separate sessions for both sucrose and cocaine responding. Rats subsequently underwent a multiple schedule of reinforcement for sucrose and cocaine, in which rats had access to the lever paired with sucrose (15 min) or cocaine (2 h), followed by a timeout (20 s) and availability of the other reinforcer. The order of the reinforcers was pseudo-randomized across animals to ensure an equal number of subjects (n=4) underwent each reinforcer order.

Pavlovian Conditioning

The second behavioral paradigm involved Pavlovian conditioning for sucrose reward, as described previously [2]. Rats (n=4) underwent extensive training (9 d) to discriminate between a cue that predicted sucrose delivery (CS+), a cue paired with reward omission (CS-), and two separate CS+ presentations without reward presentation (CS+NR). Voltammetric recordings of cue responses were made on the tenth day of training.

ICSS

Following surgery, rats (n=5) were trained in ICSS as previously described [3]. Each training session began with white noise, house and cue lights, and lever extension. Rats were primed with electrical stimulation (24 biphasic pulses, 60 Hz, 75-150 μA) as they approached the lever until the rat acquired ICSS (FR1 schedule continuous reinforcement). Rats underwent two separate training sessions (2 min) on a minimum of three days before voltammetric recordings.

On the recording day, freshly prepared Ag/AgCl reference and carbon-fiber microelectrode were inserted and voltammetric recordings were begun. Subjects were allowed to press a lever continually for electrical stimulation, for two minutes or a minimum of 50 presses. Subject C experienced an electrode break during behavior, preventing full collection of this data set. However, sufficient data was collected in subject C to build a training set for PCA-ILS.

FSCV

Glass-sealed carbon-fiber microelectrodes, 90-110 μm exposed length, were inserted into micromanipulators that was placed in the implanted guide cannula. The microelectrode was lowered to the brain region of interest where robust dopamine release was identified. The microelectrode and reference electrode were connected to a head-mounted amplifier attached to a commutator (Med-Associates, St. Albans, VT) allowing unrestricted movement. Behavioral events (cues, lever extension) were controlled with a MedAssociates system. FSCV data was displayed as two-dimensional color plots with time as the abscissa, the applied potential as the ordinate, and the current in false color.

Data Analysis

Statistical tests were conducted using commercial software (Statistica, Tulsa, OK; GraphPad Software, La Jolla, CA). Significance was tested at $\alpha=0.05$.

Training sets for dopamine and pH were built according to guidelines described previously.[4-7] Training sets consisted of five cyclic voltammograms for both dopamine and pH changes that spanned the amplitudes obtained during behavioral experiments. Normally, the sets were from the same animal with the same electrode and instrumentation as the behavioral data to ensure they included noise typical of each electrode and recording site. The CVs were collected during electrical stimulations that were not part of the behavioral data. K-matrices (see supplementary material) for dopamine and pH were calculated for each set to aid in qualitative analyte identification. Currents were converted to concentrations using external calibration factors (10 nA/μm at the peak oxidation potential for dopamine, -40 nA/pH unit at E_{QH} for pH[8]).

To characterize pH changes during each lever press for sucrose and cocaine, voltammetric data was divided into 20-second snippets surrounding lever presses. pH changes were calculated every 100 ms, and averaged into 500 ms bins during statistical analysis. If animals responded more for one reinforcer than another, data was truncated to provide an equal number of trials for each reinforcer for each subject.

For rats undergoing Pavlovian conditioning, two training sets were built for each subject: one using CVs for dopamine and pH obtained during electrical stimulation, and a second set using CVs from naturally occurring transients. Time blocks (centered ± 5 s surrounding cue onset) were constructed and

peak dopamine concentrations at cue onset or delivery of unexpected sucrose were obtained from local maxima in the dopamine concentration versus time traces in each time block taken 0-3 s following cue onset. The time point of each transient was recorded to ensure both training sets were analyzing the same event. Dopamine transients that fell below the limit of detection (3*RMS) during analysis with the electrical stimulation training set were excluded from data analysis.

For ICSS, data were analyzed in 10-s blocks (± 5 s around each lever press) and the peak concentrations were recorded. Each snippet was digitally background subtracted at local minima in the current versus time trace at the peak oxidation potential for dopamine, usually two to three seconds before each lever press. In cases where several presses were in rapid succession, the same local minima were used for the adjacent dopamine transients. Composite training sets were constructed using a locally written program using LabVIEW (National Instruments, Austin, TX). CVs for both dopamine and pH were selected at random from each training sets A-E. Due to the large number of possible composite training sets (5^{10}), the number of training sets was limited to 10,000. This process was repeated with larger training set sizes (n=2, 3, and 4 CV standards from each training set for both DA and pH). Resulting K-matrices and Q_{α} values were recorded and averaged for each training set size.

(1) Cameron, C. M.; Wightman, R. M.; Carelli, R. M. *Neuropharmacology* **2014**, *86*, 319.
(2) Saddoris, M. P.; Carelli, R. M. *Biol Psychiatry* **2014**, *75*, 156.
(3) Garris, P. A.; Kilpatrick, M.; Bunin, M. A.; Michael, D.; Walker, Q. D.; Wightman, R. M. *Nature* **1999**, *398*, 67.
(4) Heien, M. L. A. V.; Johnson, M. A.; Wightman, R. M. *Analytical Chemistry* **2004**, *76*, 5697.
(5) Keithley, R. B.; Heien, M. L.; Wightman, R. M. *Trends Analyt Chem* **2009**, *28*, 1127.
(6) Keithley, R. B.; Carelli, R. M.; Wightman, R. M. *Anal Chem* **2010**, *82*, 5541.
(7) Keithley, R. B.; Wightman, R. M. *ACS Chem Neurosci* **2011**, *2*, 514.
(8) Takmakov, P.; Zachek, M. K.; Keithley, R. B.; Bucher, E. S.; McCarty, G. S.; Wightman, R. M. *Anal Chem* **2010**, *82*, 9892.

Glossary of Terms in PCA-ILS. Important terminology in principal component analysis (PCA-ILS) with fast-scan cyclic voltammetry (FSCV)

- **Training Set** – Training sets are collections of cyclic voltammograms (CVs) collected from experimental data to build a calibration model for multiple analytes of interest. Training sets consist of multiple analytes, with experimental CVs spanning current ranges similar to those to be predicted in the unknown data. Ideally, the standards closely resemble the analyte of interest, and avoid sources of other systematic current (ex. pH contributions in a DA CV, large glitches, etc.) However, selected CVs should be representative of stochastic noise present during that particular FSCV recording session, or the model will have a poor tolerance for noise in analyzed data.
- **Principal components (PCs) –** For each training set, the total number of PCs calculated is equal to the number of standards. Thus, for a standard training set with five standards for both DA and pH, ten principal components are calculated. In FSCV, PCs are potential-dependent representations of variance captured in the experimental CVs provided for the training set. These PCs are separated into primary and secondary PCs.
    - **Primary PCs** – Primary principal components reflect signals that capture a significant amount of variance present in the cyclic voltammograms provided in the training set.
    - **Secondary PCs –** Secondary principal components are PCs determined to only describe noise. The data contained in these secondary PCs is used to calculate the tolerance for residual analysis (see $\mathbf{Q_\alpha}$ section below).
    - **Rank –** The number of primary components retained by the PCA-ILS model built from the training set. This can be determined in different ways, but our lab uses Malinowski's F-test to select the primary components that capture the most variance without retaining more primary components than necessary
- **K-matrix** – The K-matrix serves as a general representation of the characteristic shape for a particular analyte in an individual training set. The point of calibration (ex. $E_{p,a}$ for dopamine, E_{QH} for pH) is scaled to a user-defined external calibration factor. It can be used as a diagnostic tool to confirm that the training set model has adequately isolated analytes of interest. The K-matrix is

calculated using matrix algebra with the relevant principle components and regression coefficients.

- **Residual analysis** – Experimental data (e.g. current) unaccounted for by the PCA-ILS model reflects variance in the data that is insufficiently modeled in the experimental training set. In most cases, these currents arise from chemical and electrical noise.
    - **Q_t –** For any experimental CV, the contributions from principal components (e.g. DA, pH) are subtracted from the total current. The remaining currents at each applied potential are squared and summed to give the Q_t value for that particular cyclic voltammogram
    - **Q_α** – The threshold for residual analysis. If Q_t for any experimental cyclic voltammogram exceeds Q_α, it can be stated that, at a user defined confidence level (α), there is a significant amount of current present in the data that cannot be captured by the primary PCs. This indicates that this model is inadequate for concentration prediction, and the experimental CV(s) must be discarded. It depends primarily on the discarded secondary PCs (more secondary PCs, higher Q_α). Generally, more consistent CV standards (ex. small current range, identical shapes) will lead to a diminished tolerance for noise, and a lower Q_α value.

Table S-1. Training sets built in different subjects predict different peak dopamine concentrations than training sets built within subject. Five different data sets (n = # of electrically evoked dopamine transients) were analyzed with five different training sets (including the training set built within subject, in bold). The mean maximum concentration ± SEM of the dopamine transients are expressed in nM. Asterisks denote significance level from control within-subject training set (Repeated measures one-way ANOVA, Dunnet's multiple comparisons, * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001). The average absolute percent difference in predicted concentration compared to the prediction with the correct training set for each individual transient is included in parentheses (± SEM). These values contain both overestimations and underestimations in predicted peak dopamine concentration.

Data Set →	A (n = 52)	B (n = 57)	C (n = 17)	D (n = 65)	E (n = 60)
Training Set A	**103 ± 6 nM**	264 ± 26 nM****	224 ± 11 nM****	177 ± 18 nM****	107 ± 6 nM****
Training Set B	117 ± 6 nM****	**296 ± 28 nM**	224 ± 12 nM****	184 ± 21 nM****	286 ± 28 nM****
Training Set C	89 ± 6 nM****	257 ± 24 nM****	**269 ± 13 nM**	194 ± 19 nM****	242 ± 11 nM****
Training Set D	87±6 nM****	262 ± 26 nM****	282 ± 13 nM**	**237 ± 19 nM**	203 ± 21 nM****
Training Set E	107 ± 6 nM****	286 ± 28 nM****	242 ± 11 nM***	203 ± 21 nM****	**578 ± 34 nM**
No PCR	130 ± 6 nM****	296 ± 27 nM	204 ± 20 nM****	154 ± 21 nM****	524 ± 36 nM****

Table S-2. Characteristics of dopamine K-matrices for various subjects for two different training set construction methods: electrically-evoked or naturally occurring dopamine transients. Minor differences were seen in the peak potentials, while notable differences observed in the peak current ratio. The most significant difference between constructed training sets were Q_α values, which were systematically lower for transients constructed only with naturally occurring transients. Relatively low correlation coefficients for SOCC1 and SOCC3 were due to difficulty in obtaining clean cyclic voltammograms from naturally occurring transients, leading to broader oxidation peaks (SOCC1) and minor ionic fluctuations on the anodic scan (SOCC2).

Subject	**Training Set**	**r^2**	**Q_α (nA^2)**	**$E_{p,a}$ (V)**	**$E_{p,c}$ (V)**	**$I_{p,a}/I_{p,c}$**
SOCC1	Electrical	0.882	199.8	0.63	-0.26	2.3
	Sucrose		191.9	0.64	-0.26	6.3
SOCC2	Electrical	0.883	473.1	0.66	-0.19	3.2
	Sucrose		413.1	0.66	-0.32	3.5
SOCC3	Electrical	0.957	289.2	0.59	-0.25	3.9
	Sucrose		144.6	0.60	-0.23	2.1
SOCC4	Electrical	0.923	311.6	0.58	-0.18	5.9
	Sucrose		127.4	0.59	-0.20	3.2