Microdialysis in the Rat Striatum: Effects of 24-hr Dexamethasone Retrodialysis on Evoked Dopamine Release and Penetration Injury

Kathryn M. Nesbitt, Erika L. Varner, Andrea Jaquins-Gerstl, and Adrian C. Michael

Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, USA

Table of Contents

SUPPORTING INFORMATION

EXPERIMENTAL

Fast Scan Cyclic Voltammetry. Carbon fiber electrodes were constructed by threading a single carbon fiber (7 µm diameter, T650, Cytec Carbon Fibers LLC., Piedmont, SC) through borosilicate capillaries (0.58 mm I.D., 1.0 mm O.D., Sutter Instruments, Novato, CA). The capillaries were pulled to fine tips around the carbon fiber with a vertical puller (Narishing Tokyo, Japan). Carbon fibers were glued in place with a low viscosity epoxy (Spurr Epoxy, Polysciences Inc., Warrington, PA) and cured overnight at 70 ºC. The exposed carbon fiber was cut to a length of 400 µm for in vivo studies or 800 µm for detection at the outlet of microdialysis probes. Capillaries were backfilled with mercury and a nichrome wire (Goodfellow, Oakdale, PA) was placed into the mercury to make an electrical connection.

Fast scan cyclic voltammetry (FSCV) was executed using a computer controlled EI-400 potentiostat (Ensman Instruments, Bloomington, IN) with CV Tarheels version 4.3 software (Michael Heien, University of Arizona, Tucson AZ). A triangular waveform was applied as a linear sweep (vs Ag/AgCl) from 0 V to 1 V, then to -0.5 V, and then back to the resting potential of 0 V at a scan rate of 400 V/s. Scans were performed at a frequency of 2.5 Hz unless otherwise noted. Background subtracted voltammograms were used to quantify dopamine (DA) on the initial potential sweep between 0.6 V and 0.8 V. The DA current was converted to concentrations by post-calibrations with freshly prepared standard solutions of DA (Sigma Aldrich, St. Louis, MO) dissolved in nitrogen purged aCSF.

Electrochemical Pretreatment for Outlet Detection. In addition to the isopropyl alcohol pretreatment, 800 µm electrodes were electrochemically pretreated (0-2V vs. Ag/AgCl at 200 V/s for 3 s) exactly 10 min before each collection. The electrode was pulled out of the outlet

before each pretreatment and re-lowered immediately after it was complete. To reduce resistance, the electrode was positioned so that majority of the carbon fiber was inside the outlet. The pretreatment causes a drift in the signal, which is noticeable when detecting low concentrations close to the detection limit (~20-30 nM).[1] For this reason the pretreatment was completed exactly 10 min before each collection at the outlet, followed by a 60 Hz waveform scan for 60 s to help stabilize the drift. The pretreatment had no impact on the brain tissue because the in vivo electrode was outside of the path of the electrochemical cell.

Surgical and Stimulation Procedures. All procedures involving animals were approved by the Institutional Animal Care and Use of Committee of the University of Pittsburgh. Male Sprague-Dawley rats (250-350 g, Hilltop, Scottsdale, PA) were anesthetized with isoflurane (0.5 % by volume, Henry Schein Animal Health, Elizabethtown, PA). Rats were wrapped in a heating blanket (37°C) and placed in a stereotaxic frame. The incisor bar was adjusted so the dorsal ventral measurements at lambda and bregma were no more than 0.01 mm apart (flat skull). For all voltammetry experiments, the reference and stimulating electrodes were placed in the same positions in the brain. Reference electrodes were connected to the brain via a salt bridge. Bipolar stimulating electrodes were lowered into the medial forebrain bundle (MFB) until maximum DA release was observed (4.3 mm posterior and 1.2 mm lateral from bregma). Electrically evoked DA release was recorded by FSCV during stimulation of the MFB (stimulus waveform: biphasic, square, constant current pulses 300 μA pulse height, 4 ms pulse width).

24 hr Microdialysis Probe Implantation Procedure. A small craniotomy was made over the striatum. Microdialysis probes were perfused with aCSF or DEX using a syringe pump. Probes were lowered slowly (5 μm/sec) into the striatum (1.6 mm anterior and 2.5 mm lateral from bregma) over the course of approximately 23 min using a micropositioner (Model 2660,

David Kopf Instruments, Tujunga, CA) to final position of 7 mm below dura. Probes were secured with bone screws and acrylic cement. Anesthesia was removed and animals were placed in a Raturn Microdialysis Bowl Stand-Alone System (MD-1404, BASI, West Lafayette, IN) for one day.

Electrode Placement for Voltammetry Next to Microdialysis Probes after 24 hr. After 24 hr animals were re-anesthetized and placed back in the stereotaxic frame. Without disturbing the probe, holes were drilled for the reference electrode, stimulating electrode and both carbon fiber electrodes. One electrode (E1) was aimed lateral to the probe so that the tip of the carbon fiber was 70 μm and the base 100 μm from the probe. The second electrode (E2) was aimed 1 mm posterior to the probe (0.45 mm anterior from bregma, 2.5 mm lateral from midline, and 5 mm below dura). A stimulating electrode was placed into the MFB and evoked DA was recorded (Fig. S1).

Voltammetry at the Probe Outlet after 4 hr. Anesthetized rats were placed in a stereotaxic frame and adjusted to flat skull. Three holes were drilled and the dura was carefully removed to expose the brain. A carbon fiber electrode was inserted into the striatum (0.7 mm anterior to bregma, 2.5 mm lateral from bregma, and 5.0 mm below dura), and a stimulating electrode was lowered to the MFB. Three evoked DA responses were recorded in 20 min intervals to establish a stable, pre-probe response. The electrode was removed and a microdialysis probe was inserted using a micropositioner (5 μm/sec) in the same location except 7.0 mm below dura. After 2 hr, evoked DA release was monitored in the outlet. Following three stimulations, nomifensine and raclopride were sequentially administered and evoked DA monitored.

Voltammetry at the Probe Outlet after 24 hr. The anesthetized rats underwent aseptic surgery and a microdialysis probe was lowered as described above. After a 24 hr recovery the rats were re-anesthetized and returned to the stereotactic frame. A 400 μm carbon fiber electrode was inserted into the striatum (0.45 mm anterior to bregma, 3.5 mm lateral from bregma, and 5.0 mm below dura), the stimulating electrode was positioned at the MFB, and detection at the outlet and in vivo were completed the in same order as the post-probe procedure in the 4 hr study.

Fluorescent Dexamethasone Procedure. Microdialysis probes were implanted for 4 hr during which dexamethasone fluorescein (10 μM) was perfused through the probe (n=3 rats). Horizontal sections (30 μm) were taken along the probe tract (130 slices per rat). Three random sections from each rat were imaged, thresholded and masked. Using NIS Element Advanced Research software random line measurements were perform in the area defined as a positive fluorescent signal. Nine different line measurements were made from each image for a total of 81 measurements.

Defining TH and DAT Colocalization. Fluorescence microscopy was used to examine the colocalization of tissue labeled for tyrosine hydroxylase (TH) and dopamine transporters (DAT). For each probe track, images of both TH and DAT were collect using sequential mode, allowing for images to be merged and a composite image created. Since it is difficult to visualize the degree of colocalization from a pair of images, an important alternative is to display the intensities of the pairs of homologous pixels in a 2D scatterplot. The two antibodies were analyzed for the degree of colocalization by measuring the equivalent pixel position in each of the acquired images by generating a 2D-scatterplot (Fig. 4a). Each axis covers the range of intensities of the fluorophores, in our case Cy3 and CY5 (respectively, TH and DAT). The scatterplot shows the frequency of occurrence between the pair of intensities which reveals any

correlation between the fluorophores. The relationship between the intensities in the two images is calculated by linear regression. The slope of this linear approximation provides the rate of association of the two fluorophores.[2] Following the generation of the scatterplot it is possible to quantitatively evaluate colocalization between the fluorophores (TH and DAT). Values calculated for the scatterplot using NIS Element Advanced Research software include Pearson's Correlation Coefficient (PCC), and Manders' Overlap Coefficient (MOC). Pearson's Correlation and Manders' Overlap are mathematically similar differing in the use of either absolute intensities (Manders') or the deviation from the mean (Pearson's).[2, 3] Pearson's Correlation is well defined and is an accepted means for describing overlap between image pairs. It's computed values are between -1 to 1 with -1 being no overlap, 1 being perfect overlap and 0 representing random distributions between images. Only the similarities of shapes between images are account for not their intensities. PCC is defined as:[4, 5]

$$PCC = \frac{\sum_i (R_i - \bar{R}) * (G_i - \bar{G})}{\sqrt{\sum_i (R_i - \bar{R})^2 * \sum_i (G_i - \bar{G})^2}}$$

where R_i= intensity in red channel, $\bar{R}$= average intensity in red channel, G_i= intensity in green channel and $\bar{G}$= average intensity in green channel

MOC is also used to describe overlap however this method does not perform any pixel averaging functions like that of PCC therefore values range from 0 to 1. This method is also not sensitive to intensity variations between images. MOC is defined as:[4]

$$MOC = \frac{\sum_1 (R_i * G_i)}{\sqrt{\sum_1 R_i^2 * \sum_i G_i^2}}$$

Thresholding Images. Specifying correct threshold limits is a crucial procedure for image analysis. The point is to determine which pixels will and will not be included in the

analysis, therefore distinguishing pixels to be analyzed from background. Fig. S4 illustrates this procedure by means of an example. In the center of each image is the probe track, each channel corresponds to a specific antibody (red=TH and blue=DAT), and the far right column is the overlay of both channels. The top row (a) is unprocessed raw data, certain pixels in this row appear very bright (especially toward the center around the probe track) and other pixels are very dim. The build in "smart threshold function" of the NIS Element Advanced Research software automatically disregards these pixels and applies a mask to only the pixels to be used in the analysis. In our case, the mask is colored in white (bottom row (b)). Since all the images are imported into the software at the same time they are batch processed thereby allowing for all images to be analyzed in the same manner avoiding user bias.

FIGURES

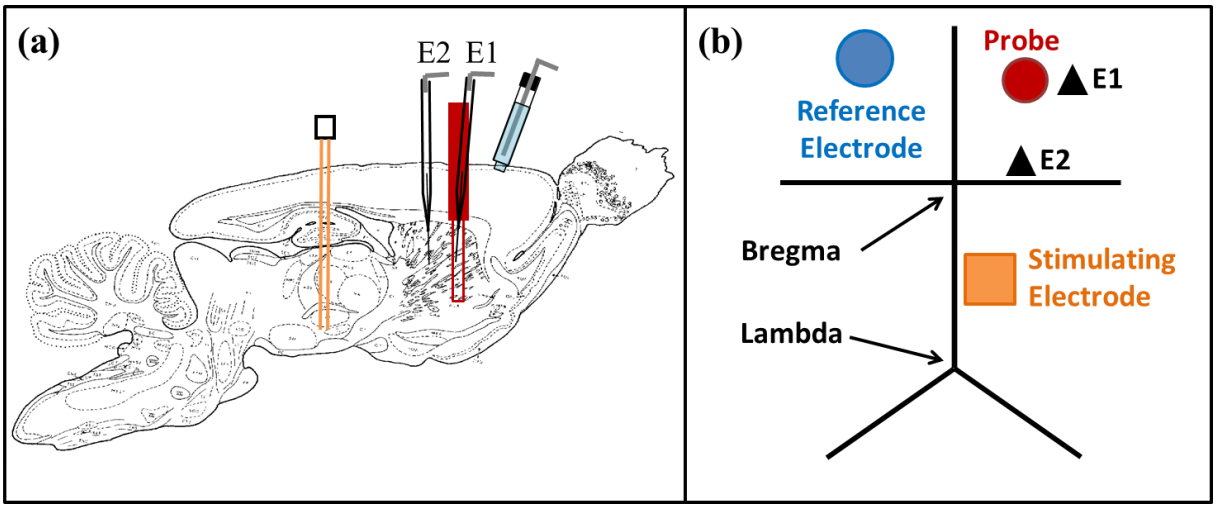

Figure S1. Device placement in the brain after 24 hr probe implantation for evoked DA detection next to probes. (a) Represents a sagittal brain slice illustrating the microdialysis probe (red) expanding the length of the dorsal striatum. The reference electrode (blue) was in contact with the surface of the brain, a stimulating electrode (orange) was positioned in the medial forebrain bundle. E2 was aimed 1 mm posterior to the probe and E1 was aimed at a 5° angle 70-100 µm lateral to the probe. (b) Top-down view of device placement.

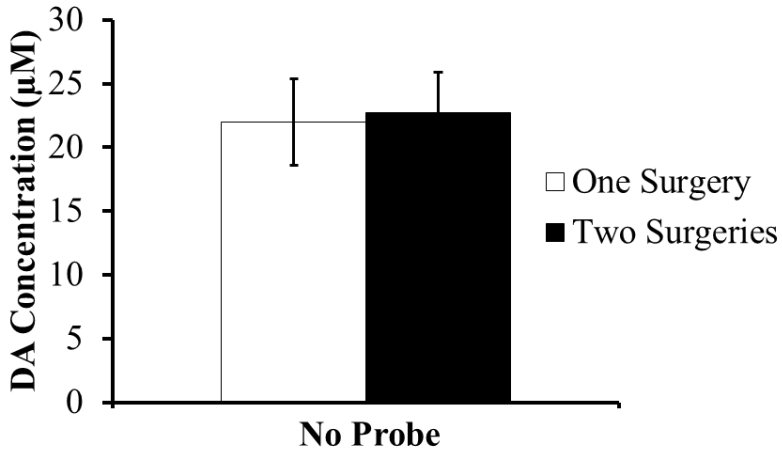

Figure S2. Maximum average (±SEM) evoked DA measured in two separate groups of rats during only one surgery (white) and after a second surgery (black). There was no significant difference in maximum evoked DA release after the second surgery *T*-test: $t(9)=0.15$, $p>0.05$.

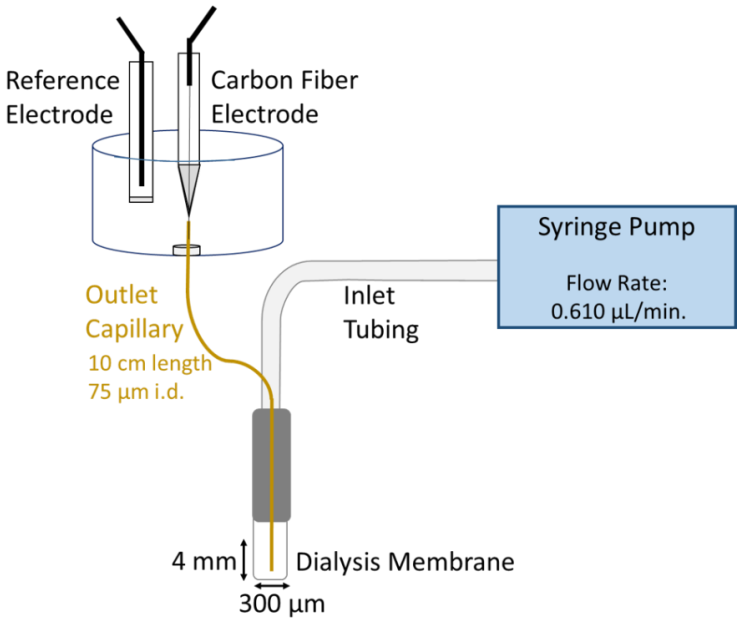

Figure S3. Diagram for voltammetry at the microdialysis probe outlet.

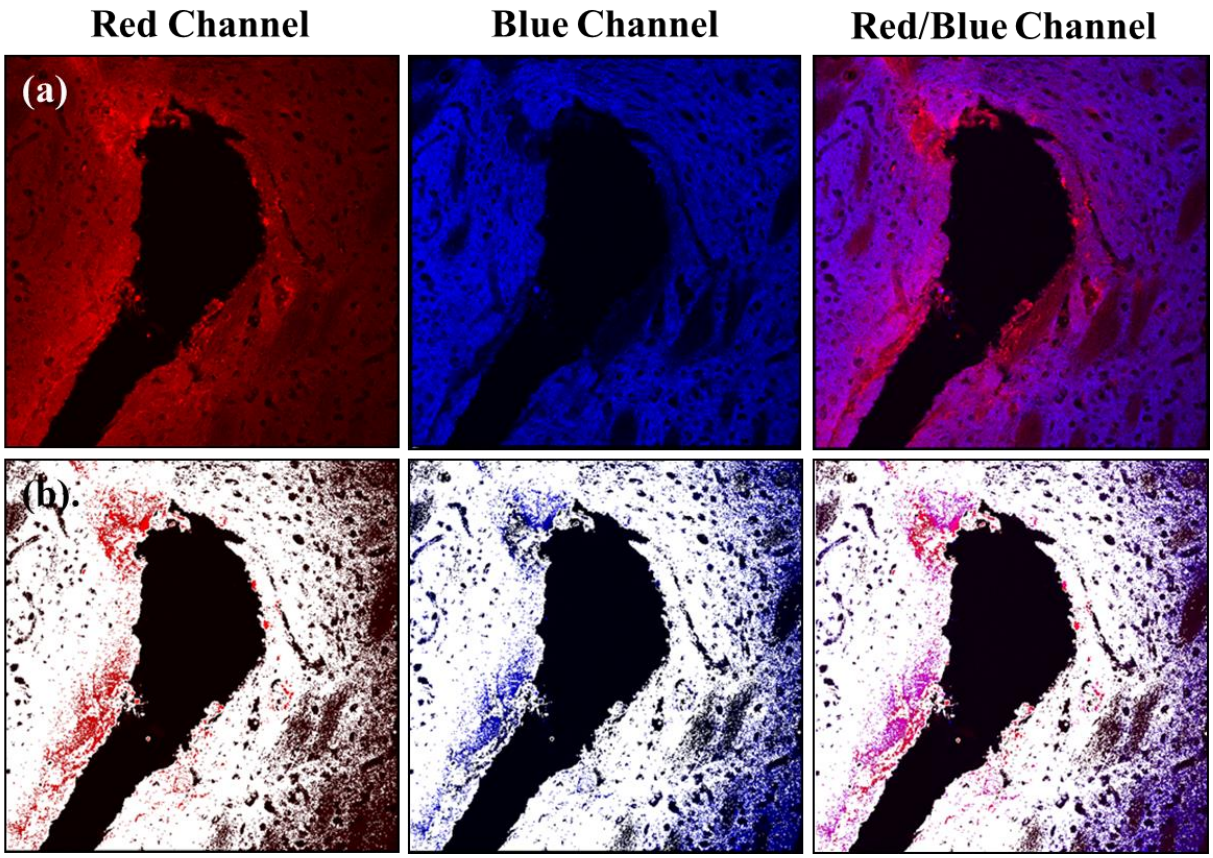

Figure S4. Example of the thresholding process by NIS Element Advanced Research software. The brain tissue contains a probe track in the center of each image. Columns represent individual channels. The last column is overlaid channels of the red and blue. Row (a) represents raw images and row (b) is the images after thresholding. Only the area in white is used in analysis. Pixels outside this region are not considered as they are either over or under the threshold limit.

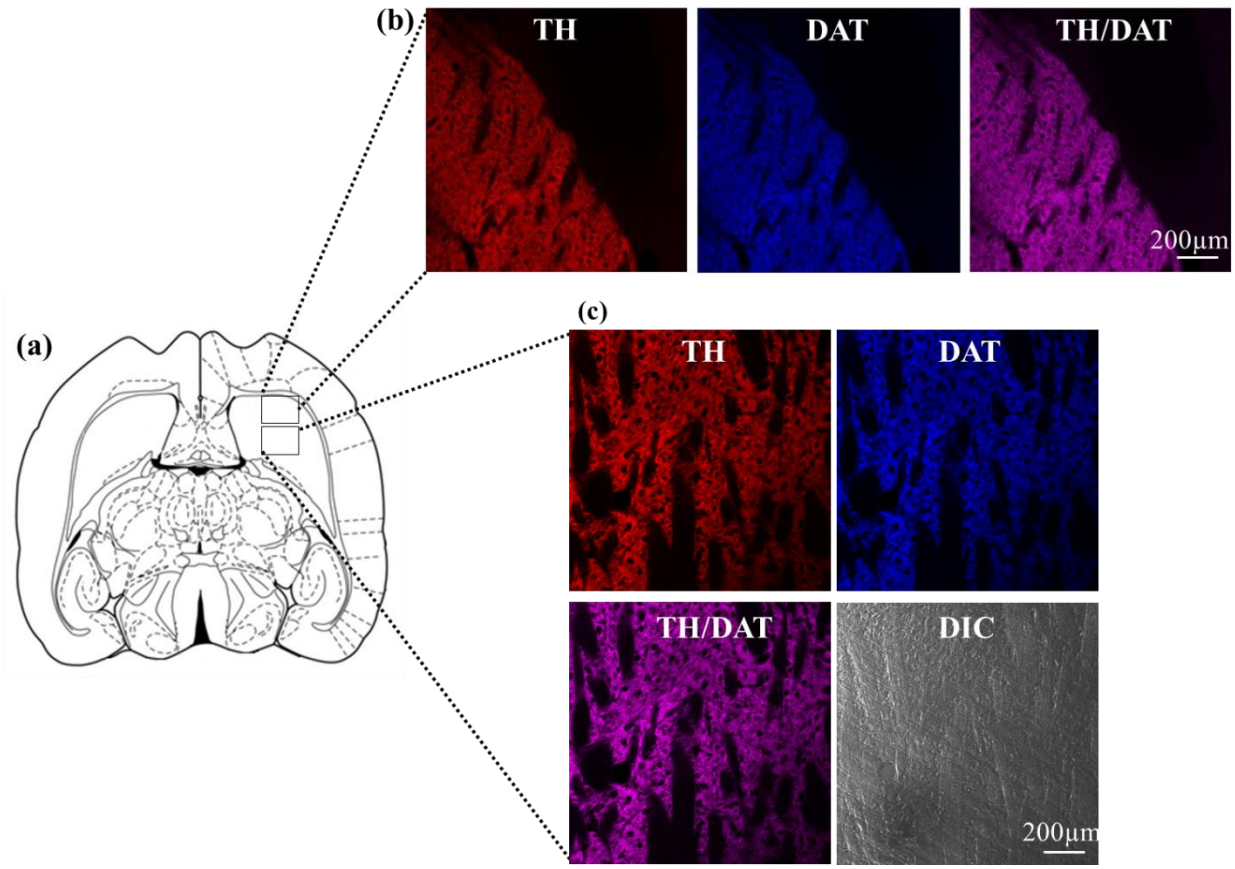

Figure S5. Labeling scheme of striatal tissue. (a) Horizontal slices cut 30 µm thick. (b) and (c) are representative images of tissue stained with TH and DAT antibodies and the corresponding overlay. (b) Indicates the edge of the striatum showing that only the striatum is stained with these antibodies. (c) Also includes the differential interference contrast image (DIC). Scale bars are 200 µm.

REFERENCES

(1) Lu, Y., Peters, J. L., and Michael, A. C. (1998) Direct comparison of the response of voltammetry and microdialysis to electrically evoked release of striatal dopamine, *J. Neurochem. 70*, 584-593.

(2) Costes, S. V., Daelemans, D., Cho, E. H., Dobbin, Z., Pavlakis, G., and Lockett, S. (2004) Automatic and quantitative measurement of protein-protein colocalization in live cells, *Biophys. J. 86*, 3993-4003.

(3) Adler, J., and Parmryd, I. (2010) Quantifying colocalization by correlation: the Pearson correlation coefficient is superior to the Mander's overlap coefficient, *Cytometry A. 77*, 733-742.

(4) Manders, E. M., Stap, J., Brakenhoff, G. J., van Driel, R., and Aten, J. A. (1992) Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy, *J. Cell Sci. 103 (Pt 3)*, 857-862.

(5) Manders, E. M. M., Verbeek, F. J., and Aten, J. A. (1993) Measurement of co-localization of objects in dual-colour confocal images, *J. Microsc. 169*, 375-382.