The effect of dexamethasone on gliosis, ischemia, and dopamine extraction during microdialysis sampling in brain tissue

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Supplementary Information

Materials and Methods

Reagents. All reagents were used as-received from the indicated supplier. All solutions were prepared in ultrapure water (Nanopure, Barnstead Inc, Dubuque, IA) and filtered with Iso-Disc TM filters, (N-25-2 Nylon 25mm x 0.2µm, Supelco, Bellefonte; PA). Bovine serum albumin (BSA), paraformaldehyde, polyvinyl alcohol, glycerol,1, 4-diazabicyclo [2.2.2] octane (DABCO), Triton-X 100, dopamine (DA) and ascorbic acid were from Sigma (St. Louis; MO); 2-methybutane was from Alfa Aesar (Ward Hill, MA); fluorescent beads were from Molecular Probes (0.1 µm diameter, FluoSpheres® carboxylate-modified polystyrene microspheres suspended (2% solids) in water with 2mM sodium azide; Eugene, OR); primary antibody for glial fibrillary acidic protein (GFAP) in 0.5% BSA was from BD Biosciences Pharmingen (San Diego, CA); secondary antibody (goat anti-mouse IgG, CY3 in 0.5% BSA) was from Jackson Immunoresearch (West Grove, PA); isoflurane was from Halocarbon Products Corporation (North Augusta, SC); dexamethasone sodium phosphate (9-fluoro-11β,17,21-trihydroxy-16α-

methylpregna-1,4-diene-3,20-dione 21-(dihydrogen phosphate) disodium salt, 20mg/5ml, American Regent Incorporated, Shirley, NY) was purchased from the University of Pittsburgh Presbyterian Hospital Pharmacy. All other salts and reagents were from Fisher Scientific (Pittsburgh, PA). Phosphate buffered saline (PBS) contained 155 mM NaCl and 100 mM phosphate adjusted to pH 7.4. Artificial cerebrospinal fluid (aCSF) contained 144 mM Na^+, 1.2 mM Ca^{2+}, 2.7 mM K^+, 152 mM Cl^-, 1.0 mM Mg^{2+}, and 2.0 M PO_4^{3-} and was adjusted to pH 7.4.

Animals and surgical procedures. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Male Sprague-Dawley rats (Hilltop; Scottsdale, PA) were anesthetized with isoflurane and wrapped in a 37^oC homoeothermic blanket (EKEG Electronics; Vancouver, BC, Canada) during an aseptic stereotaxic surgical procedure to position a guide cannula (MD-2251, Bioanalytical Systems, Inc. (BASi), West Lafayette, IN) over the striatum. The guide cannula was anchored in place with bone screws and acrylic cement and animals were given four days for post-operative recovery before experiments continued. All animals regained or exceeded their pre-surgical body weight by the end of the post-operative recovery period.

Microdialysis procedures. Prior to use, microdialysis probes (BASi MD-2204) were sterilized with ethylene oxide and flushed for 1 hr with aCSF. The probes were connected via FEP tubing (MF-5164, BASi) to a 1.0 ml gas-tight syringe (Hamilton). Rats were re-anesthetized briefly with isoflurane and returned to the stereotax while the stereotaxic carrier was used to slowly (~30 min) lower the probes through the guide cannula and into the striatum. The probes were perfused with aCSF at a flow rate of 0.610 µL/min for 5 days by means of a syringe pump

(Harvard Apparatus). In one group of rats, the probes were perfused with plain aCSF. In a second group of rats, the aCSF contained dexamethasone. The dexamethasone concentration was 10 µM for the first 24 hrs of the perfusion and 2 µM for the remainder of the experiment: preliminary experiments showed that continued perfusion with 10-µM dexamethasone profoundly agitated the rats causing them to damage the probe or connecting tubing and, in one instance, injuring themselves. The biological half life of dexamethasone is 36-54 hrs, [1] so it is likely that the continuous perfusion led to a cumulative dosing effect.

HPLC analysis. The microdialysis samples were analyzed by capillary HPLC coupled to a highly sensitive electrochemical detector. The mobile phase was delivered with a NanoLC Ultra pump (Eksigent Technologies, Dublin, CA) at 2 µL/min without the use of a splitter. The sample injection valve (VICI Valco Instruments, Houston, TX) had a 500-nL fused silica capillary sample loop. The fused silica capillary columns (5.5-6.5 cm long, 100 µm i.d., and 365 µm o.d.) were packed in-house with Waters 2.6 µm XTerra C18 MS stationary phase (Waters, Milford MA) by the slurry method. Mobile phases were prepared by mixing acetonitrile (4%) with aqueous buffer (96%) containing 100 mM sodium acetate, 0.15 mM disodium EDTA, and 18 mM sodium octyl sulfate adjusted to pH 4 with acetic acid. The mobile phase was passed through a 0.20 µm Nylon filter prior to use (Fisher Scientific, Pittsburgh, PA). A short length of 25 µm i.d. fused silica capillary acted as a connecter between the column and a radial-flow electrochemical detector with a BASi radial style auxiliary electrode, a thin (13 µm) Teflon spacer and a lab-made glassy carbon working electrode block. A BASi Epsilon potentiostat controlled the detection potential at +0.7 V vs. Ag/AgCl (3 M NaCl). This system separated dopamine from

other electroactive species in less than 3 minutes (Supplementary Fig 1). Detection limit is 1.0 nM (three times signal to noise) and quantification limit is 3.2 nM (ten times signal to noise). Calibration curves were established on the same day as analysis with freshly prepared standard solutions (Supplementary Fig 1, inset).

Dopamine extraction curves. Dopamine extraction curves were obtained by perfusing the probes with 0, 100, 250, or 1000 nM dopamine and 50 µM ascorbate as a preservative. This range of dopamine concentrations is wider than typically used: most studies of dopamine extraction are confined to the 0-200 nM range[2, 3] , although serotonin and norepinephrine have been studied over the 0-400 nM range[4]. The selection of a wider concentration range here was based on preliminary results that indicated the non-linear character of the dopamine extraction curves obtained in the presence of dexamethasone (see main paper for further discussion). The wider concentration range allowed a more thorough investigation of the nonlinear character of the extraction curves. After changing the composition of the perfusion fluid, probes were perfused for 1 hr, after which a 1-hr sample was collected into a glass microvial containing 4 µL of 0.5 M acetic acid (the acetic acid acidified the samples and protected the dopamine from air-oxidation during the 1-hr collection). Samples were stored on dry ice and analyzed in triplicate by HPLC as soon as possible after sample collection, usually on the same day as collection. The results were used to construct dopamine extraction curves, as explained in the main paper. The extraction curves were analyzed by linear or quadratic regression with OriginPro 7.5 (OriginLabs, Northampton, MA).

Tissue fixation and processing. After 5 days of continuous microdialysis, rats underwent transcardial perfusion with 160 ml PBS, 160 ml of 4% paraformaldehyde in PBS, and 50 ml of a 0.1% suspension of fluorescent beads (0.1 µm diameter) in PBS. The brain was submerged in 4% paraformaldehyde for 2 hours and then in 30% sucrose overnight. The brain was frozen by dipping in liquid nitrogen-cooled 2-methylbutane and stored at -80°C until sliced horizontally in a cryostat into 30-µm thick sections. The sections were mounted onto glass slides and stored at -20°C until further use.

Immunofluorescence protocol and fluorescence microscopy. Standard protocols were used to label the thin tissue sections with antibodies for glial fibrillary acidic protein, GFAP, a well-known marker for astocytes, one type of glial cell involved in gliosis. Sections were also treated with DAPI to aide in the visualization of neuronal and glial nuclei. Fluorescence microscopy (Olympus BX61, Olympus; Melville, NY) was performed with a 10X or 20X objective and appropriate filter sets (Chroma Technology; Rockingham, VT) for visualization of the beads, GFAP antibody, and DAPI-labeled nuclei. The images were analyzed with the Metamorph/Fluor 7.1 software package (Universal Imaging Corporation; Molecular Devices) and quantified with Metamorph and OriginPro.

Supplementary Results

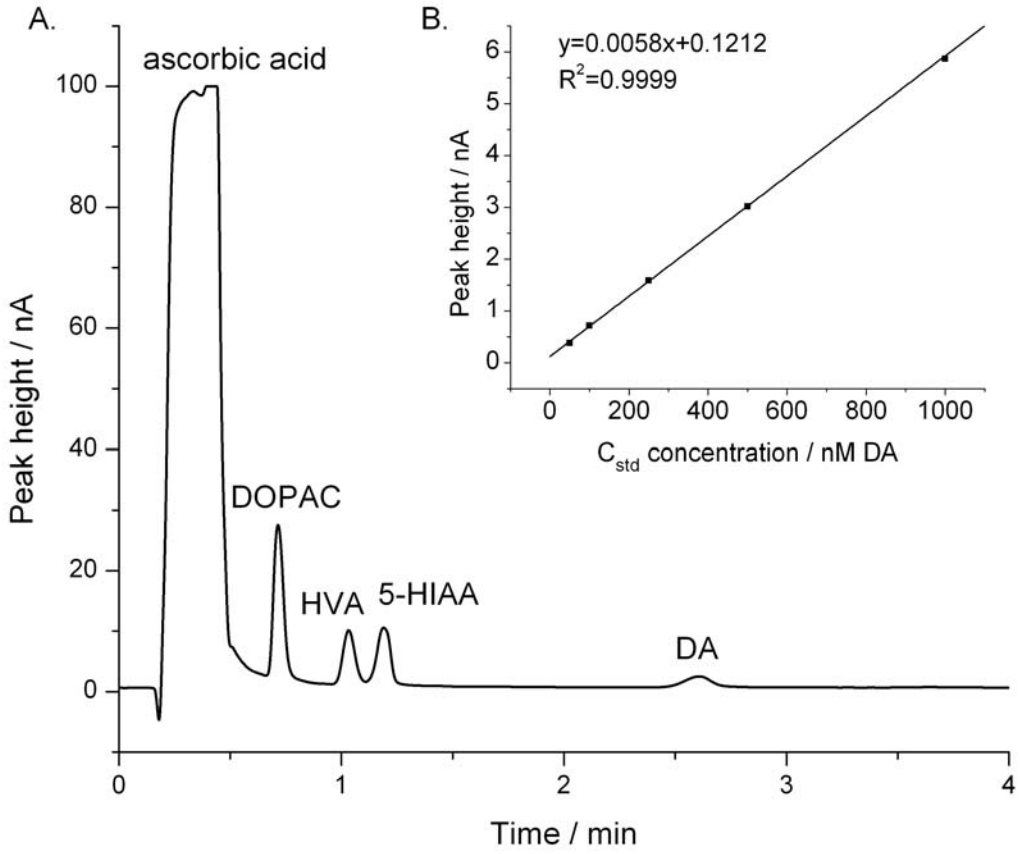
Microscopy of non-implanted control tissues. Images of 30-µm thick horizontally-cut sections of the non-implanted rat striatum (control tissue) exhibit the normal structure and morphology of this brain region (Supplementary Fig 2). DAPI-labeled nuclei (blue,

Supplementary Fig 2A) are distributed uniformly with no obvious voids or clusters. Bead-laden blood vessels (green, Supplementary Fig 2B) are spaced on-average 60 μm apart, with some vessels appearing in profile and some appearing in cross-section. Most vessels are capillaries but larger vessels are observed occasionally. The beads are confined to the vessels and do not reach the interstitial spaces, because brain endothelial cells form tight junctions. GFAP-labeled glial cells (red, Supplementary Fig 2C) appear star-like, hence the name astrocyte, are sparsely distributed, and exhibit numerous fine processes. An overlay image of the blood vessels and astrocytes shows points of contact (yellow, Supplementary Fig 2D), consistent with the role glia play in transporting substances from vessels to brain cells. The differential interference contrast (DIC) image (Supplementary Fig 2F) shows that the tissue is uniform and free of defects.

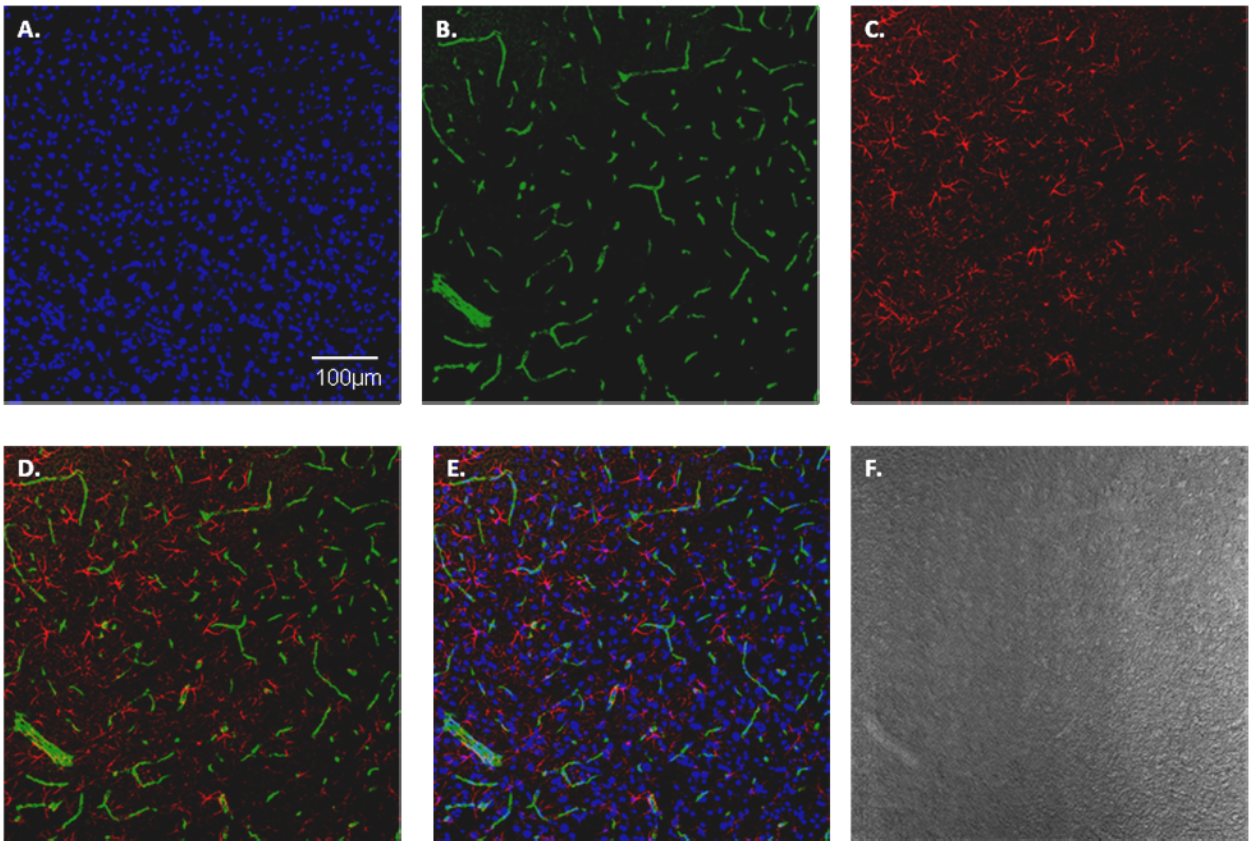
Color-coded intensity plots: For quantitative image analysis, we used Metamorph software to convert the fluorescent GFAP images to color-coded surface intensity plots (Supplementary Fig 3): the intensity range was 0-255 (low-high). In non-implanted tissues (Supplementary Fig 3A) occasional spots of medium intensity (yellow) correspond to normal glial cells. In tissues perfused without dexamethasone (Supplementary Fig 3B), the increased number and intensity of the GFAP spots reflect both the proliferation and enlargement of the glial cells. These features are absent in the tissues perfused with dexamethasone (Supplementary Fig 3C).

Dopamine extraction curves: Supplementary Table 1 reports the numerical values of $C_{out,c}$ and C_{nnf} for dopamine measured in the rat striatum. $C_{out,c}$ was determined by HPCL

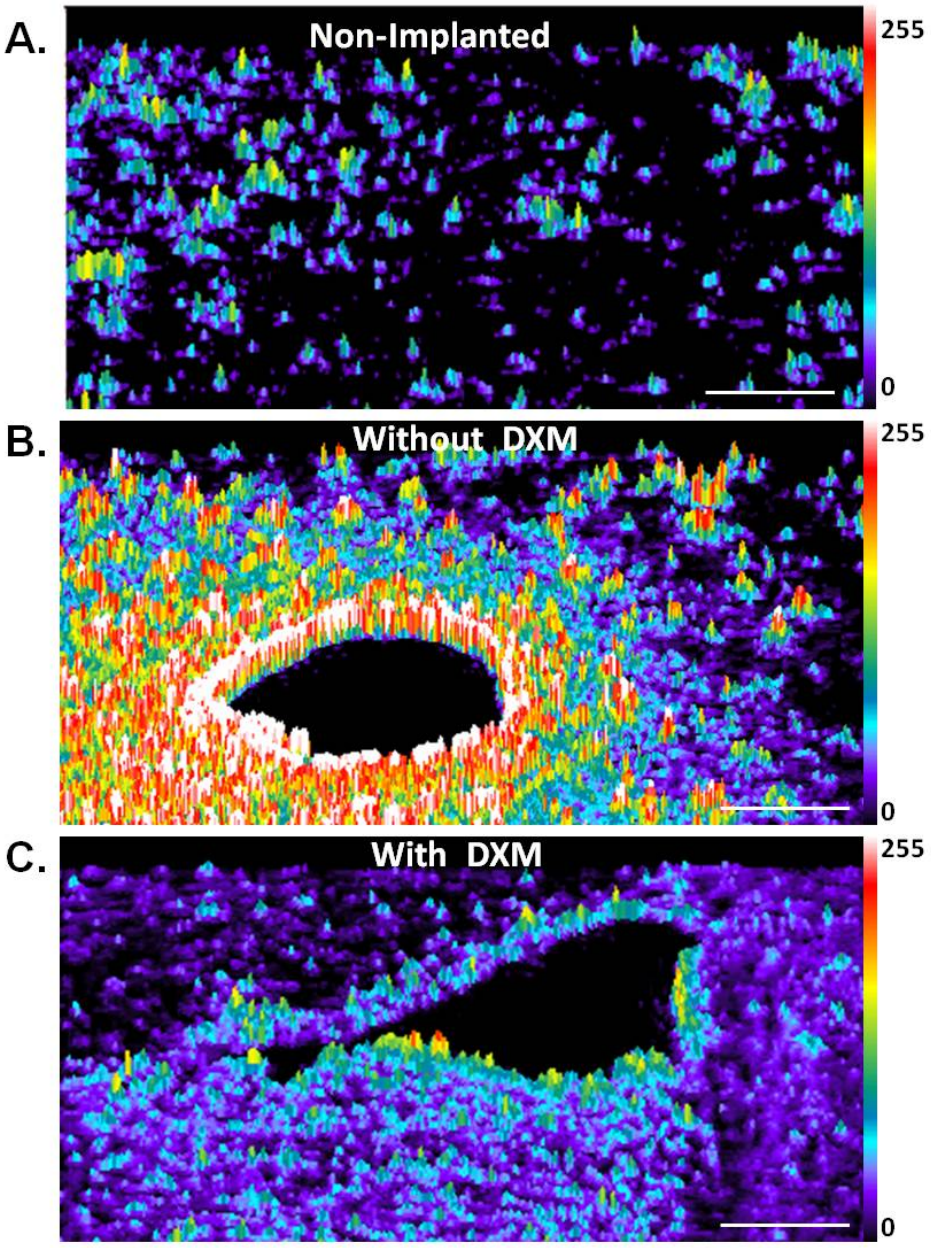
analysis of dialysate samples collected when C_{in} was zero. C_{nnf} was determined by regression analysis of the extraction curves. Linear regression of the results obtained without dexamethasone produced r^2 values greater than 0.999. However, in the case of the results obtained with dexamethasone, high regression coefficients required quadratic regression.



Supplementary Fig 1. A) A typical chromatogram of a brain dialysate sample obtained by capillary HPLC coupled to a radial-flow electrochemical detector with a thin (13 µm) Teflon spacer. This sample was obtained 1 day after probe implantation: the probe was perfused with dexamethasone and dopamine (1000 nM). B) A typical calibration curve for dopamine.



Supplementary Fig. 2. Microscopy of non-implanted striatal tissue. A) nuclei labeled with DAPI, B) blood vessels labeled with fluorescent beads, C) glial cells (astrocytes) labeled with GFAP antibody, D) overlay of B and C (note yellow pixels indicating contact between glial cells and blood vessels), E) overlay of A, B, and C, F) DIC image.



Supplementary Fig. 3. A color-plot representation of the GFAP images of non-implanted striatal tissue (A) and striatal tissue dialyzed without (B) and with (C) dexamethasone. Pixel intensities are color coded from 0 to 255 as indicated on the color scales next to the images. Scale bars = 100µm.

Supplementary Table 1.

Parameter	Day 1		Day 4	
	Without DXM	With DXM	Without DXM	With DXM
	N = 3	N = 6	N = 3	N = 3
Conventional Microdialysis Concentration ($C_{out,c}$), nM[a]	9.19 ± 3.49	11.94 ± 5.36	16.49 ± 8.85	19.11 ± 3.41
No-net-flux Concentration (C_{nnf}), nM[b]	9.34 ± 7.17	16.26 ± 1.54	19.07 ± 4.19	19.37 ± 1.61
Extraction Fraction (E)[c]	0.901 ± 0.010		0.858 ± 0.008	
Regression Equation[c] $Y = C_{in} - C_{out}$, $X = C_{in}$	$Y = 0.901X - 9.02$	$Y = -2.1\times10^{-4}X^2 + 0.76X - 15.78$	$Y = 0.858X - 10.02$	$Y = -4.6\times10^{-4}X^2 + 1.20X - 25.07$
Regression Coefficient (r^2)[c]	0.9996	0.9997	0.9997	0.9987

a: Determined by direct measurement.
b: Determined by regression of data from individual animal.
c: Determined by regression of averages across animals.

Supplement References:

(1) Marcus, H. J.; Carpenter, K.; Price, S., J.; Hutchinson, P. J. *J. Neurooncol.* **2010**, *97*, 11-23.

(2) Bungay, P. M.; Morrison, P. F.; Dedrick, R. L.; Chefer, V. I.; Zapata, A. In *Handbook of Microdialysis: Methods, Applications, and Perspectives*; Westerink, B. H. C., Cremers, T. I. F. H., Eds.; Elsevier: Amsterdam, The Netherlands, 2007, pp 131-167.

(3) Smith, A. D.; Justice, J. B. J. *J. Neurosci. Methods* **1994**, *54*, 75-82.

(4) Cosford, R. J. O.; Vinson, P.; Kukoyi, S.; Justice, J. B. J. *J. Neurosci. Methods* **1996**, 39-47.