SUPPLEMENTAL MATERIAL for "**Inhaled Nitric Oxide Improves Outcome After Successful Cardiopulmonary Resuscitation in Mice.**"

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Supplemental Methods

MRI Acquisition—Magnetic resonance imaging data were obtained using a 9.4 Tesla horizontal-bore magnet (Bruker Biospin Corp., Billerica MA) equipped with a custom surface coil for transmission and reception of radio frequencies for MRI of mouse brain. During imaging, mice were anesthetized by 2% isoflurane administered through a nose cone. Following image localization, multi-slice diffusion-weighted images (DWI) were acquired using a conventional spin-echo pulse sequence with an echo time of 26 ms, a

repetition time of one second, sixteen contiguous slices of 750 microns, and an isotropic resolution of 260 microns in the image plane. Two coronal diffusion weightings ("b values" of 154 and 1294 sec/mm^2) were acquired every three minutes, and six pairs of diffusion values were acquired for each animal in order to reduce motion artifacts (e.g., due to respiration) by averaging.

MRI Analysis—For each animal, volumetric MRI data were aligned to the coordinate space of the Allen Mouse Brain atlas[1] using publicly available software developed by one of the authors (JBM: www.nitrc.org/projects/jip). Briefly, digitized slides of the Allen Mouse Brain "Reference Atlas" were segmented manually into gray matter, white matter, and cerebrospinal fluid in order to form a template for automated alignment of T2-weighted MR brain images from a cohort of mice. Subsequently, data from each animal were aligned to this cross-subject MRI template using automated adjustment of linear and non-linear transformations. Linear "affine" alignment (6-parameter rigid-body transformation plus 3 uniform inflations and 3 uniform skews) was followed by adjustment of three-dimensional distortion fields to reduce residual alignment errors due to MRI artifact or anatomical variance. All MRI data were registered into brain volumes with a slice thickness of 500 microns and an in-plane resolution of 250 microns.

Because DWI is sensitive to motion, respiration produced subtle artifacts in images with heavy diffusion weighting. To minimize these artifacts, each series of six b-values were averaged using a weighting function equal to the inverse of the global residue with respect to mean in order to produce a single time-averaged brain volume for each b-value. Subsequently, the apparent diffusion coefficient (ADC) for each brain

voxel was computed using the standard formulation for the MRI signal: $S = S_0 \exp(-b * ADC)$. Brains maps of average ADC were computed for each subject group, and the average ADC value within each region of interest was computed for each animal for entry into histograms and statistical tables.

MRI Regions of Interest—Anatomical regions of interest were determined by the Allen Brain Atlas and cross-subject maps of average ADC value in the two subject groups. These regions included (1) whole brain, (2) whole caudoputamen (blue outline in Supplemental Figure 1), and (3) whole hippocampus, as defined from the Allen Brain Atlas. Based on our pilot studies, two additional bilaterally symmetric regions of interest were defined: (1) lateral cortex (red outlines in Supplemental Figure 1) and (2) ventral lateral hippocampus (green outlines in Supplemental Figure 1).

Histological studies—Four days after CA/CPR, mice were sacrificed, and brains were perfusion-fixed in 4% formalin in PBS and embedded in paraffin. Brains were cut with a microtome in coronal planes including the hippocampus (6 μm thickness). Activation of caspase 3 was assessed by immunohistochemistry in paraffin-embedded brain sections obtained 96h after CA/CPR using a rabbit monoclonal antibody against cleaved caspase 3 (1:80, Cell Signaling) according to the protocol recommended by the manufacturer. Cleaved caspase 3-positive neurons in the CA1 sector of the hippocampus were manually counted by an investigator blinded to the treatment group, and the number of these neurons per square millimeter of examined area was reported.

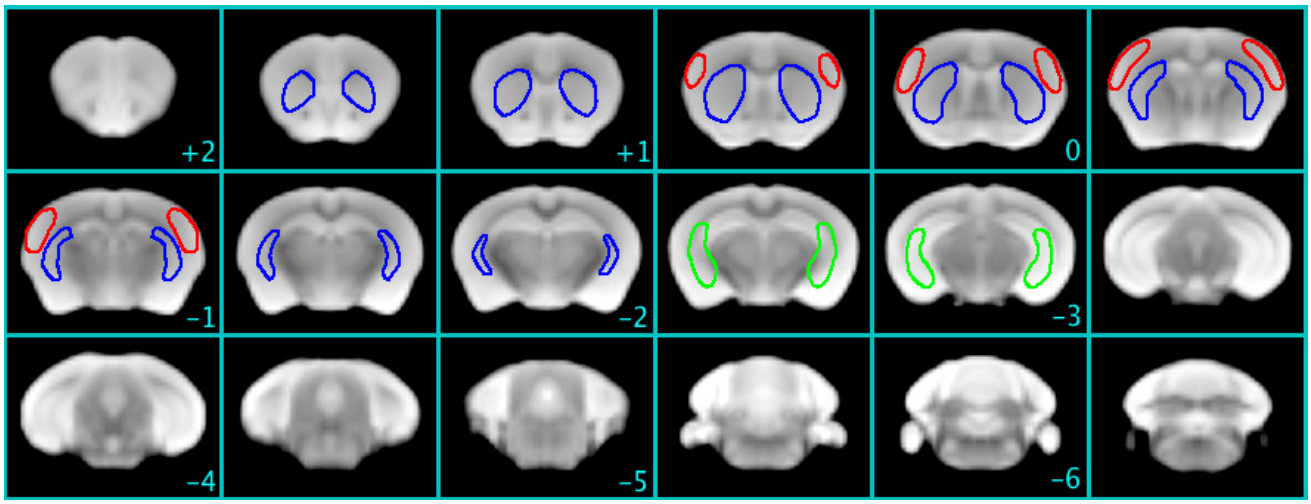
Measurements of gene expression—Total RNA was extracted from cortex of mice 24h after CA/CPR or sham surgery using the illustra RNA spin Mini kit (GE Healthcare, Waukesha, WI), and cDNA was synthesized using MMLV-RT (Promega, Madison, WI). Tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), gp91phox (NOX2), and 18S ribosomal RNA transcript levels were measured by real-time PCR using a Realplex 2 system (Eppendorf, Westbury, NY). The following primer sets were used: TNF-α (5'-CAGCCTCTTCTCATTCCTGC-3', 5'-GGTCTGGGCCATAGAACTGA-3'), IL-1β (TaqMan, Applied Biosystems), IL-6 (5'-CCGGAGAGGAGACTTCACAGA-3', 5'-CAGAATTGCCATTGCACAAC-3'), NOX2 (5'-CTGCTCTCCTTTCTCAGGGGT-3', 5'-GTGTGCAGTGCTATCATCCAA-3'), and 18S rRNA (5'-CGGCTACCACATCCAAGGAA-3', 5'-GCTGGAATTACCGCGGCT-3'). Changes in the relative gene expression normalized to levels of 18S rRNA were determined using the relative C_T method. The mean value of samples from control mice was set as 1.

Supplemental Table 1. Group characteristics before cardiac arrest and in the first hour after cardiac arrest and CPR

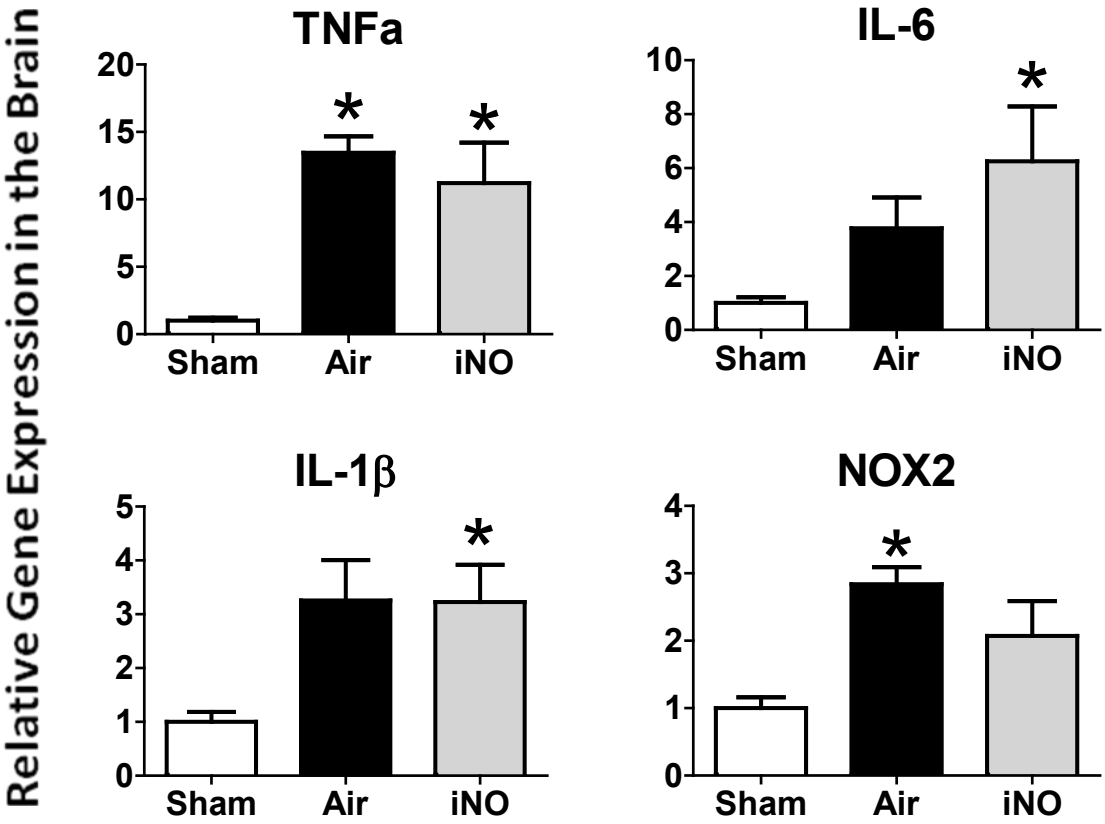
	Air (n=13)	Inhaled NO (n=13)
Weight, g	24.6±0.5	24.3±0.4
HR before CA, bpm	594±18	587±11
MAP before CA, mmHg	123±3	123±3
Total dose of Epinephrine, µg	0.9±0.1	0.8±0.0
CPR time to ROSC, s	262±16	255±14
HR at ROSC, bpm	523±5	512±3
MAP at ROSC, mmHg	106±7	111±8
HR at 60 min after CPR, bpm	323±18	294±12
MAP at 60min after CPR, mmHg	49±3	48±2

Values are mean±SEM. Air, mice subjected to cardiac arrest and CPR that breathed air; Inhaled NO, mice subjected to cardiac arrest and CPR that breathed NO; CA, cardiac arrest; HR, heart rate; MAP, mean arterial pressure; CPR, cardiopulmonary resuscitation; ROSC, return of spontaneous circulation. No differences were found statistically significant.

Supplemental Figures and Figure legends



Supplemental Figure 1. Representative fast spin-echo images derived from the mouse showing the definition of the regions employed for analysis. Slices are labeled with respect to bregma in the coordinate space of the Allen Mouse Brain Atlas. Using the nomenclature employed in the manuscript, colored outlines identify caudoputamen (blue), lateral cortex (red), and ventral lateral hippocampus (green).



Supplemental Figure 2. Expression of genes encoding tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β, and gp91phox (NOX2, a subunit of NADPH oxidase) in the brain cortex of sGCα1$^{-/-}$ mice 24h after sham surgery (Sham), after CA/CPR and breathing air (Air), or after CA/CPR and breathing NO (iNO) starting 1h after CPR for 23h. N=4-8. *P<0.05 vs Sham.

Supplemental References

1. Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, Boe AF, Boguski MS, Brockway KS, Byrnes EJ, Chen L, Chen L, Chen TM, Chi Chin M, Chong J, Crook BE, Czaplinska A, Dang CN, Datta S, Dee NR, Desaki AL, Desta T, Diep E, Dolbeare TA, Donelan MJ, Dong HW, Dougherty JG, Duncan BJ, Ebbert AJ, Eichele G, Estin LK, Faber C, Facer BA, Fields R, Fischer SR, Fliss TP, Frensley C, Gates SN, Glattfelder KJ, Halverson KR, Hart MR, Hohmann JG, Howell MP, Jeung DP, Johnson RA, Karr PT, Kawal R, Kidney JM, Knapik RH, Kuan CL, Lake JH, Laramee AR, Larsen KD, Lau C, Lemon TA, Liang AJ, Liu Y, Luong LT, Michaels J, Morgan JJ, Morgan RJ, Mortrud MT, Mosqueda NF, Ng LL, Ng R, Orta GJ, Overly CC, Pak TH, Parry SE, Pathak SD, Pearson OC, Puchalski RB, Riley ZL, Rockett HR, Rowland SA, Royall JJ, Ruiz MJ, Sarno NR, Schaffnit K, Shapovalova NV, Sivisay T, Slaughterbeck CR, Smith SC, Smith KA, Smith BI, Sodt AJ, Stewart NN, Stumpf KR, Sunkin SM, Sutram M, Tam A, Teemer CD, Thaller C, Thompson CL, Varnam LR, Visel A, Whitlock RM, Wohnoutka PE, Wolkey CK, Wong VY, Wood M, Yaylaoglu MB, Young RC, Youngstrom BL, Feng Yuan X, Zhang B, Zwingman TA, Jones AR. Genome-wide atlas of gene expression in the adult mouse brain. *Nature*. 2007;445:168-176.