Nemeth et al., "Uptake of dendrimer-drug by different cell types in the hippocampus after hypoxic-ischemic insult in neonatal mice: effects of injury, microglial activation and hypothermia."

Supplementary Materials

Table S1: Materials

Material	Vendor	Cat #	Antibody dilution
Rabbit anti-Iba1	Wako Chemicals (Osaka, Japan)	019-19741	1:1000
Rabbit anti-NeuN-Alexa Fluor® 488	Abcam (Cambridge, MA USA)	ab190195	1:100
Rabbit anti-GFAP	Dako North America, Inc. (Carpenteria, CA USA)	Z033429-2	1:5000
Rat anti-CD68	Bio-Rad Antibodies (Kidlington, Oxford, UK)	MCA1957	1:1000
Goat anti-rabbit IgG-Alexa Fluor® 488	Jackson ImmunoResearch (West Grove, PA USA)	111-545-144	1:500
Donkey anti-rat IgG-Alexa Fluor®488	Jackson ImmunoResearch (West Grove, PA USA)	712-586-153	1:500
DAPI	Invitrogen/Life Technologies (Eugene, OR USA)	D1306	1 µg/mL
10% Formalin	Fisher Chemical (Fair Lawn, NJ USA)	SF100-4	
Cresyl violet	Electron Microscopy Sciences (Hatfield, PA, USA)	12780	
L.A.B. Solution	Polysciences (Warrington, PA USA)	24310	
DAKO fluorescence mounting medium	Dako North america, Inc. (Carpenteria, CA USA)	S302380-2	
Love mash	Bio-Serv (Flemington, NJ USA)	S3823P	
Genesis Hova-Bator incubator	GQF Manufacturing Co., Inc. (Savanna, GA USA)	1588	
IBM SPSS Statistics	IBM Corporation (Armonk, NY USA)		
Axiovision® 4.8.2	Carl Zeiss Microscopy, LLC (Thornwood, NY USA)		

Animal surgery and postoperative care: detailed procedures

Ten lactating female CD-1 mice with litters of 10 pups were obtained at postnatal day (P) 6 (day of birth = P1, Charles River Laboratories) and housed with food and water *ad libitum*. On P7, 4 male and 4 female pups per litter were anesthetized with isoflurane (4% induction; 1-1.5% maintenance), and the right common carotid artery was permanently ligated. Pups recovered 75-90 minutes at 36 °C before hypoxia (10% O_2, balance N_2, 36 °C, 15 min).

Immediately following HI, mice were randomized to 6 h of normothermia (ambient temperature 36.5 °C) or hypothermia (ambient temperature 30.5 °C), with 2 males and 2 females per litter in each temperature group. Remaining mice served as uninjured controls. Pups were placed in individual chambers on an aluminum platform within a Genesis Hova-Bator incubator (GQF Manufacturing Co). Pilot studies showed that 7 days after HI, brain injury was less severe in hypothermic mice (Supplementary Figure S2; $p < 0.05$) and body temperatures stabilized within 20 min and remained at ~33 °C in the hypothermic group and ~36.5 °C in the normothermic group for the 6 h period of hypothermia/normothermia. Therefore, in the present study, incubator temperatures were set at 30.5 °C and 36.5 °C for hypothermia and normothermia, respectively, and were monitored throughout (range 29.4-31.3 °C and 36.1-37.0 °C). Core body temperature was monitored 30 min and 6 h after HI, using a microthermistor probe (mean at 6 h: 31.6 °C and 35.4 °C).

To determine the effect of injection time on uptake of D-NAC, mice were injected with Cy5-D-NAC (10 mg/kg; *i.p.*) at 0, 6, or 24 h after HI (Table 1). Dendrimer-conjugated NAC has been extensively tested for *in vivo* safety and stability; this dose has resulted in both cellular uptake and functional recovery in neonatal models of ischemic and inflammatory brain injury.[25,26] Mice were perfused with 10% formalin, 24 h after injection of Cy5-D-NAC (24, 30, or 48 h after HI). Brains were post-fixed for 4 h, cryoprotected, frozen, and stored at -80 °C.

When pups were removed for surgery, the dam's diet was supplemented with Love mash™ soft pellets (Bio-Serv, Flemington, NJ), to relieve stress and enhance milk production. Health of

pups was confirmed by examination (including confirmation of milk bands) and by weights prior to surgery and at perfusion. Each litter was examined for normal nursing behavior 15 to 30 minutes after return to the dam and before pups were removed for perfusion, 24, 30 and 48 h after HI.

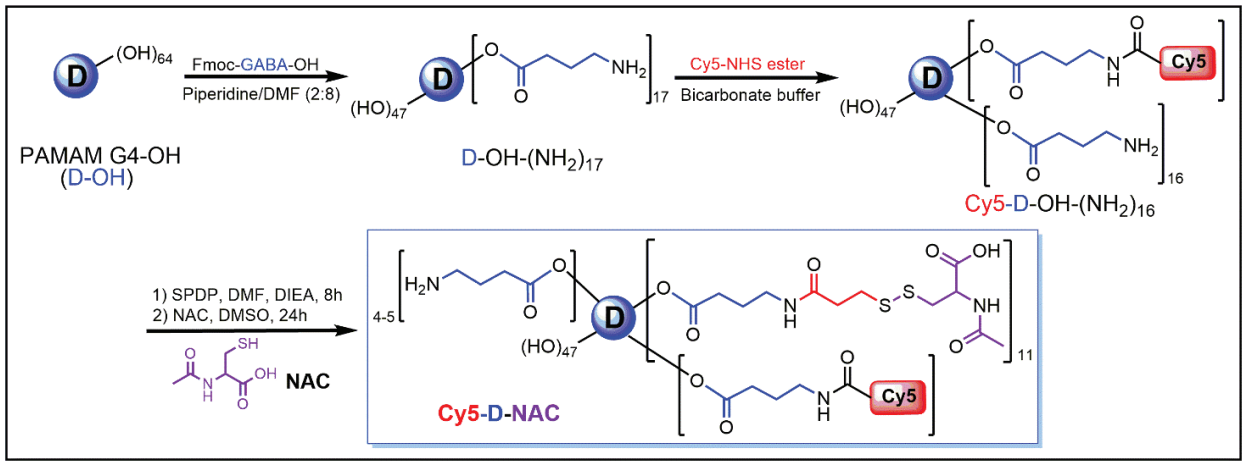

Figure S1. Preparation of the Cy5-D-NAC conjugate. A bifunctional dendrimer was reacted with Cy5, followed by reaction of linker and NAC in two steps.

Preparation of Cy5-labeled D-NAC conjugate

The bifunctional dendrimer was first prepared following an established protocol.[26,28] This bifunctional dendrimer with 17 free amine groups on the surface (100 mg) was dissolved in 10 mL of sodium bicarbonate buffer (pH 8.5) at room temperature. The reaction mixture was cooled to 0 °C and Cy5-NHS ester (3.5 mg) was added and dissolved in 1 mL of DMSO. The reaction mixture was stirred overnight at room temperature after which the reaction was terminated and lyophilized to get the crude product. Finally, the crude product was dialyzed against DI water overnight (MW cutoff = 1000 Da) with successive change of water. The resulting reaction mixture was then lyophilized to get Cy5-labeled bifunctional dendrimer (92 mg). Without further purification, the intermediate (75 mg, 0.00458 mmol) was dissolved in DMF (20 mL), DIEA (16 mg, 0.1283 mmol) was added, and the reaction mixture was cooled to 0 °C. The heterobifunctional linker SPDP (40 mg, 0.1283 mmol) dissolved in DMF (5 mL) was added to the reaction mixture and stirred for 8 h. N-acetyl cysteine (20 mg, 0.1283 mmol) dissolved in DMSO (5 mL) was added, and the reaction mixture was stirred overnight at room temperature. The solvent was evaporated at 25 °C under high vacuum and dialyzed in DMF (membrane MW CO 1000 Da) for 16 h. The resulting solvent was evaporated at 25 °C under high vacuum and again dialyzed in DI water for 8 h. Finally, the conjugate was purified using GPC fractionation to obtain pure Cy5-D-NAC (40 mg). The synthetic scheme is depicted in Figure S1.

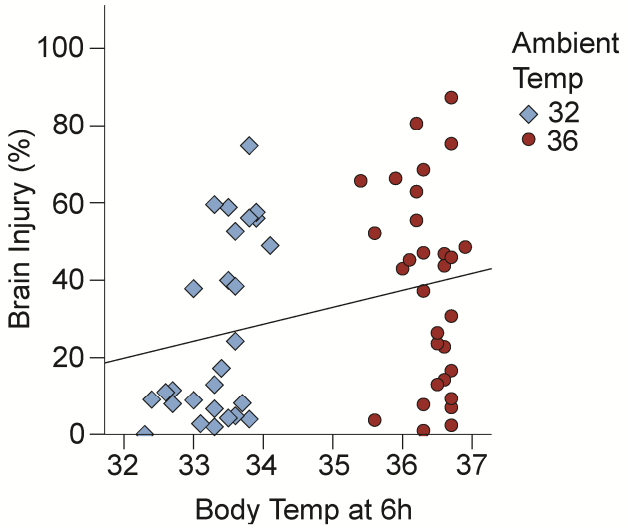

Figure S2. Hypothermic treatment reduced brain injury, when examined 7 days after HI in P7 CD1 mice. Immediately after HI, mice were maintained at 36°C (normothermia, 15 male, 15 female) or 32°C (hypothermia, 13 male, 14 female) for 6 h. To determine whether this hypothermic treatment was effective in reducing brain injury, we used a volumetric method to quantify brain injury, 7 days after HI. The volumes of the ipsilateral, ligated hemisphere and the contralateral uninjured hemisphere were measured 7 days after HI in a series of Nissl-stained sections throughout the forebrain (240 micron section interval). Brain injury was expressed as a percentage of the contralateral, uninjured hemisphere: Brain injury (%) = ((contralateral-ipsilateral)/contralateral x 100). At a survival time after HI of 7 days, brain injury was less severe in hypothermic mice ($p < 0.05$, one-tailed T) and injury was significantly correlated with rectal temperature at 6 h (Pearson's $r = 0.28$, $p < 0.05$).

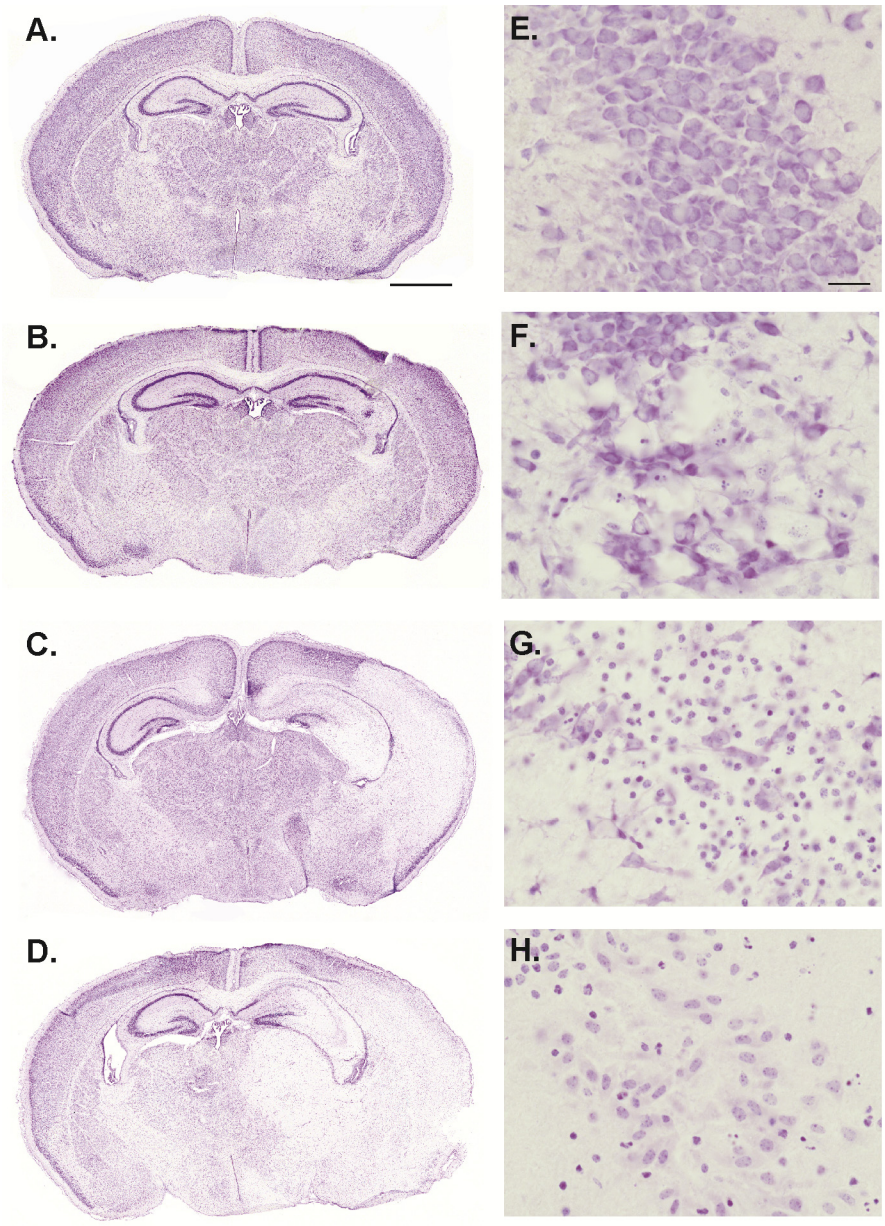

Figure S3. Histopathologic brain injury 24 to 48 h after HI on P7: Mice subjected to HI on P7 were euthanized 24h after injection of Cy5-D-NAC (24 to 48h after HI). Gross histopathology and cellular injury were evaluated in a series of cresyl-violet Nissl-stained sections (120 μm section interval) throughout the forebrain, examined at low magnification (A-D) and high magnification (E-H, pyramidal cell layer in CA3). Examples typical of each level of injury scored are shown: (A, E) no apparent injury, score = 0; (B, F) mild injury within the hippocampus, score = 1; (C, G) moderate injury within the hippocampus and cortex, striatum, or thalamus, score = 2; (D, H) severe injury, with extensive confluent infarction or tissue cavitation, score = 3. At this early time point, there was no difference in injury score among treatment groups. (Kruskal-Wallis test, $p = 0.208$). A-E: Bar = 1 mm; E-H: Bar = 25 μm.

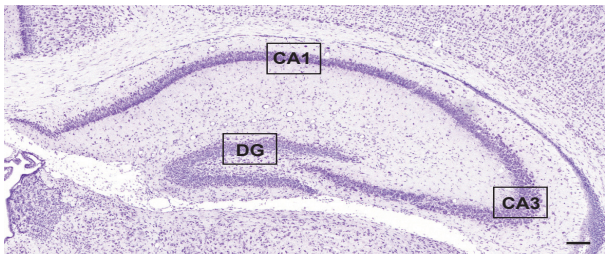

Figure S4. Hippocampal regions of interest for uptake analysis. The hippocampus was selected for uptake analysis. In each subject, three sections were selected at comparable levels of the dorsal hippocampus. In each section, three images were collected as shown, centered on the principal cell layers of CA1, CA3, and dentate gyrus (DG). Scale bar = 100 μm.

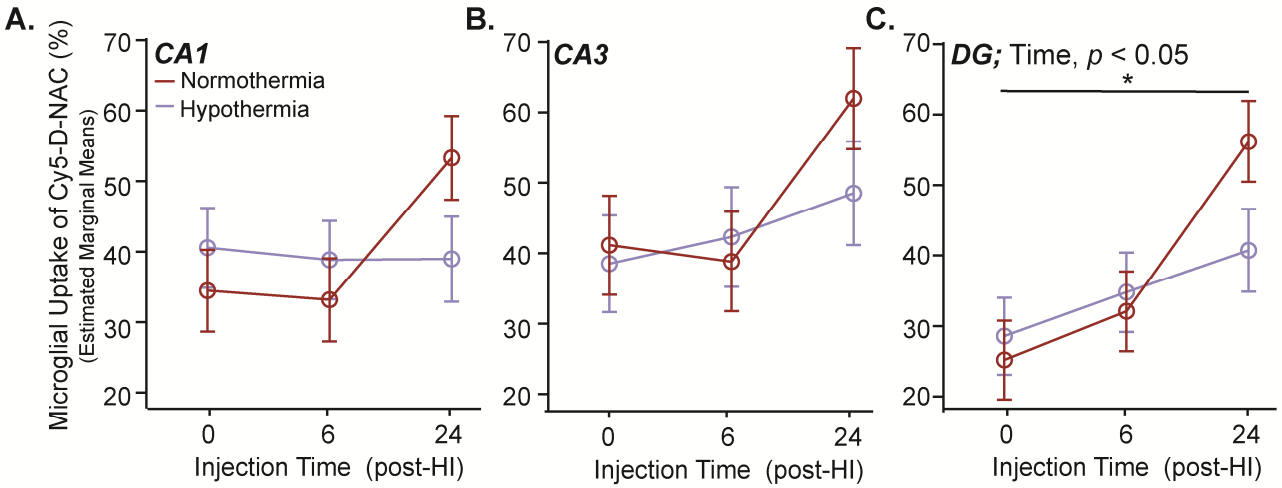

Figure S5. Microglial uptake of Cy5-D-NAC, by hippocampal region. Patterns of microglial uptake of Cy5-D-NAC are shown for each hippocampal region analyzed. No effects of injection time or temperature were detected in CA1 (A). (B) In contrast, in the CA3 there was a trend towards an effect of injection time on uptake (p = 0.07). (C) In the DG, there was a significant effect of injection time on Cy5-D-NAC uptake ($F_{2,71}$ = 7.01, $p < 0.05$). Further contrast analysis reveals that microglial uptake was significantly greater in the DG after injection at 24 h than at 0 h, regardless of hypothermic treatment. Estimated marginal means ± s.e.m. are shown.

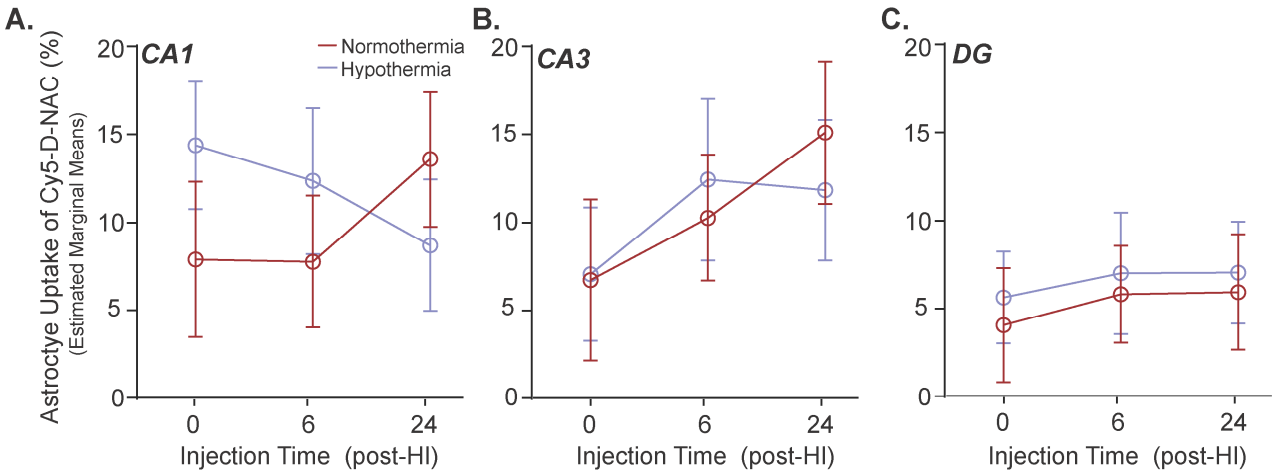

Figure S6. Astrocyte uptake of Cy5-D-NAC, by hippocampal region. Patterns of astroctye uptake of Cy5-D-NAC are shown for each hippocampal region. Cy5-D-NAC was detected in astrocytes in each region of the hippocampus; however, no significant effects of injection time or temperature were detected in the CA1 (A), CA3 (B), or the DG (C). Estimated marginal means ± s.e.m. are shown

Table S2.

Summary of cell counts and Cy5-D-NAC-positive cells, by cell type. (mean ± s.e.m.)

	Microglia (Iba1+)				Neurons (NeuN+)				Astrocytes (GFAP+)			
	Normothermic		Hypothermic		Normothermic		Hypothermic		Normothermic		Hypothermic	
	Total	Cy5-D-NAC+	Total	Cy5-D-NAC+	Total	Cy5-D-NAC+	Total	Cy5-D-NAC+	Total	Cy5-D-NAC+	Total	Cy5-D-NAC+
0 h	223.25 ± 29.5	73.92 ± 18.9	233.62 ± 26.5	100.31 ± 15.3	1025.54 ± 48.5	30.77 ± 11.0	812.4 ± 63.2	18.07 ± 4.0	163.63 ± 27.0	12.00 ± 6.0	114.07 ± 15.7	12.64 ± 3.4
6 h	320.17 ± 28.7	92.17 ± 21.7	276.23 ± 38.5	116.92 ± 27.6	966.92 ± 39.0	11.31 ± 3.1	925.82 ± 42.2	17.91 ± 4.9	158.08 ± 23.1	11.92 ± 4.4	143.22 ± 22.8	15.44 ± 5.3
24 h	392.08 ± 62.2	189.33 ± 44.8	388.09 ± 46.0	163.09 ± 24.5	903.69 ± 42.3	22.15 ± 5.1	837.36 ± 83.0	8.27 ± 2.4	144.75 ± 28.7	15.17 ± 5.1	157.40 ± 32.0	19.60 ± 9.9