

Pre-conditioning induces the precocious differentiation of neonatal astrocytes to enhance their neuroprotective properties

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ABSTRACT

Hypoxic preconditioning reprogrammes the brain's response to subsequent H/I (hypoxia–ischaemia) injury by enhancing neuroprotective mechanisms. Given that astrocytes normally support neuronal survival and function, the purpose of the present study was to test the hypothesis that a hypoxic preconditioning stimulus would activate an adaptive astrocytic response. We analysed several functional parameters 24 h after exposing rat pups to 3 h of systemic hypoxia (8% O₂). Hypoxia increased neocortical astrocyte maturation as evidenced by the loss of GFAP (glial fibrillary acidic protein)-positive cells with radial morphologies and the acquisition of multipolar GFAP-positive cells. Interestingly, many of these astrocytes had nuclear S100B. Accompanying their differentiation, there was increased expression of GFAP, GS (glutamine synthetase), EAAT-1 (excitatory amino acid transporter-1; also known as GLAST), MCT-1 (monocarboxylate transporter-1) and ceruloplasmin. A subsequent H/I insult did not result in any further astrocyte activation. Some responses were cell autonomous, as levels of GS and MCT-1 increased subsequent to hypoxia in cultured forebrain astrocytes. In contrast, the expression of GFAP, GLAST and ceruloplasmin remained unaltered. Additional experiments utilized astrocytes exposed to exogenous dbcAMP (dibutyryl-cAMP), which mimicked several aspects of the preconditioning response, to determine whether activated astrocytes could protect neurons from subsequent excitotoxic injury. dbcAMP treatment increased GS and glutamate transporter expression and function, and as hypothesized, protected neurons from

glutamate excitotoxicity. Taken altogether, these results indicate that a preconditioning stimulus causes the precocious differentiation of astrocytes and increases the acquisition of multiple astrocytic functions that will contribute to the neuroprotection conferred by a sublethal preconditioning stress.

Key words: cell death, glutamate, glutamine synthetase, excitotoxicity, stroke, transporter.

INTRODUCTION

Preconditioning refers to a paradigm whereby exposing cells or an organ or organism to a sublethal insult provides protection against a subsequent insult that would normally produce injury. Although both acute and long-term preconditioning have been demonstrated, several studies have confirmed that the protective effect of an episode of hypoxic preconditioning occurs when the interval between the preconditioning event and the subsequent lethal event is ~24 h. Hypoxic preconditioning in the neonatal rat (8% O₂, for 3 h) was originally shown to provide tolerance to H/I (hypoxia–ischaemia) brain injury in the neonatal rat brain: this phenomenon has subsequently been demonstrated for the adult brain (Gidday et al., 1994; Vannucci et al., 1998; Bergeron et al., 2000; Jones and Bergeron, 2001). Since preconditioning provides dramatic neuroprotection there is much interest in understanding the molecular mechanisms

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Abbreviations: CNS, central nervous system; CP, ceruloplasmin; dbcAMP, dibutyryl-cAMP; DMEM, Dulbecco's modified Eagle's medium; EAAT-1, excitatory amino acid transporter-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; HBSS, HEPES-buffered salt solution; H/I, hypoxia–ischaemia; IL-1, interleukin-1; LDH, lactate dehydrogenase; MCT-1, monocarboxylate transporter-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; P6, postnatal day 6; TBS, Tris-buffered saline.

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underlying the cell retention that is achieved in order that it might be mimicked using pharmacological agents. Several studies have shown that hypoxic exposure alters gene expression and that it activates several intracellular signalling pathways, some of which may contribute to the adaptive responses observed after hypoxia (Semenza, 2000; Bernaudin et al., 2002; Ran et al., 2005).

Many studies seeking to identify mechanisms of hypoxic preconditioning-induced adaptive responses have focused on the neuronal response to this insult. For instance, investigators have shown increased levels of anti-apoptotic Bcl-2, increased levels of HIF-1 (hypoxia-inducible factor-1)-regulated genes, and an increased capacity of neurons to sequester calcium (Gidday, 2006). However, astrocytes serve many essential physiological functions in the CNS (central nervous system) on which neurons depend for their survival and peak functional performance. These include inducing the formation and maintenance of the blood-brain barrier, clearing extracellular potassium that accumulates with neuronal activity and collecting and metabolizing excitatory amino acids (Sofroniew and Vinters, 2010). Astrocytes also store energy in the form of glycogen that can be metabolized to pyruvate for their own survival or to lactate for export to neurons for additional fuel during energy crises, such as during H/I (Swanson and Choi, 1993). Astrocytes also produce enzymes to detoxify metals and xenobiotics (Sofroniew and Vinters, 2010). Additionally astrocytes, and especially perivascular astrocytes, express ceruloplasmin, which is a multifunctional protein that serves as an amine oxidase, an antioxidant and a ferroxidase. Ceruloplasmin expression increases when astrocytes become activated (Kuhlow et al., 2003). A subset of these astroglial functions are induced in adult astrocytes in response to hypoxic or chemical preconditioning (Romera et al., 2004, 2007; Kawahara et al., 2005; Hoshi et al., 2006, 2010; Yamada et al., 2006; Zhang et al., 2007; Weller et al., 2008; Yu et al., 2008). Therefore we were interested in establishing whether immature astrocytes would respond to a preconditioning event by enhancing their expression of the proteins that support these essential support functions, which, in turn, would enable them to better support neurons to survive a subsequent lethal excitotoxic challenge.

To test this hypothesis, we studied the status of multiple indicators of the astrocytic physiological state, including GFAP (glial fibrillary acidic protein), the neurotrophic, calcium-binding protein, S100b, GS (glutamine synthetase), EAAT-1 (excitatory amino acid transporter-1; also known as GLAST), MCT-1 (monocarboxylate transporter-1) and CP (ceruloplasmin) levels 24 h after a 3 h hypoxic exposure (a time corresponding to the beginning of a subsequent H/I insult in previous preconditioning experiments). We were particularly interested in evaluating GS and GLAST as indices of glial glutamate handling capacity, as levels of glutamate have been shown to increase to as high as 500 μ M after H/I in the newborn and neurons are extremely vulnerable to excitotoxic death in the immature brain (Hagberg et al.,

1993). To determine whether these changes were direct responses of astrocytes to hypoxia, we evaluated the effects of hypoxia on enriched cultures of forebrain astrocytes. Finally, astrocytes exposed to exogenous dbcAMP (dibutyryl-cAMP) – which mimics some but not all of the preconditioning responses – were utilized to determine whether alterations in glutamate handling would influence neuronal survival. Altogether, our results indicate that a preconditioning stimulus elevates multiple astrocytic functions that endow them with an enhanced capacity to protect neurons from a subsequent challenge.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals and laboratory reagents were purchased from either Fisher Scientific (Pittsburgh, PA, U.S.A.) or Sigma (St. Louis, MO, U.S.A.). Eagle's minimum essential media and DMEM/F12 [DMEM (Dulbecco's modified Eagle's medium) with nutrient F12 mixture in the ratio 1:1] were purchased from Mediatech/Life Technologies (Fisher Scientific). Fetal bovine serum was purchased from Tissue Culture Biologicals (Tulare, CA, U.S.A.). Calf serum was purchased from Hyclone (Logan, UT, U.S.A.). Neurobasal and B27 supplements were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Cytosine β -D-arabinofuranoside and dbcAMP were purchased from Sigma. Rabbit polyclonal antibodies against cow GFAP (catalogue no. Z0334) were purchased from Dako (Carpinteria, CA, U.S.A.). Mouse anti-S100b was purchased from Sigma (catalogue no. SAB1402349). Mouse monoclonal antibody against GS (catalogue no. MAB302) was purchased from Millipore (Temecula, CA, U.S.A.). Rabbit polyclonal anti-GS antibodies were purchased from Sigma (catalogue no. G2781), mouse monoclonal anti-EAAT-2 antibodies were purchased from BD Biosciences (catalogue no. 611654; San Jose, CA, U.S.A.) and mouse monoclonal anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were purchased from Millipore (catalogue no. MAB374). Mouse anti-CP was purchased from BD (catalogue no. 611488). Secondary antibodies for immunofluorescence were donkey anti-rabbit Dylight 488 or donkey anti-mouse IgG Dylight 549 from Jackson ImmunoResearch (West Grove, PA, U.S.A.). TRIZOL[®] was purchased from Molecular Research (Bethesda, MD, U.S.A.), and reverse transcriptase reaction reagents, buffers and enzymes were purchased from Sigma or Qiagen (Valencia, CA, U.S.A.). D-[³H]aspartate and [³⁵S]UTP were purchased from New England Nuclear (Boston, MA, U.S.A.).

Hypoxic preconditioning

Wistar rat pups at P6 (postnatal day 6, where P1 is the day of birth) underwent hypoxic preconditioning as described

previously (Gidday et al., 1994; Vannucci et al., 1998). Control animals were maintained at 37°C for 3 h under atmospheric conditions. To determine the effect of a subsequent H/I episode, P7 rat pups underwent permanent unilateral (right) carotid artery ligation, followed 2 h thereafter by 60 min of systemic hypoxia as described previously (Vannucci and Vannucci, 2005). The interval from the onset of hypoxic preconditioning to the onset of cerebral H/I was 24 h and the animals were allowed to survive for an additional 24 h. All experiments on animals were carried out in accordance with institutional guidelines and subsequent to IACUC committee approvals, and the present authors further attest that all efforts were made to minimize the number of animals used and their suffering.

In situ hybridization

Animals were anaesthetized with a mixture of ketamine (75 mg/kg) and xylazine (5 mg/kg) prior to intracardiac perfusion with 4% (w/v) paraformaldehyde. Brains were postfixed with 4% paraformaldehyde overnight and then cryoprotected for at least 24 h in 30% sucrose in 0.1 M phosphate buffer (pH 7.4). The brain samples were frozen in embedding medium (O.C.T.; Sakura Finetek, Torrance, CA, U.S.A.) on a solid CO₂/ethanol slush. Brains were sectioned at 12 µm and thaw mounted on to SuperfrostPlus™ slides and then placed at -80°C. *In situ* hybridization using a ³⁵S-labelled riboprobe for GFAP was performed as previously described (Vannucci et al., 1998).

Immunofluorescence

Vibratome sections (50 µm) were cut on a Ted Pella 1000 series vibratome, incubated in 0.2% Triton X-100 in TBS (Tris-buffered saline; pH 7.4) for 30 min and then blocked for 1 h with 10% (v/v) donkey serum, 10% (w/v) BSA and 0.05% Triton X-100 in TBS. Sections were incubated in mouse anti-S100b (Sigma) and rabbit anti-GFAP (Dako; diluted 1:500) and incubated for 24 h at 4°C, followed by extensive rinses in TBS containing 1.5% NaCl and 0.05% Triton X-100. Secondary antibodies were incubated for 24 h at 4°C (diluted 1:400). The secondary antibodies were carefully examined to ensure that there was no cross-talk between fluorescent dyes or cross-reactivity between secondary antibodies. No signal above background was obtained when the primary antibodies were replaced with pre-immune sera. The sections were then washed, counterstained with DAPI (4',6'-diamidino-2-phenylindole; 1 µg/ml; Sigma) for 5–10 min and coverslipped with Fluorogel (Electron Microscopy Sciences, Hatfield, PA, U.S.A.). Then 5 µm z-stacks were captured using a Zeiss LSM-510 microscope. Z-series projections were generated, rotated by 10° and assembled in a montage using Adobe Photoshop CS-2.

Astrocyte cultures

Primary astrocyte cultures were prepared from 1–2-day-old rat pups using standard methods (Levison and McCarthy,

1991). For the experiments described in Figure 3, enriched astrocyte cultures were prepared using the shaking method and then transferred to a chemically defined medium for 5 days to enhance their maturation (S.W. Levison, unpublished data). On day 6, the astrocytes were subjected to hypoxia (8% O₂) for 3 h, in a Forma O₂/CO₂ incubator (Forma Scientific, Marietta, OH, U.S.A.) and then maintained under atmospheric conditions (21% O₂/5% CO₂) for 24 h. For the experiments described in Figures 4–6, astrocyte cultures were prepared as described by Hamby et al. (2006), save for the use of EGF (epidermal growth factor) and antibiotics. Once the astrocytes reached confluence, cultures were treated once with 8 µM cytosine β-D-arabinofuranoside for 4–8 days to substantially reduce microglial contamination. Thereafter, cultures were maintained in growth medium (Hamby et al., 2006).

For activated astrocyte studies, monolayers of primary rat astrocytes were maintained for 7–8 days in growth medium alone or in growth medium containing 250 µM dbcAMP, both of which were replenished every 2–3 days prior to experimentation.

Neuronal cultures

Neuronal cultures were prepared by enzymatically and mechanically dissociating cortices of E15 (embryonic day 15) mouse cerebrum and plating the resulting cells at 6 hemispheres per plate in neurobasal medium supplemented with B27 and 50 i.u. (international units)/ml penicillin/50 µg/ml streptomycin. Glutamine (1.5 mM) was added just prior to use. At 2 days after plating, cultures were treated once with 1 µM β-D-arabinofuranoside for an additional 2 days. The medium was partially replenished (1/2 volume exchange) twice per week and on the day of experimentation [DIV (days *in vitro*) 8]. All cultures were kept at 37°C in a humidified 6% CO₂-containing atmosphere.

Immunoblotting

Western blots from tissue homogenates

First, 10–15 µg of protein was separated on 7% Tris/acetate polyacrylamide gels electrophoresed at 150 V for 80 min and transferred at 300 mA for 80 min to nitrocellulose membranes. The membranes were stained with 0.1% Ponceau S in 5% acetic acid to confirm proper transfer of proteins. Then, membranes were blocked for 1 h in 10% non-fat dried skimmed milk diluted in PBS-T (0.05% Tween 20 in PBS). Membranes were incubated overnight at 4°C in primary antibody diluted in 1% BSA/PBS-T. After incubation with the primary antibody, the blots were extensively washed with PBS-T for 30 min and then incubated for 1.5 h at room temperature (20°C) with secondary antibody conjugated with horseradish peroxidase diluted in 1% BSA/PBS-T. The membranes were then washed extensively in PBS-T for 30 min prior to visualization using Renaissance™ chemiluminescence (NEL104; NEN Life Science, Boston, MA, U.S.A.).

Blots were probed with rabbit anti-EAAT-1 (1:1000; Alpha Diagnostic, San Antonio, TX, U.S.A.), mouse anti-GS (1:2000; Millipore) or rabbit anti-GFAP (1:5000; Dako), rabbit anti-ceruloplasmin (1:10000; Dako) or rabbit anti-MCT-1 [1:8000; a gift from Dr Ian Simpson (Department of Neural and Behavioral Sciences, Penn State College of Medicine, Hershey, PA, U.S.A.) (Vannucci and Simpson, 2003)]. The blots were visualized using the RenaissanceTM chemiluminescence reagent from NEN. Images were obtained and quantified using a UVP imaging system with LabWorks software (UVP, Upland, CA, U.S.A.).

Western blots from astrocyte cultures

Cultures grown in 15 mm multi-well dishes (Falcon Primaria, Becton Dickinson, Lincoln Park, NJ, U.S.A.) were washed with 0.4 ml of ice-cold PBS. Then, 50–100 μ l of lysis buffer containing 1% Nonidet P40, 5 mM EDTA, 5 mM iodoacetamide and 1 \times CompleteTM protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) in TBS was added to each well. Plates were placed on ice for 30 min while rocking and cells were harvested by gentle scraping. Cellular debris was pelleted (10000 *g*, 10 min and 4°C) and cell lysates were stored at –20°C. Protein concentration was determined using the BCA assay (Pierce, Rockford, IL, U.S.A.). First, 5–15 μ g of protein was separated by SDS/10% PAGE under reducing conditions and electrophoretically transferred to nitrocellulose. Membranes were washed twice with water (20 ml, 5 min) and proteins of interest were detected using the primary antibodies at 1 μ g/ml visualized using species-specific WesternBreeze Immunodetection kits (Invitrogen) as per the manufacturer's instructions. Results were recorded on an X-ray film (Fujifilm, Tokyo, Japan). Digitized images were analysed by computer-assisted densitometry (Gel-Pro analyzer; Media Cybernetics) and protein levels were normalized to their respective GAPDH levels.

Measurement of excitatory amino acid transport

D-[³H]aspartate was used as the substrate for glutamate transporters as described and characterized previously (Vidwans and Hewett, 2004). Cells were washed twice with HBSS (Hepes-buffered salt solution; 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 20 mM Hepes, 15 mM glucose and 0.01 mM glycine) and rocked for 10 min at room temperature. The buffer was then fully exchanged with HBSS containing D-[³H]aspartate (final concentration: 0.1 μ Ci/ml) and 50 μ M of unlabelled aspartate as a carrier. Uptake was terminated after 3 min by washing three times with ice-cold choline stop buffer containing 116 mM choline chloride, 0.8 mM MgSO₄, 1 mM KH₂PO₄, 10 mM Hepes, 5 mM KOH, 10 mM glucose, 0.9 mM CaCl₂ and 5 mM of non-radioactive D-aspartate (4°C). This time point was within the linear range of uptake for both dbcAMP-treated and -untreated cultures (results not shown). Culture wells were subsequently aspirated dry and cells were lysed by the addition of 400 μ l

of warm 0.2% SDS. The amount of accumulated radioactivity was estimated in 50% of the cell lysate via liquid scintillation counting (Packard TriCarb 4000 scintillation counter). Total protein content per well was determined using the BCA assay (Pierce). Readings of c.p.m. are expressed as c.p.m./mg of protein.

Toxicity bioassay

Astrocytes were washed twice and then incubated for 45–90 min with a medium containing glutamate: the final well volume equalled 75 or 100 μ M. Culture medium from six wells was removed, pooled and then combined with the neuronal plating medium and added to the pure neuronal cultures for the next 16–18 h. Cell viability was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] reduction assay (Lobner, 2000), with minor modifications. MTT (3 mg/ml in PBS) was added to each well and cultures were placed in a humidified, 6% CO₂-containing incubator at 37°C. At least 4 h later, the medium was aspirated and 400 μ l of acidic propan-2-ol was added to dissolve the formazan salt. A 200 μ l portion was transferred to a 96-well plate and attenuation was measured at 540 nm using a reference wavelength of 690 nm (Spectramax Plus³⁸⁴; Molecular Devices). Neuronal cell death was measured in parallel from the same cells by determination of levels of LDH (lactate dehydrogenase) released into the cell culture medium prior to the addition of MTT (Uliasz and Hewett, 2000). Data are expressed as a percentage of total neuronal LDH activity (defined as 100%), which was determined in each experiment by assaying the supernatant of parallel cultures exposed for 16–18 h to 75 or 100 μ M glutamate.

Statistical analyses

Data were analysed using one-way ANOVA followed by the Student–Newman Keul's test or the Student's *t* test to detect significant differences between two means with $P < 0.05$.

RESULTS

To assess how hypoxic preconditioning might affect the astrocytes in the immature brain, we first performed immunofluorescence for GFAP and for S100b. In the neocortex of control animals, GFAP staining was sparse and those cells that were GFAP immunoreactive in the gray matter had long radial processes (Figures 1A and 1C). A subset of these cells was S100b immunoreactive (Figures 1B and 1C). In the animals exposed to 3 h of hypoxia 24 h earlier, the GFAP immunoreactive cells were now more intensely immunoreactive and they possessed multipolar morphologies (Figures 1D and 1F). S100b immunoreactivity also was markedly increased, and in most of S100b-immunoreactive

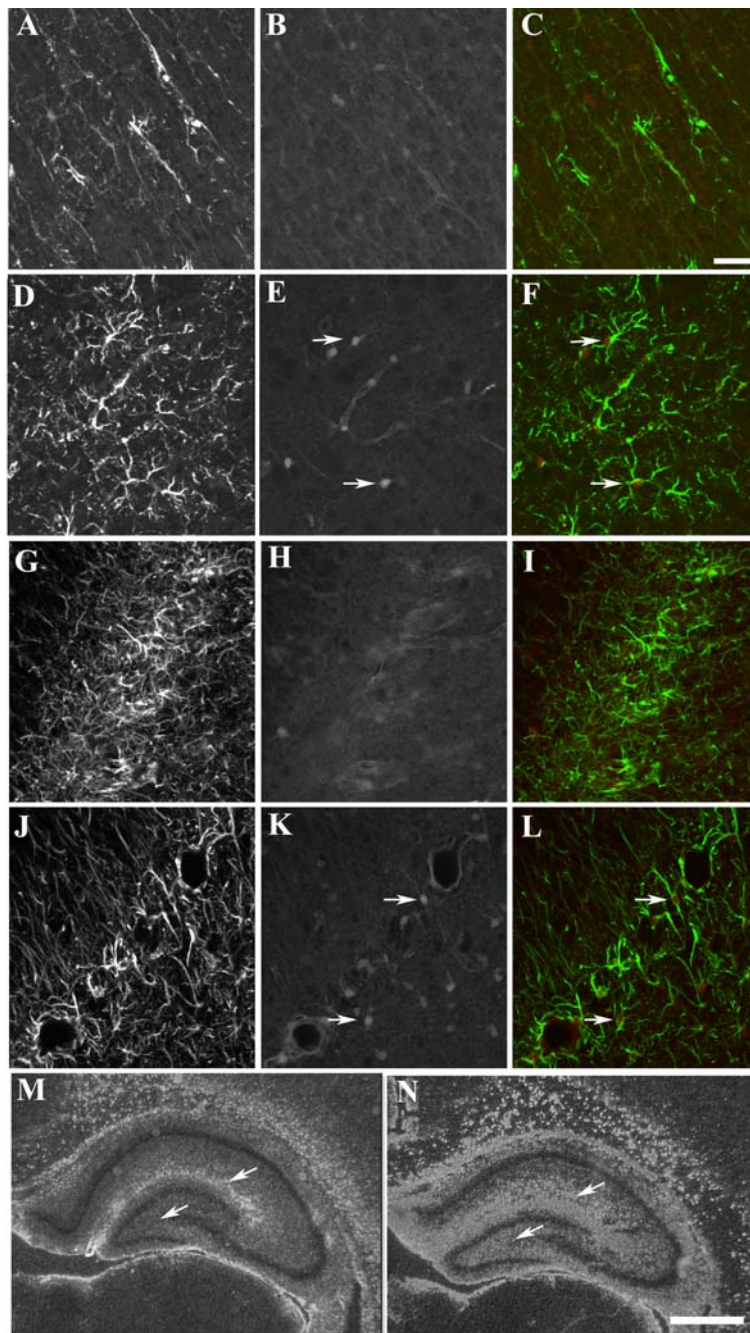


Figure 1 Increase in GFAP and S100B expressions in the preconditioned neonatal rat brain
 Vibratome sections from normal (A–C, G–I, M) and preconditioned (D–F, J–L, N) brains at the level of the hippocampus were processed for GFAP (green) immunofluorescence in combination with S100B (red) from neocortex (A–F) and hippocampal CA2 regions (G–L). Images are Z-stacks from 1 μ m confocal Z-sections through 5 μ m. Arrows in (E, F, K, L) point to S100B immunoreactive astroglial nuclei. Other sections were processed for *in situ* hybridization using a 35 S-labelled riboprobe for GFAP, 24 h after exposure to hypoxia. Arrows in (M, N) point to the dentate gyrus and CA2 regions of the hippocampus where the intensity of GFAP mRNA expression, shown here as white silver grains from the *in situ* hybridization, was significantly higher in the preconditioned brains. Scale bars: (A–L) 50 μ m and (M, N) 1 mm.

cells the S100b was located in the nucleus (Figures 1E and 1F). GFAP and S100b immunoreactivity were also increased in the CA1–3 layers of the hippocampus and in the dentate gyrus of the hippocampus (compare Figures 1G and 1I with

Figures 1J and 1L). Again, S100b was nuclear and not cytoplasmic (Figures 1H–1L). Reflecting the increase in GFAP immunoreactivity, the expression of GFAP mRNA as demonstrated by increased hybridization signal was increased

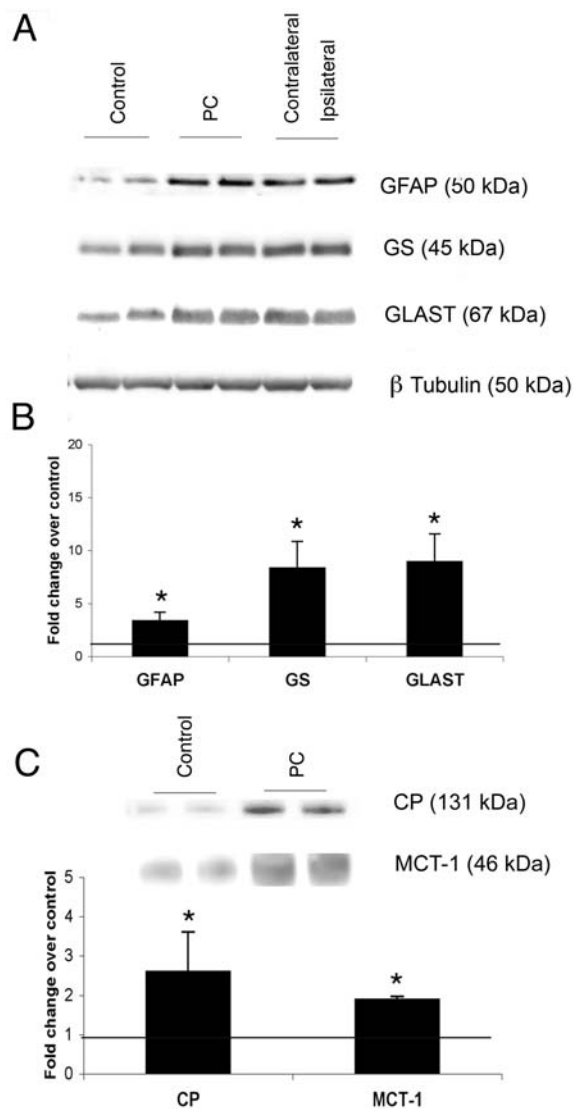


Figure 2 Effect of hypoxic preconditioning on GFAP, GS, GLAST, ceruloplasmin and MCT-1 expression levels in the neonatal rat brain

(A) GFAP, GS and GLAST expression was analysed by Western blotting, with protein samples extracted from a wedge-shaped section of preconditioned brains composed of the neocortex, white matter and a portion of the striatum as described previously (Vannucci et al., 2004). Equivalent regions of the ipsilateral and contralateral hemispheres of preconditioned animals subjected to H/I for 60 min and control animals were analysed. Blots were re-probed for β -tubulin to establish equal protein loading. (B) Densitometric measurements were carried out on individual immunoblots for each antibody tested and values represent the means \pm S.E.M. for six animals. The solid line represents the normalized values of the controls. (C) Western-blot analysis of CP and MCT-1. The fold change in expression levels of CP and MCT-1 in preconditioned animals over the control is shown. * $P < 0.05$ using the Student's *t* test.

in the hippocampus and the overlying white matter (Figures 1M and 1N). Sections were also stained for GS, but it was difficult by immunofluorescence to detect changes in GS (results not shown).

Western blotting showed that there was a 3-fold increase in the level of GFAP and an 8-fold increase in the level of GS compared with controls 24 h after hypoxic preconditioning

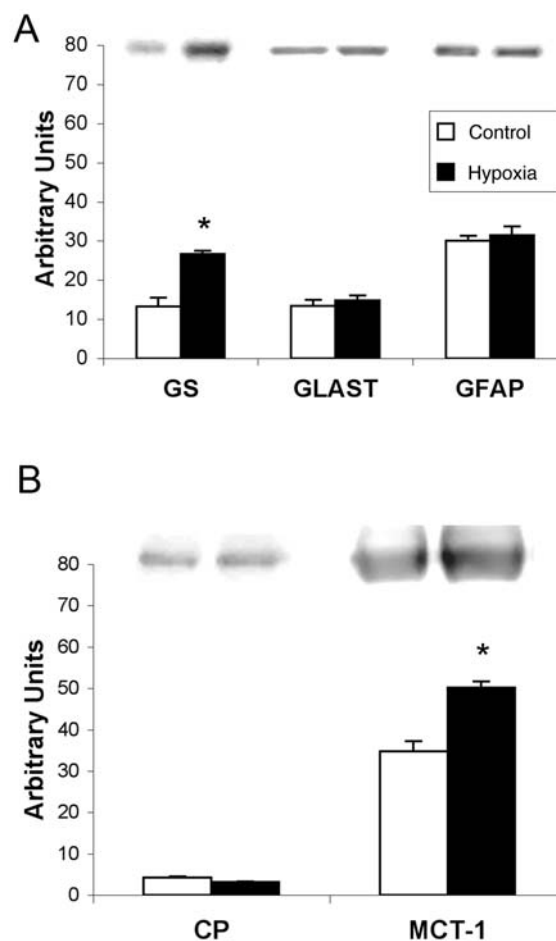


Figure 3 Effect of hypoxia in regulating the expression of astrocyte-specific enzymes and transporters is selective *in vitro*

Western blot showing the expression of GFAP, GS and GLAST (A) and CP and MCT-1 (B) in astrocytes exposed to either 21% O₂/5% CO₂ (atmospheric condition) or 8% O₂ (hypoxic condition) for 3 h *in vitro*. Results are representative of results obtained from three independent experiments where each condition was evaluated in duplicate. Densitometric measurements were carried out on individual immunoblots for each antibody tested and the values represent the means \pm S.E.M. for three separate experiments. * $P < 0.05$ by the Student's *t* test.

(Figures 2A and 2B). Whereas preconditioning *in vivo* is viewed as a milder insult that, by definition, does not damage the brain, prolonged H/I produces cell death and causes significant astrocyte activation. To evaluate the extent of glial activation, we exposed a group of preconditioned animals to an additional episode of 60 min of H/I. Contrary to our expectations, there was no additional increase in the expression of GFAP and GS in brains from preconditioned animals that were subsequently subjected to H/I (Figures 2A and 2B).

As glutamate transporters are essential for buffering extracellular glutamate, thereby protecting neurons from excitotoxic stresses under both physiological and pathological conditions, we next tested whether the preconditioning paradigm induced the glutamate transporter EAAT-1 (GLAST). GLAST, like GS, was induced 8-fold in the brains of

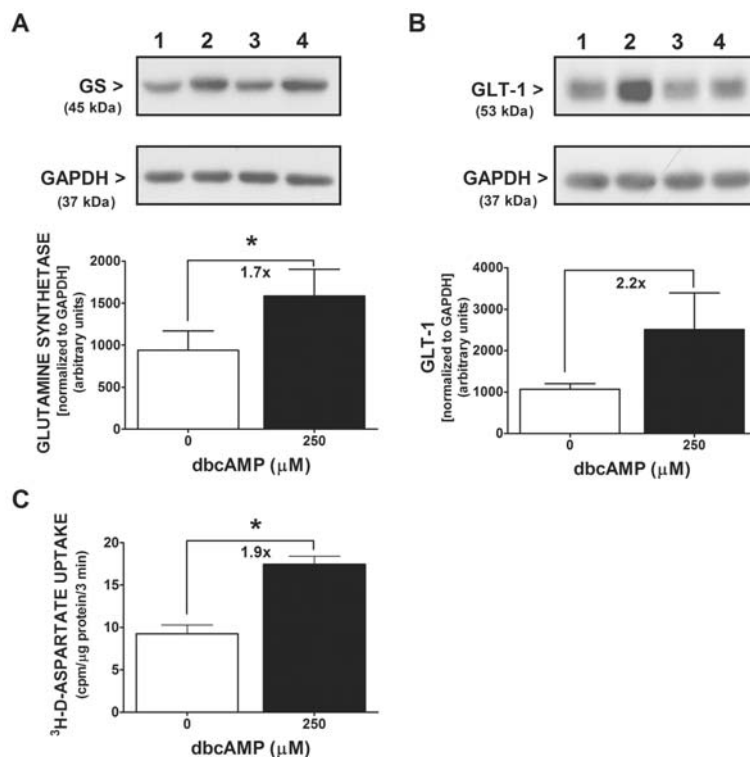


Figure 4 dbcAMP increases astrocytic glutamate uptake and metabolism proteins

Rat astrocytes were treated with a medium alone (lanes 1 and 3) or with a medium containing 0.25 mM dbcAMP (lanes 2 and 4) for 7 days. Then, 5 μg (A) or 15 μg (B) of protein was analysed by Western blotting for GS (A) and GLT-1 (B). Densitometric measurements were carried out on individual immunoblots for each antibody tested. Values represent the means ± S.E.M. for eight individual experiments. Values below the bars represent the mean fold increase in protein expression that followed dbcAMP treatment. * $P < 0.05$ by the unpaired Student's *t* test following Welch's correction for unequal variances. (C) Astrocyte cultures were treated with dbcAMP (0.25 mM) for 8 days. The uptake of D-³H]aspartate (0.1 μCi/ml) was measured at 3 min. Results shown are expressed as c.p.m./μg of protein per 3 min and are the means ± S.E.M. ($n = 12$ culture wells from three separate experiments). The value above the bar represents the mean fold increase of D-³H]aspartate uptake after treatment with dbcAMP. * $P < 0.05$ by the unpaired Student's *t* test ($P < 0.05$).

preconditioned animals as compared with naïve controls (Figures 2A and 2B). As seen with GFAP and GS, the level of GLAST in the hemispheres of preconditioned animals subjected to H/I for 60 min was comparable to the induced level of GLAST in the animals exposed to the preconditioning stimulus (Figure 2A).

Astrocytes maintain high intracellular concentrations of certain antioxidants and play an important role in antioxidant defences in the brain. As oxidative injury is an important component of H/I cell death, we assessed whether the antioxidant potential of astrocytes might be altered by a preconditioning stimulus. Indeed, a 2-fold increase in the expression of ceruloplasmin was observed in the preconditioned neonatal rat brain as compared with the normal control (Figure 2C). As levels of lactate increase during H/I and acidosis can be neurotoxic, we analysed the expression of MCT-1. MCT-1 levels increased by 2-fold in neonatal rat brains subjected to hypoxic preconditioning at 24 h of recovery as compared with naïve control (Figure 2C).

To determine whether these changes are a direct response of astrocytes to hypoxia, we placed highly enriched cultures of

forebrain type 1 astrocytes (A2B5-negative/GFAP-positive) produced from the neonatal rat brain into a reduced oxygen environment (8% O₂) for 3 h and analysed protein expression 24 h after returning the cells to an atmospheric environment. Western-blot analyses revealed a 2-fold increase in the levels of GS (Figure 3A) as well as MCT-1 in the astrocytes subjected to hypoxia as compared with the control (Figure 3B). Interestingly, the expression of GFAP, GLAST and ceruloplasmin did not change after hypoxia (Figures 3A and 3B), indicating that some, but not all, of the responses observed in the preconditioned brain may occur in a cell-autonomous manner.

To determine whether activated astrocytes would protect neurons from a subsequent glutamate challenge, we assessed their excitatory amino acid buffering capabilities. For these studies, astrocytes were treated with dbcAMP, which has been shown to increase astrocytic morphological complexity, GFAP and vimentin immunoreactivity as well as other functional properties of activated astrocytes (Fedoroff et al., 1984; Eddleston and Mucke, 1993). dbcAMP was chosen since the *in vitro* hypoxia was insufficient to induce glutamate transporter expression, whereas dbcAMP is known to do so (Gegelashvili

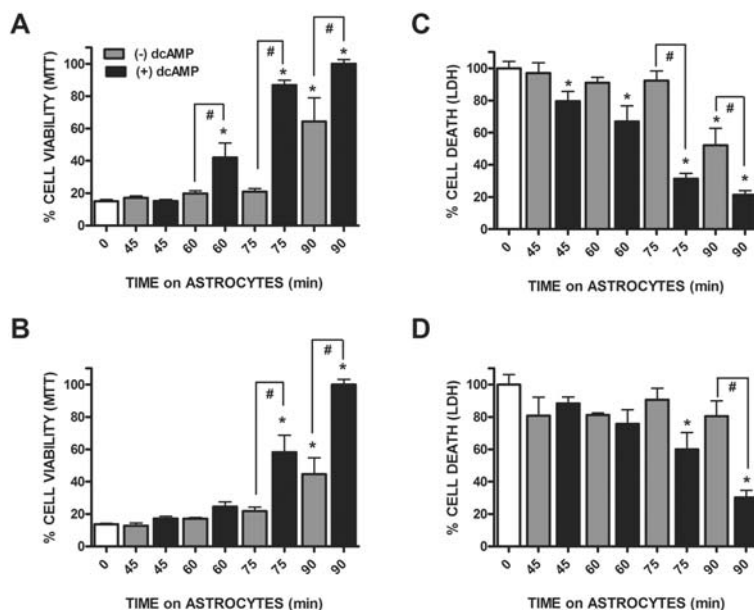


Figure 5 **dbcAMP-preconditioned astrocytes have an enhanced capacity to protect neurons from glutamate excitotoxicity**
 Rat astrocytes were treated with a medium alone [(-)dbcAMP] or with a medium containing 0.25 mM dbcAMP [(+) dbcAMP] for 8 days. Glutamate was added to the astrocytes for 45–90 min after which the buffer was collected and transferred to highly enriched murine neuronal cultures. After 16–18 h, neuronal cell viability was determined using the MTT (A, B) or LDH assay (C, D) ($n=4-6$ from three separate experiments). An asterisk indicates a value significantly different from the control (0 min), which were cultures exposed to a final concentration of 75 μ M (A, C) or 100 μ M (B, D) glutamate for 16–18 h. The symbol '#' indicates a significant between-group difference. Results were analysed using one-way ANOVA followed by the Student–Newman–Keul's test after an appropriate transformation of percentile data ($P<0.05$).

et al., 1996; Swanson et al., 1997). Astrocytes stimulated with dbcAMP adopted a stellate morphology that was accompanied by a 1.7-fold increase in GS and a 2.2-fold increase in GLT-1 expression (Figures 4A and 4B). This increase in glutamate clearing proteins was accompanied by a 1.9-fold increase in functional excitatory amino acid uptake as measured using D- $[^3\text{H}]$ aspartate (Figure 4C). dbcAMP did not increase levels of CP in these rat astrocyte cultures (Supplementary Figure S1 available at <http://www.asnneuro.org/an/003/an003e062add.htm>).

To determine whether the dbcAMP-stimulated astrocytes would prevent neuronal cell death on challenge using an excitotoxic stimulus, HBSS containing glutamate was added to the astrocytes for 45–90 min, after which the buffer was collected and transferred to pure neuronal cultures. After 16–18 h, neuronal cell viability was determined using MTT (Figures 5A and 5B) and LDH (Figures 5C and 5D) assays. Highly enriched neuron cultures exposed to glutamate-containing medium transferred from dbcAMP-treated astrocytes were two to four times less likely to die than cells exposed to glutamate-containing medium transferred from untreated astrocytes (Figure 5). Of note, this protective effect was both time and concentration dependent such that higher concentrations (Figures 5B and 5D) of glutamate took a longer time to become detoxified than lower concentrations (Figures 5A and 5C). For example, only 60% of the neurons were viable as assessed by the MTT assay after being incubated with a medium that originally contained 100 μ M glutamate and was detoxified

by the dbcAMP-treated astrocytes for 75 min, whereas 90% of the neurons were viable when 75 μ M glutamate was used (Figures 5B and 5D compared with Figures 5A and 5C).

DISCUSSION

Multiple pathogenetic mechanisms contribute to H/I injury to the brain, such as inadequate blood flow autoregulation, elevated extracellular excitatory amino acids, altered cerebral metabolism, accumulation of toxic metabolites, elevated intracellular calcium, release of cytokines and prostaglandins, iron accumulation and overproduction of free radicals. Hypoxic preconditioning can mitigate the detrimental effects of H/I on neurons by inducing endogenous adaptive mechanisms that can protect the perinatal brain from subsequent injury. Many *in vivo* and *in vitro* studies were designed to determine how preconditioning induces adaptive responses in neurons. Despite the importance of astrocytes in maintaining brain homeostasis, fewer studies have been conducted to distinguish the direct effects of hypoxic preconditioning on astrocytes, which might then provide neuroprotection. Of those studies that have evaluated astroglial responses to preconditioning stimuli, except for a few (Brucklacher et al., 2002; Jones and Bergeron, 2004;

Cimarosti et al., 2005), most have studied astrocytes in the adult brain (Trendelenburg and Dirnagl, 2005). Therefore the present study was designed to determine how immature astrocytes would respond to a preconditioning stimulus and to test the hypothesis that preconditioning paradigms induce adaptive responses from astrocytes, enabling them to protect neurons from the assortment of noxious signals generated after a more severe hypoxic episode.

Our results on preconditioning-induced tolerance provide several novel insights: (i) hypoxic preconditioning leads to the precocious differentiation of neocortical astrocytes; (ii) preconditioning induces nuclear S100B, which has been implicated in glial differentiation; (iii) preconditioning increases the expression of astrocytic GS and GLAST, mediators crucial for detoxifying extracellular glutamate; (iv) preconditioning induces the expression of ceruloplasmin and MCT-1, thereby enhancing lactate transport and antioxidant functions of astrocytes respectively; (v) hypoxic preconditioning strongly activates astrocytes since no further activation was solicited after a subsequent exposure to 60 min of H/I; (vi) a subset of these responses are cell autonomous as they are elicited by exposing astrocytes *in vitro* to hypoxia; (vii) changes in glutamate transporter expression correlate with the buffering capability of astrocytes for glutamate and protect neurons from an excitotoxic insult.

Progress has been made in understanding the role of astrocytes in ischaemic injury (Nedergaard and Dirnagl, 2005). There is solid evidence that astrocytes can critically influence neuronal survival during ischaemia and other brain insults. Astrocytes can protect neurons by providing trophic support, for example by releasing erythropoietin, which can inhibit neuronal apoptosis (Ruscher et al., 2002). Oxygen–glucose deprivation or treating astrocytes with pharmacological agents that stabilize hypoxia-inducing factors increases the production and secretion of VEGF (vascular endothelial growth factor) and erythropoietin (Chavez et al., 2006; Chu et al., 2010). Astrocytes also scavenge oxygen free radicals via glutathione, metallothioneins or ceruloplasmin (Trendelenburg et al., 2002). Furthermore, studies have shown that the glycogen accumulates in the brain after a traumatic or metabolic insult, which might be protective (Folbergrova et al., 1996). Astrocytes are the principal repositories of glycogen in the brain, and experimentally induced increases in astrocytic glycogen protect neurons *in vitro* from ischaemia and glucose deprivation (Swanson and Choi, 1993; Wender et al., 2000). Recent studies in immature brain have shown that hypoxic preconditioning increases brain glycogen and this additional pool of metabolic fuel delays energy depletion during H/I (Brucklacher et al., 2002). These studies provided the impetus for the present study to test the involvement of astrocytes in hypoxic preconditioning-induced neuroprotection.

However, in the neonatal brain, many astrocytes are immature and thus might be less competent to provide neuroprotection. Using confocal microscopy for GFAP we were struck by the finding that there were far fewer GFAP-positive

radial cells in the neocortices of rats exposed to hypoxia 24 h earlier compared with controls. Moreover, when we stained for S100B, which is expressed by mature astrocytes, we found that S100B staining was increased. However, whereas S100B is expressed in both the nuclei and processes of mature astrocytes, S100B was solely nuclear. Studies of S100B function in gliogenesis have shown that S100B is expressed at high levels in oligodendrocyte progenitors that are beginning to differentiate and that S100B is lost as they mature. In S100B-null mice, oligodendrocyte maturation is delayed, supporting the conclusion that S100B promotes oligodendrocyte differentiation. In contrast, S100B begins to be expressed by astrocyte precursors during the first week of life in rats and mice and is expressed at even higher levels in mature astrocytes. In neither lineage has it been established how S100B promotes differentiation; however, S100B has been shown to increase the activity of the nuclear kinase NDR (nuclear Dbf2-related) (Deloulme et al., 2004).

Our findings are similar to those of Sizonenko et al. (2007), who found that H/I brain damage in P3 rat pups disrupted the normal radial glial architecture, distorted the radial pattern of Nestin immunostaining, increased GFAP-immunopositive astrocytes and reduced neocortical fractional anisotropy by magnetic resonance imaging (Sizonenko et al., 2007). H/I at P3 produces neuronal cell death, which complicates any interpretation of the causes of the alterations in the radial glial cells. With 3 h of hypoxia at P5, there is virtually no increase in neuronal cell death; therefore, our results suggest that neonatal hypoxia is sufficient to induce the premature differentiation of radial glial cells into astrocytes.

Our GFAP *in situ* hybridization, immunohistochemistry and Western-blot results revealed that hypoxia alone was sufficient to elevate GFAP expression in the preconditioned brain compared with the normal. Whereas the function of GFAP has long been rather elusive, there are recent studies showing that GFAP interacts with GLAST through a PDZ domain on GLAST and that increasing levels of GFAP will increase the levels of GLAST as well as [³H]aspartate uptake (Sullivan et al., 2007a). Furthermore, GFAP and GLAST levels both decrease subsequent to neonatal hypoxic brain injury, and the regions where these proteins are lost correspond to brain areas that are most susceptible to injury. Preconditioning, by inducing levels of GFAP, may thus prevent the loss of GFAP during the H/I insult, thus preserving levels of GLAST, which in turn would limit glutamate accumulation and excitotoxicity (Sullivan et al., 2007b).

Astrocytes have the innate capacity to promote neuronal survival and have been shown to limit neuronal death from excitotoxins (Rosenberg and Aizenman, 1989), oxidants (Wilson, 1997; Dringen et al., 2000) and other stressors. Glutamate is not only the predominant excitatory neurotransmitter in the CNS (Fonnum, 1984; Anderson and Swanson, 2000; Danbolt, 2001) but it is also a potent neurotoxin whose excitotoxicity has been implicated in triggering neuronal death in ischaemia (Takagi et al., 1993; Vannucci et al., 1999). The rapid removal of glutamate from

the extracellular space is required for the survival and normal functioning of neurons, and the energy-dependent glial glutamate transporter has been shown to be important for sustaining neuronal functions (Anderson and Swanson, 2000; Voutsinos-Porche et al., 2003). Moreover, glutamate is predominantly converted into glutamine in astrocytes by the enzyme GS and returned to neurons in order to replenish the presynaptic neurotransmitter pool to maintain synaptic transmission (Sibson et al., 2001). GS also increases after ischaemia (Petito et al., 1992). Therefore the capacity of astrocytes to reduce extracellular levels of glutamate can dramatically impact the extent of neuronal damage after an insult. Given the complications associated with administering NMDA (*N*-methyl-D-aspartate) receptor antagonists to the developing brain, enhancing astrocytic glutamate detoxification may represent a novel alternative means of providing neuroprotection after a developmental brain injury (Ikonomidou et al., 1999).

Glutamate transporters are necessary in order to maintain resting levels of glutamate, and effective glutamate uptake by astrocytes prevents glutamate neurotoxicity (Rothstein et al., 1996; Tanaka et al., 1997). Previous studies have shown that β -lactam antibiotics, which are potent stimulators of glutamate transporters, offer neuroprotection by increasing the transporter expression (Rothstein et al., 2005). Moreover, the expression of the glutamate transporter EAAT-2 increases significantly in the cortex 24 h after hypoxic preconditioning (Cimarosti et al., 2005). We therefore measured the levels of GS and GLAST to understand whether preconditioning regulates the astrocytic capacity to handle elevated glutamate that would, in turn, be instrumental in shaping the kinetics of glutamatergic synaptic activity during subsequent H/I. Immunoblot analysis revealed an 8-fold increase in the levels of both GS and GLAST in the forebrain of the preconditioned animals as compared with the normal. Since GS and GLAST are almost exclusively produced by astrocytes (Danbolt, 2001), our results suggest that astrocytes could play a crucial role in hypoxic preconditioning-mediated neuroprotection by metabolically reducing excess glutamate in the preconditioned brain. Furthermore, our results show that once induced, the levels of GS and GLAST are maintained, suggesting a long half-life, so that they are readily available to detoxify the extraordinarily high levels of glutamate that are released subsequent to an H/I episode.

Increased iron accumulation and overproduction of free radicals have been associated with neuronal damage caused by H/I (Palmer et al., 1999; Wallin et al., 2000). Our *in vivo* studies revealed a 2-fold increase in the levels of ceruloplasmin in the preconditioned brain. As ceruloplasmin is a multifunctional protein that is expressed by astrocytes and that can serve as an amine oxidase, an antioxidant or a ferroxidase, a testable prediction (which future studies could evaluate) is that higher levels of ceruloplasmin should protect neural cells from oxidative stress (Patel et al., 2002).

To investigate whether all these changes are a direct response of astrocytes to hypoxia, we studied the effects of

reduced oxygen on primary astrocyte cultures. Similar to the results obtained *in vivo*, exposure to hypoxia *in vitro* increased the levels of GS and MCT-1, although to a lesser extent. However, in contrast with the *in vivo* condition, there was no change in the levels of GFAP, GLAST or ceruloplasmin. Thus hypoxia regulates the expression of a subset of astrocyte-specific enzymes and transporters. These differences are likely attributable to the inability of astrocyte cultures to faithfully mimic the *in vivo* environment. In support of this hypothesis, we have observed that reducing the volume of medium overlying cultured astrocytes reduces MCT-1 expression as well as glucose utilization, consistent with the interpretation that under standard *in vitro* culture conditions the astrocytes are hypoxic due to poor solubility of O₂ in the culture medium (S.W. Levison and I. Simpson, unpublished data). Moreover, it has been well established that there are multiple astrocyte populations *in vivo*. Hence, these cells may not adequately model the responses of every type of astrocyte *in vivo*.

That GLAST and ceruloplasmin were not induced in the astrocytes *in vitro* in direct response to hypoxia suggests that additional signals are necessary for their induction. One likely possibility is that signals from activated microglia are necessary. Indeed in earlier studies we showed that IL-1 (interleukin-1) induced ceruloplasmin and that ceruloplasmin was not induced in mice lacking the IL-1 type 1 receptor. However, the observation that neither ceruloplasmin nor GLAST was induced in the mixed brain cultures where microglia were certainly present would argue against this hypothesis.

At a critical threshold, H/I will overwhelm the normal capacity of astrocytes to maintain metabolic homeostasis, resulting in the depletion of energy substrates, neuronal overexcitation and accumulation of toxic by-products of metabolism that are detrimental to neuronal viability. Our studies add to the emerging body of literature that supports the view that preconditioning triggers metabolic reprogramming by inducing the expression of proteins involved in the supply and demand pathways of metabolism, thus shifting the threshold where damage will occur. Investigating the mechanisms by which astrocytes respond to hypoxic preconditioning could be a productive focus for future research on the mechanisms of neuroprotection, which could lead to the identification of new small molecules that could activate astrocytes, mimicking the preconditioning stimulus with therapeutic benefits. However, caution is needed when applying these findings to newborns as it is conceivable that the precocious transformation of radial glial cells into mature astrocytes could have a deleterious rather than a beneficial outcome.

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