

Doxycycline inhibits proinflammatory cytokines but not acute cerebral cytogenesis after hypoxia–ischemia in neonatal rats

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Background: Neonatal hypoxia–ischemia (HI) is a major cause of perinatal brain injury and is associated with a spectrum of neuropsychiatric disorders. Although very few treatment options are currently available, doxycycline (DOXY) has been reported to be neuroprotective in neonatal HI. Our objective was to investigate the effects of DOXY on neonatal brain development in normal and HI rat pups. We hypothesized that DOXY would inhibit microglial activation but that developmentally important processes, including cytogenesis and trophic responses, would not be impaired. **Methods:** To investigate the putative neurodevelopmental consequences of DOXY administration in a clinically relevant animal model of HI, we performed a time-course analysis such that postnatal rat pups received DOXY (10 mg/kg) or vehicle immediately before HI ($n \geq 6$). We then assessed cytogenesis, proinflammatory cytokines, brain-derived neurotrophic factor (BDNF) and matrix metalloproteinases regionally and longitudinally. **Results:** We found that DOXY significantly inhibits neuroinflammation in the frontal cortex, striatum and hippocampus; decreases interleukin-1 β (IL-1 β) and tumour necrosis factor- α (TNF- α); and augments BDNF following HI. In addition, DOXY-treated pups have significantly fewer 2-bromo-5-deoxyuridine (BrdU)-positive cells in the subventricular zone 6 hours post-HI. However, DOXY does not persistently affect cytogenesis in the subventricular zone or dentate gyrus up to 7 days post-HI. The BrdU-positive cells not expressing markers for mature neurons colabel with nestin, an intermediate filament protein typical of neuronal precursors. **Limitations:** Our study investigates “acute” neurodevelopment over the first 7 days of life after HI injury. Further long-term investigations into adulthood are underway. **Conclusion:** Taken together, our results suggest the putative clinical potential of DOXY in the management of neonatal cerebral HI injury.

Introduction

Hypoxia–ischemia (HI) is a common form of perinatal brain damage and is a major etiological contributor to cerebral palsy, epilepsy, autism, attention deficit hyperactivity disorders and learning, cognitive and intellectual disabilities.¹ In Canada, 16% of deaths in neonatal intensive care units result from cerebral injury sustained during an HI event,² and infants that survive HI insults have resultant neuropsychiatric complications diverse in their progression, presentation and contribution to long-term disability. Currently, there are no pharmacotherapies approved for the treatment of HI brain damage. However, in recent years, the contribution of neuroinflammation to cerebral HI has been investigated and several anti-inflammatory therapies have been examined. Minocycline has been documented to be neuroprotective in neonatal rats after HI³ and in many other in vivo models of

cerebral ischemia and neurodegeneration.^{4–7} Minocycline, however, is not approved for use in neonates and has a significant side-effect profile.⁸ Doxycycline (DOXY), on the other hand, is approved for use in neonates and, compared with other drugs in its class and other pharmacotherapies investigated for the putative treatment of neonatal HI, has a lesser side-effect profile.⁹ We have previously reported the significant acute and subacute neuroprotective and anti-inflammatory properties of DOXY in neonatal HI brain injury. It significantly decreases cleaved caspase-3 protein expression, decreases microglial activation and modulates cerebral amino acids after HI in neonatal rats.^{10,11}

The anti-inflammatory properties of DOXY are independent of its antimicrobial actions, and it is thought to exert its beneficial effects after cerebral injury in part through the inhibition of microglial activation. Whereas persistent microglial activation has been reported to be neurotoxic, some

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microglial responses such as the production of neurotrophins, the attraction/differentiation of neural precursors and the removal of toxic products limit injury and enhance repair.^{12–16} However, inflammation associated with microglial activation has also been shown to impair both basal and insult-induced neurogenesis.¹⁷ Thus, the inhibition of microglia, via an anti-inflammatory therapy, may have consequences extending far beyond a reduction in neuroinflammation alone, especially in the developing nervous system.

Despite conflicting reports on the effects of minocycline on neurogenesis after cerebral ischemia in adults,^{4,7,17} the effects of a tetracycline on cytotogenesis following neonatal HI have not been examined. In addition, there is a tendency to draw conclusions on the effects of inflammatory blockade on neurogenesis/cytogenesis in adult animals¹⁸ and compare or transpose these data to the neonatal central nervous system. However, these comparisons providing useful background knowledge are not appropriate. Thus, we undertook a time-course evaluation of the impact of DOXY administration on cell proliferation and differentiation in the context of clinically relevant mild HI brain injury in neonatal rats. Because cell fate of multipotent progenitors and newborn cells is determined by specific environmental cues¹⁹ and there is an intimate connection between the trophic and toxic factors released by microglia, we also analyzed the major pro-inflammatory cytokines (tumour necrosis factor- α [TNF- α], interleukin-1 β [IL-1 β]), a neurotrophin that mediates neuroprotection (brain-derived neurotrophic factor [BDNF]) and gelatinases that have dual nature in development and repair (matrix metalloproteinases [MMP]-2/-9) in regions vulnerable to HI and important to the genesis and survival of new cells following neonatal HI brain injury.

Methods

Animals and surgical procedures

As previously described, we used a reproducible, well characterized procedure combining unilateral common carotid artery occlusion with systemic hypoxia to produce mild HI brain injury in Sprague Dawley rat pups.^{10,11} The paradigm produces unilateral brain damage as a consequence of acute reduction in blood flow and oxygenation mimicking the disruption in nutrient delivery that is the primary cause of the neurologic injury during the perinatal period.²⁰ Briefly, on postnatal day 7, we anesthetized pups weighing between 12 and 17 g with a mixture of isoflurane (4.5% induction, 1.5% maintenance) and oxygen (O₂) balanced with nitrogen (N₂). On the anterior ventral surface, we made a small lateral incision at the base of the neck, and the right common carotid artery was exposed, isolated and permanently ligated. The entire surgical procedure lasted no longer than 7 minutes. After a 2-hour recovery period in their home cages, we placed rat pups in a humidified hypoxia chamber filled with premixed 8% O₂ balanced with N₂ for 1 hour. Normothermia was maintained in the chamber at 37.5°C. After the hypoxic period, we returned the pups to their dams until euthanasia. In addition, SHAM-operated controls underwent the same

surgical procedure with the exception of the ligation of the common carotid artery, and at no time were these SHAMs subjected to hypoxia. All procedures described are in accordance with the guidelines set forth by the Canadian Council on Animal Care and approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta.

Drug administration and tissue preparation

We administered DOXY (Sigma, 10 mg/kg) or saline vehicle intraperitoneally as a one-time dose immediately before HI. The dose selected was based on previous experiments and dose–response studies performed in our laboratory.^{10,11} This dose of DOXY is below, but approaches, the lowest end of dosing commonly used for antimicrobial effects in children. We euthanized the pups by decapitation 3, 6 or 24 hours or 7 days after HI ($n = 6$). We chose an acute survival paradigm as injury-induced cellular proliferation is most pronounced during the earliest phases after HI and the immediate postnatal period.²¹ To label proliferating cells and ensure sufficient labelling of proliferating cells in the immediate post-HI survival period, pups received 30 mg/kg of 5-bromo-2-deoxyuridine (BrdU; Sigma) intraperitoneally every 12 hours. The first dose of BrdU was concomitant with administration of DOXY or vehicle. Pups enrolled in the 7-day survival arm of this investigation received BrdU every 12 hours beginning on postnatal day 12 until euthanasia on postnatal day 14.²² This paradigm allowed evaluation of cell accumulation and is reflective of the week-long cellular response to HI. Upon euthanasia, brains were removed and flash frozen in isopentane on dry ice; the samples were stored at –80°C until analysis.

Enzyme-linked immunosorbent assays (ELISA)

We used a separate group of pups for ELISA and gelatin zymography assays. In this arm of the study, we administered DOXY or vehicle as described; euthanized pups by decapitation 3, 6, 12 or 24 hours or 7 days after HI ($n = 8$); removed the brains and rapidly dissected the frontal cortex, striatum and hippocampus. At the time of assay, regional samples were homogenized in 100 μ L ddH₂O. This homogenate was aliquoted for both ELISA and gelatin zymography. We assessed protein amounts in the homogenate using a bicinchoninic acid protein assay (Sigma), and neurotrophin, proinflammatory cytokine and gelatinase activity were normalized to the protein amounts. We purchased commercially available DuoSet ELISA kits for IL-1 β , TNF- α and BDNF from R&D Systems, and we performed these assays according to the protocols provided by the manufacturers.

Gelatin zymography

For gelatin zymography, we added 30 μ L of homogenate to 75 μ L lysis buffer containing 50 mM Tris–HCl, 320 mM sucrose, 1 mM dithiothreitol, 10 μ g/mL leupeptin, 10 μ g/mL soybean trypsin inhibitor and 2 μ g/mL aprotinin. After centrifugation at 12 000 relative centrifugal force for 5 minutes, supernatant was collected and combined with 20 μ L

gelatin sepharose in a tube that was rotated at 4°C for 1 hour. After further centrifugation and the addition of binding and elution buffers, we combined prepared samples with loading dye according to protein amounts; samples were loaded on precast Novex 10% zymogram (gelatin) gels (Invitrogen) and run at 150 V for 2.0–2.5 hours at 4°C. We loaded purified human MMP standards (Oncogene) in 1 lane at a final protein concentration of 10 µg/100 µL. Upon separation by electrophoresis, we incubated the gels in renaturing buffer (Triton-X100 2.5% v/v) for 1 hour and then in incubating buffer solution (50 mM Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂) at 37.5°C for 4 days. We stained the gels with Coomassie blue R-250 for 1 hour and then destained them accordingly. Matrix metalloproteinase activation appeared as transparent bands on a blue background. An MCID image analysis system digitally captured images of gels. We quantified bands by densitometry using Adobe Photoshop Elements and then reversed images of representative gels for display in the figures.

Immunohistochemistry

For immunohistochemical analyses, we obtained serial coronal sections (20 µm) throughout the brain using a cryostat. We collected 11 sections for each animal in reference to specific structural landmarks and corresponding to the following stereotaxic coordinates in the developing rat brain:

6.8 mm to 2.0 mm from Bregma.²³ We performed immunostaining using antibodies recognizing neurons (Alexa-Fluor 488 anti-neuronal nuclei [NeuN], Chemicon, 1:100), cleaved caspase-3 (rabbit anti-cleaved caspase-3, Cell Signaling Technology, 1:1000), BrdU (mouse anti-BrdU, Roche, 1:600), nestin (mouse anti-nestin, Chemicon, 1:100), proliferating cells (mouse anti-Ki-67, Dako, 1:100), activated microglia (mouse anti-ED-1, Serotec, 1:100) and astrocytes (Alexa-Fluor 488 anti-gial fibrillary acidic protein [GFAP], Chemicon, 1:500).

Sections immunostained for or with BrdU underwent a DNA denaturation step in 2M HCl for 60 minutes at 37.5°C immediately after fixation. We then neutralized sections in 0.1 M sodium borate (2 × 5 min) and phosphate-buffered saline before proceeding with the immunohistochemical procedure previously described.^{10,11}

Quantification of BrdU-positive cells

We counted numbers of BrdU-positive cells in 6 animals at each time point and in each treatment group in the medial subventricular zone and dentate gyrus. To perform cell counts, we identified locations using specific landmarks (Fig. 1A and E) and corresponding to reference sections of the medial striatum and medial hippocampus included in the aforementioned stereotaxic coordinates.²³ Under the 10× microscope objective, an investigator blind to the treatment groups and end points obtained a photomicrograph of the

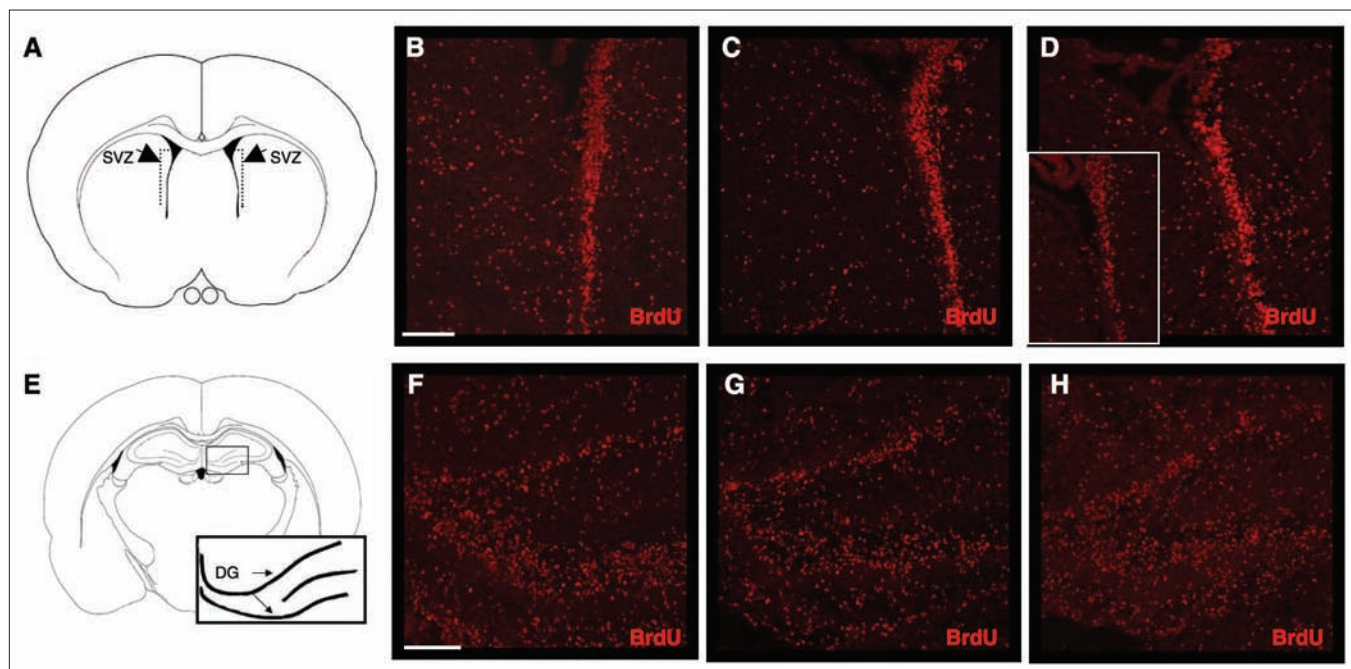


Fig. 1: Administration of doxycycline (DOXY) significantly decreases the number of 5-bromo-2-deoxyuridine (BrdU)-positive cells in the subventricular zone (SVZ) 6 hours following hypoxia-ischemia (HI) but not 7 days post-HI, in neonatal rats. Representative photomicrographs illustrate the number, pattern and distribution of BrdU-positive cells in the subventricular zone (reference section A) of (B) SHAM rat pups and rat pups 24 hours after HI and treatment with (C) saline vehicle or (D) DOXY, where the inset shows significantly fewer BrdU-positive cells in DOXY-treated pups 6 hours post-HI. Additional photomicrographs illustrate the number, pattern and distribution of BrdU-positive cells in the dentate gyrus (DG; reference section E) of (F) SHAM rat pups and rat pups 48 hours after HI and treatment with (G) saline vehicle or (H) DOXY. Scale bars = 50 µm.

designated fields and then counted all BrdU-positive nuclei in these designated areas and presented them as numbers of cells (in hundreds). A second investigator blind to all conditions also performed cell counts on a subset of randomly selected sections to conform to standard interjudge reliability practices.

Statistical analyses

All results are expressed as means and standard deviations. We compared normally distributed data differences between 2 groups using Student *t* tests. Where appropriate, and when variances between 2 groups were significantly different, we used a Welch correction. One-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test on significant main effects and interactions allowed the analysis of multiple groups. Statistical significance was $p < 0.05$, according to the general convention for probability values.

Results

Consistent with a mild injury of this nature and our previous publications,^{10,11} we observed significant diffuse and patchy neuronal cell loss with concomitant microglial activation throughout the dorsal-lateral cortex, striatum, hippocampus and thalamus, ipsilateral to carotid ligation (data not shown). Evidence of complete infarction, cystic lesions, severe ventriculomegaly and/or complete ablation of certain regions (i.e., the dentate gyrus or subventricular zone) were not observed in any animal in this study.

BrdU immunoreactivity

We observed BrdU immunoreactivity throughout the neonatal rat brain in both SHAM and HI animals at all time points investigated. The BrdU-positive cells were clearly identifiable as bright illuminations above background, and the staining of these cells was nuclear and often punctate. Although we observed numerous BrdU-positive cells in the cortex and thalamus, most were concentrated in the subventricular zone and dentate gyrus (Fig. 1). Because BrdU immunoreactivity was so intense and widespread, we confirmed antibody specificity by examining BrdU immunoreactivity in HI-naïve adult animals in which sparse immunoreactivity was localized solely to the dorsal subventricular zone (data not shown). Postnatal day 7 rat pups had significantly more BrdU-positive cells throughout the brain than HI-naïve adults. In addition, Ki-67 immunostaining corroborated BrdU immunoreactivity (data not shown).

We quantified the numbers of BrdU-positive cells in the major zones of proliferation: the subventricular zone and dentate gyrus. Three hours after HI there were no significant changes in the numbers of BrdU-positive cells of either DOXY or vehicle-

treated pups in the subventricular zone (Fig. 2A) or dentate gyrus (data not shown). Continued analyses revealed that treatment with DOXY significantly decreased the number of BrdU-positive cells in the subventricular zone 6 hours post-HI (Fig. 2B, $F_{3-16} = 7.32, p = 0.003$). We found that SHAM animals treated with DOXY also had significantly fewer BrdU-positive cells in the subventricular zone at 6 hours (Fig. 2B, $F_{3-16} = 7.32, p = 0.003$). Twenty-four hours post-HI, DOXY-treated HI pups also had fewer BrdU-positive cells in the subventricular zone compared with vehicle-treated HI pups, although this decrease failed to reach statistical significance (Fig. 2C, $T_{10} = 2.15, p = 0.06$). When the time course was extended to 7 days post-HI, there was no sustained change in the number of BrdU positive cells observed in any experimental group in either the subventricular zone (Fig. 2D) or dentate gyrus (data not shown).

Identifying the phenotype of the progeny

To identify the phenotype of the BrdU-positive cells, we performed immunochemical analyses. At no point along the investigated time period did BrdU-positive cells colocalize with an antibody against neuronal nuclei (NeuN), a marker of postmitotic neurons (Fig. 3A–D, Q, R). To identify the

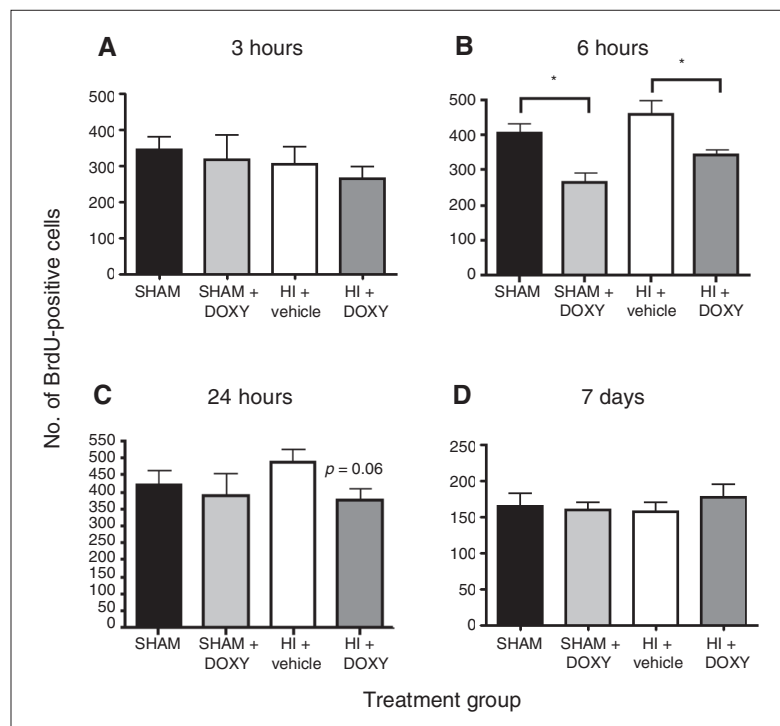


Fig. 2: Histograms of the number of 5-bromo-2-deoxyuridine (BrdU)-positive cells in the medial subventricular zone (A) 3 hours, (B) 6 hours, (C) 24 hours and (D) 7 days after hypoxia-ischemia (HI) in SHAM rat pups, SHAM pups treated with doxycycline (DOXY) and HI pups treated with vehicle or DOXY. (B) *Treatment with DOXY significantly decreases the number of BrdU-positive cells in both treated SHAM and HI animals at 6 hours. (C) Twenty-four hours post-HI, treatment with DOXY also decreases the number of BrdU-positive cells, although this decrease is not statistically significant. (D) By 7 days post-HI, there are no significant increases or decreases between any groups. Values represent means and standard deviations ($n = 6$).

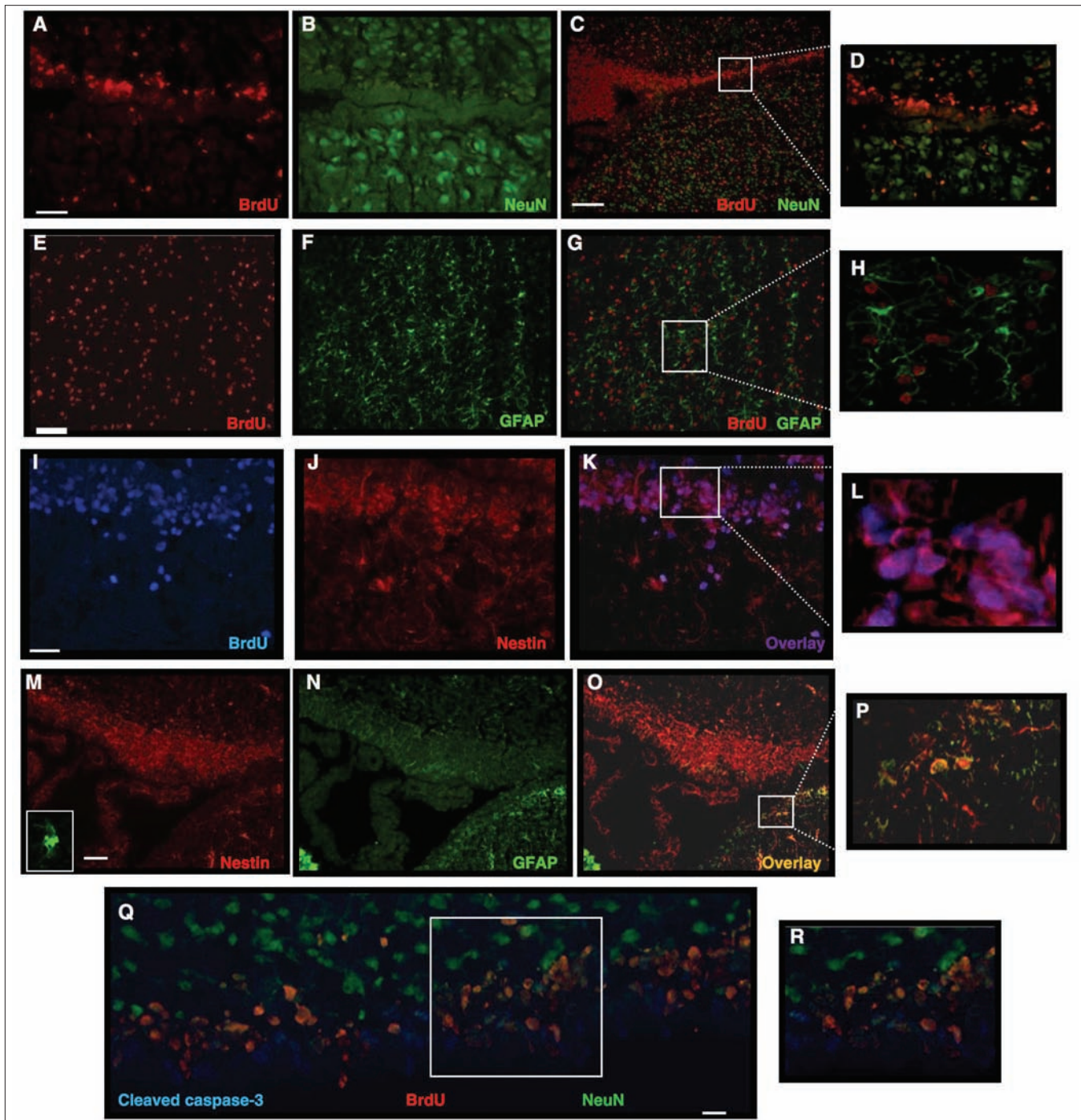


Fig. 3: 5-Bromo-2-deoxyuridine (BrdU)-positive cells significantly colocalize with nestin, a marker of immature precursors, acutely following hypoxia–ischemia (HI) in neonatal rats. (**K** and **L**) Photomicrographs indicate that most BrdU-positive cells in the subventricular zone after HI colocalize with the immature precursor marker nestin. (**A**) BrdU-positive cells, (**B**) neuronal nuclei (NeuN)-positive cells and (**C**) a low magnification overlay image of the entire subventricular zone. (**D**) A higher magnification overlay image from the inset in (**C**) of BrdU- and NeuN-positive cells shows lack of colocalization. Scale bar = 50 μ m in **C**, 10 μ m in **A**, **B**, **D**. Double immunostaining for (**E**) BrdU (scale bar = 20 μ m) and (**F**) glial fibrillary acidic protein (GFAP) show that most BrdU-positive cells in HI rat pups do not colocalize with GFAP (**G**, inset **H**). (**I**–**L**) Photomicrographs of (**I**) BrdU (scale bar = 10 μ m) and (**J**) nestin immunostaining after HI, illustrating that most BrdU-positive cells in the subventricular zone colocalize with the intermediate filament protein, nestin (**K**, inset **L**). Most (**M**) nestin-positive cells (scale bar = 20 μ m) in the subventricular zone are also negative for GFAP (**N**). Overlay of images **M** and **N** are shown in **O** and inset **P** with few nestin/GFAP-positive cells (yellow/orange) shown medially. Inset in **M** depicts a nestin-positive neuron at high magnification (green). Representative photomicrograph of sections triple-stained for NeuN (green), BrdU (red) and cleaved caspase-3 (blue) 7 days post-HI are shown in **Q** and **R** (scale bar = 10 μ m).

putative phenotype of the BrdU-positive cells, we performed double labelling with GFAP (Fig. 3E–H), nestin (a marker of immature multipotent precursors, Fig. 3I–L) and ED-1 (data not shown). Nestin is present in neural-specific intermediate filament proteins and can also be expressed by astrocytes and radial glia during development. Throughout the time course, it was clear that most BrdU-positive cells did not colocalize with GFAP or ED-1, and Figure 3, panels E–H are representative of the typical pattern of BrdU and GFAP double immunostaining over 7 days. The typical pattern and distribution of nestin-positive cells in the subventricular zone is shown in Figure 3, panels J and M. Colocalization experiments consistently revealed that more than 90% of BrdU-positive cells in the subventricular zone double-label with nestin (Fig. 3K–L). In addition, most nestin-positive cells in the subventricular zone were negative for GFAP (Fig. 3O and P).

Identifying new cells versus cells re-entering the cell cycle

To conclude that BrdU cells examined and quantified were “new” and not damaged cells re-entering the cell cycle, we demonstrated an absence of cell death markers in the BrdU-positive cells. Figure 4, panels A–D depict BrdU/cleaved caspase-3 immunoreactivity 7 days post-HI. Acutely after HI, we identified few BrdU/cleaved caspase-3 positive cells in either the subventricular zone or dentate gyrus (data not shown), and by 7 days post-HI there was a significant number of cleaved caspase-3 positive cells existing independently (Fig. 4). However, as clearly depicted in Figure 4, panels C and D, most BrdU-positive cells in the proliferative zones do not colocalize with cleaved caspase-3. This observation was consistent throughout the time course. Figure 3, panels Q and R depict triple labelling performed with antibodies against BrdU, NeuN and cleaved caspase-3 7 days post-HI. As depicted in Figure 3Q, numerous NeuN-positive cells (green) and cleaved caspase-3 cells (blue) exist on their own in the subventricular zone and surrounding striatum. Additionally, BrdU/cleaved caspase-3 positive cells (purple) and small numbers of NeuN/cleaved caspase-3 positive cells (blue/green) are shown. This pattern of immunostaining was consistent and replicated in the dentate gyrus (data not shown).

Levels of IL-1 β , TNF- α , BDNF and gelatinase activity

Three hours post-HI, BDNF was significantly decreased in the frontal cortex and striatum ipsilateral to carotid ligation in all HI pups compared with SHAM animals (Fig. 5C, $F_{3-28} = 6.14$, $p = 0.002$; Fig. 5F, $F_{3-28} = 3.81$, $p = 0.021$). In the hippocampus ipsilateral to carotid ligation, vehicle-treated pups had significantly increased IL-1 β compared with SHAM (Fig. 5G, $F_{3-28} = 3.97$, $p = 0.018$). Treatment with DOXY significantly attenuated this increase (Fig. 5G, $F_{3-28} = 3.97$, $p = 0.018$). Significant changes in gelatinase activity were evidenced by regional differences in MMP-9 and MMP-2 following HI. In some animals, treatment with DOXY attenuated the activities of both MMP-9 and MMP-2 in the frontal cortex and hippocampus compared with vehicle (zymogram, Fig. 6A), although these changes failed to reach statistical significance.

Six hours post-HI, TNF- α in the frontal cortex and hippocampus and striatal IL-1 β were significantly increased (Fig. 7B, $F_{3-28} = 4.47$, $p = 0.011$; Fig. 7H, $F_{3-28} = 4.17$, $p = 0.015$). The administration of DOXY normalized the levels of these proinflammatory cytokines, as DOXY-treated pups had significantly lower levels of IL-1 β and TNF- α compared with vehicle-treated pups in these brain regions (Fig. 7B, D, H, all $p < 0.05$). Pups treated with DOXY also had significantly lower levels of TNF- α in the striatum (Fig. 7E, $F_{3-26} = 3.02$, $p = 0.048$). The activity of MMP-9 in the striatum and frontal cortex was also significantly increased (Fig. 8A and B). In the hippocampus, all HI pups had significantly elevated levels of BDNF compared with SHAM pups (Fig. 7I, $F_{3-28} = 8.19$, $p < 0.001$). Treatment with DOXY further increased BDNF levels in HI pups compared with vehicle-treated pups (Fig. 7I, $F_{3-28} = 8.19$, $p < 0.001$). Twenty-four hours after HI, IL-1 β and BDNF were significantly increased in the hippocampus of all HI pups (data not shown).

Seven days post-HI, we identified longer-term changes in IL-1 β , TNF- α and BDNF levels. In the frontal cortex, HI pups had significantly elevated levels of IL-1 β and BDNF (Fig. 9A, $F_{3-33} = 9.14$, $p = 0.019$; Fig. 9C, $F_{3-33} = 6.68$, $p = 0.001$). Treatment with DOXY significantly increased IL-1 β in HI pups compared with pups treated with vehicle (Fig. 9A, $F_{3-33} = 9.14$, $p = 0.002$). In the striatum, a distinct and consistent pattern

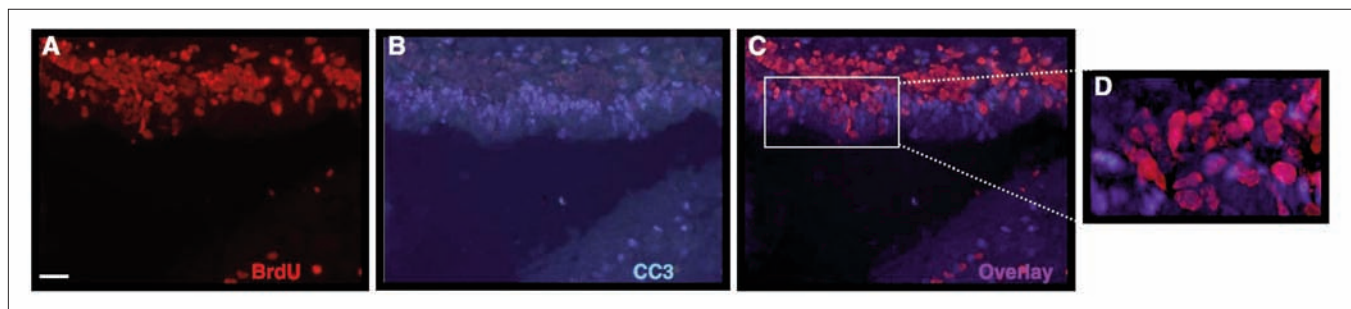


Fig. 4: 5-Bromo-2-deoxyuridine (BrdU)-positive cells following hypoxia-ischemia (HI) in neonatal rats do not significantly colocalize with cleaved caspase-3, an end effector of apoptosis. Representative photomicrographs illustrating that most BrdU-positive cells in the subventricular zone 7 days post-HI are negative for cleaved caspase-3, an effector of apoptotic cell death. (A–D) Few BrdU/cleaved caspase-3 (CC3)-positive cells (purple) exist in the subventricular zone 7 days post-HI. However, (C, D) most BrdU-positive cells in the subventricular zone do not colocalize with the end effector of apoptosis. (A) BrdU-positive cells, (B) CC3-positive cells lining the lateral ventricle, (C) an overlay image of A and B and (D) an image from the inset depicting that BrdU/CC3 colocalizations appear as purple cells lining the lateral ventricle (scale bar = 10 μ m).

immersed as vehicle-treated pups had significantly increased IL-1 β , TNF- α and BDNF compared with SHAM pups (Fig. 9D–F, all $p < 0.05$). Treatment with DOXY reliably and significantly reduced the levels of TNF- α and BDNF (Fig. 9E, $F_{3,32} = 4.31, p = 0.011$; Fig. 9F, $F_{3,32} = 9.52, p = 0.020$). The administration of DOXY also decreased and normalized the levels of IL-1 β in the striatum ($p = 0.05$). In the hippocampus, both DOXY- and vehicle-treated pups had significantly elevated levels of IL-1 β 7 days post-HI (Fig. 9G, $F_{3,33} = 3.15, p = 0.038$). As depicted in the histograms and zymogram in Figure 10, changes in the activity of the gelatinases 7 days post-HI were restricted to the hippocampus. Increased MMP-9 and MMP-2

activity was present in both DOXY- and vehicle-treated pups.

Discussion

Our data show that 1) a single administration of DOXY acutely affects cytotgenesis after HI in the subventricular zone, but does not lead to any persistent change in the number of BrdU-positive cells in either the subventricular zone or dentate gyrus 1 week after HI; 2) most BrdU-positive cells present in the first week post-HI are positive for the immature neuronal marker nestin; 3) most newly born cells exist independently of cleaved caspase-3; 4) a one-time dose of

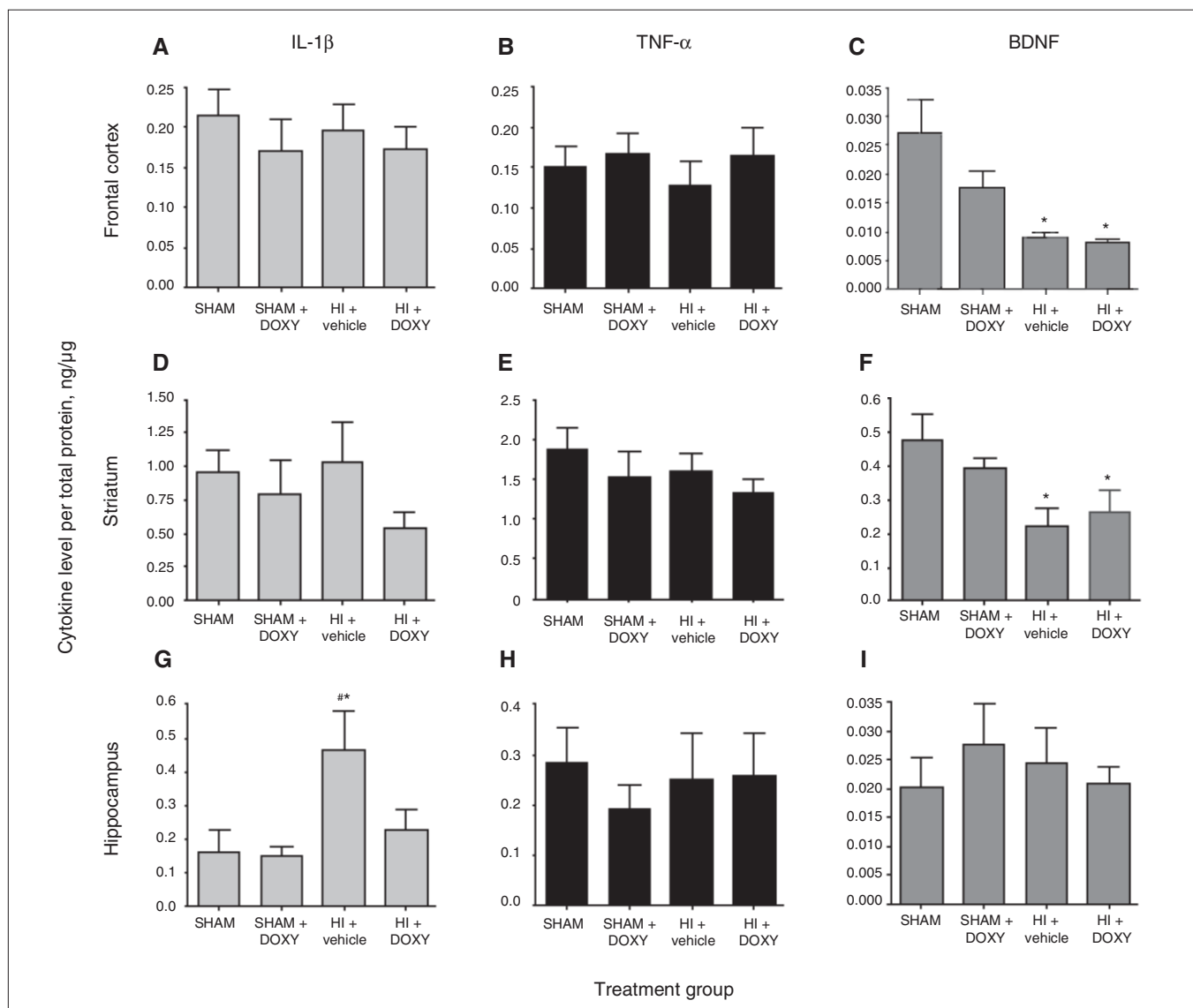


Fig. 5: Levels of interleukin-1 β (IL-1 β), tumour necrosis factor- α (TNF- α) and brain-derived neurotrophic factor (BDNF) in the frontal cortex, striatum and hippocampus ipsilateral to common carotid artery ligation 3 hours after hypoxia–ischemia (HI) in neonatal rats. (C, F) Histograms show that neonatal HI, independent of treatment with doxycycline (DOXY) or saline vehicle, causes significant reductions in BDNF in both the frontal cortex and striatum as compared with BDNF levels measured in SHAM pups. In addition, (G) vehicle-treated pups have significantly elevated IL-1 β in the hippocampus 3 hours post-HI, and this elevation is significantly attenuated by the administration of DOXY. *Significantly different from SHAM. ##Significantly different from DOXY-treated HI pups. Values represent means and standard deviations ($n = 8$).

DOXY significantly inhibits neuroinflammation up to 7 days post-HI; and 5) the response to DOXY administration is regional and time-dependent as evidenced by TNF- α , IL-1 β and BDNF levels following injury.

Acute cerebral cytogenesis in the neonatal rat brain following HI

In previous studies, we reported the neuroprotective and anti-inflammatory effects of DOXY after neonatal HI using the same clinically relevant animal model described here. In these prior investigations, DOXY significantly improved neuronal survival, decreased microglial activation, decreased cleaved caspase-3 protein expression and modulated cerebral amino acid neurotransmitters. It is because inflammation, apoptosis and amino acid neurotransmitters have roles in both brain development and neonatal HI pathology that further investigation into the consequence of inhibiting these processes was warranted. In the present investigation we demonstrated that numerous BrdU-positive cells exist throughout the brains of both SHAM and HI animals, with highest density localized to the subventricular zone and dentate gyrus. The numbers of BrdU-positive cells observed was consistent with the developmental stage of the pups; BrdU is

a deoxythymidine analogue and is incorporated into new DNA during the S-phase of the cell cycle. The number of cells labelled by a single injection of BrdU is limited by its bioavailability (half-life = 2 h) and the number of cells in S-phase during this brief period. Thus when marking the entire population of newly formed cells during a time span, repeated pulses of BrdU are required.²⁴

Before the studies outlined herein, the effects of the tetracyclines on cell genesis, DNA synthesis and impact on eventual neurogenesis after neonatal HI had not been studied. Investigations conducted in adult animals reveal that anti-inflammatory agents can be effective in restoring neurogenesis after cranial irradiation, epilepsy and ischemic stroke.^{7,17,18,25} Although further investigation with chronic survival is required, our data suggest that the acute time course of cerebral cytogenesis after HI is likely not affected by the administration of DOXY as the number of BrdU-positive cells are not significantly different between DOXY- and vehicle-treated animals 7 days post-HI. Taking these findings together with previous data indicating that DOXY improves neuronal survival, it is possible that the administration of DOXY in this model preserves the normal pattern of cerebral cytogenesis.

Despite the 7-day trend in BrdU immunoreactivity, DOXY did significantly reduce the number of BrdU-positive cells in

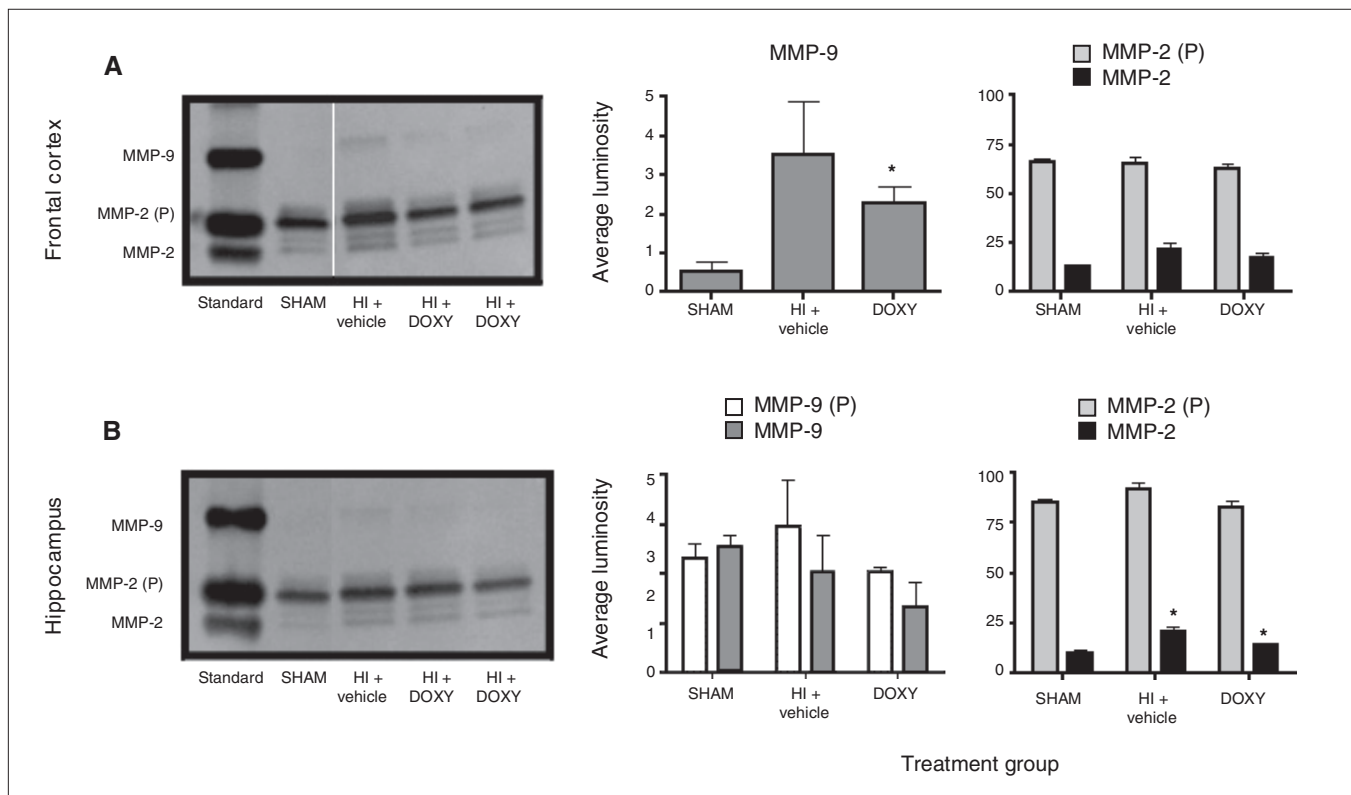


Fig. 6: Levels of matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2) in the frontal cortex and hippocampus ipsilateral to common carotid artery ligation 3 hours after hypoxia-ischemia (HI) in neonatal rats. (A) The HI pups treated with doxycycline (DOXY) have significantly increased MMP-9 activity (P) in the frontal cortex compared with SHAM pups 3 hours post-HI. (B) Both vehicle- and DOXY-treated pups have significantly increased MMP-2 in the hippocampus. Treatment with DOXY tended to decrease the activities of both forms of MMP-9 and active MMP-2 in the frontal cortex and hippocampus, although these decreases failed to reach significance. *Significantly different from SHAM. Values represent mean luminosity and standard deviation (n = 3).

the subventricular zone 6 hours post-HI. Although this reduction did not persist, it is almost certainly related to DOXY administration itself, as even DOXY-treated SHAM animals had significantly fewer BrdU-positive cells compared with vehicle-treated SHAM animals. This finding is likely related to peak levels of DOXY in the brain and thus maximal impact on activated microglia.¹⁰ Support for this conclusion is provided in Figure 7: DOXY-treated pups have significantly less IL-1 β and TNF- α compared with vehicle-treated pups in the striatum at 6 hours. In addition, other investigators have suggested that activated microglia have a

deleterious effect on newly formed cells mediated by the action of IL-1 β , TNF- α , nitric oxide and/or reactive oxygen species.¹⁷ With respect to our data (Fig. 7E), of special interest is the reduction in TNF- α observed with DOXY as this decrease occurred in the absence of TNF- α changes in any other experimental group, most notably the vehicle-treated HI pups. However, these results must be interpreted cautiously as we investigated a small proportion of a large inventory of microglial effectors.

Further analyses of BrdU immunoreactivity in the subventricular zone reveal that neither DOXY-treated nor vehicle-

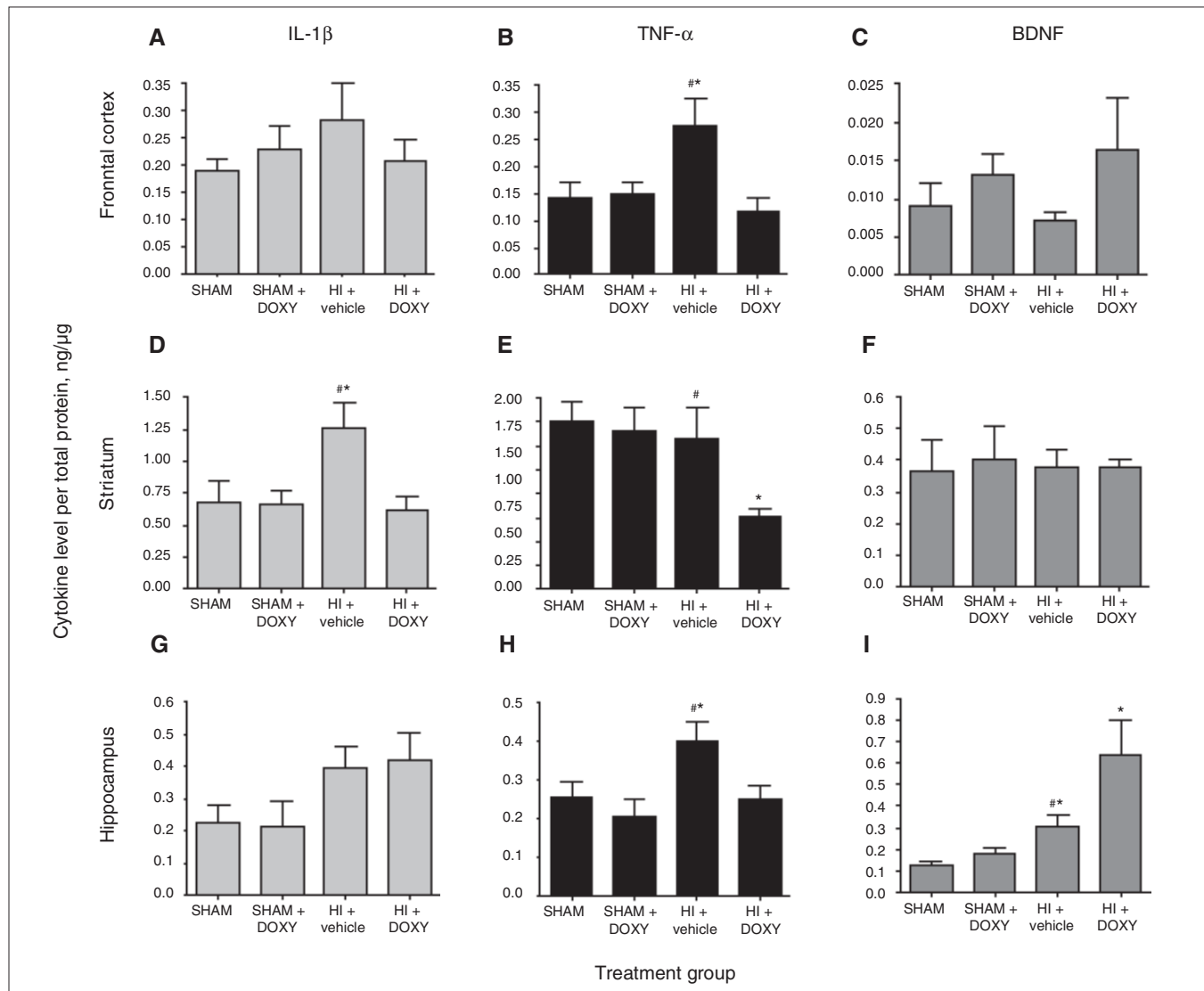


Fig. 7: Levels of interleukin-1 β (IL-1 β), tumour necrosis factor- α (TNF- α) and brain-derived neurotrophic factor (BDNF) in the frontal cortex, striatum and hippocampus ipsilateral to common carotid artery ligation 6 hours after hypoxia-ischemia (HI) in neonatal rats. (D) Histograms show that IL-1 β is significantly increased in the striatum of vehicle-treated pups 6 hours post-HI and that the administration of doxycycline (DOXY) significantly attenuates this increase. (E) In addition, DOXY-treated pups have significantly lower levels of TNF- α in the striatum 6 hours post-HI compared with vehicle-treated pups. (B, H) Treatment with DOXY normalizes TNF- α in the frontal cortex and hippocampus. (I) BDNF levels are significantly increased in HI pups in the hippocampus, and those pups treated with DOXY have significantly increased BDNF compared with vehicle-treated pups. *Significantly different from SHAM. #Significantly different from DOXY-treated HI pups. Values represent means and standard deviations ($n = 8$).

treated pups had any significant increases or decreases in the number of BrdU-positive cells compared with SHAM controls. In previous investigations, acute reductions in BrdU immunoreactivity and depletion of neural progenitors have been demonstrated following moderate HI.²⁶ These findings were not replicated in our study primarily because we used a milder injury model. For our study, we titrated the injury such that the subventricular zone/striatum and dentate gyrus/hippocampus were not completely infarcted or obliterated to experimentally replicate a common clinical picture of HI brain injury. In relation to mild HI injuries, it is highly unlikely that our findings represent changes in cell density due to surrounding tissue loss as opposed to changes in the absolute numbers of new cells present.²² Similarly, it was important to demonstrate BrdU uptake in the absence of apoptotic markers.²⁷ This was vital, as a recent finding suggests that HI preferentially triggers neurons to re-enter the cell cycle and resume apoptosis-associated DNA synthesis.²⁷ As shown in Figure 4, very few BrdU-positive cells in the subventricular zone colocalized with cleaved caspase-3, an end effector of caspase-dependent apoptotic cell death. This was consistent throughout the 7-day time course examined, indicating that most BrdU-positive cells studied and quantified in this investigation were likely newborn cells and not those triggered to re-enter the cell cycle as a part of HI cell death cascades.

In our investigation, the phenotypic identification of newly generated cells at the time of euthanasia showed that significant numbers of BrdU-positive cells colocalized with nestin, representative of an intermediate stage of development. It is plausible that these cells are an uncommitted neural precursor, and further evidence for this is found in Figure 3E–H, where it is demonstrated that BrdU-positive cells do not significantly colocalize with GFAP or ED-1. Nestin is a marker of neural progenitor cells, and as the brain matures the expression of nestin wanes. Classically, it is believed that the maturation of newborn cells, from proliferation in the subgranular zone to migration and differentiation in the granular layer, takes about 4 weeks in the adult rat dentate gyrus.²⁸ The acute nature of the time course described here allowed only for study of DOXY's effects after HI when cell proliferation is maximal in terms of injury response and developmental stage. Thus, extension of this time course to approximately 30 days post-HI would be required to know the proportion of nestin-positive cells that eventually became mature neurons. NeuN is a soluble protein localized to the nucleus and in the cytoplasm of postmitotic neurons,²⁹ and in the investigated time course we did not observe significant BrdU/NeuN colocalizations but we noted evidence of satellite cells (closely opposed NeuN-positive and BrdU-positive cells, Fig. 3Q, R). Although little information is known about the terminal differentiation of

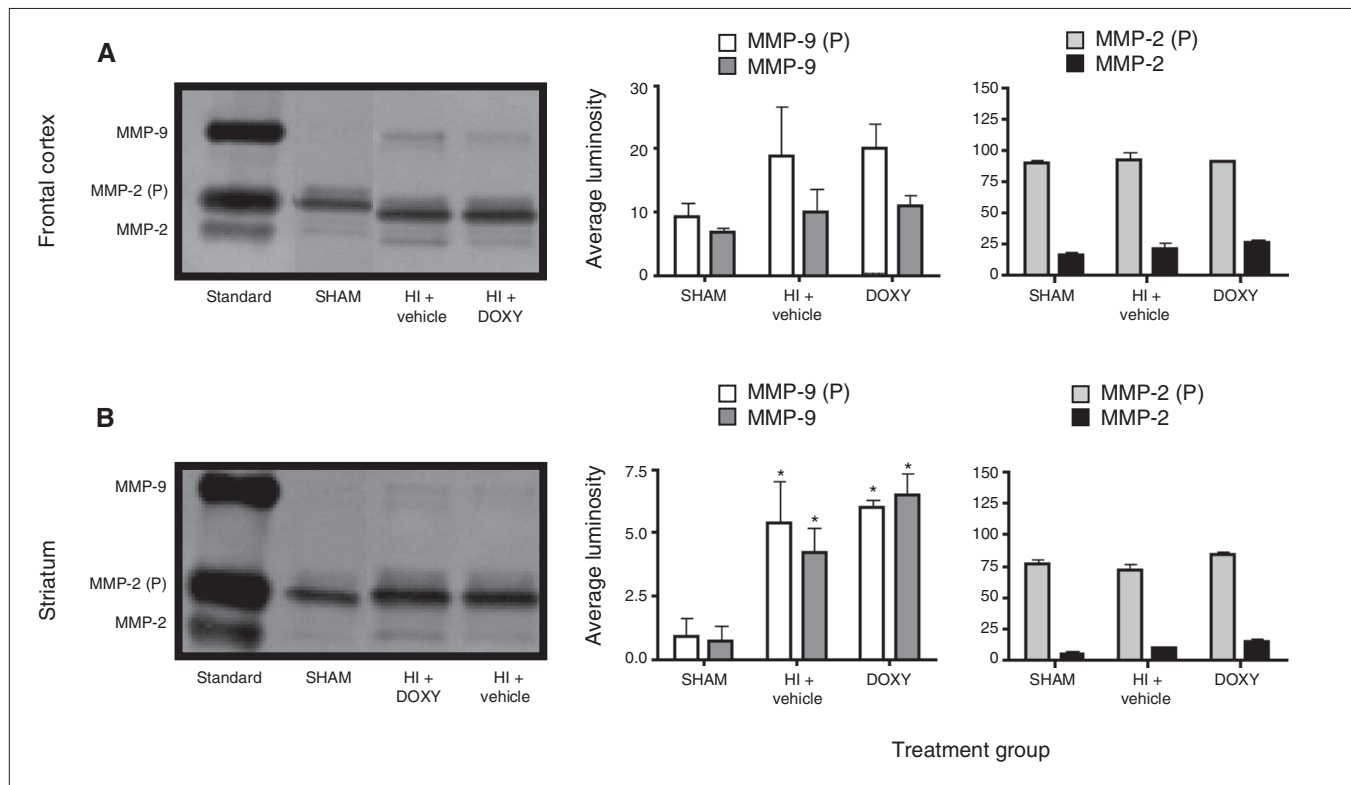


Fig. 8: Levels of matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2) in the frontal cortex and striatum ipsilateral to common carotid artery ligation 6 hours after hypoxia-ischemia (HI) in neonatal rats. (A, B) Zymograms and histograms demonstrate that HI pups treated with either DOXY or vehicle have significantly increased MMP-9 activity compared with SHAM pups in (B) the striatum 6 hours post-HI. (A) Zymogram showing that in the frontal cortex, vehicle-treated pups exhibited increased MMP-9. *Significantly different from SHAM. Values represent mean luminosity and standard deviation ($n = 3$).

neurons in the neonatal brain after injury, the lack of significant NeuN/BrdU colocalization was the expected result given the time required for the expression of this antigen to appear.^{23,30,31}

Doxycycline inhibits neuroinflammation in a temporal and region-specific manner along the tested time course

It is clear that neuroinflammation is capable of inhibiting cerebral cytotgenesis by a variety of mechanisms, including

stimulation of the hypothalamic–pituitary–adrenal axis with subsequent elevation in glucocorticoids, alterations in progenitor cell-neurovasculature interactions, or the direct effects of activated microglia and their secreted effectors.¹⁸ Thus, we investigated 2 major proinflammatory cytokines, BDNF and MMP-2/MMP-9, along the same time course for which we studied cytotgenesis. In concert with previous *in vitro* reports indicating that DOXY is capable of modulating neuroinflammation after hypoxia,³² treatment with DOXY significantly

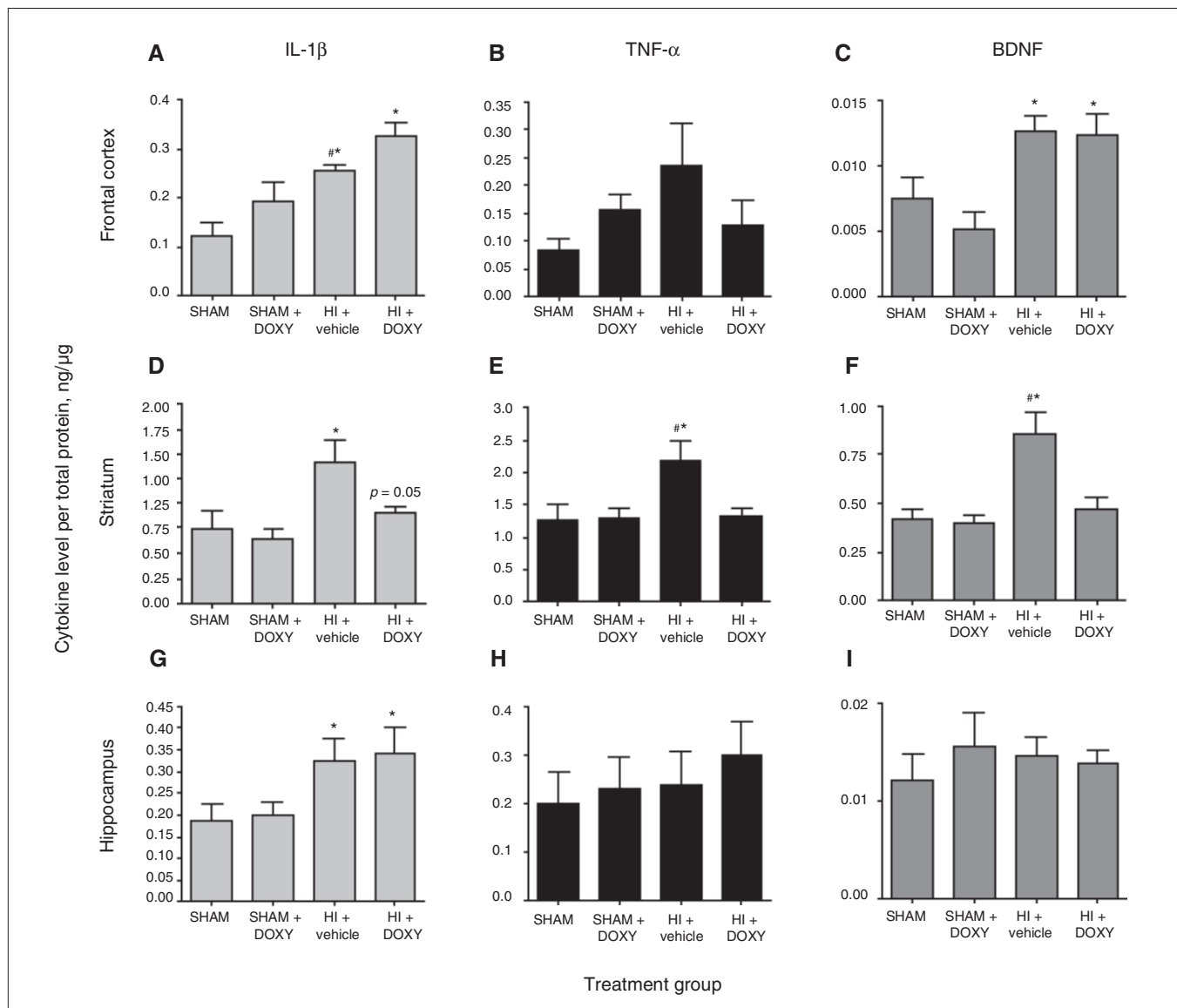


Fig. 9: Levels of interleukin-1 β (IL-1 β), tumour necrosis factor- α (TNF- α) and brain-derived neurotrophic factor (BDNF) in the frontal cortex, striatum and hippocampus ipsilateral to common carotid artery ligation 7 days after hypoxia-ischemia (HI) in neonatal rats. (A, C) Seven days post-HI, there were significant elevations in IL-1 β and BDNF in the frontal cortex of both vehicle- and DOXY-treated pups. (A) Pups treated with doxycycline (DOXY) had significantly higher levels of IL-1 β compared with those treated with vehicle. (G) All HI pups also had significantly increased IL-1 β in the hippocampus compared with SHAMs. (D, E, F) In the striatum, levels of IL-1 β , TNF- α and BDNF were significantly increased in vehicle-treated pups compared with SHAMs. (E and F) Treatment with DOXY normalized these increases, as there was significantly less TNF- α and BDNF in the DOXY-treated pups compared with vehicle-treated pups. (D) Treatment with DOXY also reduced IL-1 β in the striatum 7 days post-HI, although this decrease failed to reach significance ($p = 0.05$). *Significantly different from SHAM. #Significantly different from DOXY-treated HI pups. Values represent means and standard deviations ($n = 8$).

decreased levels of TNF- α in every brain region examined 6 hours post-HI. These data highlight the temporal role of the proinflammatory cytokines in the evolution and propagation of neuroinflammation after HI, with IL-1 β being upregulated in response to injury first at 3 hours (Fig. 5) followed by increases in TNF- α at 6 hours (Fig. 7). Six hours post-HI, BDNF increased in the hippocampus in response to HI, and most interestingly, treatment with DOXY further increased BDNF to levels that were significantly different from those in the vehicle-treated pups. Although the mechanism of this is presently unknown, BDNF has been documented to be neuroprotective after neonatal HI and is known to activate protective cascades in neurons and glia.³³⁻³⁶ Microglia are not the sole source of BDNF in the brain, and this DOXY-dependent increase in BDNF may be a result of the augmentation of many cell types and suggests that DOXY's ability to inhibit microglia does not affect their protective capacity. This is especially relevant when it is considered that DOXY significantly decreases the number of activated microglial cells following HI¹¹ but is still capable of increasing levels of BDNF as shown here. These data are also consistent with previous studies on the effects of tetracyclines on microglia in vitro.³⁷

To our knowledge, our data are the first to show that a single dose of DOXY given before HI persistently reduces proinflammatory cytokine and BDNF levels in 3 brain regions vulnerable to HI 1 week beyond the initial injury. In the striatum, IL-1 β , TNF- α and BDNF were each significantly increased in vehicle-treated pups compared with SHAM animals up to 7 days after HI. The administration of DOXY significantly attenuated these increases in TNF- α and BDNF induced by HI. This pattern was also observed with IL-1 β , although the reduction caused by DOXY just failed to reach statistical significance ($p = 0.05$). The regional and temporal changes in BDNF and the cytokines are representative of the multiphasic events in the post-HI inflammatory response. Although DOXY inhibits microglial activation and decreases neuroinflammation, our compiled data do not show significant DOXY-dependent MMP inhibition. Other investigators

have documented variability in MMP inhibition by tetracyclines and suggest that single dosing of these drugs may not be sufficient to inhibit MMP activity.³⁸

Limitations

The data presented in this manuscript expand the current understanding of the anti-inflammatory properties of DOXY and reaffirm the multiphasic nature of inflammation following HI. However, future studies addressing the contribution and role of peripheral inflammatory cells such as neutrophils and T-cells and cytokines such as IL-6 after HI and in response to treatment with DOXY are warranted as the infiltration of systemic immune cells after injury were not addressed in the present study. This investigation is also limited by nature of the 7-day survival time course studied. The data presented here only documents "acute" neurodevelopment in neonatal rats. Thus, in studies not described here, investigations expanding the time course presently studied, with additional cell-specific markers and repeated dosing of DOXY in a postinjury dosing regimen are underway in our laboratory.

Conclusion

We detail that that DOXY inhibits neuroinflammation in a significant temporal- and regional-specific manner along a 7-day post-HI time course using a clinically relevant neonatal animal model of mild HI. We show that a single dose of DOXY given before HI persistently reduces proinflammatory cytokine levels 7 days postinjury and that DOXY does not consistently inhibit cerebral cytogenesis in either the subventricular zone or dentate gyrus up to 7 days post-HI. The results of this investigation reveal novel insights into the neuropharmacology of DOXY, provide the foundation for future studies on the long-term effects of DOXY administration in the developing nervous system and, together with other work published on DOXY, suggest the putative clinical potential of this therapeutic agent.

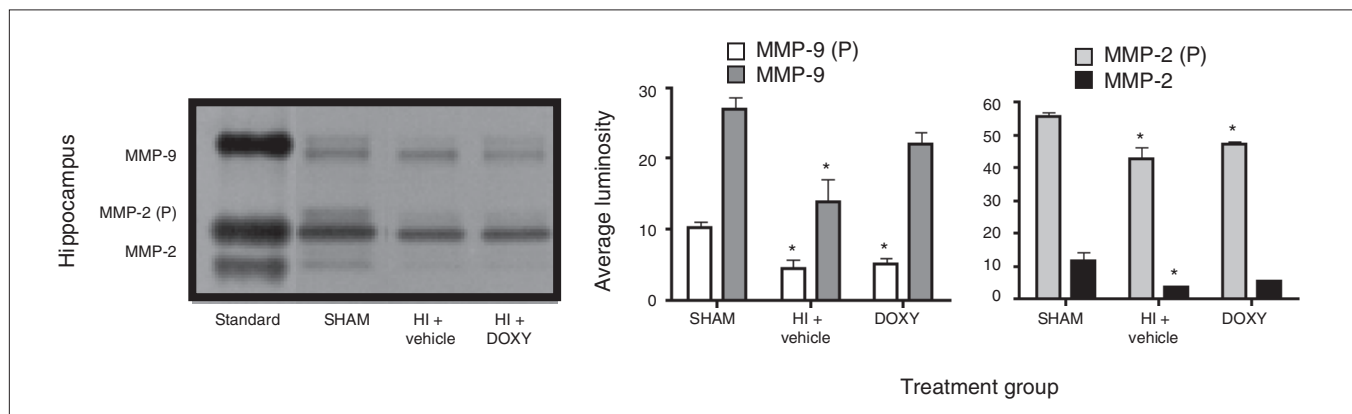


Fig. 10: Levels of matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2) in the hippocampus ipsilateral to common carotid artery ligation 7 days after hypoxia-ischemia (HI) in neonatal rats. Representative zymogram and histograms illustrate that vehicle-treated pups have significantly increased MMP-9 activity in the hippocampus compared with SHAM animals. The activities of pro-MMP-9 and pro-MMP-2 are significantly decreased in pups treated with doxycycline (DOXY). *Significantly different from SHAM. Values represent mean luminescence and standard deviation ($n = 3$).

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Competing interests: None declared.

Contributors: Dr. Jantzie acquired the data. Both Drs. Jantzie and Todd designed the study, analyzed the data, wrote and reviewed the article and approved its publication.

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