Minocycline attenuates hypoxia–ischemia-induced neurological dysfunction and brain injury in the juvenile rat

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Abstract
To investigate whether minocycline provides long-lasting protection against neonatal hypoxia–ischemia-induced brain injury and neurobehavioral deficits, minocycline was administered intraperitoneally in postnatal day 4 Sprague–Dawley rats subjected to bilateral carotid artery occlusion followed by exposure to hypoxia (8% oxygen for 15 min). Brain injury and myelination were examined on postnatal day 21 (P21) and tests for neurobehavioral toxicity were performed from P3 to P21. Hypoxic–ischemic insults resulted in severe white matter injury, enlarged ventricles, deficits in the hippocampus, reduction in numbers of mature oligodendrocytes and tyrosine hydroxylase-positive neurons, damage to axons and dendrites, and impaired myelination, as indicated by the decrease in myelin basic protein immunostaining in the P21 rat brain. Hypoxic–ischemic insult also significantly affected physical development (body weight gain and eye opening) and neurobehavioral performance, including sensorimotor and locomotor function, anxiety and cognitive ability in the P21 rat. Treatments with minocycline significantly attenuated the hypoxia–ischemia-induced brain injury and improved neurobehavioral performance. The protection of minocycline was associated with its ability to reduce microglial activation. The present results show that minocycline has long-lasting protective effects in the neonatal rat brain in terms of both hypoxia–ischemia-induced brain injury and the associated neurological dysfunction.

Introduction
Hypoxia–ischemia (HI) plays an important role in the pathogenesis of periventricular leukomalacia, a form of white matter disease closely associated with cerebral palsy (Volpe, 2001; Hagberg et al., 2002). Increasing evidence has demonstrated that late oligodendrocyte progenitors, which are the predominant oligodendrocyte lineage during the peak period of periventricular leukomalacia (i.e. 24–32 weeks of gestation), are the major target of brain injury in infant brain with periventricular leukomalacia (Back et al., 2001, 2002). In the rat, late oligodendrocyte progenitor cells represent the predominant oligodendrocyte lineage stage in the cerebral hemispheres between postnatal day 2 (P2) to P7 (Back et al., 2002). In our previous studies, we have developed a neonatal rat model to mimic the HI condition through bilateral carotid artery occlusion followed by exposure to hypoxia (8% oxygen for 10–20 min) in P4 rats. Using this rat model, we found that HI insult caused severe white matter damage, oligodendrocyte injury, hypomyelination and axonal impairment in the neonatal rat (Lin et al., 2004; Fan et al., 2005a; Cai et al., 2006). These white matter changes were similar to injuries found in infant brains with periventricular leukomalacia.

Minocycline, a semi-synthetic second-generation tetracycline derivative that crosses readily into most body tissues, including the cerebrospinal fluid (Tikka et al., 2001; Tikka & Koistinaho, 2001; Tomas-Camardiel et al., 2004), has been shown to provide protection in ischemic brain injury and other models of neuronal degenerative diseases (Yrjanheikki et al., 1998, 1999; Arvin et al., 2002; Wang et al., 2003; Tomas-Camardiel et al., 2004; Fan et al., 2005b, c). However, the opposite results have also been reported. Whereas minocycline was found to provide partial protection against HI brain injury in the neonatal rat, it worsened brain injury induced by the same insult in neonatal mice (Tsuji et al., 2004). Minocycline has also been shown to impair oligodendrocyte progenitor cell responses and remyelination in a non-immune model of demyelination in rats (Li et al., 2005). Therefore, the neuroprotective effects of minocycline on neonatal hypoxic–ischemic brain injury are still controversial. In our recent studies, we found that minocycline alleviated HI-induced white matter injury examined shortly after the HI insult (Cai et al., 2006). Periventricular leukomalacia and other white matter injuries in newborn infants have long-term effects on physical, visual, motor, sensory, cognitive and social development in human infants (Hagberg et al., 2002). A recent study shows that although post treatment with minocycline reduced brain infarct size, it failed to improve motor and cognitive functional alterations in a rat model of stroke (Ahtoniemi et al., 2005). It is unknown whether minocycline offers long-term protection in our neonatal rat model. The current study is designed to determine the long-lasting effects of minocycline on HI-induced brain injury and deficits in neurobehavioral performance in juvenile rats after the neonatal HI insult.

Materials and methods

Chemicals
Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO, USA). Monoclonal mouse antibodies against rat myelin basic protein (MBP), microtubule-associated protein (MAP1) or tyrosine hydroxylase (TH), adenomatous polyposis coli (Clone CC1) (APC-CC1), and ED1 or CD11b (OX42)
were purchased from ImmunoStar (Hudson, WI, USA), Chemicon (Temecula, CA, USA), Calbiochem (San Diego, CA, USA) and Serotec (Raleigh, NC, USA), respectively.

Animals
Timed pregnant Sprague–Dawley rats arrived in the laboratory on day 19 of gestation. Animals were maintained in an animal room on a 12-h light/dark cycle and at constant temperature (22 ± 2 °C). The day of birth was defined as the P0. After birth, the litter size was adjusted to 12 pups per litter to minimize the effect of litter size on body weight and brain size. All procedures for animal care were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center.

Surgery procedures and animal treatment
The surgery procedures were performed as previously described (Lin et al., 2004; Fan et al., 2005a; Cai et al., 2006). The operation was performed in P4 rats. Pups were lightly anesthetized with isoflurane (4% induction, 1.5% maintenance). Bilateral common carotid artery occlusion was performed with 8-0 silk sutures under a surgical microscope. After the wound was sutured, animals were placed on a warm heating pad (35–37 °C) for recovery from anesthesia. Sham-operation (surgery without bilateral common carotid artery occlusion) was conducted in the littermates to serve as the controls. All animals survived the operation.

Both bilateral common carotid artery occlusion and sham-operated animals were further divided into two groups: one received intraperitoneal (i.p.) injections of minocycline and the other received i.p. injection of sterile PBS (pH, 7.4). Minocycline (45 mg/kg) in PBS or PBS alone was administered 12 h before and immediately after the bilateral common carotid artery occlusion or the sham-operation and then every 24 h for 3 days. The hypoxic exposure (8% oxygen/92% nitrogen) was performed in a chamber maintained at 37 °C for 15 min. The duration of hypoxic exposure after bilateral common carotid artery occlusion treatment was chosen based on the results reported previously, which reproducing resulted in preferential white matter injury in 85–92% of rat pups (Lin et al., 2004). Sham-operated rats were also exposed to hypoxia for 15 min. Because neonatal rats exposed to hypoxia alone do not show any brain injury (Rice et al., 1981), the current study did not include a group with only the hypoxic exposure. Each dam had the same litter size (12 pups). Equal numbers of rat pups (three male and three female pups) for each treatment group were from six different litters. There were six pups in each group. Seventeen days after the operation (P21), rat pups were anaesthetized with 4% isoflurane and killed by transcardiac perfusion with physiological saline followed by 4% paraformaldehyde for brain section preparation.

Behavioral testing
The developmental test battery used was based on the tests for neurobehavioral toxicity (Altman & Sudarshan, 1975; Hermans et al., 1992). Behavioral tests including righting reflex, wire hanging maneuver, cliff avoidance test and locomotor activity were performed for all rat pups from P3 to P21 for 1, 4, 1 and 10 min, respectively, as previously described (Fan et al., 2005a, c). The elevated plus-maze test was performed on P19 for 5 min and the passive avoidance test was performed on P20 and P21 for 2 min for each session. Body weights of rat pups were recorded daily. The time of eye opening was recorded also.

Righting reflex
This test is a reflection of subcortical maturation (Altman & Sudarshan, 1975; Hermans et al., 1992). Pups were placed on their backs, and the time required to turn over on all four feet and touch the platform was measured; the cut-off time was 60 s.

Wire hanging maneuver
This maneuver tests neuromuscular and locomotor development (Altman & Sudarshan, 1975; Hermans et al., 1992). Pups suspended by their forelimbs from a horizontal rod (5 × 5 mm² area, 35 cm long, and 50 cm high between two poles) tend to support themselves with their hind limbs, preventing them from falling and aiding in progression along the rod. The cut-off time was 240 s. A sawdust-filled box at the base served as protection for the falling pups.

Cliff avoidance test
This test assesses the integration of exteroceptive input (vibrissae) and locomotor output (Altman & Sudarshan, 1975; Shen et al., 1991; Hermans et al., 1992). Pups placed on the edge of a platform (20 × 20 cm) with forepaws and chest extending over the edge tend to move away by backing up or turning to the side. Avoidance was scored by reflex latency between being placed on the edge and turning the body sideways with forepaws or turning the head away. If the pup did not make any response within 60 s or fell off from the platform, it was recorded as 60 s.

Locomotor activity
This test measures the activity and habituation response of animals on placement in a novel environment (Hermans et al., 1992; Tien et al., 2003). Locomotor activity was measured by using the Video Tracking System SMART-2000 (San Diego Instruments, Inc., San Diego, CA, USA). Pups were placed in the activity chambers in a quiet room with dimmed light. The total distance traveled by the animal was recorded during a 10-min testing period.

Passive avoidance
Passive avoidance gives information about learning and memory capabilities as well as maturation of the inhibitory process (Hermans et al., 1992). The passive avoidance procedure consists of two sessions. In the first session (P20), rats were trained in a step-down type of passive avoidance apparatus. The experimental chamber (30 × 30 × 40 cm) was made of plexiglass. The floor of the chamber was made of parallel 2-mm-caliber stainless steel rods spaced 1 cm apart and connected with an electric shock generator. The safe part was a piece of wooden board (8 × 25 × 2.5 cm) placed at a corner of the chamber above the metal rods. Each animal was placed initially on the safe platform. When the rat stepped down onto the floor, it received a foot shock (1 s, 0.5 mA). Although the rats repeatedly stepped up and down, they eventually remained on the board. The number of shocks required to retain an individual animal on the board for 2 min was recorded as a measure of acquisition of passive avoidance. The second session was carried out 24 h after the first session (P21). The rat was placed on the safe board and steel rods were not connected with the electric shock generator. The retention latency, i.e. the time elapsed before the rat stepped down to the grid floor, was recorded as a measure of the retention of passive avoidance. If the rat did not
step down to the grid floor within 2 min, a ceiling score of 2 min was assigned.

**Elevated plus-maze test**

The elevated plus-maze test is used to assess anxiolytic behavior (Agmo & Belzung, 1998; Sasaki et al., 2002). The procedure is based on rodents’ natural tendency to avoid open space and it dose not contain any experimenter-controlled aversive element. Nevertheless, exposure to it is stressful for the subjects (Agmo & Belzung, 1998). The plus-maze consists of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 10 cm) emanating from a common central platform (5 × 5 cm) to form a plus shape. The entire apparatus was elevated to a height of 40 cm above the floor. A video camera and illumination-lamps were mounted on the ceiling. The anxiety-related behaviors for each animal were recorded for a period of 5 min by a VCR-recording system on P19. At the beginning of the test, the rat was placed on the central platform with its head facing an open arm. The parameters recorded were the numbers of open arm or enclosed arm entries (arm entry defined as all four paws into an arm), and the total time each animal spent in various sections of the maze (open arms, center, enclosed arms). The results were expressed as the number of open arm or enclosed arm entries and the percentage of the time spent in open arms or enclosed arms (time spent in open arms or enclosed arms divided by the sum of time spent in either arm).

**Brain Injury**

Elevated plus-maze test was used to assess anxiolytic behavior. Agmo and Belzung (1998) described the procedure for the elevated plus-maze test. The test consists of two open arms and two enclosed arms, each arm being 30 cm long and 5 cm wide. The rats were placed on the central platform at the start of the test, and their behavior was recorded for 5 minutes. The parameters recorded were the number of entries into open arms, enclosed arms, and the total time spent in each arm. The results were expressed as the percentage of time spent in each arm.

**Immunohistochemistry studies**

Brain injury was estimated based on the results of hematoxylin and eosin (H&E) staining and immunohistochemistry in consecutive frozen coronary sections at a thickness of 10 μm prepared from the P21 rat brain (17 days after the HI insult). Because variation in section orientation may significantly affect the estimate of thickness of the corpus callosum, careful attention was given to trimming and placement of the brain tissue on the cutting stage to ensure that the cutting surface of the tissue was perpendicular to the horizontal brain plane. When the cutting surface was found to be not vertical to the horizontal plane, these sections were not used to estimate thickness of the corpus callosum or the MBP staining. Increasing evidence indicates that not only white matter injury, but also neuronal and axonal injury are involved in periventricular leukomalacia (Inder et al., 1999; Back & Rivkees, 2004). Therefore, in addition to the injury to oligodendrocytes and myelination, we also examined axonal injury with the MAP1 antibody and dopamine neuron injury with the TH antibody. For immunohistochemistry, the final concentrations of the primary antibodies were diluted as follows: APC-CC1 (1 : 20), MBP (1 : 100), MAP1 (1 : 200), TH (1 : 20,000), ED1 (1 : 200) and CD11b (OX42; 1 : 100). Mature oligodendrocytes were identified with APC-CC1, which immunostains oligodendrocyte cell bodies without labeling of oligodendrocyte processes or astrocytes (Murtie et al., 2005). MAP1 provides selective staining of neurons and stronger staining of axons and dendrites. Microglia were detected using lectin histochemistry, as well as by CD11b (OX42) immunostaining, which recognizes both the resting and the activated microglia. ED1 immunostaining detected the activated microglia or macrophages. After incubation with biotinylated second antibodies for 1 h at room temperature, brain sections were further incubated with Cy3-conjugated avidin or the avidin–horseradish peroxidase system (ABC kit from Vector Laboratories, Burlingame, CA, USA) for an additional 1 h in the dark at room temperature. For immunofluorescence staining, DAPI (100 ng/mL) was simultaneously used to identify nuclei in the final visualization. Sections incubated in the absence of primary antibody were used as negative controls. Biotinylated tomato lectin was used at a concentration of 10 μg/mL and the results were visualized using the Vector ABC system.

**Quantitation of immunostaining data and statistics**

To compare the cell number among the treatment groups, positively stained cells were counted. Our previous studies indicate that this neonatal rat model of HI produces preferential white matter injury primarily in the forebrain (Cai et al., 2001, 2006; Lin et al., 2004). APC-CC1+ cell counting was performed in the forebrain sections at the bregma level. TH+ cell counting was performed in the midbrain sections at a level one-third rostral from the lambda to the bregma. A digital microscopic image for each brain section was captured at the singular area where the APC-CC1+ cells were most abundant or at the substantia nigra where the TH+ cells were most abundant. Three brain sections at the same brain level were counted bilaterally in each animal. Axonal and dendritic lengths were estimated in the MAP1-stained sections from the forebrain at the bregma level for the cortex area or in those from the diencephalon at a level one-half rostral from the lambda to the bregma for the hippocampal area. The lengths of axons and dendrites in the whole image were measured using NIH Image software in three brain sections at the same brain level and the average of the measures was calculated bilaterally for a single brain. The mean thickness of the corpus callosum has been suggested to be related to neuropsychological outcome (Nosarti et al., 2004). The thickness of the corpus callosum at the bregma level in each rat brain was determined in H&E-stained sections at the centre of the corpus callosum with the NIH Image software. Perinatal asphyxia has been reported to reduce the thickness of corpus callosum MBP staining (Kohlhauser et al., 2000). We found in our preliminary study that HI resulted in a thinner MBP staining at the subcortical white matter tract. To examine quantitatively effects of HI on the thickness of the subcortical MBP staining, the mean thickness of the subcortical MBP staining under the forelimb area of the corpus in a microscopic image at the bregma level (coordinates according to Paxinos & Watson, 1998) was determined by use of the NIH Image software. The thickness of MBP staining was measured at the beginning, the middle and the end of the area (see Fig. 4 for positions) and the average of the three measures was calculated bilaterally for a single brain. Cell number or thickness of the corpus callosum and the MBP staining were taken from the average of three sections for each brain. Results are presented as the mean ± SEM of six animals. The immunostaining and the neurobehavioral performance data were analysed by one-way ANOVA followed by the Student–Newman–Keuls test. Results with a P < 0.05 were considered statistically significant.
Results
Minocycline improved neurobehavioral deficits induced by HI

Physical development
HI insult retarded somatic development. Compared with the control group, HI insult on P4 resulted in a lower body weight from P5 to P21 (Fig. 1A). Minocycline treatment significantly reduced the HI-induced weight loss in rats. The day of eye opening for the sham + PBS and sham + minocycline groups was P15.3 ± 0.1 and P15.3 ± 0.2, respectively. HI insult delayed the day of eye opening (P17.3 ± 0.3). Minocycline treatment significantly shortened the HI-induced delay in eye opening in the rat (P16.2 ± 0.3).

Righting reflex
As show in Fig. 1B, the HI group exhibited significantly longer mean latency time as compared with the control group from P5 to P15. Recovery from this impairment started on P16. Minocycline treatment significantly shortened the HI-induced increase in righting reflex latency. By P8, there was no difference in righting reflex between the control and the HI + minocycline groups.

Wire hanging maneuver
The righting ability of the rat increased with age. The mean latency time of the HI group was significantly less than that of the control group from P6 to P16 (Fig. 1C). Minocycline treatment significantly increased the wire hanging latency of the rat from P6. It was not until P17 that the latency time in the HI group increased to the level of the control group, whereas that in the HI + minocycline group reached the control level by P8.

Cliff avoidance test
All pups from the control group succeeded in the cliff avoidance test by P11 and the avoidance latency decreased with age (Fig. 1D). It was not until P14 that pups from the HI group had cliff avoidance responses with a success rate at 83%, whereas minocycline treatment advanced cliff avoidance responses of all pups from the HI + minocycline group by 3 days (P11). The avoidance response latency in the HI group reached the level of the control group by P18, whereas that in the HI + minocycline group reached the level of the control group by P14.

Locomotor activity
Locomotor activity, as measured by the total crossing distance of an individual rat during a 10-min period, increased with age until P15 in all groups (Fig. 2A). HI treatment resulted in hyperactivity from P12 to P18 as compared with the control group. Minocycline treatment prevented the HI-induced hyperactivity in the HI + minocycline group from P12 to P14, and reduced the HI-induced increases in locomotor activity from P15 to P17.

Passive avoidance
The number of electric foot shocks needed to retain the rat on the safe board was significantly increased in the HI group at P20 (Fig. 2B). HI also reduced the retention latency to step down from the board the next day (P21) as compared with the control group. Minocycline treatment significantly improved HI-induced learning and memory deficits.

Elevated plus-maze test
As shown in Fig. 2C and D, a greater number of entries into the open, but not the enclosed arm was observed in the HI group as compared with the control group at P19. HI insult also increased the proportion of time spent in the open arms while it decreased the proportion of time spent in the enclosed arms. Minocycline administration inhibited HI-induced less anxiety-like behavior during the elevated plus-maze test.

Minocycline reduced brain injury induced by HI
Consistent with our previous results (Fan et al., 2005a), HI insult caused white matter rarefaction or severe necrosis at the corpus callosum area in P21 rat brains (Fig. 3C and G). Although the injury was largely in the white matter, necrotic neuronal cells in the cortex and the hippocampus were also observed in severely injured HI rat brains. Enlargement of ventricles and reduction in white matter size were observed in the HI rat brain 17 days after HI insult, as measured by the ventricle size index and the white matter size index (Fig. 3C and G, Table 1), but not in the control group (Fig. 3A and E, Table 1). Minocycline treatment reduced HI-induced brain injury, ventricle enlargement and white matter reduction (Fig. 3D and H, Table 1).
Treatment with minocycline also significantly alleviated the HI-induced reduction in the corpus callosum thickness at the bregma level (Table 1).

Minocycline ameliorated loss of mature oligodendrocytes and hypomyelination induced by HI

Mature oligodendrocytes were identified with APC-CC1. APC-CC1-positive staining was primarily observed in the corpus callosum and the subcortical white matter tract at the bregma level and in the internal capsule areas. HI insult significantly reduced the number of APC-CC1-positive cells (Fig. 4C), as compared with that in the Sham + PBS or Sham + minocycline rat brain (Fig. 4A and B). Minocycline treatment attenuated the HI-induced reduction in the number of mature oligodendrocytes in the P21 rat brain (Fig. 4D). The detailed APC-CC1 cell counting data are given in Table 1.

Oligodendrocytes are myelin-producing cells, and reduction in their number may result in hypomyelination. MBP staining was used as a marker of myelination in the P21 rat brain. White matter rarefaction (indicated by asterisks), eosinophilic coagulation necrosis (indicated by arrows) and ventricle dilation (V) in the forebrain and the diencephalon levels were found in the HI group (C and G). Minocycline attenuated hypoxia–ischemia-induced brain injury (D and H). Two levels of coronal sections of the pup brain in each group are presented: (I) forebrain sections: including striatum at the bregma level (A–D) and (II) diencephalon sections: including hippocampus at a level one-half rostral from the lambda to the bregma (E–H). Scale bar (in A), 200 μm.

Oligodendrocytes are myelin-producing cells, and reduction in their number may result in hypomyelination. MBP staining was used as a marker of myelination in the P21 rat brain. MBP-positive staining was clearly detected in the P21 sham rat brain, primarily in the corpus callosum, the subcortical white matter tract and the internal capsule areas. The mean thickness of the subcortical white matter MBP staining under the forelimb area of the cortex was measured for comparison (Table 1). HI resulted in severe impairment of MBP staining at the subcortical white matter tract (Fig. 4G). HI insult reduced the thickness of the MBP-positive staining (107.0 ± 16.3 μm) as compared with the Sham + PBS group (178.7 ± 6.5 μm) and the Sham + minocycline group (189.7 ± 8.0 μm) (Table 1). Minocycline treatment significantly ameliorated HI-induced hypomyelination, as indicated by an increased thickness of MBP staining (158.2 ± 5.3 μm).

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Minocycline attenuated HI-induced enlargement of ventricles, reduction in size of the white matter, thickness of the corpus callosum, axonal lengths and subcortical MBP staining, loss of oligodendrocytes and tyrosine hydroxylase neurons, and microglial activation in the rat brain 17 days (P21) after the hypoxic–ischemic insult

**Table 1.** Minocycline attenuated HI-induced enlargement of ventricles, reduction in size of the white matter, thickness of the corpus callosum, axonal lengths and subcortical MBP staining, loss of oligodendrocytes and tyrosine hydroxylase neurons, and microglial activation in the rat brain 17 days (P21) after the hypoxic–ischemic insult

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>Hypoxia-ischemia</th>
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<tr>
<td></td>
<td>PBS</td>
<td>Minocycline</td>
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<tr>
<td>Ventricle size index (%)</td>
<td>1.68 ± 0.10</td>
<td>1.79 ± 0.14</td>
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<tr>
<td>White matter size index (%)</td>
<td>6.21 ± 0.19</td>
<td>6.07 ± 0.18</td>
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<tr>
<td>Corpus callosum thickness in H&amp;E staining (μm)</td>
<td>234.8 ± 10.2</td>
<td>229.0 ± 6.2</td>
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<tr>
<td>Subcortical MBP staining (μm)</td>
<td>178.7 ± 6.5</td>
<td>189.7 ± 8.0</td>
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<tr>
<td>Oligodendrocytes (APC-CC1+) (cells/mm²)</td>
<td>1456 ± 95</td>
<td>1498 ± 59</td>
</tr>
<tr>
<td>Cortical axon and dendrite length (μm)</td>
<td>102.2 ± 3.7</td>
<td>103.7 ± 3.9</td>
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<tr>
<td>Hippocampal axon and dendrite length (μm)</td>
<td>45.0 ± 2.0</td>
<td>42.7 ± 2.5</td>
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<tr>
<td>Tyrosine hydroxylase neurons (cells/mm²)</td>
<td>61.0 ± 3.7</td>
<td>64.4 ± 1.8</td>
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<tr>
<td>Activated lectin-positive cells (cells/mm²)</td>
<td>13.3 ± 6.9</td>
<td>8.9 ± 4.4</td>
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<tr>
<td>Activated microglia in OX42 staining (cells/mm²)</td>
<td>31.1 ± 11.2</td>
<td>28.9 ± 8.7</td>
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<tr>
<td>ED1-positive cells (cells/mm²)</td>
<td>44.4 ± 5.6</td>
<td>48.9 ± 5.6</td>
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Brain sectioning and immunostaining were performed as described in the Methods. Sections from the forebrain at the bregma level were used in H&E and MBP staining. Counting of APC-CC1+, lectin+/ ED1+ cells (activated microglia/macrophages) was performed in the periventricular area of sections at the bregma level. H&E-stained sections were scanned by a densitometer, and the areas of ventricles, the whole subcortical white matter tract and the whole brain section were measured. The ratio between the areas of ventricles or the white matter and that of the whole brain section was calculated as the ventricle size index or the white matter size index, respectively. CD11b (OX42)-positive cells with an enlarged cell body and blunt processes (as indicated in Fig. 7C) were considered as activated microglia. MAP1 staining was performed in sections from the forebrain at the bregma level (for changes in cortical area) and in sections from the diencephalon at a level one-half rostral from the lambda to the bregma (for changes in hippocampal area). TH+ cell counting was performed in the substantia nigra area of the midbrain sections at a level one-third rostral from the lambda to the bregma. Data are presented as the mean ± SEM of six animals for each group. Data were analysed by one-way ANOVA followed by the Student–Newman–Keuls test. *P < 0.05 compared with the Sham + PBS group. †P < 0.05 compared with the HI + PBS group.

Minocycline attenuated HI-induced axonal and dendritic damage in the rat brain

Whereas neurons in the cerebral cortex, hippocampus and cerebellum had very weak immunoreactivity for MAP1, axons and dendrites were detected by strong MAP1 immunostaining. In the Sham + PBS rat brain, the average length of axons and dendrites in the cortex and the hippocampal CA1 regions was 102.2 ± 3.7 and 45.0 ± 2.0 μm, respectively (Fig. 5A and E, Table 1). As shown in Fig. 5C and G, HI resulted in injuries to axons and dendrites of the cortical and hippocampal neurons, as indicated by the beaded axonal profiles and the shorter average length of axons and dendrites (33.5 ± 1.7 μm and 10.7 ± 1.5 μm, respectively) (Fig. 5C and G, Table 1). Minocycline treatment prevented the HI-induced injury to axons and dendrites (Fig. 5D and H, Table 1).

Minocycline reduced loss of TH neurons induced by HI

Positive staining of TH was used to detect dopamine neurons in the substantia nigra. All TH-positive cells in the midbrain sections at a level one-third rostral from the lambda to the bregma were counted bilaterally in three sections for each animal. In the P21 control rat brain, TH-positive cells were more predominant in the lateral regions of the substantia nigra (Fig. 6A). HI significantly reduced the number of TH-positive neurons in the rat brain (Fig. 6C). Minocycline treatment attenuated the HI-induced reduction in the number of TH-positive neurons (Fig. 6D). Counts of TH-positive cells are given in Table 1.

Minocycline reduced microglial activation induced by HI in the rat brain

To investigate mechanisms involved in the protective effects of minocycline, we examined microglial activation in the rat brain following HI insult. HI stimulated activation of microglia, as indicated by lectin histochemistry in the corpus callosum area at the bregma level. In the Sham + PBS, Sham + minocycline and HI + minocycline groups, most microglia were at the resting stage with a ramified shape (Fig. 7A, B and D), whereas those in the HI group were activated, round in shape and in significantly increased number, on both sides of the corpus callosum and the subcortical white matter tract (Fig. 7C, Table 1). Minocycline treatment significantly reduced the number of activated microglia in the rat brain (Table 1).

Results of HI-induced microglial activation and effects of minocycline on this activation demonstrated by lectin staining were further confirmed by CD11b (OX42) (Fig. 7E–H, Table 1) and by ED1 immunostaining (Fig. 7I–L, Table 1).

Discussion

Although neuroprotective effects of minocycline have been reported in many in vivo and in vitro animal models of human neurodegenerative diseases, protection of minocycline against neonatal hypoxic–ischemic brain injury is still controversial. Although marked protection of minocycline against the neonatal rat HI brain injury has been reported previously (Arvin et al., 2002), a recent study shows that minocycline only provides partial protection against HI brain injury in the neonatal rat and it worsens brain injury induced by the same insult in neonatal mice (Tsuji et al., 2004). Furthermore, post treatment with minocycline failed to improve motor and cognitive functional alterations in a rat model of stroke, although it reduced brain infarct size (Ahtoniemi et al., 2005). Data from the present study suggest that minocycline itself when used at a dose of 45 mg/kg does not have apparent adverse effects, as indicated by the indistinguishable body weight gain and neurobehavioral performance between the Sham + PBS and Sham + minocycline groups. Results from the present study not only confirm our previous observations that minocycline protects the neonatal rat brain from HI injury induced by bilateral carotid artery occlusion followed by 15 min of hypoxic exposure (Cai et al., 2006), but also further demonstrate that minocycline has long-lasting...
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I. APC-CC1

A. Sham+PBS

B. Sham+mino

C. HI+PBS

D. HI+mino

II. MBP

E. Sham+PBS

F. Sham+mino

G. HI+PBS

H. HI+mino

Fig. 4. Representative photomicrographs of (I) APC-CC1 staining and (II) MBP staining in the P21 rat brain. Hypoxia–ischemia and minocycline treatments were performed as described in the Methods. APC-CC1- or MBP-positive staining was detected in the P21 Sham + PBS (A and E) and the Sham + minocycline rat brains (B and F), respectively. Hypoxia–ischemia resulted in loss of APC-CC1- or MBP-positive staining (C and G). Minocycline attenuated hypoxia–ischemia-induced loss of APC-CC1- or MBP-positive staining (D and H). The thickness of MBP staining was measured at the locations indicated by arrows. Scale bar (in A), 200 μm.

I. MAP1-Cortex

A. Sham+PBS

B. Sham+mino

C. HI+PBS

D. HI+mino

II. MAP1-Hippocampus

E. Sham+PBS

F. Sham+mino

G. HI+PBS

H. HI+mino

Fig. 5. Representative photomicrographs of MAP1 staining in the P21 rat brain. Hypoxia–ischemia and minocycline treatments were performed as described in the Methods. MAP1-positive staining was detected in the P21 Sham + PBS (A and E) and Sham + minocycline rat brains (B and F). Hypoxia–ischemia caused axonal and dendritic impairment as indicated by the beaded (arrows indicated in C) or shorter of MAP1-positive staining (C and G). Minocycline attenuated the HI-induced beaded or shorter MAP1 staining (D and H). Two levels of coronal sections of the pup brain in each group are presented: (I) forebrain sections: including striatum at the bregma level (A–D) and (II) diencephalon sections: including hippocampus at a level one-half rostral from the lambda to the bregma (E–H). Scale bar (in A), 50 μm.

Minocycline protection on HI-induced brain injury and improves neurobehavioral performance in juvenile rats after the neonatal HI insult. Compared with the HI brain injury from the Rice et al. (1981) model (unilateral carotid artery ligation followed by 2–3 h of hypoxic exposure) or with the focal cerebral infarct induced by middle cerebral artery occlusion (Ahtoniemi et al., 2005), the injury in our model is less severe and mostly occurs in the white matter (Lin et al., 2004). This is an indication that the protective effects of minocycline on neonatal HI brain injury may depend on the severity of the initial insult. The different consequences of minocycline treatment may also depend on the timing of treatment. In the present study, the initial minocycline treatment was started 12 h before HI. It will be important for future studies to determine whether post treatment will also provide similar protection against HI injury and neurobehavioral deficits.

Delayed behavioral disturbances resulting from neonatal HI have been found in other studies (Buwalda et al., 1995; Nyakas et al., 1996). Perinatal HI-induced behavioral disturbances have been grouped into two categories. In the first, the focus is on hyperactivity whereas in the second it is on learning and memory deficits (Nyakas et al., 1996). The transiently increased locomotor activity found in the HI group in the current study is an indication of hyperactivity. Locomotor activity is associated with the activity and habituation response of animals on placement in a novel environment (Hermans et al., 1992). Impulsivity and hyperactivity are major signs of attention deficit–hyperactivity, a behavioral disorder affecting 8–12% of children worldwide (Biederman & Faraone, 2005). Although there is no direct evidence, it has been hypothesized that attention deficit–hyperactivity disorder may be associated with selective damage to dopaminergic neurons (Shaywitz et al., 1976, 1984). Numerous studies have shown that locomotor activity disorders are associated with an abnormal level of dopamine content in the rat brain concomitant with substantia nigra injury (Bakos et al., 2004) or in...
mice lacking expression of TH (Nishii et al., 1998; Kobayashi & Sano, 2000). Neonatal HI has been demonstrated to decrease TH-positive neurons in the substantia nigra of neonatal (P9) (Oo et al., 1995) and adult rat brain (P56–P84) (Burke et al., 1992). Results from the present study show that neonatal HI results in impaired TH immunoreactivity of dopamine neurons in the substantia nigra and the associated transient hyperactivity in the juvenile rat, and that while minocycline reduces the injury to dopamine neurons in the substantia nigra, it also improves HI-induced hyperactivity. Although attribution of attention deficit–hyperactivity disorder solely to a selective dopaminergic dysfunction is oversimplified (Nyakas et al., 1996), our data suggest that there might be a close link between locomotor activity and the dopamine neuronal injury in the substantia nigra.

The passive avoidance data from the current study confirm the existence of HI-induced learning and memory deficits. The HI-induced cognitive dysfunction was also reported in other studies (Ikeda et al., 2001; Simonova et al., 2003; Ten et al., 2003). In the current study, the exact anatomic basis of brain injury for the HI-induced cognitive dysfunction is unknown. The motor and cognitive dysfunction has also been linked with childhood hydrocephalus, in which ventricles are dilated and periventricular white matter, axons and myelin are the primary targets of injury (Del Bigio, 2004). In a rat model of hydrocephalus induced by kaolin injection, learning disability as determined by the water maze test has been found to be associated with the degree of ventricle dilation, and an early shunting surgery, which reduces the ventricular dilation, prevented the behavioral disability (Del Bigio et al., 1997). In the present study,

![Fig. 6. Representative photomicrographs of TH staining in the P21 rat brain.](image)

![Fig. 7. Representative photomicrographs of (I) lectin staining, (II) CD11b (OX42) and (III) ED1 staining in the P21 rat brain.](image)
enlarged bilateral ventricles and impairment of axons and myelin were observed in the HI rat brain. Regardless of whether the ventricular enlargement is caused by the paucity of cerebral myelin (Volpe, 2001) or by increased intracranial pressure (Del Bigio et al., 1997), the similarity in the pattern of brain injury between the rat model of hydrocephalus (Del Bigio et al., 2003) and the current study suggests that axonal and dendritic damage at the hippocampus and the ventricle enlargement induced by HI may contribute significantly to the HI-induced learning and memory deficits found in the present study.

Results from the current elevated plus-maze test also show that HI insult increased the number of open arm entries and the proportion of the time spent in the open arms while decreasing the proportion of the time spent in the enclosed arm, behaviors indicative of a decreased anxiety level. A reduction in HI-induced anxiety-like behaviors has been reported in other perinatal asphyxia animal models (Hoeger et al., 2000; Caputa et al., 2005; Plamondon & Khan, 2005). Anxiety-like behavior was found to be associated with the levels of dopamine and its metabolites in the mouse brain following ischemia (Winter et al., 2005). In the current study, although HI-induced alterations in anxious behaviors and the improvement of these behaviors following minocycline treatment are found to be positively linked with the injury in dopamine neurons in the substantia nigra, as indicated by the number of TH+ neurons, to elucidate the basis of the reduced anxiety-like behaviors induced by HI, further studies are required.

The precise mechanisms underlying the protective effect of minocycline on HI-induced brain injury are unknown. Minocycline (10 mg/kg, i.v.) has been found to prevent a rapid drop in the mean arterial blood pressure induced by lipopolysaccharide in rats, but not to affect the mean arterial blood pressure when used alone (Hsiao et al., 2002). Our preliminary studies show that bilateral common carotid artery occlusion in P4 rats results in a 47.2% reduction in cortical blood flow, as determined by a laser Doppler device (Z. Cai, unpublished data). It is unknown if minocycline has an effect on the HI-induced reduction in cerebral perfusion in the present study. At present it is impossible to monitor the physiological responses in neonatal rats following minocycline treatment. However, Xu et al. (2004) reported that minocycline protected the adult rat brain from injury induced by middle cerebral artery occlusion, but did not affect mean arterial blood pressure. The neuroprotection of minocycline has also been attributed to many other factors, including inhibition of matrix metalloproteinase 9 (Brundula et al., 2002), iNOS (Amin et al., 1996; Yrjanheikki et al., 1998) or caspase-1 and caspase-3 (Sanchez Mejia et al., 2001; Arvin et al., 2002) and the anti-apoptotic property of minocycline (Stirling et al., 2004; Wang et al., 2004). As reported by other investigators (Yrjanheikki et al., 1998; Tikka et al., 2001; Wu et al., 2002; Tomas-Camardiel et al., 2004), our current data indicate that the protective effects of minocycline on HI-induced brain injury and neurobehavioral dysfunction may be associated with the inhibition of microglial activation. Our previous studies have shown that white matter injuries, including oligodendrocyte injury, in the HI rat brain were accompanied by increased activation of microglia/macrophages, as indicated by ED1 and CD11b (OX42)-positive immunostaining (Cai et al., 2001, 2006), and that the short-term protection of minocycline was probably due to the reduction in HI-induced oxidative and nitrosative stress, which is largely the result of inhibition of microglial activation (Cai et al., 2006).

In summary, neonatal HI results not only in injury to axons, oligodendrocytes and myelin in the current rat model, but also in long-term neurological dysfunction. Minocycline attenuates HI-induced brain injury and improves impaired neurobehavioral performance in juvenile animals. In evaluation of the efficacy of a pharmacological treatment on HI-induced brain injury, it is equally important to assess histopathological evidence and the improvement of neurobehavioral performance.

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Abbreviations
APC-CC1, adenomatus polyposis coli (Clone CC1); HI, hypoxia–ischemia; MBP, myelin basic protein; MAP1, microtubule-associated protein; P, postnatal day; TH, tyrosine hydroxylase.

References

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