Inhibition of Neuroinflammation Prevents Injury to the Serotonergic Network After Hypoxia-Ishemia in the Immature Rat Brain

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Abstract

The phenotypic identities and characterization of neural networks disrupted after neonatal hypoxia-ischemia (HI) in the preterm brain remain to be elucidated. Interruption of the central serotonergic (5-hydroxytryptamine [5-HT]) system can lead to numerous functional deficits, many of which match those in human preterm neonates exposed to HI. How the central serotonergic network is damaged after HI and mechanisms underlying such injury are not known. We used a Postnatal Day 3 rat model of preterm HI and found parallel reductions in the 5-HT transporter expression, 5-HT levels and numbers of 5-HT-positive dorsal raphe neurons 1 week after insult. Post-HI administration of minocycline, an inhibitor of activated microglia, attenuated HI-induced damage to the serotonergic network. Minocycline effects seemed to be region specific, that is, where there was microglial activation and increases in tumor necrosis factor-α and interleukin β. The concurrent improvement in serotonergic outcomes suggests that inhibition of neuroinflammation prevented damage to the serotonergic neurons rather than affected the regulation of 5-HT or serotonin transporter. These data elucidate the mechanisms of serotonergic network injury in HI, and despite the known adverse effects associated with the use of minocycline in neonates, postinsult administration of minocycline may represent a novel approach to counter neuroinflammation and preserve the integrity of the central serotonergic network in the preterm neonate.

Key Words: Hypoxia-ischemia, Microglia, Neonatal, Neuroinflammation, Raphe, Serotonin, Serotonin transporter.

INTRODUCTION

Premature birth and exposure to a hypoxic-ischemic (HI) insult are major factors that contribute to mortality and lifelong morbidity in newborns. There is currently no therapeutic intervention that can minimize HI brain injury. The development of treatment strategies is restricted by the lack of knowledge of affected neuronal networks and the mechanisms that underpin HI-induced neurodegeneration. The serotonergic network has a diverse range of central functions, and its disruption is purported to underlie numerous physiological and behavioral changes in disease states such as epilepsy, depression, movement disorders, autism, and sudden infant death syndrome (SIDS) (1–4). Many of these deficits match those observed in HI-affected neonates (5–8). Furthermore, serotonergic fibers pervasively innervate the brain, including HI-damaged forebrain regions. Indeed, in human neonates with HI encephalopathy, brainstem tryptophan hydroxylase is reduced (9, 10) and there is injury to the dorsal brainstem where serotonergic cell bodies are located (11). We recently demonstrated that the expression of the serotonin transporter (SERT), the major regulator of serotonin (5-hydroxytryptamine [5-HT]) availability, and 5-HT-positive raphe cell bodies are both decreased in the brainstem after preterm HI in the rodent (12). However, it is not known whether forebrain SERT and 5-HT changes occur in concert with brainstem injury. To understand the pathogenesis of functional deficits in the HI-affected neonate, it is necessary to determine how a preterm HI insult affects major components of the central serotonergic network.

The maintenance or progression of brain injury after neonatal HI can occur via secondary mechanisms of which neuroinflammatory processes, particularly the activation of microglia, are pivotal. Activated microglia can multiply, migrate to sites of injury, release cytotoxic substances such as proinflammatory cytokines, and persist in the neonatal brain for weeks after neonatal HI (13–16). Minocycline (a second-generation tetracycline) is a potent inhibitor of microglia (17, 18) and attenuates neurodegeneration after neonatal HI (19–22), but its effectiveness to alleviate serotonergic damage in neonates has not been studied. In the adult rat, minocycline reverses 3-nitropropionic acid neurotoxicity–induced changes in 5-HT levels (23) and curtails the 3,4-methylenedioxyamphetamine–induced reduction in SERT expression (24). Although minocycline has successfully attenuated neurodegeneration in adult clinical trials (25–27), minocycline...
is not administered in human neonates because tetracyclines have historically been associated with adverse effects such as teeth discoloration and stunting bone growth (28–31). Nevertheless, the inhibitory effects on neuroinflammation of minocycline might provide protection to the neonatal brain, and understanding its effects may elucidate the mechanisms underpinning HI-induced neonatal brain injury.

We hypothesized that HI in the immature brain disrupts the serotonergic system and that inhibiting neuroinflammation would prevent this brain injury. Using a model of preterm HI in the Postnatal Day 3 (P3) rat pup, we determined how neonatal HI disrupts the serotonergic network, focusing on the impact of P3 HI on SERT. We also investigated the potential role of neuroinflammation in producing P3 HI-induced damage to the serotonergic network using minocycline. Minocycline was administered postinsult because this period is compatible with the timing of clinical recognition of affected neonates.

MATERIALS AND METHODS

Animals

Experiments were performed on Sprague-Dawley dams and their pups (10–12 per litter) in accordance with ethical approvals stipulated by the University of Queensland Animal Ethics Committee. To limit the possible effects of circadian rhythms, experiments were performed between 9 AM and 11 AM. Dams and pups were housed in animal facilities maintained at 22°C and on a 12-hour light-dark cycle with food and water accessible ad libitum. Efforts were made to minimize the number of animals used and their suffering.

Hyoxic-Ischemic Insult and Administration of Minocycline

On P3, pups were randomly assigned to 1) control + saline, 2) P3 HI + saline, 3) P3 HI + minocycline, or 4) control + minocycline groups. A total of 6 litters were used. The HI insult was performed as previously described (12, 20, 32). Briefly, pups were anesthetized using isoflurane (2%; Baxter, Deerfield, IL). The right common carotid artery was isolated and ligated permanently. The wound was sutured, and the pups recovered for 20 minutes on a heating pad before being exposed to 6% O2 for 30 minutes at 37°C in a humidified chamber. Pups then recovered on a heating pad and breathed room air before being returned to the dam. A P3 rat model is analogous to the preterm human brain at approximately 24 to 28 weeks’ gestation in terms of the cortical organization, number of synapses, and neurochemical development (33, 34). This P3 HI model incurs typical pathological features (e.g., encephalopathy, neuronal loss, and hypomyelination) seen in premature human neonates (6, 20, 32, 34–37). The control group underwent the same procedures, but the carotid artery was not ligated, and they were exposed to room air instead of hypoxia. Each group had approximately equal distribution of males and females. The mortality rate was less than 3.5%.

Minocycline (Sigma-Aldrich, Castle Hill, New South Wales, Australia) was administered 2 hours after hypoxia (45 mg/kg, intraperitoneally [i.p.]) and then every 24 hours from P4 to P9 (22.5 mg/kg, i.p.). Minocycline is a second-generation tetracycline that crosses the blood-brain barrier after systemic administration (38–40) and has anti-inflammatory effects in the CNS based primarily on its ability to inhibit immune mediators, particularly microglia (18, 41). We have previously shown that this minocycline regimen reduces numbers of activated microglia after P3 HI (20, 42). The control + saline and P3 HI + saline groups received equivalent volumes of sterile saline i.p. On P10, animals were killed by sodium pentobarbital overdose (80 mg/kg, i.p.; Lethabarb, Virbac, France).

Immunohistochemistry

Animals were perfused via the heart with 1% sodium nitrate solution (in 0.1 mol/L PBS, pH 7.4), followed by 4% formaldehyde (in 0.1 mol/L PBS, pH 7.4). Brains were postfixed in 4% buffered formaldehyde and stored in 10% sucrose (in 0.1 mol/L PBS, pH 7.4 at 4°C). The numbers for each group were as follows: 1) control + saline (n = 5), 2) P3 HI + saline (n = 5), 3) P3 HI + minocycline (n = 5), and 4) control + minocycline (n = 3). Serial coronal forebrain (40 μm) and brainstem (50 μm) sections were cut using a freezing microtome. Two 1-in-4 series of forebrain sections and two 1-in-4 series of brainstem sections were exposed to an immunoperoxidase technique (43, 44) to visualize SERT protein, 5-HT-positive raphe neurons, and the microglial cell marker ionized calcium-binding adapter molecule 1 (Iba-1) (12, 20, 42, 45).

The Iba-1 is expressed specifically in microglia and is not found in astrocytes, oligodendrocytes, or neurons (46). Although Iba-1 is also present in macrophages, macrophages are not usually found in the brain parenchyma and can be distinguished morphologically from Iba-1-positive microglia. It is weakly expressed in ramified and resting microglia and upregulated in activated microglia, enabling Iba-1 immunopositivity to be used as a marker of microglial activation (47). Series of sections from each experimental group were processed simultaneously to minimize possible variability in staining. Briefly, forebrain sections were incubated for 48 hours in goat anti-SERT (1:2500; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or rabbit anti-Iba-1 (1:10,000; WAKO Pure Chemical Industries, Osaka, Japan), followed by a 2-hour incubation in a biotinylated donkey anti-rabbit or anti-goat (1:400; Jackson Immunoresearch, West Grove, PA). Sections were then incubated for a further 2 hours in a solution of avidin–biotin–horseradish peroxidase complex (ABC) Vector Elite Kit, Burlingame, CA) before being exposed to a nickel 3,3-diaminobenzidine chromogen solution. To identify 5-HT-positive raphe neurons, sections were incubated for 48 hours in rabbit anti–5-HT antibody (1:7500; gift from Professor D. Pow, University of Queensland, Queensland, Australia), followed by a 2-hour incubation in a biotinylated donkey anti-rabbit (1:400). Sections were then incubated for a further 2 hours in a solution of avidin–biotin–horseradish peroxidase complex (ABC) Vector Elite Kit, Burlingame, CA) before being exposed to a nickel 3,3-diaminobenzidine chromogen solution. To identify 5-HT-positive raphe neurons, sections were incubated for 48 hours in rabbit anti–5-HT antibody (1:7500; gift from Professor D. Pow, University of Queensland, Queensland, Australia), followed by a 2-hour incubation in a biotinylated donkey anti-rabbit (1:400). Sections were then incubated for a further 2 hours in a solution of ABC before being exposed to a 3,3-diaminobenzidine solution (nickel omitted) to visualize amber-colored 5-HT-positive raphe neurons. A second series of brainstem sections underwent dual immunoperoxidase procedure to visualize Iba-1 and 5-HT-positive neurons. Sections were immunolabeled for Iba-1 (as previously described), but subsequently incubated in anti–5-HT (as previously described) and the peroxidase reaction was terminated.
to only lightly label 5-HT–positive raphe neurons an amber color. This procedure allowed for counts of Iba-1–positive microglia to be performed in serotonin raphe subdivisions. All sections were mounted on chrome-alum–subbed slides, dehydrated in alcohol, cleared in xylene, and coverslipped.

Analysis of Immunohistochemistry

To determine the effect of P3 HI and minocycline on cerebral and brainstem hemisphere size ipsilateral to the carotid ligation, photomicrographs were taken of 3 consecutive sections in the forebrain (160-μm intervals) between 2.7 and 1.7 mm relative to bregma and in the brainstem (200 μm intervals) from −7.3 to −8.3 mm relative to bregma (48). Images of brain sections were acquired using an Olympus microscope (BX41) and photographed with a CCD camera (Olympus DP70). Using the software program Analysis Life Science Research, the outlines of the left and right hemispheres were traced, and the percentage change in right hemisphere area relative to the left was calculated for each section and averaged for all 3 sections.

The distribution of SERT in the cytoplasm and plasma membrane of fibers, cell bodies, and axon terminals (49) makes it an excellent marker of serotonergic fibers in the brain (50, 51). We focused on forebrain regions innervated by serotonergic fibers and known to be affected after preterm HI in rodent models (37, 52). Photomicrographs (200 μm × 200 μm) were taken of the motor (bregma 2.2 mm) and somatosensory (bregma 2.2 mm) cortex, lateral hypothalamus (bregma −4.16 mm), ventrolateral thalamus (bregma −4.16 mm), and horizontal limb of the diagonal band (bregma 0.48 mm) (48). For each brain region, 3 sequential serial sections (160 μm apart) were analyzed using the commercial software National Institutes of Health ImageJ, as described (59). The area density was obtained and expressed as a percentage of 200 μm². Threshold images were obtained with visual comparison being made to the original gray-scale images to ensure that the threshold tool effectively resolved all labeled fibers. Particle analysis was then carried out to determine the SERT-positive area. The mask tool was used to confirm that all labeled fibers were detected and measured.

Serotonergic 5-HT–positive raphe neurons were counted in 2 subdivisions of the dorsal raphe defined as the ventrolateral part (level −7.64 mm to −8.3 mm relative to bregma, using a grid of 360 μm × 360 μm) and the dorsal part (level −8.3 mm to −7.3 mm relative to bregma, using a grid of 360 μm × 360 μm) (48). These regions were chosen because we found that these dorsal raphe subdivisions show the greatest loss after P3 HI (12). The grid was placed over the subdivision, and 5-HT–labeled neurons were only included if the nucleus could be discriminated. A total count representative of the dorsal raphe was obtained by adding counts from the ventrolateral and dorsal parts.

Activated microglia were identified as Iba-1–positive microglia with distinct morphology; that is, dense labeling, thickened processes, and retraction of processes toward the cell body (60). Counts of total and activated microglia were obtained in the somatosensory cortex, thalamus, and dorsal raphe in 3 consecutive sections in the right hemisphere.

Total Protein Preparation

For protein analyses and ELISA, another group of pups was killed with sodium pentobarbionate (80 mg/kg, i.p.) on P10. Brains were rapidly excised, and specific areas of the brain were collected and immediately frozen and stored at −80°C. The numbers in each group were as follows: 1) control + saline (n = 10), 2) P3 HI + saline (n = 10), 3) P3 HI + minocycline (n = 9), and 4) control + minocycline (n = 9). Total protein extracts were obtained 1 week after P3 HI from 3 brain regions: 1) frontal cortex (+4.0 to 0.0 mm relative to bregma), 2) thalamus (0.0 to −5.0 mm relative to bregma), and 3) brainstem (−7.0 to −12.0 mm relative to bregma) to determine 5-HT and cytokine levels and SERT protein expression. We specifically chose brain regions that elicited losses of SERT-positive immunolabeling and are known to be damaged after neonatal HI. To extract total protein, the brain tissue from each area was gently homogenized in ice-cold distilled H₂O and centrifuged at 1,500 × g for 5 minutes at 4°C to remove cell debris. The supernatant was recovered and frozen at −80°C. Total protein was determined using a commercial bicinchoninic acid kit (Pierce, Rockford, IL).

Western Blotting

Western blotting was carried out to detect the levels of SERT protein expression 1 week after P3 HI on total protein extracts from frontal cortex, thalamus, and brainstem regions. Samples (20 μg per well) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis using non-denaturing conditions. Separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), blocked with 5% nonfat dried milk in TBS-Tween 20 0.1%, and then incubated in the goat anti-SERT antibody (1:2000) overnight at 4°C. The SERT was detected using horseradish peroxidase–conjugated anti-goat immunoglobulin G (1:10,000; Sigma) and visualized using enhanced chemiluminescence (Amersham, Sydney, New South Wales, Australia) on x-ray film (Super RX, FujiFilm Australia, New South Wales, Australia). Each blot was stripped using Re-blot Plus (Millipore, Macquarie Park, New South Wales, Australia) and reprobed with β-actin (1:20,000;Imagenex, San Diego, CA) to quantify SERT levels relative to β-actin protein. Chemiluminescence was eliminated for SERT when the antibody was preincubated with the control peptide (1:2000; Santa Cruz Biotechnology Inc). Bands were quantified using commercial software (National Institutes of Health ImageJ).

ELISA

Serotonin concentrations in total protein samples (120 μg) from the frontal cortex, thalamus, and brainstem regions were determined using a commercial ELISA kit (Alpco Diagnostics, Salem, NH). Standard dilutions of 5-HT and samples were acetylated for 30 minutes at room temperature and then applied to a microtiter plate where the antigen was bound, in accordance with the manufacturers’ instructions. Values of brain 5-HT levels were determined as nanograms of 5-HT per milligram of total protein and expressed as a percentage change from control values on P10. The assay detection range was
from 0.2 to 1.2 ng/mg protein; the intra-assay variation was less than 7.0%.

To measure brain levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), total protein samples (160 μg) were assayed from the frontal cortex, thalamus, and brainstem for all experimental groups with commercially available quantikine rat-specific ELISA kits (R&D Systems, Minneapolis, MN). The optical density was measured at 450 nm, and the amounts of TNF-α and IL-1β were calculated using the assay standard curves. The quantikine assays were sensitive to 5 pg/mL. The intra-assay and interassay coefficients of variation were less than 5% and less than 10% for TNF-α and less than 6% and less than 9% for IL-1β.

Data Analysis
All analyses were performed blind to the observer. Unless stated otherwise, data are presented as mean ± SEM. All statistical analyses were performed using PRISM 5 (GraphPad, San Diego, CA). Statistical analyses of results were evaluated using analysis of variance followed by Student t-tests. Statistical significance was set at p < 0.05.

RESULTS
Damage to the Serotonergic Network After P3 HI
In the forebrain of control animals (Fig. 1), SERT-positive immunolabeling was predominantly localized on fibers, and highly varicose fibers were often observed (Fig. 1A, C, G) (61, 62). These observations are consistent with reports that SERT is distributed in the plasma membrane throughout the cell body, axon, dendrites, and terminals of serotonergic neurons (49). Thus, SERT is a very good indicator of the magnitude of brain serotonergic innervation (50).

After P3 HI, SERT-positive immunolabeling was decreased in several areas of the brain versus labeling in control animals (Fig. 1). After P3 HI, SERT-positive fibers in regions such as the motor cortex and the horizontal limb of the diagonal band (Fig. 1D, H) often lacked the beaded varicose appearance seen in control sections, or they displayed fewer varicosities (Fig. 1B). After P3 HI, SERT immunoreactivity was substantially decreased in the somatosensory cortex, motor cortex, ventrolateral thalamus, lateral hypothalamus, diagonal band compared with that in control animals (Fig. 1A–J). In areas such as the lateral hypothalamus (Fig. 1F), SERT-positive fibers seemed to be clumped, grouped in smaller bundles, and had processes that were shorter than those in controls; these are features suggestive of neurodegeneration. In the dorsal raphe nuclei (Fig. 1K, L), SERT-positive immunolabeling primarily indicated neuronal cell body profiles, thereby confirming previous findings that SERT immunolabeling is virtually exclusively found on 5-HT neurons in the brainstem dorsal raphe nuclei (12, 50). After P3 HI, there were reduced numbers of SERT-positive neuronal profiles in the dorsal raphe nuclei versus control animals ipsilateral to the carotid ligation (Fig. 1L). Quantification of the SERT-positive immunolabeling in each brain region was performed (Table). For all regions, there was a significant decrease in SERT-positive label in P3 HI animals versus controls.

To further quantify changes in SERT labeling patterns and determine 5-HT and cytokine levels in the same areas, we extracted tissue from specific regions and used Western blotting to determine how P3 HI affected SERT ipsilateral to the carotid ligation (Fig. 2A). We selected brain areas that encompassed the major regions of serotonergic innervation that are known to be disrupted after neonatal HI and that displayed disrupted SERT immunolabeling. On P10, P3 HI SERT protein expression was reduced compared with controls in the frontal cortex, thalamus, and brainstem by 34.4%, 23.5%, and 23.4%, respectively (Fig. 2A, B). Western blotting using the control peptide revealed no SERT protein expression.

We next examined whether 5-HT levels were also altered on P10 in the same brain regions. Postnatal Day 3 HI significantly decreased 5-HT levels ipsilateral to the carotid ligation in the frontal cortex, thalamus, and brainstem by 15.0%, 24.9%, and 17.7%, respectively, compared with control animals (Fig. 2C).

Minocycline Treatment Attenuated P3 HI–Induced Damage to the Serotonergic Network
In animals subjected to P3 HI, there was an 18.4% ± 3.43% reduction in cerebral hemisphere size ipsilateral to the carotid ligation compared with the nonligated side (Fig. 3A). Thus, the severity of brain injury after P3 HI was similar to that produced in our previous studies using the same model (12, 32, 42). There was also no significant difference in brain

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TABLE. Area Density Measurements of 5-Hydroxytryptamine Transporter Immunolabeling in Forebrain Regions and Dorsal Raphe Nuclei

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Control</th>
<th>P3 HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatosensory cortex</td>
<td>7.8 ± 1.3</td>
<td>1.7 ± 0.2**</td>
</tr>
<tr>
<td>Motor cortex</td>
<td>6.0 ± 1.0</td>
<td>1.7 ± 0.6*</td>
</tr>
<tr>
<td>Lateral hypothalamus</td>
<td>16.7 ± 0.9</td>
<td>12.4 ± 0.6*</td>
</tr>
<tr>
<td>HLDB</td>
<td>10.0 ± 1.6</td>
<td>4.1 ± 0.5*</td>
</tr>
<tr>
<td>Ventrolateral thalamus</td>
<td>14.0 ± 1.9</td>
<td>6.7 ± 0.8*</td>
</tr>
<tr>
<td>Dorsal raphe nuclei</td>
<td>5.7 ± 0.9</td>
<td>2.1 ± 0.3*</td>
</tr>
</tbody>
</table>

Data are expressed as percentages of 200 μm² area. *p < 0.01, **p < 0.001.

HLDB, horizontal limb of the diagonal band; P3 HI, Postnatal Day 3 hypoxia-ischemia.
size between the control, minocycline P3 HI, and minocycline-only groups on P10. Thus, 1 week of minocycline treatment prevented the P3 HI-induced reduction in brain size. Because a primary region of interest in the current study is the raphe nuclei, we investigated possible changes in brainstem hemisphere size ipsilateral to the carotid ligation after P3 HI. In contrast to the forebrain, brainstem hemisphere size at the level of the dorsal raphe did not change after P3 HI or after minocycline treatment. These data are consistent with previous findings in the brainstem, whereby P3 HI did not alter brainstem hemisphere size at the level of the obex (32) or dorsal raphe (12).

To examine whether inhibiting microglia can alter P3 HI-induced reductions in SERT protein expression, 5-HT, and dorsal raphe counts, we administered minocycline for 1 week after the HI insult. Western blotting analyses revealed no difference in SERT protein expression levels between control and minocycline-treated HI animals in the 3 brain regions examined (Fig. 3B–E). Thus, minocycline treatment prevented the decrease in SERT expression after P3 HI. Minocycline administration alone did not alter SERT protein expression (Fig. 3B–E).

Minocycline treatment also prevented the P3 HI-induced decrease in 5-HT levels in the frontal cortex (Fig. 3F) and thalamic regions (Fig. 3G). In the brainstem, there was no difference in 5-HT levels between saline- and minocycline-treated P3 HI animals (Fig. 3H). In addition, there was no difference between saline- and minocycline-only-treated control animals in all 3 areas examined. Thus, minocycline administration alone did not elevate basal levels of 5-HT in any of the brain regions examined.

To determine the effect of minocycline on the number of serotonergic dorsal raphe neurons, counts of 5-HT–positive neurons were performed in control (Fig. 4A, B), P3 HI (Fig. 4C, D), and P3 HI animals treated with minocycline (Fig. 4E, F). Treatment with minocycline after P3 HI virtually abolished P3 HI–induced reduction in numbers of 5-HT–positive neurons on P10 ipsilateral to the carotid ligation compared with the contralateral nonligated side (Fig. 4G). There was no difference in numbers of 5-HT–positive neurons in control and minocycline-only groups (Fig. 4G).

Minocycline Inhibited Neuroinflammatory Mediators After P3 HI

We next determined how minocycline influenced numbers of Iba-1–positive microglia in the frontal cortex, thalamic, and brainstem regions (Fig. 5). After P3 HI, Iba-1–positive microglia seemed thickened with shorter processes and dense cell body indicative of activation (Fig. 5B). At 1 week after P3 HI, the number of Iba-1–positive microglia was significantly higher than controls in the frontal cortex (345%; Fig. 5D), thalamus (160%; Fig. 5E), and the dorsal raphe nuclei (153%; Fig. 5F) ipsilateral to the carotid ligation. Treatment with minocycline completely abolished the P3 HI-induced increase in numbers of activated microglia in all 3 regions (Fig. 5C–F).

In animals subjected to P3 HI, there was a significant increase over control levels in both frontal cortex TNF-α (Fig. 6A) and IL-1β levels (Fig. 6D); however, only TNF-α levels (Fig. 6B) and not IL-1β levels (Fig. 6E) were significantly elevated in the thalamic tissue after P3 HI. Neither cytokine changed in the brainstem after P3 HI (Fig. 6C, F). The P3 HI–induced increases in TNF-α and IL-1β in the frontal cortex were prevented by 1-week minocycline treatment (Fig. 6A, D). Regardless of the brain region examined, minocycline administration alone had no effect on TNF-α and IL-1β levels.

DISCUSSION

We present novel evidence that neonatal HI disrupts the serotonergic network and that a posts insult anti-inflammatory
intervention prevents this injury. The concomitant reductions in SERT, 5-HT levels, and 5-HT–positive raphe neurons suggest that serotonergic network injury is a consequence of degrading serotonergic neurons that project to the HI-damaged forebrain. It was striking that minocycline prevented the P3 HI–induced changes in SERT expression as well as 5-HT levels and numbers of dorsal raphe neurons. Our findings shed light on a potential treatment that targets microglia to prevent degeneration of the serotonergic network in the injured neonatal brain.

We have identified the serotonergic system as a pervasive network that is disrupted after neonatal HI. The dorsal raphe nuclei constitute the primary sources of this extensive forebrain serotonergic neural network (1, 52, 62), and our findings substantiate reports that P3 HI or asphyxia in the immature rat brain incurs significant losses of serotonergic dorsal raphe nuclei (12, 63). The parallel reductions in 5-HT levels and SERT suggest that there was reduced availability of 5-HT for release as well as limited reuptake of 5-HT. Consistent with this, ischemia in P7 rat pups concurrently attenuates 5-HT and the 5-HT metabolite, 5-hydroxyindoleacetic acid, suggesting that there is disruption of the serotonergic neuronal network rather than direct modulation of SERT itself or of serotonergic metabolism (64). The losses of serotonergic brainstem raphe neurons concur with our recent findings (12) and support data from human studies (9–11). The concomitant and parallel change to key features of the serotonergic network leads us to speculate that this P3 HI–induced disruption most likely occurs as a consequence of loss or damage to serotonergic neurons.

In general, greater losses of SERT and reductions in 5-HT occurred in the forebrain regions versus brainstem raphe nuclei. These data suggest that the sequence of damage to serotonergic fibers may have been initiated in the forebrain core/penumbra areas of the HI-injured brain. Ligation of the common carotid artery affects a vascular field that primarily encompasses forebrain regions. The brainstem is thought to be outside of this vascular field and thus spared of ischemia in HI models. Indeed, brainstem blood flow tends to increase during HI (65); there was also no change in brainstem area after P3 HI, consistent with our previous report (32). Damage to forebrain sites may impact remote brain regions, such as the dorsal raphe nuclei, by virtue of the neural network that connects them. Serotonergic cell bodies in the dorsal raphe nuclei may degenerate after P3 HI brain injury, for example, by retrograde degeneration of serotonergic fibers that innervate primary injury regions or via damage to descending neural inputs that project to the dorsal raphe nuclei. The regional differences in the degree and general appearance of SERT loss may reflect these putative mechanisms. In addition, the impact of HI in a particular brain region may be dictated by differences in the distribution and density of the serotonergic innervation to specific brain regions and/or differing regional vulnerabilities to HI. Nevertheless, damage to the remote brainstem raphe nuclei may evolve via secondary injury mechanisms such as neuroinflammation.

Our data suggest that increased numbers of activated microglia and elevated levels of IL-1β and TNF-α may constitute a secondary mechanism responsible for serotonergic network damage after perinatal HI. Consistent with reports in the adult rat brain (23, 24), treatment with minocycline alone did not increase expression of SERT or elevate 5-HT levels in the neonatal brain. Therefore, it is unlikely that minocycline had a direct action on SERT to regulate 5-HT release. We postulate that the P3 HI–induced upregulation of neuroinflammatory mediators in the brain was critical for minocycline to effectively reduce damage to the serotonergic system rather than a direct regulatory effect on 5-HT neurotransmission per se. Post-HI minocycline treatment attenuated the P3 HI–induced serotonergic system changes, thereby demonstrating that the affected serotonergic system is responsive to anti-inflammatory interventions. Relatively minor changes in HI-induced numbers of activated microglia and levels of pro-inflammatory cytokines were apparent in the brainstem compared with the forebrain regions examined, suggesting that the minocycline effects were region specific and probably dependent on the degree of neuroinflammation. This notion further supports the hypothesis that serotonergic fiber networks in the forebrain may be damaged by neuroinflammatory processes which then, in turn, lead to the loss and/or interruption of raphe 5-HT neurons. It remains to be investigated whether forebrain neuroinflammation after neonatal HI initiates subsequent serotonergic neuronal damage in the remote brainstem via retrograde degeneration and/or target deprivation mechanisms. Indeed, minocycline prevents neonatal HI-induced injury to oligodendrocyte precursor cells (20–22) and may, therefore, contribute to preserving normal myelination and axonal function.

Using the same P3 HI model, we previously saw greater numbers of activated microglia in white matter regions compared with the brain nuclei examined in the present study (20). This may reflect different degrees of neuroinflammation in white versus gray matter, different timing of increases in the number of activated microglia, the degree of tissue HI, and the amalgamation of various neurodegenerative, neuroprotective, and recovery processes occurring in different brain regions 1 week after P3 HI. Microglia can switch from a resting to an active state within 10 minutes after insult and remain activated for weeks after neonatal HI. Activated microglia amass in ischemic brain regions and produce excessive levels of pro-inflammatory cytokines and reactive oxygen and nitrogen species that can be neurotoxic (13, 15, 16, 20, 21, 66, 67). The period of sustained neuroinflammation in the brain after neonatal HI is a crucial time in the neonate because it coincides with the progression of secondary brain injury and worsening neurodevelopmental outcomes (66–71). Furthermore, because of the difficulty in assessing injury in premature neonates, clinical diagnosis of perinatal HI in the preterm neonate is often not made until 3 days after birth, well into the secondary injury phase. This critical post-HI period, therefore, represents an appropriate time to administer neuroprotective agents, such as minocycline, to stem the progression of serotonergic injury.

In addition to the inhibition of activated microglia and the production and release of proinflammatory cytokines, the efficacy of minocycline to produce neuroprotection in models of HI can be attributable to several properties (31). Minocycline can modulate chemokines and chemokine receptors expressed on microglia and produce anti-inflammatory actions (72, 73). During inflammation, matrix metalloproteinases are activated and can cause tissue damage, including disruption of...
the blood-brain barrier. In adult ischemia models, minocycline inhibits matrix metalloproteinases and assists in maintaining the integrity of the cerebral vasculature and the blood-brain barrier (74–76). Minocycline also attenuates neonatal HI-induced markers of apoptotic cell death (21) and upregulates anti-apoptotic proteins (77).

This is the first study to characterize simultaneously the preterm HI-induced damage of major components of the serotonergic network throughout the brain. Understanding how the serotonergic system is affected by neonatal HI is fundamental to pinpointing the potential neural basis for HI-induced functional deficits. Serotonergic disruption may underpin neurological impairments such as hyperactivity and cognitive, learning, memory, cardiorespiratory, and attention deficits observed in preterm children who have experienced neonatal HI (6–8, 78, 79). Alterations of serotonergic function during the perinatal period alter serotonergic neurotransmission and the sensitivity of serotonergic systems to future stressors (80–82). In addition, current theories implicate a disrupted 5-HT neurocircuitry in the brainstem raphe nuclei that may contribute to cardiorespiratory instability in neonates and increased susceptibility to SIDS (4, 10, 83, 84). Premature birth is a significant risk factor for SIDS (85), and exposure to an HI insult is one mechanism that may be sufficient to alter brainstem serotonergic function and render neonates susceptible to later respiratory complications and possibly SIDS (10, 84).

FIGURE 4. Photomicrographs of coronal brainstem sections through the ventrolateral dorsal raphe nucleus immunolabeled for 5-hydroxytryptamine (5-HT) to identify serotonergic raphe neurons (A–F). Example sections are contralateral (A, C, E) and ipsilateral (B, D, F) to the carotid ligation from a control (A, B), a Postnatal Day 3 hypoxia-ischemia (P3 HI) animal (C, D), and a P3 HI animal administered minocycline (Mino) for 1 week (E, F). (G) Effects of Mino in control and P3 HI animals on numbers of 5-HT–positive neurons. Scale bars = 50 μm. ***p < 0.001 control versus P3 HI animals; ††p < 0.01 P3 HI versus P3 HI Mino-treated animals.

FIGURE 3. Effects of postinsult minocycline (Mino) treatment on cerebral hemisphere size (A), 5-hydroxytryptamine (5-HT) transporter (SERT) protein expression (B–E) and brain 5-HT levels (F–H) in control and Postnatal Day 3 hypoxia-ischemia (P3 HI) animals. Examples of Western blots for SERT protein and β-actin expression (B) are from samples of thalamic tissue. Histograms present quantification of SERT protein expression (C–E) and brain 5-HT (F–H) in the frontal cortex, thalamus, and brainstem 1 week after P3 HI. *p < 0.05, **p < 0.01 control versus P3 HI animals; †p < 0.05, ††p < 0.01 P3 HI versus P3 HI Mino-treated animals.
By targeting P3 HI–induced activated microglia, minocycline treatment could be a novel therapy to minimize serotonergic changes after neonatal HI and preserve the integrity of brain 5-HT neurocircuitry. Caution is warranted, however, because although minocycline is an excellent tool to block activated microglia and has been tested in numerous clinical trials in adults, its use in neonates has been avoided because of reported adverse effects (28–31). Minocycline is a second-generation tetracycline that does not produce adverse effects commonly associated with other tetracyclines, such as gastrointestinal irritability, because it is rapidly absorbed from the gut and has a long half-life (14 hours). Furthermore, minocycline is excreted primarily via nonrenal routes and is, therefore, often the tetracycline of choice in patients with renal dysfunction (30). Recent evidence also suggests that minocycline can improve hyperbilirubinemia-induced brain injury and vestibular and hearing problems in the neonate (86–88). New analogs of minocycline that are devoid of side effects in neonates may be required before translation to the clinic can be achieved. Nevertheless, this study has implications for neonatal HI injuries that involve childhood and adult serotonergic functional deficits, the effects of neuroinflammation in generating injury to the serotonergic system, and for identifying potential drug targets to ameliorate damage to the serotonergic system.
FIGURE 6. Effects of Postnatal Day 3 hypoxia-ischemia (P3 HI) and minocycline (Mino) administration on tumor necrosis factor-α (TNF-α) (A–C) and interleukin-1β (IL-1β) (D–F) levels in the brain 1 week after insult. *p < 0.05, **p < 0.01 control versus P3 HI animals; †p < 0.05 P3 HI versus P3 HI Mino-treated animals.
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