Published in final edited form as:

Neuroscience. 2010 June 16; 168(1): 240–252. doi:10.1016/j.neuroscience.2010.03.024.

INTERLEUKIN-1 β -INDUCED BRAIN INJURY AND NEUROBEHAVIORAL DYSFUNCTIONS IN JUVENILE RATS CAN BE ATTENUATED BY α -PHENYL-*N*-TERT-BUTYL-NITRONE

L.-W. FAN^a, L.-T. TIEN^b, B. ZHENG^a, Y. PANG^a, P.G. RHODES^a, and Z. CAI^{a,*}

^a Department of Pediatrics, Division of Newborn Medicine, University of Mississippi Medical Center, Jackson, MS 39216, USA

^b School of Medicine, Fu-Jen Catholic University, Hsin-Chuang, Taipei County, Taiwan

Abstract

Our previous study showed that perinatal exposure to interleukin-1 β (IL-1 β), an inflammatory cytokine, induces acute injury to developing white matter in the neonatal rat brain, and α -phenyl-*n*-tert-butyl-nitron (PBN), a free radical scavenger and antioxidant, protects against IL-1 β -induced acute brain injury. The objective of the present study was to further examine whether perinatal exposure to IL-1 β resulted in persistent brain damage and neurological disabilities, and whether PBN offers lasting protection. Intracerebral injection of IL-1 β (1 μ g/kg) was performed in postnatal day 5 (P5) Sprague-Dawley rat pups and PBN (100 mg/kg) or saline was administered intraperitoneally 5 min after IL-1 β injection. Perinatal IL-1 β exposure significantly affected neurobehavioral functions in juvenile rats. Although some neurobehavioral deficits such as performance in negative geotaxis, cliff avoidance, beam walking, and locomotion were spontaneously reversible, sustained deficits such as poor performance in the vibrissa-elicited forelimb-placing test, the pole test, the passive avoidance task, and the elevated plus-maze task were still observable at P21. Perinatal IL-1 β exposure resulted in persistent brain damage including enlargement of ventricles, loss of mature oligodendrocytes, impaired myelination as indicated by the decrease in myelin basic protein immunostaining, axonal and dendritic injury, and loss of hippocampal CA1 neurons and tyrosine hydroxylase positive neurons in the substantia nigra and ventral tegmental areas of the rat brain. Treatments with PBN provided lasting protection against the IL-1 β -induced brain injury and improved the associated neurological dysfunctions in juvenile rats, suggesting that prompt treatments for brain injury induced by perinatal infection/inflammation might have important long-term consequences.

Keywords

interleukin-1 β ; neurobehavioral deficits; axonal and dendritic injury; dopamine neuron; antioxidant

Accumulating evidence indicates that maternal or placental infection or inflammation, which occurs in approximately 30% of all preterm births (Goldenberg et al., 2008; Goncalves et al., 2002) and hypoxia-ischemia (Hagberg et al., 2002b; Huleihel et al., 2004; Rezaie and Dean

*Corresponding author: Dr. Zhengwei Cai, Department of Pediatrics, Division of Newborn Medicine, University of Mississippi Medical Center, Jackson, MS 39216-4504, USA, Tel.: +1-601-984-2786, Fax: +1-601-815-3666, zcai@ped.umsmed.edu (Z. Cai).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

2002; Volpe, 2003) are major contributors to perinatal brain injury, such as periventricular leukomalacia (PVL), a predominant form of injury in the premature infant brain (Dammann and Leviton, 1997, 2004; Hagberg et al., 2002b; Volpe, 2003). Increased concentrations of inflammatory cytokines such as tumor necrosis factor- α (TNF α), interleukin-1 β (IL-1 β) and IL-6 are frequently observed in the infant brain with PVL (Kadhim et al., 2001, 2003; Yoon et al., 1997b), and in the cord blood (Yoon et al., 1996) or amniotic fluid (Yoon et al., 1997a) from the birth of infants with PVL. Therefore, it has been proposed that inflammatory cytokines derived from maternal infection are mediators between maternal infection and perinatal brain injury like PVL (Dammann and Leviton, 1997, 2004; Hagberg et al., 2002b; Volpe, 2003). However, direct evidence linking increased inflammatory cytokines with persistent brain injury and neurological dysfunction is still lacking.

In our previous work, we found that intracerebral injection of IL-1 β resulted in acute white matter and neuronal injury in the neonatal rat (Cai et al., 2004; Fan et al., 2009). Co-administration of lipopolysaccharide (LPS), which is responsible for most of the inflammatory effects of infection from gram-negative bacteria, with IL-1 receptor antagonist (IL-1ra), but not with antibodies to TNF α , protected the rat brain from LPS-induced white matter injury (Cai et al., 2003). These data provide evidence that IL-1 β may play an important role in neonatal brain injury associated with infection/inflammation. We also found that treatment with α -Phenyl-*n-tert*-butyl-nitron (PBN), a nitron-based spin trapping compound and an antioxidant (Floyd et al., 2002), can provide protection against IL-1 β -induced acute white matter and neuronal injury (Fan et al., 2009). However, it remains to be elucidated whether IL-1 β has long-lasting effects on brain injury and neurological functions and whether PBN offers lasting protection. The present study was designed to examine effects of PBN on perinatal IL-1 β exposure-induced brain injury and neurobehavioral dysfunctions in juvenile rats.

EXPERIMENTAL PROCEDURES

Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO., USA). Recombinant rat IL-1 β was purchased from R&D System (Minneapolis, MN). Monoclonal mouse antibodies against adenomatus polyposis coli (Clone CC1) (APC-CC1); myelin basic protein (MBP), microtubule-associated protein 1 (MAP1), MAP2, neuron-specific nuclear protein (NeuN), or tyrosine hydroxylase (TH); and OX42 (CD11b) were purchased from Calbiochem (San Diego, CA), Chemicon (Temecula, CA) and Serotec (Raleigh, NC), respectively.

Animal treatment and surgical procedures

Timed pregnant Sprague-Dawley rats arrived in the laboratory on day 19 of gestation. Animals were maintained in a room with a 12-h light/dark cycle and at constant temperature ($22 \pm 2^\circ$ C). The day of birth was defined as postnatal day 0 (P0). After birth, the litter size was adjusted to twelve 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. Every effort was made to minimize the number of animals used and their suffering.

Intracerebral injection of IL-1 β to 5-day old rat pups of both sexes was performed as previously described (Cai et al., 2004). Under light anesthesia with isoflurane (1.5%), IL-1 β (1 μ g/kg) in sterile saline containing 0.1% BSA (total volume of 2 μ l) was administered to the rat brain at the location of 1.0 mm posterior and 1.0 mm lateral to the bregma, and 2.0 mm deep to the scalp at the left hemisphere in a stereotaxic apparatus with a neonatal rat adapter. The dose of

IL-1 β was chosen based on the peak concentrations of IL-1 β achieved in the rat pup brain following LPS administration as reported previously (Cai et al., 2003, 2004; Pang et al., 2003) and on the published data (Holmin and Mathiesen, 2000). The injection site was located at the area just above the left cingulum. The control rats were injected with the same volume of sterile saline containing 0.1% BSA. This cohort of animals was in addition to the cohort that we used for acute brain injury studies in previous report (Fan et al., 2009). All animals survived the intracerebral injection.

Both IL-1 β - and saline-injected animals were further divided into two groups: one received intraperitoneal (i.p.) injections of α -Phenyl-*n*-tert-butyl-nitrone (PBN) and the other sterile saline. PBN (100 mg/kg) or vehicle alone was administered 5 min after the IL-1 β injection. Our previous study showed that this dosing and timing of PBN administration protects the neonatal rat brain from LPS-induced (Fan et al., 2008b, 2008c) and hypoxia-ischemia-induced white matter injuries (Lin et al., 2004, 2006). Sixteen days after the injection (P21), juvenile rats were killed by transcardiac perfusion with normal saline followed by 4% paraformaldehyde for brain section preparation. Brain development in P21 rats is thought to be equivalent to that in one-year old human infant (Hagberg et al., 2002a), an age at which many brain development disorders become apparent. Twelve rat pups (6 male and 6 female pups) were included in each treatment group for behavioral tests. Frozen coronal brain sections at 10 μ m of thickness from 6 pups (3 male and 3 female pups) of each group were prepared in a cryostat for immunohistochemistry.

Behavioral testing

The behavioral tests were performed as previously described (Fan et al., 2005, 2006, 2008a, 2008d) with modifications. The developmental test battery used was based on the tests for neurobehavioral toxicity (Altman et al., 1971; Altman and Sudarshan, 1975; Hermans et al., 1992). The negative geotaxis, cliff avoidance test, beam walking test, pole test, and locomotion were performed for all rat pups from P3 to P21. The vibrissa-elicited forelimb-placing test and plus maze were performed in all rat pups on P20. The passive avoidance was performed in all rat pups on P20 (learning trial) and P21 (memory trial).

Negative geotaxis—This test is believed to test reflex development, motor skills and vestibular labyrinth and cerebellar integration (Altman and Sudarshan, 1975; Hermans et al., 1992). Rats placed on a 25° incline with their head pointing down the slope tend to turn to face upward and begin to crawl up the slope. Each pup was given three trials a day and the time spent for a turn of 180° upward was recorded. The cut-off time was 60 sec.

Cliff avoidance test—This test is used to assess the integration of exteroceptive input (vibrissae) and locomotor output (Altman and Sudarshan, 1975; Hermans et al., 1992; Shen et al., 1991). Pups placed on the edge of a platform (20 $\times$ 20 $\times$ 20 cm) with forepaws and chest extending over the edge tend to move away by backing up with forepaws or their body and head turning sideways. Avoidance was scored by reflex latency, the time between edge placement and body repositioning. Each pup was given three trials a day. If the pup did not make any response within 60 sec or fell off from the platform, it was recorded as 60 sec.

Beam walking test—Motor coordination and balance were assessed by measuring the ability of animals to traverse a graded series of narrow beam to reach an enclosed safety platform (Altman and Sudarshan, 1975; Karl et al., 2003). The test consisted of elevated platforms connected by a 60 cm long square wood beam with a width of 1.2 cm. The beam was placed horizontally, 50 cm above the bench surface, with one end mounted on a narrow support and the other end attached to a goal box with a litter of pups. A sawdust-filled box was placed underneath serving as protection for the falling pups. A light (60 Watt) was positioned above

the start of the beam. A litter of pups was placed on the goal box, and one pup at a time was removed and placed on the starting platform. Each pup was given three trials a day. The time spent on the beam for each pup that traversed the beam and joined their littermates was recorded and the cut-off time was 60 sec.

Locomotor activity—This test measures the activity and habituation response of animals on placement in a novel environment (Hermans et al., 1992). Locomotor activity was measured from P3 to P21, using the Video Tracking System SMART-2000 (San Diego Instruments, Inc., San Diego, CA., USA). Pups were placed in the activity chambers (30 × 45 × 40 cm) in a quiet room with dimmed light. The total distance traveled by the animal was recorded during a 10-min testing period.

Pole test—This test was used to assess the maturation of ascending and descending skills (Altman and Sudarshan, 1975). The rat was confronted with a situation in which it had to turn around and climb down a pole. For this purpose, a wooden stick (diameter: 1.7 cm, length: 40 cm) with a cork ball on its top (diameter: 3 cm) was installed vertically on a platform with a sawdust-filled box at the base served as protection for the falling pups. The rat was placed directly under the ball at the top of the pole with its head held upwards. The mature pattern of descending consists of turning around on the rod, which consists of the release of grip with the forepaws, rotation of the trunk, and support of the body during this maneuver by the hindpaws, then descent with the head in the leading position. Each pup was given three trials a day and the time to turn round and to reach the platform at the bottom was measured performance latency (cut-off time: 60 sec). Maturation of skill was assessed by a score defined as following: 0, falling down; 1, descending down with head up; 2, descending down with the release of grip with the forepaws; 3, descending down with head turning around.

Vibrissa-elicited forelimb-placing test—This test uses stimulation of the rat's vibrissae to trigger a placing response to measure forelimb placing deficit (De Ryck et al., 1992; Frost et al., 2006; Schallert and Woodlee, 2005). Rats use their vibrissae to gain bilateral information about the proximal environment and this information is integrated between the hemispheres. All animals were tested on P20. The animal was gently held by its torso, allowing the forelimbs to hang free. Independent testing of each forelimb was induced by gently brushing vibrissae of the corresponding side on the edge of a tabletop once per trial for 10 trials. The percentage of trials in which the rat successfully places its forepaw onto the tabletop is recorded for each side. Intact animals place the forelimbs of both sides quickly onto the counter top with 100% success in all variants of this test. If an animal struggled during testing, the data were not included in the overall analysis.

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 from each other and connected with an electric shock generator. The safe part was a piece of wood 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 hours 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 anxiety behavior (Agmo and Belzung, 1998; Schmitt et al., 2002). The procedure is based on rodents' natural tendency to avoid open space and it does not contain any experimenter-controlled aversive element. Nevertheless, exposure to it is stressful for the subjects (Agmo and Belzung, 1998). The plus-maze consists of two open arms ($30 \times 5 \times 0.25$ cm) and two enclosed arms ($30 \times 5 \times 10$ 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 at the ceiling. The anxiety-related behaviors for each animal were recorded for a period of 5 min by a VCR-recording system on P20. 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, and 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).

Immunohistochemistry studies

Brain injury was estimated based on the results of Nissl staining and immunohistochemistry in consecutive brain sections prepared from rats sacrificed 16 days (P21) after the intracerebral injection. For immunohistochemistry, the final concentrations of the primary antibodies were as the following: APC-CC1, 1:20; MBP, MAP1, MAP2, NeuN, or TH, 1:100; and OX42, 1:200. Mature oligodendrocytes (OLs) were identified with APC-CC1. Anti-MBP staining was used as a marker of myelination. Anti-MAP1 provides selective staining of neuronal axons and dendrites. Anti-MAP2 provides selective staining of neuronal dendritic processes. NeuN detects the neuron-specific nuclear protein which primarily localizes in the nucleus of the neurons with slight staining in the cytoplasm. Microglia were detected using OX42 immunostaining, which recognizes both the resting and the activated microglia. Sections were incubated with primary antibodies at 4 °C overnight and further incubated with secondary antibodies conjugated with fluorescent dyes (FITC, rhodamine, Cy2 or Cy3) for 1 h in the dark at room temperature. DAPI (100 ng/ml) was used simultaneously to identify nuclei in the final visualization. Sections incubated in the absence of primary antibody were used as negative controls. Positive staining of TH was used to detect dopamine neurons in the substantia nigra (SN) and ventral tegmental area (VTA). For dual labeling studies using TH antibody in combination with biotin conjugated NeuN antibody, sections were first incubated with mouse TH antibody followed by rhodamine-labeled anti-mouse secondary antibody. Prior to a further incubation with biotin conjugated NeuN antibody, sections were incubated with 1% mouse serum for 1 h to block any excessive anti-mouse IgG. After incubation with biotin conjugated NeuN antibody, sections were further incubated with FITC-labeled avidin for 1 h in the dark at room temperature. The resulting sections were examined under a fluorescent microscope at appropriate wavelengths. Our previous studies demonstrated that this procedure does not cause interference between TH and NeuN staining (Fan et al., 2008b, 2008d).

Estimate of the size of ventricle

To compare the size of lateral ventricles, Nissl stained sections at the bregma level were scanned by a densitometer (Bio-Rad, Hercules, CA) and areas of the left and right ventricles as well as that of the whole brain section were measured (Fan et al., 2005). The ratio between the area of the left or the right ventricle and that of the whole brain section was calculated as the ventricle size index.

Quantification of immunostaining data and statistics

Immunostaining was performed in brain sections from the forebrain at the bregma level and in the diencephalon sections at levels 1/2 and 2/3 rostral from the lambda to the bregma (the middle dorsal hippocampus). Three brain sections at the bregma level or at each of the two hippocampal levels were taken for cell counting or other measurements, and the average from the three sections was calculated for a single brain. TH+ cell counting was performed in the midbrain sections at a level 1/3 rostral from the lambda to the bregma. Most immunostaining data were quantified by counting of positively stained cells. When the cellular boundary was not clearly separated, numbers of DAPI-stained nuclei from the superimposed images were counted as the cell number. Three digital microscopic images were randomly captured at the cingulum (the bregma level, APC-CC1 positive OLs, OX42), the parietal cortex (NeuN, OX42), CA1 region of the hippocampus (NeuN) or at the SN and VTA (TH, OX42) where the TH+ cells were most abundant for each section. The number of positively stained cells in the three images was counted and averaged. Three sections at the bregma level or at each of the two hippocampal section levels were examined by an observer blind to the treatment and the mean value of cell counting was used to represent one single brain. For convenience of comparison among the treatment groups, results were standardized as the average number of cells/mm (CA1) or cells/mm² (parietal cortex, SN and VTA). Lengths of all MAP1 positive staining in the parietal cortex and CA1 region of the three brain sections at each of the two hippocampal levels were measured, using the NIH image software and the average was calculated bilaterally for a single brain. In addition to cell counting, OX42 staining as well as MAP2 staining were quantified by calculating the percentage area of OX42 or MAP2 positive staining in the whole image frame, respectively, using the NIH image software. This method has been successfully used to quantify the density of cortical serotonin transporter-immunoreactive fiber networks (Maciag et al., 2006) and the density of cortical MAP2 staining (Fan et al., 2008a). For measurement of subcortical MBP staining at the bregma level, MBP stained sections were scanned by a densitometer (Bio-Rad, Hercules, CA). MBP positive staining in the corpus callosum and the subcortical white matter tract was outlined over the whole brain section and the area and mean optical density (OD) of MBP staining were determined using NIH software. The optical density of the area outside the outlined region (MBP negative) was measured for background. This value was subtracted from the signal OD. The corrected OD was used to represent the intensity of MBP positive staining. The quantified data were presented as the mean±SEM and analyzed by one-way ANOVA followed by Student-Newman-Keuls test.

The behavioral data were presented as the mean±SEM and analyzed by one-way ANOVA (for passive avoidance, plus-maze and forelimb-placing tests) or by two-way repeated measures ANOVA for data from tests conducted continuously at different postnatal days, followed by Student-Newman-Keuls test. Results with a $p < 0.05$ were considered statistically significant.

RESULTS

PBN attenuated neurobehavioral deficits induced by perinatal IL-1 β exposure

No significant difference was observed between the male and female rats within the same treatment group from P6 to P21. Therefore, data from rats of both sexes were combined and presented here.

Perinatal IL-1 β exposure caused neurobehavioral deficits. Some of the behavioral deficits in IL-1 β exposed rats were reversible and recoverable. As shown in Fig. 1, the response latency of the control rats decreased with age in the negative geotaxis (Fig. 1A), the cliff avoidance test (Fig. 1B) and the beam walking test (Fig. 1C). Two-way ANOVA showed that both the treatment and age had significant effects on rat performance in these tests. A delayed decline

in response latency in these tests was observed in IL-1 β exposed-rats as compared to the control group, and PBN treatment prevented such a delay (Figs. 1A–C). The deficits in response latency in the IL-1 β exposed rats eventually recovered by P15–P21. Perinatal IL-1 β exposure also resulted in transient hyperactivity, as measured by the increase in the total crossing distance of an individual rat during a 10-min period in an open field (Fig. 1D) from P13 to P17. Although PBN treatment prevented the IL-1 β -induced hyperactivity in the IL-1 β +PBN group, the hyperactivity in the IL-1 β group was spontaneously reversed by P18. On the other hand, neurobehavioral deficits in some tests were still observable in the IL-1 β -exposed group and PBN treatment prevented these deficits.

Pole Test—Pups from the control group started to succeed in the pole test (descending from a vertically placed round wood pole with a length of 40 cm and a diameter of 1.7 cm) from P17 (with a success rate of 66.7%). While the maturation of skill increased with age, the performance latency in the pole test decreased with age (Fig. 2A and 2B). Two-way ANOVA showed that both the treatment and age significantly affect maturation skill and performance latency in rats. It was not until P19 that pups from the IL-1 β -injected group succeeded in the pole test (with a success rate of 33.3%). Neither the maturation of skill nor the performance latency of pole test in the IL-1 β -injected group reached the control group level by P21, whereas the responses of all pups in the IL-1 β +PBN group were not different from the control group.

Vibrissa-elicited Forelimb-placing Test—All pups from the control group succeeded in both the same side and the crossing side vibrissa-elicited forelimb-placing test (100%) on P20 (Fig. 2C). The success rate of the vibrissa-elicited forelimb-placing test in the IL-1 β -injected group was significantly lower (same side, 25.4 $\pm$ 2.2%; crossing side, 17.6 $\pm$ 2.5%) than that in the control group ($P<0.05$). The IL-1 β +PBN group exhibited the same performance level as the control group (100%) on P21.

Passive Avoidance—As shown in Fig 2D, the number of electric foot shocks required to retain the rat on the safe board was significantly increased in the IL-1 β group (saline, 4.5 $\pm$ 0.4; IL-1 β , 11.1 $\pm$ 0.6 shocks, $P<0.05$). IL-1 β also reduced the retention latency to step down from the board the next day (P21) as compared to the control group (saline, 115 $\pm$ 3 sec; IL-1 β , 19 $\pm$ 8 sec, $P<0.05$). PBN treatment significantly improved IL-1 β -induced learning and memory deficits (P20, 5.0 $\pm$ 0.5 shocks; P21, 107 $\pm$ 9 sec, $P<0.05$ vs the IL-1 β group).

Elevated Plus-maze Test—As shown in Fig. 2E, a higher number of entries into the open arm was observed in the IL-1 β -injected group (8.1 $\pm$ 0.4) as compared with the control group (4.1 $\pm$ 0.3) at P20. IL-1 β administration also increased the step-through latency into the open arm (saline, 12.2 $\pm$ 0.8%; IL-1 β , 31.8 $\pm$ 2.0%, $P<0.05$) while decreased the step-through latency into the enclosed arm (saline, 87.8 $\pm$ 0.80%; IL-1 β , 68.2 $\pm$ 2.0%, $P<0.05$) (Fig. 2F). PBN treatment improved IL-1 β -induced less anxiety-like behavior in the elevated plus-maze test (number of entries into the open arm, 4.1 $\pm$ 0.3; step-through latency into the open arm, 11.3 $\pm$ 1.4%, $P<0.05$ vs the IL-1 β group).

PBN attenuated IL-1 β -induced ventricle enlargement

IL-1 β administration caused dilatation of bilateral ventricles at the bregma level, and the dilatation in the ipsilateral (the left) ventricle was more prominent than in the contralateral side in the P21 rat brain (Fig. 3B and Table 1). Treatment with PBN significantly reduced the enlargement of ventricles following IL-1 β injection (Fig. 3C and Table 1).

PBN ameliorated IL-1 β -induced loss of mature oligodendrocyte and hypomyelination

Mature OLs were identified with APC-CC1 antibody. APC-CC1 positive cells were primarily observed in the cingulum (Figs. 3D–3F), the corpus callosum and the subcortical white matter

tract at the bregma level and in the internal capsule area. IL-1 β exposure significantly reduced the number of APC-CC1 positive cells (653.3 ± 71.1 cells/mm², $P < 0.05$) (Fig. 3E and Table 1), as compared to that in the saline (1184.4 ± 35.7 cells/mm²) or saline+PBN rat brain (1215.6 ± 42.6 cells/mm²). PBN treatment attenuated the IL-1 β -induced reduction in mature OLs in the P21 rat brain (1122.2 ± 42.8 cells/mm², $P < 0.05$) (Fig. 3F and Table 1).

OLs are myelin-producing cells and reduction in OLs 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 control rat brain, primarily in the corpus callosum (Fig. 3G), the subcortical white matter tract, and the internal capsule areas. The area and the mean optical density of MBP staining in the cingulum and the adjacent subcortical white matter tract ventral to the forelimb region of the somatosensory cortex were measured for comparison (Table 1). IL-1 β injection significantly reduced both the area and mean optical density of MBP staining in this region (Fig. 3H and Table 1). PBN treatment significantly ameliorated IL-1 β -induced decreases in MBP staining in terms of both the area and the mean optical density of MBP positive staining (Fig. 3I and Table 1). This finding was consistent with the APC-CC1+ cell counting data.

PBN decreased IL-1 β -induced axonal and dendritic damage

We have found in our previous study that perinatal IL-1 β exposure resulted in up-regulation of β -amyloid precursor protein (APP), a marker of axonal injury, in the rat brain one day after IL-1 β injection (Fan et al., 2009). To further verify whether IL-1 β -induced axonal injury persists, MAP1 immunostaining was used as a marker for detection of IL-1 β -induced injury to axons and dendrites in the P21 rat brain. IL-1 β exposure resulted in damage to axons and dendrites of the neurons, as indicated by 64% or 71% reduction in lengths of MAP1 positive immunostaining in layer V of parietal cortex (Fig. 4B) and in the hippocampal CA1 region at the middle dorsal hippocampal level, respectively, as compared to the control group (Fig. 4A, Table 1). PBN treatment attenuated the IL-1 β -induced injury to axons and dendrites in these areas (Fig. 4C and Table 1).

Neuronal dendritic processes were also identified by MAP2 immunostaining. IL-1 β treatment reduced dendrite density, as determined by the percentage of area in the image, by about 50% in the cingulum area adjacent to the parietal cortex, the hippocampal CA1 region (Fig. 4E and Table 1), and in layer V of parietal cortex (Table 1). PBN treatment prevented the induction of dendritic loss by IL-1 β (Fig. 4F and Table 1).

PBN ameliorated IL-1 β -induced neuronal injury

Nissl Staining showed that neonatal IL-1 β exposure reduced the number of hippocampal neurons in the CA1 region, but not in layer V of parietal cortex (Table 1). Treatment with PBN prevented the IL-1 β -induced loss of hippocampal neurons in the CA1 region (Table 1). The Nissl staining data were confirmed by neuron-specific nuclear protein (NeuN) immunostaining. Consistent with the Nissl staining data, IL-1 β treatment reduced the number of NeuN+ neurons in the hippocampal CA1 region (Fig. 4K) by about 19% as compared to the control group (Fig. 4J and Table 1), but not in layer V of parietal cortex (Figs. 4H&G, and Table 1). PBN treatment prevented the IL-1 β -induced reduction in the number of NeuN+ neurons in the hippocampal CA1 region (Fig. 4L and Table 1).

PBN reduced losses of tyrosine hydroxylase-immunoreactive neurons following IL-1 β

Positive staining of TH was used to detect dopamine neurons in the SN and VTA. Double labeling with TH and NeuN antibodies was performed and all TH positive and NeuN positive cells in the three midbrain sections for each animal (at levels 1/3 rostral from the lambda to the bregma) were counted bilaterally. In the P21 control rat brain, TH positive cells were more

predominant in the compact and the lateral regions of the SN (Fig. 5A) and VTA (Fig. 5G) in the ventral midbrain. IL-1 β treatment significantly reduced the number of TH positive neurons in both the SN (59.9 ± 1.7 vs 41.7 ± 1.7 cells/mm², $P < 0.05$) and the VTA regions (109.7 ± 6.4 vs 64.9 ± 2.8 cells/mm², $P < 0.05$) (Figs. 5B & 5H and Table 1). However, IL-1 β exposure did not significantly alter the total number of NeuN positive cells in these regions (Figs. 5D–F and Table 1), suggesting that IL-1 β did not result in actual neuronal cell death in this area. IL-1 β treatment decreased the ratio between the number of TH positive cells and the number of NeuN positive cells, from $71.1 \pm 2.0\%$ to $49.4 \pm 2.0\%$ in the SN of the rat brain. Therefore, the reduction in the ratio seems to be a consequence of phenotypic suppression of TH expression rather than the actual neuronal cell death. PBN treatment attenuated the IL-1 β -induced reduction in the number of TH positive neurons (Figs. 5C & 5I, and Table 1) and maintained the ratio between the TH positive and the NeuN positive cells at a level similar to that for the control group ($72.5 \pm 1.8\%$).

Protection of PBN was also associated with suppression of microglial activation

We found in our previous study (Fan et al., 2009) that protection of PBN against IL-1 β -induced brain injury in the neonatal rat was associated with suppression of microglial activation. In the present study, we further examine the link between the protection of PBN and the suppression of microglial activation in the juvenile rat brain, using two methods to quantify OX42 staining data. In the control rat brain, few OX42 positive cells were detected and most of those cells were in a resting status with a small rod shaped soma with fine and ramified processes (Figs. 6A & 6D, and Table 1). A significantly increased number of OX42+ cells were found not only in the cingulum and the cortex of the rat brain 16 days after IL-1 β injection (Fig. 6B and Table 1), but also in the SN and the VTA (1.96- and 2.15-fold increase, respectively) (Figs. 6E, 6H and Table 1). Many of these OX42+ cells showed typical features of activated microglia, i.e. bright staining of an elongated or a round shaped cell body with blunt or no processes (arrows indicated in Figs. 6B and 6E) (Kreutzberg, 1996; Godoy et al., 2008). PBN treatment reduced the number of OX42+ cells, most of which were at the resting status, following IL-1 β injection in the cingulum area (Fig. 6C), the cortex (Table 1), the SN (Figs. 6F, 6I and Table 1), and VTA (Table 1) to a level close to that in the control group. OX42 staining was also quantified by calculating the percentage area that contains OX42 immunostaining. Higher percentage of OX42+ immunostaining area was also observed in the cingulum, the cortex, the SN and the VTA of the IL-1 β -exposed rat brain (Table 1). OX42+ immunostaining in these brain regions of the PBN treated group (Table 1) was similar to that in the control rat brain, indicating that PBN treatment attenuated the IL-1 β -induced activation of microglia.

DISCUSSION

Increased evidence indicates that the development of neurological functions may be affected by maternal and/or neonatal exposure to infectious agents and such exposures are particularly associated with the subsequent development of sensory, motor and cognitive disturbances in adulthood (Bilbo et al., 2005, 2006; Hagberg et al., 2002b; Rezaie and Dean, 2002; Volpe, 2003). Consistent with data reported from these studies, the major findings from the present study are that perinatal exposure to IL-1 β results in not only acute injury in the neonatal brain as we reported previously (Cai et al., 2004; Fan et al., 2009), but also sustained brain injury as well as neurobehavioral deficits in juvenile rats, and that PBN treatment provides protection against the lasting effects of IL-1 β exposure. Our data indicate that some impairments of neurobehavioral performance (Fig. 1) appear to be temporary and reversible, suggesting that IL-1 β only delays development of these functions. However, there are neurobehavioral deficits that are still observable at P21, such as performance in the vibrissa-elicited forelimb-placing test (sensorimotor), the pole test (maturation of ascending and descending skills), the passive avoidance task (learning and memory), and the elevated plus-maze task (anxiety-like response).

This is an indication that some damaging effects of IL-1 β in this model are long-lasting and might not be eventually overcome by compensatory mechanisms.

Data from the current study suggest that the neurobehavioral deficits in juvenile rats might be explained by IL-1 β -induced persisting brain injury. In the present study, IL-1 β exposure resulted in loss of mature OLs (APC-CC1+) and hypomyelination in the corpus callosum (Fig. 3). OLs are myelin-producing cells, and loss of mature OLs (APC-CC1+) may lead to impaired myelination of axons (Osterhout et al., 2002; Rezaie and Dean, 2002). Many clinically relevant disorders, including poor sensorimotor and neuromuscular activity, and abnormal motor coordination, could be attributed to the destruction of myelin sheath in the central nervous system (Franco-Pons et al., 2007; Frost et al., 2006; Kerschensteiner et al., 2004). The normal neurobehavioral performance depends directly on the degree of dendritic arborization and synaptic connectivity (Ba and Seri, 1995). Therefore, IL-1 β -induced alterations in the connection of neurons, as indicated by the abnormal cortical and hippocampal axons and dendrites (Fig. 4), may also contribute to the deficits in neurological reflexes as well as decrements in early sensorimotor and locomotor neurobehavioral performance. The hippocampus is associated with cognitive function, emotional behavior and anxiety modulation (Bilbo et al., 2005; Lynch et al., 2004; Spolidorio et al., 2007). The IL-1 receptors are distributed throughout the brain and with high density in the hippocampus (Cunningham and De Souza, 1993; Parnet et al., 2002). It is also known that the hippocampus is vulnerable to immune-related alterations and important to the pathology of behavioral and cognitive disorders (Bilbo et al., 2005; Lynch et al., 2004). The neuronal and axonal injury in the hippocampal CA1 region (Fig. 4) found in the juvenile rat brain following perinatal exposure to IL-1 β and the learning and memory, and anxiety-like behavioral deficits support the link between anatomic structural alterations and neurological functions. In the present study, perinatal IL-1 β exposure also results in decreased number of TH positive neurons (a marker of dopaminergic neuron) in the SN and VTA of the rat brain (Fig. 5). Dopaminergic (DA) neurons in the SN are involved in the control of voluntary movements and postural reflexes and DA neurons in the VTA are involved in modulation of rewarding and cognitive behaviors (Prakash and Wurst, 2006). Although the number of NeuN positive cells in the SN and VTA regions remained unchanged in the IL-1 β -exposed rat (Fig. 5E and Table 1), suggesting that it might be an indication of phenotypic suppression of TH expression in these neurons rather than actual neuronal cell loss in the P21 rat brain, reduction in the number of TH+ cells in the SN and VTA following perinatal IL-1 β exposure is an important indicator of impairment in the dopaminergic system, which may contribute to the motor dysfunction observed in the current study. The protection of PBN on IL-1 β -induced sustained brain injury and the concomitant improvement of neurobehavioral performance in these animals provide further evidence for the association between the anatomical abnormalities and neurobehavioral deficits in IL-1 β -exposed rats.

Results from the present study demonstrate that treatment with PBN has lasting protective effects on IL-1 β -induced brain injury, although the mechanisms involved are not completely understood. PBN is one of the most widely used nitron-based antioxidants to trap and stabilize free radicals in biological systems (Floyd et al., 2002). The neuroprotective action of PBN has been observed in the LPS-induced white matter injury (Fan et al., 2008a, 2008b, 2008c, 2008d), LPS-mediated septic shock models (Endoh et al., 2001; Sang et al., 1999), animal models of hypoxia-ischemia (Lin et al., 2004, 2006), stroke (Behl and Moosmann, 2002) and other neuronal degenerative diseases (Floyd et al., 2000, 2002). Data reported by other investigators indicate that multi effects of PBN are possibly involved in its neuroprotection. PBN may form a spin adduct with free radicals (Green et al., 2003, Lee and Park, 2005), suppress ROS production from mitochondria (Floyd and Hensley, 2000), and inhibit activation of nuclear factor-kappa B (NF- κ B, a transcription factor for a wide variety of pro-inflammatory cytokine genes) (Kotake et al., 1998; Sang et al., 1999) and the induction of pro-inflammatory cytokines and iNOS (Kotake et al., 1998; Lin et al., 2006; Sang et al., 1999). PBN also has

anti-apoptosis properties (Lee and Park, 2005; Li et al., 2001). Our recent study has shown that PBN protects the neonatal brain from IL-1 β -induced acute white matter injury and that the protection of PBN is associated with its anti-oxidation and anti-inflammatory properties as indicated by the decreased elevation of oxidation products and the reduced number of activated microglia in the neonatal rat brain treated with PBN following IL-1 β exposure (Fan et al., 2009). An interesting finding from the current study is that perinatal IL-1 β exposure results in persistent inflammatory response as indicated by sustained activation of microglia in many brain regions in the juvenile rat brain, and PBN treatment prevents such a response (Fig. 6). The activated microglia are a major source of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Colton and Gilbert, 1993; Chao et al., 1992; Fan et al., 2009), both of which may put neurons and OLs at risk of oxidative and nitrosative damage (Colton and Gilbert, 1993; Chao et al., 1992; Thorburne and Juurlink, 1996). Therefore, the lasting protective effects of PBN on IL-1 β -induced brain injury are still likely associated with its anti-oxidative and anti-inflammatory properties.

Another interesting finding from the present study is that IL-1 β exposure has long-lasting effects on dopaminergic neurons in the SN and VTA, an area that is located far from the site of IL-1 β injection in the forebrain. At the present time, it is unclear how an injection of IL-1 β at the forebrain causes injury in distant brain regions. Several possibilities could be involved. IL-1 β may diffuse from the site of injection along with a gradient of IL-1 β concentrations. The increased IL-1 β level in the distant brain regions may subsequently stimulate responses from neural cells in those regions, such as activation of microglia (as shown in Figure 6 and Table 1) and astrocytes. It has been reported that astrocytes may also become a source of ROS/RNS when exposed to IL-1 β (Thornton et al., 2006). To verify this possibility, confirmation of the existence of such a gradient of IL-1 β concentrations in the rat brain is needed. Alternatively, activated microglia at the site of injection may migrate to distant brain regions (Carbonell et al., 2005) or secondary signaling molecules generated at the IL-1 β injection site may mediate the injury in distant brain regions. Degeneration of SN neurons in the adult brain is a hallmark of Parkinson's disease and the dysfunction of VTA neurons is involved in the addictive disorders and schizophrenia (Prakash and Wurst, 2006). Several studies (Bilbo et al., 2006; Ling et al., 2004; Landrigan et al., 2005) have indicated that perinatal exposure to infectious agents in the rat may increase risks for development of neurodegenerative diseases in adulthood. Koprach et al. (2008) and Godoy et al. (2008) have recently shown that IL-1 β increases susceptibility of DA neurons to degeneration or exacerbates DA neuron degeneration in animal models of Parkinson's disease, suggesting IL-1 β may play an important role in the degeneration of DA neurons. Results from the current study indicate that although a single exposure to IL-1 β during the perinatal stage does not cause actual death of DA neurons, it is sufficient to induce lasting alterations in the DA neuronal system, which may facilitate degeneration of DA neurons to be triggered by other environmental factors in the adult brain and lead to Parkinson's disease and/or other movement disorder (Koprach et al., 2008; Ling et al., 2004; Prakash and Wurst, 2006). Although the exact consequences of perinatal IL-1 β exposure on development of neurodegenerative diseases in later life remain unknown, it is worth further investigation.

Acknowledgments

The authors thank Drs. Ing K. Ho, Tangeng Ma, and Stanley Smith for their generosity in providing access to laboratory equipment that was essential for the completion of these experiments. This work was supported by NIH grant HD 35496, NS 54278, Newborn Medicine Funds and a research grant from the Department of Pediatrics, UMC, Jackson, MS, USA.

Abbreviations

APC-CC1	adenomatus polyposis coli (Clone CC1)
DA	dopamine
HP	hippocampus
MBP	myelin basic protein
NeuN	neuron-specific nuclear protein
IL1- β	interleukin 1- β
IL-1ra	IL-1 receptor antagonist
LPS	lipopolysaccharide
MAP1	microtubule-associated protein 1
MAP2	microtubule-associated protein 2
OL	oligodendrocyte
OD	optical density
PBN	α -Phenyl- <i>n</i> -tert-butyl-nitrone
P5	postnatal day 5
PVL	periventricular leukomalacia
SN	substantia nigra
TNF α	tumor necrosis factor- α
TH	tyrosine hydroxylase
VTA	ventral tegmental area

References

- Agmo A, Belzung C. The role of subtypes of the opioid receptor in the anxiolytic action of chlordiazepoxide. *Neuropharmacology* 1998;37:223–232. [PubMed: 9680247]
- Altman J, Sudarshan K. Postnatal development of locomotion in the laboratory rat. *Anim Behav* 1975;23:896–920. [PubMed: 1200422]
- Altman J, Sudarshan K, Das GD, McCormick N, Barnes D. The influence of nutrition on neural and behavioral development. 3. Development of some motor, particularly locomotor patterns during infancy. *Dev Psychobiol* 1971;4:97–114. [PubMed: 5162548]
- Ba A, Seri BV. Psychomotor functions in developing rats: ontogenetic approach to structure-function relationships. *Neurosci Biobehav Rev* 1995;19:413–425. [PubMed: 7566743]
- Behl C, Moosmann B. Oxidative nerve cell death in Alzheimer's disease and stroke: antioxidants as neuroprotective compounds. *Biol Chem* 2002;383:521–536. [PubMed: 12033440]
- Bilbo SD, Biedenkapp JC, Der-Avakian A, Watkins LR, Rudy JW, Maier SF. Neonatal infection-induced memory impairment after lipopolysaccharide in adulthood is prevented via caspase-1 inhibition. *J Neurosci* 2005;25:8000–8009. [PubMed: 16135757]
- Bilbo SD, Rudy JW, Watkins LR, Maier SF. A behavioural characterization of neonatal infection-facilitated memory impairment in adult rats. *Behav Brain Res* 2006;169:39–47. [PubMed: 16413067]
- Cai Z, Lin S, Pang Y, Rhodes PG. Brain injury induced by intracerebral injection of interleukin-1 β and tumor necrosis factor- α in the neonatal rat. *Pediatr Res* 2004;56:377–384. [PubMed: 15201401]

- Cai Z, Pang Y, Lin S, Rhodes PG. Differential roles of tumor necrosis factor- α and interleukin-1 β in lipopolysaccharide-induced brain injury in the neonatal rat. *Brain Res* 2003;975:37–47. [PubMed: 12763591]
- Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *J Immunol* 1992;149:2736–2741. [PubMed: 1383325]
- Carbonell WS, Murase SI, Horwitz AF, Wandell JW. Infiltrative microgliosis: activation and long-distance migration of subependymal microglia following periventricular insults. *J Neuroinflamm* 2005;2:5.
- Colton CA, Gilbert DL. Microglia, an in vivo source of reactive oxygen species in the brain. *Adv Neurol* 1993;59:321–326. [PubMed: 8380516]
- Cunningham ET Jr, De Souza EB. Interleukin 1 receptors in the brain and endocrine tissues. *Immunol Today* 1993;14:171–176. [PubMed: 8499077]
- Dammann O, Leviton A. Inflammatory brain damage in preterm newborns--dry numbers, wet lab, and causal inferences. *Early Hum Dev* 2004;79:1–15. [PubMed: 15282118]
- Dammann O, Leviton A. Maternal intrauterine infection, cytokines, and brain damage in the preterm newborn. *Pediatr Res* 1997;42:1–8. [PubMed: 9212029]
- De Ryck M, Van Reempts J, Duytschaever H, Van Deuren B, Clincke G. Neocortical localization of tactile/proprioceptive limb placing reactions in the rat. *Brain Res* 1992;573:44–60. [PubMed: 1576535]
- Endoh H, Kato N, Fujii S, Suzuki Y, Sato S, Kayama T, Kotake Y, Yoshimura T. Spin trapping agent, phenyl N-tert-butyl-nitron, reduces nitric oxide production in the rat brain during experimental meningitis. *Free Radic Res* 2001;35:583–391. [PubMed: 11767416]
- Fan LW, Chen RF, Mitchell HJ, Lin RCS, Simpson KL, Rhodes PG, Cai Z. α -Phenyl-n-tert-butyl-nitron attenuates lipopolysaccharide-induced brain injury and improves neurological reflexes and early sensorimotor behavioral performance in juvenile rats. *J Neurosci Res* 2008a;86:3536–3547. [PubMed: 18683243]
- Fan LW, Lin S, Pang Y, Rhodes PG, Cai Z. Minocycline attenuates hypoxia-ischemia-induced neurological dysfunction and brain injury in the juvenile rat. *Eup J Neurosci* 2006;24:341–350.
- Fan LW, Mirchell HJ, Rhodes PG, Cai Z. Alpha-phenyl-n-tert-butyl-nitron attenuates lipopolysaccharide-induced neuronal injury in the neonatal rat brain. *Neuroscience* 2008b;151:737–744. [PubMed: 18191905]
- Fan LW, Mirchell HJ, Tien LT, Rhodes PG, Cai Z. Interleukin-1 β -induced brain injury in the neonatal rat can be ameliorated by α -phenyl-n-tert-butyl-nitron. *Exp Neurol* 2009;220:143–153. [PubMed: 19682987]
- Fan LW, Mirchell HJ, Tien LT, Zheng B, Pang Y, Rhodes PG, Cai Z. α -phenyl-n-tert-butyl-nitron reduces lipopolysaccharide-induced white matter injury in the neonatal rat brain. *Dev Neurol* 2008c;68:365–378.
- Fan LW, Tien LT, Mitchell HJ, Rhodes PG, Cai Z. α -Phenyl-n-tert-butyl-nitron ameliorates hippocampal injury and improves learning and memory in juvenile rats following neonatal exposure to lipopolysaccharide. *Eup J Neurosci* 2008d;27:1475–1484.
- Fan LW, Pang Y, Lin S, Tien LT, Ma T, Rhodes PG, Cai Z. Minocycline reduces lipopolysaccharide-induced neurological dysfunction and brain injury in the neonatal rat. *J Neurosci Res* 2005;82:71–82. [PubMed: 16118791]
- Floyd RA, Hensley K, Bing G. Evidence for enhanced neuro-inflammatory processes in neurodegenerative diseases and the action of nitrones as potential therapeutics. *J Neural Transm Suppl* 2000;60:387–414. [PubMed: 11205157]
- Floyd RA, Hensley K, Forster MJ, Kelleher-Anderson JA, Wood PL. Nitrones as neuroprotectants and antiaging drugs. *Ann NY Acad Sci* 2002;959:321–329. [PubMed: 11976206]
- Floyd RA, Hensley K. Nitron inhibition of age-associated oxidative damage. *Ann NY Acad Sci* 2000;899:222–237. [PubMed: 10863542]
- Franco-Pons N, Torrente M, Colomina MT, Vilella E. Behavioral deficits in the cuprizone-induced murine model of demyelination/remyelination. *Toxicol Lett* 2007;169:205–213. [PubMed: 17317045]

- Frost SB, Barbay S, Mumert ML, Stowe AM, Nudo RJ. An animal model of capsular infarct: endothelin-1 injections in the rat. *Behav Brain Res* 2006;169:206–211. [PubMed: 16497394]
- Godoy MC, Tarelli R, Ferrari CC, Sarchi MI, Pitossi FJ. Central and systemic IL-1 exacerbates neurodegeneration and motor symptoms in a model of Parkinson's disease. *Brain* 2008;131:1880–1894. [PubMed: 18504291]
- Green AR, Ashwood T, Odergren T, Jackson DM. Nitrones as neuroprotective agents in cerebral ischemia, with particular reference to NXY-059. *Pharmacol Ther* 2003;100:195–214. [PubMed: 14652110]
- Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet* 2008;371:75–84. [PubMed: 18177778]
- Goncalves LF, Chaiworapongsa T, Romero R. Intrauterine infection and prematurity. *Ment Retard Dev Disabil Res Rev* 2002;8:3–13. [PubMed: 11921380]
- Hagberg H, Ichord R, Palmer C, Yager JY, Vannucci SJ. Animal models of developmental brain injury: relevance to human disease. *Dev Neurosci* 2002a;24:364–366. [PubMed: 12640174]
- Hagberg H, Peebles D, Mallard C. Models of white matter injury: comparison of infectious, hypoxic-ischemic, and excitotoxic insults. *Ment Retard Dev Disabil Res Rev* 2002b;8:30–38. [PubMed: 11921384]
- Hermans RH, Hunter DE, McGivern RF, Cain CD, Longo LD. Behavioral sequelae in young rats of acute intermittent antenatal hypoxia. *Neurotoxicol Teratol* 1992;14:119–129. [PubMed: 1593986]
- Holmin S, Mathiesen T. Intracerebral administration of interleukin-1 β and induction of inflammation, apoptosis, and vasogenic edema. *J Neurosurg* 2000;92:108–120. [PubMed: 10616089]
- Huleihel M, Golan H, Hallak M. Intrauterine infection/inflammation during pregnancy and offspring brain damages: possible mechanisms involved. *Reprod Biol Endocrinol* 2004;2:7. [PubMed: 14969589]
- Kadhim H, Tabarki B, De Prez C, Sebire G. Cytokine immunoreactivity in cortical and subcortical neurons in periventricular leukomalacia: are cytokines implicated in neuronal dysfunction in cerebral palsy? *Acta Neuropathol (Berl)* 2003;105:209–216. [PubMed: 12557006]
- Kadhim H, Tabarki B, Verellen G, De Prez C, Rona AM, Sebire G. Inflammatory cytokines in the pathogenesis of periventricular leukomalacia. *Neurology* 2001;56:1278–1284. [PubMed: 11376173]
- Karl T, Pabst R, von Horsten S. Behavioral phenotyping of mice in pharmacological and toxicological research. *Exp Toxicol Pathol* 2003;55:69–83. [PubMed: 12940631]
- Kerschensteiner M, Bareyre FM, Buddeberg BS, Merkler D, Stadelmann C, Bruck W, Misgeld T, Schwab ME. Remodeling of axonal connections contributes to recovery in an animal model of multiple sclerosis. *J Exp Med* 2004;200:1027–1038. [PubMed: 15492125]
- Koprich JB, Reske-Nielsen C, Mithal P, Isacson O. Neuroinflammation mediated by IL-1 β increases susceptibility of dopamine neurons to degeneration in an animal model of Parkinson's disease. *J Neuroinflamm* 2008;5:8.
- Kotake Y, Sang H, Miyajima T, Wallis GL. Inhibition of NF-kappaB, iNOS mRNA, COX2 mRNA, and COX catalytic activity by phenyl-N-tert-butyl-nitron (PBN). *Biochim Biophys Acta* 1998;1448:77–84. [PubMed: 9824673]
- Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* 1996;19:312–318. [PubMed: 8843599]
- Landrigan PJ, Sonawane B, Butler RN, Transande L, Callan R, Droller D. Early environmental origins of neurodegenerative disease in later life. *Environ Health Perspect* 2005;113:1230–1233. [PubMed: 16140633]
- Lee JH, Park JW. The effect of alpha-phenyl-N-t-butyl-nitron on ionizing radiation-induced apoptosis in U937 cells. *Free Radic Res* 2005;39:1325–1333. [PubMed: 16298862]
- Lin S, Cox HJ, Rhodes PG, Cai Z. Neuroprotection of alpha-phenyl-n-tert-butyl-nitron on the neonatal white matter is associated with anti-inflammation. *Neurosci Lett* 2006;405:52–56. [PubMed: 16876321]
- Lin S, Rhodes PG, Lei M, Zheng F, Cai Z. alpha-Phenyl-n-butyl-nitron attenuates hypoxic-ischemic white matter injury in the neonatal rat brain. *Brain Res* 2004;1007:132–141. [PubMed: 15064144]

- Ling Z, Chang QA, Tong CW, Leurgans SE, Lipton JW, Carvey PM. Rotenone potentiates dopamine neuron loss in animals exposed to lipopolysaccharide prenatally. *Exp Neurol* 2004;190:373–383. [PubMed: 15530876]
- Li PA, He QP, Nakamura L, Csiszar K. Free radical spin trap alpha-phenyl-N-tert-butyl-nitron inhibits caspase-3 activation and reduces brain damage following a severe forebrain ischemic injury. *Free Radic Biol Med* 2001;31:1191–1197. [PubMed: 11705697]
- Lynch AM, Walsh C, Delaney A, Nolan Y, Campbell VA, Lynch MA. Lipopolysaccharide-induced increase in signalling in hippocampus is abrogated by IL-10 a role for IL-1 beta? *J Neurochem* 2004;88:635–646. [PubMed: 14720213]
- Maciag D, Simpson KL, Coppinger D, Lu Y, Wang Y, Lin RC, Paul IA. Neonatal antidepressant exposure has lasting effects on behavior and serotonin circuitry. *Neuropsychopharmacology* 2006;31:47–57. [PubMed: 16012532]
- Osterhout DJ, Marin-Husstege M, Abano P, Casaccia-Bonnel P. Molecular mechanisms of enhanced susceptibility to apoptosis in differentiating oligodendrocytes. *J Neurosci Res* 2002;69:24–29. [PubMed: 12111812]
- Pang Y, Cai Z, Rhodes PG. Disturbance of oligodendrocyte development, hypomyelination and white matter injury in the neonatal rat brain after intracerebral injection of lipopolysaccharide. *Brain Res Dev Brain Res* 2003;140:205–214.
- Parnet P, Kelley KW, Bluth RM, Dantzer R. Expression and regulation of interleukin-1 receptors in the brain. Role in cytokines-induced sickness behavior. *J Neuroimmunol* 2002;125:5–14. [PubMed: 11960635]
- Prakash N, Wurst W. Development of dopaminergic neurons in the mammalian brain. *Cell Mol Life Sci* 2006;63:187–206. [PubMed: 16389456]
- Rezaie P, Dean A. Periventricular leukomalacia, inflammation and white matter lesions within the developing nervous system. *Neuropathology* 2002;22:106–132. [PubMed: 12416551]
- Sang H, Wallis GL, Stewart CA, Kotake Y. Expression of cytokines and activation of transcription factors in lipopolysaccharide-administered rats and their inhibition by phenyl N-tert-butyl-nitron (PBN). *Arch Biochem Biophys* 1999;363:341–348. [PubMed: 10068457]
- Schallert, T.; Woodlee, MT. Motor systems: orienting and placing. In: Whishaw, IQ.; Kolb, B., editors. *The behaviour of the laboratory rat: A handbook with tests*. New York: Oxford University Press; 2005. p. 129-140.
- Schmitt U, Luddens H, Hiemke C. Anxiolytic-like effects of acute and chronic GABA transporter inhibition in rats. *J Neural Transm* 2002;109:871–880. [PubMed: 12111474]
- Shen Y, Isaacson RL, Smotherman WP. The behavioral and anatomical effects of prenatal umbilical cord clamping in the rat and their alteration by the prior maternal administration of nimodipine. *Rest Neurol Neurosci* 1991;3:11–22.
- Spolidorio PC, Echeverry MB, Iyomasa M, Guimaraes FS, Del Bel EA. Anxiolytic effects induced by inhibition of the nitric oxide-cGMP pathway in the rat dorsal hippocampus. *Psychopharmacology (Berl)* 2007;195:183–192. [PubMed: 17661019]
- Thorburne SK, Juurlink BH. Low glutathione and high iron govern the susceptibility of oligodendroglial precursors to oxidative stress. *J Neurochem* 1996;67:1014–1022. [PubMed: 8752107]
- Thornton P, Pinteaux E, Gibson RM, Allan SM, Rothwell NJ. Interleukin-1-induced neurotoxicity is mediated by glia and requires caspase activation and free radical release. *J Neurochem* 2006;98:258–266. [PubMed: 16805812]
- Volpe JJ. Cerebral white matter injury of the premature infant--more common than you think. *Pediatrics* 2003;112:176–180. [PubMed: 12837883]
- Yoon BH, Jun JK, Romero R, Park KH, Gomez R, Choi JH, Kim IO. Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1beta, and tumor necrosis factor-alpha), neonatal brain white matter lesions, and cerebral palsy. *Am J Obstet Gynecol* 1997a;177:19–26. [PubMed: 9240577]
- Yoon BH, Romero R, Kim CJ, Koo JN, Choe G, Syn HC, Chi JG. High expression of tumor necrosis factor- α and interleukin-6 in periventricular leukomalacia. *Am J Obstet Gynecol* 1997b;177:406–411. [PubMed: 9290459]

Yoon BH, Romero R, Yang SH, Jun JK, Kim IO, Choi JH, Syn HC. Interleukin-6 concentrations in umbilical cord plasma are elevated in neonates with white matter lesions associated with periventricular leukomalacia. *Am J Obstet Gynecol* 1996;174:1433–1440. [PubMed: 9065108]

Fig. 1.

PBN attenuated IL-1 β -induced dysfunction in neurological reflexes, as indicated by preventing the delayed decline of mean latency times in the negative geotaxis (A), the cliff avoidance test (B), the beam (1.2 $\times$ 1.2 cm²) walking test (C), and in locomotion activity (D) in the IL-1 β -exposed rat. Results are expressed as the mean $\pm$ SEM of twelve animals in each group. Two-way ANOVA showed that both neonatal treatment and age had significant effects on animal performance. * $P < 0.05$ represents a significant difference for the IL-1 β group as compared with the control and other groups.

Fig. 2. PBN attenuated IL-1 β -induced deficits in motor coordination, as determined by maturation of skills (A) and performance latency (B) in the pole test, by early sensorimotor behavioral performance in the vibrissa-elicited forelimb-placing test (C), by learning and memory deficit in the passive avoidance test on P20 and P21 (D), and by less anxiety-like behavior in the elevated plus-maze test on P20 (E&F). Results are expressed as the mean $\pm$ SEM of twelve animals in each group. * P<0.05 represents a significant difference for the IL-1 β group as compared with the control and other groups.

Fig. 3.

Representative photomicrographs of Nissl staining (A–C), APC-CC1 staining (D–F) and MBP staining (G–I) in the P21 rat brain. A, Nissl stained brain sections (at the bregma level) show normal morphology from the control group. B, IL-1 β exposure resulted in enlarged ventricles and the dilatation in the ipsilateral (left) ventricle was more prominent. C, PBN attenuated IL-1 β -induced ventricle enlargement. IL-1 β administration resulted in loss of APC-CC1 positive OLs (E) and a reduction in MBP positive staining (H), as compared with the control group (D & G, respectively). PBN attenuated IL-1 β -induced loss of APC-CC1+ cells and the reduction in MBP positive staining (F & I). The scale bar in A represents 500 μ m for A–C and G–I, and that in D represents 50 μ m for D–F.

Fig. 4.

Effects of PBN treatment on IL-1 β induced axonal, dendritic and neuronal injury in the cortex (A–C & G–I) and the hippocampus (D–F & J–L) of the P21 rat brain. IL-1 β caused axonal and dendritic impairment as indicated by the short beaded MAP1 positive staining in layer V of the parietal cortex (B), as compared to that in the control rat brain (A). PBN attenuated IL-1 β -induced abnormalities in MAP1 staining (C). IL-1 β caused dendritic impairment as indicated by the loss of MAP2 staining in the hippocampus (E) (measured by the percentage area of image that contained MAP2 immunoreactivity), as compared to the control group (D). PBN attenuated the IL-1 β -induced loss of MAP2 staining (F). IL-1 β also reduced the number of NeuN stained neurons in the hippocampal CA1 region (K), as compared with that in the control rat brain (J). PBN attenuated IL-1 β -induced reduction in number of NeuN stained neurons in this region (L). However, no differences in the number of NeuN positive neurons were found among the control (G), the IL-1 β -exposed (H) and the PBN+IL-1 β (I) groups in layer V of the parietal cortex. The scale bar shown in A represents 50 μ m.

Fig. 5.

Representative photomicrographs of TH staining in the rat brain 16 days (P21) after IL-1 β injection. TH positive neurons were abundant in the SN (A) and the VTA (G) areas of the midbrain sections at a level 1/3 rostral from the lambda to the bregma in the control rat brain (A). IL-1 β injection caused a loss of TH positive staining (B & H). PBN attenuated IL-1 β -induced loss of TH positive staining (C & I). Double labeling with TH and NeuN antibodies (D–F) shows that IL-1 β injection caused a reduction in TH positive cells (red), but not in the number of NeuN (green) positive cells (E) (D, E and F are magnified part indicated by the white box in A, B and C, respectively). The scale bar shown in A represents 200 μ m for A–C and G–I, and that in D represents 50 μ m for D–F.

Fig. 6

Fig. 6.

Representative photomicrographs of microglia in the rat brain 16 days (P21) after IL-1 β injection. As shown by OX42 immunostaining (A–C, cingulum area; D–I, SN), most microglia were at the resting status with a small rod shaped soma and ramified processes in the control group (arrow indicated in A & D). Numerous activated microglia with a round shaped cell body and blunt processes were observed in the rat brain after IL-1 β injection (arrows indicated in B & E). PBN treatment significantly reduced number of activated microglia stimulated by IL-1 β (C & F). Merged images showed that OX42 positive activated microglia (red) were found around TH+ cells (green) in the SN of the IL-1 β exposed rat brain (H), but much less OX42 positive microglia were found in this area in the control (G) and IL-1 β +PBN groups (I). The scale bar in A represents 50 μ m.

Table 1PBN attenuated IL-1 β -induced rat brain injury 16 days (P21) after IL-1 β injection

Treatment	Saline	Saline+PBN	IL-1 β	IL-1 β +PBN
Ventricle size index in bregma level				
Left ventricle (%)	0.23 $\pm$ 0.04	0.27 $\pm$ 0.04	0.92 $\pm$ 0.04*	0.24 $\pm$ 0.06 [#]
Right ventricle (%)	0.16 $\pm$ 0.03	0.22 $\pm$ 0.03	0.53 $\pm$ 0.06*	0.16 $\pm$ 0.04 [#]
Number of OLs in cingulum area				
APC-CC1+ (cells/mm ²)	1184.4 $\pm$ 35.7	1215.6 $\pm$ 42.6	653.3 $\pm$ 71.1*	1122.2 $\pm$ 42.8 [#]
MBP staining				
Area (mm ²)	2.62 $\pm$ 0.11	2.59 $\pm$ 0.12	2.14 $\pm$ 0.09*	2.65 $\pm$ 0.10 [#]
Density (OD)	0.163 $\pm$ 0.004	0.162 $\pm$ 0.004	0.109 $\pm$ 0.005*	0.147 $\pm$ 0.008 [#]
MAP1 stained axons and dendrites				
Parietal cortex (length, μ m)	180.8 $\pm$ 6.1	179.5 $\pm$ 5.3	64.9 $\pm$ 9.2*	176.4 $\pm$ 6.5 [#]
HP-CA1 (length, μ m)	82.2 $\pm$ 1.9	86.5 $\pm$ 3.7	23.3 $\pm$ 3.0*	83.5 $\pm$ 4.1 [#]
MAP2 stained dendrites				
% of area in cingulum area	9.0 $\pm$ 0.5	8.6 $\pm$ 0.5	6.1 $\pm$ 0.5*	9.1 $\pm$ 0.7 [#]
% of area in parietal cortex	6.8 $\pm$ 0.4	6.9 $\pm$ 0.3	3.0 $\pm$ 0.2*	7.0 $\pm$ 0.3 [#]
% of area in HP-CA1	17.3 $\pm$ 0.9	16.7 $\pm$ 0.7	8.5 $\pm$ 0.6*	15.9 $\pm$ 1.3 [#]
Neurons in parietal cortex				
Nissl+ cell (cell/mm ²)	1371.8 $\pm$ 60.3	1386.7 $\pm$ 53.3	1382.2 $\pm$ 61.7	1364.2 $\pm$ 39.7
NeuN+ cell (cell/mm ²)	1137.8 $\pm$ 48.9	1171.1 $\pm$ 51.9	1146.7 $\pm$ 38.8	1155.6 $\pm$ 50.2
Neurons in HP-CA1				
Nissl+ cell (cell/mm)	209.2 $\pm$ 4.7	213.3 $\pm$ 3.5	179.6 $\pm$ 4.5*	210.4 $\pm$ 4.3 [#]
NeuN+ cell (cell/mm)	229.6 $\pm$ 6.3	231.7 $\pm$ 4.6	186.7 $\pm$ 6.4*	229.6 $\pm$ 4.3 [#]
Neurons in SN and VTA				
TH+ cells in SN (cell/mm ²)	59.9 $\pm$ 1.7	60.4 $\pm$ 1.3	41.7 $\pm$ 1.7*	61.4 $\pm$ 1.5 [#]
NeuN+ cells in SN (cell/mm ²)	84.3 $\pm$ 1.2	83.9 $\pm$ 1.6	84.4 $\pm$ 1.4	84.7 $\pm$ 1.3
TH+ cells in VTA (cell/mm ²)	109.7 $\pm$ 6.4	106.9 $\pm$ 5.6	64.9 $\pm$ 2.8*	107.8 $\pm$ 4.7 [#]
NeuN+ cells in VTA (cell/mm ²)	142.9 $\pm$ 3.7	140.4 $\pm$ 3.8	127.5 $\pm$ 5.7	137.9 $\pm$ 4.2
Activated microglia (cell/mm ²)				
OX42+ in cingulum area	284.4 $\pm$ 27.7	268.9 $\pm$ 21.1	513.3 $\pm$ 20.3*	264.4 $\pm$ 23.2 [#]
OX42+ in parietal cortex	157.8 $\pm$ 13.5	162.2 $\pm$ 12.6	528.9 $\pm$ 37.5*	164.4 $\pm$ 9.5 [#]
OX42+ in SN	197.8 $\pm$ 19.9	180.0 $\pm$ 17.1	584.4 $\pm$ 43.5*	175.6 $\pm$ 17.4 [#]
OX42+ in VTA	202.2 $\pm$ 19.0	182.2 $\pm$ 17.4	637.8 $\pm$ 38.4*	193.3 $\pm$ 26.2 [#]
Activated microglia (OX42+)				
% of area in cingulum area	4.7 $\pm$ 0.3	4.4 $\pm$ 0.4	15.0 $\pm$ 0.8*	5.1 $\pm$ 0.4 [#]
% of area in parietal cortex	2.3 $\pm$ 0.3	2.7 $\pm$ 0.3	11.2 $\pm$ 0.9*	2.3 $\pm$ 0.4 [#]
% of area in SN	4.1 $\pm$ 0.4	4.2 $\pm$ 0.3	15.2 $\pm$ 0.8*	4.5 $\pm$ 0.4 [#]

Treatment	Saline	Saline+PBN	IL-1 β	IL-1 β +PBN
% of area in VTA	3.6 $\pm$ 0.3	3.6 $\pm$ 0.4	14.9 $\pm$ 1.1 *	4.1 $\pm$ 0.5 [#]

Intracerebral injection of IL-1 β and intraperitoneal PBN treatment were performed as described in the text. The ventricle size index and the area and OD of MBP staining were determined by a densitometer as described in methods. Cell counting of APC-CC1+ was performed in the cingulum area of the forebrain at the bregma level. MAP1, MAP2, Nissl, and NeuN staining was performed in sections from the forebrain at the bregma level and in the hippocampal area of the diencephalon sections. TH+ cell or NeuN+ cell counting in the substantia nigra (SN) and ventral tegmental area (VTA) was performed in the midbrain sections at the level 1/3 rostral from the lambda to the bregma. OX42+ cell counting was performed in the same levels as TH staining. OX42 positive cells with an elongated or a round shaped cell body and blunt processes (as indicated in Fig. 6B&6E) were considered as the activated microglia. Data are presented as the mean $\pm$ SEM (mm², OD, μ m, % of area, cell/mm², or cell/mm) of six animals for each group. Data were analyzed by one-way ANOVA followed by Student-Newman-Keuls test.

* P<0.05 compared with the saline group.

[#] P<0.05 compared with the IL-1 β group.