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Neonatal lipopolysaccharide exposure induces long-lasting learning impairment, less anxiety-like response and hippocampal injury in adult rats

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Abstract

Infection during early neonatal period has been shown to cause lasting neurological disabilities and is associated with the subsequent impairment in development of learning and memory ability and anxiety-related behavior in adults. We have previously reported that neonatal lipopolysaccharide (LPS) exposure resulted in cognitive deficits in juvenile rats (P21); thus, the goal of the present study was to determine whether neonatal LPS exposure has long-lasting effects in adult rats. After an LPS (1 mg/kg) intracerebral (i.c.) injection in postnatal day 5 (P5) Sprague-Dawley female rat pups, neurobehavioral tests were carried out on P21 and P22, P49 and P50 or P70 and P71 and brain injury was examined at 66 days after LPS injection (P71). Our data indicate that neonatal LPS exposure resulted in learning deficits in the passive avoidance task, less anxiety-like (anxiolytic-like) responses in the elevated plus-maze task, reductions in the hippocampal volume and the number of NeuN+ cells, as well as axonal injury in the CA1 region of the middle dorsal hippocampus in P71 rats. Neonatal LPS exposure also resulted in sustained inflammatory responses in the P71 rat hippocampus, as indicated by an increased number of activated microglia and elevation of interleukin-1 β content in the rat hippocampus. This study reveals that neonatal LPS exposure causes persistent injuries to the hippocampus and results in long-lasting learning disabilities, and these effects are related to the chronic inflammation in the rat hippocampus.

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Keywords

Lipopolysaccharide; chronic inflammation; hippocampus; learning dysfunction; anxiolytic behavior

INTRODUCTION

Maternal, placental or neonatal infection/inflammation has been shown to induce neonatal brain injury and it may be associated with the consequent neurological disorders and functional disability in later life (Hagberg et al., 2002, Volpe, 2003, Hagberg et al., 2012). Increasing evidence indicates that acute inflammation can be shifted to a chronic inflammatory state and/or adversely affect brain development (Williamson et al., 2011, Hagberg et al., 2012). Using this neonatal rat model, we have found that neonatal exposure to LPS resulted in sensory, motor, emotional and cognitive impairment in juvenile rats (P21) (Fan et al., 2005, Fan et al., 2008).

Our previous studies have shown that neonatal exposure (P5) to LPS through an i.c. injection in rats can produce chronic inflammation and injury in the nigrostriatal dopaminergic system, as indicated by the phenotypic suppression of tyrosine hydroxylase expression from neurons in the substantia nigra (SN), impairment of nigrostriatal neuron connectivity and neurobehavioral deficits in the LPS-exposed rats (Fan et al., 2005, Fan et al., 2008, Fan et al., 2011a, Fan et al., 2011b). On the other hand, neonatal LPS exposure did not cause actual death of dopaminergic neurons in the SN and the LPS-induced motor dysfunction was spontaneously recoverable by adult ages (P70) (Fan et al., 2011a, Fan et al., 2011b). We also observed that neonatal LPS exposure (P5) lead to learning and memory deficits in the passive avoidance task, less anxiety-like (anxiolytic-like) responses in the elevated plus maze task, and axonal injury in the CA1 region of the middle dorsal hippocampus in the juvenile male and female rats (P21) (Fan et al., 2005, Fan et al., 2008). However, it is unclear whether neonatal exposure to LPS can produce chronic inflammation and neuronal damage in other brain regions, such as hippocampus, and cause hippocampal related neurological disability in adults. Thus, the present study was designed to examine whether neonatal LPS exposure can produce chronic inflammation and persistent neuronal injury in hippocampus, and long-lasting cognitive deficits in adult rats.

Our preliminary data showed that neonatal LPS infection induced great learning impairments in P49 female rats. To eliminate a possible effect of gender difference, female rats was used in this study. The hippocampal formation not only mediates processes associated with learning and memory but also anxiety and fear (Spolidorio et al., 2007, Zarrindast et al., 2012). The abnormal neurobehavioral performance in avoidance learning and memory, and locomotor activity has also been linked with dopaminergic neuronal injury in many studies (Nishii et al., 1998, Denenberg et al., 2004). Therefore, the learning and memory task, behaviors of locomotion and anxiety-like response were determined in this study.

EXPERIMENTAL PROCEDURES

Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO., USA). Monoclonal mouse antibodies against neuron-specific nuclear protein (NeuN) or microtubule-associated protein 1 (MAP1), and OX42 (CD11b) were purchased from Millipore (Billerica, MA, USA) and Serotec (Raleigh, NC), respectively. ELISA kits for immunoassay of rat interleukin-1 β (IL-1 β) (RLB00), interleukin-6 (IL-6) (R6000B) and

tumor necrosis factor- α (TNF α) (RTA00) were purchased from R&D Systems (Minneapolis, MN, USA).

Animals

Timed pregnant Sprague-Dawley rats arrived in the laboratory on day 19 of gestation. Animals were maintained in an animal room on a 12-h light/dark cycle and at constant temperature ($22 \pm 2^\circ\text{C}$). 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 or Fu Jen Catholic University. Every effort was made to minimize the number of animals used and their suffering.

Surgery procedures and animal treatment

Intracerebral injection of LPS to 5-day old Sprague-Dawley female rat pups was performed as previously described (Cai et al., 2003, Pang et al., 2003, Fan et al., 2005). Under light anesthesia with isoflurane (1.5 %), LPS (1 mg/kg, from *Escherichia coli*, serotype 055: B5) in sterile saline (total volume of 2 μL) was administered to the rat brain at the location of 1.0 mm posterior and 1.0 mm left to the bregma, and 2.0 mm deep to the skull surface in a stereotaxic apparatus with a neonatal rat adapter. The dose of LPS was chosen based on the previously reported results which produced brain injury (Cai et al., 2003, Pang et al., 2003, Fan et al., 2005). 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. All animals survived the intracerebral injection.

Each dam had the same litter size (12 pups) and equal numbers of LPS-treated and saline-treated rat pups were included in a litter. The pups were weaned at P21 and four rats (2 LPS-treated and 2 saline-treated) per cage were housed after weaning. Thirty-six rats (eighteen rats from each group) were used in the present study. Equal numbers of rat pups (6 pups) were included in LPS or saline injection group for behavioral tests for three experiment groups (P21, P49, P70). Sixty six days after the injection (P71), rats were sacrificed by transcardiac perfusion with normal saline followed by 4 % paraformaldehyde for brain section preparation. Frozen coronal brain sections at 10 μm of thickness from 6 rats of each group were prepared in a cryostat for immunohistochemistry staining. The other 6 rats from each group were used for preparation of free-floating coronal brain sections at 40 μm of thickness in a sliding microtome (Leica, SM 2000R, Wetzlar, Germany) for stereological estimates of the size of cerebrum, ventricle, white matter, and hippocampus. For determination of the content of pro-inflammatory cytokines in the hippocampus, six P71 rats from each group were sacrificed by decapitation to collect fresh brain tissue.

Behavioral testing

The behavioral tests were performed as previously described (Fan et al., 2005, Fan et al., 2008, Fan et al., 2011a, Fan et al., 2011b) with modifications. As shown in Fig. 1, three groups were including in the present study. Group 1: sixteen days after the injection (P21), rats (6 rats in each group) were performed by open field, elevated plus-maze and passive avoidance (learning trial) tests. The memory trials were tested on P22, P50 and P71. Group 2: forty-four days after the injection (P49), rats (6 rats in each group) were performed by open field, elevated plus-maze and passive avoidance (learning trial) tests. The memory trials were tested on P50 and P71. Group 3: sixty-five days after the injection (P70), rats (6 rats in each group) were performed by open field, elevated plus-maze and passive avoidance (learning trial) tests. The memory trials were tested on P71.

Locomotion (open field)

This test measures the activity and habituation response of animals on placement in a novel environment (Hermans et al., 1992). Locomotor activity was measured on P21, P49 or P70, using the ANY-maze Video Tracking System (Stoelting Co., Wood Dale, IL, USA). Pups were placed in the activity chambers ($42 \times 25 \times 40 \text{ cm}^3$) in a quiet room with dimmed light. The total distance traveled by the animal was recorded during a 10-min testing period (Fan et al., 2005, Fan et al., 2008, Fan et al., 2011a, Fan et al., 2011b). The number of rearing events including exposure rearing responses (body inclined vertically with hindpaws on the floor of the activity cage and forepaws on the wall of the chamber) and sniffing-air responses (rearing in the open area of the active chamber) were counted during the first 5-min testing period. The summation of exposure rearing and sniffing-air responses reflect vertical activity which has been used apart from locomotion, as a reliable criterion for motor activity during their exposure to novelty (Antoniou et al., 2004).

Passive avoidance

Passive avoidance involves the learned inhibition of a natural response and gives information about learning and memory capabilities (Olton, 1973, Rodier, 1977, Hermans et al., 1992). The passive avoidance procedure consists of two sessions (Fan et al., 2005, Fan et al., 2008). In the first session (P21, P49 or P70), rats were trained in a step-down type of passive avoidance apparatus. The experimental chamber ($30 \times 30 \times 40 \text{ cm}^3$) 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 ($8 \text{ cm} \times 25 \text{ cm} \times 2.5 \text{ cm}$) 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 (stimulus amplitude: 80 volts; 1 second duration) using Isolated Square Wave Stimulator (#7092-611, Phipps and Bird, Inc., Richmond, VA). 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 1, 29 or 50 days after the first session (P22, P50 or P71). 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 elevated plus-maze test was performed as previously described (Fan et al., 2005, Fan et al., 2008) with modifications.

The plus-maze consists of two open arms ($50 \text{ cm} \times 10 \text{ cm} \times 1.5 \text{ cm}$ walls) and two enclosed arms ($50 \text{ cm} \times 10 \text{ cm} \times 40 \text{ cm}$ high walls) emanating from a common central platform ($10 \text{ cm} \times 10 \text{ cm}$) to form a plus shape. The entire apparatus was elevated to a height of 50 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 P21, P49 or P70.

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 66 days (P71) after the intracerebral injection. For immunohistochemistry staining, primary antibodies were used in the following dilutions: MAP1 or NeuN, 1:100; and OX42, 1:200. NeuN detects the neuron-specific nuclear protein which primarily localizes in the nucleus of the neurons with slight staining in the cytoplasm. MAP1 provides selective staining of neuronal axons and dendrites. Activation of microglia was 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 or rhodamine) 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. The resulting sections were examined under a fluorescent microscope at appropriate wavelengths.

Determination of IL-1 β , IL6 and TNF α protein by ELISA

Three major proinflammatory cytokines, IL-1 β , IL-6 and TNF α , were determined by ELISA Immunoassay, #RLB00, sensitivity <5 pg/ml; Rat IL-6 Immunoassay, #R6000B, sensitivity = 14~36 pg/ml; Rat TNF Immunoassay, #RTA00, sensitivity <5 pg/ml; R&D Systems Inc., Minneapolis, MN) as previously described (Cai et al., 2003, Pang et al., 2003, Fan et al., 2011b). Briefly, rats were sacrificed by decapitation and the fresh hippocampal tissues from each rat were collected 66 days after LPS injection (P71). Tissues were homogenized by sonication in 1 ml ice-cold PBS (pH 7.2) and centrifuged at 12,000g for 20 min at 4°C. The supernatant was collected and the protein concentration was determined by the Bradford method. The protein concentration in all samples was adjusted to 4 mg/ml. 100 μ l of each sample (50 μ l per well, duplicate) was needed for each ELISA test. ELISA was performed following manufacturer's instructions and data were acquired using a 96-well plate reader (Bio-Tek Instruments, Inc., VT). The cytokine contents were expressed as pg cytokines/mg protein.

Estimate of the volume of cerebrum and hippocampus

The stereological estimates of the total volume of cerebrum, ventricles, white matter, striatum and hippocampus were determined in the P71 rat brain following the methods described previously (Gundersen and Jensen, 1987). The fifty-six equally spaced thick (40 μ m) sections that were to be used in the analysis came from a one in six series. Nissl stained sections were scanned by a densitometer (Bio-Rad, Hercules, CA) and area of the ventricles, white matter, striatum, and hippocampus as well as that of the whole brain section (cerebellum) were outlined and determined using NIH image software in each of the fifty-six sections (Fan et al., 2005, Fan et al., 2008, Fan et al., 2011a). The Cavalieri principle (Gundersen and Jensen, 1987) was used to estimate the reference volumes.

Quantification of immunostaining data

Nissl, NeuN, MAP1 and OX42 stained sections was performed in the hippocampal area of the diencephalon sections at levels 1/2 and 2/3 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.

In the present study, CA1 neuronal changes were primarily observed in the hippocampus of the diencephalon following LPS exposure. Therefore, unless otherwise stated, three digital microscopic images were randomly captured at the CA1 region of the hippocampus were most abundant for each section. The number of positively stained cells in the three images was averaged. Three sections at each of the two section levels were examined by an observer blind to the treatment and the mean value of cell counting or length was used to represent one single brain. For convenience of comparing results among the treatment groups, results were standardized as the average number of Nissl or NeuN+ cells/mm (CA1) or the average number of OX42+ cells/mm² (CA1). Lengths (μ m) of all MAP1 positive staining in the CA1 region of the three brain sections at each of the two brain levels were measured, using the NIH image software and the average was calculated bilaterally for a single brain. In response to LPS challenge, the number of OX42+ microglia increases and the size of these cells become bigger. We developed a method to quantitatively measure these changes, i.e, using computer software to determine the percentage area that contains OX42 positive staining in the entire area of the captured image (Fan et al., 2011b). 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 MAP1 and MAP2 staining (Fan et al., 2011b). In addition to cell counting, OX42 staining as well as MAP1 staining was quantified by this method in the present study, using the NIH image software.

Statistical analysis

The behavioral data from elevated plus-maze task and quantitative data from immunostaining and ELISA were presented as the mean $\pm$ SEM and analyzed by Student's t-test. Results with a $p < 0.001$ were considered statistically significant. The other behavioral data were presented as the mean $\pm$ SEM and analyzed 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 $p < 0.001$ were considered statistically significant.

RESULTS

Neonatal LPS exposure-induced locomotor deficits were spontaneously recoverable

Neonatal LPS exposure resulted in decreases in body weight [$F(1, 30) = 28.407$, $p < 0.001$] (P21, $t(10) = 8.984$, $p < 0.001$; and P49, $t(10) = 7.080$, $p < 0.001$) (Fig. 2A) and increases in the vertical activity [$F(1, 30) = 30.484$, $p < 0.001$] at P21 [$t(10) = -5.425$, $p < 0.001$] and P49 [$t(10) = -9.192$, $p < 0.001$], as indicated by the increased number of rearing events (exposure rearing responses and sniffing-air responses) (Fig. 2C), as compared to the saline-treated group. There were no significant differences in the total crossing distance of an individual rat during a 10-min period in an open field on P21, P49 and P70 [$F(1, 30) = 0.002$, $p = 0.964$, ns] (Fig. 2B). However, the reduction in body weight and the increases in the vertical activity were spontaneously reversible, and by P70 no significant differences in locomotion between the two groups were observed (Figs. 2A to 2C).

Passive avoidance

As shown in Fig 3, the number of electric foot shocks required to retain the rat on the safe board was significantly increased in the LPS group at P21 [$t(10) = -10.541$, $p < 0.001$] (Fig. 3A, left panel), P49 [$t(10) = -10.541$, $p < 0.001$] (Fig. 3B, left panel), and P70 [$t(10) =$

−7.278, $p < 0.001$] (Fig. 3C left panel). LPS exposure reduced the retention latency to step down from the board the next day P22 [$F(1, 20) = 455.747$, $p < 0.001$; P22, $t(10) = 33.678$, $p < 0.001$] (Fig. 3A, right panel) and P50 [$F(1, 10) = 69.431$, $p < 0.001$; P50, $t(10) = 6.037$, $p < 0.001$] (Fig. 3B, right panel) as compared to the control group. The neonatal LPS-induced learning deficits was sustained in P70, however, the LPS-induced memory deficits was recoverable (P71) as determined by passive avoidance task [$t(10) = 1.577$, $p = 0.146$, ns] (Fig. 3C, right panel).

Elevated plus-maze test

A higher number of entries into the open arm were observed in the LPS-injected group as compared with the control group at P21 [$t(10) = -9.428$, $p < 0.001$] (Fig. 4A, left panel), P49 [$t(10) = -9.820$, $p < 0.001$] (Fig. 4B, left panel), and P70 [$t(10) = -6.548$, $p < 0.001$] (Fig. 4C, left panel). LPS administration increased the step-through latency into the open arm at P21 [$t(10) = -13.373$, $p < 0.001$] (Fig. 4A, right panel), P49 [$t(10) = -8.876$, $p < 0.001$] (Fig. 4B, right panel), and P70 [$t(10) = -6.118$, $p < 0.001$] (Fig. 4C, right panel) while decreasing the step-through latency into the enclosed arm at P21 [$t(10) = 13.373$, $p < 0.001$] (Fig. 4A, right panel), P49 [$t(10) = 8.876$, $p < 0.001$] (Fig. 4B, right panel), and P70 [$t(10) = 6.118$, $p < 0.001$] (Fig. 4C, right panel). Therefore, neonatal LPS exposure-induced less anxiety-like behavior sustained in adults (P70) as determined by the elevated plus-maze test.

Neonatal LPS exposure resulted in persistent ventricle enlargement and reduction of hippocampus volume

Nissl staining showed that neonatal LPS exposure caused persistent dilatation of bilateral ventricles [$t(10) = -12.304$, $p < 0.001$] (Figs. 5B, 5D and 5E). LPS exposure decreased the volume of cerebrum [$t(10) = 4.241$, $p = 0.002$], white matter [$t(10) = 3.783$, $p = 0.004$], striatum [$t(10) = 5.889$, $p < 0.001$], and hippocampus [$t(10) = 8.401$, $p < 0.001$] at P70 rats (Figs. 5D and 5E), as compared with the control group (Figs. 5C and 5E).

Neonatal LPS exposure induced losses of neurons in hippocampal CA1 region

LPS exposure induced a decrease in number of Nissl stained neurons in the hippocampal CA1 region at the middle dorsal hippocampus level [$t(10) = 13.183$, $p < 0.001$] (Figs. 6B and 6E), as compared with that in the control rat brain (Figs. 6A and 6E). This result was confirmed by NeuN immunostaining (Figs. 6C, 6D and 6E). NeuN detects the neuron-specific nuclear protein which primarily localizes in the nucleus of the neurons with slight staining in the cytoplasm. The data of NeuN staining showed a similar reduction in the number of hippocampal CA1 neurons [$t(10) = 9.528$, $p < 0.001$] (Figs. 6D and 6E), as compared to that in the control rat brain (Figs. 6C and 6E).

Neonatal LPS exposure induced persistent axonal damage in hippocampal CA1 region

Neuronal axonal processes were identified by MAP1 immunostaining. As shown in Fig. 7B, neonatal LPS exposure resulted in abnormal axon morphology (twisted and beaded appearance) in the hippocampal CA1 region of P71 rat brains, and significantly reduced axonal length [$t(10) = 11.409$, $p < 0.001$] (Figs. 7B and 7C, left panel) and density [$t(10) = 6.227$, $p < 0.001$] (Figs. 7B and 7C, right panel).

Neonatal LPS exposure induced a sustained increase in microglial activation

Neonatal LPS exposure resulted in a sustained increase in microglial activation in the P71 rat brain, as indicated by the number and morphology of the OX42 positive cells. In the control rat brain, some 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 (arrows

indicated in Fig. 8A). A significantly increased number of OX42+ cells were found in the hippocampal CA1 region of the LPS-exposed rat brain [$t(10) = -12.229$, $p < 0.001$] (Figs. 8B and 8C, left panel). 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 Fig. 8B) (Kreutzberg, 1996). OX42 staining was quantified by measuring the percentage area that contains OX42 immunostaining in the captured images. Higher percentage of OX42+ immunostaining area was observed in hippocampal CA1 region of the neonatal LPS-exposed rat brain [$t(10) = -8.712$, $p < 0.001$] (Figs. 8B and 8C, right panel).

Neonatal LPS exposure induced a sustained increase in inflammatory responses

IL-1 β , a major proinflammatory cytokine, was undetectable in the saline-injected rat brain, but was still significantly increased in the hippocampus of the rat brain 66 days after LPS injection [$t(10) = -22.371$, $p < 0.001$] (Fig. 8D). However, IL-6 [$t(10) = 1.386$, $p = 0.196$, ns] and TNF α [$t(10) = -0.815$, $p = 0.434$, ns] were almost undetectable in the hippocampus of the P70 control or LPS-exposed rat brain (Fig. 8D).

DISCUSSION

Consistent with our previous results that i.c. injection with LPS induces decreases in body weight (Fig. 2A) and increases in the vertical activity at P21 and P49 (Fig. 2C) (Fan et al., 2011a, Fan et al., 2011b). The rearing responses reflect vertical activity which has been used apart from locomotion, as a reliable criterion for motor activity during their exposure to novelty (Antoniou et al., 2004). Those LPS-induced deficits are spontaneously recoverable by adult ages (P70) (Fan et al., 2011a, Fan et al., 2011b). Rearing is a behavior often associated with stress (Beale et al., 2011). It may be influenced by hypothalamo-pituitary-adrenal axis than hippocampus. Thus, we found that rearing event subsided over time even though increased inflammation, reduced brain volume, and neuritis dysfunction persisted in the hippocampus of rats. However, the effect of neonatal LPS treatment on neuroendocrine immune system should be further investigated.

The cognitive and anxiety-related deficits are still observed in adult female rats (P70) following neonatal LPS exposure. The present results shows that neonatal LPS exposure lead to learning (P21, P49 and P70) and memory deficits (P22 and P50) in the passive avoidance task (Fig. 3), less anxiety-like responses (P21, P49 and P70) in the elevated plus maze task (Fig. 4), reduction of the hippocampal volume (Fig. 5) and NeuN+ cells (Fig. 6), and axonal injury in the hippocampal CA1 region (Fig. 7) in the adult rats (P70). Since there was no significant difference in horizontal locomotion among groups at P21, P49 and P70 (Fig. 2B), it is unlikely that differences in horizontal locomotion contribute to the impaired avoidance performance and anxiety-related deficits of the LPS group. The hippocampus plays important roles in the process of learning, memory and anxiety (Spolidorio et al., 2007, Zarrindast et al., 2012), and the hippocampal injury observed in the LPS-treated group may contribute to the poor performance of the LPS group in the avoidance and elevated plus maze tasks. Neonatal LPS exposure resulted in sustained inflammatory responses in the P71 rat hippocampus, as indicated by an increased number of activated microglia (Figs. 8B and 8C) and elevation of interleukin-1 β content in the rat hippocampus (Fig. 8D). Therefore, data from the present study further demonstrate that LPS-induced hippocampal chronic inflammation and neuronal injury are not only persisted but also linked with hippocampal-related neurobehavioral deficits in adult rats. Our data showed that neonatal LPS exposure caused sustained inflammatory responses in hippocampus, hippocampal injury, learning and memory deficits in both male and female rats. There was no significant difference in anxiety-like responses between the LPS- and saline-treated male rats in P49 and P70 (Tien et al., unpublished data). One of the possible reasons may be due to the sex differences in

the colonization and function of glia between males and females. Adult females have significantly more microglia with thicker, longer branches than males within the hippocampus, cortex, and amygdale, and progesterone may influence glial function (Schwarz and Bilbo, 2012). To clarify whether neonatal LPS exposure may have the effect of gender difference on hippocampal injury and hippocampal-related neurobehavioral deficits, the further study will be needed.

Combining with our previous finding, neonatal LPS exposure causes the neuronal damage and axonal injury not only in the substantia nigra (Fan et al., 2011b) but also in the hippocampus persisted in adult (P71) rats (Figs. 5 to 7). It has been suggested that perinatal exposure of rats to LPS leads to impaired learning and psychotic-like behavior in mature offspring, together with an aberrant forms of synaptic plasticity in the hippocampal CA1 region (Escobar et al., 2011). This might result in later-occurring brain dysfunctions, such as learning deficits in rats (Escobar et al., 2011). The hippocampal CA1 dopaminergic system is also important for modulating the emotional behavior and anxiolytic-like responses (less anxiety-like responses) (Spolidorio et al., 2007, Zarrindast et al., 2012). Accumulated data indicate that a long-term hippocampal inflammation induces the anxiolytic-like responses (Fan et al., 2005, Fan et al., 2008, Hein et al., 2012) and infection early in life protects against stressor-induced depressive-like symptoms in adult rats (Bilbo et al., 2008). The above reports are similar with our present finding that neonatal LPS exposure causes persistent hippocampal CA1 injury, learning deficits and anxiolytic-like responses in adult rats.

However, the neonatal LPS exposure induced memory deficits were recoverable at P71 rats (Fig. 3C). It has been reported that an early-life infection leads to cognitive impairment in conjunction with an inflammatory challenge in young adulthood and it might also accelerate the cognitive decline associated with aging (16 months) (Bilbo et al., 2006, Bilbo, 2010). Our previous data indicate that although neonatal LPS-induced motor neurobehavioral impairment is spontaneously recoverable (Fan et al., 2011b), perinatal brain inflammation may enhance adult susceptibility to the development of neurodegenerative disorders triggered later on by environmental toxins at an ordinarily non-toxic or sub-toxic dose (Fan et al., 2011a). Therefore, the LPS exposure-induced persistent injury to the hippocampus and the chronic inflammation may represent the existence of silent neurotoxicity, i.e. perinatal inflammation-induced memory impairments in animal models are not observable in the adult life unless other environmental insults or immune challenges occur in the adult (Costa et al., 2004, Bilbo et al., 2006, Fan et al., 2011a). It remains unknown as to how perinatal exposure to LPS leads to an increased risk for development of hippocampus-related neurological disorders and the further study is needed.

The persistent inflammatory response has been found in the rat hippocampus following neonatal LPS exposure, as indicated by the sustained activation of microglia and the increased expression of inflammatory cytokine, IL-1 β , in the P71 rat hippocampus (Fig. 8). We have reported previously that following neonatal LPS exposure similar inflammatory responses were observed in neonatal rats (P6), juvenile (P21) rats, or striatum and SN regions of adult rats and these inflammatory responses were associated with neurological dysfunction in these animals (Cai et al., 2003, Pang et al., 2003, Fan et al., 2005, Fan et al., 2008, Fan et al., 2011b). Microglia, the major resident immune cells in the brain, are detectable at very early time points in the central nervous system (CNS) of embryos, but the largest population of newborn microglia emerges in late gestation and early postnatal period in both human and rats (Pont-Lezica et al., 2011, Harry and Kraft, 2012). In Adults, quiescent microglia switch to an activated phenotype in response to pathogen invasion or tissue damage and thereby promote an inflammatory response including release of free radicals, cytokines, and lipid metabolites (Dutta et al., 2008, Glass et al., 2010, Qian et al.,

2010). However, microglia differentiate into fully mature (ramified) microglia by P14 in rats, and exist primarily in an amoeboid/phagocytic state during early brain development and can respond vigorously to infection or injury (Bilbo and Schwarz, 2009, Pont-Lezica et al., 2011, Harry and Kraft, 2012). Therefore, the hypothesis has been proposed that subsets of glia are permanently maintained in an activated or primed state into adulthood as a consequence of neonatal infection (Bilbo and Schwarz, 2009). Our previous study also show that neonatal LPS exposure results in sustained activated microglia in the P70 rat SN (Fan et al., 2011b), and the primed microglia can produce dopaminergic neuron loss and eventually Parkinson's disease-like neurological deficits triggered by a small dose of rotenone (Fan et al., 2011a). Inhibition of microglia activation by minocycline provides protective effects in the neonatal rat brain on LPS-induced hippocampal injury, chronic inflammation, and learning and memory deficits on P21 rats (Fan et al., 2005). Therefore, neonatal LPS exposure induces chronic microglia activation may contribute the hippocampus-related deficits in adult rats. Although the long-lasting effect of minocycline (P70) need to be further studied, our present data provide the important information regarding to the links between neonatal LPS exposure-induced chronic microglia activation and hippocampus-related behavioral performance alteration in later life.

Our current data indicate that not only increases in the number of activated microglia were found in the hippocampal CA1 region, but also increased contents of IL-1 β were detected in the hippocampus of the P71 rat brain (Fig. 8). Inflammatory responses establish feed-forward loops and uncontrolled sustained inflammation may result in production of neurotoxic factors that amplify underlying neurodegenerative disease states, including Parkinson's disease and Alzheimer's disease (Dutta et al., 2008, Glass et al., 2010, Qian et al., 2010). Cytokines such as interleukin IL-1 β , TNF α and IL-6 are produced by glia within the CNS, and are implicated in synaptic formation and scaling, long-term potentiation, and neurogenesis (Bilbo and Schwarz, 2009). Cytokine receptors are distributed throughout the brain and with high density in the hippocampus (Cunningham and De Souza, 1993, Parnet et al., 2002); therefore, the hippocampus may be particularly vulnerable to infection (inflammation) and important to the pathology of behavioral and cognitive disorders (Lynch et al., 2004, Bilbo et al., 2006). Results from the present study show that neonatal exposure to LPS results in damage to the hippocampus and to axons in the CA1 region in the adult rat brain and causes associated cognitive disorders support such a possibility.

The hippocampal formation is an important site to mediate processes associated with learning, memory, anxiety and fear (Spolidorio et al., 2007, Zarrindast et al., 2012). Recently, it has been proposed that inflammation has long-term consequences and could speculatively modify the risk of a variety of neurological disorders, including cerebral palsy, autism spectrum disorders, schizophrenia, multiple sclerosis, cognitive impairment, Parkinson disease, and Alzheimer's disease (Hagberg et al., 2002, Bilbo and Schwarz, 2009, Boksa, 2010, Williamson et al., 2011, Burd et al., 2012, Hagberg et al., 2012). In the present study, we have found that neonatal LPS exposure caused the persistent injury to the hippocampus with long-lasting learning deficits but not rearing of locomotion or memory. Our data reveal that neonatal LPS exposures cause long-lasting learning deficits mediated by a long-term activation of microglia in the hippocampus. Other related behaviors such as rearing of locomotion or memory seem partly affected by damage of the hippocampus during the development of the rats. In order to understand the effects of chronic inflammation on behavioral functions in the CNS, more studies including neuroendocrine or brain regional immune system should be investigated. It is interesting to study the interactions between the behaviors and brain damages induced by chronic inflammations by using our LPS animal model.

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Abbreviations

CNS	central nervous system
ELISA	enzyme-linked immunosorbent assay
IL1-β	interleukin 1- β
IL6	interleukin 6
LPS	lipopolysaccharide
MAP1	microtubule-associated protein 1
NeuN	neuron-specific nuclear protein
P5	postnatal day 5
TNFα	tumor necrosis factor- α

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]
- Antoniou K, Papathanasiou G, Panagis G, Nomikos GG, Hyphantis T, Papadopoulou-Daifoti Z. Individual responses to novelty predict qualitative differences in d-amphetamine-induced open field but not reward-related behaviors in rats. *Neuroscience*. 2004; 123:613–623. [PubMed: 14706774]
- Beale KE, Murphy KG, Harrison EK, Kerton AJ, Ghatei MA, Bloom SR, Smith KL. Accurate measurement of body weight and food intake in environmentally enriched male Wistar rats. *Obesity (Silver Spring)*. 2011; 19:1715–1721. [PubMed: 21233804]
- Bilbo SD. Early-life infection is a vulnerability factor for aging-related glial alterations and cognitive decline. *Neurobiol Learn Mem*. 2010; 94:57–64. [PubMed: 20388544]
- 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]
- Bilbo SD, Schwarz JM. Early-life programming of later-life brain and behavior: a critical role for the immune system. *Front Behav Neurosci*. 2009; 3:14.
- Bilbo SD, Yirmiya R, Amat J, Paul ED, Watkins LR, Maier SF. Bacterial infection early in life protects against stressor-induced depressive-like symptoms in adult rats. *Psychoneuroendocrinology*. 2008; 33:261–269. [PubMed: 18164556]
- Boksa P. Effects of prenatal infection on brain development and behavior: a review of findings from animal models. *Brain, Behavior, and Immunity*. 2010; 24:881–897.
- Burd I, Balakrishnan B, Kannan S. Models of fetal brain injury, intrauterine inflammation, and preterm birth. *Am J Reprod Immunol*. 2012; 67:287–294. [PubMed: 22380481]
- 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]
- Costa LG, Aschner M, Vitalone A, Syversen T, Soldin OP. Developmental neuropathology of environmental agents. *Annu Rev Pharmacol Toxicol*. 2004; 44:87–110. [PubMed: 14744240]
- Cunningham ET, De Souza EB. Interleukin 1 receptors in the brain and endocrine tissues. *Immunol Today*. 1993; 14:171–176. [PubMed: 8499077]

- Denenberg VH, Kim DS, Palmiter RD. The role of dopamine in learning, memory, and performance of a water escape task. *Behav Brain Res*. 2004; 148:73–78. [PubMed: 14684249]
- Dutta G, Zhang P, Liu B. The lipopolysaccharide Parkinson's disease animal model: mechanistic studies and drug discovery. *Fundam Clin Pharmacol*. 2008; 22:453–464. [PubMed: 18710400]
- Escobar M, Crouzin N, Cavalier M, Quentin J, Roussel J, Lante F, Batista-Novais AR, Cohen-Solal C, De Jesus Ferreira MC, Guiramand J, Barbanel G, Vignes M. Early, time-dependent disturbances of hippocampal synaptic transmission and plasticity after in utero immune challenge. *Biological Psychiatry*. 2011; 70:992–999. [PubMed: 21377655]
- 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]
- Fan LW, Tien LT, Lin RC, Simpson KL, Rhodes PG, Cai Z. Neonatal exposure to lipopolysaccharide enhances vulnerability of nigrostriatal dopaminergic neurons to rotenone neurotoxicity in later life. *Neurobiol Dis*. 2011a; 44:304–316. [PubMed: 21798348]
- Fan LW, Tien LT, Mitchell HJ, Rhodes PG, Cai Z. Alpha-phenyl-n-tert-butyl-nitrone ameliorates hippocampal injury and improves learning and memory in juvenile rats following neonatal exposure to lipopolysaccharide. *Eur J Neurosci*. 2008; 27:1475–1484. [PubMed: 18364024]
- Fan LW, Tien LT, Zheng B, Pang Y, Lin RC, Simpson KL, Ma T, Rhodes PG, Cai Z. Dopaminergic neuronal injury in the adult rat brain following neonatal exposure to lipopolysaccharide and the silent neurotoxicity. *Brain, Behavior, and Immunity*. 2011b; 25:286–297.
- Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms underlying inflammation in neurodegeneration. *Cell*. 2010; 140:918–934. [PubMed: 20303880]
- Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. *J Microsc*. 1987; 147:229–263. [PubMed: 3430576]
- Hagberg H, Gressens P, Mallard C. Inflammation during fetal and neonatal life: implications for neurologic and neuropsychiatric disease in children and adults. *Ann Neurol*. 2012; 71:444–457. [PubMed: 22334391]
- 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*. 2002; 8:30–38. [PubMed: 11921384]
- Harry GJ, Kraft AD. Microglia in the developing brain: a potential target with lifetime effects. *Neurotoxicology*. 2012; 33:191–206. [PubMed: 22322212]
- Hein AM, Zarcone TJ, Parfitt DB, Matousek SB, Carbonari DM, Olschowka JA, O'Banion MK. Behavioral, structural and molecular changes following long-term hippocampal IL-1 β overexpression in transgenic mice. *J Neuroimmune Pharmacol*. 2012; 7:145–155. [PubMed: 21748283]
- 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]
- Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci*. 1996; 19:312–318. [PubMed: 8843599]
- 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 β ? *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]
- Nishii K, Matsushita N, Sawada H, Sano H, Noda Y, Mamiya T, Nabeshima T, Nagatsu I, Hata T, Kiuchi K, Yoshizato H, Nakashima K, Nagatsu T, Kobayashi K. Motor and learning dysfunction during postnatal development in mice defective in dopamine neuronal transmission. *J Neurosci Res*. 1998; 54:450–464. [PubMed: 9822156]
- Olton DS. Shock-motivated avoidance and the analysis of behavior. *Psychol Bull*. 1973; 79:243–251. [PubMed: 4633560]

- 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, Bluthé 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]
- Pont-Lezica L, Bechade C, Belarif-Cantaut Y, Pascual O, Bessis A. Physiological roles of microglia during development. *J Neurochem*. 2011; 119:901–908. [PubMed: 21951310]
- Qian L, Flood PM, Hong JS. Neuroinflammation is a key player in Parkinson's disease and a prime target for therapy. *J Neural Transm*. 2010; 117:971–979. [PubMed: 20571837]
- Rodier PM. Correlations between prenatally-induced alterations in CNS cell populations and postnatal function. *Teratology*. 1977; 16:235–246. [PubMed: 73229]
- 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]
- Schwarz JM, Bilbo SD. Sex, glia, and development: Interactions in health and disease. *Horm Behav*. 2012; 62:243–253. [PubMed: 22387107]
- 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*. 2007; 195:183–192. [PubMed: 17661019]
- Volpe JJ. Cerebral white matter injury of the premature infant-more common than you think. *Pediatrics*. 2003; 112:176–180. [PubMed: 12837883]
- Williamson LL, Sholar PW, Mistry RS, Smith SH, Bilbo SD. Microglia and memory: modulation by early-life infection. *J Neurosci*. 2011; 31:15511–15521. [PubMed: 22031897]
- Zarrindast MR, Nasehi M, Pournaghshband M, Ghorbani-Yekta B. Dopaminergic system in CA1 modulates MK-801 induced anxiolytic-like responses. *Pharmacol Biochem Behav*. 2012

1. Neonatal LPS exposure results in long-lasting learning deficits in adult rats
2. Neonatal LPS exposure causes long-lasting anxiolytic responses in adult rats
3. Neonatal LPS exposure causes persistent hippocampal injuries in adult rats
4. Neonatal LPS exposure results in chronic inflammatory responses in adult rats

Fig. 1.

Procedures of behavior tests in three experimental groups after LPS injection on P5 rats.

Group 1: sixteen days after the injection (P21), rats were performed by open field, elevated plus-maze and passive avoidance (learning trial) tests. The memory trials were tested on P22, P50 and P71. Group 2: forty-four days after the injection (P49), rats were performed by open field, elevated plus-maze and passive avoidance (learning trial) tests. The memory trials were tested on P50 and P71. Group 3: sixty-five days after the injection (P70), rats were performed by open field, elevated plus-maze and passive avoidance (learning trial) tests. The memory trials were tested on P71.

Fig. 2.

LPS-induced the body weight change (A) and effects in locomotor activity, as determined by the open field test in rats, including total distance traveled (B) and rearing responses (C) in the rat. The results are expressed as the mean $\pm$ SEM of six animals in each group, and analyzed by two-way repeated measures ANOVA for data from tests conducted continuously at different postnatal days. * $P < 0.001$ represents significant difference for the LPS group as compared with the saline group on the same postnatal day.

Fig. 3. LPS-induced learning and memory deficit, as determined by passive avoidance, 16 (P21) (A), 44 (P49) days (B), and 65 days (P70) (C) after the injection. The results are shown as the number of electric foot shocks required to retain the rat on the safe board (left panel) and the retention latency to step down from the board on the next day (right panel). The results are expressed as the mean $\pm$ SEM of six animals in each group, and analyzed by Student's t-test (learning, left panel) or two-way repeated measures ANOVA for data from tests conducted continuously at different postnatal days (memory, right panel). * $P < 0.001$ represents significant difference for the LPS group as compared with the saline group on the same postnatal day.

A. P21 **B. P49** **C. P70** **Fig. 4.**

LPS-induced less anxiety-like behavioral, as determined by the elevated plus-maze test, 16 days (P21) (A), 44 days (P49) (B), and 65 days (P70) (C) after the injection. The results are shown as the numbers of open arm or enclosed arm entries (left panel) and the percentage of 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) (right panel). The results are expressed as the mean $\pm$ SEM of six animals in each group, and analyzed by Student's t-test. * $P < 0.001$ represents significant difference for the LPS group as compared with the saline group.

Fig. 5. Representative photomicrographs of Nissl staining in the rat brain 66 days (P71) after LPS injection. The forebrain sections at the bregma level (A, B) and diencephalon sections including hippocampus at a level 1/2 rostral from the lambda to the bregma (C, D) were used. Figs. A and C: Nissl stained brain sections show normal morphology from the control group. Figs. B and D: LPS exposure resulted in enlarged ventricles (B) and a decreases size of hippocampus (D) in LPS group. The scale bar shown in A represents 500 μm. Fig. E: stereological estimates of the volume of cerebrum and different regions were performed as described in materials and methods. The results are expressed as the mean ± SEM of six animals in each group, and analyzed by Student's t-test. * $P < 0.005$ represents significant difference for the LPS group as compared with the saline group.

Fig. 6.

Representative photomicrographs of Nissl (A, B) and NeuN staining (C, D) in the rat brain 66 days (P71) following LPS injection. LPS exposure induced a decrease in number of Nissl stained neurons in the hippocampal CA1 region at the middle dorsal hippocampus level (B), as compared with that in the control rat brain (A). NeuN staining revealed a similar reduction in the number of hippocampal CA1 neurons (D), as compared to that in the control rat brain (C). The scale bars shown in A represent 50 μ m. E: Quantitation of Nissl+ and NeuN+ cells were performed as described in materials and methods. The results are expressed as the mean $\pm$ SEM of six animals in each group, and analyzed by Student's t-test. * $P < 0.001$ represents significant difference for the LPS group as compared with the saline group.

Fig. 7.

Representative photomicrographs of MAP1 immunostaining in the P71 rat brain. LPS caused axonal impairment as indicated by the shorter MAP1 positive staining and less percentage of areas stained with MAP1 antibody within the hippocampal CA1 region at the middle dorsal hippocampus level (B), as compared with that in the control rat brain (A). The scale bar shown in A represents 50 μ m. Quantitation of the length of MAP1+ axon staining (C, left panel) and the percentage area of image that contained MAP1+ staining (C, right panel) in the hippocampal CA1 region were performed. Lengths (μ m) of all MAP1 positive staining in the CA1 region of the three brain sections at each of the two brain levels were measured, using the NIH image software and the average was calculated bilaterally for a single brain. The percentage area that contains MAP1 positive staining in the entire area of the captured image was measured using computer software. The results are expressed as the mean $\pm$ SEM of six animals in each group, and analyzed by Student's t-test. * $P < 0.001$ represents significant difference for the LPS group as compared with the saline group.

Fig. 8.

Representative photomicrographs of microglia (A and B) and LPS-stimulated increases in inflammatory cytokines (D) in the rat brain 66 days (P71) after LPS injection. As shown by OX42 immunostaining (A) in hippocampal CA1 region at the middle dorsal hippocampus level, a few microglia at the resting status with a small rod shaped soma and ramified processes were found in the hippocampal CA1 region of the control rat brain (the arrow shown in A). Numerous activated microglia (the arrow shown in B) with a round or elongate shaped cell body and blunt processes were observed in the hippocampal CA1 region of the rat brain with neonatal LPS exposure. The scale bar in A represents 50 μm . Quantitation of the number of OX42+ cells (C, left panel) and the percentage area of image that contained OX42 staining in the hippocampal CA1 region (C, right panel) were performed as described in materials and methods. The results are expressed as means $\pm$ SEM of six animals in each

group, and analyzed by Student's t-test. LPS exposure stimulated increases in an inflammatory cytokine (IL-1 β) in the hippocampal CA1 region of the rat brain (D). IL-1 β concentrations were determined by ELISA, as described in materials and methods. Data are presented as means $\pm$ SEM of six samples in the unit of pg/mg protein. * $P < 0.001$ represents significant difference for the LPS group as compared with the saline group.