Published in final edited form as:

Brain Behav Immun. 2011 February ; 25(2): 286–297. doi:10.1016/j.bbi.2010.09.020.

Dopaminergic neuronal injury in the adult rat brain following neonatal exposure to lipopolysaccharide and the silent neurotoxicity

Lir-Wan Fan^a, Lu-Tai Tien^b, Baoying Zheng^a, Yi Pang^a, Rick C. S. Lin^c, Kimberly L. Simpson^c, Tangeng Ma^d, Philip G. Rhodes^a, and Zhengwei Cai^{a,*}

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

^bSchool of Medicine, Fu-Jen Catholic University, Hsin-Chuang, Taipei County 24205, Taiwan

^cDepartments of Anatomy, Psychiatry & Human Behavior, University of Mississippi Medical Center, Jackson, MS 39216, USA

^dDepartment of Pharmacology & Toxicology, University of Mississippi Medical Center, Jackson, MS 39216, USA

Abstract

Our previous studies have shown that neonatal exposure to lipopolysaccharide (LPS) resulted in motor dysfunction and dopaminergic neuronal injury in the juvenile rat brain. To further examine whether neonatal LPS exposure has persisting effects in adult rats, motor behaviors were examined from postnatal day 7 (P7) to P70 and brain injury was determined in P70 rats following an intracerebral injection of LPS (1 mg/kg) in P5 Sprague-Dawley male rats. Although neonatal LPS exposure resulted in hyperactivity in locomotion and stereotyped tasks, and other disturbances of motor behaviors, the impaired motor functions were spontaneously recovered by P70. On the other hand, neonatal LPS-induced injury to the dopaminergic system such as the loss of dendrites and reduced tyrosine hydroxylase immunoreactivity in the substantia nigra persisted in P70 rats. Neonatal LPS exposure also resulted in sustained inflammatory responses in the P70 rat brain, as indicated by an increased number of activated microglia and elevation of interleukin-1 β and interleukin-6 content in the rat brain. In addition, when challenged with methamphetamine (METH, 0.5 mg/kg) subcutaneously, rats with neonatal LPS exposure had significantly increased responses in METH-induced locomotion and stereotypy behaviors as compared to those without LPS exposure. These results indicate that although neonatal LPS-induced neurobehavioral impairment is spontaneously recoverable, the LPS exposure-induced persistent injury to the dopaminergic system and the chronic inflammation may represent the existence of silent neurotoxicity. Our data further suggest that the compromised dendritic mitochondrial function might contribute, at least partially, to the silent neurotoxicity.

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*Corresponding author: Dr. Zhengwei Cai, Ph.D., 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@umc.edu (Z. Cai).

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Conflict of Interest Statement: All authors declare that there are no conflicts of interest.

Keywords

Lipopolysaccharide; Motor disturbances; Substantia nigra; Tyrosine hydroxylase; Interleukin-1 β ; Methamphetamine; dendritic mitochondria

1. Introduction

Perinatal exposure to an endotoxin, lipopolysaccharide (LPS), has been shown to increase the risk for dopaminergic disorders in animal models of Parkinson's disease, schizophrenia, autism and cerebral palsy (Boksa, 2010; Dutta et al., 2008; Feleder et al., 2010; Ling et al., 2002, 2004, 2006). In our previous studies, we developed a neonatal rat model of central exposure to LPS through an intracerebral (i.c.) injection in postnatal day 5 (P5) rats to study white matter injury associated with the infection/inflammation in immature brain (Cai et al., 2003; Fan et al., 2005a; Pang et al., 2003). White matter injury is a major cause of mortality and morbidity in preterm infants (Goldenberg et al., 2008) and the major cellular target of white matter injury in preterm infants has been found to be late oligodendrocyte progenitors, the predominant oligodendrocyte lineage in the brain of preterm infants at 23–32 gestation wk and also in P2-P7 rat brain (Back et al., 2002). Therefore, we choose to challenge the P5 rat brain with LPS. Unexpectedly, we found in this model that LPS exposure induced not only white matter injury around the ventricle areas as well as sensory, motor, emotional and cognitive impairment (Fan et al., 2005a, 2008c), but also dopaminergic neuronal injury in the substantia nigra (SN), suggesting possible involvement of dopaminergic impairment in this neonatal rat model of central inflammation-induced neurological dysfunction (Fan et al., 2005b, 2008b, 2008d). However, accumulating data have implicated that perinatal infection/inflammation-induced neurological impairments in animal models are not observable in the adult life unless other environmental insults or immune challenges occur in the adult (Bilbo et al., 2006; Fortier et al., 2004; Tenk et al., 2007). It remains unknown as to how perinatal exposure to LPS leads to an increased risk for development of dopaminergic system-related neurological disorders. Therefore, the first object of the present study was to investigate in our model whether neonatal LPS exposure-induced neurological dysfunction and injury to the dopaminergic system persist into adult life.

The concept of silent toxicity was originally proposed by Reuhl (1991) and defined as “persistent morphological and/or biochemical injury, which remains clinically unapparent unless unmasked by experimental or natural processes”. This concept has recently been introduced as the proposed underlying mechanism for adult diseases with a possible fetal origin (Godfrey and Barker, 2001; Costa et al., 2004), i.e. that exposure to environmental agents during development may cause damage or alteration in developmental programming and the resulting functional deficits may become apparent only later in life. In the current study, we found that neonatal LPS exposure-induced injury to the dopaminergic system and chronic central inflammation was persistent, while neurobehavioral impairments were spontaneously reversible and not manifested in the adult animal. It is unknown what the consequences of the persisting injury to the dopaminergic system and the chronic central inflammation are and whether they represent silent neurotoxicity, which may show apparent manifestations in later life. Methamphetamine (METH)-induced hyperactivity has been used to test functions of the dopaminergic system (Karila et al., 2010; Wakuda et al., 2008; Wallace et al., 1999). A lesion in the dopaminergic system, such as dysregulation of presynaptic striatal dopamine, alteration of central dopamine turnover, or dopaminergic receptor sensitization, was evidenced by enhanced behavioral reaction upon METH administration in the animals (Fortier et al., 2004; Karila et al., 2010; Wakuda et al., 2008; Wallace et al., 1999, 2001). The second objective of the present study was to determine

whether neonatal LPS exposure results in silent neurotoxicity in motor functions of the dopaminergic system in the adult rat brain, as tested by a METH challenge.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO., USA). Monoclonal mouse antibodies against microtubule-associated protein 1 (MAP1), MAP2, or neuron-specific nuclear protein (NeuN); and OX42 (CD11b) were purchased from Chemicon (Temecula, CA) and Serotec (Raleigh, NC), respectively. Polyclonal rabbit antibody against tyrosine hydroxylase (TH) was purchased from Chemicon (Temecula, CA., USA). ELISA kits for immunoassay of rat IL-1 β , IL-6 and TNF α were purchased from R&D Systems (Minneapolis, MN).

2.2. Animals

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 ($22 \pm 2^\circ\text{C}$). The day of birth was defined as postnatal day 0. 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.

2.3. Surgery procedures and animal treatment

In order to eliminate a possible gender difference, only male rats were used in the present study. Intracerebral injection of LPS to 5-day old Sprague-Dawley male rat pups was performed as previously described (Cai et al., 2003; Fan et al., 2005a; Pang et al., 2003). 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 scalp at the left hemisphere in a stereotaxic apparatus with a neonatal rat adapter. The injection was completed in 5 min and the needle was kept in this position for an additional 2 min and then retrieved slowly out of the brain. The wound was sutured and the pups were placed on a thermal blanket (34°C - 35°C) for recovery before being returned to their dams. The dose of LPS was chosen based on the previously reported results which produced reproducible brain injury (Cai et al., 2003; Fan et al., 2005a; Pang et al., 2003). 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.

Forty five rats from each group were used in the present study. Twelve rats in each group were used for behavioral tests from P7 to P70. These 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 were prepared from 6 rats for each group in a cryostat for immunohistochemistry staining. The remaining 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 total number of neurons in the substantia nigra (SN) and ventral tegmental

area (VTA). For determination of the content of pro-inflammatory cytokines or mitochondrial complex I activity in the whole brain or in the SN+VTA, six P6 rats and twelve P70 rats from each group were sacrificed by decapitation to collect fresh brain tissue on respective days. Three additional rats from each group were sacrificed for electron microscopic (EM) study on P70 as previously described (Fan et al., 2008a). The rest of twelve rats from the LPS- or saline-injected group were further divided into two groups: one received a subcutaneous (s.c.) injection of methamphetamine (METH) (0.5 mg/kg) (6 male rats) and the other with saline (6 male rats) on P70.

2.4. Behavioral testing

The behavioral tests were performed as previously described (Fan et al., 2005b, 2008a, 2008d, 2010) 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). Body weight of rat pups was recorded weekly. Behavioral tests including the locomotion (distance traveled and rearing), stereotypy, movement initiation test, vibrissa-elicited forelimb-placing test, pole test, and tapered/ledged beam walking test were used to determine motor functions. All animals were tested and video taped in the same order once a week from P7 to P70.

2.4.1. Locomotion and stereotypy—This test measures the activity and habituation response of animals on placement in a novel environment (Hermans et al., 1992; Tien et al., 2003). Locomotor activity was measured 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. 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). The stereotyped behaviors including: standing (on all four feet, essentially motionless and not actively sniffing), grooming (washing the face or any other part of its body with the forepaws, with the mouth generally in contact with the body), scratching (raising of hindpaw to touch any part of its body), head-swinging (standing on all four feet, moving its head from side to side), sniffing (not moving, but sniffing parts of the walls or floor of the apparatus) and freezing (standing on all four feet, freezing position, completely inactive, i.e. head oriented forwards and eyes fixed at a point of the upper side of the cage) were quantified during the first 5-min testing period. Quantification of stereotyped behaviors was achieved by counting the frequency of discrete episodes and the summation of all stereotyped responses during the testing period was scored for each rat (Antoniou et al., 1998, 2004; Gentry et al., 2004).

2.4.2. Vibrissa-elicited forelimb-placing test—This test is used to measure forelimb placing deficit upon stimulation of the rat's vibrissae to trigger a placing response (De Ryck et al., 1992; Schallert and Woodlee, 2005; Woodlee et al., 2005). Rats use their vibrissae to gain bilateral information about the proximal environment and this information is integrated between the hemispheres. In the cross-midline test of forelimb placing, the animal was gently held by its torso, but was turned sideways so that the vibrissae were perpendicular to the surface of the table. The downwardly oriented limb was gently restrained by the experimenter as the downwardly oriented vibrissae were brushed against a table edge once per trial for 10 trials. The percentage of trials in which the rat successfully places its forepaw onto the tabletop was 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. Damages to the nigrostriatal system lead to a decrease in the successful rate of placing in the contralesional limb in this test, consistent with parkinsonian akinesia.

2.4.3. Movement initiation test—Movement initiation for each forelimb was assessed to test the forelimb akinesia (Fleming et al., 2004; Schallert and Woodlee, 2005; Tillerson et al., 2002). The animal was held by its torso with its hindlimbs and one forelimb lifted above the surface of the table so that the weight of the animal's body was supported by one forelimb alone. The animal was allowed to initiate stepping movements in a 60 sec period for one forelimb and then the other in a balanced order. Bedding from the animal's homecage was placed on the table near the animal to encourage movement. Animals with nigrostriatal damage typically take longer time to initiate a movement compared to control animals. The time to initiate one step was recorded for each forelimb and initiation times for both forelimbs were averaged together to create one score.

2.4.4. 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: 3 cm, length: 50 cm) with a cork ball on its top (diameter: 7 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 animal was given three trials weekly and the time to turn round and to reach the platform at the bottom was measured performance latency (cut-off time: 60 sec).

2.4.5. Tapered/ledged beam walking test—Sensorimotor functions of forelimbs and hindlimbs were tested using a tapered/ledged beam (Schallert and Woodlee, 2005; Zhao et al., 2005). Foot-faults (slips) made with the hindlimbs can be measured as an index of hindlimb function. The tapered/ledged beam consists of the staging area (6×15 cm), trapezoid walking beam (6 and 1.5 cm wide and 135 cm long) and offloading area (1.5×15 cm). The entire apparatus was elevated to a height of 50 cm above the floor. The beam walking apparatus consisted of a tapered beam with underhanging ledges (2 cm wide, dropped 2 cm below the upper beam surface) on each side to permit foot faults without falling. Bedding from the animal's homecage was placed on the end of beam to encourage movement. A bright light was placed above the start point to motivate the rats to traverse the beam. The rats' performance was video taped and later analyzed by calculating the slip ratio of the forelimb and hindlimb (number of slips/number of total steps). The time spent on the beam for each animal that traversed the beam and joined their littermates was recorded. Steps onto the ledge were scored as a full slip and a half slip was given if the limb touched the side of the beam. The slip ratio for both hindlimbs were averaged together to create one score. The mean of three trials was used for statistical analyses.

2.4.6. Methamphetamine-induced locomotion—Locomotor activity was measured in the activity chamber as described above. On P70 experimental day, rats were placed in the chambers 1 hour before the administration of saline or METH to acclimatize them to their surroundings. Baseline levels of locomotion were determined during the 10 min prior to METH or saline administration (−10 to 0 min). After rats received saline or METH (0.5 mg/kg, s.c.), their activities were recorded for 1 hour. The total distance traveled by the animal was determined during every 10-min testing period for 1 hour. Rating of rearing events

(including exposure rearing and sniffing-air responses) and stereotyped behaviors (including standing, grooming, scratching, head-swinging, sniffing and freezing responses) were determined at the first minute of every 10-min intervals during 60 min after METH or saline administration. The results from six 10-min intervals were summed as one score.

2.5. Immunohistochemistry studies

Brain injury was estimated based on the results of Hematoxylin and Eosin (H&E) staining, Nissl staining and immunohistochemistry in brain sections prepared from rats sacrificed 65 days (P70) after the intracerebral injection.

For immunohistochemistry staining, primary antibodies were used in the following dilutions: MAP1, MAP2, or NeuN, 1:100; and OX42, or TH, 1:200. MAP1 provides selective staining of neuronal axons and dendrites. MAP2 provides selective staining of neuronal dendritic processes. TH was used to detect dopamine neurons in the SN and VTA. 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. When double-labeling was required, primary antibodies from different hosts were used in combination with appropriate secondary antibodies, which were against the immunoglobulin from the corresponding hosts. The resulting sections were examined under a fluorescent microscope at appropriate wavelengths.

2.6. 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 as previously described (Cai et al., 2003; Fan et al., 2005a; Pang et al., 2003). Briefly, SN +VTA and striatum tissues from each rat were collected 65 days after LPS injection (P70). 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. 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.

2.7. Brain sample preparation for electron microscopic study

Brain samples for the EM study were prepared following the procedure described previously (Fan et al., 2008a). Briefly, three P70 rats from each group were perfused with saline followed by 3.5% paraformaldehyde plus 0.5% glutaraldehyde in 0.1 M PBS. The brains were post-fixed in the same fixative overnight at 4°C. Brains were then cut coronally into 50–100 μ m sections with a vibratome (Lancer). A small area/block (~1 mm $\times$ 1 mm) in the region of the SN was dissected out with a No. 10 blade. Typically, two to three blocks were dissected from each brain. Each was processed using standard EM osmication with *en bloc* staining procedures. They were flat embedded in epon, attached to beam capsules, trimmed and cut into ultrathin sections. Tissue was collected onto grids coated with formvar, and then further stained with lead citrate and uranyl acetate. Materials were examined and randomly photographed with a Leo Biological transmission electron microscope by an investigator blind to the treatment. Typically, more than 40 photomicrographs were obtained from each case and then analyzed for their abnormal ultrastructural characteristics.

2.8. Determination of mitochondrial complex I activity

Complex I activity was determined by a spectrophotometric assay based on the quantification of the rate of oxidation of the complex I substrate NADH to ubiquinone as described by Champy et al. (2004) and Hoglinger et al. (2005) with modification. The whole brain (without the cerebellum) of the P6 rat or the SN+VTA area in the P70 rat brain was isolated, frozen in liquid nitrogen, and stored at -80°C . The frozen brain tissue was homogenized mechanically, sonicated on ice in 10 mM Tris-HCl (pH 7.2), containing 225 mM mannitol, 75 mM saccharose and 0.1 mM EDTA, and then centrifuged ($600 \times g$) for 20 min at 4°C , to obtain post-nuclear supernatants. The optical density of the supernatants (40 μg sample protein) in 1 ml of an assay mixture was spectrophotometrically recorded at a wavelength of 340 nm for 200 seconds at 37°C . The assay mixture was a potassium phosphate buffer (25 mM, pH 7.5) containing 2 mM potassium cyanide, 5 mM magnesium chloride, 2.5 mg/ml bovine serum albumin, 2 μM antimycin A, 100 μM decylubiquinone and 300 μM NADH. The proportion of NADH oxidation sensitive to an excess of rotenone (10 μM) was attributed to the complex I. This procedure minimizes the dissociation of rotenone from the complex I because of the use of small buffer volumes, maintenance at low temperatures and rapid analysis. The specific activity (nmol NADH oxidation/min/mg protein) of Complex I (NADH-ubiquinone oxidoreductase) was calculated using a molar extinction coefficient $\epsilon_{340\text{nm}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ (Chen et al., 2005). Enzyme activities were expressed as nmol/min/mg of brain tissue. {Complex I activity = [Rate (min^{-1}) / $\epsilon_{340\text{nm}}$ ($6.22 \text{ mM}^{-1} \text{ cm}^{-1}$)]/0.040 mg}

2.9. Estimate of the size of ventricle and the size of striatum

To compare the size of lateral ventricles and striatum, HE stained sections at the bregma level and 1/3 rostral from the lambda to the bregma were scanned by a densitometer (Bio-Rad, Hercules, CA) and areas of the left ventricle, right ventricle, striatum as well as that of the whole brain section were outlined and determined using NIH image software (Fan et al., 2005a). The ratio between the area of the left ventricle, right ventricle, or striatum and that of the whole brain section was calculated as the ventricle or striatum size index.

2.10. Quantification of immunostaining data and statistics

HE and Nissl staining as well as immunohistochemistry were performed in brain sections at the bregma level and the level 1/3 rostral from the lambda to the bregma. Three consecutive brain sections at the bregma level were taken for measurement of the ventricle or striatum size index and the average of measures was calculated for a single brain. OX42+ cell counting in the SN and VTA was performed in the midbrain sections at a level 1/3 rostral from the lambda to the bregma. Three digital microscopic images were randomly captured for each section at the parietal cortex (the bregma level, NeuN, MAP1, OX42 and Nissl), or at the SN and VTA (MAP2, and OX42). The number of positively stained cells in the three images was counted and averaged. Three sections at the bregma level or at the midbrain level were examined by an observer blind to the treatment and the mean value of cell counting or axonal length 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^2 (parietal cortex, SN and VTA). Lengths (μm) of all MAP1 positive staining in the parietal cortex of the three brain sections 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 larger. 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., 2010). This method has also 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). In addition to cell

counting, OX42 staining as well as MAP1 and MAP2 staining were quantified by this method in the present study, using the NIH image software.

The stereological estimates of the total number of TH+ or NeuN+ neurons (*est N*) were determined in the SN and VTA regions of the P70 rat brain following the methods described by Ling et al. (2006) and Lokkegaard et al. (2001). The nine equally spaced thick (40 μ m) sections in the midbrain level that were to be used in the analysis came from a one in six series. The numbers of TH+ or NeuN+ cells were counted in each of the nine sections, which cover the entire SN and VTA region. The Cavalieri principle (Gundersen and Jensen, 1987) was used to estimate the reference volumes, *est V(ref)*, and the volume density, *est N_V*. The product of the two is an estimate of total number of cells in this region: *est N* = *est V(ref)* $\times$ *est N_V*.

Quantified data from immunostaining, mitochondrial complex I activity, and ELISA were presented as the mean $\pm$ SEM and analyzed by Student's t-test. Results with a $p < 0.05$ were considered statistically significant. The 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 or by two-way ANOVA for METH-induced locomotion, followed by Student-Newman-Keuls test. Results with a $p < 0.05$ were considered statistically significant.

3. Results

3.1. Neonatal LPS exposure-induced neurobehavioral deficits were spontaneously recoverable

Neonatal LPS exposure resulted in decreases in body weight [$F(1, 220) = 33.518$, $p < 0.001$] (from P14 to P63, $p < 0.05$) (Fig. 1A) and several behavioral deficits in the rat. However, the reduction in body weight and the behavioral deficits were spontaneously reversible, and by P70 all of the tested behaviors in the LPS-injected group reached the level of the control group (Figs. 1B–1 I).

3.1.1 Locomotion and stereotypy—Neonatal LPS-treatment significantly increased the total crossing distance of an individual rat during a 10-min period in an open field (Fig. 1B) [$F(1, 220) = 45.707$, $p < 0.001$] on P14, P35 and P42. Neonatal LPS-treatment also increased the vertical activity from P14 to P49 or P56, as indicated by the increased number of rearing events (exposure rearing responses and sniffing-air responses) (Fig. 1C) [$F(1, 220) = 67.41$, $p < 0.001$] and the increased stereotype behaviors (Fig. 1D) [$F(1, 220) = 100.451$, $p < 0.001$], as compared to the saline-treated group. By P63–P70, no significant differences in locomotion and stereotype behaviors between the two groups were observed.

3.1.2. Vibrissa-elicited forelimb-placing test—All rats from the control group succeeded in vibrissa-elicited forelimb-placing test (100%) from P21 (Fig. 1E). The success rate of the vibrissa-elicited forelimb-placing test in the LPS-injected group was significantly lower than that in the control group [$F(1, 220) = 539.476$, $p < 0.001$] from P21 to P63 ($p < 0.001$) (Fig. 1E). This sensorimotor deficit in neonatal LPS-exposed rats was not present by P70.

3.1.3. Movement initiation test—Rats from the control group initiated stepping movements in 1–5 sec period for one forelimb in a balanced order (Fig. 1F) from P21. The neonatal LPS-exposed rats took longer time to initiate a movement [$F(1, 220) = 94.013$, $p < 0.001$] between P21 to P56 ($p < 0.001$) (Fig. 1F), indicating the forelimb akinesia (prolongation of the reaction time). However, the differences in movement initiation between the LPS-exposed and the control groups vanished by P63 to P70.

3.1.4. Pole test—By P35 all control rats succeeded in the pole test, but neonatal LPS-exposed rats performed with only a success rate of 8.3%. The performance latency in the pole test decreased with age [$F(10, 220) = 248.428, p < 0.001$] (Fig. 1G). The performance latency of pole test in the neonatal LPS-injected group was longer than that in the control group from P28 to P56 [$F(1, 220) = 157.767, p < 0.001$]. The LPS-induced bradykinesia (prolongation of the movement time) was recoverable and the performance latency of pole test in the LPS-exposed group reached the level of the control group by P63 (Fig. 1G).

3.1.5. Tapered/ledged beam walking test—All P21 and older rats from the control group succeeded in the tapered/ledged beam walking test in less than 10 sec period with no foot-faults (slips) made with the hindlimbs, measured as an index of hindlimb function (Fig. 1H). The neonatal LPS-exposed rats made more foot-faults (slips) with the hindlimbs [$F(1, 220) = 138.577, p < 0.001$] from P21 to P56 (Fig. 1H). The neonatal LPS-injected group also need longer time to complete the task (beam walking latency) than that in the control from P21 to P56 [$F(1, 220) = 132.398, p < 0.001$] P21 to P56 (Fig. 1I). No differences in the performance of this task were observed between the two groups by P63–P70.

3.2. Neonatal LPS exposure resulted in persistent ventricle enlargement and axonal and dendritic damage

Neonatal LPS administration caused persistent dilatation of bilateral ventricles [left, $t(10) = -13.012, p < 0.001$; right, $t(10) = -7.82, p < 0.001$] and the dilatation in the ipsilateral (the left) ventricle was more prominent than in the contralateral side in the P70 rat brain [$t(10) = -8.951, p < 0.001$] (Figs. 2B and 2C). LPS exposure also decreased the size of striatum at the bregma level, as indicated by the striatum size index (left, $7.53 \pm 0.13\%$; right, $8.63 \pm 0.23\%$), as compared with control group (left, $11.68 \pm 0.25\%$; right, $11.78 \pm 0.18\%$) [left, $t(10) = 14.911, p < 0.001$; right, $t(10) = 11.045, p < 0.001$]. Nissl Staining showed that neonatal LPS exposure did not change the density of parietal cortical neurons in the P70 rats [$t(10) = 1.839, p = 0.096, ns$] and this result was confirmed by NeuN immunostaining [$t(10) = -0.635, p = 0.54, ns$]. Neuronal axonal and dendritic processes were identified by MAP1 immunostaining. As shown in Fig. 2E, neonatal LPS exposure resulted in abnormal axon morphology (twisted and beaded appearance) in the parietal cortex at the bregma level of P70 rat brains, and significantly reduced axonal length [$t(10) = 39.635, p < 0.001$] and density [$t(10) = 6.586, p < 0.001$] (Figs. 2E and 2F, compared to the control Fig. 2D). Neonatal LPS exposure also reduced the dendritic density, as identified by MAP2 immunostaining, in both the SN [$t(10) = 6.757, p < 0.001$] and the VTA [$t(10) = 5.671, p < 0.001$] regions of the P70 rat brain (Figs. 2H and 2I, compared to the control Fig. 2G).

3.3. Neonatal LPS exposure induced loss of tyrosine hydroxylase-immunoreactive neurons

TH immunoreactive neurons were used to identify dopamine neurons in the SN and the VTA. In the P70 control rat brain, TH positive cells were more predominant in the compacta and the lateral regions of the SN (Fig. 3A) and VTA in the ventral midbrain. The total number of TH+ neurons (*est N*) was estimated in the rat substantia nigra using the stereological estimates as described in Methods. Neonatal LPS exposure significantly reduced the number of TH positive neurons in both the SN [$t(10) = 14.139, p < 0.001$] and the VTA [$t(10) = 18.023, p < 0.001$] regions of the rat brain (Figs. 3B and 3C).

Our previous studies indicated that neonatal LPS exposure reduces the number of TH positive neurons in these areas, but does not result in actual neuronal cell death in the P6 and P21 rat brain (Fan et al. 2008a, 2008b). Consistent with our data in the P6 and P21 rat brain, neonatal LPS treatment significantly reduced the number of TH+ neurons, but did not significantly alter the total number of NeuN+ cells in both the SN [$t(10) = 0.441, p = 0.669$,

ns] and the VTA [$t(10) = -0.427$, $p = 0.678$, ns] regions in the P70 rat brain (Figs. 3D–H). Therefore, the reduction in the number of TH positive cells is a consequence of phenotypic suppression of TH expression, rather than actual neuronal cell death. Thus, neonatal LPS exposure decreased the ratio between the number of TH+ cells and the number of NeuN+ cells in the SN [$t(10) = 11.200$, $p < 0.001$; from $84.2 \pm 0.4\%$ to $56.0 \pm 2.5\%$] and VTA [$t(10) = 9.627$, $p < 0.001$; from $70.9 \pm 1.9\%$ to $50.7 \pm 1.0\%$] of the rat brain (Fig. 3I).

3.4. Neonatal LPS exposure induced a sustained increase in microglial activation and inflammatory responses

Consistent with our previous data in the P21 rat brain (Fan et al., 2005b), neonatal LPS exposure resulted in a sustained increase in microglial activation in the P70 rat brain, as indicated by the number and morphology of the OX42 positive cells. 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 (Fig. 4A, red). A significantly increased number of OX42+ cells were found not only in the cingulum [$t(10) = -8.458$, $p < 0.001$] (155.6 ± 8.9 and 375.6 ± 24.4 cells/mm² for saline and LPS groups, respectively) and the cortex [$t(10) = -8.000$, $p < 0.001$] (80.0 ± 9.1 and 222.2 ± 15.3 cells/mm² for saline and LPS groups, respectively), but also in the SN [$t(10) = -10.614$, $p < 0.001$] (100.0 ± 11.8 and 273.3 ± 11.3 cells/mm² for saline and LPS groups, respectively) and the VTA of the LPS-exposed rat brain [$t(10) = -8.673$, $p < 0.001$] (111.1 ± 6.6 and 275.6 ± 17.8 cells/mm² for saline and LPS groups, respectively) (Figs. 4B–4C). 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. 4B) (Kreutzberg, 1996; Godoy et al., 2008). OX42 staining was also quantified by measuring the percentage area that contains OX42 immunostaining in the captured images. Higher percentage of OX42+ immunostaining area was observed in the SN [$t(10) = -13.291$, $p < 0.001$] ($1.98 \pm 0.17\%$ and $7.45 \pm 0.38\%$ for saline and LPS groups, respectively) and the VTA [$t(10) = -9.869$, $p < 0.001$] ($2.37 \pm 0.20\%$ and $7.91 \pm 0.52\%$ for saline and LPS groups, respectively) of the neonatal LPS-exposed rat brain (Fig. 4D). A comparable increase in percentage of OX42+ immunostaining area was also observed in the cingulum and the cortex areas of the neonatal LPS-exposed rat brain (data not shown).

Two major proinflammatory cytokines, IL-1 β and IL-6 were undetectable in the saline-injected rat brain, but were still significantly increased in both the SN+VTA [IL-1 β , $t(10) = -7.378$, $p < 0.001$; IL-6, $t(10) = -3.156$, $p = 0.01$] and the striatum [IL-1 β , $t(10) = -6.714$, $p < 0.001$; IL-6, $t(10) = -3.789$, $p = 0.004$] regions of the rat brain 65 days after LPS injection (Fig. 4E). However, TNF α was undetectable in the above regions of the P70 control or LPS-exposed rat brain (data not shown).

3.5. Neonatal LPS exposure resulted in an enhanced behavioral reaction upon stimulation with methamphetamine

The above results indicate that while neonatal LPS-exposure induced body weight loss and motor neurobehavioral deficits were spontaneously recoverable along with growth (Fig. 1), the resulting brain damage, especially to injury to the dopaminergic system, as well as the chronic inflammation persist (Figs. 2–4). To investigate potential consequences of the sustained injury on dopaminergic motor function, METH was administered to P70 rats. METH promotes the release of dopamine and induces increases in motor activity (Karila et al., 2010). Two-way ANOVA showed that adult (P70) METH treatment and the interaction of neonatal (P5) LPS treatment x adult (P70) METH treatment have significant effects on the total distance traveled [$F(1, 20) = 212.437$, $p < 0.001$; $F(1, 20) = 29.949$, $p < 0.001$, respectively, Fig. 5A], rearing responses [$F(1, 20) = 191.32$, $p < 0.001$; $F(1, 20) = 46.957$, $p < 0.001$, respectively, Fig. 5B], and stereotyped behaviors [$F(1, 20) = 278.472$, $p < 0.001$;

$F(1, 20) = 30.311, p < 0.001$, respectively, Fig. 5C]. Consequences of the persistent lesion in dopaminergic system (or its silent neurotoxicity) was evidenced by the enhanced behavioral reaction to METH, including distance traveled [$\Delta_{(\text{Saline+METH} - \text{Saline+Saline})} = 4517.3 \pm 386.9$ cm, $\Delta_{(\text{LPS+METH} - \text{LPS+Saline})} = 9949.1 \pm 516.8$ cm; $t(10) = -8.413, p < 0.001$, as indicated by the narrow bars in Fig. 5A], rearing responses [$\Delta_{(\text{Saline+METH} - \text{Saline+Saline})} = 18.3 \pm 1.5$ events, $\Delta_{(\text{LPS+METH} - \text{LPS+Saline})} = 54.3 \pm 1.7$ events; $t(10) = -15.247, p < 0.001$, narrow bars in Fig. 5B], and stereotyped behaviors [$\Delta_{(\text{Saline+METH} - \text{Saline+Saline})} = 21.8 \pm 1.0$ events, $\Delta_{(\text{LPS+METH} - \text{LPS+Saline})} = 43.3 \pm 1.5$ events; $t(10) = -11.542, p < 0.001$, narrow bars in Fig. 5C], between the control and the neonatal LPS-exposed groups.

3.6. Neonatal LPS exposure induced dendritic mitochondrial damage and reductions in mitochondrial complex I activity

To investigate possible mechanisms involved in the persistent consequences of neonatal LPS exposure, we examined the ultra structures of dendrites and mitochondrial complex I activity in the substantia nigra area. Those nigra dendrites were examined using an electron microscopy. The typical dendritic morphology containing cytoplasm and organelles such as mitochondria (pointed by an arrow) from a saline-treated animal is shown in Fig 6A. In contrast, a rather large dendrite from a LPS-treated animal exhibited vacant contents including lack of normal mitochondria profiles (Fig 6B). The arrow in Fig 6B points to those organelles exhibiting membrane debris of the degenerating process. The astro in Fig 6B points an adjacent dendrite revealing rather normal mitochondria ultrastructures, suggesting that not all dendrites are degenerating.

Mitochondrial complex I activity is given as the amount of NADH oxidized per minute per milligram protein in homogenates of the whole brain of P6 rat and the SN+VTA areas in the P70 rat brain. Neonatal LPS exposure resulted in a decrease in mitochondrial complex I activity in the P6 rat brain [$t(10) = 4.289, p = 0.002$], and this decrease persisted in the SN +VTA area of the P70 rat brain [$t(10) = 3.266, p = 0.008$] (Fig. 6C).

4. Discussion

Perinatal exposure to an endotoxin, LPS, has been shown to increase the risk for dopaminergic system injury in animal models and is associated with the subsequent development of sensory, motor and cognitive disturbances in adulthood (Bakos et al., 2004; Boksa et al., 2010; Breivik et al., 2002; Dutta et al., 2008; Feleder et al., 2010; Ling et al., 2002, 2004, 2006). Our previous studies showed that neonatal LPS exposure in P5 rat brain, somewhat equivalent to the third trimester human fetus in terms of brain development (Craig et al., 2003; Feleder et al., 2010), caused white matter injury, neurological deficits, alterations in dopaminergic neurons, as well as motor disturbance in juvenile rats (P21) (Fan et al., 2005b, 2008a, 2008d). Data from the present study demonstrate that neonatal LPS exposure-induced deficits in locomotion, stereotype tasks, and other motor behaviors (Fig. 1) may last a long period. However, along with development all of the tested behaviors in the LPS-injected group reached the level of the control group by P70, indicating that perinatal LPS exposure-induced motor dysfunction may spontaneously recover. The exact mechanism for the spontaneous recovery of motor function is still unknown at the present time. One of the possible causes might be that inflammation probably not only initiates damage but also activates endogenous repair mechanism, such as release of neurotrophin factors (Malaeb and Dammann, 2009). Nevertheless, these observations are consistent with studies reported by other investigators who reported that perinatal infection/inflammation-induced disturbance of motor behaviors, including locomotion, are not observed in the young adults (P70) (Bilbo et al., 2006; Fortier et al., 2004; Tenk et al., 2007).

On the other hand, results from the present study show that brain injuries resulted from neonatal LPS exposure, including enlargement of bilateral ventricles, alterations in axons and dendrites in the parietal cortex and the SN as well as loss of TH immunoreactivity in dopamine neurons in the SN and VTA areas persist in P70 rats (Figs. 2~3). Increased ventricle volume has been observed in many pathologic conditions such as hydrocephalus and periventricular leukomalacia (McCrea and Ment, 2008). Increased cranial pressure or loss of surrounding tissues and reduced tension could be two possible reasons for this observation. Experimentally induced hydrocephalus by injection of kaolin into the cisterna magna has been shown to cause white matter loss and motor and cognitive dysfunction in rats (Del Bigio et al., 1997;2003), but a shunting procedure prevented the behavioral dysfunction and ventricle dilation (Del Bigio et al., 1997). Spontaneous recovery of LPS-induced neurobehavioral dysfunction was observed in P70 rats, which still had enlarged ventricles in the present study, indicating ventricle dilatation may not have direct connections with motor dysfunction observed in our study. As shown in Fig 2B, the dilated ventricles in the LPS-treated rat brain were still surrounded entirely by ependymal cells, although the ependymal layer was thinner than in the control brain. There was no apparent tissue damage area surrounding the ventricles. This might be an indication of the injury inside the ventricles. At present, the exact mechanisms leading to LPS-induced ventricle enlargement remain to be determined. Ling et al. (2002,2004,2006) reported that animals exposed to LPS prenatally (E10.5) are born with fewer than normal dopamine neurons and sustain fewer in adulthood. However, our data show that the number of NeuN positive cells in the SN and VTA regions (most neurons in these areas are normally TH+ neurons) remains unchanged in the LPS-exposed rat (Figs. 3E-F) suggesting that neonatal LPS exposure on P5 caused phenotypic suppression of TH expression rather than actual neuronal cell loss in the P70 rat brain. The present study also shows a sustained decrease in MAP2+ staining in the SN, an indicator of the damage of dopaminergic dendrites. The rat dopaminergic system develops both pre- and post-natally, with the processes of mitosis and phenotype specification of dopaminergic neurons occurring prenatally while its receptor development and brain growth spurt occurring predominantly in the postnatal period (Burke, 2003;Ries et al., 2009). It is likely that exposure to LPS in the rat at different stages of dopaminergic development may contribute to the different pattern of dopaminergic system injury seen in the present study as compared to that reported by Ling et al. (2002,2004,2006).

Another sustained alteration in the rat brain following neonatal LPS exposure in the present study was the persistent inflammatory response, as indicated by the sustained activation of microglia and the increased expression of inflammatory cytokines, IL-1 β and IL-6 in the P70 rat brain (Fig. 4). We have reported previously that following neonatal LPS exposure similar inflammatory responses were observed in neonatal (P6) or juvenile (P21) rats and these inflammatory responses were associated with neurological dysfunction in these animals (Cai et al., 2003;Fan et al., 2005a;2008c;Pang et al., 2003). Data from the current study indicate that these inflammatory responses still exist even 65 days after the LPS exposure. Microglia, the major resident immune cells in the brain, 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). In the present study, activated microglia were detected in the SN region, an area far away from the injection site of LPS (the cygulum). An interesting question is what the source of the increased microglia is. The increased number of activated microglia in the SN region could be a result of migration of microglia from the surrounding areas or even outside the CNS, or a result of increased activation of local microglia through increased proliferation following diffusion of LPS or other mediators (such as inflammatory cytokines) to this region from the injection site. Since comparable increases in the number of OX42+ cells were also found in the cingulum and the cortex and increased contents of IL-1 β and IL-6 were detected in the SN area of the P70 rat

brain, both possibilities exist. To answer this question precisely, more investigations are needed. 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). It is also known that the SN is vulnerable to immune-related alterations and is important to the pathology of behavioral and motor disorders (Dutta et al., 2008;Glass et al., 2010;Ling et al., 2006;Qian et al., 2010). In the current study, although the sustained inflammatory responses could be accompanying events associated with persistent brain injury, data from the present study indicate that neonatal LPS exposure-induced dopaminergic injury and chronic inflammation in the adult brain are apparently not associated with any motor impairment tested during later life. What then are the potential consequences of the persistent dopaminergic injury and chronic inflammation in the adult brain? Neurobehavioral responses to the challenge of METH in these adult animals may have provided an appropriate answer to this question. METH is known to increase locomotor activity via enhancement of dopamine release and therefore, METH-induced locomotion test is used to assess the dopaminergic function as a behavioral indicator of activity in the dopamine pathway (Karila et al., 2010;Wakuda et al., 2008;Wallace et al., 1999). In the current study, the LPS-exposed rats showed significantly increased METH-induced locomotion and stereotypy compared to that of the control rat (Fig. 5), suggesting that the dopaminergic system in the LPS-exposed rat brain is more sensitive to METH stimulation. Enhanced locomotion responses upon METH stimulation have been shown to be indicators of dopaminergic lesions, such as dysregulation of presynaptic striatal dopamine, alteration of central dopamine turnover, or dopaminergic receptor sensitization (Fortier et al., 2004;Karila et al., 2010;Wakuda et al., 2008;Wallace et al., 1999,2001). Therefore, the enhanced METH-induced reaction in the LPS-exposed animal observed in the current study suggests the existence of functional alterations in the dopaminergic system of the LPS-exposed rat brain, although they do not show motor abnormality for the time-being. It is likely that the persisting dopaminergic injury and inflammatory conditions observed in the neonatal LPS-exposed rat brain in the current study represent a silent toxic status, i.e. although individuals with these persisting injuries may not have apparent motor dysfunction, they may manifest more severe dopaminergic lesions and motor dysfunction upon stimulation in later life. Such a silent toxic status has been hypothesized to be responsible for the enhanced adult susceptibility to environmental factors known to be involved in the induction of neurodegenerative disorders and/or alterations of the normal aging process (Bilbo et al., 2006;Fortier et al., 2004;Tenk et al., 2007).

The mechanisms that are responsible for the "silent neurotoxicity" are unknown at the present time. Although validation is needed, our data suggest one of the possibilities. MAP2 staining indicates that neonatal LPS exposure resulted in a sustained dendritic injury in the SN region (Fig. 2). To further address this issue, we examined the ultrastructures of dendrites in this area. Our EM study showed some dendrites are rather vacant and containing membrane debris from the LPS-treated rat brain while dendrites with normal appearance of mitochondria are also present in the adjacent area (Fig. 6B). The significantly reduced mitochondrial complex I activity (Fig. 6C) supports the notion that dendritic mitochondrial function in the LPS-treated rat brain is partially impaired. It has been shown that normal neurobehavioral performance depends directly on the degree of dendritic arborization and synaptic connectivity (Ba and Seri, 1995). In the absence of further stimulation, behaviors of LPS-treated animals with partially damaged SN dendritic mitochondrial function may appear to be normal. When challenged with METH, the already injured dendritic mitochondria become incapable to deal with such additional stress and the dopaminergic system could be further injured, thus leading to abnormal motor responses. In brief, the compromised dendritic mitochondrial function in the LPS-exposed rat brain may contribute, at least partially, to mechanisms that are responsible for the silent toxicity.

Acknowledgments

This work was supported by funds from the NIH (HD 35496 and NS 54278), Newborn Medicine Funds and a research grant from the Department of Pediatrics, UMC.

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Fig. 1.

Effects of neonatal LPS-exposure on growth and motor behaviors from P7 to P70 rats. A, the body weight change. B~D, locomotor activity and stereotyped behaviors, as determined in the open field test, including total distance traveled (B), rearing responses (C), and stereotyped behaviors (D). E, the vibrissa-elicited forelimb-placing test. F, the movement initiation test. G, the pole test. H~I, the tapered/ledged beam walking test as determined by slip step ratio (H) and performance latency (I). The results are expressed as the mean $\pm$ SEM of twelve animals in each group, and analyzed by two-way repeated measures ANOVA for data from tests conducted continuously at different postnatal days. * $P < 0.05$ represents significant difference for the LPS group as compared with the saline group on the same postnatal day.

Fig. 2.

Representative photomicrographs of HE staining (A and B), MAP1 immunostaining (D and E), and MAP2 immunostaining (G and H) in rat brain sections prepared 65 days (P70) after LPS or saline injection. The forebrain sections at the bregma level (A, B, D and E) and the midbrain sections at a level 1/3 rostral from the lambda to the bregma (G and H) were used. HE stained brain sections from the control group show normal morphology (A). LPS exposure resulted in enlarged ventricles and the dilatation in the ipsilateral (the left as shown in B) ventricle was more prominent in the LPS group (B). LPS caused axonal and dendritic impairment as indicated by the short beaded MAP1 positive staining in the cortex at the bregma level (E) as compared to that in the control rat brain (D). LPS also caused dendritic impairment as indicated by the loss of MAP2 immunostaining (measured by the percentage area of image that contained MAP2 immunoreactivity in the SN (H) and VTA). The scale bar shown in A represents 500 μ m for A and B. The scale bars shown in D represents 50 μ m for D, E, G, and H. Quantitation of the ventricle size index (C), MAP1+ axon and dendrite staining (F), and MAP2+ dendrite staining (I) were performed as described in Methods. The results are expressed as the mean $\pm$ SEM of six animals in each group, and analyzed by Student's t-test. * $P < 0.05$ represents significant difference for the LPS group as compared with the saline group. # $P < 0.05$ represents significant difference for the left ventricle as compared with the right ventricle in the LPS group.

Fig. 3.

Representative photomicrographs of TH (A and B), NeuN (D and E), and NeuN double staining with TH antibody (G and H) in the rat brain 65 days (P70) after LPS injection. TH and NeuN positive staining was detected in the SN and VTA areas of the midbrain sections at a level 1/3 rostral from the lambda to the bregma in the rat brain. LPS exposure reduced the number of TH positive neurons in the SN (B) and the VTA (C), but did not change the number of NeuN neurons in the SN (E) and VTA as compared to that in the control rat brain (D). Double labeling with TH and NeuN antibodies (G and H) shows that LPS exposure caused a reduction in TH positive cells (red), but not in the number of NeuN (green) positive cells (H) (yellow color shown the merged color of red and green). The scale bars shown in A represents 200 μm for A and B. The scale bar shown in D represents 50 μm for D, E, G and H. Stereological estimates of the number of TH+ cells (C), number of NeuN+ cells (F), and the ratio between TH+ cells and NeuN+ cells (I) in the SN and the VTA areas were performed as described in Methods. The results are expressed as the mean $\pm$ SEM of six animals in each group, and analyzed by Student's t-test. * $P<0.05$ represents significant difference for the LPS group as compared with the saline group.

Fig. 4.

Representative photomicrographs of microglia (A and B) and LPS-stimulated increases in inflammatory cytokines (E) in the rat brain 65 days (P70) after LPS injection. As shown by OX42 immunostaining (A, red) in SN, a few microglia at the resting status with a small rod shaped soma and ramified processes were found in the SN of the control rat brain (a magnified image of the arrow indicated OX42+ cell is shown in the insert in A). Numerous activated microglia (a magnified image of the arrow indicated OX42+ cell is shown in the insert in B) with a round or elongate shaped cell body and blunt processes were observed in the SN of the rat brain with neonatal LPS exposure, where significantly reduced number of TH+ cells (green) was observed. The scale bar in A represents 50 μ m for A and B.

Quantitation of the number of OX42+ cells (C) and the percentage area of image that contained OX42 staining (D) in the SN and the VTA were performed as described in Methods. The results are expressed as the mean $\pm$ SEM of six animals in each group, and analyzed by Student's t-test. LPS exposure stimulated increases in inflammatory cytokines (IL-1 β and IL-6) in the SN+VTA and the striatum areas of the rat brain (E). IL-1 β and IL-6 concentrations were determined by ELISA, as described in Methods. Data are presented as the mean $\pm$ SEM of six samples in the unit of pg/mg protein. * P<0.05 represents significant difference for the LPS group as compared with the saline group.

Fig. 5. Neonatal LPS-exposure resulted in enhanced METH-induced locomotor activity and stereotyped behaviors, as determined by the open field test, including total distance traveled (A), rearing responses (B), and stereotyped behaviors (C) in the rat. The results are expressed as the mean $\pm$ SEM of six animals in each group and analyzed by two-way ANOVA. * $P < 0.05$ represents significant difference for the saline+METH group as compared with the saline+saline group. # $P < 0.05$ represents significant difference for the LPS+METH group as compared with the LPS+saline group. The narrow bars represent the increment in METH-stimulated locomotor activity within the saline- or the LPS-exposed group. & $P < 0.05$ represents significant difference between the increment in METH-increased

locomotor activity within the LPS group as compared to that within the saline-injected group.

Fig. 6.

Representative examples of electron photomicrographs of nigra dendrites (A&B), as well as the enzymatic activity of mitochondrial complex I in the rat brain at P70 (C). The typical nigra dendritic morphology containing cytoplasm and organelles such as mitochondria (pointed by an arrow) from a saline-treated animal is shown in Fig 6A. Fig 6B shows a rather large dendrite with vacant contents including lack of normal mitochondria profiles from a LPS-treated animal. The organelles within the dendrite exhibit membrane debris of the degenerating process (an arrow pointed in 6B). However, an adjacent dendrite still shows rather normal mitochondria ultrastructures (the astro pointed in 6B). The scale bar shown in A represents 1 μm for both A and B. Neonatal LPS exposure reduced the

enzymatic activity of mitochondrial complex I in the whole brain homogenates from P6 rats (left panel of 6C) and in the SN+VTA homogenates from P70 rats (right panel of 6C). The results are expressed as the mean $\pm$ SEM of six animals from each group, and analyzed by Student's t-test. * P<0.05 represents significant difference for the LPS group as compared with the saline group.