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Melatonin ameliorates brain injury induced by systemic lipopolysaccharide in neonatal rats

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Abstract

Our previous study showed that lipopolysaccharide (LPS)-induced brain injury in the neonatal rat is associated with nitrosative and oxidative stress. The present study was conducted to examine whether melatonin, an endogenous molecule with antioxidant properties, reduces systemic LPS-induced nitrosative and oxidative damage in the neonatal rat brain. Intraperitoneal (i.p.) injection of LPS (2 mg/kg) was administered to Sprague–Dawley rat pups on postnatal day 5 (P5), and i.p. administration of melatonin (20 mg/kg) or vehicle was performed 5 minutes after LPS injection. Sensorimotor behavioral tests were performed 24 h after LPS exposure, and brain injury was examined after these tests. The results show that systemic LPS exposure resulted in impaired sensorimotor behavioral performance, and acute brain injury, as indicated by the loss of oligodendrocyte immunoreactivity and a decrease in mitochondrial activity in the neonatal rat brain. Melatonin treatment significantly reduced LPS-induced neurobehavioral disturbances and brain damage in neonatal rats. The neuroprotective effect of melatonin was associated with attenuation of LPS-induced nitrosative and oxidative stress, as indicated by the decreased nitrotyrosine- and 4-hydroxynonenal-positive staining in the brain following melatonin and LPS exposure in neonatal rats. Further, melatonin significantly attenuated LPS-induced increases in the number of activated microglia in the neonatal rat brain. The protection provided by melatonin was also associated with a reduced number of inducible nitric oxide synthase (iNOS)+ cells, which were double-labeled with ED1 (microglia). Our results show that melatonin prevents the brain

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injury and neurobehavioral disturbances induced by systemic LPS exposure in neonatal rats, and its neuroprotective effects are associated with its impact on nitrosative and oxidative stress.

Keywords

melatonin; lipopolysaccharide; nitrosative and oxidative damage; mitochondria; microglia

Introduction

Periventricular leukomalacia (PVL) is a white matter disease of premature infants that is frequently related with the destruction of immature oligodendroglia and axons (Back et al., 2001, Pleasure et al., 2006). Increasing evidence indicates that perinatal infection or inflammation and hypoxia-ischemia are major contributors to PVL and result in the subsequent development of impaired neurological outcomes (Harden et al., 1996, Rezaie and Dean, 2002, Volpe, 2003). Our previous studies have shown that neonatal exposure (postnatal day 5, P5) to lipopolysaccharide (LPS) through an intracerebral (i.c.) injection results in brain inflammation and white matter and neuronal injury in rats, which was closely associated with the increased nitrosative and oxidative stress following LPS exposure (Fan et al., 2008b, Fan et al., 2008c). The inflammatory response that ensues because of the initial occult exogenous oxidative/nitrosative stress becomes a secondary endogenous source of reactive oxygen species (ROS) and reactive nitrogen species (RNS). A previous study also showed that nitrosative and oxidative injury to premyelinating oligodendrocytes (OLs) was present in the infant brain with PVL (Haynes et al., 2003).

Melatonin (*N*-acetyl-5-methoxytryptamine) has been reported to have neuroprotective actions through its ability to detoxify species mediating oxidative and nitrosative damage (ROS and RNS) and to prevent activation of the pro-inflammatory enzymes, inducible nitric oxide synthase (iNOS), and cyclooxygenenase-2 (COX-2) (Brzozowska et al., 2009, Esposito and Cuzzocrea, 2010). Clinically, melatonin has been used in pediatrics to treat respiratory distress syndrome, bronchopulmonary dysplasia, seizure disorders and to reduce oxidative stress in sepsis and asphyxia (Gitto et al., 2011, Sanchez-Barcelo et al., 2011, Aversa et al., 2012, Chen et al., 2012). Thus, melatonin may potentially protect against brain injury induced by infection or inflammation, by attenuating the induction of nitrosative and oxidative stress in neonates. However, the effect of melatonin on brain damage induced by proinflammatory factors in neonates has not yet been tested.

Our previous study showed that intraperitoneal (i.p.) LPS exposure, which is more clinically relevant, causes pathological brain damage in the cortex, hippocampus, and striatum of neonatal rats; this damage is comparable to that caused by its intracerebral (i.c.) injection (Cai et al., 2013). In the present study, we further investigated whether systemic LPS exposure causes brain injury through activation of brain oxidative and/or nitrosative stress damage. Further, the effect of melatonin on LPS-induced nitrosative and oxidative damage was studied in the neonatal rat brain.

Experimental Procedures

Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO, U.S.A.).

Mouse monoclonal antibodies against late oligodendrocyte (OL) progenitor cell marker O4 (O4) or α-amyloid precursor protein (APP) and ED1 were purchased from Millipore (Billerica, MA, U.S.A.) and Serotec (Raleigh, NC, U.S.A.), respectively. Rabbit polyclonal antibodies against nitrotyrosine (NT), 4-hydroxynonenal (4-HNE), or inducible nitric oxide synthase (iNOS) and ionized calcium binding adapter molecule 1 (Iba1) were obtained from Chemicon, Alexis (San Diego, CA, U.S.A.) and Wako Chemicals USA (Irvine, CA, U.S.A.), respectively. The enzyme-linked immunosorbent assay (ELISA) kit for immunoassay of rat interleukin-1β (IL-1β) was purchased from R&D Systems (Minneapolis, MN, U.S.A.).

Animals

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 ± 2 °C). The day of birth was defined as postnatal day 0 (P0). After birth, the litter size was adjusted to 12 pups per litter to minimize the effect of litter size on body weight and brain size. All procedures for animal care were conducted in accordance with the National Institutes of Health (NIH) 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.

Animal treatment

An i.p. injection of LPS (2 mg/kg, from *Escherichia coli*, serotype 055: B5) was administered to 5-day-old (P5) Sprague–Dawley rat pups of both sexes. The control rats were injected with the same volume of sterile saline (0.1 mL). All animals survived the injection. Both LPS- and saline-injected animals were further divided into two groups: one received i.p. injections of melatonin and the other received vehicle. Melatonin (20 mg/kg) was dissolved in 5% ethanol in saline (Ozdemir et al., 2007, Olivier et al., 2009) and administered 5 minutes after LPS injection. A total of 72 rats from six litters were used in the present study. One pup from each litter was assigned to each of 12 groups, so there were six pups in each group (n = 6). Behavioral tests were performed on P6. After the behavioral tests, two-thirds of the rats were killed by decapitation to collect fresh brain tissue, which was used for mitochondrial complex I activity and ELISA assays. The remaining rats were killed by transcardiac perfusion with normal saline followed by 4% paraformaldehyde treatment for brain section preparation. Free-floating 40-μm–thick coronal brain sections were prepared using a freezing microtome (Leica, SM 2000R, Wetzlar, Germany) for immunohistochemistry.

Behavioral testing

Behavioral tests were performed as described previously with certain modifications (Fan et al., 2008a, Fan et al., 2013). The developmental test battery was based on previously well-established tests for neurobehavioral toxicity (Altman et al., 1971, Hermans et al., 1992). The righting reflex and wire-hanging maneuver were performed by all rat pups at P6.

Righting reflex

This test is believed to reflect muscle strength and subcortical maturation (Altman et al., 1971, Hermans et al., 1992). Pups were placed on their backs, and the time required to turn over onto all four feet and touch the platform was measured. The cut-off time was 60 sec.

Wire-hanging maneuver

This maneuver tests neuromuscular and locomotor development (Altman and Sudarshan, 1975, Hermans et al., 1992). Pups are suspended by their forelimbs from a horizontal rod (5 × 5 mm^2 in cross-section, 35-cm long, between two 50-cm–high poles). A sawdust-filled box at the base served as protection for the falling pups. Suspension latencies were recorded, and the cutoff time was 120 s.

Immunohistochemistry

Brain injury was estimated based on the results of immunohistochemistry performed on consecutive brain sections of rats killed 1 day (P6) after LPS injection. For immunohistochemical staining, primary antibodies were used at the following dilutions: O4, 1 μg/mL; 4-HNE or Iba1, 1:500; ED1, iNOS, 1:200; and APP or NT, 1:100. O4 was used to detect late OL progenitor cells in the white matter. Upregulation of APP was used as a marker of axonal injury. Positive staining for NT or 4-HNE was used as a marker of products of RNS or lipid peroxidation, respectively. Microglia were detected using Iba1 immunostaining, which recognizes both resting and activated microglia, and by ED1 immunostaining, which detects activated microglia or macrophages. Sections were incubated with primary antibodies at 4 °C overnight and then with secondary antibodies conjugated with fluorescent dyes (Alexa Fluor® 555, 1:500 or Alexa Fluor® 488, 1:200; Invitrogen, Carlsbad, CA, U.S.A.) for 1 h in the dark at room temperature. 4′, 6-Diamidino-2-phenylindole (DAPI) (100 ng/mL) was used to visualize nuclei. Sections incubated in the absence of primary antibody were used as negative controls. When double-labeling was performed, primary antibodies from different hosts were used in combination with secondary antibodies against the immunoglobulin from the corresponding hosts. The resulting sections were examined under a fluorescent microscope (Olympus, BX60) at appropriate wavelengths.

Determination of mitochondrial complex I activity

Complex I activity was determined using a spectrophotometric assay based on the quantification of the rate of oxidation of the complex I substrate nicotinamide adenine dinucleotide (NADH) to ubiquinone as described in previous studies (Champy et al., 2004, Hoglinger et al., 2005), with minor modifications. Brain tissue from each pup was collected 6 or 24 h after LPS injection. Frozen brain tissue was homogenized mechanically, sonicated

on ice in 10 mM Tris-HCl buffer (pH 7.2) containing 225 mM mannitol, 75 mM saccharose, and 0.1 mM ethylenediaminetetraacetic acid (EDTA), and then centrifuged (600 × *g*) for 20 min at 4 °C to obtain postnuclear supernatants. The optical density of the supernatants (40 μg of sample protein in 1 mL assay mixture) was spectrophotometrically recorded at a wavelength of 340 nm for 200 s 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 complex I activity. This procedure minimizes the dissociation of rotenone from complex I by the use of small buffer volumes, low temperatures, and rapid analysis. The specific activity (nmol NADH oxidized min^{-1} mg^{-1} protein) of complex I (NADH-ubiquinone oxidoreductase) was calculated using a molar extinction coefficient $\varepsilon_{340\ nm}$ = 6.22 mM^{-1} cm^{-1} (Chen et al., 2005). Enzyme activities were expressed as nmol/min/mg of brain tissue. Complex I activity was calculated as follows: Complex I activity = [Rate (min^{-1})/ε_{340nm} (6.22 mM^{-1} cm^{-1})]/0.040 mg.

Determination of IL-1β protein level by ELISA

The level of proinflammatory cytokine IL-1β was assayed by ELISA, as previously described (Cai et al., 2013, Fan et al., 2013, Kaizaki et al., 2013). Briefly, brain tissue from each pup was collected 24 h after LPS injection, based on a previous report that the LPS-stimulated increase in inflammatory cytokines in the rat brain reaches a peak value at that time point (Pang et al., 2003). Brain tissues were homogenized by sonication in 1 mL ice-cold PBS (pH 7.2) and centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatant was collected, and the protein concentration was determined by the Bradford method. ELISA was performed according to the manufacturer's instructions, and data were acquired using a 96-well plate reader (Bio-Tek instruments, Inc., VT, U.S.A.). The cytokine contents were expressed as picogram cytokines per milligram of protein.

Quantification of data and statistics

Our previous studies indicate that neonatal i.p. LPS injection produces white matter injury primarily in the cingulum in the forebrain (Fan et al., 2013). Therefore, brain sections at the bregma level were used to assess all pathological changes caused by systemic LPS injection. Most immunostaining data were quantified by counting positively stained cells. When the cellular boundary was not distinct, numbers of DAPI-stained nuclei from the superimposed images were counted. Three digital microscopic images were randomly captured in each of three sections, and the number of positively stained cells in the three images was counted and averaged (cells/mm^2). The mean value of cell counts from the three brain sections was used to represent each brain. For ease of comparison among the treatment groups, results were standardized as the average number of cells/mm^2. APP, NT, or 4-HNE staining was quantified using the NIH image software to determine the percentage area containing APP, NT, or 4-HNE positive staining in the entire area of the captured image (Cai et al., 2013, Fan et al., 2013, Kaizaki et al., 2013). In response to LPS challenge, the number of Iba1-positive microglia increases and the somas of the cells become larger. In addition to cell density, Iba1 immunoreactivity was also quantified by calculating the percentage area of the whole image showing Iba1 immunostaining (Cai et al., 2013, Fan et al., 2013, Kaizaki et al., 2013).

Data from behavioral testing, immunostaining, mitochondrial complex I activity, and ELISA assay were presented as the mean ± SEM and analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Results with $p < 0.05$ were considered statistically significant.

Results

Melatonin improved neurobehavioral deficits induced by LPS exposure

Compared to saline injection, LPS injection in P5 rats resulted in sensorimotor behavioral deficits at P6 (Figs. 1A and 1B). No gender differences in LPS-induced sensorimotor behavioral dysfunction were observed in the rats. Melatonin treatment significantly improved sensorimotor behavioral performance following LPS exposure (Figs. 1A and 1B).

Righting reflex

As shown in Fig. 1A, on P6, significantly longer mean latency times were seen in the LPS-injected group compared to the control group ($p < 0.05$). Melatonin treatment significantly shortened the LPS-induced increase in righting reflex latency ($p < 0.05$) (Fig. 1A).

Wire-hanging maneuver test

On P6, the mean latency time of the LPS-injected group was significantly lesser than that of the control group ($p < 0.05$) (Fig. 1B). The reduction in wire-hanging latency was much less prominent in the LPS + melatonin group than in the LPS group ($p < 0.05$), and there was no difference in wire-hanging maneuver between the control and LPS + melatonin groups (Fig. 1B).

Melatonin attenuated the reduction of OLs following LPS exposure

Consistent with our previous study (Fan et al., 2013), systemic LPS exposure (i.p.) resulted in white matter injury, as indicated by the reduction in O4+ staining. No gender differences in LPS-induced white matter injury were observed in the P6 rat brain. Late OL progenitor cells (O4+/O1−) are the main OL population in the P6 rat brain (Back et al., 2001). Abundant O4+ cells, in which positive staining was primarily localized to the cell membrane and processes, were observed in the P6 control rat brain (Fig. 2A, red), mostly in the subcortical white matter. LPS-induced injury to developing OLs was primarily found in the cingulum (Fig. 2B, red). LPS treatment resulted in pyknotic O4+ cells in this area (Fig. 2B, red, arrows indicated). Melatonin treatment reduced the number of pyknotic OLs (Fig. 2C). Compared to the control group, the LPS group had significantly reduced number of O4+ cells in the cingulum area in the P6 brain ($p < 0.05$) (Fig. 2D). Melatonin treatment significantly attenuated this LPS-induced loss of O4+ cells in the P6 rat brain ($p < 0.05$) (Fig. 2D).

Melatonin decreased LPS-induced axonal damage

Upregulation of APP, an early sign of axonal and neuronal lesions (Meng et al., 1997, Deguchi et al., 1999), was determined by immunostaining the striatum of P6 rats. APP expression was barely detectable in the control rat brain (Fig. 3A). Beaded APP

immunostaining was observed in the cingulum white matter (Fig. 3B), cortex, and striatum of the LPS-exposed brain ($p < 0.05$; Fig. 3D). Melatonin treatment attenuated the LPS-induced injury to axons in the above areas, as indicated by weak APP immunostaining, which was similar to that observed in control rats ($p < 0.05$; Figs. 3C and 3D).

Melatonin attenuated LPS-induced decreases in mitochondrial complex I activity

Mitochondrial complex I activity was measured as the amount of NADH oxidized per minute per milligram protein in homogenates of whole brains of rats 24 h after LPS injection. Consistent with our previous study (Fan et al., 2013), systemic LPS exposure reduced enzymatic activity of mitochondrial complex I after 24 h (P6; $p < 0.05$; Fig. 4). Melatonin treatment attenuated this decrease ($p < 0.05$; Fig. 4).

Melatonin protected against LPS-induced nitrosative and oxidative damage in the rat brain

NT is a product of the reaction of tyrosine residues of proteins with the RNS peroxynitrite, and is a marker of nitrosative stress (Haynes et al., 2003). 4-HNE, an aldehyde product of the reaction between ROS and the lipid component of cellular membranes, is a marker of oxidative stress (Haynes et al., 2003, Raza and John, 2006). Positive staining for NT or 4-HNE was rarely found in control rat brains (Figs. 5G and 5H). In the current study, systemic LPS exposure resulted in nitrosative and oxidative damage similar to that induced by i.c. LPS injection, as reported previously (Fan et al., 2008b, Fan et al., 2008c). Increased expression of NT (Figs. 5A, 5C and 5G) and 4-HNE (Figs. 5D, 5F and 5H) was observed in the cingulum white matter, cortex, and striatum of the rat brain 24 h after LPS injection ($p < 0.05$; Figs. 5G and 5H). Many of these cells were O4+ cells in the white matter of the cingulum (Figs. 5C and 5F). Melatonin treatment significantly decreased the NT- and 4-HNE-positive staining in the cingulum white matter, cortex, and striatum of the rat brain 24 h after LPS injection ($p < 0.05$; Figs. 5G and 5H).

Melatonin decreased LPS-induced increase in microglial activation and inflammatory responses

Activated microglia are a major source of ROS and RNS (Chao et al., 1992, Colton and Gilbert, 1993), which may put OLs at risk of oxidative and nitrosative damage (Thorburne and Juurlink, 1996). In the control rat brain, a few Iba1+ cells were detected, and most of those cells were in a resting state with a ramified shape (indicated by arrows in Fig. 6A). Significantly increased numbers of activated microglia, indicated by bright staining of an elongated or a round shaped cell body with blunt or no processes, were found in the cingulum white matter (Fig. 6B), cortex, and striatum of the rat brain 24 h after LPS injection ($p < 0.05$) (Fig. 6G). Iba1 staining was also quantified by measuring the percentage area with Iba1 immunostaining in the captured images. A higher percentage of Iba1-immunostained area was observed in the cingulum, cortex, and striatum of the LPS-exposed rat brain (Fig. 6H). Melatonin treatment following LPS injection reduced the number of activated microglia and percentage of Iba1-immunostaining area ($p < 0.05$) (Figs. 6C, 6G, and 6H). Double-labeling showed that many activated microglia (ED1+, Fig. 6D) were iNOS-expressing cells (146.67 ± 16.87) (Figs. 6E and 6F). No iNOS+ cells were detected in

the control rat brain, and iNOS+ cells were rarely found in the brains of rats treated with saline + melatonin (8.89 ± 4.44, $p < 0.05$).

Systemic LPS exposure resulted in inflammatory responses in the rat brain, as evidenced by an increase in IL-1β immunostaining (Fig. 7). Concentrations of IL-1β, a major proinflammatory cytokine, in the sera and brains of LPS-exposed rats were significantly increased compared to those in control rat brains at 24 h ($p < 0.05$) (Fig. 7). Melatonin attenuated the induction of serum IL-1β by LPS ($p < 0.05$) (Fig. 7). However, IL-1β concentrations in the brains of rats treated with saline + melatonin remained elevated at 24 h ($p < 0.05$) (Fig. 7).

Discussion

The present results reveal that developing brains are under oxidative and nitrosative stress following systemic LPS injection. Thus, systemic LPS exposure causes neural damage through induction of oxidative and nitrosative stress as well as by increasing i.c. LPS levels (Fan et al., 2008b) in neonatal rats.

Melatonin, a highly effective antioxidant and a free radical scavenger (Esposito and Cuzzocrea, 2010, Mauriz et al., 2012), has been shown to provide neuroprotective effects in an LPS-mediated model of septic shock (Srinivasan et al., 2010), a hypoxia-ischemia model (Shinozuka et al., 2013), and in other degenerative neurological disorders (Bonnefont-Rousselot and Collin, 2010). Melatonin possesses direct antioxidant properties for detoxifying ROS and RNS, and plays an indirect antioxidant role by supporting superoxide dismutase and glutathione peroxidase activities (Bonnefont-Rousselot and Collin, 2010). In the current study, melatonin treatment significantly decreased the expression of NT and 4-HNE in the rat brain following LPS injection, suggesting that melatonin protects the developing brain through its action as an antioxidant. In addition, melatonin may preserve the integrity of mitochondria from oxidative damage by reducing oxygen consumption, membrane potential, and superoxide anion production (Leon et al., 2004, Lopez et al., 2009). Results from the present study show that melatonin not only reduced LPS-induced neurobehavioral disturbance and brain damage, but also prevented LPS-induced decrease in mitochondrial complex I activity in the neonatal rat brain (Fig. 4).

Several studies have indicated that melatonin may decrease the production of pro-inflammatory cytokines and inhibit the expression the pro-inflammatory enzymes iNOS and COX-2 in cells of the liver and lung of LPS-treated rats (Crespo et al., 1999, Esposito and Cuzzocrea, 2010, Mauriz et al., 2012, Lin et al., 2013). It has been suggested that melatonin protects against developing white matter damage by inhibiting microglial activation and oligodendroglial maturation, and these effects appear to be melatonin-receptor dependent (Olivier et al., 2009). One study reported that melatonin reduced inflammation and promoted subsequent myelination in the white matter of a model of neonatal stroke (left middle cerebral artery electrocoagulation followed by occlusion of both common carotid arteries for 50 min, in P7 Wistar rats), but was not able to reduce cortical infarct volume and oxidative stress, as indicated by 3-NT immunostaining. The lack of observation of an antioxidative effect of melatonin in that neonatal stroke model may be because either the melatonin target

in the affected region was absent or the cortical injury was too severe to be reversible (Villapol et al., 2011). In addition, melatonin may promote early phases of inflammation by stimulating pro-inflammatory mediators on the one hand, and contribute to its attenuation on the other hand (Radogna et al., 2010). Therefore, the ambiguous results reported in different animal models may be attributable to the opposing effects of melatonin on the early phase of inflammation. Further detailed study is needed to understand the effects of melatonin on pro-inflammatory and anti-inflammatory pathways in the brain.

Previous studies have indicated that ROS generation is increased in women during pregnancy, and newborn infants have lower levels of plasma antioxidants compared to adults. Therefore, newborns, especially those delivered preterm, may be more prone to oxidative stress than older individuals (Gitto et al., 2009, Aversa et al., 2012). Our current results show that melatonin attenuates the brain injury and neurobehavioral disturbances induced by systemic LPS exposure in neonates; the protective effects of melatonin are associated with its anti-nitrosative and anti-oxidative properties. It seems likely that melatonin treatment will be useful in the management of neonates with nitrosative and oxidative stress related problems. However, further biochemical investigations and long-term studies are required to define the most effective applications for melatonin.

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1. LPS resulted in impairment of sensorimotor behaviors in neonatal rats
2. LPS caused nitrosative and oxidative stress damages in neonatal rat brain
3. Melatonin attenuated LPS-induced neurobehavioral disturbance in neonatal rats
4. Melatonin protects against LPS-induced stress damages in neonatal rat brain

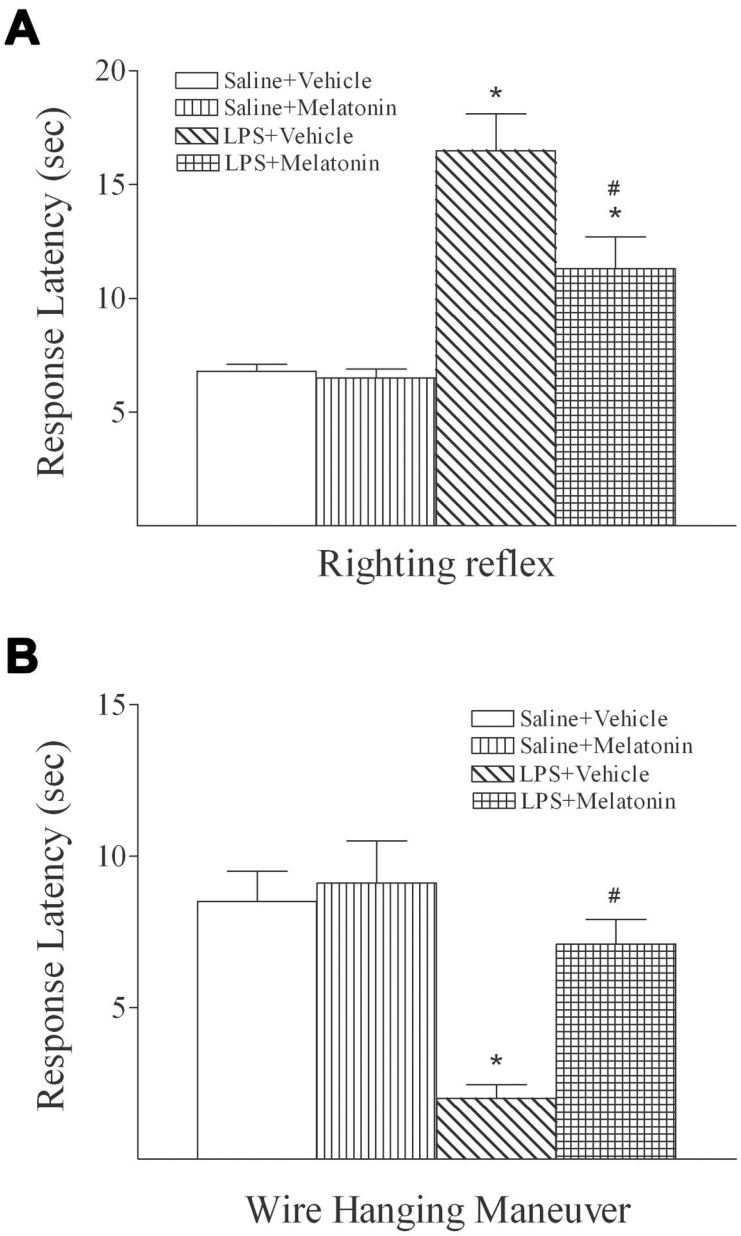

Fig. 1.

Melatonin attenuated systemic LPS-induced elongation of mean latency times in righting reflex (A) and reduction of mean latency times in the wire hanging maneuver (B) in the rats. The results are expressed as the mean ± SEM of six animals in each group, and analyzed by one-way ANOVA. *$p < 0.05$ represents a significant difference for the LPS + Vehicle group or LPS + Melatonin group as compared with the Saline + Vehicle group. $^{\#}p < 0.05$ represents a significant difference for the LPS + Melatonin group as compared with the LPS + Vehicle group.

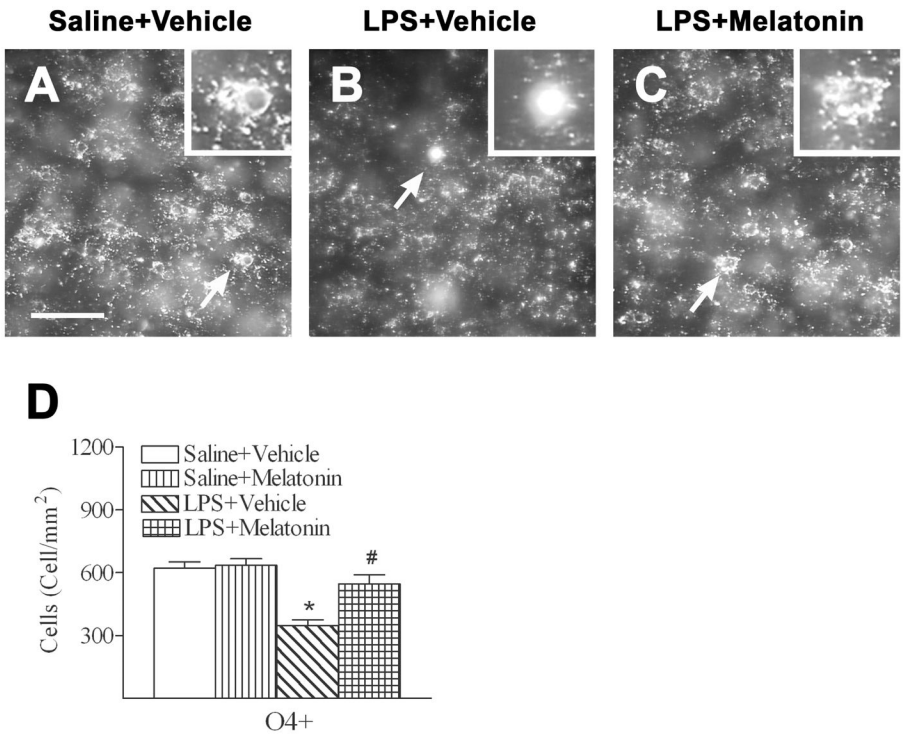

Fig. 2.

Representative photomicrographs of O4 immunostaining in rat brain 24 hours (P6) after LPS injection. Abundant O4 positive cells, which had positive staining at the cell membrane and processes, were found in the cingulum white matter of the brain sections at the bregma level in P6 control rat brains (A). LPS injection resulted in pyknotic O4 cells with condensed cell body and immunoreactivity in the cytoplasm (arrow indicated in B) and reduced the number of normal O4 positive cells. Inserts in A, B and C are magnified parts indicated by the white boxes. Melatonin attenuated LPS-induced loss of O4 positive cells (C). Quantitation of the O4 positive cells (D) was performed as described in Methods. The results are expressed as the mean ± SEM of six animals in each group, and analyzed by one-way ANOVA. $*p < 0.05$ represents a significant difference for the LPS + Vehicle group as compared with the Saline + Vehicle group. $^{\#}p < 0.05$ represents a significant difference for the LPS + Melatonin group as compared with the LPS + Vehicle group. The scale bar shown in A represents 50 μm for A to C.

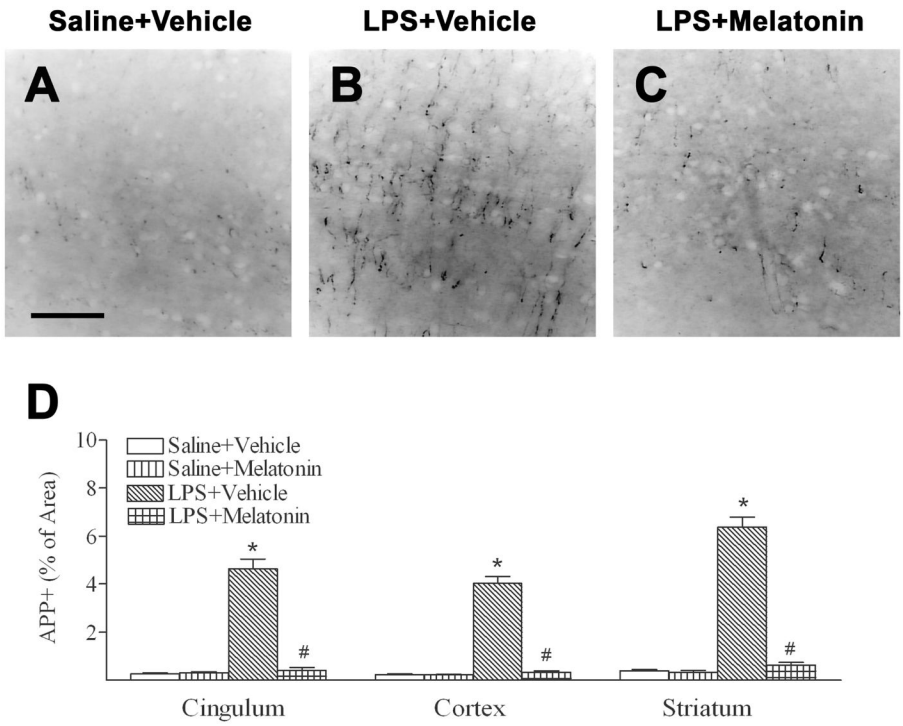

Fig. 3.

Representative photomicrographs of APP immunostaining in the rat brain 24 hours (P6) after LPS injection. Weak APP positive staining was detectable in the brain sections at the bregma level of control brain (A). The beaded APP staining was found in the cingulum white matter (B) of the LPS-exposed brain. Melatonin treatment attenuated the LPS-induced injury to axons in the cingulum white matter (C). Quantitation of the percentage area of image that contained APP+ staining in the cingulum white matter, cortex and striatum were performed as described in Methods. The results are expressed as the mean ± SEM of six animals in each group, and analyzed by one-way ANOVA. $*p < 0.05$ represents a significant difference for the LPS + Vehicle group as compared with the Saline + Vehicle group. $^{\#}p < 0.05$ represents a significant difference for the LPS + Melatonin group as compared with the LPS + Vehicle group. The scale bar shown in A represents 50 μm for A to C.

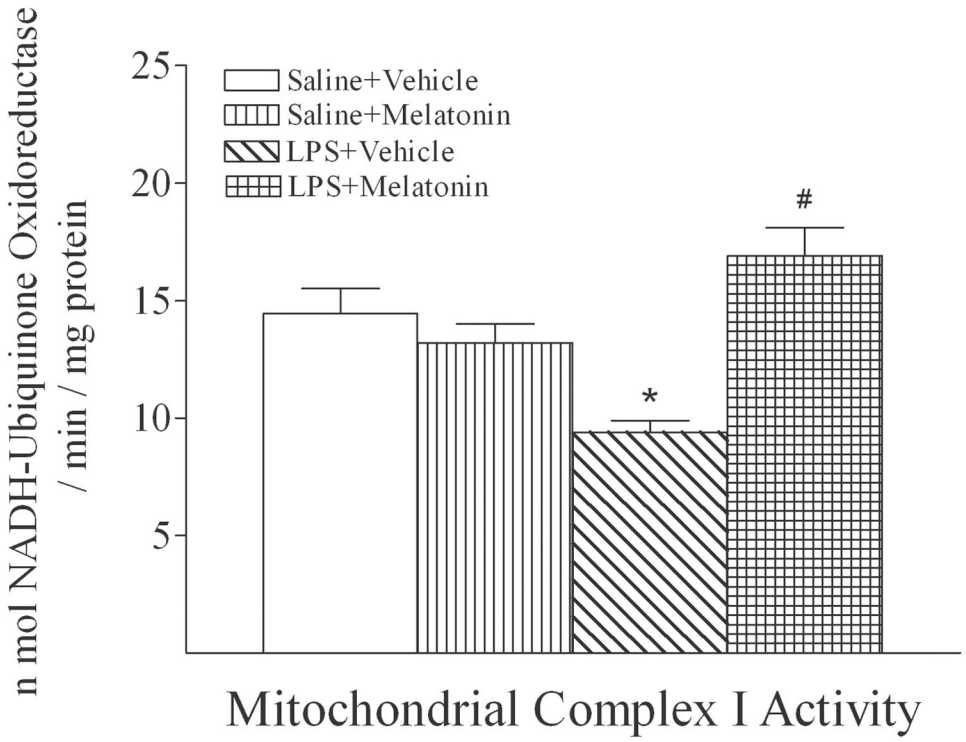

Fig. 4.

Melatonin attenuated the systemic LPS-induced a reduction in the enzymatic activity of mitochondrial complex I in the rat brain 24 hours (P6) after LPS injection. LPS exposure reduced enzymatic activity of mitochondrial complex I in 24 hours after LPS injection. Melatonin treatment attenuated the LPS-induced decrease in mitochondrial complex I activity in P6 rats. The results are expressed as the mean ± SEM of six animals in each group, and analyzed by one-way ANOVA. $*p < 0.05$ represents a significant difference for the LPS + Vehicle group as compared with the Saline + Vehicle group. $^{\#}p < 0.05$ represents a significant difference for the LPS + Melatonin group as compared with the LPS + Vehicle group.

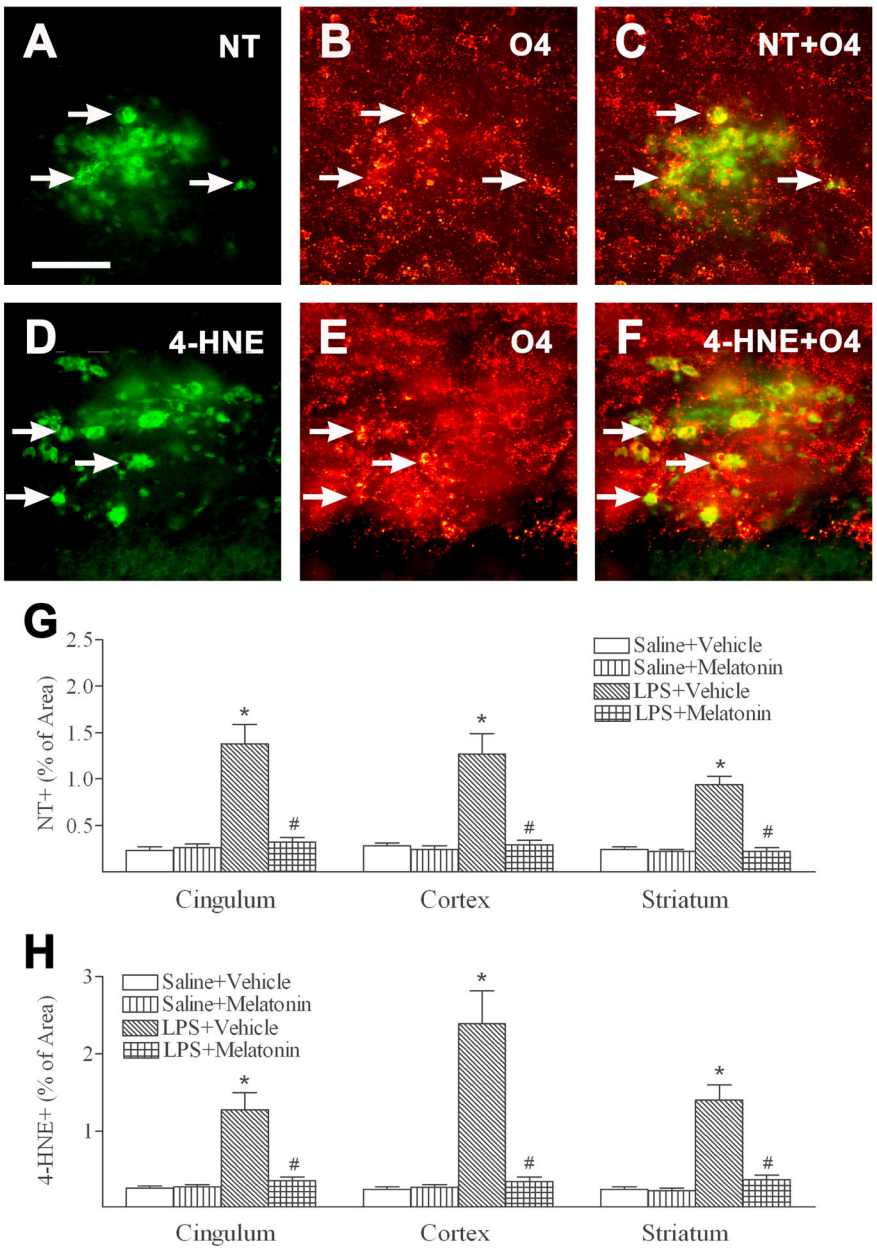

Fig. 5.

Representative photomicrographs of NT, or 4-HNE staining in the rat brain 24 h after LPS injection. The NT (G) or 4-HNE staining (H) was rarely found in the brain sections at the bregma level of control brain. The nitrosative (B, NT+) and oxidative damage (E, 4-HNE+) was found in the cingulum white matter of the LPS-exposed brain. Double-labeling showed that many NT positive cells (A) in the LPS-injected rat brain were O4+ cells (B) in cingulum white matter. C is a merged image of A and B. Double-labeling also showed that many 4-HNE positive cells (D) in the LPS-injected rat brain were O4+ cells (E) in cingulum white matter. F is a merged image of D and E. Melatonin treatment attenuated the LPS-induced NT (G) and 4-HNE staining (H) in the cingulum white matter. Quantitation of the percentage area of image that contained NT+ (G) or 4-HNE+ staining (H) in the cingulum

white matter, cortex and striatum were performed as described in Methods. The results are expressed as the mean ± SEM of six animals in each group, and analyzed by one-way ANOVA. $^{*}p < 0.05$ represents a significant difference for the LPS + Vehicle group as compared with the Saline + Vehicle group. $^{\#}p < 0.05$ represents a significant difference for the LPS + Melatonin group as compared with the LPS + Vehicle group. The scale bar shown in A represents 50 μm for A to F.

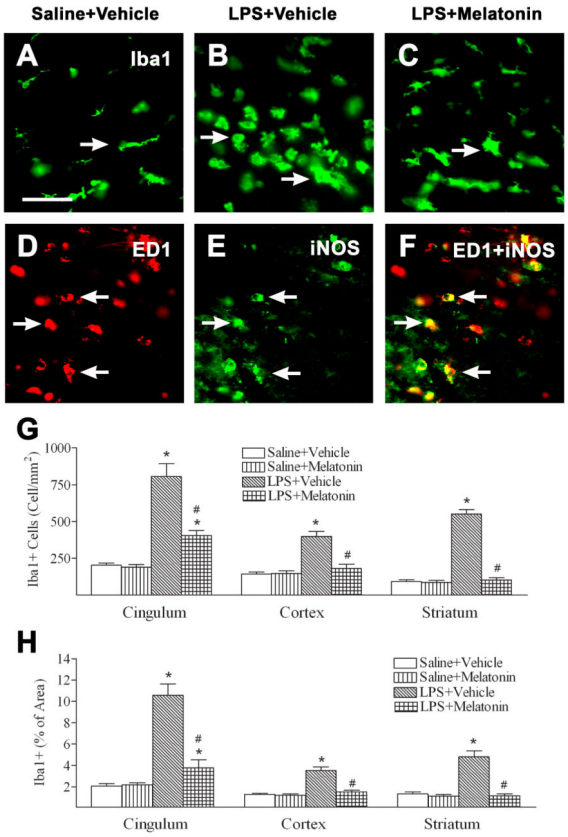

Fig. 6.

Representative photomicrographs of microglia in the rat brain 24 hours (P6) after LPS injection. As shown by Iba1 immunostaining in the cingulum white matter (A), a few microglia at the resting status with a small rod shaped soma and ramified processes (arrows indicated in A) were found in the control rat brain. Numerous activated microglia showing bright staining of an elongated or a round shaped cell body with blunt or no processes (arrows indicated in B and C) were observed in the cingulum white matter (B) of the rat brain with neonatal LPS exposure. Melatonin treatment reduced the number of activated microglia stimulated by LPS in the above areas (C). Double-labeling showed that ED1 positive activated microglia (D) in the LPS-injected rat brain were iNOS expressing cells (E). F is a merged image of D and E. Quantitation of the number of Iba1+ cells (G) and the percentage area of image that contained Iba1 staining (H) in the cingulum white matter, cortex, and striatum were performed as described in Methods. The results are expressed as the mean ± SEM of six animals in each group, and analyzed by one-way ANOVA. $*p < 0.05$ represents a significant difference for the LPS + Vehicle group or LPS + Melatonin group as compared with the Saline + Vehicle group. $^{\#}p < 0.05$ represents a significant difference for the LPS + Melatonin group as compared with the LPS + Vehicle group. The scale bar shown in A represents 50 μm for A to F.

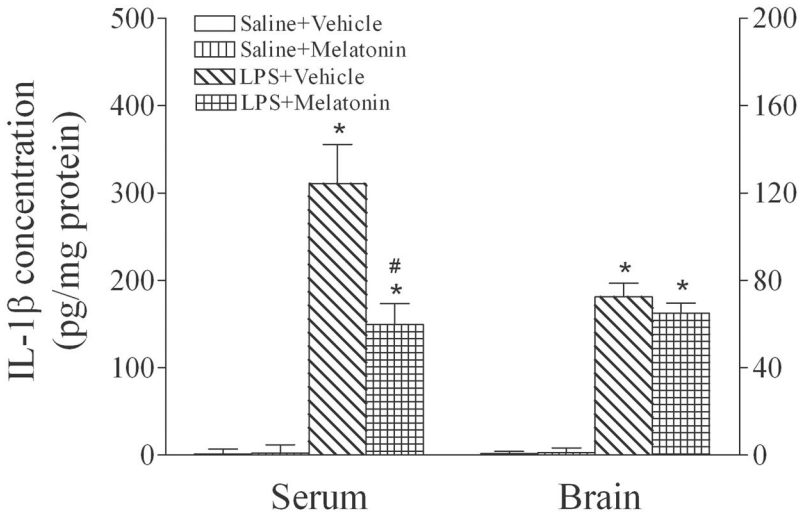

Fig. 7.

Melatonin attenuated systemic LPS-stimulated increases in inflammatory cytokines (IL-1β) in the rat brain 24 hours after LPS injection. IL-1β concentrations were determined by ELISA kit and presented in the unit of pg/mg protein, as described in Methods. Twenty-four hours following LPS injection, serum and brain levels of IL-1β were elevated as compared with the Saline + Vehicle group. Treatment with melatonin attenuated induction of serum IL-1β contents by LPS. The results are expressed as the mean ± SEM of six animals in each group, and analyzed by one-way ANOVA. $*p < 0.05$ represents a significant difference for the LPS + Vehicle group or LPS + Melatonin group as compared with the Saline + Vehicle group. $^{\#}p < 0.05$ represents a significant difference for the LPS + Melatonin group as compared with the LPS + Vehicle group.