Dexamethasone and betamethasone protect against LPS-induced brain damage in the neonatal rats

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Abstract

The aim of this study is to test whether dexamethasone (Dex) and betamethasone (Beta), two of the most commonly used corticosteroids, protect against lipopolysaccharide (LPS)-induced white matter damage and neurobehavioral dysfunction. LPS or sterile saline was injected into the brain white matter of rat pups at postnatal day 5 (P5) and Dex or Beta was given intraperitoneally to the rat pups 1 h before the LPS microinjection. Brain inflammatory response, brain damage, and myelination were examined at P6, P8 and P14. Neurobehavioral tests were performed from P3 through P22. Our results demonstrate that Dex and Beta markedly diminish the LPS-induced brain inflammatory response, restore myelin basic protein (MBP) expression and alleviate lateral ventricle dilation. Both corticosteroids demonstrate significant protection against most of LPS-induced behavioral deficits, including those in rearing, vibrissa-elicited forelimb-placing, beam walking, learning and elevated plus-maze test. Notably, only Beta improved the locomotion and stereotype dysfunction. In contrast to their beneficial effects, neither drug prevented LPS-induced delay in body weight gain from P6 through P21. Our study suggests that if their adverse effects are minimized, corticosteroids may be the potential candidate drugs to prevent brain damage in premature infants.

Introduction

Periventricular leukomalacia (PVL) is the most common form of brain injury in preterm infants and results in high mortality and morbidity in this group of infants. The etiology of PVL is still unclear but there is considerable evidence that maternal infection/inflammation and hypoxia-ischemia are two major contributory factors (1–3). After intracerebral delivery of lipopolysaccharide (LPS), we have previously reported PVL-like neuropathological changes (4) and neurobehavioral deficits in the neonatal rat (5). Later, we further showed that suppression of microglial activation by minocycline is protective against LPS-induced oligodendrocyte (OL) damage (6). As brain inflammation is also associated with neuronal

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damage in hypoxia-ischemia animal models (7), anti-inflammation is one of the plausible strategies to prevent and/or treat PVL in the future.

Corticosteroids are potent anti-inflammatory drugs and have been used clinically to treat septic shock and other inflammatory diseases for half a century. Antenatal corticosteroid therapy has also been used to reduce the incidence of respiratory distress syndrome and other complications of preterm birth (8). In a recent large population clinical study, Lee et al. (9) reported that antenatal exposure of both dexamethasone (Dex) and betamethasone (Beta) have tended to reduce the incidence of PVL and intraventricular hemorrhage. Dex and Beta are two corticosteroids used to accelerate lung maturation in the perinatal period. Therefore, the aim of this study is to test whether these two corticosteroids may protect against brain white matter damage and neurobehavioral deficits following LPS exposure.

Materials and Methods

1. Chemicals

LPS (055:B5), Dex and Beta were obtained from Sigma Chemical Co (St. Louise, MO). Antibodies used were obtained from the following sources: O4, ED1, Glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) from Millipore (Temecula, CA); CD43 from AbD Serotec (Raleigh, NC) and inducible nitric oxide synthase (iNOS) from Cell Signaling Technology (Danvers, MA).

2. Animal surgery and treatment

Myelination in rats occurs exclusively after birth (10), and the developmental stage of OLs in the rat brain at P2 to P7 is roughly equivalent to that in the human at 24 to 32 gestational weeks (11), a developmental window with a high incidence of PVL. Therefore, we used a P5 rat model in which PVL-like brain injury has been consistently documented (4, 5). Briefly, Sprague–Dawley rat pups at P5 were anesthetized with isoflurane (4% induction, 1.5% maintenance) and placed in a stereotaxic apparatus with an adapter for neonatal rats (David Kopf, Tujunga, CA). The intracerebral injection was performed using a 10 μl precise syringe (World Precision Instruments, Inc., Sarasota, FL) in the following coordination: 1.0 mm posterior and 1.0 mm lateral to the bregma, and 2.5 mm deep to the skull surface. LPS (1 μg/ per animal) or the vehicle (for the control) at the volume of 2 μl were injected into the left brain hemisphere over a period of 5 min. Dex or Beta (both used at 0.5 mg/kg body weight) was given to rat pups intraperitoneally 1 h before LPS injection. The doses are equivalent to clinical uses to treat premature infants.

To minimize variations in body and brain size, the litter size was adjusted to 10 pups per litter. The experimental procedure was approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and, in addition, was in accordance with the guidelines of the National Institutes of Health on the care and use of laboratory animals.

3. Histologic examination and immunohistochemistry

At P6, P8 and P14, rat brains were fixed by transcardiac perfusion with 4% paraformaldehyde. Frozen sections (10 μm) or free-floating sections (40 μm, for O4 immunohistochemistry) consecutively were prepared at the level of the bregma. Hematoxylin and eosin (H&E)-stained sections were examined under a light microscope for any alterations in histopathology. Immunohistochemistry was performed using a standard protocol as previously described (4, 5). The final concentrations of the antibodies were diluted as follows: O4 (1:100), ED1 (1:200), MBP (1:100), CD43 (1:200) and iNOS (1:100). Images were captured with a CCD camera (Oly-750, Olympus), and superimposed using Adobe Photoshop (version 7.0) software, if necessary.

To compare the size of lateral ventricles, H&E-stained sections at the bregma level were scanned by a densitometer (Bio-Rad, Richmond, CA) to acquire digital images for area measurement, which was done using the Quantity One software (Bio-Rad, version 4.5.2). The areas (cm^2) occupied by the left and right ventricles, as well as that by the whole brain section were outlined and measured. Ventricle size index was calculated as a ratio of the area of each ventricle to that of the whole brain hemispheres.

4. Quantification of immunohistochemistry data

Three consecutive brain sections in each animal were used for cell counting. O4+, ED1+ and iNOS+ cells were counted in the entire periventricular white matter area (including corpus callosum and cingular white matter). The mean value was calculated from 3 slides of the same animal and averaged. MBP immunohistochemistry was quantified using ImageJ software (NIH). Images of MBP immunostaining (primarily at the corpus callosum) from three consecutive sections at the bregma level were acquired under a low-power microscopic view (10×). The ratio of the area occupied by MBP immunostained fibers to that of the entire image frame was calculated, and was defined as the myelin index (see Fig. 3.) Cell counting and myelin index assessment was performed in a blinded manner in that the individuals who performed the procedure were unaware of the test conditions.

5. Behavioral tests

The developmental test battery used was based on the behavioral tests for neurotoxicity (12, 13), which were performed from P3 to P21. Behavioral tests were performed by an individual who was unaware of the test conditions. Body weights of rat pups were recorded daily (from P3 to P21). Eight animals were included in each treatment group.

1) Locomotor activity—Locomotor activity was measured by the Video Tracking System-SMART (2000 San Diego Instruments, Inc., San Diego, CA). Pups were placed in the activity chambers in a quiet room with a dimmed light. The total distance traveled by the animals was recorded during a 10 min testing period.

The numbers of rearing events including exposure rearing response (body inclined vertically with hindpaws on the floor of the activity cage and forepaws on the wall of the cage) and sniffing-air response (rearing in the open area of the active cage) were counted. The summation of exposure rearing and sniffing-air responses reflects vertical activity which has

been used apart from locomotion, and has been reported as a reliable criterion for assignment of rats into groups during their exposure to novelty (14).

2). Stereotypy—The stereotyped behaviors including standing (on all four feet, essentially motionless and no active sniffing), grooming (washing the face or any other parts of its body with the forepaws, with the mouth generally in contact with the body), scratching (raising of hindpaws to touch any part of its body), head-swinging (standing on all four feet and moving its head from side to side), sniffing (sniffing parts of the walls or floor of the apparatus), and freezing (standing on all four feet in a 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 of testing period.

3). Elevated plus-maze test—The plus-maze consists of two open arms (30×5×0.25 cm) and two enclosed arms (30×5×10 cm) emanating from a common central platform (5×5 cm) to form a plus shape. The anxiety-related behaviors were recorded by a video camera for a period of 5 min on P19. The parameters used for data analysis were the numbers of open or enclosed arm entries (arm entry defines as all four paws into an arm), and the time the animals spent in the various sections of the maze (open arms, center, enclosed arms).

4). Beaming walking test—Motor coordination and balance were assessed by measuring the ability of the animals to traverse a narrow beam to reach an enclosed safety platform. The time spent on the beam for each pup to traverse the beam and join their littermates was recorded, and the cut-off time was set at 60 s. Test was performed on P20.

5). Vibrissa-elicited forelimb-placing test—This test uses stimulation of the rat's vibrissae to trigger a placing response to measure forelimb-placing deficit (15). Rats use their vibrissae to gain bilateral information about the proximal environment, and this information is integrated between the hemispheres. The test was performed on P21. The animal was gently held by its torso, allowing the forelimbs to hang free. Independent testing of each forelimb was induced by gently brushing vibrissae of the corresponding side on the edge of a tabletop once per trial for 10 trials. The percentage of trials in which the rat successfully placed its forepaw onto the tabletop was recorded for each side. Intact animals placed the forelimbs of both sides quickly onto the countertop, with 100% success in all variants of this test. If an animal struggled during testing, the data were not included in the overall analysis.

6). Passive avoidance (Learning and memory tests—The passive avoidance procedure consists of two sessions designed to test learning and memory. In the first session (P20), rats were trained to learn how to avoid an electric shock. The number of shocks required to retain an individual animal on a safe board for 2 min was recorded as a measure of acquisition of passive avoidance. The second session was carried out 24 h after the first session (P21). The rat was placed on the safe board and steel rods were not connected with the electric shock generator. The retention latency, i.e. the time elapsed before the rat stepped down to the grid floor, was recorded as a measure of the retention of passive avoidance. If the rat did not step down to the grid floor within 2 min, a ceiling score of 2 min was assigned.

6. Statistics

One Way Analysis of Variation followed by post-hoc Tukey test was used to determine statistical significance among treatments, and the level of significance was set at $p < 0.05$.

Results

1. Dex and Beta suppressed the LPS-induced inflammatory response in the rat brain

Consistent with our previous observations, LPS induced an acute inflammatory response characterized by microglia/macrophage activation and polymorphonuclear leukocyte (PMN) infiltration after 24 h treatment (Fig. 1). The increased ED1+ cells and infiltrated PMNs were primarily detected in the periventricular white matter, and to a less extent, the subventricular zone, the cerebral cortex and the striatum. iNOS+ cells were found exclusively in the periventricular white matter area in the LPS-treated rat brain (Fig. 2B, shown in low power magnification). Double-labeling immunohistochemistry showed that iNOS is expressed by both CD43+ PMNs and ED1+ microglia, but not GFAP+ astrocytes and NG2+ OL progenitor cells (Fig. 2E–2H). The inflammatory response was significantly suppressed by both Dex and Beta treatment, as shown by a decreased number of PMNs (Fig 1G&H) and ED1+ microglia/macrophage (Fig. 1K&L). iNOS+ cells were significantly reduced by both corticosteroid treatments (Control: 0±0; LPS: 27.7±4.1; Dex: 1.0±0.6; Beta: 1.0±0.5; mean±SEM). It appears that Dex and Beta have very similar efficacy in suppressing brain inflammation. No differences between Dex or Beta and the control were noted.

2. Dex and Beta prevented LPS-induced loss of pre-oligodendrocytes (pre-OLs) and myelination deficits

O4+ pre-OLs are the major OL lineage in the P5 rat brain (11), therefore we used O4 immunohistochemistry to quantify changes of pre-OLs. Three days after LPS treatment, the number of O4+ pre-OLs was significantly diminished. Some O4+ cells appeared to be undergoing degeneration, as shown by the condensed O4 immunostaining in the soma and by fragmented processes (Fig. 3B). Later at P14, MBP immunostaining showed a marked hypomyelination in LPS-treated rats (Fig. 3G&H), compared to the well-developed myelination in the control group (Fig. 3E&F). The number of pre-OLs (Fig. 3M) and MBP immunostaining (Fig. 3N) were significantly improved in both Dex and Beta treatment groups. No differences between Dex or Beta and the control groups were noted.

3. Dex and Beta attenuated LPS-induced lateral ventricle dilation

One of the most consistent pathological features in LPS-treated rat brain is the bilateral dilation of lateral ventricles (4, 5). As shown in Table 1, the lateral ventricles were significantly dilated in LPS-treated rat brain when compared to the control. Both Dex and Beta significantly decreased the ventricle dilation by LPS. Dex appears to be slightly more effective than Beta although this difference is not statistically significant (p>0.05).

4. Dex and Beta improved LPS-induced behavioral deficits

The long-term neurological functions of rats were evaluated by a battery of behavioral tests. Locomotor activity increased with age until P15 in all groups. LPS-treated rats showed hyperactivity on P15 as compared to the controls (Fig. 4A). Beta but not Dex, significantly prevented LPS-induced hyperactivity. The stereotypy (Fig. 4B) and rearing activity (Fig. 4C) were significantly higher in LPS-exposed rats (only observed at P21 and P14, respectively) than the control. Both corticosteroids showed recovery on rearing activity, but only Beta showed improvement on stereotypy activity.

Motor-coordination was evaluated by a beam walking test. LPS-treated rats showed a significant increase of performance latency as compared to the control rats (Fig. 4D, tested in both 3.5×3.5 and 1.4×1.4 cm^2 beam). The sensomotor function, as evaluated by the vibrissa-elicited forelimb-placing test on P20, was also impaired in LPS-treated rats (Fig. 4E, significant decrease in success rate). Both corticosteroids showed significant improvement on these two tests.

The plus-maze test showed that LPS-treated animals exhibit a less anxiety-like behavior, as demonstrated by a significantly increased number of entries into the open field (Fig. 4F). The passive avoidance test showed that the number of electric foot shocks required for retaining the rats on the safe board significantly increased in LPS-treated pups at P20, suggesting that the learning ability was impaired in these animals. LPS also tended to reduce the retention latency to step down from the board the next day (P21) as compared with the control group, but did not reach a statistically significant level (Fig. 4G). Both Dex and Beta significantly prevented LPS-induced less anxiety-like behavior and learning deficits.

5. Dex and Beta failed to improve LPS-induced body weight loss

LPS treatment resulted in an immediate arrest in body weight gain in the first 24 h. Body weight started to increase at 48 h and continued to increase with a similar trajectory as that of the control rats, but there never was a catch-up growth compared to the control. Neither Dex nor Beta treatment prevented the LPS-induced delay in body weight gain. Dex or Beta alone (n=8) delayed normal weight gain, in a similar pattern of the rats treated with LPS (Fig. 4H).

Discussion

This study shows that both Dex and Beta prevented LPS-induced brain damage in the neonatal rat. This is presumed to be associated with their powerful anti-inflammatory properties.

Our previous in vivo studies have suggested that activated microglia play a central role in LPS-induced brain damage in neonatal rats (5, 6). In line with these findings, the current study demonstrates that the neuroprotection against LPS-induced brain damage by Dex and Beta is associated with a marked decrease in the inflammatory response. LPS up-regulates iNOS expression in both activated microglia and PMNs, and this is significantly suppressed by steroid treatment (Fig 2). Therefore, the neuroprotective effects of Dex and Beta may be attributed, at least partially, to their suppression of NO and oxidative species. The role of

NO in LPS-induced OL damage has been recently demonstrated by an in vitro study which showed that NO and subsequent reactive nitrogen species (RNS) produced by LPS-activated microglia can lead to acute pre-OL lysis, while a delayed OL degeneration is mediated by proinflammatory cytokines such as TNFα (16). The role of PMNs in the inflammatory brain damage is less studied compared to microglia, but they likely contribute to a substantial extent by producing toxic factors such as proinflammatory cytokines (17) and reactive oxygen species (18). Therefore, we speculate that suppression of inflammatory mediators and RNS may underlie the major neuroprotive mechanism by the corticosteroids used in this study.

Lateral ventricle dilation is frequently observed in PVL infants. Importantly, we have consistently documented enlarged lateral ventricles in our LPS intracerebral injection model, and we have used this neuropathology as a general indicator of brain damage (4, 5). It is generally thought that an increase in intraventricular pressure, or loss of surrounding tissue and a subsequent space-filling by ventricular enlargement are two potential causes of ventricle dilation.

Although the white matter is predominantly damaged in our LPS model, we recently found that the gray matter, e.g., the dopaminergic system, is also compromised (19). The many behavioral deficits in LPS-exposed rats are likely due to both white and gray matter damage. Our goal for neurobehavioral tests was to utilize these tools as general measures for assessing the neuroprotection by corticosteroids. Most of the behavioral deficits were ameliorated by both steroids, except that the locomotor and stereotypy activity were selectively improved by Beta and not Dex. The reason for this disparity between these two corticosteroids, which showed very similar anti-inflammatory effects, is not known at this time. However, we can speculate that this may be associated with the adverse neurological effects of Dex (see below), rather than lack of neuroprotection.

Although Dex and Beta-mediated neuroprotection may be largely due to their anti-inflammatory activity, it may also result from their direct protection on OLs. For instance, in vitro studies have demonstrated that corticosteroids are protective against cytokine-induced blockage of survival and differentiation of OL progenitor cells (20) and cell death (21). If this is also true in vivo, it can explain why Dex and Beta show nearly complete protection against brain damage in the current study. Interestingly, Ikeda et al. (22) have reported that up-regulation of endogenous corticosteroids by LPS is associated with neuroprotection against subsequent hypoxic-ischemic brain damage (known as LPS preconditioning), which further supports this notion.

Dex and Beta are the only two corticosteroids currently used antenatally to accelerate fetal lung maturation, especially in those pregnant women with imminent delivery of an infant at 24 to 34 weeks gestation. During the past decade, concerns have being raised regarding their potential adverse effects on development (particularly Dex). There are numerous studies suggesting that Beta has less adverse effects than Dex when used prenatally. For instance, in a large population study conducted by the National Institute of Child Health and Human Development Neonatal Research Network, it was reported (9) that Beta was associated with a reduced risk for neonatal death and with trends of decreased risk for other adverse neonatal

outcomes compared with Dex. Later, a study from the same group further showed that antenatal Beta exposure was associated with reduced risks, while Dex exposure was associated with increased risk of hearing impairment, neurodevelopmental impairments and a decreased likelihood of unimpaired status, compared with the control group (23). Consistent with these clinical data, our animal study shows that while providing similar efficacy in suppressing inflammation, Beta is more effective than Dex in decreasing LPS-induced behavioral deficits.

One of the adverse effects of corticosteroids on development is a reduced body weight and/or head circumference (24). In line with these clinical reports, our animal study showed that both corticosteroids significantly reduced body weight in the presence or absence of LPS. It should be noted that the severity of these adverse effects correlates with the dose of corticosteroids used (25). Although a single dose usually is given prenatally, multiple doses sometimes are used prenatally without much confirmed benefit (8). In this study, the doses of Dex and Beta used are clinical relevant but at the high end. It is possible that by reducing the currently used dose Dex and Beta, the adverse effects can be minimized. Since the most common reason for preterm labor is maternal infection (which can also result in fetal cerebral inflammation), these neonates with evidence of active infection may need to be excluded from corticosteroid therapy. In brief, we demonstrate the neuroprotection of corticosteroids against inflammatory brain damage, suggesting that they may be potential candidates in preventing brain damage in premature infants

Acknowledgments

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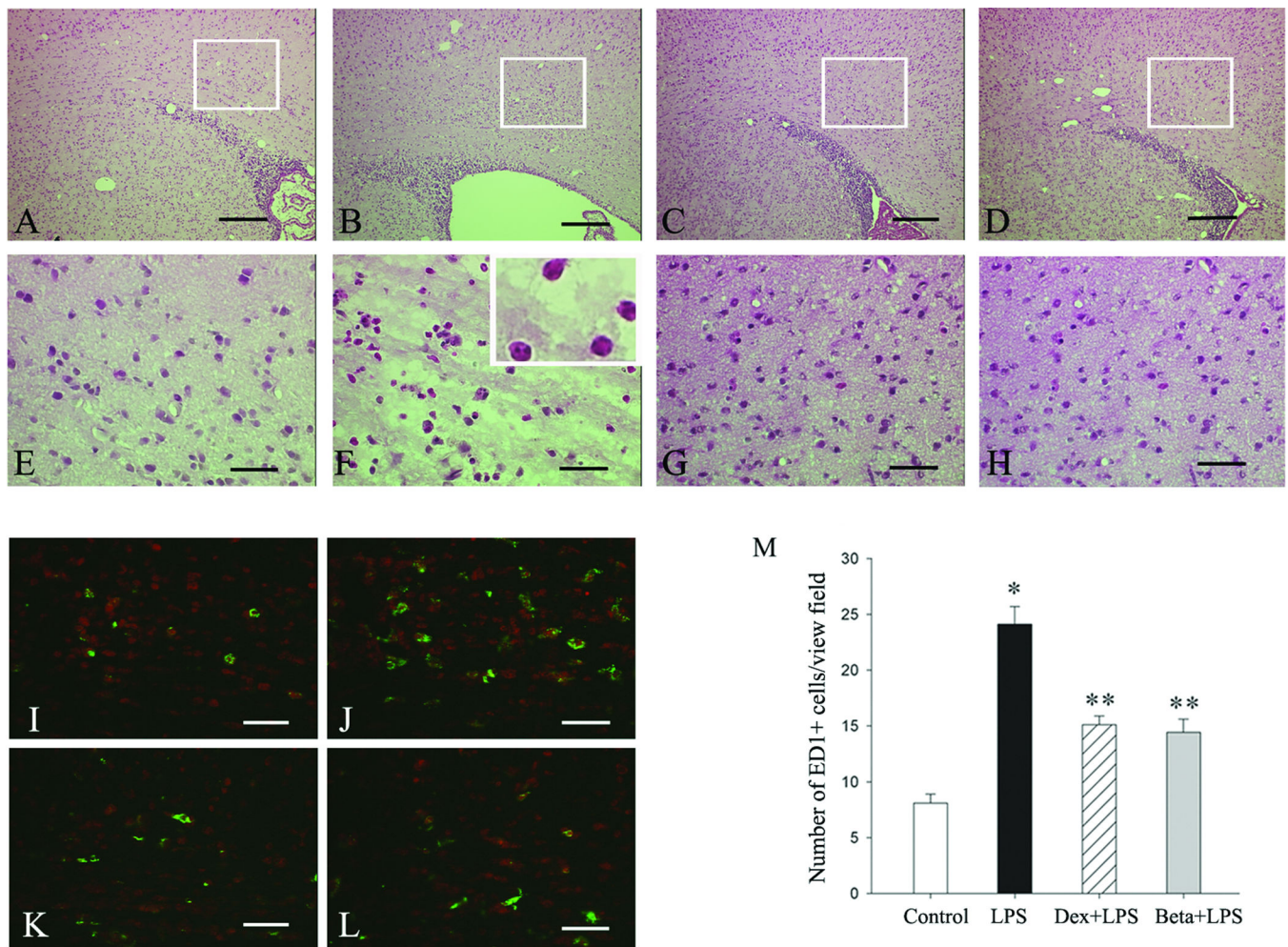
Fig 1.

Dex and Beta suppress LPS-induced PMN infiltration and microglia activation in the P6 rat brain. A–H: H&E staining for examination of PMN infiltration. No PMNs were found in the control rat brain (A&E), while LPS induced a marked recruitment of PMN into brain parenchyma especially in the brain white matter (B&F). The typical PMNs show horse shoe-like nuclei that can be readily identified (magnified in the insert in F). Both Dex (C&G) and Beta (D&H) significantly reduced PMN infiltration. Images in E–H are higher magnifications of white boxes in the corresponding images from A–D. I–L: ED1 immunostaining for examination of microglia activation. ED1+ cells (green) were primarily found in the periventricular white matter in both the control (I) and LPS-treated rat brain (J), but their numbers were significantly increased after LPS treatment (quantitative counting data shown in M). Both Dex (K) and Beta (L) significantly suppressed microglia activation. Sections were counterstained with Propidium iodide (red). Data shown are mean±SEM from eight animals. Scale bar: in A to D: 250 μm, E to L: 100 μm. *p<0.01 vs control; ** p<0.01 vs LPS.
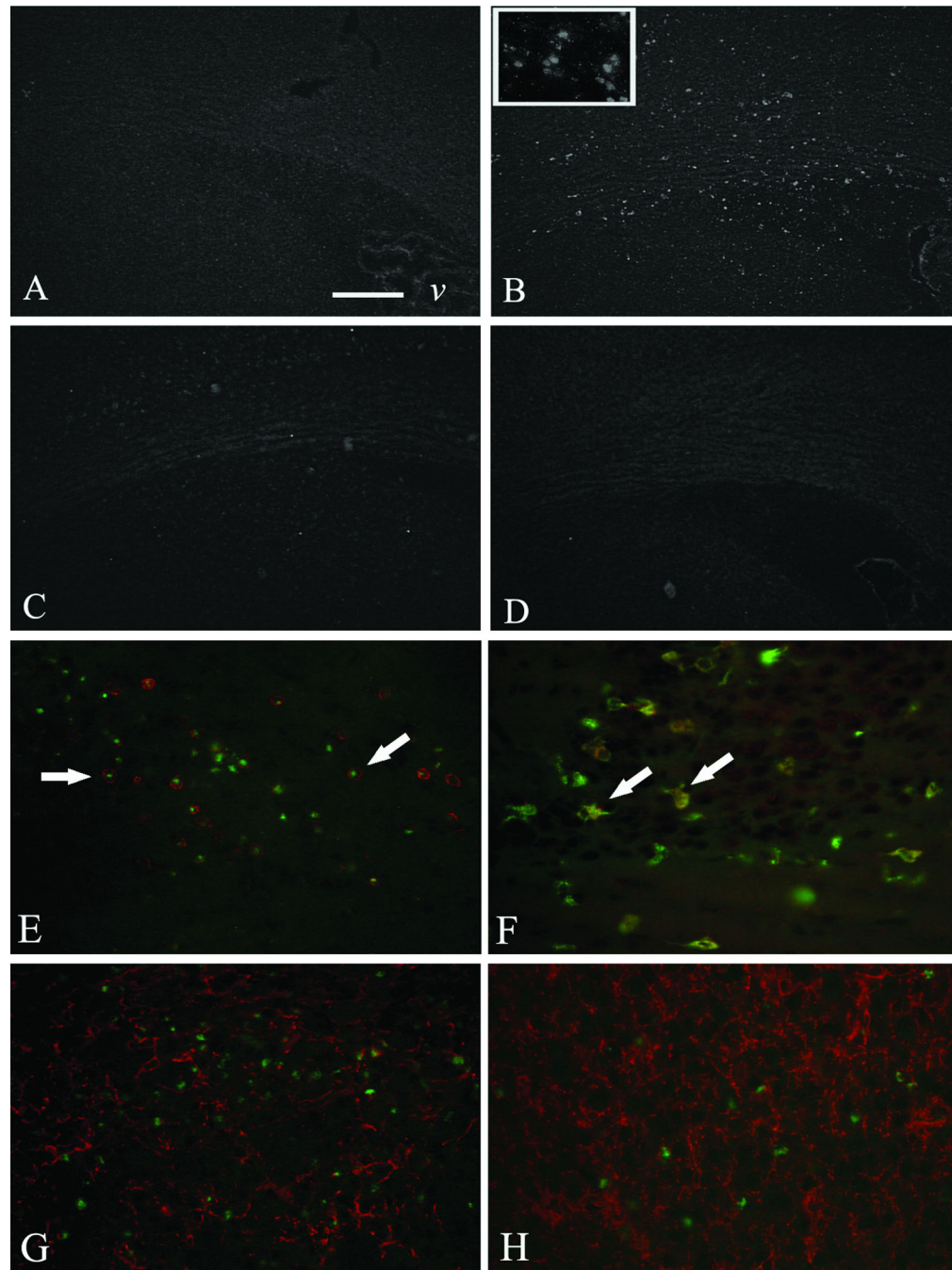
Fig 2.

Dex and Beta suppress LPS-induced iNOS expression in the rat brain. Extensive iNOS+ cells were found in the LPS-treated (B, the white box highlights iNOS+ cells in higher magnification), but not the control rat brain (A), 24 h after treatment. The induction of iNOS was significantly reduced by Dex (C) and Beta (D) treatment. Double-labeling shows that the majority of iNOS+ cells (green) co-localize with CD43+ PMNs (red in E) and ED1+ microglia/macrophages (red in F), but not GFAP+ astrocytes (red in G) or NG+ OL

progenitor cells (red in H). Arrows in E and F delineate co-labeled cells. Scale bar: A, B, C&D: 250 μm; E, F, G&H: 100 μm. v: lateral ventricle.
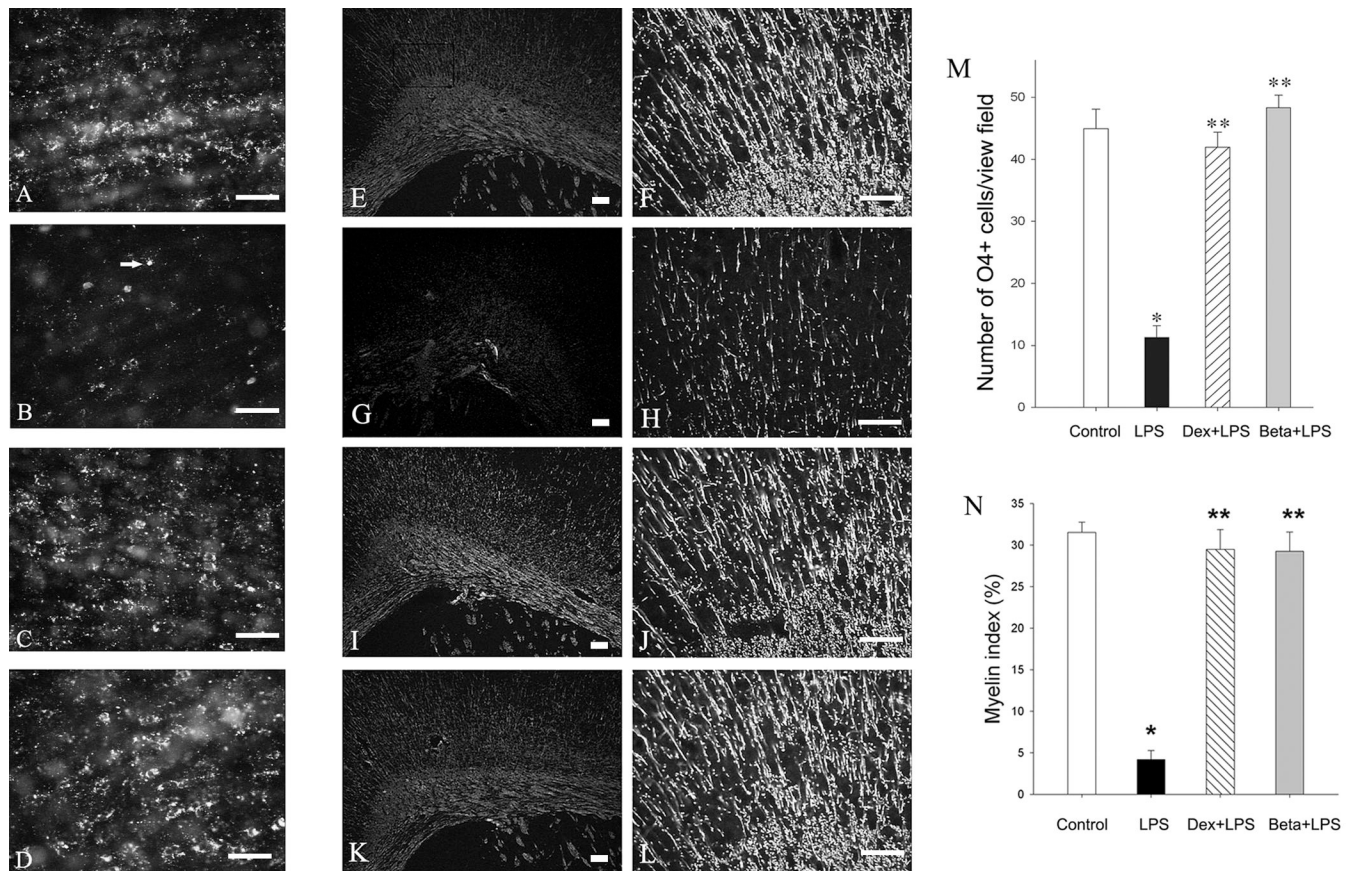
Fig 3.
Dex and Beta attenuate LPS-induced loss of pre-OLs at P8 and hypomyelination at P14. A–
D: O4 immunohistochemistry. Numerous pre-OLs were revealed by O4 immunostaining in
the white matter of the control rat brain (A), while their numbers were significantly reduced
by LPS exposure (B). Some OLs appeared to undergo degeneration (arrow). Dex (C) and
Beta (D) significantly prevented loss of O4+ cells in the white matter after LPS treatment.
B–L: MBP immunohistochemistry. At P14, LPS induced severe hypomyelination as shown
by reduced areas and intensity of MBP immunoreactivity (G&H), which is in contrast to the
rather well-myelinated brain white matter track in the controls (E&F). Dex (I&J) and Beta
(K&L) significantly attenuated LPS-induced hypomyelination. Scale bar: 100 μm. M:
quantification of O4+ cell counting. N: Myelin index. Data are shown as mean±SEM from
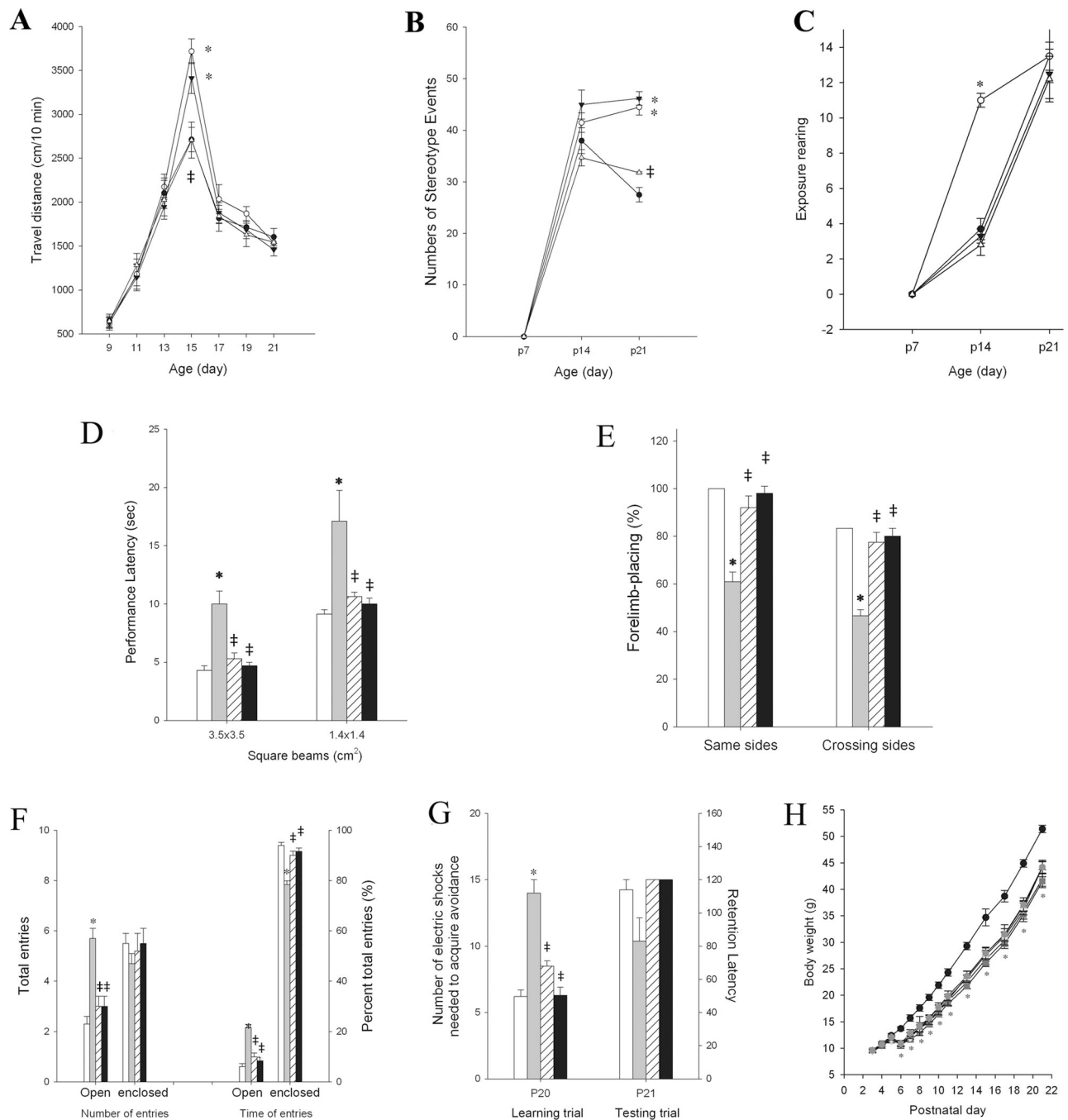eight animals. *p<0.01 vs control; ** p<0.01 vs LPS.

Fig 4.
Effects of corticosteroids treatment on LPS-induced neurobehavioral deficits and body
weight loss. A–C: locomotor activity tests. LPS significantly increased locomotor activity at
P15 (A), stereotype behavior at P21 (B) and exposure rearing at P14 (C). Both
corticosteroids reversed LPS-induced increase in exposure rearing, but only Beta
significantly attenuated LPS-induced increase in locomotor and stereotype activity. D and E:
motor function tests. LPS-treated rats showed impaired motor function, as indicated by
increased time during beam walking (D). LPS also impaired the sensory motor function as

shown by the reduced success rate of vibrissa-elicited forelimb-placing (E). Both steroids showed recovery in beam walking and vibrissa-elicited forelimb-placing tests. F: anxiety test. LPS induced a less anxiety-like behavior (significantly increased number of entries and times spend in the open field during the elevated maze test) in the rats, which was significantly ameliorated by Dex and Beta treatment. G: learning and memory test. LPS-treated rats showed deficits in learning during the passive avoidance test. The learning deficits were significantly ameliorated by Dex and Beta. It should be noted that the time stayed on the safe platform by Dex and Beta-treated rats all exceeded 120 sec (the cut-off value), their retention latency were simply assigned to 120 sec, therefore, no standard errors were obtained from these two groups. H: body weight changes. Neither of the corticosteroids protect against LPS-induced arrest in body weight gain. Further studies showed that Dex and Beta themselves cause delayed body weight gain similar to that by LPS. Line plots in Fig. A, B, C and H: control (●), LPS (○), Dex+LPS (▼), and Beta+LPS ( ); Bar plots in Fig. D, E, F and G: control (open bar), LPS (gray bar), Dex+LPS (striped bar), and Beta+LPS (black

Table 1

Lateral ventricle size index in the neonatal rat brain

Treatment	Ventricle size index (%)	
	Left ventricle	**Right ventricle**
Control	0.49±0.21	0.42±0.18
LPS	4.85±1.03[*]	3.85±0.61[*]
Dex+LPS	1.09±0.57[**]	0.75±0.42[**]
Beta+LPS	1.19±0.21[**]	0.99±0.63[**]

Changes in the lateral ventricle size in the P15 rat brain are estimated by a ventricle size index, which is defined as the ratio between the area of each ventricle and the area of the whole brain section measured by Quantity One software. The left side was injected with LPS or sterile saline.

Data are shown as mean±SD. and analyzed by one-way ANOVA followed by post-hoc Turkey test. A total of 7 rats were used in each group.

[*] $P<0.01$ vs control

[**] $P<0.01$ vs LPS for the same side of ventricle.