MISS ANA PAULA DOMÍNGUEZ RUBIO (Orcid ID : 0000-0003-4499-8397)

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Maternal administration of melatonin exerts short- and long-term neuroprotective effects on the offspring from lipopolysaccharide-treated mice

Ana Paula Domínguez Rubio[1], Fernando Correa[1], Julieta Aisemberg[1], Damián Dorfman[2], María Victoria Bariani[1], Ruth Rosenstein[2], María Zorrilla Zubilete[3], Ana María Franchi[1]

[1]Universidad de Buenos Aires. Consejo Nacional de Investigaciones Científicas y Técnicas. Laboratorio de Fisiopatología de la Preñez y el Parto. Centro de Estudios Farmacológicos y Botánicos (CEFyBO). Facultad de Medicina. Ciudad Autónoma de Buenos Aires. [2]Universidad de Buenos Aires. Consejo Nacional de Investigaciones Científicas y Técnicas. Laboratorio de Neuroquimíca Retiniana y Oftalmología Experimental. Centro de Estudios Farmacológicos y Botánicos (CEFyBO). Departamento de Bioquímica Humana. Facultad de Medicina. Ciudad Autónoma de Buenos Aires. [3]Universidad de Buenos Aires. Consejo Nacional de Investigaciones Científicas y Técnicas. Laboratorio de Neuropsicofarmacología del estrés. Centro de Estudios Farmacológicos y Botánicos (CEFyBO). Departamento de Farmacología. Facultad de Medicina. Ciudad Autónoma de Buenos Aires.

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Corresponding Author:
Dr. Ana María Franchi.
Centro de Estudios Farmacológicos y Botánicos (CEFyBO).
Facultad de Medicina (Universidad de Buenos Aires).
Paraguay 2155, Piso 16.
C1121ABG, Ciudad Autónoma de Buenos Aires. Argentina.
Tel: +54 11 4508 3680 ext 102 - Fax: +54 11 4508 3680 ext 111
E-mail: anafranchi2000@gmail.com

ABSTRACT

Preterm birth is a major contributor to early and delayed physical and cognitive impairment. Epidemiological and experimental data indicate that maternal infections are a significant and preventable cause of preterm birth. Recently, melatonin has been suggested to exert neuroprotective effects in several models of brain injury. Here we sought to investigate whether the administration of melatonin is able to prevent LPS-induced fetal brain damage in a model of lipopolysaccharide (LPS)-induced preterm labor. For this purpose, 15-days pregnant BALB/c mice received intraperitoneally two doses of lipopolysaccharide (LPS) or vehicle, the first one at 10:00 h (0.26 mg/kg) and the second at 13:00 h (0.52 mg/kg). On day 14 of pregnancy, a group of mice was subcutaneously implanted with a pellet of 25 mg melatonin. This experimental protocol resulted in 100% of preterm birth and pup death in the LPS group and a 50% of term birth and pup survival in the Melatonin+LPS group. In the absence of melatonin, fetuses from LPS-treated mothers showed histological signs of brain damage, microglial/macrophage activation, and higher levels of IL-1β, iNOS and nNOS mRNAs as well as increased histone acetyltransferase activity and histone H3 hyperacetylation. In contrast, antenatal administration of melatonin prevented LPS-induced fetal brain damage. Moreover, when behavioral traits were analyzed in the

offspring from Control, Melatonin and Melatonin+LPS, no significant differences were found, suggesting that melatonin prevented LPS-induced long-term neurodevelopmental impairments. Collectively, our results suggest that melatonin could be a new therapeutic tool to prevent fetal brain damage and its long-term consequences induced by maternal inflammation.

1 INTRODUCTION

About 1 in 10 live births is a preterm birth [1]. Despite medical advances in obstetrics and neonatology, preterm birth remains one of the leading causes of perinatal brain injury and subsequent neurological disabilities, such as cerebral palsy, blindness, hearing loss, and cognitive and neurobehavioral disorders [2,3]. Epidemiological and experimental data point towards maternal infection and inflammation as key factors in triggering preterm labor and it has been suggested that at least 40% of all premature deliveries are due to mothers with intrauterine infection [4]. Maternal systemic inflammation could affect the fetal brain development in the short- and long-term, with important implications for the neurological outcome in preterm infants [5]. Indeed, maternal exposure to lipopolysaccharide (LPS), a main component of Gram-negative bacteria cell-wall, elicits a strong inflammatory response by leukocytes and other cells in uterus, placenta and fetal tissues, leading to the production of pro-inflammatory mediators such as cytokines, chemokines, prostaglandins and reactive oxygen and nitrogen species [1,6–10]. Increased production of pro-inflammatory molecules by fetal tissues is known as fetal inflammatory response syndrome [11] which contributes to preterm brain injury [12].

Melatonin (*N*-acetyl-5-methoxytryptamine), the main product of the pineal gland, is a conserved indolamine derived from tryptophan with pleiotropic effects in the neuroimmuno-endocrine system. Melatonin exerts its effect through a variety of mechanisms [13]. Besides its interaction with well-characterized G-protein coupled receptors, melatonin has intrinsic antioxidant properties and acts as a free radical scavenger and broad spectrum antioxidant [13]. Moreover, experimental evidence suggests that melatonin induces increased expression of antioxidant enzymes such as glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase [14,15]. It has been shown that melatonin administration exerts anti-inflammatory effects by regulating cytokine production by immune cells (reviewed in [16]), therefore improving the course of diseases with an inflammatory etiology [17].

Several reports indicate an important role for melatonin in reproductive physiology [18]. Maternal plasma melatonin levels are high during late pregnancy and it has been suggested its contribution to gestation maintenance by stimulating progesterone production [19,20] and maintaining uterine quiescence [21]. More recently, cumulative data suggest that maternal treatment with melatonin has protective effects against fetal brain damage and promotes fetal survival [8,9,22,23]. Indeed, it has been shown that maternally administered melatonin readily crosses the placenta and reaches fetal circulation easily [24], with virtually no toxic effects on fetal development. However, the exact mechanisms by which melatonin exerts neuroprotective effects remain poorly understood.

We have previously shown that maternal administration of melatonin prevented LPS-induced preterm labor in 50% of the cases [9]. Here we sought to investigate whether antenatal administration of melatonin could also prevent the fetal brain damage associated with maternal exposure to LPS. In the present study, we investigated the effect of antenatal

administration of melatonin on fetal brain damage and neurobehavioral outcome in a model of LPS-induced maternal inflammation.

2 MATERIALS AND METHODS

2.1 Reagents

LPS from *Escherichia coli* (serotype 05:B55), anti-β-actin antibody and melatonin were purchased from Sigma Chemical Co. (St Louis, MI, USA). Alexa 568-conjugated donkey anti-goat secondary antibody and Alexa 488-conjugated donkey anti-mouse conjugated secondary antibody were from Molecular Probes (Thermo-Fisher Scientific; Waltham, MA, USA). Anti-acetylated histone H3 antibody, goat polyclonal anti-ionized calcium binding adaptor molecule 1 (Iba-1), mouse monoclonal anti-ED-1, donkey anti-mouse secondary horse radish peroxidase (HRP)-conjugated antibody and donkey anti-goat secondary horse radish peroxidase (HRP)-conjugated antibody were purchased from Abcam Inc. (Cambridge, MA, USA). Western blotting reagents and nitrocellulose membranes (Trans-Blot, 0.45μm) were from Bio-Rad Inc. (Hercules, CA, USA) and molecular weight marker was purchased by GE Healthcare Bio-Science Corp. (Piscataway, NJ, USA). Trizol reagent and ribonuclease inhibitor were from Genbiotech (Buenos Aires, Argentina). Ultrapure water, DTT, RNAse free DNAse I, Moloney Murine Leukemia virus reverse transcriptase (MMLV-RT), random primers, dNTPs and GoTaq DNA polymerase were purchased from Promega (Madison, USA).

2.2 Animals

Female BALB/c mice from our own colony were housed in a standard animal room with food and water *ad libitum*, in controlled conditions of humidity, temperature (21 ± 2°C), and luminosity (200 lux), under a 12 h light/dark lighting schedule (lights on at 7:00 h). For mating, a single male mouse was mated with two nulliparous females of the same strain. Time of pregnancy was determined by visual inspection of the vaginal plug, which was defined as day 0 of pregnancy. Under our animal facility conditions, normal term labor occurred on day 19 of gestation. Animals were killed in a carbon dioxide chamber, and all efforts were made to minimize animal suffering. The experimental procedures reported here were approved by the Animal Care Committee of the Center for Pharmacological and Botanical Studies, National Research Council, and by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) from the School of Medicine, University of Buenos Aires (Nº 1163/2016), and were carried out in accordance.

2.3 Treatments

As shown in Fig. 1, mice were randomly allocated into four experimental groups (Control, Melatonin, LPS and Melatonin+LPS). BALB/c females on day 14 of pregnancy were anaesthetized with ketamine hydrochloride (110 mg/kg) and xylazine hydrochloride (10 mg/kg) and subcutaneously implanted with a single pellet of melatonin (25 mg in 3% w/v vegetable oil) compressed in a metallic punch of 4 mm diameter and 3 mm length or were sham-operated without pellet implantation, as previously described [9]. On day 15 of pregnancy, mice received intraperitoneally (i.p.) two doses of LPS or vehicle, the first one at 10:00 h (0.26 mg/kg in 0.1 ml of sterile saline solution) and the second at 13:00 h (0.52

mg/kg in 0.1 ml vehicle). Animals were closely observed for any signs of morbidity (piloerection, decreased movement, and diarrhea), vaginal bleeding, and preterm delivery. The beginning of preterm delivery was defined by the delivery of the first pup. Normal term labor occurs on day 19 of gestation and the day of parturition was defined as postnatal day 0. On postnatal day 1, all litters were examined externally and sexed. Litters were used to study physical development during lactation. At postnatal day 21, the offspring were weaned and female pups were removed from the cage. Male littermates were housed together and tested sequentially in three behavioral tests at postnatal day 90. The treatment of LPS does not result in living offspring due to their prematurity

A second set of mice were euthanized in a carbon dioxide chamber on day 15 of pregnancy, 2 h after the second injection of vehicle or LPS. Fetuses were immediately removed, and brains were stored in Trizol at -80°C for RT-PCR studies. A third set of mice was euthanized 5 h after the second injection of vehicle or LPS. Fetuses were immediately removed and brains were dissected and stored at -80°C for Western blot and HAT activity analysis, or stored in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C overnight for histological or immunofluorescence assay.

2.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Four fetal brains from the same litter were pooled and homogenized in a glass homogenizer on ice in 1 ml of Trizol reagent and total RNA was isolated according to the manufacturer's recommendations (Invitrogen). Following extraction, 8 µg of RNA were treated with RNAse-free DNAse I to digest contaminating genomic DNA. Total mRNA was synthesized to cDNA using MMLV-RT, random primers and RNAse inhibitors. PCR was

performed with 4 µg of cDNA and specific primers designed using the Primer3 Software package and checked for self-complementarity with OligoCalc Software package. The sequences of primers used in this experiment were as follows: IL-1β forward: 5'-GCAACTGTTCCTGAACTC-3′; IL-1β reverse: 5′-CTCGGAGCCTGTAGTGCA-3′; nNOS forward: 5´-AGCACCTACCAGCTCAAGGA–3´; nNOS reverse: 5´-ATAGTGATGGCCGACCTGAG–3´; iNOS forward: 5´-CACCTTGGAGTTCACCCAGT–3´; iNOS reverse: 5´-ACCACTCGTACTTGGGATGC–3´; β-actin forward: 5´-TGTTACCAACTG GGACGACA–3´; β-actin reverse: 5´-TCTCAGCTGTGGTGGTGAAG–3´. PCR cycle parameters were as follows: for IL-1β, 35 cycles of 94°C 30 s, 55°C 1 min, 72°C 1 min; for nNOS and iNOS, 35 cycles of 94°C 30 s, 60°C 1 min, 72°C 1 min; for β-actin, 30 cycles of 94°C 40 s, 57°C 30 s, 72°C 1 min.

PCR products were loaded into a 2% agarose gel and bands were visualized on a transilluminator after ethidium bromide staining. Images were taken using a digital camera Olympus C-5060 and analyzed using the Image J software package (open source, NIH). The relative mRNA level was normalized to β-actin and results were expressed as relative optical density (IL-1β/β-actin; nNOS/β-actin; iNOS/βactin).

2.5 Nuclear Extract Isolation and Western Blot

Six fetal brains from the same litter were pooled and homogenized in a glass homogenizer on ice in 200 µl of homogenization buffer (10 mMHepes, 0.4 mMKCl, 1X cocktail of protease inhibitors, 10 µM DTT, 10 µM leupeptin, 0.2% v/v PMSF) and left on ice for 20 min. Next, the homogenates were centrifuged at 3000 rpm for 10 min at 4°C and the supernatants were discarded. The pellets were re-suspended in 200 µl of ice-cold 0.2 N HCl

and left on ice for 20 min, with a vigorous vortexing every 3 min. The nuclear extracts were sonicated and centrifuged at 13000 rpm for 10 min at 4°C. The supernatants were removed and neutralized with 1 M Tris-base pH 8. Protein concentration was determined by Bradford assay [25] and supernatant were stored at -80°C until further use. Protein samples (50 µg/lane) were loaded on 6-20% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gradient gel and electrotransferred to a nitrocellulose membrane. Nonspecific binding sites were blocked by incubating the membrane with 5% skim milk in 1X phosphate-buffered saline (PBS) buffer for 1 h. Next, membranes were incubated with the primary antibodies anti-acetylated histone H3 (1:3000) or anti-β-actin (1:4000) overnight at 4°C. The next day, membranes were washed with 0.1% T-PBS (10 mMTris, 100 mMNaCl and 0.1% Tween 20, pH 7.5) and incubated with the appropriate species-specific HRP-conjugated secondary antibodies (1:5000) for 1 h at room temperature. Membranes were developed by chemiluminescence (ECL Western Blotting Analysis System; GE Healthcare). The images for immunoreactive bands were acquired using the ImageQuant blot documentation instrument and analyzed using the Image J software package. The relative protein level was normalized to β-actin and results are expressed as relative optical density (acetylated histone H3/β-actin).

2.6 Histone Acetyl Transferase (HAT) activity

Six fetal brains from the same litter were pooled and homogenized in a glass homogenizer in 200 µl of homogenization buffer (50 mMTris-Base, 150 mMNaCl, 1% Nonidet P40, 0.5% Sodium deoxycholate, 0.1% SDS, pH 7.4). The nuclear extracts were obtained as previously described. HAT activity was estimated with the Activity Colorimetric Assay Kit according to the manufacturer's instructions (Sigma-Aldrich). The reaction was

incubated for 3 hours and absorbance was measured with an ELISA reader at 440 nm. All measurements were performed in duplicate. Enzyme activity is reported in nmol/µg nuclear protein/min.

2.8 Tissue morphology

Fetal brains were fixed immediately in 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C, washed, processed and embedded in paraffin. Sections of 5 µm in thickness were prepared and stained with hematoxylin–eosin (H/E) and then examined and photographed under light microscope Nikon Eclipse 200 connected to a video camera attached to a computer.

2.9 Immunofluorescence

Fetal brains were fixed immediately in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C overnight and transferred to 20% sucrose for 72 h. Brain samples were frozen and 5 µm sections were obtained with a cryostat and mounted onto charged slides. Antigen retrieval was performed in paraffin sections by heating the slides at 90°C for 30 minutes in citrate buffer (pH 6.3). Tissue was permeabilized with 0.1% Triton X-100 in PBS solution for 20 min. Sections were pre-incubated with 2% normal horse serum for 1 h, and then incubated overnight at 4°C with the primary antibody (goat polyclonal anti-Iba-1 (1:500) and/or mouse monoclonal anti-ED-1 (1:200)). After several washings, sections were incubated with the appropriate secondary antibody (Alexa 568-conjugated donkey anti-goat (1:500) or Alexa 488-conjugated donkey anti-mouse (1:500)) for 2 h at room temperature. Speficitiy was confirmed by omitting the primary antibody. Nuclei were stained with Hoechst.

Sections were mounted with an antifade mounting medium (Vectashield®, Vector Laboratories; Burlingame, CA, USA) and viewed using an epifluorescence microscope (Olympus BX-50; Tokyo, Japan), connected to a video camera attached to a computer. Comparative digital images from different samples were obtained using identical exposure time, brightness, and contrast settings.

2.10 Physiological maturation

The following three physical parameters were assessed as day of appearance in three male and three female from each litter during lactation. Pinna detachment: record the age at which time both pinnae are detached (defined as the complete separation of the pinna from the cranium). Incisor eruption: record the age when the upper and lower teeth can be observed. Eye opening: record the age when both eyes are open. Pups were observed daily before afternoon and removed from mothers for observation and immediately returned to their home cage.

2.11 Behavioral evaluation

Only male offspring were tested as adults (postnatal day 90) to avoid the behavioral effects of pubertal hormones and the estrous cycle. All behavioral testings were performed during the light period (between 12:00 and 16:00 h) under dim light illumination. One hour before each test, mice were habituated to the new illumination setting. Two males from each litter were randomly tested in the holding room during four days, first in the open field, then in the elevated plus maze, and finally in the inhibitory avoidance test (first day: day 1 of open field; second day: day 2 of open field test; third day: elevated plus maze test and day 1 of

inhibitory avoidance test; fourth day: day 2 of inhibitory avoidance). Each apparatus was cleaned with 70% ethanol and dried between sessions.

2.12 Open field test

Open-field test was performed using a square chamber (40 X 40 X 450 cm) made of grey polyvinylchloride (PVC). The floor of the open field was uniformly divided into 16 equal sized squares of 10 X 10 cm. Four squares were considered as the center. Mice were exposed to the open field 5 min. Twenty four hours after, mice were re-exposed to the chamber for another 5 min. A video camera were placed 2 m above the maze. Each animal was individually placed in the center of the chamber in each session. The behavioural parameters recorded during 5 min were: (1) locomotion: the number of horizontal lines an animal crossed; (2) rearing: the number of times an animal was standing on its hind legs with forelegs in the air or against the wall; (3) latency to the first entry into the center; (4) number of entries into de center; (5) number of self-grooming. Animals were re-exposed to the open field 24 h after the initial trial. Sessions were recorded using a video camera (Sony DCB-DVD810). Habituation to a novel environment is believed to be one the most elementary forms of non-associative learning, known to depend on hippocampus in which the repeated exposure to the same environment induces a reduction in the exploratory behaviour.

2.13 Elevated plus maze test

Each mouse was placed in the center of a plus-shaped runway elevated 50 cm above the floor and containing four arms (two open without walls and two enclosed by 30 cm high walls) 50 cm long and 10 cm wide. The number of arm entries and the amount of time spent in the open and closed arms are recorded over a 5 min period were scored for each mouse

using a video camera (Sony DCB-DVD810). Arm entry was defined as each time a mice entered an arm with its four paws. Time spent in the open arms and number of entries into opened and closed arms were scored. Immediately after testing, animals were returned to their home cage.

2.14 Inhibitory avoidance test

The apparatus consisted of two equal compartments (60 X 60 X 37 cm) separated by a guillotine door (20 X 20 mm). One was illuminated with a 60 W lamp and the other was dark. In the pre-training session, each mouse was allowed to explore the illuminated compartment for 30 s and then the dark compartment for others 30 s (guillotine door was closed). In the training session, each animal was placed in the illuminated compartment with the sliding door opened. The latency (maximum of 300 s) to enter the dark compartment (all four paws) was measured. As soon as the animal was in the dark compartment, the door was closed and a foot shock (0.5 mA, for 3 s) was delivered. The floor of the dark compartment consists of a stainless steel grid, through which a continuous current can be delivered. The animal was removed from dark compartment 10 s after the foot shock. In the test session, performed twenty four hours after the training session, each animal was placed in the illuminated compartment with the sliding door opened. The latency to enter the dark compartment was recorded. Twenty four hours later, retention was evaluated. Each animal was placed in the lit compartment again facing the wall opposite to the dark compartment, ant the time it took for the mice to cross to that side was recorded as retention latency. If the animal did not cross in a period of 300 s, it was removed and returned to its cage. No shock was delivered in the retention test.

2.15 Data analysis and statistical procedures

The data obtained from the fetal brain were analyzed by means of a two-way ANOVA with interactions (LPS and melatonin) in a completely randomized design. The assumptions of normality and homoscedasticity were studied analytically by the Shapiro–Wilks and the Levene tests, respectively. The data of physical developments, latency and center square entries (open field test), and the data of elevated plus maze test were analyzed by a two-way nested ANOVA (treatment, litter) in a completely randomized design. The assumptions of normality and homoscedasticity were studied analytically by the Shapiro–Wilks test and the Levene test, respectively. The data of line crossing, rearing and grooming (open field test) and latency (inhibitory avoidance test) was analyzed by four-way nested ANOVA with repeated measures (treatment, litter, pup and time) in a completely randomized design. The assumptions of normality, homogeneity of variances, and sphericity were studied analytically by Shapiro–Wilks test, Box test, and Mauchly test, respectively. We considered the pregnant female or the litter as the experimental unit. We used more than one animal from each litter to study physical development and behavioral tests in order to better control “error” variation. Moreover, using sub-samples allowed us to avoid increasing the number if pregnant mice (nested ANOVA). Tukey’s test was used for post-hoc comparisons. Results were considered significant at $p<0.05$. Means with a common letter are not significantly different ($p>0.05$). All statistical analyses were performed using the statistical program Infostat (Universidad de Córdoba, Córdoba, Argentina).

3 Results

Figure 2 shows the effect of maternal LPS in the absence or presence of melatonin on fetal brain structure. Maternal exposure to LPS provoked a disorganization of the fetal cerebral parenchyma and meninges. Moreover, an increased leukocyte infiltration was observed in fetal brains from LPS-treated mothers in the absence of melatonin. These changes were substantially reduced in animals treated with Melatonin+LPS, in which the brain structure was preserved and leukocyte infiltration was lower.

Microglia/macrophages in the fetal brain were analyzed by Iba-1 and ED1 immunostaining, as shown in Figure 2 (panels E to H). An increase in Iba-1 and ED-1 immunoreactivity was observed in fetal brains from animals treated with LPS in the absence of melatonin, whereas melatonin decreased the effect of LPS on these parameters. Since maternal administration of LPS induced the activation of fetal microglial/macrophage cells, we proceeded to analyze the mRNA levels of pro-inflammatory mediators in these fetal brains and whether the co-administration of melatonin exerted anti-inflammatory effects. IL-1β, inducible nitric oxide synthase (iNOS) and neuronal NOS (nNOS) mRNA levels were assessed at 2 h after the last administration of LPS. As shown in Fig. 3, maternal administration of melatonin significantly prevented the increase in IL-1β (Fig. 3A), iNOS (Fig. 3B) and nNOS (Fig. 3C) mRNA levels in the fetal brains from LPS-treated mothers.

In order to analyze the involvement of epigenetic changes in the effect of melatonin, fetal brains were collected at 5 h after the last maternal injection of LPS and tissues were processed for histone acetyltransferase (HAT) activity assay and the acetylation pattern of histone H3 determination. Maternal administration of LPS increased the fetal cerebral HAT enzymatic activity and melatonin prevented this effect (Fig. 4A). LPS-induced changes in

HAT activity led to an increased acetylation pattern of histone H3, which was prevented by maternal administration with melatonin (Fig. 4B and 4C).

Maternal infections during pregnancy interfere with normal fetal brain development and may have long-lasting consequences on the offspring. Therefore, we proceeded to study different parameters of physical and behavioral development in the offspring of animals treated with LPS and/or melatonin during pregnancy. As already mentioned, our model of maternal administration of the endotoxin induces 100% of preterm birth and pup death [9,26] with melatonin preventing these deleterious effects in 50% of the cases [9]. Therefore, we lacked the group of mice from LPS-treated mothers for the following studies. The offspring from Melatonin+LPS mothers showed no differences in physical development parameters (the day of pinna detachment, incisor eruption and opening of the eyes) when compared to the offspring from Control or Melatonin-treated animals (Table 1). In the following experiments, we performed behavioral tests in said progeny when they reached adulthood. In the Open Field test, the offspring of Melatonin+LPS treated mice showed no differences in horizontal activity or “number of line crosses” (Fig. 5A), vertical activity or “number of rearing” (Fig. 5B), latency to center (Fig. 5C), center square entries (Fig. 5D) or number of grooming (Fig. 5E) when compared to the Control or Melatonin-treated animals. Next, we performed the Elevated Plus Maze test (EPM) which evaluates anxiety-like behavior [27]. As shown in Fig. 6, there were no significant differences in the number of closed arms entries (Fig. 6A), open arms entries (Fig. 6B) and the time spent in the open arms (Fig. 6C) among all the experimental groups. Finally, we performed the Inhibitory Avoidance Test (PA) which is a fear-motivated test classically used to assess memory and learning [28]. There

were no significant differences among groups in the latency to enter into the dark compartment in the testing day were observed.

4 DISCUSSION

The present study demonstrates that antenatal maternal administration of melatonin prevents LPS-induced damage to the fetal brain. Prophylactic treatment with melatonin resulted not only in short-term beneficial effects, such as a preservation of brain structure, decreased microglial/macrophage activation, reduced production of pro-inflammatory mediators and prevention of epigenetic changes in the fetal brain; but also in long-term effects, such as preservation of behavioral profile in the offspring from LPS-treated mothers. Despite advances in perinatal and neonatal care, the mortality and morbidity due to brain injuries in premature babies remain significant [2,3]. Maternal infection and inflammation are associated not only with an increased risk of preterm labor [29], but also have a critical role in fetal brain damage [1,30]. Infection-induced activation of the maternal immune system leads to a fetal neuroinflammatory response that disrupts the normal fetal central nervous system development with long-term consequences for the offspring, such as periventricular leukomalacia, cerebral palsy and other motor and/or cognitive neurodevelopmental disorders [1,31,32]. Therefore, developing new strategies to protect the immature brain against injury during perinatal and neonatal periods is of paramount importance.

Previous results from our lab have shown that LPS administration to BALB/c pregnant mice induced 100% of premature parturition and intra-uterine fetal death without affecting maternal survival [9,33]. However, antenatal treatment with melatonin prevented in 50% of cases the initiation of preterm labor and protected the offspring from LPS-induced

deleterious effects, since they were born alive and their body weights did not differ from control mice [9]. Moreover, the postnatal physical development (pinna detachment, incisor eruption and eye opening) was similar to control animals. Chen et al. [22] have shown that maternally administered melatonin resulted in fetal protection against LPS-induced intrauterine death and intrauterine growth retardation. In agreement with other reports [6,7] increased levels of IL-1β mRNA were observed in the fetal brain from mothers treated with the endotoxin, whereas antenatal administration of melatonin exerted neuroprotective effects by reducing the LPS-induced expression of IL-1β in fetal brains. Accordingly, it has been demonstrated that melatonin downregulates IL-1β expression in LPS-stimulated RAW264.7 macrophage [34] and CRL1999 human vascular smooth muscle [35] cell lines. Conversely, Wong et al. [10] found that melatonin downregulates the LPS-induced increase of serum IL-1β levels but was unable to reduce the expression of this cytokine in neonatal rat brain; whereas Xu et al. [8] reported that melatonin differentially regulated the expression of pro-inflammatory cytokines in several maternal tissues but failed to observe an upregulation of IL-1β in the fetal brain of LPS-treated mothers. These discrepancies could be explained by the different concentrations of LPS as well as the different administration schemes of melatonin used in each study.

Excessive fetal brain production of free radical as a result of hypoxia-ischemia or inflammation is associated with tissue damage due to lipid peroxidation, nucleic acid damage, protein nitration/oxidation, mitochondrial dysfunction and eventually, cell death [36]. Large amounts of NO, mainly generated by iNOS, during inflammatory processes can be toxic [37]. The developing brain is particularly sensitive to oxidative damage due to its limited antioxidant capacity. Maternal exposure to LPS increased the fetal brain mRNA levels of

iNOS and nNOS and melatonin prevented this effect. Our results agree with several studies showing that melatonin inhibited the LPS-induced expression of different NOS isoforms in murine placenta [38], murine liver [39], neural stem cells [40], rat optic nerve [41], rat liver [42,43], rat lung [43], and neonatal rat brain [10]. Similarly, prophylactic antenatal administration of melatonin has been shown to have neuroprotective effects against oxidative damage in models of hypoxic-ischemic/reperfusion brain injury in fetal rat [44–46], mouse [47] and sheep [48,49]. In accordance to these studies, we observed that maternal administration of melatonin restored to control levels the LPS-induced activation of microglial/macrophage cells in fetal brains. Overall, the lower expression of pro-inflammatory mediators and the lower activation of glial cells resulted in a preservation of the histological structure of the fetal brain, providing further support the potential use of melatonin as neuroprotective agent in peri- and neonatology.

It has been suggested that maternal exposure to LPS might increase the risk of neurodegenerative disorders in the offspring not only by inducing acute brain damage but also by long-lasting epigenetic changes [50]. Li et al. [50] have recently reported that the offspring from mice that received a dose of LPS during pregnancy presented lower acetylation levels of histones H3 and H4 in the hippocampus at 12 and 22 months old when compared to age-matched controls. Similarly, Cao et al. [51] reported that *in utero* neuroinflammation altered the microglial expression of histone deactylases (HDAC) in fetal sheep. When exposed *in vitro* to a second challenge with LPS, these microglial cells responded differently than the microglial cells obtained from naïve sheep [51]. Our results show that LPS-induced maternal inflammation resulted in an increased activity of histone acetyltransferases (HAT) together with higher levels of histone H3 acetylation in the immature brain, whereas melatonin treatment restored both the HAT activity and the histone

H3 acetylation pattern to control levels. Conversely, Sharma et al. [52] have reported that melatonin induced histone H3 hyperacetylation in C17.2 neural stem cells only at physiological concentrations and increased mRNA expression of HDAC3, HDAC5 and HDAC7. Similarly, Yang et al. [53] have shown that melatonin exerted neuroprotective effects in a model of ischemia-reperfusion injury by upregulating the expression of silent information regulator 1 (SIRT1), a type of histone deacetylase. In contrast, we did not observe any effect of melatonin *per se* in fetal brain HAT activity or histone H3 acetylation pattern. Therefore, the exact role on melatonin in epigenetic modulation remains elusive and further studies are warranted.

Maternal inflammation adversely affects the fetal brain development and has long-lasting consequences in the adult life [1,7,30]. However, the effects that prenatal exposure to inflammation might have later in life depend on many factors, being a crucial one the developmental stage of the immature brain at the time of injury. Thus, several reports indicate behavioral alterations in the offspring of LPS-treated mothers, such as increased anxiety and reduced exploratory activity [54–58] as well as significant impairments of spatial learning and memory [50]. However, conflicting reports exist suggesting that maternal exposure to LPS might result in a diminished anxiogenic behavior [59,60]. A caveat of our LPS-induced preterm birth model is that the endotoxin induced 100% of preterm birth and pup death in the absence of melatonin. Therefore, we were unable to compare the behavioral changes produced by maternal exposure to LPS in the offspring from LPS-treated mice with those from Control, Melatonin or Melatonin+LPS group. However, we decided to study whether the offspring from Melatonin+LPS treated mice show differences in several behavioral traits to those from Control or Melatonin treated animals. Interestingly, we

observed that the offspring from Melatonin+LPS mice behaved similarly to those from Control or Melatonin groups in the tests of Open Field and Elevated Plus Maze, suggesting that these animals showed the same exploratory activity and similar levels of anxiogenic behavior. Prenatal stress has been proposed as a risk factor for cognitive alterations in the offspring, such as impaired associative memory [61]. However, the offspring from mothers treated with melatonin and LPS showed the same degree of associative memory in the test of Passive Avoidance than those from control or melatonin only treated mothers. Overall, these results suggest that antenatal treatment with melatonin could prevent the behavioral changes in the offspring associated with LPS-induced maternal inflammation [54–58]. Since melatonin is continuously released from the pellet, it may blunt the circadian rhythm of melatonin in the mother and the fetus. However, taking into account that these test were done at 90 days, it can be presumed that the animals recovered their circadian function at this time.

Despite the increasing amount of evidence suggesting a neuroprotective role of melatonin during maternal infection/inflammation [9,10,22] and intrauterine fetal growth restriction [23,48], except for one small on-going study [62], no large-scale completed randomized controlled trials have been undertaken so far [63]. The results presented here provide further support to the necessity of developing such clinical trials.

Collectively, our results provide evidence that antenatal administration of melatonin reduces fetal neuroinflammation and prevents behavioral changes in the offspring in a model of endotoxin-induced maternal inflammation.

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Author contributions

Ana Paula Domínguez Rubio contributed to concept/design, acquisition of data, data analysis/interpretation. Damián Dorfman and María Victoria Bariani contributed to data acquisition/interpretation. Fernando Correa provided reagents and contributed to drafting the manuscript. Ruth Rosenstein, María Zorrilla Zubilete and Ana María Franchi contributed to concept/design, data analysis/interpretation, and approval of the manuscript.

Conflict of interests

None to declare.

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Legends to Figures

Fig. 1. Experimental protocol of the four groups. BALB/c mice on day 14 of pregnancy were subcutaneously implanted with a single pellet of melatonin or were sham operated. The next day, mice were i.p. injected with two doses of LPS or vehicle. Sham-operated mice treated with LPS delivered prematurely on the night of day 15 of pregnancy. Mice in Control (sham-operated, vehicle), Melatonin only (melatonin pellet-implanted, vehicle) and 50% of Melatonin+LPS (melatonin pellet-implanted, LPS) groups delivered in the night of day 18 or the morning of day 19.

Fig. 2. Photomicrographs showing representative Hematoxylin-Eosin (panels A to D), and Iba-1/ED-1/Hoescht (panels E to H). In panels A to D, white arrows show infiltrating leukocytes whereas the white bars indicate the meninges. Scale bar: 50 µm. In panels E to H, white arrows show Iba-1 positive cells whereas arrowheads show ED-1/Iba-1 double positive cells. Scale bar: 100 µm. Panels I and J show the quantification of Iba-1 positive

cells and ED1/Iba-1 double positive cells per section, respectively. Data are mean ± SEM (n = 7 fetal brains per group). Different letters denote significant differences. A‡B‡C, P<0.05.

Fig. 3. Effect of maternal melatonin and LPS administration on fetal brain IL-1β (panel A), iNOS (panel B) and nNOS (panel C) mRNA levels. Fetal brains were collected two hours after the second injection of vehicle or LPS, total RNA was extracted and RT-PCR was performed for each experimental group. In the absence of melatonin, LPS significantly increased IL-1β, iNOS and nNOS mRNA levels, whereas melatonin (which had no effects *per se*) significantly decreased LPS effects. Data are mean ± SEM (n=10). Different letters denote significant differences. A‡B, *P*<0.05.

Fig. 4. Effect of maternal melatonin and LPS administration of histone acetyltransferase (HAT) activity (panel A) and histone H3 acetylation pattern (panels B and C). Fetal brains were collected five hours after the second injection of vehicle or LPS, total protein was extracted and processed accordingly to HAT activity or Western blotting assay for each experimental group. In the absence of melatonin, LPS significantly increased HAT activity and histone H3 acetylation levels, whereas melatonin (which had no effects *per se*) significantly decreased LPS effects. Data are mean ± SEM (n=8-12). Different letters denote significant differences. A‡B, *P*<0.05.

Fig. 5. Behavioral parameters of the offspring from melatonin and LPS-treated mothers. Panels A to E represent different parameters of locomotor activity and habituation memory obtained with the Open Field test. No statistical significant differences among groups were

observed on the same day of testing. P>0.05. However, all groups presented statistical significant differences between days 1 and 2 of testing (Panels A, B and E). Data are mean ± SEM (n=14). Different letters denote significant differences. A≠B, *P*< 0.05.

Fig. 6. Anxiety parameters of the offspring from melatonin and LPS-treated mothers. Panels A to C represent different parameters of anxiety-related behavior measured with the Elevated Plus Maze test. No statistical significant differences among groups were observed. *P*>0.05.

Fig. 7. Associative memory of the offspring from melatonin and LPS-treated mothers. Panel A quantifies the time spent in the light chamber before entering the dark chamber in days 1 and 2 of testing, as a measure of the associative memory in the Inhibitory Avoidance test. No statistical significant differences among groups were observed. *P*>0.05.

Table 1. Effect of maternal melatonin and LPS administration on physical developmental parameters during lactation. No differences were observed in physical developmental parameters such as time of pinna detachment, incisor eruption or eye opening among mice from Control (sham-operated, vehicle), Melatonin only (melatonin pellet-implanted, vehicle) and Melatonin+LPS (melatonin pellet-implanted, LPS) groups. Data are mean ± SEM (n=10, *P*>0.05).

Table 1. Effects of prenatal exposure to melatonin and LPS on physical development.

	Pinna detachment (day)	Incisor eruption (day)	Eye opening (day)
Control	4±0	10±0	14±0
Melatonin	4±0	10±0	14±0
Melatonin+LPS	4±0	10±0	14±0