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Melatonin modulates neonatal brain inflammation through ER stress, autophagy and miR-34a/SIRT1 pathway

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Running title: Melatonin in neonatal brain inflammation

Abstract

Maternal infection/inflammation represents one of the most important factors involved in the etiology of brain injury in newborns. We investigated the modulating effect of prenatal melatonin on the neonatal brain inflammation process resulting from maternal intraperitoneal (i.p.) lipopolysaccharide (LPS) injections. LPS (300 μg/kg) was administered to pregnant rats at gestational days 19 and 20. Melatonin (5 mg/kg) was administered i.p. at the same time as LPS. Melatonin counteracted the LPS sensitization to a second ibotenate-induced excitotoxic insult performed on postnatal day (PND) 4. As melatonin succeeded in reducing microglial activation in neonatal brain at PND1, pathways previously implicated in brain inflammation regulation such as endoplasmic reticulum (ER) stress, autophagy and silent information regulator 1 (SIRT1), a melatonin target, were assessed at the same time-point in our experimental groups. Results showed that maternal LPS administrations resulted in an increase in CHOP and Hsp70 protein expression and eIF2α phosphorylation, indicative of activation of the unfolded protein response consequent to ER stress, and a slighter decrease in the autophagy process, determined by reduced lipidated LC3 and increased p62 expression. LPS-induced inflammation also reduced brain SIRT1 expression and affected the expression

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of miR-34a, miR146a and miR-126. All these effects were blocked by melatonin. Cleaved-caspase-3 apoptosis pathway did not seem to be implicated in the noxious effect of LPS on the PND1 brain. We conclude that melatonin is effective in reducing maternal LPS-induced neonatal inflammation and related brain injury. Its role as a prophylactic/therapeutic drug deserves to be investigated by clinical studies.

KEYWORDS

melatonin, inflammation, SIRT1, endoplasmic reticulum stress, autophagy, miRNA, neonatal brain injury

1 | INTRODUCTION

Systemic inflammation/infection represents one of the main events in the pathogenesis of brain injury in both term and preterm infants.[1] Preterm infants frequently show high concentrations of pro-inflammatory cytokines in amniotic fluid and fetal blood, particularly those with chorioamniotitis[1] and vasculitis.[2] These pathological conditions are clearly associated with an increased risk for intra-partum cerebral failure, cerebral palsy and permanent neurodevelopmental handicaps.[1]

In recent years, considerable evidence has demonstrated that inflammation is linked to endoplasmic reticulum (ER) stress, characterized by the accumulation of unfolded proteins in the ER that triggers a series of signal-transduction events known as unfolded protein response (UPR). The aim of UPR is to overcome the stressful condition and restore ER homeostasis,[3] and is initiated by the activation of three specific ER stress sensors: PERK (PKR-like ER kinase), ATF-6 (activating transcription factor 6) and IRE1 (inositol-requiring enzyme 1). Through the UPR, cells inhibit translation, reverse translocation, and activate ER-specific ubiquitination enzymes (the ERAD response).[3] The UPR also leads to the activation of an inflammatory response through the induction of inflammatory cytokines.[4] This is triggered by

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both the suppression of IkB translation and subsequent NFkB activation,[5] and activation of the AP-1 (activator protein-1) pathway.[6] At the same time, the UPR causes the activation of the pro-apoptotic factor CHOP/GAD153, a member of the C/EBP family of transcription factors expressed at low levels under physiological conditions but strongly induced in response to different forms of stress. CHOP may lead to the activation of the mitochondrial pathway of apoptotic cell death for removal of the affected cell(s).[7]

UPR is strictly connected to autophagy, a dynamic physiological process that triggers the self-digestion of damaged organelles and proteins. In stressed cells, autophagy can support cell adaptation and survival, but when protein and organelle turnover overwhelms the adaptive capacity, the cell dies. During development, autophagy may have additional important functions, including the adaptation to starvation occurring during fetal-to-neonatal transition.[8] Autophagy also plays a role in inflammation and adaptive immune mechanisms by regulating the secretion of IL-1β and possibly other pro-inflammatory factors.[9] Through these mechanisms, autophagy potentially influences disease pathogenesis. Strategies aimed at modulating the UPR and autophagy may lead to therapeutic interventions for diseases associated with inflammation, including brain injury.

Melatonin (*N*-acetyl-5methoxytryptamine) is a versatile and ubiquitous molecule, well known as a potent indirect antioxidant and direct free radical scavenger.[10] Melatonin has a pronounced protective effect in several models of perinatal brain damage attributed, at least in part, to its antioxidant and free radical scavenger properties.[10] In addition, melatonin regulates energy metabolism[11] and immune function[12], acting as an immunostimulant under basal conditions and as an anti-inflammatory in the presence of an exacerbated immune response.[12] Recently, we reported that melatonin protected neonatal rats from ischemic brain damage by reducing ER stress and preserving the expression of SIRT1, a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase.[13] The latter effect appears to be of

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particular relevance since enhanced SIRT1 activity has been found to protect against various neurodegenerative disorders[14] and to inhibit NF-kB signaling and NF-kB-dependent pro-inflammatory gene expression (i.e. IL-β).[15]

SIRT1 expression and inflammatory pathways are also modulated by microRNAs (miRNAs), small non-coding RNAs (18-24 nucleotide long), a new paradigm of post-transcriptional gene expression. MicroRNAs are able to modulate gene expression by targeting mRNA and suppressing protein expression. Single miRNAs can simultaneously regulate a multitude of targets and biological networks and have been linked to all known fundamental biological pathways. Deregulation of miRNAs may contribute to the development of human diseases.[16] Among the various miRNAs, miR-34a was found to inhibit the expression of SIRT1 by repressing the translation process,[17] whereas miR-146a and miR-126 have been associated to the regulation of inflammatory processes. Inflammation-related targets of miR-146a include IL-1 receptor-associated kinase-1 (IRAK1), TNF receptor-associated factor 6 (TRAF6) and IL-1β. MiR-126 regulates the expression of vascular adhesion molecules and is implicated in ER stress expression since it can modulate the heat stress protein Hsp70.[18]

In the present study, we investigated the effects of maternally administered melatonin on LPS-induced brain cellular stress. We report here that maternal lipopolysaccharide (LPS) sensitizes the immature rat brain to a second postnatal excitotoxic injury. Furthermore, we show that LPS induces a brain inflammatory response at postnatal day-1 (PND1) associated with modulation of UPR, autophagy and miR-34a/SIRT-1 pathway. Melatonin completely reverted the LPS effects.

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2 | METHODS

2.1 | Animals and drugs

All experimental procedures were carried out in compliance with the European Community Commission directive guidelines (86/609/EEC) and have been approved by the "Val de Loire" ethical committee (n°00022.01). Time-pregnant Wistar rats were purchased from CERJ (Le Genest, France). They had free access to food and water and were bred at 22°C with a normal light cycle. LPS (E. coli, serotype 055:B5; Sigma-Aldrich, MO, USA) was diluted in saline (LPS vehicle), and was injected intraperitoneally (i.p.) at the dose of 300 µg/kg to pregnant rats at gestational day (GD) 19 and 20. Melatonin was dissolved in saline containing 2% ethanol (melatonin vehicle), and injected i.p. at the dose of 5 mg/kg to pregnant rats at GD19 and GD20, in the opposite abdominal side to LPS injections.

The experiment included the following experimental groups: 1) LPS and melatonin vehicle-treated controls (Control); 2) Melatonin 5 mg/kg and LPS vehicle (Mel); 3) LPS 300 µg/kg and melatonin vehicle (LPS); 4) LPS 300 µg/kg plus melatonin 5 mg/kg (LPS+Mel).

2.2 | Ibotenate-induced excitotoxic lesions

Ibotenate (Tocris, Bristol, UK), a glutamate agonist, was diluted in PBS containing 0.02% acetic acid and was injected at post natal day (PND) 4 in pup brains under anesthesia. Twelve pups were provided for each experimental group. As previously described,[19-20] injections were performed under a warming lamp with a 26-gauge needle on a 50-µl Hamilton syringe mounted on a calibrated microdispenser. The needle was inserted 2 mm under the external surface of the scalp skin in the fronto-parietal area of the right hemisphere, 2 mm from the midline in the lateral–medial plane and 3 mm from the junction between the sagittal and lambdoid sutures in the rostrocaudal plane. Two 1 µl boluses (5 µg of ibotenate) were

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injected at a 30 second interval. In all cases, the tip of the needle reached the periventricular white matter. After the injections, the pups were returned to their dams.

At PND8, animals were sacrificed by decapitation. Brains were rapidly removed from the skull and immersed in a 4% formaldehyde solution for 4 days at room temperature. After dewatering in successive baths of 100% ethanol and xylene for 24 hours, brains were embedded in paraffin and 16-µm thick coronal sections were performed. The maximal diameter in the sagittal axis of the lesion was measured on histological sections stained with cresyl violet, distinguishing cortical and white matter levels. Lesion size was determined by two independent investigators, blind with respect to the treatment status of the animal from which tissue had been taken.

2.3 | Immunofluorescence staining

Newborn rats were sacrificed at PND1 by decapitation, 48 hours after the last i.p. maternal injections. Brains were quickly removed from the skull and were post-fixed in 4% paraformaldehyde overnight at 4°C. After 2 days in 10% sucrose- 0.12M phosphate buffer solution, brain was embedded in a cooled 10% sucrose - 7.5% gelatin solution before freezing. Finally, brains were cut coronally in 10 µm-thick sections.

The following primary antibodies were used for immunostainings: Anti-Iba-1 (Rabbit, 1:2000, Wako, 019-197414), Anti- inducible NO synthase (iNOS) (Mouse, 1:200, BD Transduction Laboratories, 610328) and Anti- Cleaved Caspase-3 (CC3) (Rabbit, 1:300, Cell Signaling Technology, #9661).

The buffer for the antibody dilution contained 1X PBS with 1% donkey or goat serum according to secondary antibody plus 0.4% Triton X-100 to permeabilize cell membrane. After 3 rinses in 1X PBS, the antigen blocking was performed with 5% serum for 45 min.

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Then, the primary antibody was incubated overnight at room temperature. The second day, the appropriate secondary antibody was applied for 90 min (1:500 dilution for the Cy3 and Alexa 488 secondary antibodies). A counterstain by DAPI (1:10000, Sigma-Aldrich, MO, USA) labeling the nucleus was performed at the end of immunofluorescence protocol.

After the immunofluorescent staining procedure, pictures at magnification 20 focusing on cingulum were performed in each experimental group (N=5 brains/ group). Cingulum region was chosen because of its particular richness in microglia at this brain developmental stage.[21] Pictures were analyzed with NIH Image J software.

2.4 | Western blot analysis

Newborn rats were sacrificed at PND1 by decapitation, 48 hours after the last i.p. maternal injections. Brains were dissected on ice, frozen in isopentane cooled to –35°C with dry ice, and stored at –80°C until use.

Brains were sonicated in 0.4 mL lysis buffer containing 10 mM Tris, 10 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride and a complete protease inhibitor cocktail (Roche, 1 697 498) using an Ultrasonic Liquid Processor XL Sonicator (Heat SystemUltrasonic Inc.). Homogenates were centrifuged for 5 min at 18,500g (4°C) and the supernatants aspirated and stored at -80°C until used. After mixing with sodium dodecyl sulfate gel-loading buffer and heating 4 min at 95°C, samples (50 µg protein) were electrophoresed onto sodium dodecyl sulfate-polyacrylamide gel and proteins transferred to a PVDF membrane. ColorBurst™ electrophoresis marker (3 µL/gel, Sigma, C1992) was used for qualitative molecular mass determinations and for visual confirmation of blot transfer efficiency. Blots were then blocked with non-fat dry milk in TBS-T (10 mM Tris, 150 mM NaCl, pH 7.6, plus 0.1% Tween-20) and probed with the following primary antibodies: anti-CHOP (1:500, monoclonal; Santa Cruz Biotecnology, sc-7351); anti phospho(p)-eIF2α

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(1:500, monoclonal; Santa Cruz Biotecnology, sc-101670); anti-GRP78 (1:500, monoclonal; Santa Cruz Biotecnology, cs-166490); anti-Hsp70/Hsc70 (1:500, monoclonal; Santa Cruz Biotecnology, sc-24); anti-LC3 (1:1000, polyclonal; Cell Signaling Technology, #2775); anti-Beclin 1 (1:500, monoclonal; BD Transduction Laboratories, 612113); anti-p62 (1μg/mL, polyclonal; Sigma-Aldrich, P0067); anti-SIRT1 (1:1000, polyclonal; Santa Cruz Biotecnology, sc-15404). A monoclonal antibody against β-actin (1:4000, Santa Cruz Biotechnology, sc-8432) was used as control for protein gel loading. The relative intensity of the bands detected by Western blot was analyzed using Image J 1.45 software. Data were normalized to β-actin and expressed as percent of control.

2.5 | Quantitative Real Time PCR (qRT-PCR) for mature microRNAs analysis

We used the mirVana isolation kit (Applied Biosystems, Foster City, CA) following the manufacturer's recommended protocol to isolate microRNAs from the same brain homogenate supernatants used for western blot analysis. RNA was stored at -80 °C until use. Rat miR-34a, miR-126, miR-146a and U6 (reference miRNA) expressions were evaluated by using the TaqMan MicroRNA assay (Applied Biosystems). The TaqMan MicroRNA reverse transcription kit was used to reverse transcribe microRNA. Subsequently RT-qPCR was performed in 20 μL of PCR mix containing 1 μL of 20x TaqMan MicroRNA assay, which contained PCR primers and probes (5′-FAM), 10 μL of 2x TaqMan Universal PCR Master Mix No Amp Erase UNG (Applied Biosystems) and 5 μL of reverse-transcribed product. The reaction was first incubated at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The qRT-PCR was performed on a ABIPRISM 7500 Real Time PCR System (Applied Biosystems). Data were analyzed by a 7500 system software (1 1.4.0) with the automatic comparative threshold (Ct) setting for adapting baseline. Detection thresholds were set at 35 Ct. The relative amounts of miR-34a, miR-146a and miR-126 were calculated

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using the Ct method:

$\Delta Ct = Ct(miR\text{-}34a/miR\text{-}146a/miR\text{-}126) - Ct(U6)$; $2^{\Delta Ct}$

Results are expressed in the figures as fold induction relative to control values.

2.6 | Data analysis

Statistical analyses were performed by One-way ANOVA using the Prism Computer program (GraphPad Software Inc.). The Newman-Keuls multiple comparison test was used to determine differences between single treatment groups. $P<0.05$ was considered significant.

3 | RESULTS

3.1 | Melatonin effect on a second ibotenate-iduced excitotoxic insult at PND4

To assess the conditioning or sensitizing effect of prenatal LPS challenge and the potential modulating effect of melatonin, a second excitotoxic insult consisting in an intracranial injection of ibotenate, a glutamatergic agonist, was performed at PND4. Prenatal exposure to LPS led to a significant increase in cortical and white matter lesion size reflecting the sensitizing effect of inflammatory challenge in our model (Fig.1; $p<0.05$ in comparison with the control group). When melatonin was associated with LPS, cortical and white matter lesion size was the same as in the control group (Fig.1). This result suggests that melatonin prevented the sensitizing effect of inflammation to a second insult on neonatal brain. Surprisingly, prenatal melatonin injections exhibited a neuroprotective effect exclusively on white matter out of the inflammatory context (Fig. 1; $p<0.05$ in comparison with the control group).

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3.2 | Melatonin effect on microglial activation induced by LPS at PND1

Prenatal LPS injections induced a significant increase in Iba-1 positive cells within cingulum at PND1 (Fig. 2B). Iba-1 positive cells showed an amoeboid aspect reflecting the microglia activated state (Fig. 2A). In parallel, inducible NO synthase (iNOS) staining, indicating a pro-inflammatory process and acute microglial activation, was increased following the same pattern as the Iba-1 staining (Fig. 2C and D). Therefore, our prenatal inflammatory challenge led to significant microglia activation at PND1.

Prenatal melatonin injections at the same time as LPS injections induced a reduction in Iba-1 and iNOS stainings within cingulum at PND1 in comparison with pups only treated with LPS (Fig. 2A-D). Surprisingly, Iba-1+ and iNOS+ cells were less numerous in rats exposed to melatonin even without LPS challenge (Fig. 2A-D). Thus, prenatal melatonin showed a strong anti-inflammatory effect on neonatal brain whatever the inflammatory status was.

3.3 | LPS and melatonin effect on ER stress, autophagy and apoptosis

To examine whether maternal LPS caused ER stress in the neonatal brain, we assessed the expression of proteins involved in the UPR. As shown in Figure 3, LPS significantly induced the expression of CHOP, a member of the CCAAT/enhancer-binding protein family of transcription factors that is over-expressed following disruption of ER homeostasis[22] (Fig. 3A), and the phosphorylation of eIF2α (Fig. 3B), which leads to inhibition of translation in the ER.[23] LPS also increased the expression of Hsp70 (Fig. 3C), which is normally up-regulated during ER stress.[24] Surprisingly, GRP78, a member of the HSPs protein family resident in the ER and generally up-regulated after ER stress, was not increased (Fig. 3D). Maternal melatonin administration completely reverted the effects of LPS on ER stress proteins (Fig. 3A, B and C).

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Since the UPR response is strictly connected to autophagy,[25-26] we also studied the autophagy process. LPS caused a significant reduction in both lipidated LC3 (LC3 II, Fig. 4A), a microtubule-associated protein that is lipidated upon activation of autophagy, and beclin 1 (Fig. 4B), a component of the PI3K complex that is required for autophagosome formation. The effect of LPS on autophagy activation was also confirmed by the increased expression of p62 (Fig. 4C), a protein degraded during the autophagy process that serves to link ubiquitinated proteins to the autophagy machinery.[27] Melatonin blocked the effect of LPS and restored the expression of autophagy proteins to control values (Fig. 4).

To study the implication of apoptosis in LPS pathogenesis at PND1, we assessed cleaved-caspase-3 (CC3), a well-known apoptosis marker. CC3-stained cell number was not modified within cingulum at PND1 by prenatal exposure to LPS in comparison with control rats. Melatonin by itself induced a reduction in brain apoptosis at PND1. This effect was observed in both control and LPS-treated groups (Fig. 5 A and B). This result does not argue for an implication of apoptosis pathway in inflammation challenge pathogenesis in our model. Melatonin, as previously shown,[28] demonstrated an anti-apoptotic property.

3.4 | The effect of melatonin and LPS-induced inflammation on SIRT-1 and related miRNA

Since SIRT1 has been implicated in the neuroprotective effect of melatonin after ischemia,[13] we investigated if this sirtuin could also be involved in this model of prenatal inflammation. As shown in Figure 6, LPS significantly reduced the expression of SIRT1 on pups and this effect was completely reverted by melatonin.

We also investigated the modulation of miRNAs targeting IL1β, Hsp70 and SIRT1, i.e miR-146a, miR-126 and miR-34a, respectively. MiR146a (Fig. 7A) and miR126 (Fig. 7B) were significantly decreased in pups prenatally treated with LPS, effects that were completely

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blocked by melatonin (Fig. 7A and B). Interestingly, miR-34a expression, that has been demonstrated to directly target SIRT-1, was significantly increased after LPS and reduced by melatonin (Fig. 7C). The modulation pattern of these miRNAs clearly matched the pattern of the respective target proteins (Fig. 3C and Fig. 6, respectively).[29]

4 | DISCUSSION

Epidemiologic and experimental findings implicate maternal infection/inflammation in the etiology of brain injury in preterm newborns.[30] Here we show that maternal infection during late gestation and the subsequent fetal inflammation, mimicked by LPS injection, cause a pro-inflammatory state on PND1 characterized by induction of ER stress, reduction of autophagy and SIRT1 expression, modulation of related miRNAs, and sensitization to a second excitotoxic brain damage. The most important finding of the present study, however, is that melatonin administration at the same time as LPS completely reverts these effects.

4.1 | The beneficial effect of melatonin in the inflammatory context

Melatonin action has been mainly explored in hypoxic-ischemic animal models. Here, a prenatal pro-inflammatory challenge mimicking the chorioamnionitis context was used. Our results demonstrate that the placental barrier was insufficient to prevent the deleterious effects of systemic maternal inflammation/infection on the fetal brain as well as the modulating effect of melatonin. A modulating effect of melatonin on microglia activation was also recently reported in PND5 rats receiving i.p. LPS.[31] In addition, in keeping with our data, a protective effect of melatonin associated with a reduced iNOS activity was recently reported in heart mitochondrial impairment during sepsis.[32] Interestingly, the beneficial effect of melatonin on the neonatal brain was also independent on the inflammatory context, as shown by the reduction of the white matter damage after an ibotenate-induced excitotoxic

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challenge. This finding is in keeping with previous data showing a strong protective effect of melatonin on the white matter after intracranial ibotenate injection. Blocking the cAMP pathway through melatonin was implicated in this effect.[19] A sensitizing effect of inflammation in the ibotenate model was also shown after interleukine-1β i.p. injections to mouse pups.[33] These findings may argue for pleiotropic actions of melatonin and support its remarkable neuroprotective effect in various noxious contexts.

4.2 | Implication of ER stress, autophagy, SIRT-1, and related miRNA, in the neuroprotective action of melatonin

Maternal LPS exposure during pregnancy was reported to induce ER stress in the placenta and contribute to intrauterine fetal growth restriction and death.[34] In our experimental model, LPS caused ER stress in the neonatal brain, as indicated by the increased expression of CHOP and Hsp70, and by the increased phosphorylation of eIF2α. In contrast to the clear indication of UPR after LPS, we observed no increase in the expression of the molecular chaperon GRP78. Among the Hsp70 protein family, GRP78 is manly localized in the ER and is generally up-regulated during glucose deprivation-induced ER stress following an ischemic insult.[26,35-36] Hsp70 and its constitutive form Hsc70, manly localized in the cytosol and in the nucleus, are involved in chaperoning processes, including refolding of misfolded or aggregated proteins. Hsp70 is induced in response to a variety of stressful stimuli (e.g., ischemia, hyperthermia, oxidative stress and mechanical stress)[37-39] in all living organisms. Hsp70 is implicated in the formation of the immunogenic complex[40] as well as in the facilitation of the immune response to proteins and peptides, both in vivo and in vitro.[41-42] We hypothesize that the lack of activation of GRP78 and the marked activation of Hsp70 may be functional for the cellular response in inflammatory conditions. This finding indicates that different cellular pathways may be activated during different stresses depending on the

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eliciting stimuli (i.e. inflammatory versus oxygen/glucose deprivation conditions). Other factors may also condition the response including the intensity and duration of stress, the cell type, and environmental factors.

Another important finding of the present study is the modulation of autophagy in fetal/neonatal brain inflammation and after melatonin administration. Autophagy is an intracellular bulk degradation process, fundamental for the quality and quantity regulation of key intracellular biological functions.[43] Autophagy is in a dynamic equilibrium in the cell and its impairment can lead to metabolic dysfunctions and cell death. Autophagy is activated soon after birth in neonatal tissues and is essential for development and survival.[8] Aminoacids produced by autophagy recycling peroxidized proteins can be used as an energy source or, alternatively, for the synthesis of new proteins for an appropriate response to starvation, a condition that neonates face at birth.[44] We found that maternal inflammation significantly reduces autophagy in the neonatal brain, as indicated by the reduction of both beclin 1 and LC3 II expression and the increased p62 protein level. Growing evidence links defective autophagy to the pathogenesis and progression of several inflammatory diseases. Autophagy inhibition has been observed in cystic fibrosis-induced lung inflammation.[45] A defective autophagy caused cytoplasmic accumulation of mutant SOD1 and enhanced the progression of the neurodegenerative amyotrophic lateral sclerosis.[46] Conversely, autophagy activation showed cell protection in different types of metabolic, infectious, or inflammatory stresses.[47-48] Autophagy can also support apoptosis and delay necrosis in neonatal hypoxia-ischemia.[49-51] Here we found a reduced autophagy that likely represents a lack of adaptation to events, such as birth, that may require a fully efficient autophagy machinery.[8] The reduced autophagy may likely contribute to the functional consequences of the inflammatory process. Interestingly, here we found that melatonin completely reversed the effect of LPS on autophagy, in agreement with the clear link between inflammation and mitophagy, a selective form of

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autophagy that was recently demonstrated in the protective effect of melatonin in traumatic brain injury.[52] Whether or not mitophagy is involved in the inflammatory reaction induced by LPS needs to be determined.

Autophagy and apoptosis are both programmed cell death processes which beclin-1 represents a crosstalk. Caspase-3 would induce beclin-1 cleavage leading to autophagy reduction.[53] In our model, we found that LPS injections do not affect apoptotic cell death through cleaved-caspase 3 staining. This result does not support the apoptosis-induced autophagy regulation in an inflammatory context. Melatonin, conversely, exerts an antiapoptotic effect both in controls and in animals treated with LPS. The antiapoptotic effect of melatonin has been reported in several neurodegenerative conditions and may be the result of events occurring upstream to the activation of the intrinsic pathway of apoptosis, including its scavenging effect on reactive oxygen species[28] and modulation of ER stress and autophagy.[54]

An important factor involved in the protective effect of melatonin is SIRT1. Here we found that SIRT1 expression was markedly reduced after LPS. Melatonin completely reverted this reduction in agreement with our previous results obtained after hypoxia-ischemia-induced brain damage in neonates[13] and the results of other authors working with adult rats.[36] We also found that this effect was concomitant with the recovery of the autophagy process. SIRT1 is a NAD+-dependent deacetylase involved in a wide variety of biological functions. Its activation triggers transcriptional programs that enhance metabolic efficiency, upregulate mitochondrial oxidative metabolism, and increase the resistance to oxidative stress by increasing antioxidant pathways and facilitating DNA and protein repair. SIRT1 also regulates the extension of the inflammatory response[55] and the autophagy process.[56] The reduction of inflammation by SIRT1 is obtained by controlling the acetylation of the nuclear factor kappa B (NF-κB);[57] its ablation in macrophages results in NF-κB

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hyperacetylation, increasing the transcriptional activation of proinflammatory target genes.[58] Increasing SIRT1-mediated NF-κB deacetylation has been proposed as a novel approach to inflammation.[57]

Melatonin is an important regulator of immune response and seems to act as a stimulant under basal or immunosuppressive conditions and as an anti-inflammatory in the presence of an exacerbated immune response.[12] In keeping with this dual effect of melatonin, a recent study by Volt et al.[59] showed that chronic low doses of melatonin can counteract inflammaging, whereas high acute doses can counteract sepsis. Our results showing a reduced brain inflammatory response after a pharmacological dose of melatonin are in line with these findings. Melatonin also preserves NAD+ levels, contributing to the regulation of SIRT1 expression and activity.[60] The clock genes Bmall/Clock and their transcriptional regulators RORα and Rev-Erba,[61-62] that control the expression of NF-kB and of the nicotinamide phosphoribosyltransferase, that represent the rate-limiting enzyme for NAD+ biosynthesis from nicotinamide, may be involved in this effect.[63-64]

To further investigate the effects of LPS and melatonin in perinatal inflammation, we extended our experiments to the assessment of some miRNA related to inflammation and SIRT1. miR-146a was reduced by LPS and reverted to control values by melatonin. Among the validated inflammation-related targets of miR-146a, there are IL-1 receptor-associated kinase-1 (IRAK1), TNF receptor-associated factor 6 (TRAF6) and IL-1β. These targets are all involved in the processes discussed in this study: IRAK1 regulates TLR4 signaling; TRAF6-mediated NF-kB signaling requires the p62 protein to contribute to the autophagy machinery;[65] and IL-1β actively participates in inflammation. MiR-126, which shows the same expression pattern as miR-146a, modulates either the inflammation process by regulating vascular adhesion molecule expression, and modulates ER stress by Hsp70 expression. Its modulation by LPS and melatonin indicates a clear association to the target

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protein. The same miRNAs-target association was observed when we analyzed miR-34a. Several reports have shown that miR-34a regulates cell cycle progression, cellular senescence and apoptosis and that it also plays important roles in neuronal development.[66] Members of the miR-34 family have been implicated in brain trauma and epilepsy. MiR-34a inhibits SIRT1 and also regulates p53 dependent apoptosis through deacetylation and stabilization of p53.[67] The clear modulation of miR34a and SIRT1 by LPS and melatonin observed in our study and the multiplicity of effects controlled by SIRT1 strongly suggest a pivotal role of this pathway in neuroprotection and inflammation related diseases of the neonate.

5 | Conclusion

This study provides further preclinical evidence of the neuroprotective effects of melatonin in inflammation-related brain injury. There is a strong association among maternal infection/inflammation, brain damage and severe/permanent neurodevelopmental handicaps in newborns. The present data clearly indicate that melatonin may represent an effective prophylactic/therapeutic protective strategy in the perinatal clinical setting. Clinical studies using melatonin in newborns have already shown promising results,[68-70] and others are in progress.[71] In addition, women with premature rupture of membranes who are hospitalized for a long time before birth and are positive for chorioamnionitis, as well as women presenting an imminent delivery in the chorioamnionitis context could benefit from melatonin. Of course, the safety of melatonin during pregnancy needs to be assessed.

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DISCLOSURE

The authors of this paper declare that they do not have any conflict of interest.

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FIGURE LEGENDS

FIGURE 1 Effect of prenatal LPS-induced sensitization and melatonin on a second ibutenate-induced excitotoxic brain injury. Pregnant rats received LPS (300 µg/kg, i.p.) at gestational day (GD) 19 and 20 or LPS followed by 5 mg/kg melatonin (LPS+Mel). Control groups received saline (Control) or 5 mg/kg melatonin (Mel). Intracranial injection of ibotenate was performed on postnatal day (PND) 4 and rats sacrificed on PND8. Brain lesion sizes were measured in cerebral cortex and white matter. Results are reported as mean ± S.E.M. (N=12). * $p<0.05$, compared to the Control group, § $p<0.05$ compared to the LPS group, One-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

FIGURE 2 Effect of prenatal LPS and melatonin on microglia activation within cingulum 48 hours after the last maternal i.p. injection (PND1). Melatonin demonstrated a remarkable anti-inflammatory action in both control and LPS-exposed rat brain. Immunofluorescent stainings of Iba-1+ (A) and iNOS+ (C) cells, reflecting microglial cells and acute inflammatory activation respectively, were performed on pup brain at PND1. Iba-1+ (B) and iNOS+ (D) cells were counted within cingulum on 10 µm- thick coronal sections at magnification 20 in each experimental group (Control, Mel, LPS and LPS+Mel; N=5 rats/group) (scale bar= 100 µm). Results are expressed in cells/mm² and means ± S.E.M. * indicates statistically significant difference from Control bar (* $p<0.05$) and § indicates statistically significant difference from LPS bar (§ $p<0.05$ and §§ $p<0.01$) in One-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

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FIGURE 3 Effect of prenatal LPS and melatonin on endoplasmic reticulum (ER) stress markers. Western blots and quantitative evaluation of CHOP (A), phospho-eIF2α (p-eIF2α, B), Hsp70 (C) and GRP78 (D) expression, reflecting ER stress, have been performed in PND1 pup brain of each experimental group (Control, Melatonin, LPS, LPS+Melatonin; N=5 rats/group). β-actin was run as loading control. Results are reported as % of control and are the mean ± S.E.M . ** $p<0.01$ compared to the Control group, One-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

FIGURE 4 Effect of prenatal LPS and melatonin on the pup brain autophagy process at PND1. Representative Western blots and quantitative evaluation of lipidated LC3 (LC3 II, A), beclin 1 (B) and p62 (C) expression in neonatal brain. β-actin was run as loading control. Results are reported as % of control and are the mean ± S.E.M. (N=5). * $p<0.05$ compared to the Control group, One-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

FIGURE 5 Effect of prenatal LPS and melatonin on apoptosis within cingulum at PND1. A) Immunostaining of Cleaved-Caspase 3 (CC3), an apoptotic marker, was performed on frozen 10 µm- thick sections from PND1 brain of each experimental group (Control, Mel, LPS, LPS+Mel; N=5 per group). B) CC3+ cell number was obtained from pictures taken at magnification 20 focusing on cingulum (Scale bar= 100 µm). Results are expressed in cells/mm² and means ± SEM. * indicates statistically significant difference from Control bar (* $p<0.05$ and ** $p<0.01$) and § indicates statistically significant difference from LPS bar (§ $p<0.05$) in One-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

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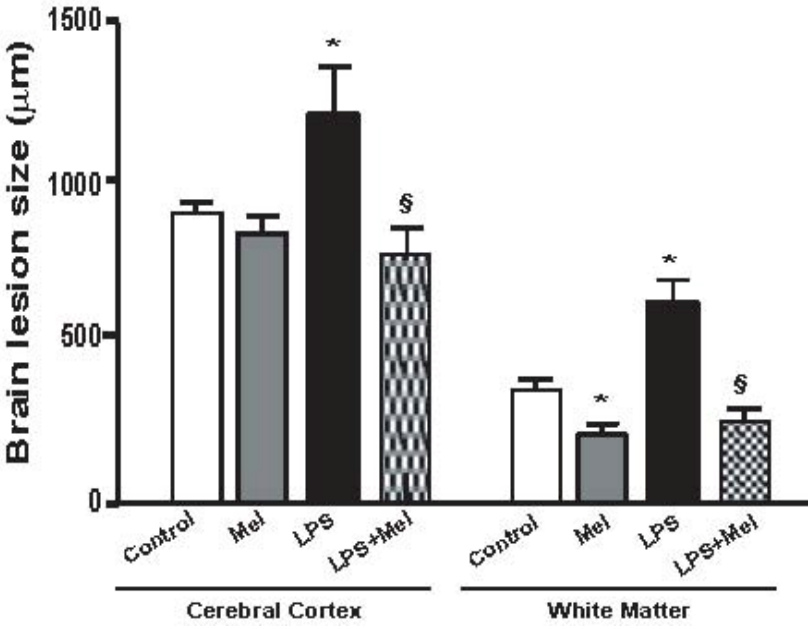
FIGURE 6 Effect of prenatal LPS and melatonin on SIRT1 expression in PND1 pup brain. Representative Western blots and quantitative evaluation of SIRT1 expression in neonatal brain. β-actin was run as loading control. Results are reported as % of control and are the mean ± S.E.M. (N=5). ** $p<0.01$ compared to the Control group, One-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

FIGURE 7 Effect of prenatal LPS and melatonin on microRNAs (miRNAs) expression. Relative expression analysis of miRNA-146a (A), miRNA-126 (B) and miRNA-34a (C) in neonatal PND1 brain performed by qRT-PCR. Results are reported as fold induction related to control values and are the mean ± S.E.M. (N=5). ** $p<0.01$ compared to the Control group, One-way ANOVA followed by Newman-Keuls Multiple Comparison Test.



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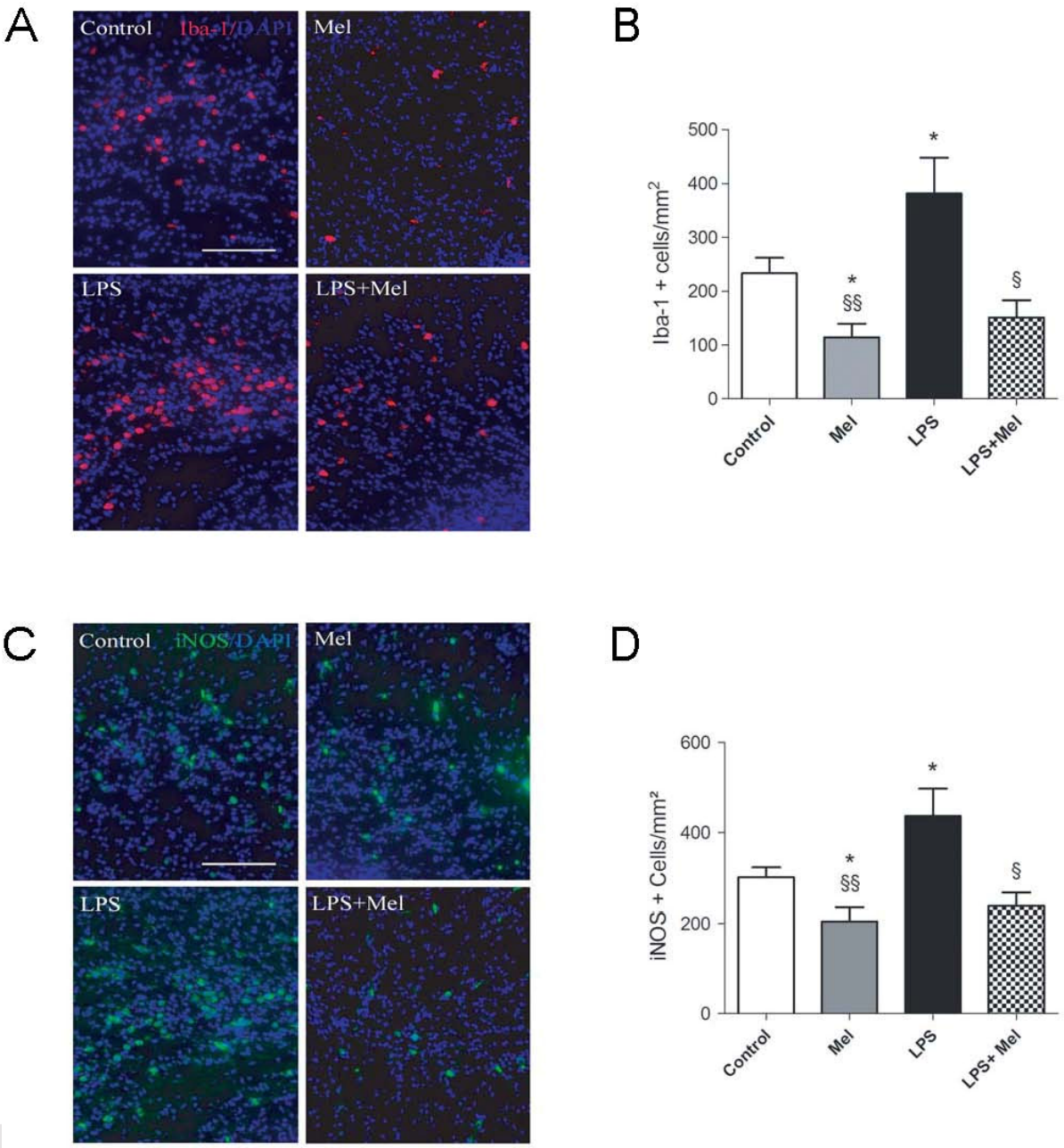

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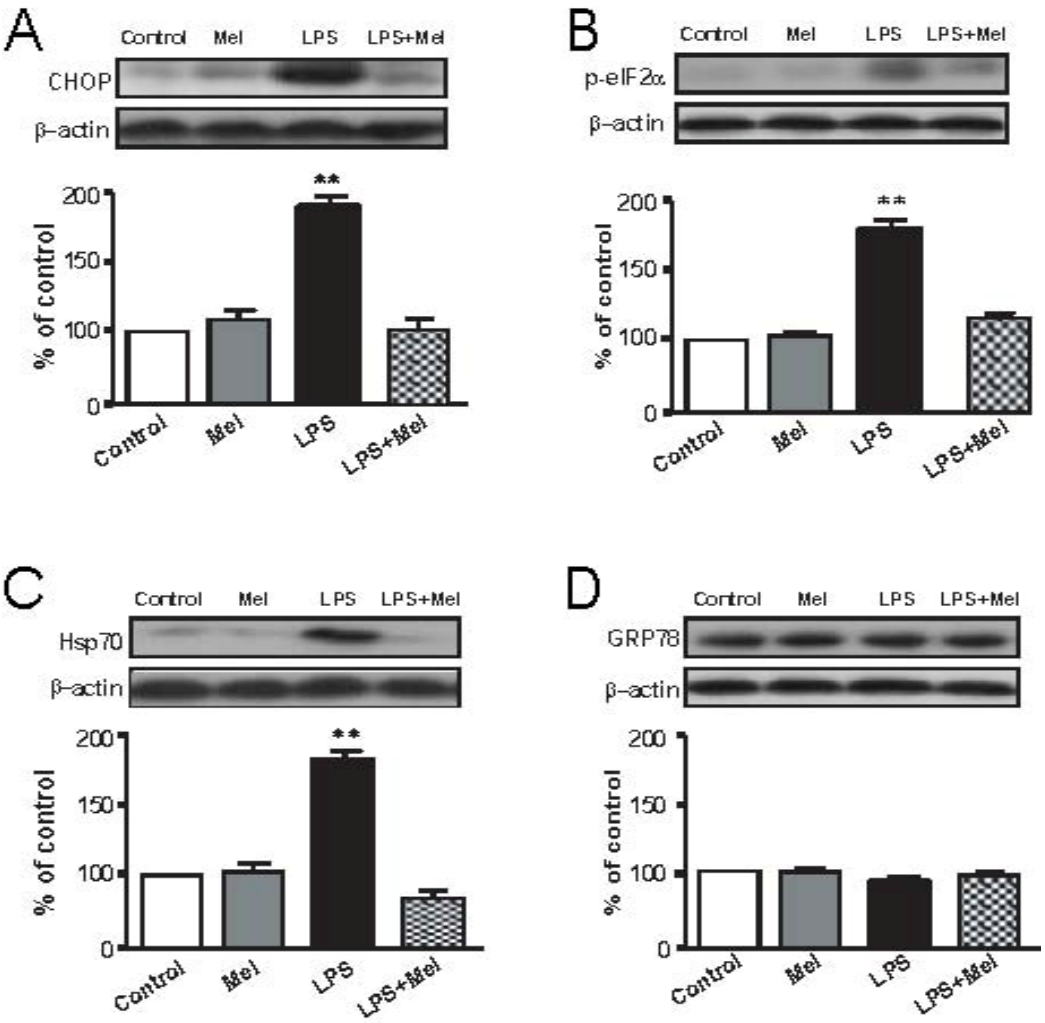

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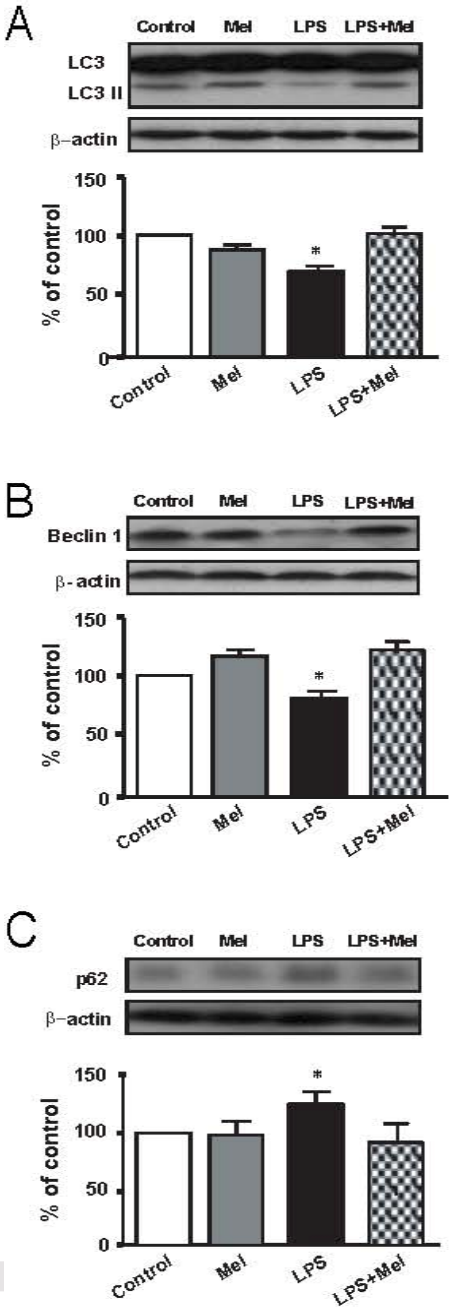

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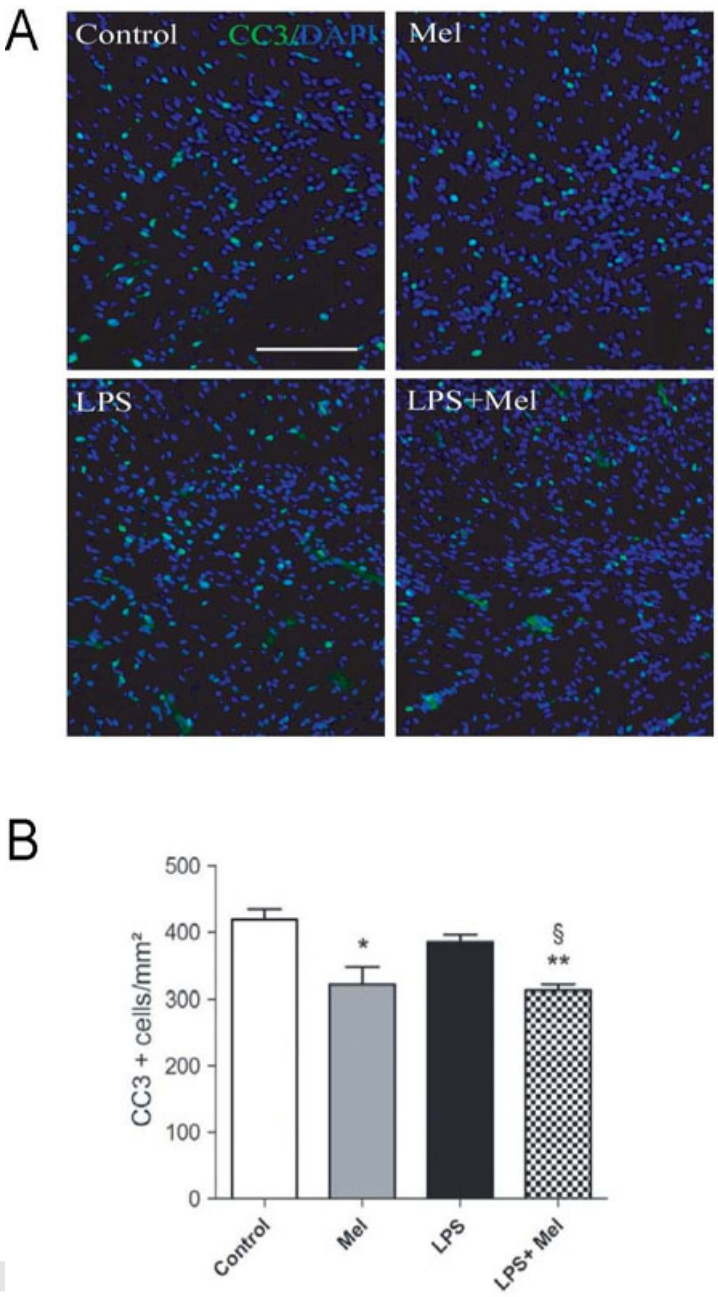

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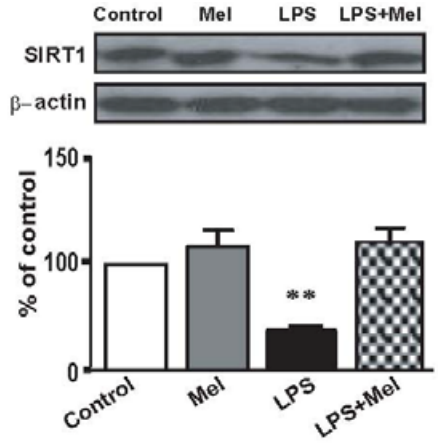

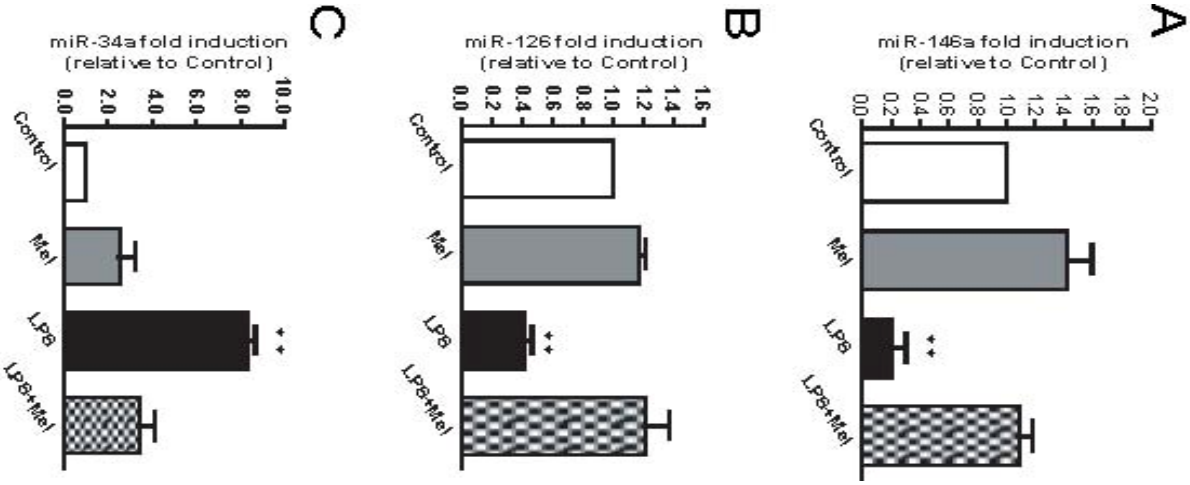

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