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

Silvia Carloni[1*], Géraldine Favrais[2,3*], Elie Saliba[2,3], Maria Cristina Albertini[1], Sylvie Chalon[3], Mariangela Longini[4], Pierre Gressens[5], Giuseppe Buonocore[4], Walter Balduini[1]

[1] Department of Biomolecular Sciences, University of Urbino "Carlo Bo", Urbino, Italy.

[2] Department of Neonatal and Pediatric intensive care, CHRU de Tours, F-37000Tours, France.

[3] INSERM U930, Université François Rabelais de Tours, F-37000 Tours, France.

[4] Department of Molecular and Developmental Medicine, Policlinico Le Scotte, University of Siena, Siena, Italy.

[5] PROTECT, INSERM, Université Paris Diderot, Sorbonne Paris Cité, F-75019 Paris, France

***Equal Contribution**

Corresponding author: Walter Balduini

Department of Biomolecular Sciences, University of Urbino "Carlo Bo" Via S. Chiara 27, 61029, Urbino (PU), Italy.

Tel. +39-0722-303526; Fax: +39-0722-303521

e-mail: walter.balduini@uniurb.it

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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 brain neonatal 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 excitotoxic insult performed on postnatal day (PND) 4. As melatonin succeeded in reducing microglial activation in neonatal brain at PND1, pathways previously implicated in the brain inflammation regulation like 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 of CHOP and Hsp70 protein expression and eIF2α phosphorylation, indicative of activation of the unfolded protein response consequent to ER stress, and a slighter decrease of autophagy process, determined by reduced lipidated LC3 and increased p62 expression. LPS-induced inflammation also reduced the brain SIRT1 expression and affected the expression 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 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 prophylactic/therapeutic drug deserves to be investigated by clinical studies.

Introduction

Systemic inflammation/infection represents one of the main events in the pathogenesis of brain injury in both term and preterm infants [1-2]. Preterm infants frequently show high concentrations of pro-inflammatory cytokines in amniotic fluid and fetal blood [3-4], particularly those with chorioamniotitis [5-6] and vasculitis [7]. These pathological conditions are clearly associated with an increased risk for intra-partum cerebral failure, cerebral palsy and permanent neurodevelopmental handicaps [8].

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 which triggers a series of signal-transduction events known as unfolded protein response (UPR). The UPR is aimed to overcome the stressful condition and restore ER homeostasis [9-10], 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) [9-10]. The UPR also leads to the activation of an inflammatory response through the induction of inflammatory cytokines [11]. This is triggered by both the suppression of IkB translation and subsequent NFkB activation [12], and activation of the AP-1 (activator protein-1) pathway [13]. 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 stresses. CHOP may lead to the activation of the mitochondrial pathway of apoptotic cell death for removal of the affected cell(s) [14-15].

UPR is strictly connected to autophagy, a physiological dynamic process that triggers the self-digestion of damaged organelles and proteins. In stressed cells, autophagy can support cell

adaptation and survival, but when proteins and organelles turnover overwhelms the adaptive capacity, the cell dyes. During development, autophagy may have additional important functions, including the adaptation to starvation occurring during fetal-to-neonatal transition [16]. Autophagy also plays a role in inflammation and adaptive immune mechanisms by regulating the secretion of IL-1β and possibly other pro-inflammatory substrates [17-18]. Through this mechanisms, autophagy potentially influences disease pathogenesis [19]. 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 potent indirect antioxidant and direct free radical scavenger [20]. 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 [21]. In addition, melatonin regulates immune function, energy metabolism [22] and the release of pro-inflammatory and anti-inflammatory cytokines [23-24]. Recently, we reported that melatonin protected neonatal rats from ischemic brain damage by reducing ER stress and preserving the expression of SIRT1 [25], a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase [26]. The latter effect appears of particular relevance since enhanced SIRT1 activity has been found to protect against different neurodegenerative disorders [27] and to inhibit NF-kB signaling and NF-kB-dependent pro-inflammatory gene expression (i.e. IL-β) [28].

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 [29]. Among the various miRNAs, miR-34a was found to inhibit the expression of SIRT1 by repressing the translation process [30], 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 it is also implicated in ER stress expression since it can modulate the heat stress protein Hsp70 [31].

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.

Material and Methods

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 abdominal opposite 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).

Ibotenate-induced excitotoxic lesion

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 [32-33], 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 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 xylen for 24 hours, brains were embedded in paraffin and 16-µm thick coronal sections were performed. The maximal diameter in the saggital 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.

Immunofluorescence staining

Newborn rats were sacrificed at PND1 by decapitation, 48 hours after 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. 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 immunofluorescent staining procedure, pictures at magnification 20 focusing on cingulum were performed in each experimental groups (N=5 brains/ group). Cingulum region has been chosen because of particular microglia richness at this brain developmental stage [34]. Pictures were analyzed with NIH Image J software.

Western blot analysis

Newborn rats were sacrificed at PND1 by decapitation, 48 hours after 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 (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 the Image J 1.45 software. Data were normalized to β-actin and expressed as percent of control.

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 homogenates 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), as previously described [35]. 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 amount of miR-34a, miR-146a and miR-126 were calculated 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.

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.

Results

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 of 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 than 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 inflammatory context (Fig. 1; $p<0.05$ in comparison with the control group).

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

Prenatal melatonin injections at the same time as LPS injections induced a reduction of 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). Then, prenatal melatonin showed a strong anti-inflammatory effect on neonatal brain whatever inflammatory status was.

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 [15] (Fig. 3A), and the phosphorylation of eIF2α (Fig. 3B), which leads to inhibition of translation into the ER [36]. LPS also increased the expression of Hsp70 (Fig. 3C), which is normally up-regulated during ER stress [37]. Surprisingly, GRP78, a member of HSPs proteins 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).

Since the UPR response is strictly connected to autophagy [38-39], we also studied autophagy process. LPS caused a significant reduction of both lipidated LC3 (LC3 II, Fig. 4A), a microtubule-associated protein that is lipidated upon activation of autophagy [40], and beclin 1 (Fig. 4B), a component of the PI3K complex that is required for the autophagosome formation [41]. 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 [42]. 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 of 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 [43], demonstrated an anti-apoptotic property.

Since SIRT1 has been implicated in the neuroprotective effect of melatonin after ischemia [25], we investigated if this sirtuin could be also 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 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) [44].

Discussion

Epidemiologic and experimental findings implicate maternal infection/inflammation in the etiology of brain injury in preterm newborns [45-46]. 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.

Melatonin action has been usually explored in hypoxic-ischemic animal models. Here, a prenatal pro-inflammatory challenge mimicking the chorioamniotitis context have been used. Our results demonstrate that the placenta 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 [47]. Interestingly, the beneficial effect of melatonin on the neonatal brain was also independent on whatever inflammatory context was, as showed by the reduction of the white matter damage after an excitotoxic 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 by melatonin was implicated in this effect [32]. A sensitizing effect of inflammation in the ibotenate model was also shown after interleukine-1β i.p. injections to mouse pups [48]. These findings may argue for a multiplicity ways of action of melatonin and support its remarkable neuroprotective effect in various noxious contexts.

Maternal LPS exposure during pregnancy was reported to induce ER stress in placenta and contribute to intrauterine fetal growth restriction and death [49]. In our experimental

model, LPS caused ER stress in 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 [39,50-51]. 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 in all living organisms (e.g., ischemia, hyperthermia, oxidative stress and mechanical stress) [52-54]. Hsp70 is implicated in the formation of the immunogenic complex [55] as well as in the facilitation of the immune response to proteins and peptides, both in vivo and in vitro [56-57]. 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 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 [58].

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 [59]. 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 [16]. 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 [60]. 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 increased of 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 [61]. A defective autophagy caused cytoplasmic accumulation of mutant SOD1 and enhanced the progression of the neurodegenerative amyotrophic lateral sclerosis [62]. Conversely, autophagy activation showed cell protection in different types of metabolic, infectious, or inflammatory stresses [63-64]. Autophagy can also support apoptosis and delay necrosis in neonatal hypoxia-ischemia [65-67]. 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 [16]. The reduced autophagy may probably contribute to the functional consequences of the inflammatory process. Interestingly, melatonin completely reversed the effect of LPS on autophagy. 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 [68]. In our model, we found that LPS injections do not affect apoptotic cell death through cleaved-caspase 3 staining. This result doesn't support the apoptosis-induced autophagy regulation in 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 [43] 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 [69]. Another possible mechanism for the antiapoptotic effect of melatonin could be through SIRT1, which was recently reported to protect against apoptosis by

promoting autophagy [70]. Here we found that SIRT1 expression was markedly reduced after LPS and melatonin completely reverted this reduction, in agreement with our previous results after hypoxia-ischemia-induced brain damage in neonates [25] and the results of other authors in adult rats [51]. Furthermore, we also found that this effect was concomitant with the recovery of the autophagy process. SIRT1, on the other hand, plays a role in a wide variety of functions in biological systems [26], including the regulation of inflammation [71]. For example, it has been demonstrated that SIRT1 provides protection against chronic inflammation by controlling the acetylation of the nuclear factor kappa B (NF-κB), and that its ablation in macrophages results in increased transcriptional activation of proinflammatory target genes [72]. Furthermore, the reduced expression and activity of SIRT1 in the adipose tissue of both rodents and humans induces cytokine/chemokine production, and monocyte and macrophage infiltration [73]. To 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 necessitates of the p62 protein to contribute to the autophagy machinery [74]; and IL-1β actively participates to inflammation. MiR-126, which shows the same expression pattern than miR-146a, modulates either the inflammation process by regulating vascular adhesion molecules expression, and modulates ER stress by Hsp70 expression. Its modulation by LPS and melatonin indicates a clear association to the target protein. The same miRNAs-target association was observed when we analyzed miR-34a. Several reports showed that miR-34a regulates cell cycle progression, cellular senescence and apoptosis and also plays important

roles in neuronal development [75]. 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 [76]. 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.

In conclusion, this study provides further preclinical evidence of the neuroprotective effects of melatonin in inflammation-related brain injury. Since there is a clear association among maternal infection/inflammation, brain damage and severe/permanent neurodevelopmental handicaps in newborns, the present data evidently indicate that melatonin may represent an effective prophylactic/therapeutic protective strategy in the perinatal clinical setting. Our results pave the way for randomized clinical trials.

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Figure legends.

Figure 1. Maternal administration of melatonin at the same time as inflammatory challenge prevented LPS sensitization to a second 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 size 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. LPS led to a significant microglia activation within cingulum at PND1, 48 hours after the last maternal i.p. injection. Melatonin demonstrated a remarkable anti-inflammatory action in both control and LPS-exposed rat brain. Imunofluorescent 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.

Figure 3. Endoplasmic reticulum (ER) stress was strongly up-regulated in PND1 pup brain following LPS maternal challenge which is completely prevented by concomitant exposure to melatonin. 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.

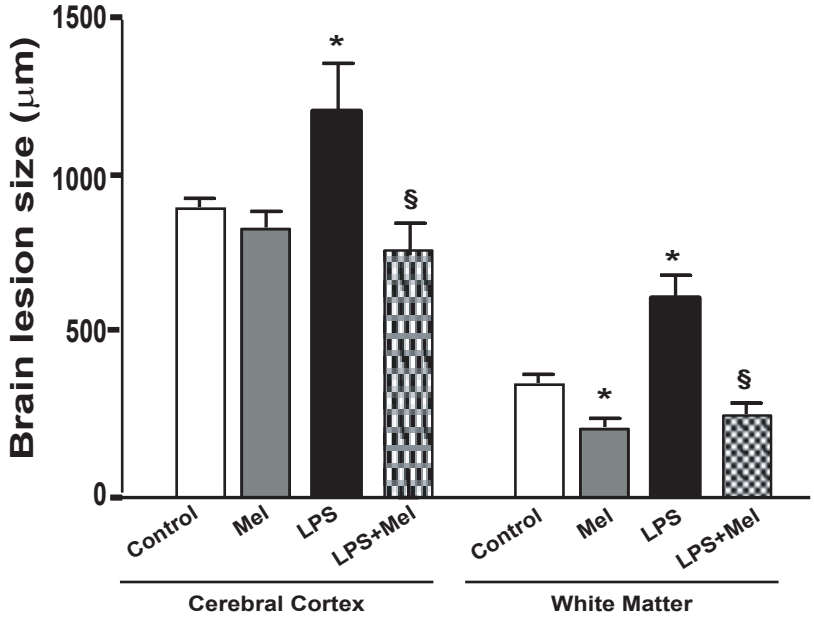
Figure 4. Late-pregnancy LPS injections played a regulation role on pup brain autophagy process at PND1 totally neutralized by concomitant melatonin injections. Representative Western blots and quantitative evaluation of lipidated LC3 (LC3 II, A), beclin 1 (B) and p62 (C) expression in neonatal brain. 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. Prenatal exposure to LPS did not induce an excess of apoptosis within cingulum at PND1. Melatonin exhibited by itself an anti-apoptotic action. 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.

Figure 6. Prenatal LPS injections induced in PND1 pup brain a significant reduction of SIRT1 expression, modulated by melatonin. 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. MicroRNAs (miRNAs) expression was modulated by melatonin during brain inflammatory condition. 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.


Brain lesion size (μm)
1500
1000
500
0
*
§
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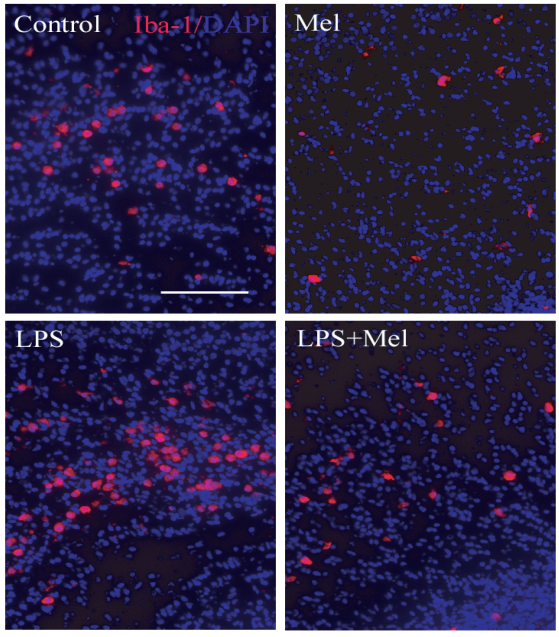
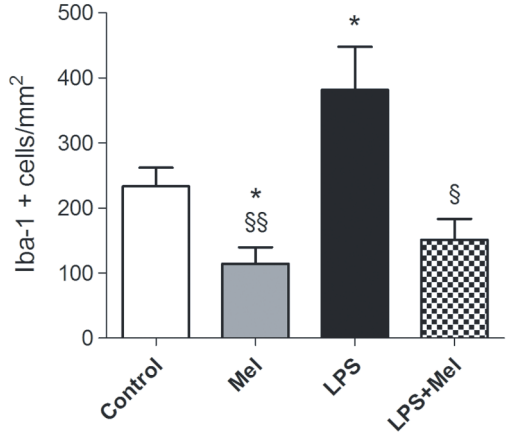
*
§
Control
Mel
LPS
LPS+Mel
Control
Mel
LPS
LPS+Mel
Cerebral Cortex
White Matter

A


Control
Iba-1/DAPI
Mel
LPS
LPS+Mel

B


Iba-1 + cells/mm2
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LPS+Mel

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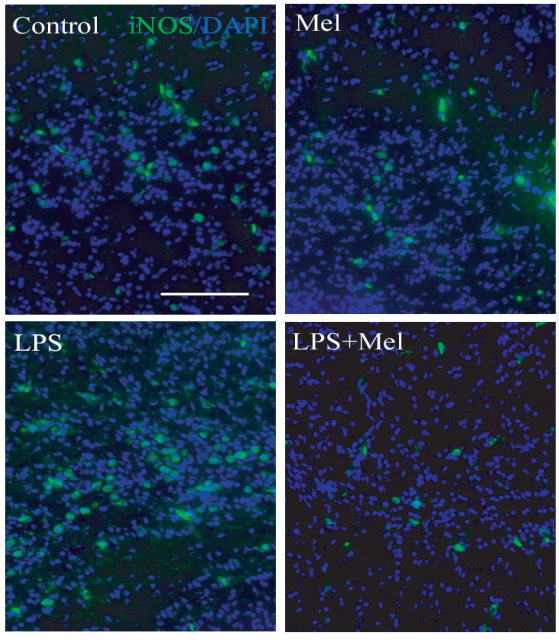
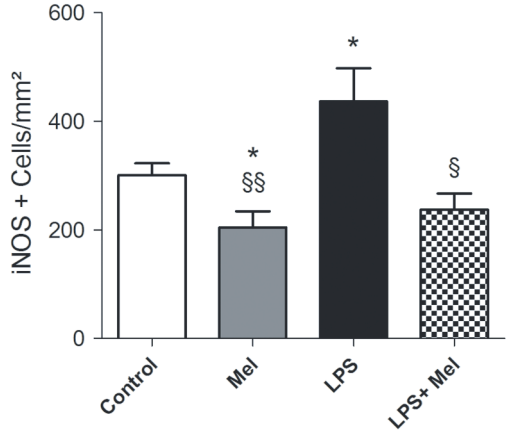

Control
iNOS/DAPI
Mel
LPS
LPS+Mel

D


iNOS + Cells/mm2
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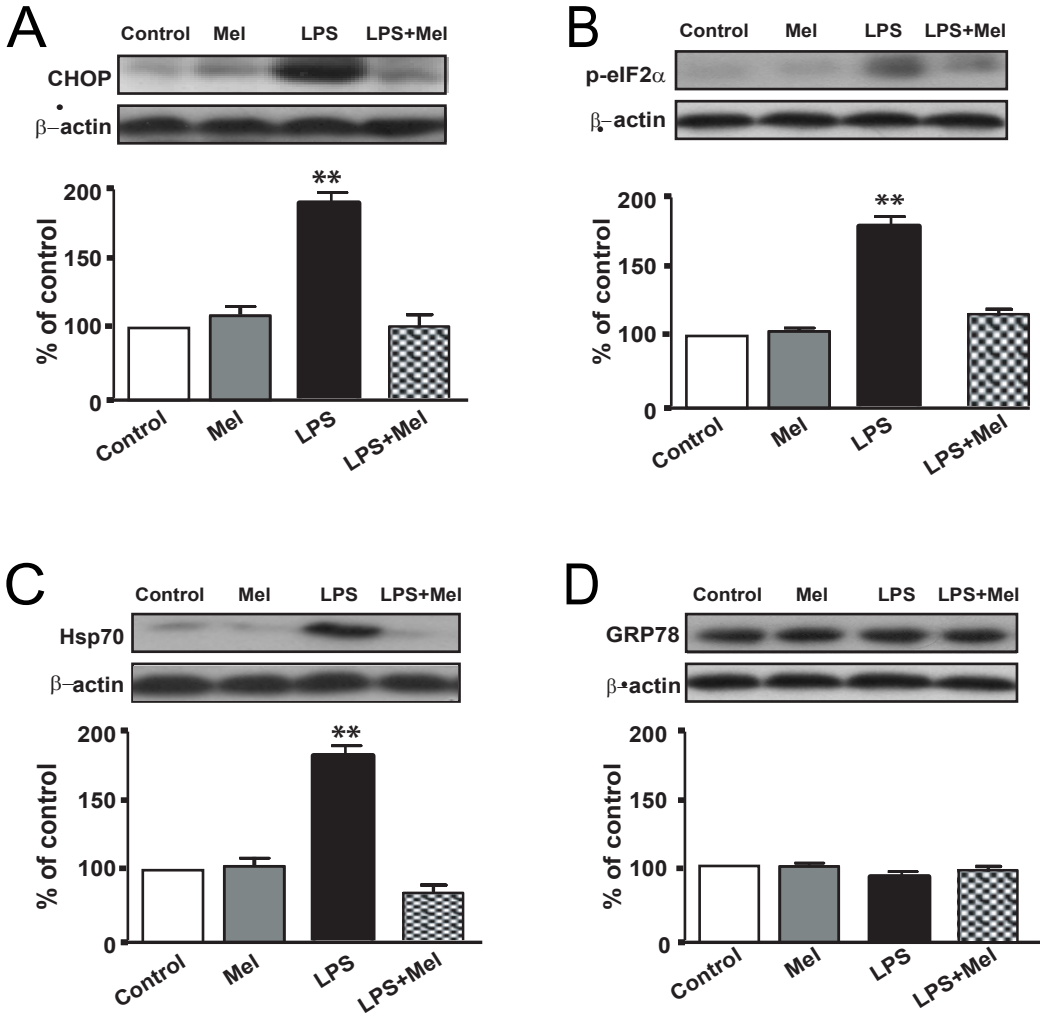
§
Control
Mel
LPS
LPS+ Mel


A
Control
Mel
LPS
LPS+Mel
CHOP
β–actin
% of control
200
150
100
0
**
Control
Mel
LPS
LPS+Mel
B
Control
Mel
LPS
LPS+Mel
p-eIF2α
β–actin
% of control
200
150
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Control
Mel
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β–actin
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LPS+Mel
GRP78
β–actin
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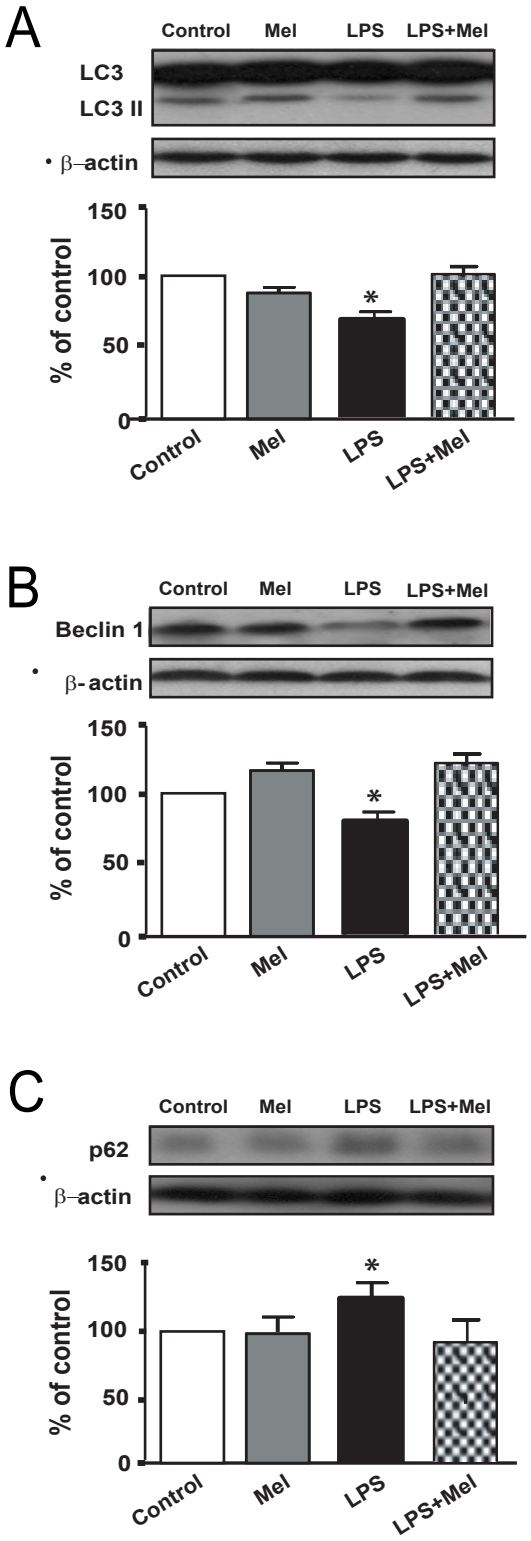
Mel
LPS
LPS+Mel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Control
Mel
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LPS+Mel
LC3
LC3 II
β–actin
150
100
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% of control
*
Control
Mel
LPS
LPS+Mel
B
Control
Mel
LPS
LPS+Mel
Beclin 1
β-actin
150
100
50
0
% of control
*
Control
Mel
LPS
LPS+Mel
C
Control
Mel
LPS
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p62
β–actin
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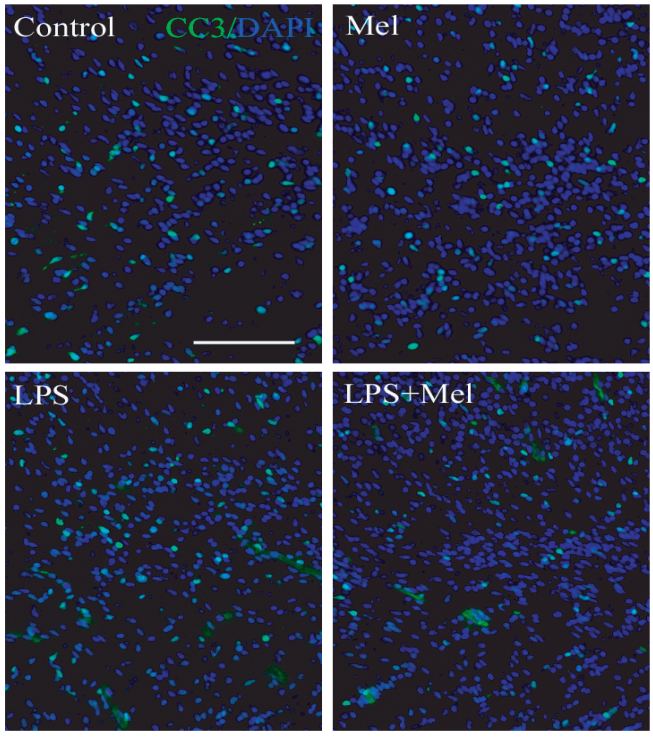
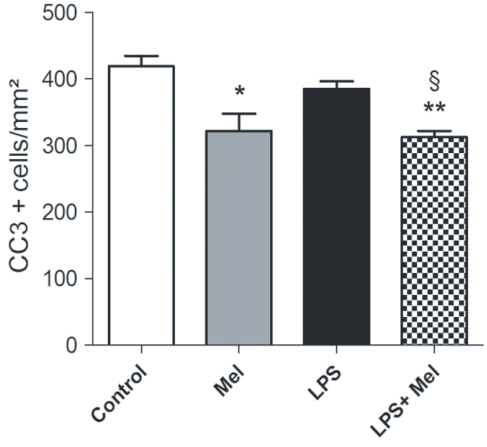
100
50
0
% of control
*
Control
Mel
LPS
LPS+Mel

A


Control
CC3/DAPI
Mel
LPS
LPS+Mel

B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500
400
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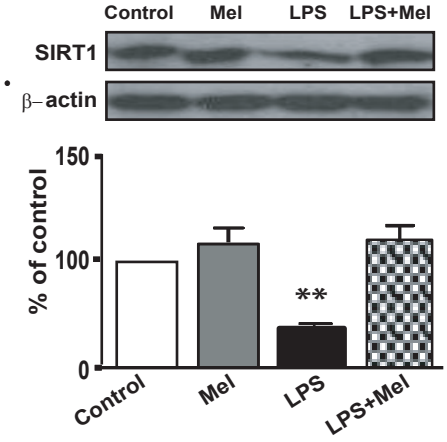
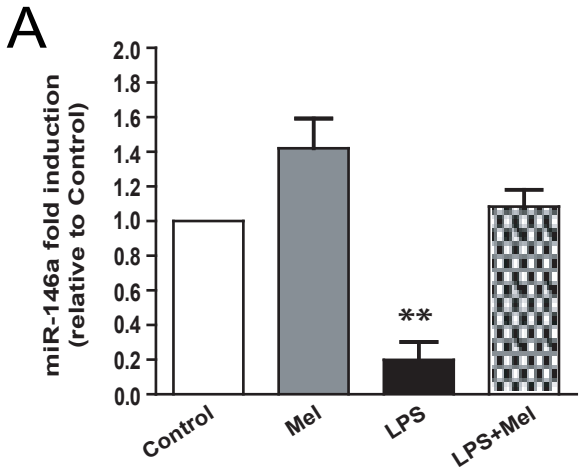
Control
Mel
LPS
LPS+ Mel


Control
Mel
LPS
LPS+Mel
SIRT1
β–actin
150
100
0
% of control
**
Control
Mel
LPS
LPS+Mel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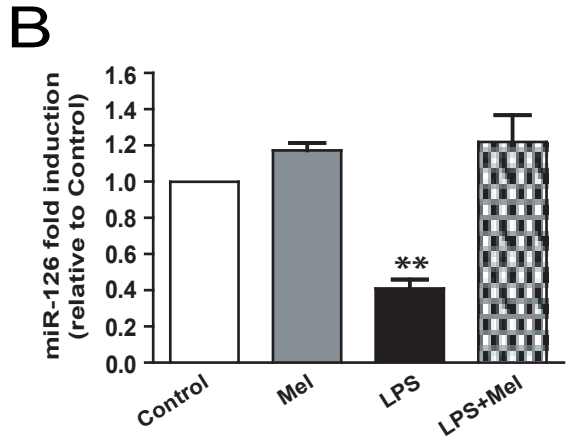
miR-146a fold induction
(relative to Control)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Control
Mel
LPS
LPS+Mel


B
miR-126 fold induction
(relative to Control)
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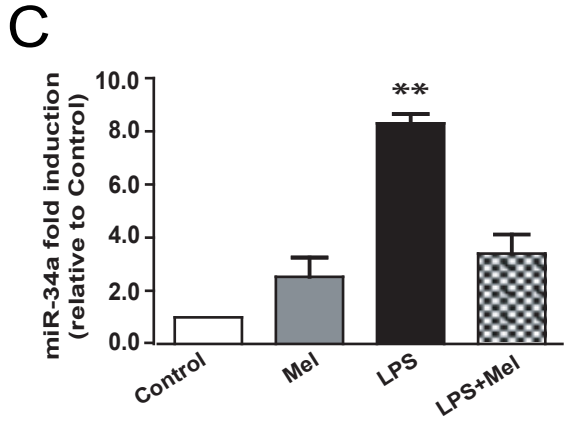
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Control
Mel
LPS
LPS+Mel


C
miR-34a fold induction
(relative to Control)
10.0
8.0
6.0
4.0
2.0
0.0
**
Control
Mel
LPS
LPS+Mel