

# Melatonin Promotes Myelination by Decreasing White Matter Inflammation After Neonatal Stroke

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**ABSTRACT:** Melatonin demonstrates neuroprotective properties in adult models of cerebral ischemia, acting as a potent antioxidant and anti-inflammatory agent. We investigated the effect of melatonin in a 7-d-old rat model of ischemia-reperfusion, leading to both cortical infarct and injury in the underlying white matter observed using MRI and immunohistochemistry. Melatonin was given i.p. as either a single dose before ischemia or a double-dose regimen, combining one before ischemia and one 24 h after reperfusion. At 48 h after injury, neither a significant reduction in cortical infarct volume nor a variation in the number of TUNEL- and nitrotyrosine-positive cells within the ipsilateral lesion was observed in melatonin-treated animals compared with controls. However, a decrease in the density of tomato lectin-positive cells after melatonin treatment was found in the white matter underlying cortical lesion. Furthermore, we showed a marked increase in the myelin basic protein-immunoreactivity in the cingulum and in the density of mature oligodendrocytes (APC-immunoreactive) in both the ipsilateral cingulum and external capsule. These results suggest that melatonin is not able to reduce cortical infarct volume in a neonatal stroke model but strongly reduces inflammation and promotes subsequent myelination in the white matter. (*Pediatr Res* 69: 51–55, 2011)

Neonatal hypoxia-ischemia is an important cause of neonatal brain injury, resulting in cerebral palsy, learning disabilities, visual field deficits, and epilepsy (1). In addition to global cerebral ischemia arising from systemic asphyxia, recent data suggest a higher incidence of focal ischemia-reperfusion leading to stroke in near-term neonates (2,3). Mechanisms of arterial ischemic injury without the confounding effect of hypoxia are not fully understood. Although therapeutic hypothermia has recently demonstrated a benefit by decreasing brain tissue injury in infants with hypoxic-ischemic encephalopathy (4,5), very little is known regarding potential neuroprotective strategies after neonatal stroke.

Melatonin, an indoleamine, is synthesized and secreted from the pineal gland relative to the circadian rhythms. Melatonin readily crosses the blood-brain barrier, and after exogenous administration, it is found in high concentrations in the brain. It was demonstrated that melatonin has neuroprotective effects through either antiapoptotic (6) or antioxidant effects (7,8) or by decreasing the excitotoxic cascade (9). Melatonin

has demonstrated neuroprotective properties in adult models of cerebral ischemia, acting as a potent antioxidant and anti-inflammatory agent (10,11). Melatonin was shown to have a potent protective effect in several models of developing white matter damage, by not only promoting oligodendroglial maturation and myelination repair but also decreasing astrogliosis and microglial activation (12–15). We previously developed a neonatal model of stroke in 7-d-old (P7) rat characterized by apoptotic cell death (16,17), inflammatory responses (18,19), and oxidative stress (20). In addition to cerebral infarct, this model induced glial activation in underlying white matter including persistent activation of microglia and astrogliosis (19,21). As the presence of activated microglia is known to impair the oligodendroglial lineage, we hypothesized that melatonin could be used as an effective neuroprotectant in this stroke model.

In summary, in this study, we show that melatonin, despite no effect on stroke lesion size, has a potent protective effect on white matter damage in P7 animals subjected to left middle cerebral artery electrocoagulation followed by a transient bilateral common carotid artery occlusion. Melatonin seems to act by promoting oligodendroglial maturation and myelination repair, together with modulation of microglial activation.

## MATERIALS AND METHODS

**Neonatal ischemia.** Full details of the study have been approved by Robert Debré Research council review board; the approval number is 2009-02. All experiments were performed in compliance with the ethical rules of INSERM. Ischemia was performed on Wistar 7-d-old rat pups (17–21 g; Janvier, Le Genest St-Isle, France) of both sexes. Briefly, anesthetized (with isoflurane, 1.5% for induction and 1% for maintenance) rats were exposed to left middle cerebral artery electrocoagulation followed by a 50-min both common carotid arteries occlusion. During ischemia and the first hour of recovery, the pups were placed in a humidified incubator at 35°C and then transferred to their mothers. Lesions were monitored at 48 h using a scale from 0 to 3, where 0 indicated no observable lesion and 1, 2, and 3 indicated small, medium, and large infarct, respectively, as previously described (22).

**Melatonin treatment.** Melatonin (Sigma Chemical Co. Aldrich) was dissolved in 1 mL of normal saline solution containing <5% DMSO. Animals subjected to the ischemia-reperfusion procedure ( $n = 36$ ) were randomly assigned to one of the three following groups: 1) control group receiving saline buffer (vehicle-treated animals,  $n = 12$ ), 2) melatonin-treated group receiving a single dose of melatonin (20 mg/kg) or its vehicle given *via* an i.p. injection 1 h before the onset of ischemia (Mel-1dose,  $n = 12$ ), and 3) melatonin-treated group receiving a double dose of melatonin 1 h before and 24 h after ischemia (Mel-2doses,  $n = 12$ ). Four animals were excluded from

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**Abbreviations:** ADC, apparent diffusion coefficient; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; P7, postnatal day 7; TL, tomato lectin

the analysis: one dead in the two groups with melatonin-treated animals, and two were excluded because of the presence of a severe iatrogenic hemorrhage in vehicle-treated animals.

**MRI acquisition.** Rat pups were imaged during ischemia and reperfusion and at 48 h. For imaging, pups were anesthetized by isoflurane 0.8% mixed in 30% O<sub>2</sub>, 70% N<sub>2</sub> and thermoregulated by a water blanket heated at 37°C. Images were acquired using a 7-T horizontal MRI device (Bruker, Germany). Apparent diffusion coefficient (ADC) maps were obtained from an EPI sequence (TE = 25 ms, TR = 3 s, *b* values = 50, 500, 1000 s/mm<sup>2</sup>, FOV 40 × 40 mm, matrix 256 × 256, repetitions = 3, 25 slices, thickness 0.5 mm, 9.5 min). T2-weighted imaging was obtained with a RARE sequence (TR = 5404 ms, TE values = 25.6, 76.8, 128, 179.2 ms, RARE factor = 4). ADC maps were defined by applying a threshold of [mean - 2SD] of the whole contralateral hemisphere ADC.

**Measurement of infarct volume.** Rat pups were killed at 48 h. Brains were removed, fixed for 5 d in 4% formol, and then embedded in paraffin and cut into 20- $\mu$ m-thick sections. Sixteen sections from anterior striatum to posterior hippocampus were selected, taken at equally spaced 0.5-mm intervals. Infarct size was determined on cresyl violet-stained sections using an image analyzer (ImageJ, National Institutes of Health, MA, <http://rsb.info.nih.gov/ij/>). Infarct volumes were expressed as the percentage of the ipsilateral hemisphere.

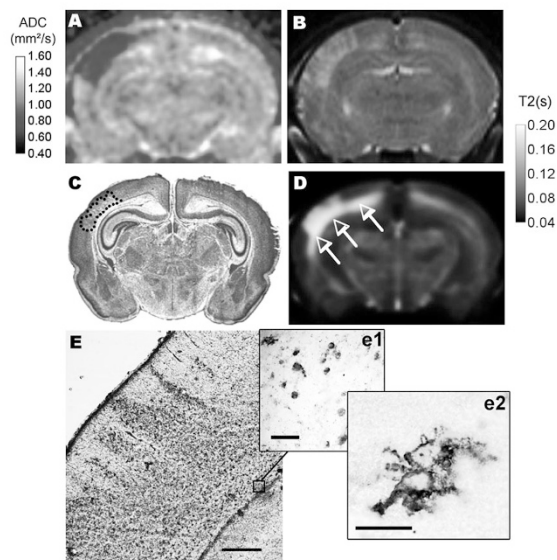
**Immunohistochemistry and TUNEL staining.** Sections (from vehicle- and melatonin two doses-treated animals) were processed for endogenous peroxidase inactivation and blocked in 0.05 M Tris-buffered saline (TBS), containing 10% FCS and 1% of Triton X-100 for 1 h. Afterward, they were incubated overnight at 4°C with either one of the following primary antibodies: tomato lectin (TL; 1:500, ABCYS, Paris, France), nitrotyrosine (NT; 1:100, Millipore, St-Quentin-en-Yvelines, France), Glial fibrillary acidic protein (GFAP; 1:500, ABCYS, Paris, France), APC (1:500, Calbiochem, St-Quentin-en-Yvelines, France), and myelin basic protein (MBP; 1:1000, Millipore, St-Quentin-en-Yvelines, France). After several washings, sections were incubated for 2 h with biotinylated anti-rabbit or anti-mouse secondary antibody, respectively. Immunolabeling with the primary antibodies was visualized using the streptavidin-biotin-peroxydase method, as previously described (23). Adjacent sections were also processed for TUNEL staining according to the manufacturer's instructions (Roche, Meylan, France).

**Cell counting and measurements of MBP-immunoreactivity.** Immunoreactive cells were counted in the ipsilateral and contralateral cingulum and external capsule in at least nine animals per group under an 40 $\times$  objective using a 0.065-mm<sup>2</sup>-grid in three to four coronal sections for each animal at 48 h of recovery. Quantitative analysis of the optical density of MBP-immunoreactive fibers was done using image-analysis software (ImageJ, National Institutes of Health) as previously described (13). Nonspecific background density was measured at each brain level in an area devoid of MBP immunostaining and subtracted from the values for the cingulum.

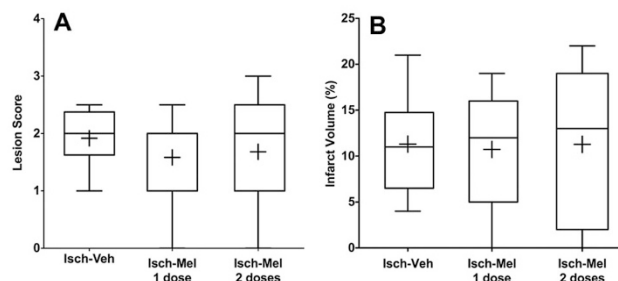
**Statistical analysis.** Infarct score and volumes are expressed as the mean  $\pm$  SD and cell counts as the mean  $\pm$  SEM. Values were analyzed by one-way ANOVA with post hoc Bonferroni test and with the nonparametric Mann-Whitney test. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, [www.graphpad.com](http://www.graphpad.com)).

## RESULTS

**Microglial activation associated with MR abnormalities in the underlying white matter after neonatal stroke.** Using MRI ADC maps and T2-weighted imaging, we observed a cortical lesion as early as 60 min after the ischemic onset (Fig. 1A) well delineated on T2W images 6 h later (Fig. 1B). The extent of the cortical lesion was similar on cresyl violet-stained sections and T2W images at 48-h postlesion (Fig. 1C and D). Interestingly, we also detected a spread of T2 abnormalities toward the subcortical white matter (indicated by arrows in Fig. 1D). At this time point, increased microglia activation was observed both in the different cortical layers and underlying white matter (Fig. 1E). Because melatonin is recognized to combine antioxidant and anti-inflammatory properties (13,24), we asked the question whether this molecule could induce neuroprotection of the injured cortex and/or white matter by modulating this inflammatory response.

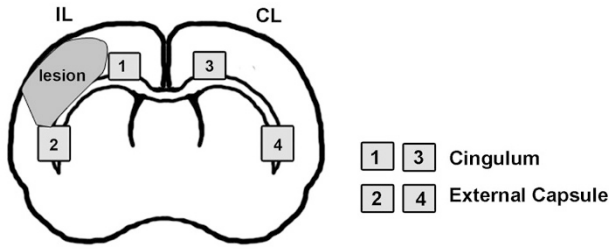


**Figure 1.** Time-course of the lesion and microglial activation after ischemia in P7 rat. (A) ADC map at 60 min after the ischemic onset showing a cortical lesion. (B) T2-weighted image at 6 h after ischemia demonstrating the cortical lesion displaying columnar aspects. (C) Cresyl violet-stained sections at 48 h showing a cortical lesion (delineated with dots). (D) T2-weighted image at 48 h showing a spread toward subcortical and white matter (white arrows). (E) Microglia recruitment (TL immunostaining) both in the cortex (note columnar aspects) and the subcortical white matter (e1, enlarged panel; e2, microglial morphology). Images (A), (B), and (D) were obtained with the use of a high-resolution matrix (0.014 × 0.014 mm/pixel). Scale bar represents 200  $\mu$ m (E), 50  $\mu$ m (e1), 20  $\mu$ m (e2).



**Figure 2.** Effect of melatonin after neonatal stroke. (A) Melatonin did not induce a significant reduction of the lesion score in the Isch-Veh ( $n = 10$ ), Isch-Mel-1-dose ( $n = 11$ ), and Isch-Mel-2-doses ( $n = 11$ ) groups. The median value for all groups was 2 and the mean (+) was 1.9, 1.7, and 1.6, respectively. (B) Percentage of infarct volume with respect to the total volume of the ipsilateral hemisphere in the Isch-Veh ( $n = 10$ ), Isch-Mel-1-dose ( $n = 11$ ), and Isch-Mel-2-doses ( $n = 11$ ) groups. The median (horizontal bar) was 11, 13, and 12 and the mean (+) was 11.3, 11.3 and 10.7, respectively. Data are expressed as the mean  $\pm$  SD.

**Melatonin did not significantly reduce cortical ischemic lesion volume.** Two different regimens of melatonin treatment were used. A first dose of melatonin (20 mg/kg, i.p.) was given immediately before the onset of ischemia and did not affect the extent of brain damage evaluated 48 h after reperfusion (mean score  $1.7 \pm 0.7$ ,  $n = 11$ ) compared with vehicle-treated animals ( $1.9 \pm 0.5$ ,  $n = 10$ ; Fig. 2A). In the double-dose regimen group, a second dose of melatonin was given 24 h after the first one. This treatment still did not reduce the lesion score (mean score  $1.6 \pm 0.8$ ,  $n = 11$ , Fig.



**Figure 3.** An illustration of cerebral hemispheres showing the lesion (gray) and counting areas: square 1 and 3 corresponding to cingulum, and square 2 and 4 to the external capsule, in the ipsilateral (IL) and contralateral (CL) hemispheres, respectively.

**Table 1.** Quantification of the number of TUNEL-, Ki67-, TL-, GFAP-, and NT-positive cells in the vehicle-treated ( $n = 10$ ) and Isch-Mel ( $n = 11$ ) groups in the IL and CL hemispheres

	Veh-IL	Mel-IL	Veh-CL	Mel-CL
<b>TUNEL</b>				
Cingulum	2.9 ± 0.3	2.7 ± 0.5	1.9 ± 0.5	2.4 ± 0.5
External capsule	4.2 ± 1.2	5.1 ± 1.3	2.0 ± 0.6	1.1 ± 0.4
<b>Ki67</b>				
Cingulum	24.8 ± 3.6	32.3 ± 2.5*	6.6 ± 1.3	11.6 ± 1.6*
External capsule	26.4 ± 3.3	24.4 ± 3.8	3.8 ± 2.7	5.4 ± 1.2
<b>TL</b>				
Cingulum	25.7 ± 1.5	18.1 ± 2.4†	14.8 ± 1.8	11.0 ± 1.4
External capsule	22.8 ± 1.3	13.8 ± 1.4†	12.8 ± 1.5	11.8 ± 0.6
<b>GFAP</b>				
Cingulum	37.5 ± 5.7	44.5 ± 9.0	14.5 ± 5.5	16.0 ± 6.9
External capsule	21.7 ± 4.1	25.5 ± 2.3	11.2 ± 4.3	11.5 ± 5.6
<b>NT</b>				
Cingulum	19.3 ± 3.6	18.1 ± 2.9	12.7 ± 3.0	13.1 ± 2.5
External capsule	16.1 ± 2.6	19.7 ± 1.0	11.0 ± 2.6	13.8 ± 1.8

Note that melatonin (two doses) induced a significantly smaller number of TL-positive cells in the IL white matter at 48-h postischemia. Data are given in mean ± SEM positive cells/0.065 mm<sup>2</sup>.

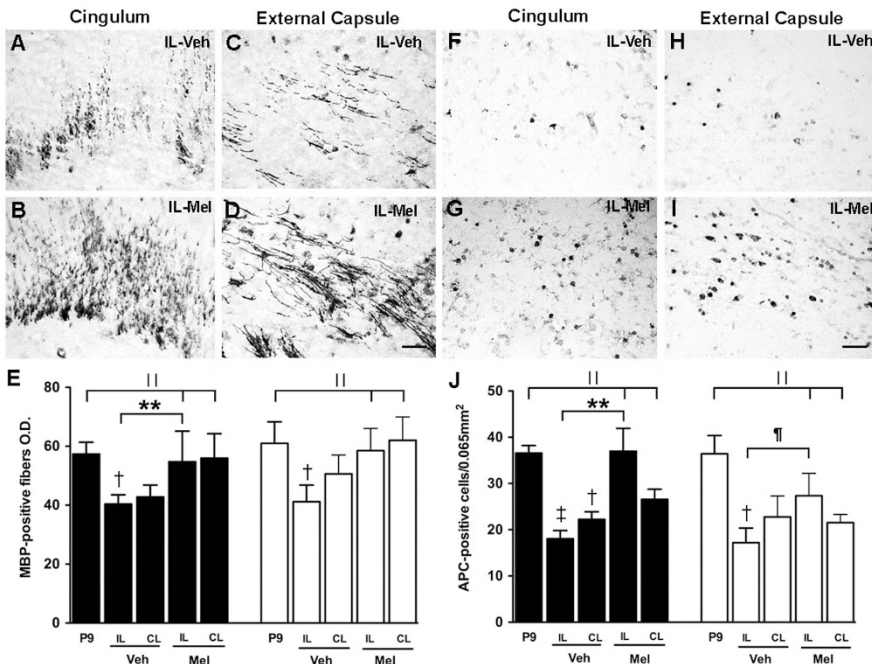
\*  $p < 0.05$  (Mann-Whitney test).

†  $p < 0.05$  (Bonferroni test).

2A). Furthermore, the melatonin treatment did not significantly affect the proportion of cortical infarct volume with respect to the total ipsilateral hemisphere volume (vehicle-treated animals, 11.3 ± 5.6%; Mel-1dose, 11.3 ± 8.3%; and Mel-2doses, 10.7 ± 6.3%, mean ± SD; Fig. 2B).

**Melatonin reduced inflammation in the underlying white matter after ischemia-reperfusion.** We next analyzed the white matter underlying cortical stroke in both the cingulum and external capsule (Fig. 3), where cell damage has been previously reported (19). Melatonin treatment was associated with a significant decrease in the density of TL-positive cells in the cingulum (by 30%,  $p < 0.05$ ) and external capsule (by 40%,  $p < 0.05$ ) when compared with vehicle-treated animals (Table 1). In contrast, no significant difference in the density of TUNEL+ cells after melatonin treatment was detected within these areas. Similarly, neither oxidative stress highlighted by 3-NT immunostaining nor astrogliosis detected using GFAP staining demonstrates a significant difference between vehicle- and melatonin-treated animals. Interestingly, melatonin treatment was associated with an increased proliferative cell density (Ki67+ cells) in both contralateral ( $p < 0.02$ ) and ipsilateral ( $p < 0.04$ ) hemispheres (Table 1).

**Melatonin treatment increases the number of myelinating fibers and mature oligodendrocytes.** As melatonin was very recently reported to promote oligodendroglial maturation in antenatal hypoxia-induced white matter injury (13), we evaluated its effect on MBP fibers density and oligodendrocyte maturation after neonatal stroke in P7 rats. Compared with P9 controls, MBP-positive fibers density was significantly diminished in both the cingulum and external capsule beneath the cortical lesion observed in ischemic animals (Fig. 4A–E). In melatonin-treated animals, MBP-positive fibers density increased by 26% ( $p < 0.05$ ) in the cingulate white matter compared with vehicle-treated rat pups (Fig. 4A, B, and E). A similar trend was found in increased MBP-immunopositive



**Figure 4.** Melatonin (two doses) attenuates the myelination defect and increases the number of mature oligodendrocytes in the cingulum and external capsule at 48 h after ischemia-reperfusion. MBP-immunoreactive fibers density was found increased in melatonin-treated animals (B) compared with vehicle-treated animals (A) in the cingulum. In contrast, no significant change in MBP-immunoreactive fibers density was observed in the external capsule after melatonin treatment (C–D). Scale bar represents 50 μm. (E) Quantitative analysis of the optical density of MBP staining in the cingulate white matter and external capsule within vehicle- and melatonin-treated animals. \*\* $p < 0.01$ ; † $p < 0.05$ ; ‡ $p < 0.01$  vs P9 controls. The number of APC-positive cells was found increased in melatonin-treated animals (G–I) compared with vehicle-treated animals (F–H) both in the cingulum and external capsule, respectively. (J) Quantitative analysis of APC-positive cells density in the cingulate white matter and external capsule from vehicle- ( $n = 10$ ) and melatonin-treated ( $n = 11$ ) animals in IL hemisphere. Melatonin significantly increased the density of mature oligodendrocytes. Data are given in mean ± SEM positive cells/0.065 mm<sup>2</sup>. ||, nonsignificant.

fibers density after melatonin treatment in the external capsule (Fig. 4C, D, and E). Similar results were obtained with the two different regimens of melatonin treatment used.

Compared with P9 controls, the mature oligodendrocytes (APC+) density was found dramatically decreased in both cingulum and external capsule underlying cortical lesion in ischemic animals (Fig. 4F and H). A higher density of mature oligodendrocytes (APC+) in the melatonin-treated animals was observed in both the cingulate white matter (by 51%,  $p < 0.01$ ) and external capsule (by 37%,  $p < 0.05$ ; Fig. 4F–J). Interestingly, melatonin treatment had no effect on the contralateral white matter injury, suggesting that melatonin was able to promote oligodendroglial maturation only during cell proliferation and/or microglial activation (see Fig. 3).

## DISCUSSION

In the neonatal stroke model used in this study, an inflammatory response of the developing white matter occurred between 24 and 72 h after ischemia-reperfusion procedure, at a developmental stage (P7) of high vulnerability to selective white matter injury (19). We have previously described in this model that cortical damage was also accompanied by a marked MBP reduction, O4+ oligodendrocytes depletion (19), and astrocyte demise (21) in subcortical white matter. In this study, we show that melatonin was not able to reduce infarct volume but was potentially able to prevent the reduction of myelin content observed within the white matter underlying cortical lesion. This effect was associated with enhanced oligodendroglial maturation and reduced microglial activation.

Endogenous melatonin was found protective (9), and there is compelling *in vivo* and *in vitro* evidence supporting the use of exogenous melatonin to protect against focal and global ischemia-reperfusion injury in adult animals (25–28). Pretreatment with a single dose significantly reduces the infarct volume by ~40% without affecting the hemodynamic parameters and regional cerebral blood flow (10). Melatonin decreases infarct size and improves neurological scores after middle cerebral artery occlusion in adult mice (29). In contrast, little is known about the effect of melatonin in neonatal hypoxic-ischemic animal models; two recent studies have suggested that melatonin could have beneficial effects and long-term neuroprotection (30) by reducing oxidative stress (31). Here, we refined these findings using a rat model of neonatal stroke, a model recognized to be of particular relevance in the setting of human neonatal stroke.

First, we tested the ability of melatonin to reduce cortical lesion induced in our stroke model administered through one or two dose regimen before ischemic damage. We hypothesized that melatonin could be a potential candidate for cortical neuroprotection, thanks to its general properties on oxidative stress and cell death. Indeed, melatonin reduces oxidative damage generated during both ischemia and reperfusion in neonatal rats and preserves mitochondrial function by improving the electron transport chain, maintaining ATP production, and restoring glutathione levels (32). Moreover, melatonin modulates reactive nitrogen species accumulation by inhibit-

ing NO synthases (33), scavenging NO, and decreasing the production of the peroxynitrite (33). In addition, melatonin can increase the antioxidative activities of superoxide dismutase (SOD) enzymes and glutathione-peroxidase (GSH-Px) in fetal rats (34). Despite these properties, we failed to observe any effect of melatonin on cortical lesion size, suggesting that either the melatonin target might not be located in the cortical plate or cortical injury may be too severe to be reversible. Accordingly, melatonin was found unable to reduce cell death and protein nitration induced by ischemia-reperfusion.

Microglia infiltration occurs over several days in the cingulum in our stroke model (19), similarly to the one observed in the acute stage of periventricular leukomalacia's pathogenesis (35). Melatonin has been demonstrated to have potent neuroprotective effect on neonatal damage mimicking lesions of the developing white matter observed in preterm humans (13,36–39). On the basis of these properties, we then focused on the potential effect of melatonin on white matter damage. Melatonin treatment reduced microglial activation and subsequently enhanced oligodendroglial maturation and myelination of cingulate white matter and external capsule. A reduction in the number of activated microglia cells in the white matter was also found in mid-gestation fetal sheep after umbilical cord occlusion and melatonin treatment (37). Similar to a previous study using a rat model of white matter damage after antenatal hypoxia-ischemia (13), melatonin has, in our model, a protective effect on ipsilateral damaged white matter through decrease of microglial activation and maintain of oligodendroglial maturation leading to a normalization of the myelination process. To date, it has been shown that the effect of melatonin include endocrine, autocrine, and paracrine actions, decreased inflammation, and antioxidant effects. However, in most of perinatal brain damage animal models reported, immune cell activation frequently occurs (35) and melatonin effect seems to be closely linked to inflammation modulation.

It is relevant to point out that the melatonin-induced preservation of myelination is associated with a significant increased of postmitotic oligodendrocytes (APC-positive cells). It was suggested that the main target of melatonin treatment could be the maturation process of the oligodendrocyte lineage (13). Conversely, assuming that the cingulate myelination deficit was associated with focal cingulate microglial activation in our stroke model, and that immature oligodendrocytes are highly vulnerable in this inflammatory context (19) as, it is reasonable to hypothesize that melatonin could restore normal oligodendrocyte functions through a reduction in white matter inflammation. Surprisingly, the effect of melatonin seems to be restricted to ipsilateral injured white matter and not found in the contralateral hemisphere. In contrast, melatonin had no effect on the reactive astrocytes density, suggesting that both microglial cells and oligodendrocytes may be the only two cell types targeted by melatonin in the developing white matter.

Finally, as previously reported in a rat model of specific white matter injury (13), we found that melatonin was able to induce cell proliferation in our stroke model. Because cell proliferation was found particularly important in the ipsilateral

lesioned hemisphere, we hypothesize that it may potentiate the effect of melatonin leading to white matter repair.

In conclusion, we highlight the potential therapeutic value of melatonin in specifically preventing white matter injury associated with neonatal stroke. In addition, our results reinforce the relationship between myelination and inflammation. It is now clear from published clinical trials that induction of hypothermia in infants who had perinatal asphyxia results in improved neurologic outcomes in survivors (40). In contrast, no neuroprotective strategy is available in the setting of neonatal stroke. Melatonin treatment might be used in high-risk pregnancies where ischemic events are common, or, because melatonin is a very safe pharmacological agent without side effects, in the hour before cesarean delivery in patients having variable or late decelerations (indications of ischemic risk). In addition, in newborn infants suffered from a stroke (with a diagnosis of seizures) melatonin could be early administered to at least reduce white matter damage.

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