

Histological study of the protective effect of melatonin on neural cells after neonatal hypoxia-ischemia

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Summary. To minimize as much as possible the neurological consequences from hypoxic-ischemic (HI) brain injury, neuroprotective strategies are urgently required. In this sense, there is growing interest in the neuroprotective potential of melatonin after perinatal asphyxia, due to its high efficacy, low toxicity and ready cross through the blood-brain barrier. Twenty six Wistar rats at postnatal day 7 were randomly assigned to: two hypoxic-ischemic groups: pups with the left common carotid artery ligated and then submitted to hypoxia (HI group) and animals that received a dose of 15 mg/kg melatonin just after the hypoxic-ischemic event and repeated twice with an interval of 24 hours (HI+MEL group). Pups without ischemia or hypoxia were used as controls (Sham group). Seven days after surgery, brains were collected and coronal sections Nissl-stained, TUNEL-labeled, or MBP- and GFAP-immunolabeled prior to determining brain infarct area, quantify surviving neurons and evaluate oligodendroglial injury and reactive astrogliosis. The number of surviving neurons showing a well preserved architecture in HI+MEL group was similar to that observed in the Sham group. Moreover, TUNEL-positive cells only appeared in the HI group. The ratio of left-to-right hemispheric MBP immunostaining showed a significant decrease in the HI group in comparison with Sham pups, which was restored after melatonin administration. Melatonin also reduced reactive gliosis. Thus, our results suggest that treatment with melatonin after neonatal hypoxia-ischemia led to a neuroprotective effect reducing cell death, white matter demyelination and reactive astrogliosis.

Key words: Hypoxia-ischemia, Melatonin, Neuroprotection, Neuron, Astrocyte, Oligodendrocyte

Introduction

Despite the advances in neonatology, hypoxia-ischemia in the perinatal period remains the single most important cause of brain injury in the newborn, leading to death or lifelong sequelae (Hamrick and Ferriero, 2003; Volpe, 2009). The severity, intensity and timing of asphyxia, as well as selective ischemic vulnerability and the immaturity of the brain, determine the extension and degree of severity of the ensuing damage (Walton et al., 1999; Ferriero, 2004). While virtually every cell is affected by asphyxia, they do not respond in the same way during hypoxia-ischemia. Thus, neurons are the most sensitive cells to the lack of O₂, and also show a selective vulnerability (Mattson et al., 1989; Johnston, 1998; Northington et al., 2001; Hilario et al., 2005). Meanwhile, astrocytes are more resistant, but hypoxia-ischemia can also alter their survival and induce a reduction in their capacity for uptaking excitatory neurotransmitters and scavenging of free radicals (FR) that finally will affect to the survival of neurons (Desagher et al., 1996; Iwata-Ichikawa et al., 1999; Chen and Swanson, 2003; Alvarez-Diaz et al., 2007). On the other hand, oligodendrocytes are particularly vulnerable to perinatal asphyxia, affecting myelination that gives rise to white matter lesions and damaging gray matter oligodendrocyte progenitors (Rothstein and Levison, 2005). These cells are the only ones involved in myelin production, so a reduced staining for myelin basic protein (MBP), the major myelin protein, is considered a hallmark of inflammation-associated diffuse white matter damage in fetal rodents (Wang et al., 2007) and preterm infants (Inder et al., 2003).

At the cellular level, hypoxia-ischemia triggers a cascade of biochemical events such as energy failure, mitochondrial dysfunction, membrane depolarization, brain edema, increase of release and inhibition of uptake of excitatory neurotransmitters such as glutamate, increase of intracellular Ca²⁺ and increase of oxidative stress that can lead to cell death (Calvert and Zhang,

2005; Blomgren and Hagberg, 2006; Hilario et al., 2006; Alonso-Alconada et al., 2010). One of the factors involved is the increase in FR generation, which is produced as a consequence of a decrease in the activity of mitochondrial complexes and ATP production. Although FR production is a normal part of cellular physiology, oxidative stress can result from an imbalance between overproduction of FRs and from a deficiency of antioxidants (Frank and Sosenko, 1987; Kelly, 1993; Saugstad, 1996). This over-production of FRs leads to damage of vital cellular components such as nucleic acids, cell membranes and mitochondria, which can finally lead to cell death (Buonocore et al., 2001; Hilario et al., 2006; Kumar et al., 2008; Weis et al., 2011).

Thus, the use of therapies centered on diminishing brain damage arising from FRs makes antioxidants an attractive target for neuroprotection. Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous indoleamine generated primarily by the pineal gland and released into the blood stream and cerebrospinal fluid. This neurohormone easily crosses the blood-brain barrier, reaching subcellular compartments with a low toxicity and high efficacy (Menendez-Pelaez and Reiter, 1993; Gupta et al., 2003; Carloni et al., 2008). It develops a wide range of physiological functions, including the removal of FRs (Tan et al., 2007) and the inhibition of the oxidation of biomolecules (Reiter, 1998). This hormone helps to maintain cell functions and survival (Reiter et al., 1994), stabilizing mitochondrial inner membranes (Garcia et al., 1999), and improving both electron transport chain activity (Acuña-Castroviejo et al., 2001) and mitochondrial integrity (Leon et al., 2004). Therefore, melatonin has shown neuroprotective effects against transient or permanent ischemic brain injury (Pei et al., 2002; Kilic et al., 2005), making it a potentially good tool for the treatment of neonatal hypoxia-ischemia (Welin et al., 2007; Carloni et al., 2008).

The aim of the present work was to evaluate histologically the protective effect of melatonin on neurons, oligodendrocytes and astrocytes after hypoxic-ischemic brain injury in neonatal rats.

Materials and methods

Animal protocols

The experimental protocol met the European Union regulations for animal research (EU 86/609). Neonatal HI was induced according to the method originally described in immature rats (Rice et al., 1981) with minor modifications. After being weighed, seven-day-old Wistar rats were anesthetized with isoflurane (4.0% for induction, 1.0% for maintenance) in oxygen, and the left common carotid artery was isolated and ligated in two locations with 6-0 surgical silk. The common carotid was then transected between the ligatures to guarantee that blood flow through the ipsilateral carotid circulation

was absent throughout the experiment. Any pups with bleeding or respiratory failure were excluded. Animals were returned to their cages and allowed to recover for 1.5-2 h. Once fully recovered from the anesthesia, the rat pups were then placed in a humidified container maintained at 36°C. HI was induced by perfusing the container with humidified 8% oxygen in nitrogen gas mixture for 2 h. After hypoxic exposure, the pups were returned to their biological dams until they were euthanized per protocol on postnatal day 14 (P14).

Experimental groups

Melatonin (Sigma-Aldrich Co. Ltd., Gillingham, UK) was dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical), diluted in normal saline to a final concentration of 5% DMSO and injected intraperitoneally according to Carloni et al. (2008): three doses of 15 mg/kg melatonin were administered starting at 5 min after the ischemic-hypoxic procedure and repeated after 24 and 48 h (MEL, n=8). The HI-injured group (HI, n=10) received a corresponding volume of normal saline containing 5% DMSO (vehicle). Sham-operated rat pups were randomly chosen from the same litters of HI rats and had neither common carotid artery ligation nor a period of hypoxia (SHAM, n=8).

Tissue processing

Seven days after surgery (on P14) rats were weighed and sacrificed via lethal injection of sodium pentobarbital and transcardially perfused with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Brains were removed and, after discarding brainstem and olfactory bulb, weighed using a high precision balance (sensitivity ± 0.001 g). The data were expressed as the whole brain tissue mass. After being immersed in the same fixative at 4°C overnight, brain tissue blocks were prepared, dehydrated and embedded in paraffin. Brains were sectioned at a thickness of 5 μ m at stereotaxic standard levels of 1.6 (1.6 mm anterior to the interaural line per Sherwood and Timiras, 1970), 2.0, and 2.3 A, at the level of mid dorsal hippocampus and thalamus, collected on polylysine-coated slides and processed for Nissl (cresyl violet) staining, TUNEL staining or GFAP immunohistochemistry. For MBP expression analysis, at least six sections per brain, three at the level of the mid-striatum and three at mid dorsal hippocampus, were assayed.

Infarction area

To quantify the extent of infarction to assess the severity of tissue injury, five-micrometer paraffin-embedded sections were stained with cresyl violet (Sigma-Aldrich Co. Ltd., Gillingham, UK) and microscope slides were scanned. The area of infarction was defined by the loss of normal cresyl violet-staining pattern. Cross-sectional areas from the rhinal sulcus to

the interhemispheric fissure of the left and right hemispheres were outlined and measured using Image J software (public domain, National Institutes of Health, <http://rsbweb.nih.gov/ij/>). Measurements, based on the intensity and uniformity of the staining, were performed by an experimenter that was blinded to the conditions of the treatment; these measurements included only intact tissue. Corresponding bihemispheric area ratios were calculated [(L:R)AREA = mean left hemisphere area to mean right hemisphere area] for each brain sample. The mean cortical infarction area $\pm$ SE at stereotaxic levels 1.6, 2.0, and 2.3 A was determined. In this neonatal stroke model, cerebral hemisphere cross-sectional area measurements obtained 7 d after the lesion provide a sensitive indicator of the extent of tissue damage, because injury is incurred at a time of rapid brain growth (Liu et al., 1996).

Assessment of brain damage

Neuronal injury

In order to determine early neuronal necrosis, consecutive pairs of brain sections were stained with cresyl violet and neurons per mm² were counted in the CA1 and CA2-CA3 areas of the hippocampus, dentate gyrus and parietal cortex (Martinez-Orgado et al., 2003) under an Olympus BX 50 light microscope (x400) by an investigator who was blind to the experimental group.

TUNEL

In situ end-labeling of fragmented DNA was carried out on brain sections using the terminal deoxynucleotidyl transferase-mediated incorporation of digoxigenin-labeled nucleotide (TUNEL) assay, applying the ApopTag Kit (Serological Corporation, USA). In brief, 5 μ m thick brain sections were deparaffinated, hydrated, pretreated with 20 μ g/ml proteinase K for 30 min at 37°C, and then incubated with TdT enzymes and digoxigenin-tagged dUTP (at dilution recommended by supplier) at 37°C for 1 hour. Finally, the reaction was revealed by peroxidase-diaminobenzidine and counterstained with hematoxylin. Involuting mammary gland sections were used as positive controls for the TUNEL method (not shown).

Immunohistochemistry

After blocking endogenous peroxidase with H₂O₂ (1%), dewaxed and rehydrated sections were incubated with mouse primary antibody to MBP (1:200, Santa Cruz Biotechnology, CA, USA) or GFAP (1:300, Dako, Denmark) overnight and then with peroxidase-labeled second antibody (HRP anti-mouse 1:100, Santa Cruz Biotechnology, CA, USA) for one hour. The sections were stained with diaminobenzidine and counterstained with hematoxylin.

White matter injury assessment

White matter injury was analyzed by densitometry measurement of MBP immunostaining pattern, using a computerized video-camera-based image-analysis system (National Institutes of Health Image software, public domain, <http://rsb.info.nih.gov/nih-image/>) according to Liu et al. (2002). Unaltered TIFF images were digitized, segmented (using a consistent arbitrary threshold -50%), and binarized (black *versus* white); then, total black pixels per hemisphere were counted, and average values were calculated per brain, expressed as pixels per hemisphere. Hemisphere areas were also outlined and measured in each section that was analyzed by densitometry. At least five sections per brain were analyzed and only sections with obvious technical artifacts related to the staining procedure were excluded. Densitometric values were expressed as ratios of left-to-right hemispheric measurements; for each brain sample, (L:R)MBP of pixels per left hemisphere to pixels per right hemisphere was calculated.

Statistical analyses

Values are presented as mean $\pm$ SEM. Differences between groups in the same brain region were studied by one-factor analysis of variance with the Bonferroni-Dunn correction (Statview, Abacus Corp., Berkeley, CA, USA). A $p < 0.05$ was considered significant.

Results

Characteristics of the experimental groups

There were no differences in body weight among the three experimental groups at postnatal day 7 (P7) (Table

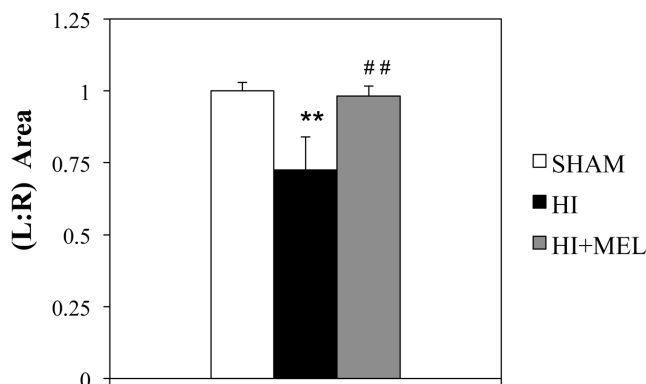


Fig. 1. Measurement of brain infarct area in 14-day-old newborn rats after hypoxia-ischemia and recovery after Melatonin administration. Bars represent the mean $\pm$ standard deviation of left hemispheric area to right hemispheric area (L:R). ** $P < 0.01$ versus Sham, ANOVA; ## $P < 0.01$ versus HI, ANOVA. SHAM, sham control pups; HI, pups asphyxiated on P7 receiving vehicle; HI+MEL, pups asphyxiated on P7 treated with melatonin.

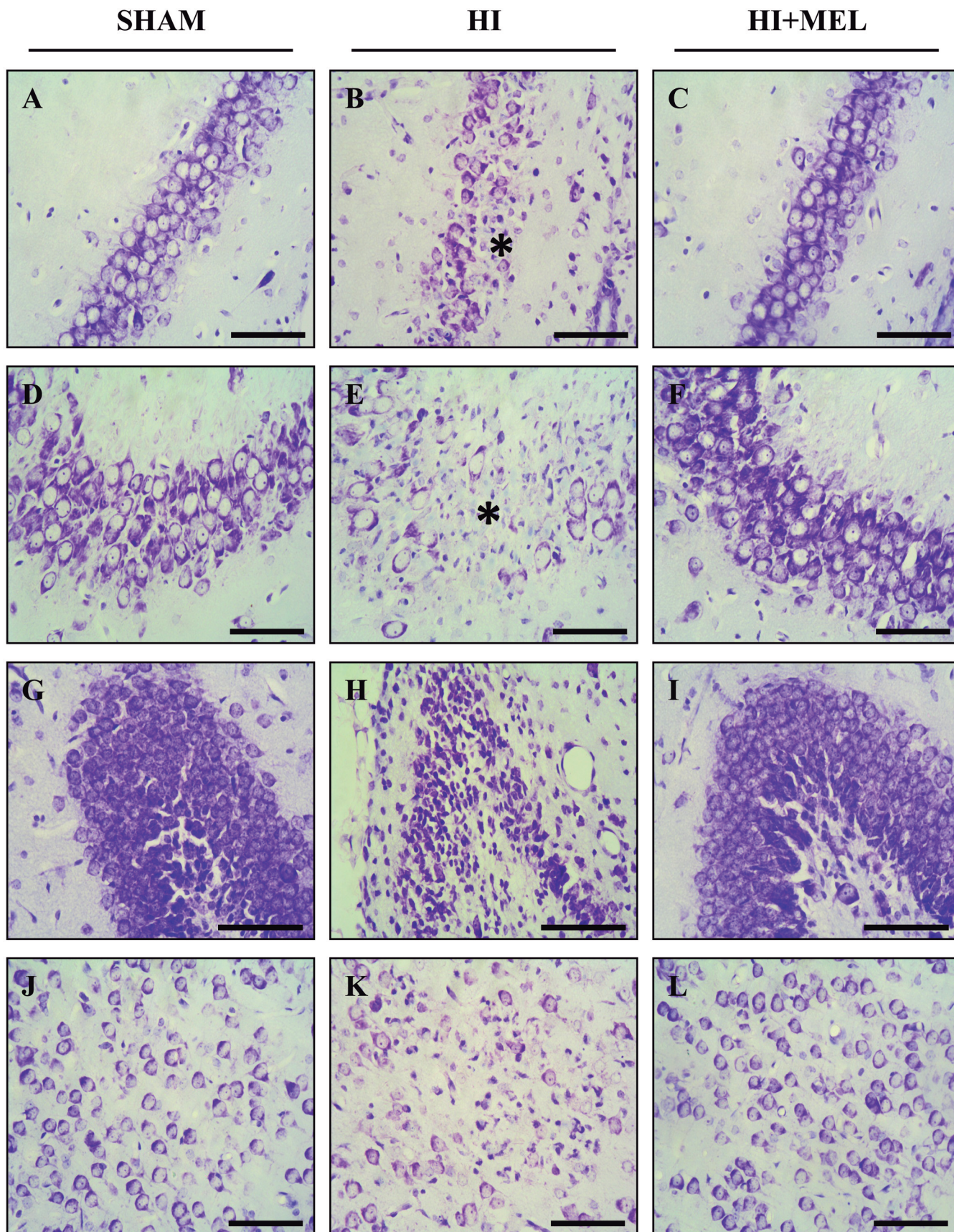


Fig. 2. Cell loss after hypoxia-ischemia and recovery after melatonin administration. Representative light microphotographs of Nissl-stained brain sections from CA1 (**A-C**), CA2/CA3 (**D-F**), DG (**G-I**) and cortex (**J-L**). Note the cell loss in CA1 and CA2/CA3 regions (asterisks). Samples were obtained from P14 animals after sham operation (SHAM: **A, D, G, J**) or after HI receiving vehicle (HI: **B, E, H, K**) or Melatonin (HI+MEL: **C, F, I, L**). CA1 and CA2/CA3 hippocampal regions; DG, dentate gyrus; Cortex, Cerebral cortex. Bar: 100 μ m.

1). HI results in a significant ($p<0.01$) lack of gain in both body (29.22 ± 1.43 g vs 23.07 ± 1.60 g) and brain (1.24 ± 0.05 g vs 0.90 ± 0.11 g) weight in the following week (P14), whereas melatonin administration avoided this decrease (31.33 ± 1.23 g; 1.20 ± 0.07 g, respectively).

Measurement of infarct area

Hypoxia-ischemia often produces cortical and subcortical cavitory infarctions over time that are not present up to 4 days after insult. Regional cross-sectional brain area measurements at the level of dorsal hippocampus and thalamus are expressed as the mean (L:R)AREA values in all groups (Fig. 1). Comparison of cerebral hemisphere ratios in the unlesioned P14 group with the HI group showed the substantial ipsilateral hemisphere damage, revealed as a significant decrease ($p<0.05$) in its (L:R)AREA, from 1 ± 0.2 to 0.72 ± 0.11 . In melatonin treated animals, we found no signs of infarct, with a (L:R)AREA of 0.98 ± 0.03 .

Assessment of brain damage

Neuronal injury

Microscopy revealed early neuronal damage induced by the hypoxic-ischemic event, with the hippocampus, the inner granule cell layers of the dentate gyrus and the fronto-temporal cortex displaying significant evidence of infarction, whereas those of non-ischemic sham animals or melatonin-treated animals did not. In brain sections stained with cresyl violet (Fig. 2), hypoxic-ischemic insult induced a significant cell loss, as marked by the asterisks. Indeed, asphyctic animals showed swollen and deformed neurons in the CA1 and CA2/CA3 areas of the hippocampus ipsilateral to the site of insult (Fig. 2B,E, respectively), and damage to dentate gyrus and cortical neurons was also observed (Fig. 2 H, K, respectively). There is no sign of cell loss or damaged neurons in the slices from animals treated with melatonin, in any of the brain regions studied (Fig. 2C,F,I,L).

Neuronal density

Seven days after hypoxic-ischemic brain injury

Table 1. Characteristics of the experimental groups.

	SHAM	HI	HI+MEL
Body weight (g) on P7	13 ± 0.4	13.4 ± 0.3	13.2 ± 0.4
Body weight (g) on P14	29.22 ± 1.43	$23.07\pm1.60^{**}$	$31.33\pm1.23^{##}$
Brain weight (g) on P14	1.24 ± 0.05	$0.90\pm0.11^{**}$	$1.20\pm0.07^{##}$

Results expressed as mean $\pm$ standard deviation. **: $P<0.01$ versus Sham, ANOVA; #: $P<0.01$ versus HI, ANOVA. P7, 7th postnatal day; P14, 14th postnatal day; SHAM, sham control pups; HI, pups asphyxiated on P7 receiving vehicle; HI+MEL, pups asphyxiated on P7 treated with Melatonin.

(P14), the neuronal densities of CA1, CA2/CA3, dentate gyrus and cortex (Fig. 3) were significantly lower in the vehicle group than in the sham group ($p<0.05$). After melatonin administration, we found that the surviving neurons showed a well preserved architecture, similar to that observed in sham group. Thus, the number of surviving hippocampal and cortical neurons was similar in melatonin-treated and sham animals, with no statistical differences in the neuronal density between these groups.

TUNEL

To determine the degree of cell damage present 7 days after H-I (P14), we also stained brain sections with TUNEL method. Although this method has been widely used to identify apoptosis, it only detects an increase in DNA fragmentation, which occurs not only in apoptotic cells, but can also occur in necrotic cells and intermediate forms of cell death. TUNEL staining was prominent in the ipsilateral hemisphere of HI animals, with a great number of TUNEL-positive cells remaining 7 days after the insult (Fig. 4B,E,H). In contrast, Melatonin treated group (Fig. 4C,F,I) showed less staining, with no signs of neurodegeneration.

Myelin basic protein immunoreactivity

MBP immunostaining pattern showed differences between groups, as shown in figure 5, which compares MBP immunostained brain sections at the level of the mid dorsal hippocampus and thalamus, evaluated at P14. When compared with sham animals (Fig. 5A,D,G), we found a substantial loss of ipsilateral MBP immunostaining in the cingulum, external capsule and adjacent striatum of animals that underwent HI (Fig. 5B,E,H). As the microphotographs show, A and B compare the cingulum and D and E compare the external

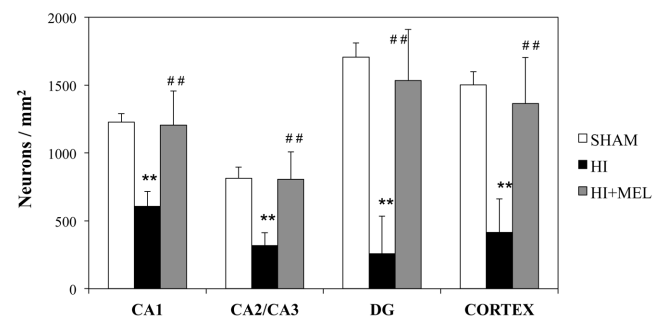


Fig. 3. Neuronal density in the CA1, CA2/CA3 and dentate gyrus areas of the hippocampus and in the parietal cortex in 14-day-old newborn rats. Neurons were counted under light microscopy after Nissl staining. Bars represent the mean $\pm$ standard deviation of neurons per mm². **: $P<0.01$ versus Sham, ANOVA; #: $P<0.01$ versus HI, ANOVA. SHAM, sham control pups; HI, pups asphyxiated on P7 receiving vehicle; HI+MEL, pups asphyxiated on P7 treated with melatonin.

capsule from sham and HI animals, respectively, and demonstrate the marked loss of MBP-immunostained axonal processes. G and H compare areas of the striatum and illustrate the marked reduction of MBP-immunoreactive axonal processes in animals subjected to HI. Melatonin treatment avoided the asymmetry in

MBP immunostaining (Fig. 5C,F,I).

Similar results were observed when we analyzed the samples obtained at the level of the mid-striatum (Fig. 6). We show a substantial loss of ipsilateral MBP immunostaining in the external capsule (B,E) and adjacent striatum (H) when compared with sham animals

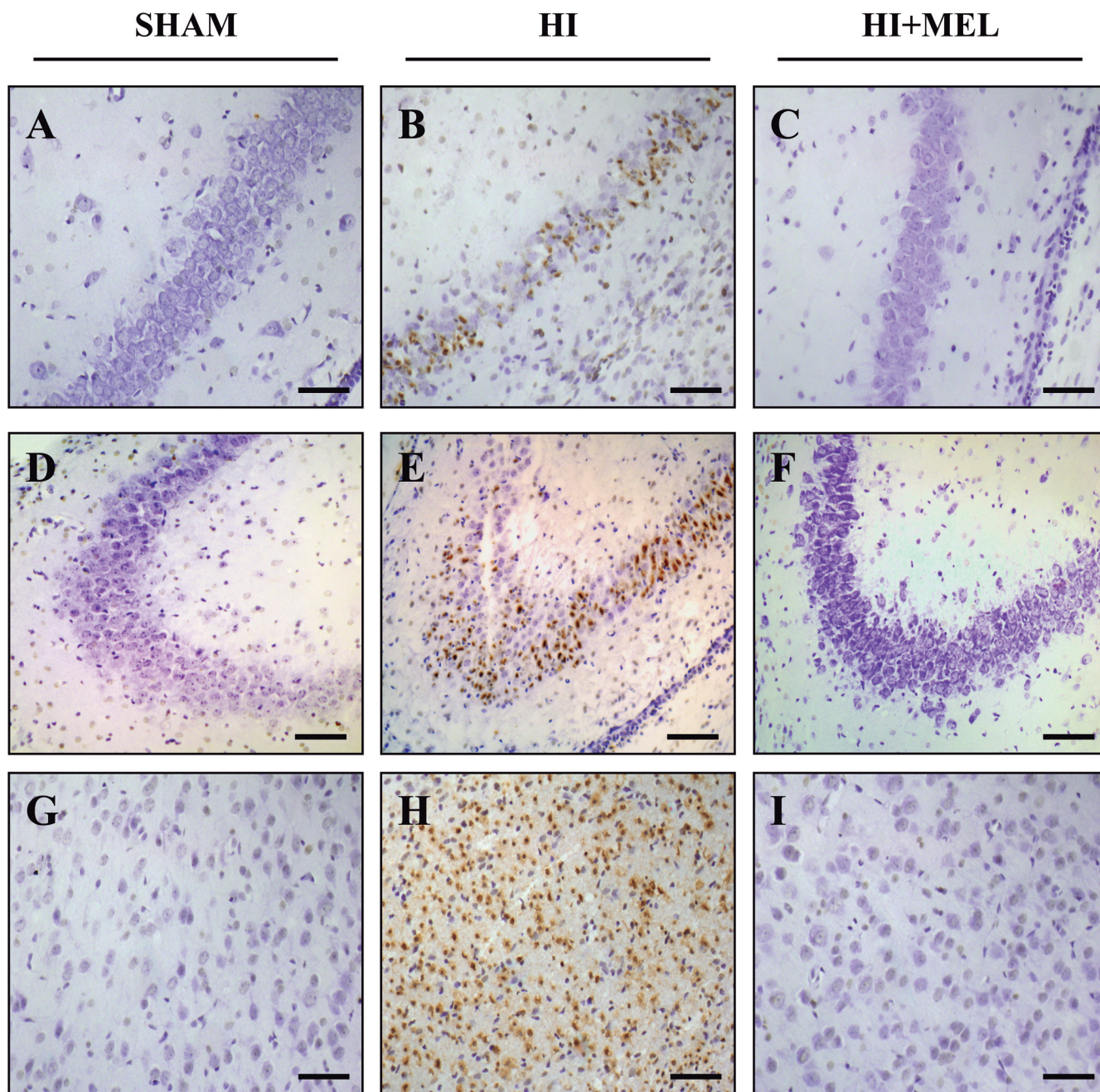


Fig. 4. Increase of TUNEL-positive cells in lesioned animals and recovery after Melatonin administration. Representative light microphotographs illustrating the TUNEL immunostaining in brain sections from CA1 (**A-C**), CA2/CA3 (**D-F**) and cortex (**G-I**). Samples were obtained from P14 animals after sham operation (SHAM: **A, D, G**) or after HI receiving vehicle (HI: **B, E, H**) or Melatonin (HI+MEL: **C, F, I**). CA1 and CA2/CA3 hippocampal regions; Cortex, Cerebral cortex. Bar: 100 μ m

(A,D,G). In this region, melatonin treatment also avoided the asymmetry in MBP immunostaining (Fig. 6C,F,I).

White matter demyelination

When we analyzed the MBP ratios between

ipsilateral (L) and contralateral (R) hemispheres, we found that there was a loss of MBP immunostaining in subcortical white matter at the level of the mid dorsal hippocampus and thalamus (Fig. 7A) and also at the level of the mid-striatum (Fig. 7B). This decrease in the (L:R)MBP ratio of HI pups was significant ($p < 0.01$) when compared with sham animals. However, the level

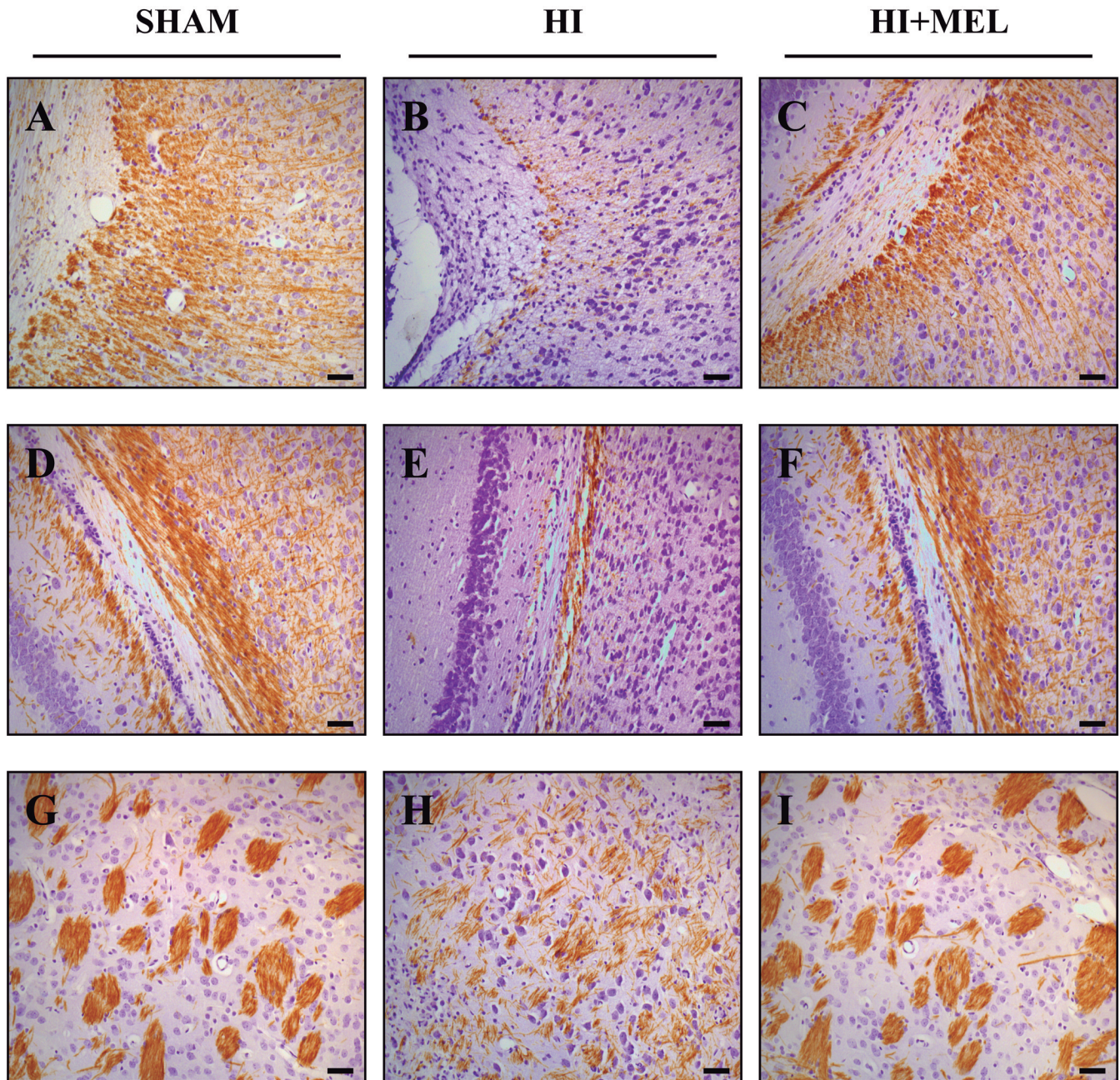


Fig. 5. Loss of MBP immunostaining at the level of the mid-dorsal hippocampus in lesioned animals and recovery after melatonin administration. Light microphotographs illustrating the disruption of MBP immunostaining in the cingulum (A-C), external capsule (D-F) and adjacent striatum (G-I). Samples were obtained from P14 animals after sham operation (SHAM: A,D,G) or after HI receiving vehicle (HI: B,E,H) or Melatonin (HI+MEL: C,F,I). Bar: 100 μ m.

of MBP remaining in the subcortical white matter of the ipsilateral hemisphere was found to be significantly higher in melatonin-treated rats than in HI rats ($p < 0.01$) in both anatomical regions. Melatonin-treated animals showed a lesser degree of MBP loss in the ipsilateral hemisphere, with (L:R)MBP ratio similar to that observed in the sham group.

Reactive astrogliosis

To test whether melatonin administration can reduce astrocyte reactivity, immunohistochemistry was performed 7 days after H-I to assess GFAP up-regulation (Fig. 8). Low levels of GFAP were detected in sham animals (Fig. 8A,D,G,J). In general, GFAP immuno-

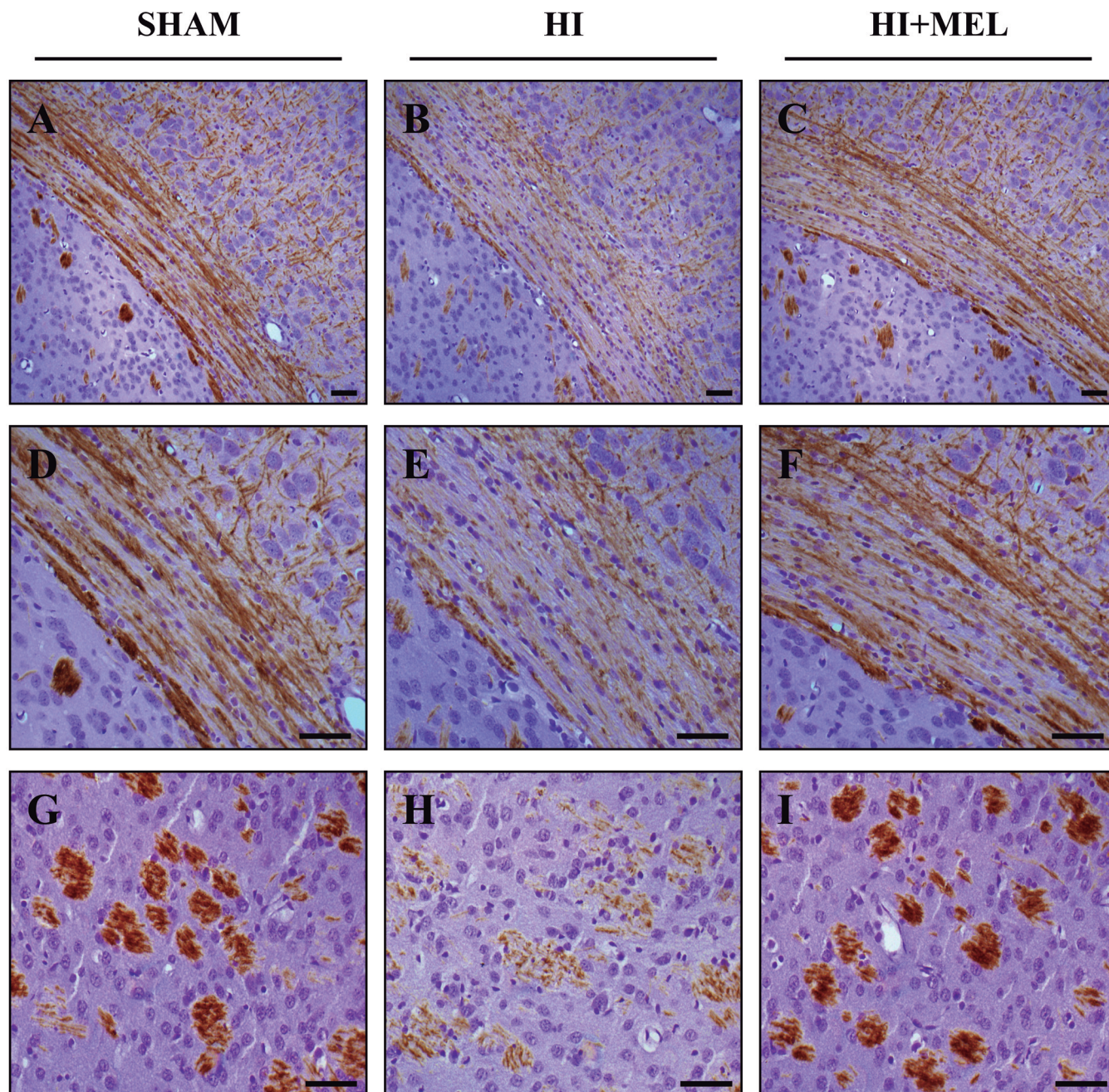


Fig. 6. Loss of MBP immunostaining at the level of the striatum in lesioned animals and recovery after melatonin administration. Light microphotographs illustrating the disruption of MBP immunostaining in the external capsule (**A-C** and **D-F**) and adjacent striatum (**G-I**). Samples were obtained from P14 animals after sham operation (SHAM, **A**, **D**, **G**) or after HI receiving vehicle (HI, **B**, **E**, **H**) or melatonin (HI+MEL, **C**, **F**, **I**). Bar: 100 μ m.

reactivity was prominent in the ipsilateral hemisphere of HI animals, in regions surrounding necrotic cells, as in CA1 and CA2/CA3 areas of the hippocampus (Fig. 8B,E, respectively), dentate gyrus (Fig. 8H) and cortex (Fig. 8K). Astrogliosis was diminished after Melatonin administration (Fig. 8C,F,I,L).

Discussion

Melatonin is a neurohormone that is being used as a neuroprotective agent in several brain injuries. In perinatal asphyxia, melatonin has been employed in different animal models mainly focusing on neurons and, to a lesser extent, astrocytes and oligodendrocytes. In this work we have made a prospective study of the protective effect that melatonin may have on neurons, astrocytes and oligodendrocytes after neonatal hypoxia-ischemia.

Clinical and experimental studies have shown that neonatal hypoxia-ischemia not only causes brain damage and neurological deficits, but also decreased somatic growth. Accordingly, we showed that somatic growth retardation was apparent in vehicle rats at P14 weeks when compared with sham, and that melatonin significantly improved body weight. Moreover, we also showed that melatonin treated pups also maintained brain weight values, suggesting a beneficial effect of this compound. The decrease in brain weight was mainly due to the loss of cerebral tissue and the subsequent formation of the cavity, an observation that agrees with other authors who described the formation of brain cavities associated with HI long episodes (Towfighi et al., 1991; Biran et al., 2006). Although recent studies have shown that melatonin was unable to reduce cortical infarct volume a short time after HI injury (48 hours) (Villapol et al., 2011), we have observed an important

decrease in the infarct area at 7 days after the injury. These results are in agreement with Carloni et al. (2008), who revealed a decrease in infarct volume at 7 and 73 days after HI in neonatal rats, and also with Pei et al. (2003), who reported that melatonin reduces infarct volume at 72 hours in adult rats. Furthermore, it is relevant to point out that melatonin-treated animals showed an increase in the number of morphologically well preserved neurons, reaching values very similar to those observed in sham animals, together with a decrease in dead cells (as Nissl staining and TUNEL-assay showed, respectively). Experimental evidence exists regarding the beneficial effects of melatonin, but the exact mechanisms by which it is neuroprotective remains a subject of investigation. Melatonin may signal through a variety of regulated pathways, including endocrine, autocrine and paracrine actions. The compound itself may exert direct antioxidant effects, scavenging FRs and/or inhibiting their formation (Rosen et al., 2006; Tan et al., 2007), and has shown to protect against excitotoxicity induced brain damage (Espinosa et al., 2000; Husson et al., 2002), an effect that can account, at least in part, for its neuroprotective effect against necrotic cell death. On the other hand, melatonin has shown the ability to reduce the number of apoptotic cells following ischemic brain injury (Sun et al., 2002; Koh, 2008). This indoleamine exerts a wide range of antiapoptotic effects, mainly targeting mitochondria, but can also enhance diverse cell survival pathways (Wang, 2009). Moreover, melatonin can up-regulate endogenous antioxidant defences (Cuzzocrea and Reiter, 2002), reduce the inflammatory response (Welin et al., 2007; Villapol et al., 2011) and promote neuroproliferation (Sotthibundhu et al., 2010; Fu et al., 2011). Regarding neonatal brain injury, sensorimotor asymmetry and learning deficits were significantly reduced after

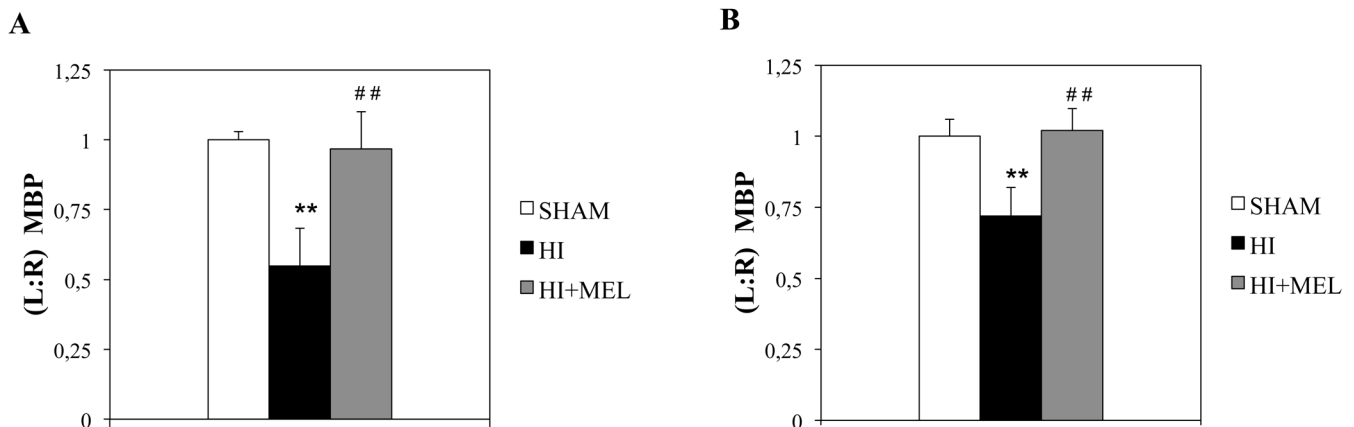


Fig. 7. Densitometric analysis of MBP immunostaining at the level of the mid dorsal hippocampus and thalamus (**A**) and at the level of the mid-striatum (**B**). MBP immunostaining in each cerebral hemisphere was estimated by computerized densitometry. For each brain sample, (L:R)MBP of pixels per left hemisphere to pixels per right hemisphere was calculated. Bars represent the mean $\pm$ standard deviation. ** $P < 0.01$ versus Sham, ANOVA; ## $P < 0.01$ versus HI, ANOVA. SHAM, sham control pups; HI, pups asphyxiated on P7 receiving vehicle; HI+MEL, pups asphyxiated on P7 treated with melatonin.

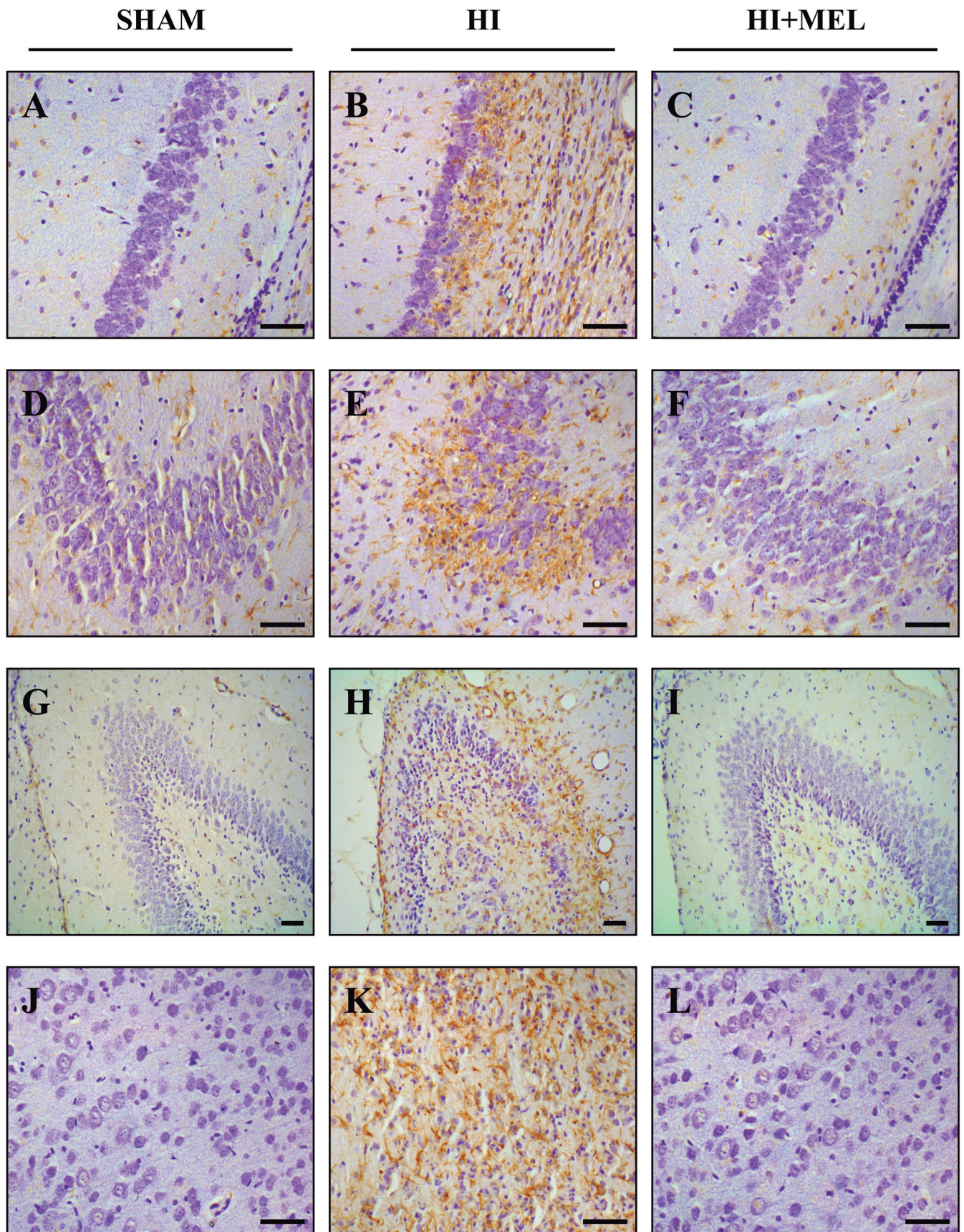


Fig. 8. Increase in reactive gliosis in lesioned animals and recovery after Melatonin administration. Representative light microphotographs illustrating GFAP immunostaining in brain sections from CA1 (**A-C**), CA2/CA3 (**D-F**), DG (**G-I**) and cortex (**J-L**). Samples were obtained from P14 animals after sham operation (SHAM: **A, D, G, J**) or after HI receiving vehicle (HI: **B, E, H, K**) or Melatonin (HI+MEL: **C, F, I, L**). (CA1 and CA2/CA3 hippocampal regions; DG, dentate gyrus; Cortex, Cerebral cortex). Bar: 100 μ m.

melatonin administration (Carloni et al., 2008), and its maternal administration abrogates inflammation and apoptotic cell death (Hutton et al., 2009a). Furthermore, melatonin can reduce hydroxyl radical levels and 4-HNE immunoreactivity (Miller et al., 2005) and microglial cell activation (Welin et al., 2007) in the fetal sheep brain. This anti-inflammatory effect may be mediated by preventing the translocation of NF- κ B to the nucleus, thus reducing the up-regulation of pro-inflammatory cytokines (Reiter et al., 2000). Other melatonin-related mechanisms are the activation of specific receptors and GABAergic pathways, its antiepileptic capacity, and the inhibition of adenylate cyclase (Husson et al., 2002).

Multiple mediators could contribute to neonatal HI oligodendroglial injury. In this neonatal stroke model, oligodendroglia is highly susceptible to glutamate-mediated injury, and hypoxia-ischemia elicits a widespread and complex inflammatory response that finally contributes to white matter injury. Oligodendrocytes are the major component of white matter and the cells for myelin formation in central nervous system (Barres, 2008), so these cells can suffer a dysfunction that alters the formation of the myelin sheath after hypoxia-ischemia. Myelin is responsible not only for protection and insulation of axons, but also for production of supportive neurotrophic factors (Lin et al., 2004). Thus, myelination deficiency will result in adverse effects on axonal function and neuronal survival leading to long-term cognitive impairment (Huang et al., 2009), such as abnormal nerve impulse conduction, cerebral palsy, long-term mental deficits, and learning disabilities in childhood. MBP is the major myelin protein, constituting 30% of the total myelin proteins, and plays a major role in myelin compaction in the central nervous system development by intercalating between phospholipidic sheets and interacting with lipids and proteolipids. Indeed, hypoxia-ischemia can induce a reduction in mRNAs encoding MBP and a subsequent decline in protein synthesis, thus representing a major mechanism that contributes to loss of MBP immunostaining on P14 (Liu et al., 2002). In this regard, our results showed that asphyctic animals displayed a great reduction in MBP immunostaining when compared with sham values, a reduction avoided by melatonin administration. Thus, melatonin could develop a protective effect on ipsilateral damaged white matter through normalization of the myelination process, a beneficial effect demonstrated in studies carried out in other animal models (Olivier et al., 2009) and using other time-periods of study (Villapol et al., 2011). These results are in agreement with others where melatonin has demonstrated a potent neuroprotective effect on neonatal damage, mimicking lesions of the developing white matter observed in preterm humans (Husson et al., 2002; Welin et al., 2007; Bouslama et al., 2007; Olivier et al., 2009).

There is growing evidence concerning the importance of astrocytes, the most abundant glial cell types in the brain, in the modulation of HI brain damage

(Takuma et al., 2004; Panickar and Norenberg, 2005). In a mild ischemic episode, neurons are more susceptible to injury while astrocytes are less affected, so those that survived adjacent to the injured tissue are able to re-establish neuronal integrity. Thus, prevention of astrocyte death is now considered a key-target for neuroprotection (Takuma et al., 2004). In response to injury, astrocytes can create new cytoplasmic processes, an event associated with the accumulation of intermediate glial filaments like glial fibrillary acidic protein (GFAP) (Panickar and Norenberg, 2005). GFAP is rarely expressed by other cell types (Eng et al., 2000) and it is used as a marker of reactive gliosis in the later phases of injury. Regarding our results, we showed that after the HI event there is an increase in GFAP immunostaining, which was restored after melatonin administration. Indeed, the number of GFAP-positive cells was apparently less after melatonin administration and the length of the processes of the astrocytes was slightly shorter than that in the HI group. In this sense, previous data have demonstrated a robust astroglial response early after HI in the neonate, with reactive astrocytes as key players in neuroinflammation and response of upstream of severe infarction (Leonardo et al., 2008a,b; Xiong et al., 2009; Hutton et al., 2009b). Although partial astrocyte activity could confer neuroprotection by scavenging reactive oxygen species and assist with reconstruction from brain injury (Sizonenko et al., 2008), excessive astrocyte activity can result in pre-oligodendrocyte myelination deficiencies and neuronal signaling impairment, due to the formation of a glial scar, which may act as a physical barrier.

Taken as a whole, our results suggest that melatonin administration after a HI brain injury in the neonatal rat may reduce brain infarct, cell death, white matter demyelination and reactive astrogliosis. Although the mechanisms by which melatonin is neuroprotective in this neonatal stroke model remain a subject of investigation, this work supports the high interest in this indoleamine as a future feasible strategy for cerebral asphyctic events.

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