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Melatonin attenuates developmental deficits and prevents hippocampal injuries in male and female rats subjected to neonatal anoxia

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[Correction added on 1 July 2024, after first online publication: The third author's name has been corrected in this version.]

Abstract

Hypoxia in preterm infants is a clinical condition that has been associated with cognitive and behavioral disturbances for which treatment strategies are strongly required. Melatonin administration following brain insults has been considered a promising therapeutic strategy due to its antioxidant and anti-inflammatory effects. Not surprisingly, it has been extensively studied for preventing disturbances following brain injury. This study evaluated the effects of melatonin on developmental disturbances, memory disruption, and hippocampal cell loss induced by neonatal anoxia in rats. Neonatal Wistar rats were subjected to anoxia and subsequently treated with melatonin. Later, maturation of physical characteristics, ontogeny of reflexes, learning and memory in the Morris water maze (MWM), and estimates of the number of hippocampal neurons, were evaluated. Melatonin treatment attenuated (1) female anoxia-induced delay in superior incisor eruption, (2) female anoxia-induced vibrissae placement reflexes, and (3) male and female anoxia-induced hippocampal neuronal loss. Melatonin also promoted an increase (5) in swimming speeds in the MWM. In addition, PCA analysis showed positive associations between the acoustic startle, auditory canal open, and free fall righting parameters and negative associations between the male vehicle anoxia group and the male melatonin anoxia group. Therefore, melatonin treatment attenuates both anoxia-induced developmental deficits and hippocampal neuronal loss.

KEYWORDS
hippocampus, isotropic fractionator, memory, reflex ontogeny

Abbreviations: A, anoxia; AMF, anoxia melatonin female; AMM, anoxia melatonin male; AVF, anoxia vehicle female; AVM, anoxia vehicle male; C, control; CEUA, Comitê de Ética no Uso de Animais; CMF, control melatonin female; CMM, control melatonin male; CVF, control vehicle female; CVM, control vehicle male; HIE, hypoxic–ischemic encephalopathy; ICB, Instituto de Ciência Biomédicas; M, melatonin; NFKB, nuclear transcription factor kappa B; P, postnatal day; PCA, principal component analysis; TNF, tumor necrosis factor; V, vehicle.

1 | INTRODUCTION

Oxygen deprivation at birth, clinically known as perinatal asphyxia, is often associated to hypoxic–ischemic encephalopathy (HIE), characterized by decreased level of consciousness, reflex movements, abnormal muscle tone, and respiratory failure (Laptook et al., 2017).

The incidence of HIE varies between 0.1 and 0.8% of live births (Kurinczuk et al., 2010). In premature births, particularly those with low birth weight, the incidence corresponds to approximately 60% of this population (Perin et al., 2022).

Prematurity may be associated with brain tissue damage, developmental delays, and poor performance at school (Quigley et al., 2012; Shah et al., 2006; Woythaler et al., 2011). When combined with HIE, prematurity may exacerbate cognitive and behavioral impairments, including attentional deficit, hyperactivity, learning disability, and cerebral palsy (Escobar et al., 1991; Gaffney et al., 1994; Medoff-Cooper et al., 2015).

In order to study HIE, we developed a rodent model of neonatal anoxia that mimics oxygen deprivation in premature infants. This global and noninvasive oxygen deprivation model promotes acute hippocampal cell death through necrosis and apoptosis in both glial and neuronal populations (Takada et al., 2011). It also reduces dentate gyrus neurogenesis, decreases hippocampal volume (Takada et al., 2011; Takada et al., 2015), and reduces cell density in the primary somatosensory cortex, leading to deficits in sensorimotor development (Kumar et al., 2017), and disturbances in learning and memory (Takada et al., 2015; Takada et al., 2016).

Hypothermia had been employed in an attempt to alleviate long-term sequelae of perinatal asphyxia (Millar et al., 2017), but has produced controversial effects in rats (Matsuda et al., 2021). Other therapeutic approaches for hypoxia have been investigated, including administration of erythropoietin, cannabidiol, allopurinol, and melatonin (Revuelta et al., 2017; Rosales-Corral et al., 2003).

Melatonin, either alone or in combination with other therapeutic agents, has been shown to attenuate brain injuries (Berger et al., 2017; Blanco et al., 2017). Melatonin's low toxicity, ability to cross the blood–brain barrier, and anti-inflammatory effects make it an excellent candidate to minimize sequelae of HIE. Indeed, melatonin administration following HIE reduced brain injury and ameliorated behavioral deficits in rodents (Berger et al., 2017; Blanco et al., 2017).

2 | MATERIAL AND METHODS

Wistar rats (*Rattus norvegicus*) from the Rat Breeding Vivarium (*ICB*, *Rede USP de Biotérios*) were housed at the Experimental Vivarium of the ICB (Department of Anatomy) and used according to a certificate approved by CEUA of the Institute of Biomedical Science, number 19/2017, and adhering to the guidelines established by CONCEA (*Conselho Nacional de Controle de Experimentação Animal*). The animals were maintained under a 12-h light/dark cycles (lights on at 07:00), at a temperature of 22 ± 1°C, with water and food ad libitum.

The complete study included four pregnant females, each paired with a male throughout the entire experiment. After birth, each litter was reduced to eight pups, aiming for an equal mix of four males and four females. All pups from the same littler were assigned to the same group. However, litters from the same female were assigned to different groups ensuring that all animals in a group came from different litters.

The experimental arrangement involved eight groups exposed to the following treatments: control vehicle male (CVM), control melatonin male (CMM), anoxia vehicle male (AVM), anoxia vehicle melatonin (AMM), control vehicle female (CVF), control melatonin female (CMF), anoxia vehicle female (AVF), and anoxia melatonin female (AMF). Males and females were analyzed separately.

The complete time course of the experiment is shown in Figure 1.

2.1 | Neonatal anoxia

Neonatal anoxia was induced using the model described by Takada et al. (2011). Briefly, 30-h-old pups weighting between 6 and 8 g were placed in a chamber at 37 ± 1°C and exposed to 100% nitrogen for 25 min. After recovery, pups were returned to the dam until weaning on postnatal day 21 (P21). Control groups underwent identical experimental conditions, except for oxygen deprivation.

2.2 | Melatonin

Melatonin (M5250; Sigma-Aldrich, São Paulo, Brazil) dissolved in a solution of 2% ethanol and 0.9% saline (vehicle) was administered intraperitoneally (15 mg/kg) 5 min, 24 h, and 48 h after anoxic insult (Carloni et al., 2008). Control subjects for melatonin received administration of the vehicle following the same time course.

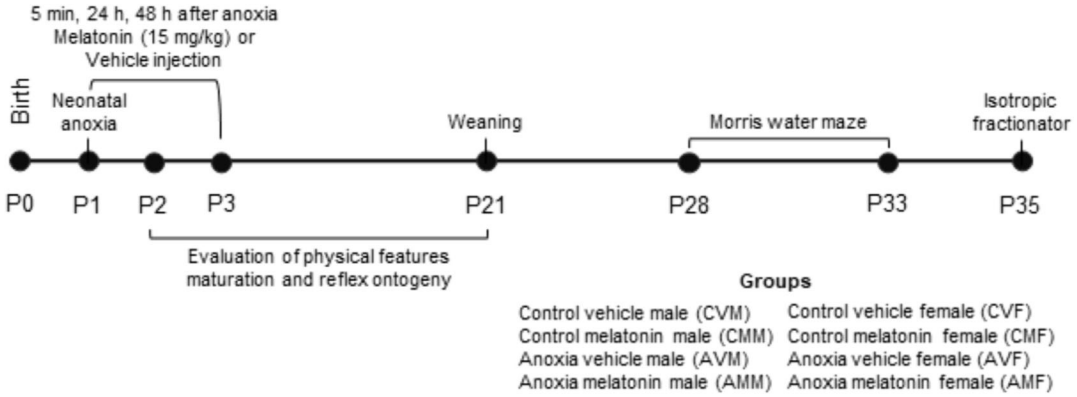

FIGURE 1 Experimental design. After birth, the animals, which were approximately 30 h old and weighed between 6 and 8 g, were submitted to anoxia or a control condition. Five minutes, 24 h, and 48 h, after insult or control condition, the animals were treated with melatonin or vehicle. From the day after insult or control condition (P2) until P21, the maturation of physical characteristics and ontogeny of reflexes were assessed. Between P28 and P33, learning and spatial memory were assessed using the Morris water maze test. On P35, the animals were euthanized to perform the isotropic fractionator technique and count cell and neuronal nuclei.

2.3 | Physical feature maturation

From P2 until weaning at P21, we monitored the maturation day of the following developmental features: ear unfolding, auditory channel opening, eyelid opening, as well as the eruption of inferior and superior incisors.

2.4 | Reflex ontogeny

From P2 until weaning at P21, we evaluated the ontogeny of the following reflexes: righting reflex, cliff avoidance, vibrissae placing, negative geotaxis, free fall righting reflex, acoustic startle response, and palmar grasp. The animals were expected to exhibit the reflex within a period of up to 10 s for three consecutive days.

2.5 | Morris water maze

Acquisition and retention of spatial reference memory were evaluated using Morris water maze. The maze consisted of a round pool measuring 200 cm in diameter and 50 cm in height, filled with water (26 ± 1°C) to a depth of 25 cm. A 9 cm in diameter platform was placed in the center of the north–east quadrant of the pool about 1 cm below the water surface. The platform was maintained in a single fix location all along training. Each trial consisted of placing the subject within the water, facing the wall near the pool border, and allowing it to search for the platform for a maximum of 120 s. If the subject did not find the platform, it was manually guided to it by the experimenter and allowed to remain there for 10 s. The animals were trained for five consecutive days, with four trials per day. The intertrial interval was 10 min. The starting point varied randomly for trial to trial. Latency, path length, and mean swimming speed to find the platform were recorded for each trial.

2.6 | Euthanasia and cell nuclei estimates

At P35, the animals were deeply anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg) and euthanized by transcardial perfusion with saline 0.9% followed by formaldehyde 4%, pH 7.4. The brain was post-fixed and later dissected to isolate the hippocampus (CA1, CA2, C3, and dentate gyrus).

The isotropic fractionator (see Herculano-Houzel & Lent, 2005) was used to estimate the number of cell nuclei. The procedure involved a chemical–mechanical dissociation of tissue in Triton X-100 1%, which disrupts the plasma membrane to obtain the suspension of homogeneously dispersed nuclei. After dissociation, this nuclear suspension was collected and stained with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI; S7113, Millipore, Temecula, USA). Then, anti-NeuN, clone A60 with conjugated Alexa Fluor 555 (MAB377A5, Millipore, Temecula, USA) was incubated for 2 h. Neuronal (NeuN+) and non-neuronal (DAPI+) cells were estimated using a Neubauer chamber and a fluorescence microscope (Eclipse 80i, Nikon).

2.7 | Statistical analysis

Physical features, maturation scores, and ontogeny of reflexes were analyzed using Kruskal–Wallis nonparametric analysis of variance, followed by pairwise

comparisons. Latency, path length, and mean swimming speed scores in the Morris water maze were analyzed using repeated measure ANOVA followed by the Tukey–Kramer test. Cell nuclei estimates were analyzed using two-way ANOVA, followed by the Tukey–Kramer test.

3 | RESULTS

3.1 | Physical feature maturation in male and female rats

Figures S2 and S3 represent the age (in days) at which physical features matured throughout the developmental period in male and female rats, respectively, based on the treatments administered. The animals' body weights were also recorded and can be found in the Supporting Information (S1).

The Kruskal–Wallis including only data of male rats revealed a significant anticipation of the day of eye opening in animals subjected to anoxia as compared with animals subjected to the control procedure ($H(3) = 8.549$, $p = 0.03$) (Figure 2c). No other physical features exhibited significant differences for male rats.

The Kruskal–Wallis including only data of female rats revealed a significant group difference in the day of superior incisor eruption ($H(3) = 9.661$; $p = 0.02$) (Figure 3c): pairwise comparisons for these scores revealed that the superior incisor eruption of the AVF subjects occurred later as compared with that in AMF subjects (Figure 3c). In contrast, CVF and CMF subjects did not differ significantly. These results indicate that anoxia delayed the superior incisor eruption and that melatonin reversed this effect. No other physical features exhibited significant differences for female rats.

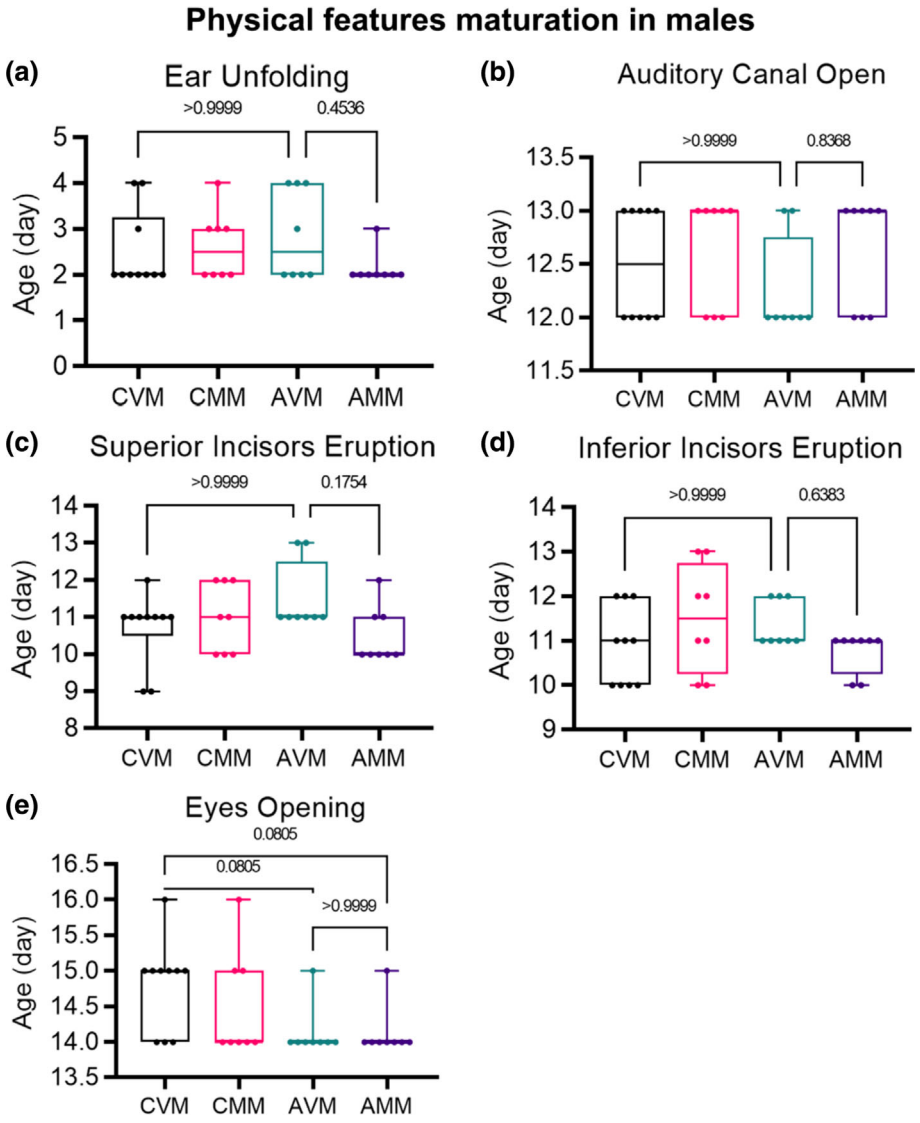

FIGURE 2 Box plots showing the age of maturation (in days) of (a) ear unfolding, (b) auditory canal open, (c) superior incisor eruption, (d) inferior incisor eruption, (e) eye opening, in male (M) rats subjected to either anoxia (A) or control (C) procedures followed by three i.p. injections of either melatonin (M) of vehicle (V), as evaluated from P2 to P21. CVM ($n = 10$), CMM ($n = 8$), AVM ($n = 8$), AMM ($n = 8$).

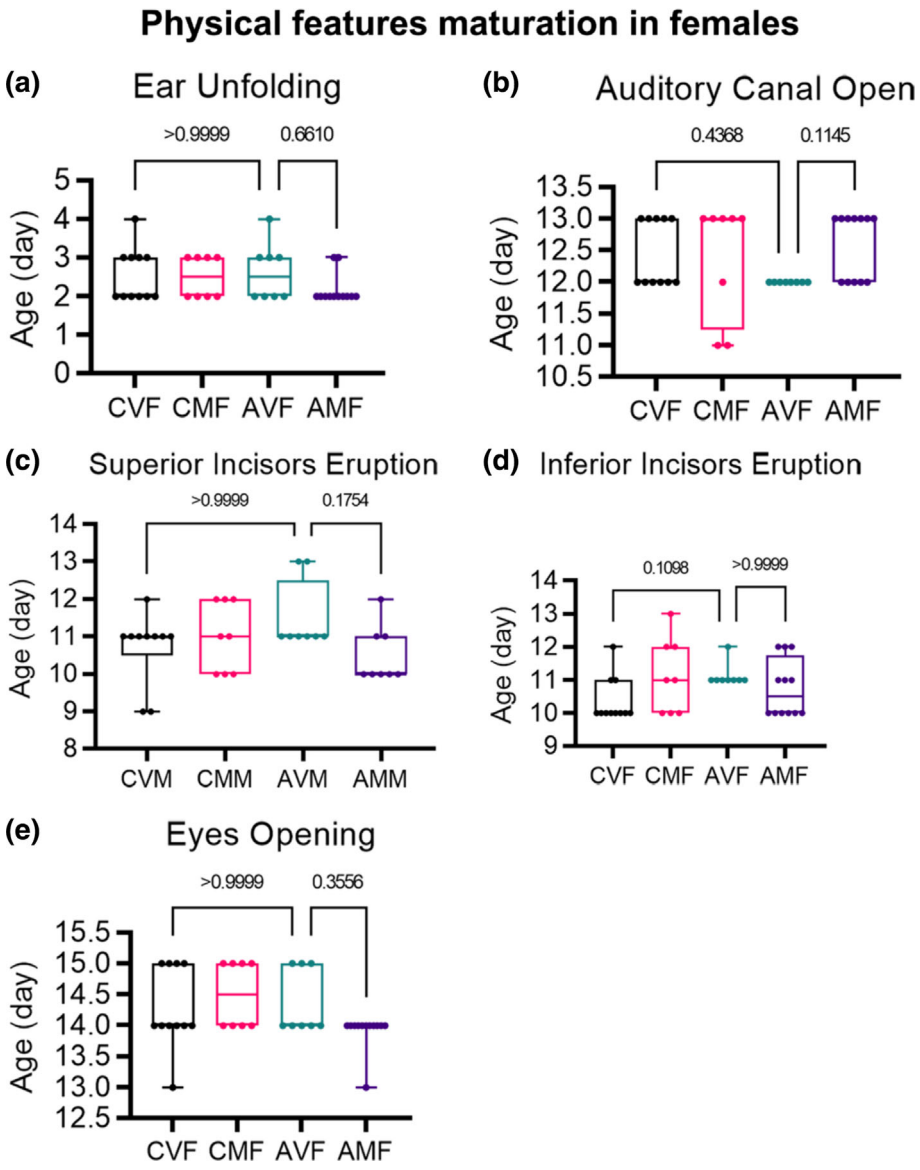

FIGURE 3 Blox plots showing the age of maturation (in days) (a) ear unfolding, (b) auditory canal open, (c) superior incisor eruption, (e) inferior incisor eruption, (e) eye opening, in female (F) rats subjected to either anoxia (A) or control (C) procedures followed by three i.p. injections of either melatonin (M) of vehicle (V), as evaluated from P2 to P21. CVF ($n = 11$), CMF ($n = 8$), AVF ($n = 8$), AMF ($n = 12$).

3.2 | Reflex ontogeny in male and female rats

The age of appearance (in days) of reflexes along the developmental period, in male and female rats, as a function of the treatments, in presented in Figures S4 and S5, respectively.

The Kruskal–Wallis test including only data of male rat revealed significant differences in both negative geotaxis reflex ($H(3) = 9.731$, $p = 0{,}02$) (Figure 4d) and palmar grasp reflex (Figure 4g). Pairwise comparisons revealed the significant differences shown in Figure 4d,g. Anoxia associated with melatonin treatment promoted earlier appearance of the negative geotaxis reflex relative to both control subjects exposed to melatonin treatment (CMM) and anoxiated subjects treated with vehicle (AVM) (Figure 4d). In contrast, anoxia delayed appearance of the palmar grasp reflex (Figure 4g), and this effect was reversed by melatonin treatment (Figure 4g). No other reflexes exhibited significant differences in their appearance for male rats.

The Kruskal–Wallis including only data of female rats revealed significant differences in both vibrissae placing ($H(3) = 13.89$; $p = 0.003$) (Figure 5c) and palmar grasp ($H(3) = 28.75$; $p < 0.0001$) (Figure 5g) reflexes. Pairwise comparisons revealed the significant differences shown in Figure 5c,g. These results may be summarized as it follows. Anoxia promoted earlier appearance of the vibrissae placing reflex (Figure 5c) that was reversed by melatonin treatment (Figure 5c). Similarly, anoxia promoted later appearance of the palmar grasp reflex (Figure 5g) that was reversed by melatonin treatment

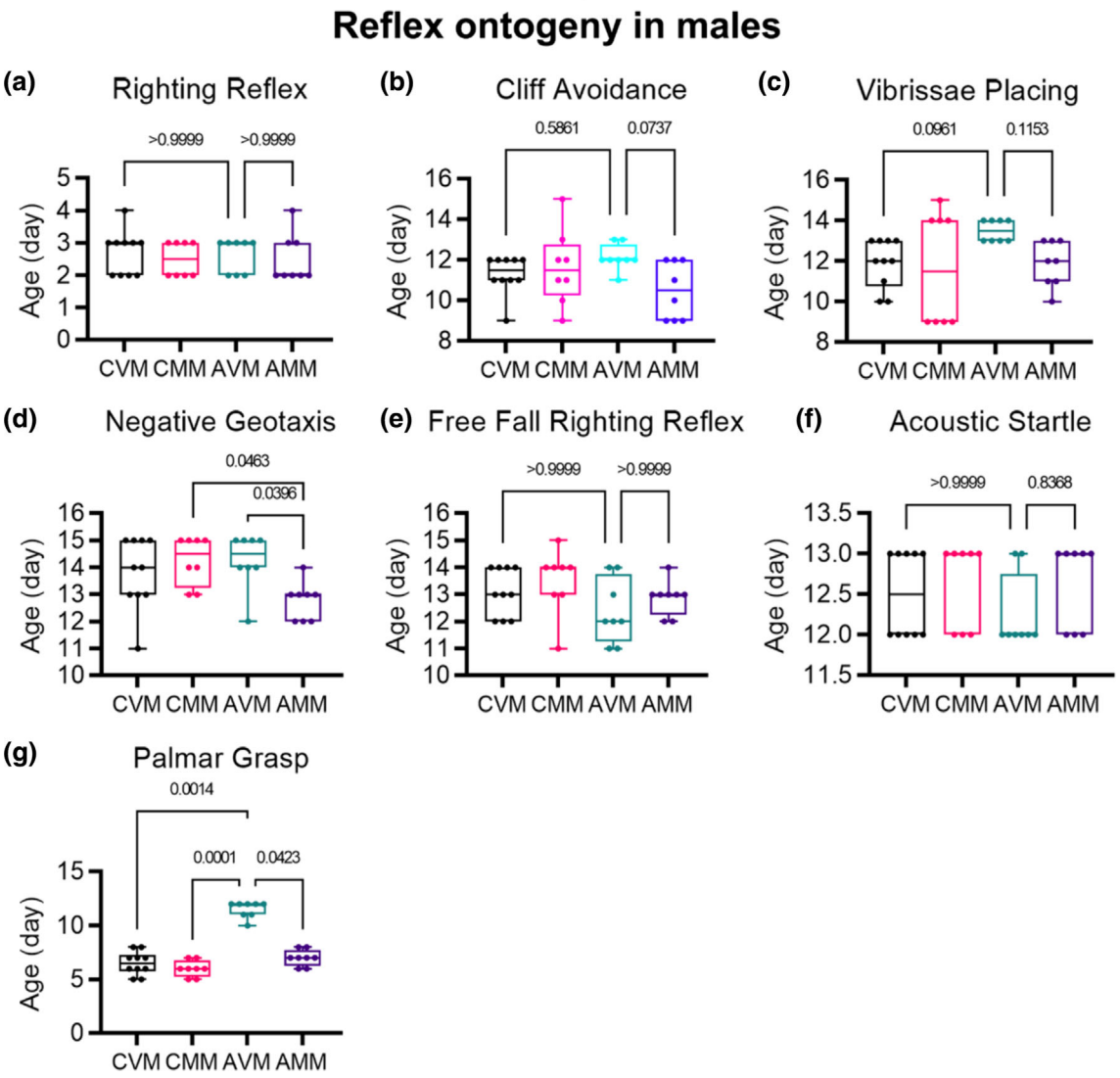

FIGURE 4 Box plot showing the age of maturation (in days) of the (a) righting reflex, (b) cliff avoidance, (c) vibrissae placing, (d) negative geotaxis, (e) free fall righting reflex, (f) acoustic startle, and (g) palmar grasp reflexes, in male rats subjected to anoxia (A) or control (C) procedures followed by three i.p. injections of either melatonin (M) of vehicle (V), as evaluated from P2 to P21. CVM ($n = 10$), CMM ($n = 8$), AVM ($n = 8$), AMM ($n = 8$).

(Figure 5g). No other reflexes exhibited significant differences in their appearance for female rats.

The PCA analysis (S6 and S7) showed positive associations between the acoustic startle, auditory canal open, and free fall righting parameters and negative associations between these and the vibrissa placement parameter in both sexes. There were negative associations between eye opening and the parameters negative geotaxis, cliff avoidance, and ear unfolding. The analysis also showed a negative association between the AVM group and the other groups (S6). Factor analysis results to identify interaction factors between stimulus × treatment and stimulus × treatment × sex are recorded in the Supporting Information (S8, S9 and S10).

3.3 | Morris water maze

Acquisition of spatial reference memory in the Morris water maze was assessed between P28 and P32. Retention of the spatial memory was assessed at P33, in a probe test session where the platform was removed from the pool. These results are shown in Figure 6.

The ANOVA including only male data revealed (1) significant main session effects for both latency ($F_{[19,600]} = 40.59$; $p < 0.0001$) (Figure 6a) and travelled distance ($F_{[19,600]} = 19.58$; p, 0.0001) (Figure 6b), (2) significant main group effects for latency ($F_{[6,600]} = 3.960$; p, 0.0082), (3) lack of significant main group effects for travelled distance ($F_{[6,600]} = 0.7932$; p, 0.4980), and (4) lack of

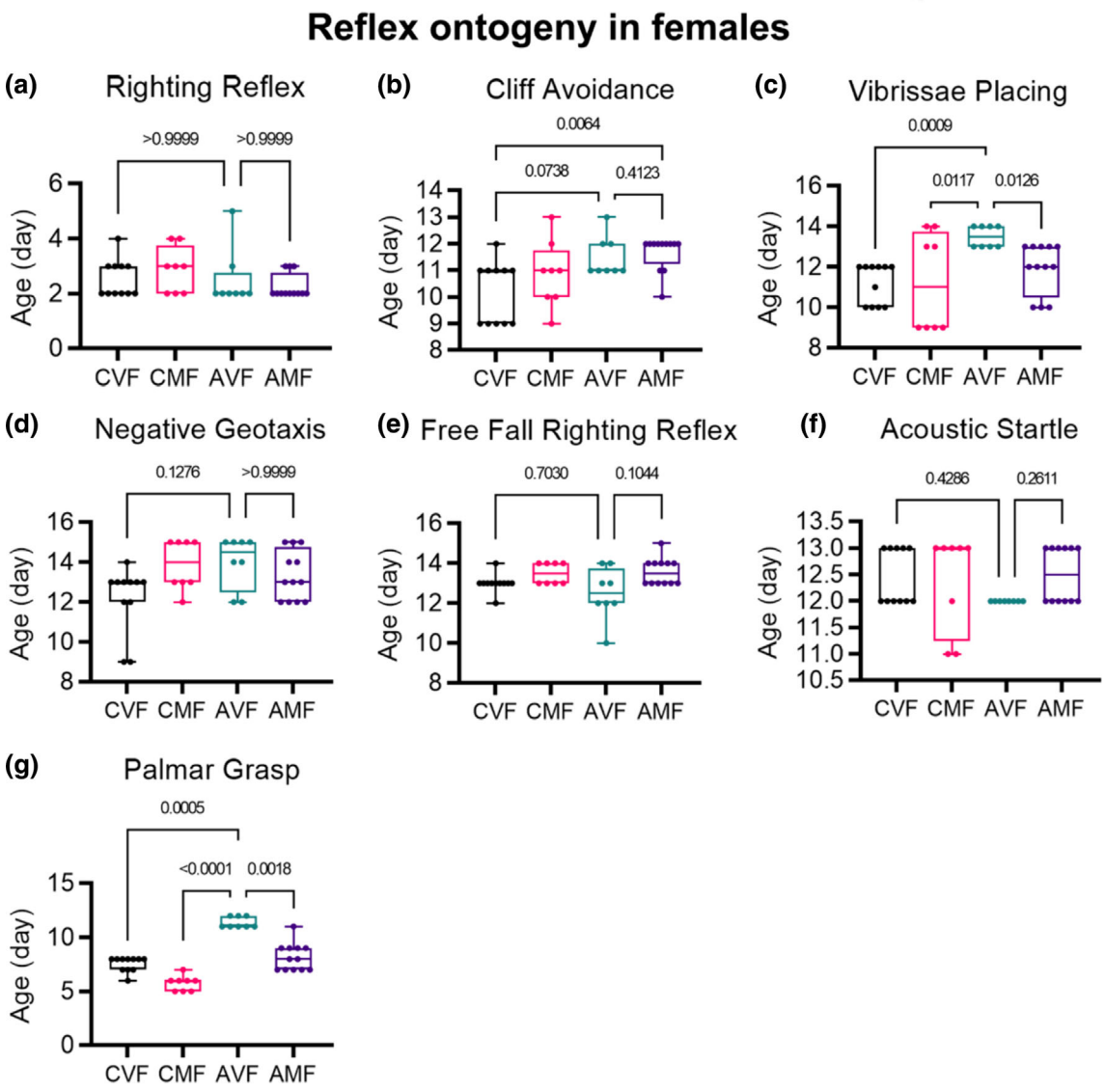

FIGURE 5 Box plots showing the age of maturation (in days) of the (a) righting reflex, (b) cliff avoidance, (c) vibrissae placing, (d) negative geotaxis, (e) free fall righting reflex, (f) acoustic startle, (g) palmar grasp reflexes, in female (F) rats subjected to either anoxia (A) or control (C) procedures followed by three i.p. injections of either melatonin (M) of vehicle (V), as evaluated from P2 to P21. CVF ($n = 11$), CMF ($n = 8$), AVF ($n = 8$), AMF ($n = 12$).

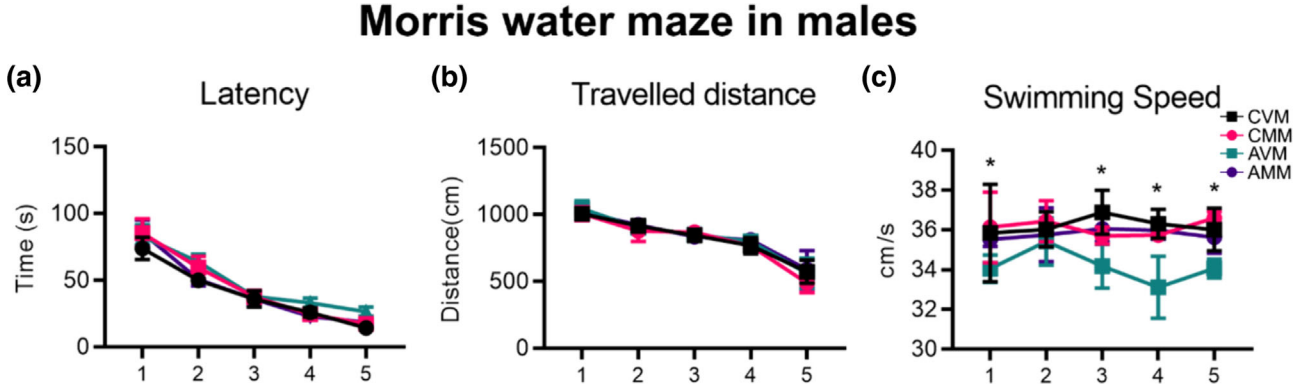

FIGURE 6 Morris water maze performance of males, evaluated from P28 to P33 to (a) latency, (b) travelled distance, and (c) swimming speed. The results are expressed as the mean ± SEM. *$p < 0.05$. CVM ($n = 10$), CMM ($n = 8$), AVM ($n = 8$), AMM ($n = 8$).

significant session × group interaction effect for both latency and travelled distance, thus indicating that all male rats equally acquired this spatial reference memory task, independently on the treatments.

These results indicate that, independently on the treatments, subjects equally acquired the spatial reference memory task.

Relative to the swimming speed along training (Figure 6c), ANOVA revealed a significant main group effect ($F_{[3,160]} = 32.51$; $p < 0.0001$). Tukey's multiple comparisons showed that the anoxic vehicle group had a lower swimming speed than the other groups on sessions 1, 3, 4, and 5 ($p \leq 0.05$; Figure 6c). These results provide evidence of the anoxia influence on swimming speed during the learning test, which could be reversed by melatonin treatment.

The ANOVA including only data of the female rats revealed (1) significant main session effects for both latency ($F_{[4.175]} = 149.2$; $p < 0.0001$) (Figure 7a) and path length ($F_{[4.175]} = 388.9$; $p < 0.0001$), (2) lack of significant group effect for both latency and travelled distance (Figure 7b), and (3) lack of significant session × group interaction effect for both latency and travelled distance, thus indicating that all female rats equally acquired this spatial reference memory task, independently on the treatments.

Tukey's multiple comparison test showed a difference between CVF × AVF on the fifth test day ($p = 0.02$). These results indicate that females of the anoxia group traveled a greater distance to reach the platform on the last day (Figure 7b). In the swimming speed parameter, there was a statistical difference between the groups ($F_{[3.175]} = 50.97$; $p < 0.0001$) (Figure 7c); Tukey's multiple comparison test showed that the vehicle anoxia group differed from the vehicle control group on the second test day ($p = 0.01$), and from all other groups on test days 1, 3, 4, and 5 ($p \leq 0.05$).

3.4 | Cell nuclei estimates

The hippocampi were individually processed at P35, using the isotropic fractionator method. The resulting nuclei suspension was stained using both DAPI, for revealing non-neuronal nuclei, and NeuN, for revealing neuronal nuclei. The estimates for both DAPI+- and NeuN+-stained nuclei are presented in Figure 8 for male rats and in Figure 9 for female rats.

The ANOVA including DAPI+ estimates of male rats hippocampi revealed a significant main group effect ($F_{[1.20]} = 9.668$; $p = 0.05$) (Figure 8a). The post hoc Tukey–Kramer test revealed the significant statistical differences presented in Figure 8a. Animals subjected to anoxia exhibited a reduction in DAPI+ hippocampal nuclei estimate (Figure 8a), and this effect was reversed by melatonin administration (Figure 8a). Similarly, ANOVA including NeuN+ estimates of male rats hippocampi revealed a significant main group effect ($F_{[1.20]} = 10.35$; $p = 0.0043$) (Figure 8b). Post hoc Tukey–Kramer test revealed the significant statistical differences presented in Figure 8b. Animals subjected to anoxia exhibited a reduction in NeuN+ hippocampal nuclei estimate (Figure 8b), and this effect was reversed by melatonin administration (Figure 8b).

DAPI+ and NeuN+ data female rats completely confirmed the results seen for male rats. The ANOVA including DAPI+ estimates of female rat hippocampi revealed a significant main group effect ($F_{[1.20]} = 10.90$; $p = 0.0036$). The post hoc test revealed the significant statistical differences presented in Figure 9a. Similarly, ANOVA including NeuN+ estimates of female rat hippocampi revealed a significant main group effect ($F_{[1.20]} = 13.20$; $p = 0.0017$) (Figure 9b). Post hoc Tukey–Kramer test revealed the significant statistical differences presented in Figure 9b.

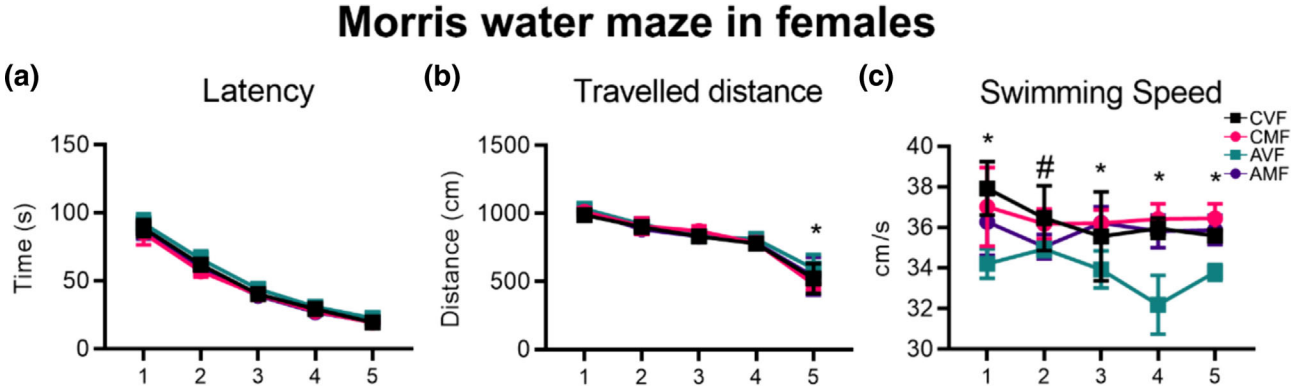

FIGURE 7 Morris water maze in females, evaluated from P28 to P33: (a) latency, (b) travelled distance, and (c) swimming speed. The results are expressed as the mean ± SEM. *$p < 0.05$. CVF ($n = 11$), CMF ($n = 8$), AVF ($n = 8$), AMF ($n = 12$). *AVF × all the groups, #AVF × CVF.

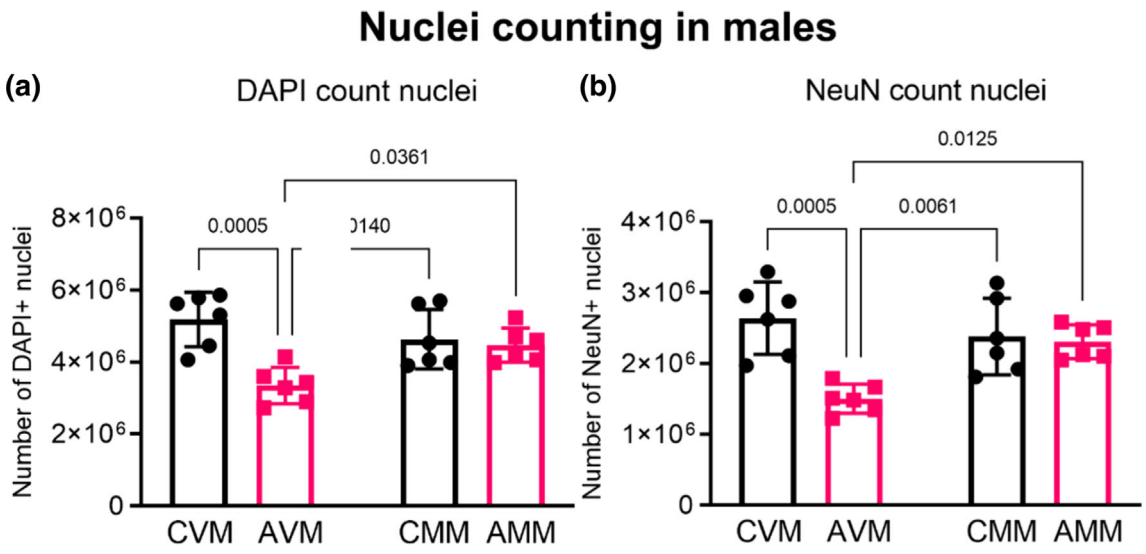

FIGURE 8 Mean cell nuclei estimate (+ SEM) of DAPI+ (a) and NeuN+ (b) stained nuclei of hippocampal cells of male rats subjected to either anoxia (A) or control (C), followed by three i.p. injections of either melatonin (M) of vehicle (V), as evaluated at P35 ($n = 6$ per group).

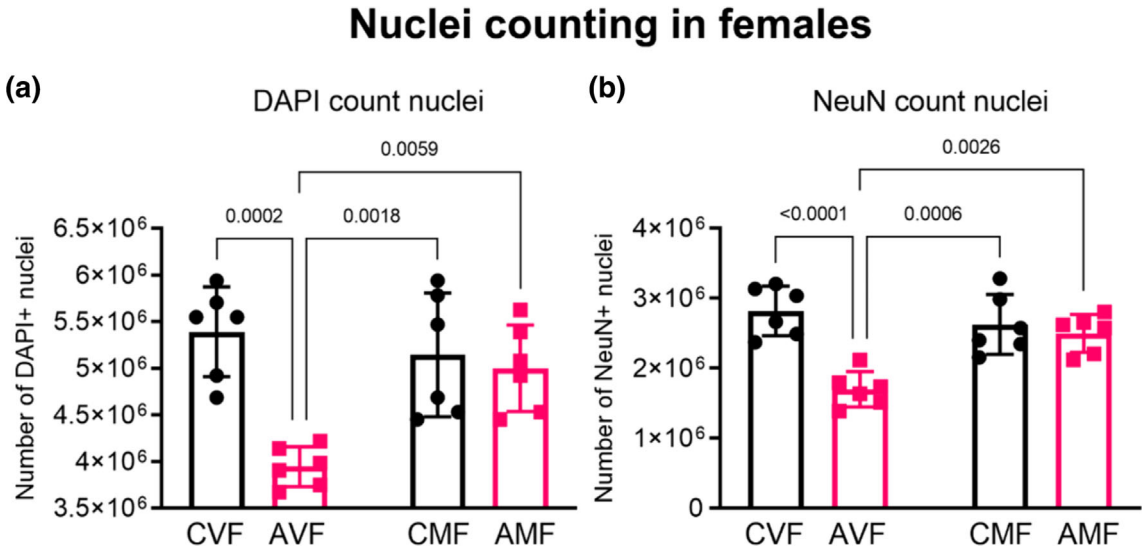

FIGURE 9 Mean cell nuclei estimate (+ SEM) of DAPI+ (a) and NeuN+ (b) stained nuclei of hippocampal cells of female rats subjected to either anoxia (A) or control (C), followed by three i.p. injections of either melatonin (M) of vehicle (V), as evaluated at P35 ($n = 6$ per group).

Thus, both male and female rats subjected to anoxia exhibited a reduction in DAPI+ and NeuN+ hippocampal nuclei estimate (Figures 8a,b and 9a,b, respectively), and this effect was reversed by melatonin (Figures 8a,b and 9a,b).

4 | DISCUSSION

In this study, we aimed to analyze the short-term neuroprotective effects of melatonin in animals submitted to neonatal anoxia, both males and females.

The neuroprotective effects of melatonin after oxygen deprivation have been demonstrated in rodents. Wang et al. (Wang et al., 2013) found that melatonin administered before and three consecutive days after hypoxia in P1 mice decreased behavioral deficits and cell death in the hippocampus, and improved memory and learning performance at P30. In rats, Carloni et al. (Carloni et al., 2008) found that the administration of 15 mg/kg 5 min, 24 h, and 48 h after hypoxia–ischemia decreased behavioral asymmetry and learning deficits in adulthood. These neuroprotective effects were also observed in piglets and sheep (Robertson et al., 2013).

The effects of oxygen deficit on development have been relatively understudied, and the exiting literature shows great variability in methodologies, particularly concerning the age of the offspring and the specific oxygen deprivation methods employed (Dell'Anna et al., 1995; Kumar et al., 2017; Lubics et al., 2005). This variability makes it challenging for replicating and comparing results across studies. However, our research group has used the neonatal anoxia model since 2011 (Takada et al., 2011), which offers a noninvasive and global approach performed in Wistar rats. This model uses rodents between 24 and 36 h after birth, roughly equivalent to a fetus at 6 months old (Semple et al., 2013). Importantly, previous studies from our group consistently revealed different sensitivities to anoxia stimuli between males and females (Cruz-Ochoa et al., 2019; Helou et al., 2021; Kumar et al., 2017; Kumar et al., 2019; Kumar et al., 2020a).

In rodent models of cerebral palsy, such as P0 neonatal anoxia followed by hindlimb restriction and combined to maternal immune activation, researchers have observed impaired righting reflex and negative geotaxis and decreased body weight compared with controls (Ho et al., 2022). Similar neurodevelopmental alterations were observed in another studies using rat neonatal anoxia combined to sensorimotor restriction, with sensorimotor changes from P4 to P10, evaluated by righting reflex latency (Samaiya et al., 2016; Samaiya et al., 2018). Prenatal hypoxia also appears to impact the sensorimotor development of the pups, resulting in delayed righting reflex and air righting compared with control groups (Piešová et al., 2020).

Conversely, mild anoxic event in P2 rats, composed by the exposition to 100% nitrogen for 10 min, did not affect the developmental milestones evaluated by the negative geotaxis, righting reflex, and cliff avoidance test, as well as the neonatal startle reflex and the eye opening (Menshanov et al., 2017).

In the hypoxic–ischemic encephalopathy rodent model at P6, the righting reflex, negative geotaxis, and cliff avoidance were positively correlated to the performance in the water maze test for learning and memory 8 weeks later, indicating that sensorimotor reflexes tested in the acute phase of perinatal hypoxic ischemic encephalopathy have strong predictive value for long-term neurofunctional outcome (Ten et al., 2003).

The sensorimotor reflexes are the basis of behavior, and the evaluation of their ontogeny provides important knowledge on the deficiencies resulting from neonatal anoxia. Although the literature presents some degree of variability, potentially due to differing experimental conditions, in our laboratory, their assessment was pretty consistent in the considered sexes (Cruz-Ochoa et al., 2019; Helou et al., 2021; Kumar et al., 2017; Kumar et al., 2019; Kumar et al., 2020b).

The treatment with melatonin depicted decreased time of installation of negative geotaxis in males and reduced the delay caused by anoxia in the placement reflex by the vibrissae in females, and in the palmar grip reflex, both in males and females. One possible explanation for this result is the role of melatonin in blocking the activity of the nuclear transcription factor kappa B (NFkB) (Xia et al., 2012). This action decreases microglial activation and nitric oxide levels, thereby attenuating the inflammatory process, as a consequence of the expression of tumor necrosis factor (TNF) and cell death, processes that are induced by oxygen deficit.

The results in the Morris water maze suggest that all animals memorized the platform's location, regardless of the stimulus, treatment, or sex. This finding is consistent with other research in this age and group (Matsuda et al., 2021). However, it is essential to consider the high number of trials/sessions used for evaluation, as it may mask potential alterations in the animals' memory. Studies show that even brain-damaged animals can learn to locate the platform when subjected to numerous tests. Even though, research with animal models of cognitive impairments has used the Morris water maze test as a resource to assess learning (Lauterborn et al., 2019).

In the swimming speed parameter, we detected that the anoxia group was slower compared with the control group of both sexes. This finding is consistent with previous research by Nyakas et al. (Nyakas et al., 1991) who also observed a decrease in the animals' motor activity 2 weeks after neonatal anoxia.

Differences in swimming speed were observed in P35 rats submitted to the same neonatal anoxia model (Kumar et al., 2019), whereas anoxia male animals swam faster than control animals. In contrast, Strata and colleagues (Strata et al., 2004) observed altered motor behavior in anoxic rats subject to neonatal oxygen deprivation combined to sensorimotor restriction. Our group did not find difference in swimming speed between P60 anoxia versus control groups (Takada et al., 2015). The transient increased motor activity in juvenile rats exposed to 100% of nitrogen neonatally has been already described (Speiser et al., 1983) (Dell'Anna et al., 1991; Speiser et al., 1998), showing that this is age dependent.

A positive action of melatonin was also revealed in the morphological structure of the hippocampus by cell counting using the isotropic fractionator method, an innovative technique in oxygen deprivation models. This method offers the advantage of being rapid and cost-effective, allowing researchers to obtain result within just one or a few days.

In addition, melatonin effects might result probably due to its role in the cascade of events that occur after neonatal anoxia, by decreasing nitric oxide levels, microglial responses, and the overproduction of pro-inflammatory mediators that culminate with the consequent cell death in the hippocampus (Carloni et al., 2008; Carloni et al., 2014; Olivier et al., 2009).

In conclusion, this study demonstrated that melatonin treatment has potential neuroprotective role in the short-term development after neonatal anoxia. In addition, melatonin appears to have a long-lasting impact on the neural hippocampal cell populations, as observed at P35. These findings suggest that further studies should be carried out to explore the clinical implementation of melatonin as a strategy in the neonatal procedures in order to avoid or minimize the deleterious effects of neonatal anoxia.

Contribution Roles Taxonomy

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CONFLICT OF INTEREST STATEMENT

The authors whose names are listed immediately below certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or nonfinancial interest in the subject matter or materials discussed in this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICAL APPROVAL

Experiments were conducted according to CEUA (Comissão de Ética no Uso de Animais) of the Institute of Biomedical Science, number 19/2017, and the rules established by the CONCEA (*Conselho Nacional de Controle de Experimentação Animal*).

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REFERENCES

Berger, H. R., Nyman, A. K. G., Morken, T. S., Vettukattil, R., Brubakk, A. M., & Widerøe, M. (2017). Early metabolite changes after melatonin treatment in neonatal rats with hypoxic-ischemic brain injury studied by in-vivo 1H MR spectroscopy. *PLoS ONE*, *12*, e0185202. https://doi.org/10.1371/journal.pone.0185202

Blanco, S., Hernández, R., Franchelli, G., Ramos-Álvarez, M. M., & Peinado, M. Á. (2017). Melatonin influences NO/NOS pathway and reduces oxidative and nitrosative stress in a model of hypoxic-ischemic brain damage. *Nitric Oxide*, *62*, 32–43. https://doi.org/10.1016/j.niox.2016.12.001

Carloni, S., Albertini, M. C., Galluzzi, L., Buonocore, G., Proietti, F., & Balduini, W. (2014). Melatonin reduces endoplasmic reticulum stress and preserves sirtuin 1 expression in neuronal cells of newborn rats after hypoxia-ischemia. *Journal of Pineal Research*, *57*, 192–199. https://doi.org/10.1111/jpi.12156

Carloni, S., Perrone, S., Buonocore, G., Longini, M., Proietti, F., & Balduini, W. (2008). Melatonin protects from the long-term consequences of a neonatal hypoxic-ischemic brain injury in rats. *Journal of Pineal Research*, *44*, 157–164. https://doi.org/10.1111/J.1600-079X.2007.00503.X

Cruz-Ochoa, N. A., Ochoa-Amaya, J. E., Pulecio, S. L., Xavier, G. F., & Nogueira, M. I. (2019). Neonatal anoxia impairs long-term energy metabolism and somatic development of Wistar rats. *International Journal of Developmental Neuroscience*, *79*, 76–85. https://doi.org/10.1016/j.ijdevneu.2019.11.001

Dell'Anna, E., Chen, Y., Loidl, F., Andersson, K., Luthman, J., Goiny, M., Rawal, R., Lindgren, T., & Herrera-Marschitz, M.

(1995). Short-term effects of perinatal asphyxia studied with Fos-immunocytochemistry and in vivo microdialysis in the rat. *Experimental Neurology*, *131*, 279–287. https://doi.org/10.1016/0014-4886(95)90050-0

Dell'Anna, M. E., Calzolari, S., Molinari, M., Iuvone, L., & Calimici, R. (1991). Neonatal anoxia induces transitory hyperactivity, permanent spatial memory deficits and CA1 cell density reduction in developing rats. *Behavioural Brain Research*, *45*, 125–134. https://doi.org/10.1016/S0166-4328(05)80078-6

Escobar, G. J., Littenberg, B., & Petitti, D. B. (1991). Outcome among surviving very low birthweight infants: A meta-analysis. *Archives of Disease in Childhood*, *66*, 204–211. https://doi.org/10.1136/ADC.66.2.204

Gaffney, G., Sellers, S., Flavell, V., Squier, M., & Johnson, A. (1994). Case-control study of intrapartum care, cerebral palsy, and perinatal death. *BMJ*, *308*, 743–750. https://doi.org/10.1136/BMJ.308.6931.743

Helou, A. Y., Martins, D. O., Arruda, B. P., de Souza, M. C., Cruz-Ochoa, N. A., Nogueira, M. I., & Chacur, M. (2021). Neonatal anoxia increases nociceptive response in rats: Sex differences and lumbar spinal cord and insula alterations. *International Journal of Developmental Neuroscience*, *81*, 686–697. https://doi.org/10.1002/JDN.10145

Herculano-Houzel, S., & Lent, R. (2005). Isotropic fractionator: A simple, rapid method for the quantification of total cell and neuron numbers in the brain. *The Journal of Neuroscience*, *25*(10), 2518–2521. https://doi.org/10.1523/jneurosci.4526-04.2005

Ho, D., Sanches, E. F., & Sizonenko, S. V. (2022). Early neurodevelopmental reflex impairments in a rodent model of cerebral palsy. *International Journal of Developmental Neuroscience*, *82*, 815–823. https://doi.org/10.1002/JDN.10235

Kumar, A. J., Martins, D. O., Arruda, B. P., Lee, V. Y., Chacur, M., & Nogueira, M. I. (2020a). Impairment of nociceptive responses after neonatal anoxia correlates with somatosensory thalamic damage: A study in rats. *Behavioural Brain Research*, *390*, 112690. https://doi.org/10.1016/j.bbr.2020.112690

Kumar, A. J., Motta-Teixeira, L. C., Takada, S. H., Yonamine-Lee, V., Machado-Nils, A. V., Xavier, G. F., & Nogueira, M. I. (2019). Behavioral, cognitive and histological changes following neonatal anoxia: Male and female rats' differences at adolescent age. *International Journal of Developmental Neuroscience*, *73*, 50–58. https://doi.org/10.1016/j.ijdevneu.2018.12.002

Kumar, A. J., Takada, S. H., Motta-Teixeira, L. C., Lee, V. Y., Xavier, G. F., & Nogueira, M. I. (2017). Sex differences in somatic and sensory motor development after neonatal anoxia in Wistar rats. *Behavioural Brain Research*, *333*, 242–250. https://doi.org/10.1016/j.bbr.2017.07.009

Kurinczuk, J. J., White-Koning, M., & Badawi, N. (2010). Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. *Early Human Development*, *86*, 329–338. https://doi.org/10.1016/j.earlhumdev.2010.05.010

Laptook, A. R., Shankaran, S., Tyson, J. E., Munoz, B., Bell, E. F., Goldberg, R. N., Parikh, N. A., Ambalavanan, N., Pedroza, C., Pappas, A., Das, A., Chaudhary, A. S., Ehrenkranz, R. A., Hensman, A. M., Van Meurs, K. P., Chalak, L. F., Hamrick, S. E. G., Sokol, G. M., Walsh, M. C., … Higgins, R. D. (2017). Effect of therapeutic hypothermia initiated after 6 hours of age on death or disability among newborns with hypoxic-ischemic encephalopathy: A randomized clinical trial. *Journal of the American Medical Association*, *318*, 1550–1560. https://doi.org/10.1001/JAMA.2017.14972

Lauterborn, J. C., Schultz, M. N., Le, A. A., Amani, M., Friedman, A. E., Leach, P. T., Gall, C. M., Lynch, G. S., & Crawley, J. N. (2019). Spaced training improves learning in Ts65Dn and Ube3a mouse models of intellectual disabilities. *Translational Psychiatry*, *9*(1), 166. https://doi.org/10.1038/s41398-019-0495-5

Lubics, A., Reglodi, D., Tamás, A., Kiss, P., Szalai, M., Szalontay, L., & Lengvári, I. (2005). Neurological reflexes and early motor behavior in rats subjected to neonatal hypoxic–ischemic injury. *Behavioural Brain Research*, *157*, 157–165. https://doi.org/10.1016/J.BBR.2004.06.019

Matsuda, V. D. V., Tejada, M. B., Motta-Teixeira, L. C., Ikebara, J. M., Cardoso, D. S., Machado-Nils, A. V., Lee, V. Y., Diccini, I., Arruda, B. P., Martins, P. P., Dias, N. M. M., Tessarotto, R. P., Raeisossadati, R., Bruno, M., Takase, L. F., Kihara, A. H., Nogueira, M. I., Xavier, G. F., & Takada, S. H. (2021). Impact of neonatal anoxia and hypothermic treatment on development and memory of rats. *Experimental Neurology*, *340*, 113691. https://doi.org/10.1016/j.expneurol.2021.113691

Medoff-Cooper, B., Rankin, K., Li, Z., Liu, L., & White-Traut, R. (2015). Multi-sensory intervention for preterm infants improves sucking organization. *Advances in Neonatal Care*, *15*, 142–149. https://doi.org/10.1097/ANC.0000000000000166

Menshanov, P. N., Bannova, A. V., & Dygalo, N. N. (2017). Anoxia ameliorates the dexamethasone-induced neurobehavioral alterations in the neonatal male rat pups. *Hormones and Behavior*, *87*, 122–128. https://doi.org/10.1016/J.YHBEH.2016.11.013

Millar, L. J., Shi, L., Hoerder-Suabedissen, A., & Molnár, Z. (2017). Neonatal hypoxia ischaemia: Mechanisms, models, and therapeutic challenges. *Frontiers in Cellular Neuroscience*, *11*, 78. https://doi.org/10.3389/fncel.2017.00078

Nyakas, C., Markel, É., Schuurman, T., & Luiten, P. G. M. (1991). Impaired learning and abnormal open-field behaviours of rats after early postnatal anoxia and the beneficial effect of the calcium antagonist nimodipine. *The European Journal of Neuroscience*, *3*, 168–174. https://doi.org/10.1111/J.1460-9568.1991.TB00077.X

Olivier, P., Fontaine, R. H., Loron, G., Van Steenwinckel, J., Biran, V., Massonneau, V., Kaindl, A., Dalous, J., Charriaut-Marlangue, C., Aigrot, M. S., Pansiot, J., Verney, C., Gressens, P., & Baud, O. (2009). Melatonin promotes oligodendroglial maturation of injured white matter in neonatal rats. *PLoS ONE*, *4*, e7128. https://doi.org/10.1371/journal.pone.0007128

Perin, J., Mulick, A., Yeung, D., Villavicencio, F., Lopez, G., Strong, K. L., Prieto-Merino, D., Cousens, S., Black, R. E., & Liu, L. (2022). Global, regional, and national causes of under-5 mortality in 2000-19: An updated systematic analysis with implications for the sustainable development goals. *Lancet Child and Adolescent Health*, *6*, 106–115. https://doi.org/10.1016/S2352-4642(21)00311-4

Piešová, M., Koprdova, R., Ujhazy, E., Kršková, L., Olexova, L., Morova, M., Senko, T., & Mojmír, M. A. (2020). Impact of prenatal hypoxia on the development and behavior of the rat

offspring. *Physiological Research*, *69*, S649–S659. https://doi.org/10.33549/PHYSIOLRES.934614

Quigley, M. A., Poulsen, G., Boyle, E., Wolke, D., Field, D., Alfirevic, Z., & Kurinczuk, J. J. (2012). Early term and late preterm birth are associated with poorer school performance at age 5 years: A cohort study. *Archives of Disease in Childhood. Fetal and Neonatal Edition*, *97*, F167–F173. https://doi.org/10.1136/ARCHDISCHILD-2011-300888

Revuelta, M., Arteaga, O., Alvarez, A., Martinez-Ibargüen, A., & Hilario, E. (2017). Characterization of gene expression in the rat brainstem after neonatal hypoxic–ischemic injury and antioxidant treatment. *Molecular Neurobiology*, *54*, 1129–1143. https://doi.org/10.1007/S12035-016-9724-6/TABLES/2

Robertson, N. J., Faulkner, S., Fleiss, B., Bainbridge, A., Andorka, C., Price, D., Powell, E., Lecky-Thompson, L., Thei, L., Chandrasekaran, M., Hristova, M., Cady, E. B., Gressens, P., Golay, X., & Raivich, G. (2013). Melatonin augments hypothermic neuroprotection in a perinatal asphyxia model. *Brain*, *136*, 90–105. https://doi.org/10.1093/BRAIN/AWS285

Rosales-Corral, S., Tan, D. X., Reiter, R. J., Valdivia-Velázquez, M., Martínez-Barboza, G., Acosta-Martínez, J. P., & Ortiz, G. G. (2003). Orally administered melatonin reduces oxidative stress and proinflammatory cytokines induced by amyloid-beta peptide in rat brain: A comparative, in vivo study versus vitamin C and E. *Journal of Pineal Research*, *35*, 80–84. https://doi.org/10.1034/J.1600-079X.2003.00057.X

Samaiya, P. K., Narayan, G., Kumar, A., & Krishnamurthy, S. (2016). Neonatal anoxia leads to time dependent progression of mitochondrial linked apoptosis in rat cortex and associated long term sensorimotor deficits. *International Journal of Developmental Neuroscience*, *52*, 55–65. https://doi.org/10.1016/J.IJDEVNEU.2016.05.005

Samaiya, P. K., Narayan, G., Kumar, A., & Krishnamurthy, S. (2018). 2,4 Dinitrophenol attenuates mitochondrial dysfunction and improves neurobehavioral outcomes postanoxia in neonatal rats. *Neurotoxicity Research*, *34*, 121–136. https://doi.org/10.1007/S12640-018-9873-7

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., & Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Progress in Neurobiology*, *106-107*, 1–16. https://doi.org/10.1016/j.pneurobio.2013.04.001

Shah, P. S., Wong, K. Y., Merko, S., Bishara, R., Dunn, M., Asztalos, E., & Darling, P. B. (2006). Postnatal growth failure in preterm infants: Ascertainment and relation to long-term outcome. *Journal of Perinatal Medicine*, *34*, 484–489. https://doi.org/10.1515/JPM.2006.094

Speiser, Z., Katzir, O., Rehavi, M., Zabarski, T., & Cohen, S. (1998). Sparing by rasagiline (TVP-1012) of cholinergic functions and behavior in the postnatal anoxia rat. *Pharmacology, Biochemistry, and Behavior*, *60*, 387–393. https://doi.org/10.1016/S0091-3057(97)00603-5

Speiser, Z., Korczyn, A. D., Teplitzky, I., & Gitter, S. (1983). Hyperactivity in rats following postnatal anoxia. *Behavioural Brain Research*, *7*, 379–382. https://doi.org/10.1016/0166-4328(83)90028-1

Strata, F., Coq, J. O., Byl, N., & Merzenich, M. M. (2004). Effects of sensorimotor restriction and anoxia on gait and motor cortex organization: Implications for a rodent model of cerebral palsy. *Neuroscience*, *129*, 141–156. https://doi.org/10.1016/J.NEUROSCIENCE.2004.07.024

Takada, S. H., dos Santos Haemmerle, C. A., Motta-Teixeira, L. C., Machado-Nils, A. V., Lee, V. Y., Takase, L. F., Cruz-Rizzolo, R. J., Kihara, A. H., Xavier, G. F., Watanabe, I. S., & Nogueira, M. I. (2015). Neonatal anoxia in rats: Hippocampal cellular and subcellular changes related to cell death and spatial memory. *Neuroscience*, *284*, 247–259. https://doi.org/10.1016/j.neuroscience.2014.08.054

Takada, S. H., Motta-Teixeira, L. C., Machado-Nils, A. V., Lee, V. Y., Sampaio, C. A., Polli, R. S., Malheiros, J. M., Takase, L. F., Kihara, A. H., Covolan, L., Xavier, G. F., & Nogueira, M. I. (2016). Impact of neonatal anoxia on adult rat hippocampal volume, neurogenesis and behavior. *Behavioural Brain Research*, *296*, 331–338. https://doi.org/10.1016/j.bbr.2015.08.039

Takada, S. H., Sampaio, C. A. G., Allemandi, W., Ito, P. H., Takase, L. F., & Nogueira, M. I. (2011). A modified rat model of neonatal anoxia: Development and evaluation by pulseoximetry, arterial gasometry and Fos immunoreactivity. *Journal of Neuroscience Methods*, *198*, 62–69. https://doi.org/10.1016/j.jneumeth.2011.03.009

Ten, V. S., Bradley-Moore, M., Gingrich, J. A., Stark, R. I., & Pinsky, D. J. (2003). Brain injury and neurofunctional deficit in neonatal mice with hypoxic-ischemic encephalopathy. *Behavioural Brain Research*, *145*, 209–219. https://doi.org/10.1016/S0166-4328(03)00146-3

Wang, Z., Liu, D., Zhan, J., Xie, K., Wang, X., Xian, X., Gu, J., Chen, W., & Hao, A. (2013). Melatonin improves short and long-term neurobehavioral deficits and attenuates hippocampal impairments after hypoxia in neonatal mice. *Pharmacological Research*, *76*, 84–97. https://doi.org/10.1016/j.phrs.2013.07.008

Woythaler, M. A., McCormick, M. C., & Smith, V. C. (2011). Late preterm infants have worse 24-month neurodevelopmental outcomes than term infants. *Pediatrics*, *127*, e622–e629. https://doi.org/10.1542/PEDS.2009-3598

Xia, Q., Chen, Z. X., Wang, Y. C., Ma, Y. S., Zhang, F., Che, W., Fu, D., & Wang, X. F. (2012). Association between the melatonin receptor 1B gene polymorphism on the risk of type 2 diabetes, impaired glucose regulation: A meta-analysis. *PLoS ONE*, *7*, e50107. https://doi.org/10.1371/JOURNAL.PONE.0050107

SUPPORTING INFORMATION

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