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HEALTH SCIENCES

Impact of prenatal lipopolysaccharide exposure on the development of rats

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Abstract: The intrauterine environment is influenced by several factors, genetic or environmental, which are essential in understanding the pathophysiological mechanisms of some diseases. In this study, the aim was to investigate the impact of prenatal lipopolysaccharide exposure on the development of rats. Fifty pregnant rats received intraperitoneal administration of lipopolysaccharide (100 µg/kg), or saline at the same dose, on the 9.5th day of pregnancy. The offspring of these rats were analyzed for indicators of brain and somatic development and maturation of physical characteristics. Reflex ontogenesis was also analyzed by vibrissae placement, negative geotaxis, palmar grasp, precipice aversion, decubitus recovery and acceleration reaction. Administration of lipopolysaccharide on the 9.5th gestational day caused delayed opening of the auditory pavilion, reduction in the length of the tail, body, cranial axes, and body weight. Thus, maternal infections can interfere in the intrauterine environment, impairing functional and structural aspects of the central nervous system, as well as the maturation of physical characteristics.

Key words: Developmental disorders, immune system, lipopolysaccharide, somatic development, sensory motor development.

INTRODUCTION

During the ontogenic period, the central nervous system (CNS) is influenced by several factors, genetic and environmental, that modulate epigenetic mechanisms and, therefore, the functioning of these systems. The impairment of these molecular mechanisms, mainly in critical periods, can cause irreversible changes, associated with neuropsychiatric disorders (Zakharova 2015, Keunen et al. 2015, Izvolskaia et al. 2018). In this context, the concept of programming was developed to explain the process by which the organism adapts to environmental insults, which generate stable changes in the phenotype, through changes in the proliferation and differentiation process (Deiró et al. 2008).

As adults, rodents exposed to adverse events during the intrauterine period — infections and pharmacological or nutritional manipulations — exhibit physiological and behavioral changes, including vulnerability to cognitive disabilities, reduced corticosterone response to stress and decreased social interaction (Izvolskaia et al. 2018). Further, the evidence indicates that these changes are not limited to animals exposed isolatedly, but also to their subsequent generation (Deiró et al. 2008).

Experimental studies usually use lipopolysaccharide (LPS), an endotoxin present in the outer membrane of gram-negative bacteria, to simulate a bacterial infection and

evaluate its consequences for offspring. LPS induces systemic inflammation and acts directly on placental cells, leading to the production of pro-inflammatory mediators, which induce microglial and astrocyte activation and, consequently, the production of cytokines in the fetal brain (Cai et al. 2000, Deiró et al. 2008, Bale et al. 2010, Zakharova 2015).

One of the ways to study the development of the CNS is to observe the ontogenesis of the reflex, since reflexes represent one of the behavioral expressions of brain function. Reflex ontogenesis encompasses visual, auditory and motor maturation, and can be affected by any environmental or organic stimulus, generating consequences for the formation of the nervous system (Soares et al. 2014). They are the result of stimuli and appear in certain periods of development, following a pre-determined order, according to the age of the animals (Deiró et al. 2008). The maturation of specific reflexes in rats has been well established, showing that disturbances at this stage of growth may point to insults in neurological development (Leite et al. 2002).

Therefore, to better understand the genesis of disorders related to neurodevelopment, which have a growing prevalence and important socio-economic impact, this study has been proposed. The aim is to investigate whether an insult during a critical period of CNS development in rats reflects structural and functional changes in their offspring's somatic and motor-sensory development.

MATERIALS AND METHODS

This was an experimental study in which the animals were kept at controlled temperature (22 ± 1°C), artificial light cycle (12 hours light/dark) and received standard commercial feed for laboratory rats and *ad libitum* water.

For the application of the prenatal inflammation protocol, 50 adults (60 days) virgin female Wistar rats (*Rattus norvegicus*) — obtained from the University of the Itajaí Valley vivarium — were used, weighing between 250 and 300g, to mate with 25 males of the same lineage and age.

For mating, at the end of the light period (7 p.m.) the females were placed in the males' cages, always using two females for each male. At 7 am the following day, pregnancy was diagnosed using biological material collected trough vaginal lavage, which consists of introducing solution of sterile 0.9% NaCl through a plastic pipette into the animal's vaginal canal. Thus, secretion is obtained for analysis under an optical microscope in order to investigate the presence of sperm next to the animal's biological material (Kirsten et al. 2010). Once the presence of sperm was confirmed, gestational day (GD) zero was considered. Next, the pregnant mothers were placed in individual cages and remained so throughout the pregnancy period.

On the 9.5th GD, the females were randomly subjected to two treatments: saline exposure (SAL group) and lipopolysaccharide exposure — obtained through phenolic extraction from *Escherichia coli*, serotype 0127: B8 (Sigma®) — at 100 µg/kg (LPS group). Both applications were intraperitoneal. LPS administration followed exposure protocol that mimics a bacterial infection (Lunardelli et al. 2014). Births happened naturally. In total, 184 animals were used, male and female, divided according to the exposure protocol. Of these, 120 were used for the assessment of somatic growth and maturation of physical characteristics and 64 for *post mortem* brain assessments. Moreover, out of the 120 animals, 20 were also evaluated for reflex ontogenesis.

Parameters analyzed

Somatic development and reflex ontogenesis were analyzed in these groups, having as reference the following parameters:

- Somatic Growth Assessment:

These measurements were obtained daily during the lactation period using a caliper with 0.01mm accuracy. Bodyweight (BW), longitudinal length (LL), tail length (TL), lateral-lateral axis (LLAxis) and skull anteroposterior axis (SAAxis) measurements were evaluated (Deiró et al. 2006, Silva 2008).

- Maturation Assessment of Physical Characteristics:

For each animal, the time in days from birth to maturation of the following characteristics was recorded: opening of the auditory pavilion (OAP), opening of the auditory conduit (OAC), eruption of the lower incisor (ELI) and eye-opening (EO) (Deiró et al. 2006, Silva 2008).

- Indicators of sensorimotor development:

From the first day of life, the consolidation of the following reflexes was analyzed: palmar grasp (PG), decubitus recovery (DR), precipice aversion (PA), negative geotaxis (NG) and acceleration reaction (AR). Reflex consolidation day was considered the first day of the sequence of three consecutive days of full onset of the expected reflex response (Deiró et al. 2006, Silva 2008).

- Brain development indicators:

Post mortem evaluations were performed on animals at 8, 15 and 21 days of age. After craniotomy, the brain was removed from the cranial box, and the following measurements were performed: weight, volume, diameter and circumference of the encephalon, cerebellar weight, anterior-posterior encephalic axis (APEA), anterior-posterior cerebellum axis (APCA), anterior-posterior brain axis (APBA), laterolateral axis of the brain (LLBA), and laterolateral axis of the cerebellum (LLCA) (Magalhães et al. 2006).

Ethical aspects

The use of animals in this study followed the Laboratory Animal Care Principles and was approved by the Animal Use Ethics Committee of the University of South Santa Catarina (protocol number 15.016.2.01.IV).

Statistical analysis

GraphPad Prism 7 was used for statistical analysis, and p-value of < 0.05 was considered to be statistically significant. Parametric data were analyzed by analysis of variance for multiple comparisons between groups (ANOVA), followed by Tukey *post-hoc*. For nonparametric data, we used the Mann-Whitney test for two independent samples.

RESULTS

The anteroposterior axis of the skull

The results of the anteroposterior cranial axis growth parameter showed statistical significance from one-way ANOVA [$F(21, 1298) = 828.9$, $p < 0.0001$]. The offspring in the LPS group showed deficits in the parameter when compared to the offspring in the SAL group, on days 1 ($p < 0.0001$), 7 ($p = 0.0407$), 9 ($p = 0.0004$) and 13 ($p = 0.0002$) postnatal.

No significant differences ($p > 0.05$) were observed when comparing the groups on the other days analyzed (Figure 1).

Laterolateral axis of the skull

In the analysis of the lateral-lateral axis measurement of the skull of the pups by the SAL and LPS groups, the one-way ANOVA showed significance [$F(21, 1298) = 16.76$, $p < 0.0001$], however during the 21 postnatal days,

no statistical difference was observed in Tukey's *post-hoc* ($p > 0.05$) (Figure 2).

Tail Length

As for tail length, the one-way ANOVA values were $F (21, 1298) = 1249$, $p < 0.0001$. Followed by Tukey's *post-hoc*, there was no difference in the comparison between the values in the SAL group and LPS group during the first 7 postnatal days ($p > 0.05$). However, on days 9 ($p = 0.0111$), 13 ($p = 0.0038$), 15 ($p < 0.0001$), 17 ($p = 0.0295$), 19 and 21 ($p < 0.0001$), the LPS group had a shorter tail length when compared to the SAL group (Figure 3).

Body length

Like the tail length, the development of the longitudinal axis of the body in the SAL group did not show a significant difference during the first 7 postnatal days when compared to the LPS group ($p > 0.05$). However, on days 9 ($p = 0.001$), 11 ($p = 0.0446$), 13 ($p < 0.0001$), 15 ($p = 0.0406$), 19 and 21 ($p < 0.0001$), the LPS group showed a reduced growth in comparison with the SAL group (Figure 4). For this parameter, the one-way ANOVA results were $F (21, 1298) = 1063$, $p < 0.0001$.

Bodyweight

Regarding body weight, the results showed statistical difference from one-way ANOVA [$F (21, 1298) = 445.2$, $p < 0.0001$]. When assessing Tukey's *post-hoc*, no significant difference was observed in weight progression between the SAL group and LPS group during the first 15 postnatal days ($p > 0.05$). However, from the 17th to the 21st postnatal day, it was observed that the LPS group had lower body weight when compared to the SAL group ($p < 0.0001$) (Figure 5).

Somatic development

Regarding somatic development, the one-way ANOVA test showed values of $F (7, 472) = 974.6$ and $p < 0.0001$. In Tukey's *post-hoc*, it was evidenced that the rats in the LPS group presented delayed opening of the auditory pavilion when compared to the SAL group ($p < 0.0001$). Regarding the other parameters analyzed, no significant differences were observed in the comparison between the two groups ($p > 0.05$) (Figure 6).

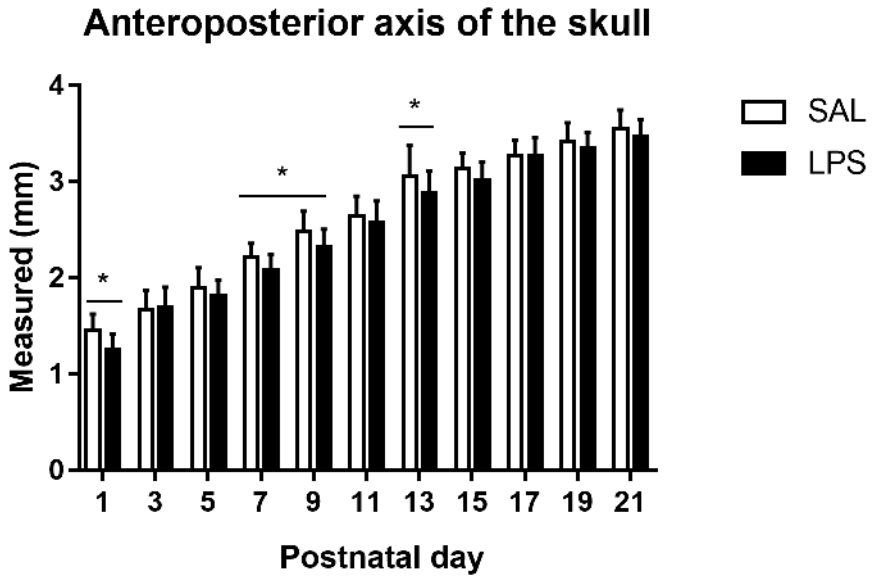

Figure 1. Effects of prenatal exposure to lipopolysaccharide (LPS), compared to the control group (SAL), on the development of rats: the anteroposterior axis of the skull (n=120). The evaluation was performed from the 1st to the 21st day of life. Data presented on average ± S.D. *p < 0.05.

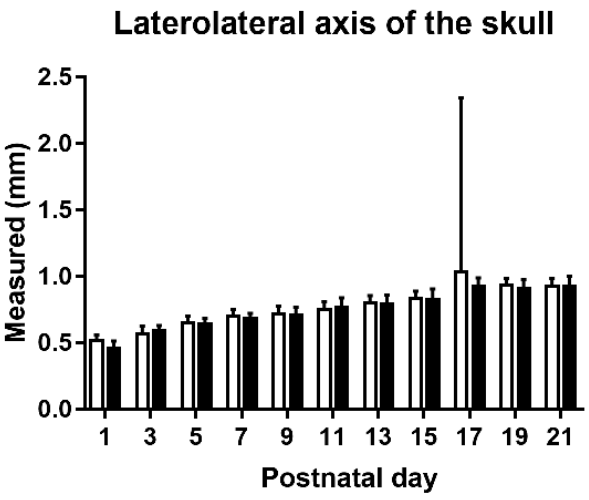

Figure 2. Effects of prenatal exposure to lipopolysaccharide (LPS), compared to the control group (SAL), on the development of rats: laterolateral axis of the skull (n=120). The evaluation was performed from the 1st to the 21st day of life. Data presented on average ± S.D. *p < 0.05.

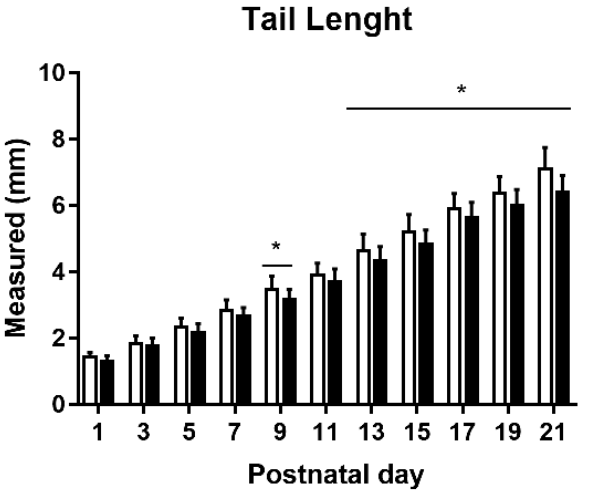

Figure 3. Effects of prenatal exposure to lipopolysaccharide (LPS), compared to the control group (SAL), on the development of rats: tail length (n=120). The evaluation was performed from the 1st to the 21st day of life. Data presented on average ± S.D. *p < 0.05.

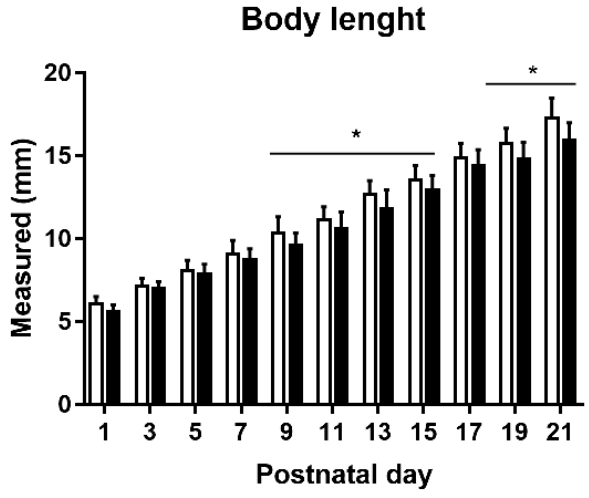

Figure 4. Effects of prenatal exposure to lipopolysaccharide (LPS), compared to the control group (SAL), on the development of rats: body length (n=120). The evaluation was performed from the 1st to the 21st day of life. Data presented on average ± S.D. *p < 0.05.

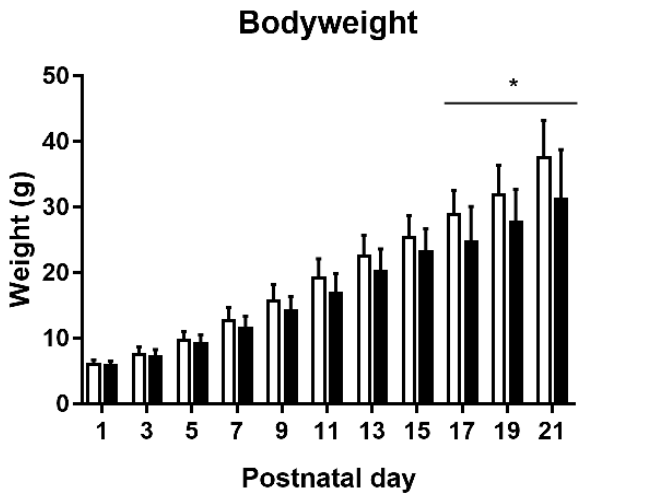

Figure 5. Effects of prenatal exposure to lipopolysaccharide (LPS), compared to the control group (SAL), on the development of rats: body weight (n=120). The evaluation was performed from the 1st to the 21st day of life. Data presented on average ± S.D. *p < 0.05.

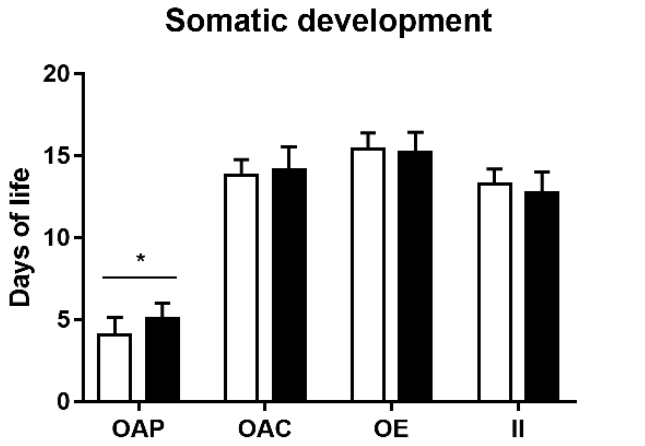

Figure 6. Effects of prenatal exposure to lipopolysaccharide (LPS), compared to the control group (SAL), on the development of rats: somatic development (n=120). The evaluation was performed from the 1st to the 21st day of life. Data presented on average ± S.D. *p < 0.05.
OAP = opening of the auditory pavilion; OAC = opening of the auditory conduit; OE = eye opening; II = eruption of lower incisors.

Reflex ontogenesis

As for reflex ontogenesis, the one-way ANOVA test resulted in the values of F (9, 90) = 92.1 and p < 0.0001. In Tukey's *post-hoc*, when comparing the SAL and LPS group, it was observed that the maturation of the decubitus recovery (p = 0.0413) and precipice aversion (p < 0.0001) parameters occurred first in the SAL group and later in the LPS group. As to other reflexes, there was no statistically significant difference (p > 0.05) (Figure 7).

Post mortem evaluation

The one-way ANOVA result for brain post-death indicators were, on the 8th day F (19, 150) = 905.7, on the 15th day F (19, 210) = 1372 and on the 21st day F (19, 220) = 714.3, with p < 0.0001.

It was observed, in the Tukey's *post-hoc*, that brain indicators APEA, LLBA, APBA, weight, diameter and encephalon circumference did not differ when the SAL and LPS groups were compared (p > 0.05). Similarly, there was no difference in APCA and cerebellar weight indicators (p > 0.05).

However, during the 8th (p < 0.0001), 15th (p < 0.0001) and 21st (p = 0.0443) post-mortem days, it was evidenced that the SAL group had a higher brain volume in comparison with the LPS group (p < 0.05) (Figures 8, 9 and 10).

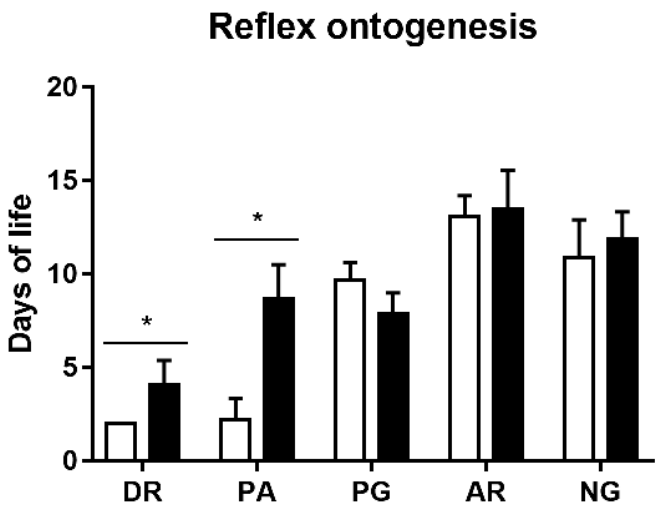

Figure 7. Effects of prenatal exposure to lipopolysaccharide (LPS), compared to the control group (SAL), on the development of rats: reflex ontogenesis (n=20). The evaluation was performed from the 1st to the 21st day of life. Data presented on average ± S.D. *p < 0.05.
DR = decubitus recovery; PA = precipice aversion; PG = palmar grasp; AR = acceleration reaction; NG = negative geotaxis.

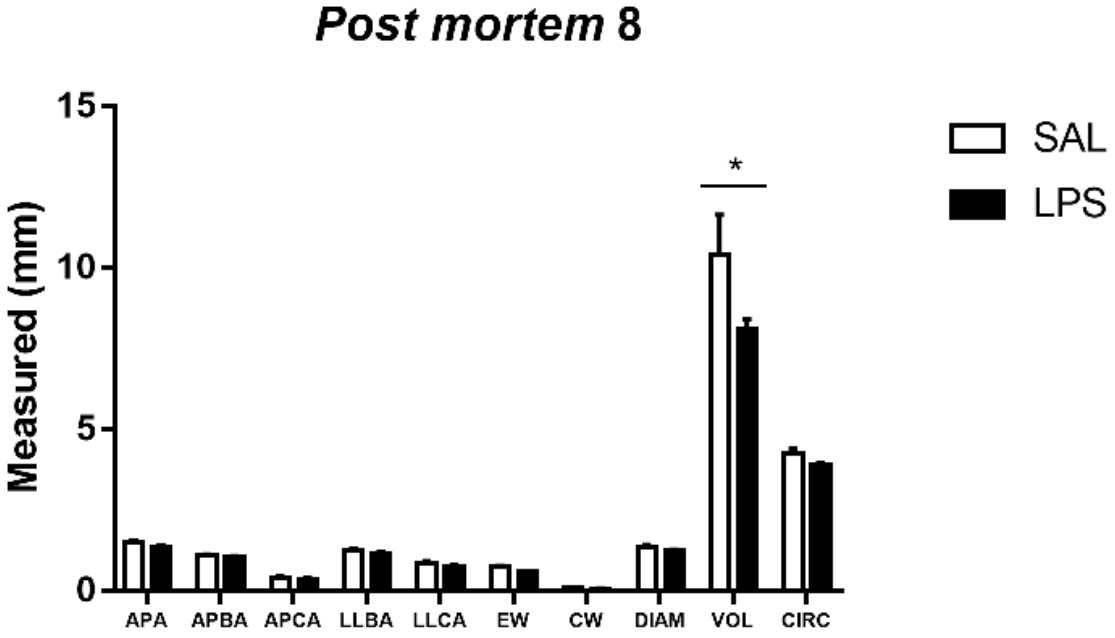

Figure 8. Effects of prenatal exposure to lipopolysaccharide (LPS), compared to the control group (SAL), on the development of rats: *post mortem* brain (n=17). The evaluation was performed on the 8th postnatal day. Data presented on average ± S.D. *p < 0.05.
APEA = anterior-posterior encephalium axis; APBA = anterior-posterior brain axis; APCA = anterior-posterior cerebellum axis; LLBA = laterolateral axis of the brain; LLCA = laterolateral axis of the cerebellum; EW = encephalic weight; CW = cerebellum weight; DIAM = diameter; VOL = volume; CIRC = circumference.

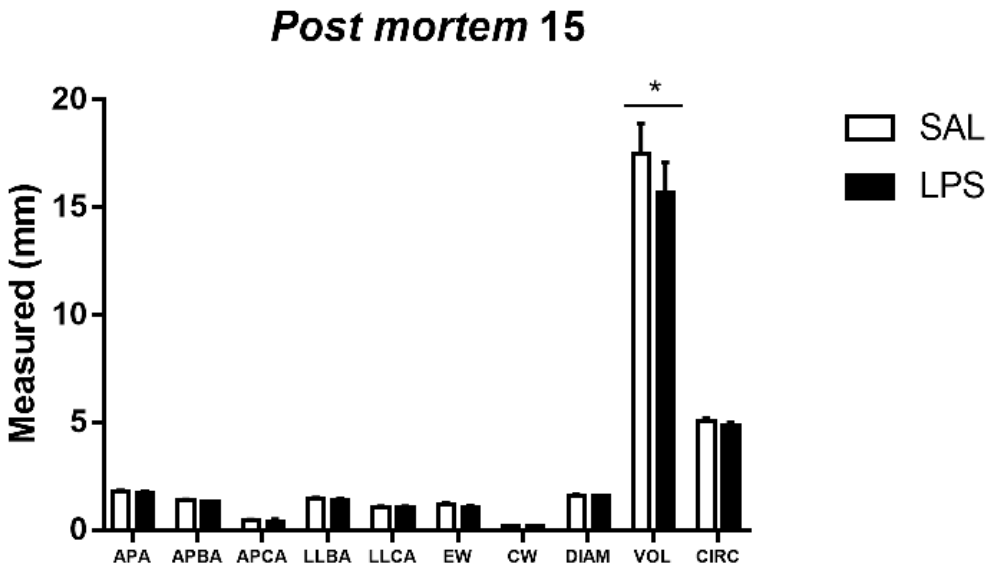

Figure 9. Effects of prenatal exposure to lipopolysaccharide (LPS), compared to the control group (SAL), on the development of rats: *post mortem* brain (n=23). The evaluation was performed on the 15th postnatal day. Data presented on average ± S.D. *p < 0.05.
APEA = anterior-posterior encephalium axis; APBA = anterior-posterior brain axis; APCA = anterior-posterior cerebellum axis; LLBA = laterolateral axis of the brain; LLCA = laterolateral axis of the cerebellum; EW = encephalic weight; CW = cerebellum weight; DIAM = diameter; VOL = volume; CIRC = circumference.

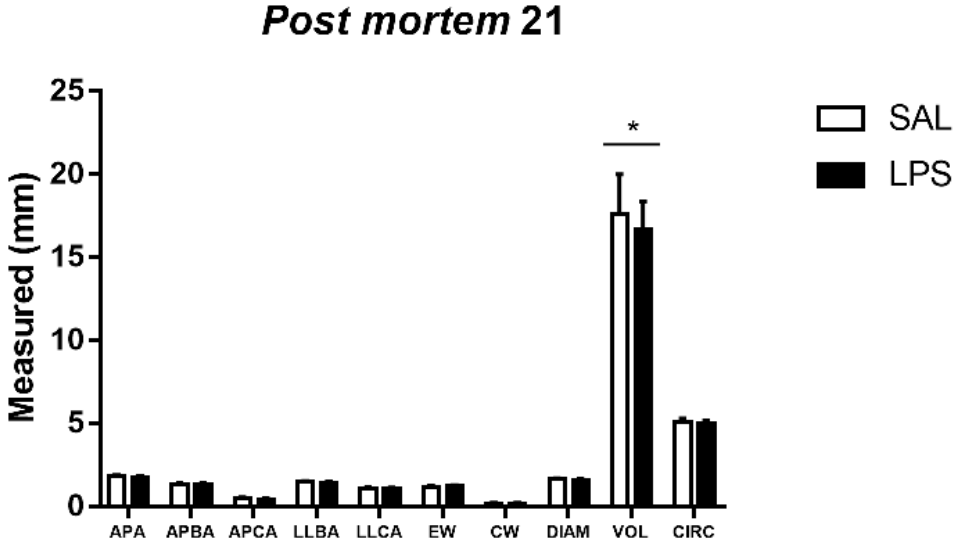

Figure 10. Effects of prenatal exposure to lipopolysaccharide (LPS), compared to the control group (SAL), on the development of rats: *post mortem* brain (n=24). The evaluation was performed on the 21st postnatal day. Data presented on average ± S.D. *p < 0.05.
APEA = anterior-posterior encephalium axis; APBA = anterior-posterior brain axis; APCA = anterior-posterior cerebellum axis; LLBA = laterolateral axis of the brain; LLCA = laterolateral axis of the cerebellum; EW = encephalic weight; CW = cerebellum weight; DIAM = diameter; VOL = volume; CIRC = circumference.

DISCUSSION

Human development is a complex process that comprises a series of changes initiated since fecundation (Moore et al. 2015). The evolutionary capacity present in humans is called phenotypic plasticity and is based on the ability that a genotype has to present different phenotypes according to the exposure to different environmental conditions. Experiences during the prenatal period are crucial for fetal health (Garland Jr & Kelly 2006). In mammals, some periods of development are extremely vulnerable to external insults. These periods are dependent on critical cellular processes, such as proliferation, migration, differentiation, synaptogenesis, myelination and apoptosis.

External stimuli, such as exposure to toxic substances and infections, are examples of environmental insults that can affect an individual's development during intrauterine life. The occurrence of the mother's immune system activation, resulting from an infection, for example, directly affects the offspring, causing structural and functional changes in the nervous system, both acute and lasting, which may predispose animals to neurological disorders in postnatal life, such as autistic spectrum and other mental disorders. Clinical and experimental observations have generated evidence indicating a multifactorial etiology, which is dependent on the time, intensity and nature of immunological exposure, environmental factors and genetic predisposition, defining long-term structural and functional changes in the offspring exposed to maternal immune activation (Estes & McAllister 2015, Scola & Duong 2017). In this study, we evaluated parameters related to the neurodevelopment of rats in the face of maternal exposure to lipopolysaccharide in a critical period of fetal development.

LPS is an important constituent of the outer membrane in gram-negative bacteria and is considered one of the most potent neuroinflammatory agents, interfering with behavioral, psychological and neuroendocrine aspects (Izvolskaia et al. 2018). In the organism, its release occurs when the bacteria multiply or are phagocyted and degraded by the defense cells (Tuin et al. 2006, Cruz-Machado 2010, Noh et al. 2014). It has been used to activate the innate brain immune response in animals, which supports the clinical use of the term LPS-induced neuroinflammation (Guerra et al. 2011). Furthermore, in mice, when administered on the eighteenth gestational day, this endotoxin suppresses the expression of factors related to neurogenesis, neuronal migration, and axonal cone growth (Liverman et al. 2006).

Reflex ontogenesis involves a simultaneous sequence of events and the participation of several regions of the CNS. It is considered an indicator of CNS development and maturation (Fox 1965, Smart & Dobbing 1971). The evaluation of reflex development enables the detection of possible changes since the beginning of the animal's life. Infections during pregnancy are associated with sensorimotor deficit related to reflex maturation. Therefore, reflexes are motor responses of the Central Nervous System to internal or external stimuli and, consequently, are survival mechanisms (Meyer 2013, Izvolskaia et al. 2018).

During ontogenesis, the development of postural control — a prerequisite for motor development, which interferes with cognitive development, verbal and non-verbal communication in children — depends on numerous neurobiological processes and the perception of the external world (Altman & Sudarshan 1975, Poltorak et al. 1998).

The assessment of motor ontogenesis is undoubtedly an important tool in the

diagnosis of deficits related to changes in neuropsychomotor development (Eickmann et al. 2002). To assess the animal's postnatal development, we measured a series of reflexological and physiological parameters. The present study has demonstrated that acute administration of LPS at a critical period of rat brain development on the 9.5th prenatal day is detrimental to developing puppies.

The various reflexes overlap each other, characterizing the simultaneous occurrence of several events in the development of the CNS (Fox 1965, Smart & Dobbing 1971). This is especially true for those that involve head movements and influence the position of the legs, such as decubitus recovery. Thus, some reflexes express labyrinthine activities and seem to be related to the animal's survival, such as food and temperature conservation (Fox 1965). In this study, prenatal exposure to toxin caused damage in reflexological parameters recumbency recovery and cliff aversion. Changes in reflex maturation indicate a correlation between biochemical and structural development and the ontogenesis of the nervous system. This abnormality may be related to disturbances in neurocellular events resulting from prenatal infection. These neurological changes have already been reported, along with the observation that immunological disorders during the early fetal phases mainly affect proliferation, differentiation, cell migration and synapse maturation (Silva 2008, Lunardelli et al. 2014). Furthermore, gestational day 9.5 is a critical period for CNS organogenesis and interferences at this stage, such as infections, can result in neurocognitive disorders.

In addition, maternal exposure to inflammation during the critical period of neurodevelopment (GD9/9.5) is capable of causing pathophysiological and behavioral disorders in the offspring, including those related to motor-sensory function, as evidenced in our research. Intrauterineexposure during a reported critical period appears to be linked to the nervous system's vulnerability to aggression. Although the CNS is known to be flexible and plastic, experimental and clinical evidence show that aggression in these periods can trigger changes in ontogenetic events (Meyer et al. 2006, Meyer et al. 2008, Meyer & Feldon 2012).

Regarding the maturation of the structures that make up the head (opening of the auditory pavilion and auditory conduit, eye-opening and incisor eruption), it is known that its delay is related to somatic growth retardation (Silva 2008). It was observed that the offspring of the neuro-inflamed group presented a delayed opening of the auditory pavilion when compared to the control group. Because of this delay in neurodevelopment, hearing may be impaired. As well as motor development. This fact may be related to neurocognitive damage resulting from maternal immune activation.

Harmful effects of LPS exposure during pregnancy on fetal neurodevelopment, aside from impairing the sensorimotor reflex, may also lead to differences in brain volume as observed in the *postmortem* analyzes found in this study (Sominsky et al. 2013). The brain volume observed at 8, 15 and 21 days of life of the SAL group was greater when compared to the group with maternal immune activation. Which is consistent with previous literature which says that there is a decrease in the overall neural cell population during the first 7 days of postnatal life in the offspring of mothers which received LPS application. Therefore, prenatal LPS exposure has specific detrimental effects on neuronal differentiation and affecting cell proliferation. After the first 7 days, it is suggested that prenatal exposure to LPS also triggers a neural proportion deficit (Graciarena et al. 2010). In this study, the decrease in brain volume from

the 8th postnatal day represents an affected neurogenesis. In this sense, it can be concluded that CNS changes resulting from prenatal immunological activation are dependent on the inflammation period as well as on the postnatal age of the offspring analyzed.

Age-related weight, height, and head circumference assessments are widely used in humans to assess growth and development, as well as nutritional status. Head circumference measurement, aside from assessing nutritional status, is an indicator of brain volume (World Health Organization 2006, 2007).

Maternal inflammation during pregnancy can cause differentiation in the offspring's body weight gain when compared to those of healthy mother (Harding et al. 2014). Body weight is considered a good indicator of physical development in rats. In the present study, the SAL group had a higher weight gain compared to the LPS group from the 17th postnatal day. The difference in weight between the groups is related to the fact that maternal immune stress is linked to the reduction in the offspring's body weight, especially in the first two weeks of life (Golan et al. 2004, Bakos et al. 2004).

The insult imposed on the mothers during the prenatal period seems to have been decisive in causing a decrease in body weight and a delay in physical development. In the present study, matrix-induced inflammation affected body growth deficit and tail length in neonatal rats. Similar results were found in previous studies, which showed delayed body growth and tail length, as well as a connection between tail length and malnutrition in neonatal rats (Barbosa & Santiago 1994, Golan et al. 2004).

There was also a decrease in the antero-posterior axis of the cranium. In rodents, the skull does not develop as a single unit but divides into two distinct regions (neurocranium and viscerocranium) (Cheverud 1982). While the viscerocranium is used during feeding and breathing, the neurocranium houses the brain and its development is mainly influenced by its growth (Young 1959, Cheverud 1982, Herring 1993). Thus, the anteroposterior axis of the brain represents the two regions, as it is measured from the anterior tip of the nasal bone to the posterior edge of the occipital bone (Miller & German 1999, Barros et al. 2018). The decrease in the animal's skull size may be associated with the decrease in brain volume observed in the LPS group.

Thereby, it is concluded that prenatal exposure to LPS in early pregnancy can interfere in the intrauterine environment, impairing functional and structural aspects of the central nervous system, as well as the maturation of physical characteristics. However, mechanisms related to immune responses need to be better clarified and there is also a need to investigate the effects of rodents'exposure to endotoxin in the adult life. It is expected that our study may help to understanding of the genesis of disorders related to neurodevelopment, which have an important socioeconomic impact and still have important therapeutic limitations.

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Abbreviations:

CNS (central nervous system), LPS (lipopolysaccharide), SAL (saline), NaCl (sodium chloride), GD (gestational day), BW (bodyweight), LL (longitudinal length), TL (tail length), LLAxis (lateral-lateral axis), SAAxis (skull anteroposterior

axis), OPA (the opening of the auditory pavilion), OAC (the opening of the auditory conduit), ELI (the eruption of lower incisor), EO (eye-opening), PG (palmar grasp), DR (decubitus recovery), PA (precipice aversion), NG (negative geotaxis), AR (acceleration reaction), APEA (anterior-posterior encephalic axis), APCA (anterior-posterior cerebellum axis), APBA (anterior-posterior brain axis), LLBA (laterolateral axis of the brain), LLCA (laterolateral axis of the cerebellum).

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MGS and JJF conceptualized and designed the study. MGS, GCD, GMS and IPA conducted and evaluated the experiments. MGS and GCD analyzed and interpreted the data as well as wrote the first draft of the manuscript. RMB supervised the work and reviewed the manuscript. All authors have read and approved the final version of the manuscript.