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AMRITA JHA KUMAR

SEX DIFFERENCES IN NEONATAL ANOXIA:
A Behavioural and Histological Study in Wistar Rats.

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A Behavioural and Histological Study in Wistar Rats.

Defense of the Thesis presented to the Pos-Graduation programme in Morphofunctional Sciences of the Institute of Biomedical Sciences at University of São Paulo, to obtain the título of Doctor in Science.

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“Life is just a certain amount of time and energy.

Putting this time and energy to maximum use for everyone’s wellbeing is all that matters.”

— Sadhguru Jaggi

LISTS OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

AF	-Anoxia female
AM	-Anoxia male
ANOVA	- Analysis of variance
CA	- Cornu ammonis
CA1	- Pyramidal neurons of CA1 in hippocampus
CA3	-Pyramidal neurons of CA3 in hippocampus
cc	-Corpus Callosum
CF	-Control female
CM	-Control male
DG	-Dentate Gyrus
EPM	-Elevated Plus Maze
H	-Hour
HI	-Hypoxia Ischaemia
MWM	-Morris Water Maze
OD	-Oxygen deprivation
PND	- Post Natal Day
SNCD	- Substantia Nigra Compact Dorsal
VPL	-Vostero Postero Lateral Thalamus Nucleus

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RESUMO GERAL

A anoxia neonatal está associada a déficits no desenvolvimento neurológico. Exploramos a diferença de sexo na anoxia neonatal, pois os deficits neurologicos e cognitivos tem sido pouco abordados em relacao ao sexo apos anóxia neonatal.

Portanto, decidimos, avaliar os efeitos a curto e medio da asfixia perinatal em modelo animal adaptado e validado. Esse estímulo não invasivo e global, pela exposição de ratos neonatos (30 h) a 100% de nitrogênio, simula as condições clínicas de bebês prematuros humanos (cerca de 6 meses de gestação), que apresentam complicacoes pela falta de oxigenio de forma adequada. Foi utilizada bateria de testes para reflexos sensorio motores, labirinto aquático de Morris, estudos de labirinto em cruz elevado e alodinia mecânica. Assim como, avaliação de alteracoes histologicas em areas neurais relacionadas aos objetivos abordados foram tambem realizadas. Os resultados mostraram alterações nos ratos submetidos a anoxia neonatal nesses testes, sendo o macho mais vulnerável ao impacto negativo do estímulo , em relacao a fêmea. Os resultados demonstram que a anoxia neonatal afeta os reflexos sensoriomotores avaliados, de forma diferenciada em machos e femeas e que sao tambem idade-dependentes e com com decrescimo no numero de celulas nas areas avaliadas. Avaliacoes comportamentais identificaram que a anoxia causou comportamento semelhante a ansiedade, menor nos animais experimentais em relacao aos controles, sendo que as femeas menos ansiosas que os machos. O machos anoxia apresentaram deficit cognitivo na memória espacial em relacao femea e respectivos controles. Foi observado decrescimo celular em regioes do hipocampo (CA1, CA3, DG) dependentes do sexo. A anoxia neonatal diminuiu significativamente o limiar a nocicepcao mecanica (alodinia) associada a alteracoes no nucleo sensorio motor do talamo (VPL) e na substancia nigra compacta. Esses resultados são relevantes na interpretação dos eventos já observados nessa condição clinica mundial e ressaltam a importancia de considerar diferencas de sexo nas pesquisas, a fim de orientar a busca por abordagens e procedimentos terapeuticos efetivos.

General Abstract

Neonatal anoxia is associated with neurodevelopmental deficits. We explored gender difference in neonatal anoxia. Only few studies have been reported describing neurological, cognitive and behavioral deficits following perinatal asphyxia along with sex difference. We therefore decided to study long term effects of perinatal asphyxia in a well- documented animal model resembling the clinical situation. This stimulus, induced by exposure of 30-hour old pups to 100% nitrogen, represents a non-invasive and global stimulus, which simulates clinical conditions of human pre-term babies (around 6 gestational months). Examinations consisted of a battery of motor and reflex tests, Morris water maze, elevated plus maze and mechanical allodynia studies. Alteration in anoxia rats were seen in these tests and male being more vulnerable for detrimental effect of the stimulus than the female. Along with development and behavior alteration histological damage in vulnerable regions linked to above deficits were seen. The present data demonstrates that neonatal anoxia, besides determining only transitory defects in behavior, profoundly affects cognitive abilities and as seeing different field (CA1, CA3, DG) of hippocampus; Substantia Nigra Compact Dorsal, and Ventroposterolateral thalamus nucleus. These results might be of relevance in the interpretation of the substrate of the cognitive impairment seen clinically. This is still a health concern worldwide, a risk factor for several mental and neurological disorders showing a delayed clinical onset. Serial neurobehavioral follow-up is still executed on the premature infants who is absent of detectable abnormalities in early children.

I General Introduction

Neonatal anoxia

Neonatal anoxia, is a medical condition resulting from deprivation of oxygen during time of birth which can affect physically but in majority affects the brain. It is considered a global clinical problem, which leads to serious and lasting consequences of mental retardation, cerebral palsy, epilepsy, hearing and visual deficiencies, along with deficities in motor and behavioural development (Hedner and Lundborg, 1980; Volpe, 1992; Dell'Anna et al., 1993; Dell'Anna et al., 1995; Dell'Anna et al., 1997; Faraone and Biederman., 1998; Vannucci et al., 1999; Cannon et al., 2002; Wainwright et al., 2004; Caputa et al., 2005; Casolini et al., 2005; Mikati et al., 2005; Rogalska et al., 2006; Majeed et al., 2007; Chen et al., 2007).

It also triggers infant's mortality and lifelong disability that affects 0.1–0.3% of full-term infants and nearly 60% of low-weight premature infants (Kurinczuk et al., 2010). The incidence of neonatal anoxic insults appears to be 2–4/1000 full-term infants, but this rate is much higher (approximately 60%) in low-weight premature newborns, making it a public health concern (Vannucci et al., 1997; Laviola et al., 2004).

The severity of these brain injury appears to be sex dependent, affecting more the male neonates, resulting in more severe neurological outcomes and poor recovery as compared to females with similar brain injuries (Waddell et al., 2016; Huang et al., 2016). Previous research showed that neonatal anoxia affected somatic growth, ontogenesis of physical development and onset of neurological reflexes in comparison to control animals, with sex differences in physical and ontogenetic reflex parameters. Male anoxic rats were more vulnerable in histological analysis at adolescence (Kumar et al., 2017). It is established that young male and female rats present asymmetries in antioxidant system properties (Sanches et al., 2013) and different molecular mechanisms of cell death (Zhu et al., 2006). Such differences might possibly be the cause of altered behavioural and morphological responses as previously described (Bona et al., 1998).

The vulnerability of the brain to the lack of oxygen turns it into the most damaged structure, especially in the border areas of the cerebral arteries and in areas of the cerebral cortex, brain stem, subcortical oligodendrial region, periventricular region and basal nuclei (Courville et al., 1950; Volpe & Pasternak., 1977; Nakamura et al., 1986; Khong et al., 2003).

Some of the neurological complications resulting from this condition are: hypoxic-ischemic encephalopathy, delayed neuromotor development, cerebral palsy, periventricular leukomalacia, mental retardation, learning disability, memory impairment, epilepsy, hyperactivity, and other behavioral changes (Vannucci et al., 2000, Funayama et al., 2005, Majeed et al., 2007, Morozova & Belosouva, 2009; Back & Rosenberg, 2014). Research on Hippocampal vulnerability to oxygen deprivation using rodent models of both hypoxia–ischemia and hypoxia (Johnston, 2001; Daval and Vert, 2004; Liu et al., 2008; Yang et al., 2011); reported that the extension of the damage appears to be related to the hippocampal plasticity, due to its neurogenese ability (Shors et al., 1989), which might minimize deficits in spatial learning and memory (Buwalda et al., 1995; Cannon et al., 2002; Caputa et al., 2005; Rogalska et al., 2006; Winocur et al., 2006).

Although cognitive changes and cerebral cell death have been extensively investigated using other animal models of oxygen deprivation, the methodological approach employed in the present study provides advantages. First, it is non-invasive global oxygen deprivation in neonatal rats up to 30 h after birth appears to parallel the experience of low-weight premature infants because the rats are subjected to anoxia at a brain maturation stage that corresponds to that of humans born prematurely (Semple et al., 2013), thus representing an analogously valid model. Second, one of the main findings following oxygen deprivation at this period in rodent models corresponds to white matter damage associated with no gross morphological alterations in gray matter (Puyal et al., 2013).

In the other hand, the injury also decreases glucocorticoids levels, altering HPA, which increases corticosterone and alter monoaminergic systems functioning (Flandreu et al., 2012; Krishnan et al., 2008). Perinatal hypoxia ischemia has shown that locomotor disorders and reduced anxiety are associated with low level of

dopamine content in the rat brain and with simultaneous substantia nigra injury (Boksa et al., 20004).

Moreover, a common brain abnormality in individuals born at very preterm is the thinning of the corpus callosum (cc), particularly of the posterior body (splenium) (Stewart et al., 1999; Peterson et al., 2000). Such injury may be partly explained by its vulnerability to hypoxic–ischaemic damage and haemorrhage but, also because of the immature stage of oligodendrocytes (Back et al., 2001).

Oxygen deprivation in human neonates.

After hypoxia, some children may recover neurologically without sequelae, while others develop permanent deficits (Bennet et al., 2000). Hypoxia also represents a state of low oxygen content in the organic tissues that can occur due to several factors, such as obstruction Physics of blood flow at any level of the body circulation or even in an environment with low oxygen concentrations in the air. This exposure of the brain to the condition of low oxygen and reduced blood supply is called Ischemic Hypoxia (HI), whose consequences include cerebral palsy and behavioral abnormalities (Lynch et al., 2009). In humans, changes in perfusion and oxygenation of the brain is the main cause of perinatal brain damage (Nyakas et al., 1996). Approximately 0.3-0.9% of newborn humans are exposed to prolonged perinatal ischemia-anoxia or anoxic injury, a period in which they are particularly susceptible to tissue damage (Tutor et al., 1996). Ischemic Hypoxia (HI) in human neonates during gestational weeks 23-32 are considered premature, they usually present damage to the developing subcortical white matter due to cell death.

Injury due to HI occurs more frequently in the context of unequivocal clinical impairment, such as placental dysfunction or prolonged labor, or premature birth and cardiorespiratory resuscitation. This condition known as periventricular leukomalacia (PVL) appears to be associated with decreased blood and consequent hypotension. Obstetric complications may also be associated with schizophrenia (Boksa et al., 2004). However, hypoxemia and acidosis are causes of cerebral palsy that are related to abnormal development in childhood, which present limitations in learning and motor

immobility (Volpe et al., 2002). In this condition, seizures and severe motor damage also occur in children, and severe cases can result in death (Tuor et al., 2012).

Central Nervous System and Oxygen Deprivation

In oxygen deprivation a complex cascade of virtually simultaneous biochemical events is triggered which promotes interruption of energy metabolism, acid-base imbalance, accumulation of oxygen-reactive species and excitatory amino acids in the extracellular space, loss of cellular water balance and onset of apoptosis (Vexler et al., 2001). It is important to point out that these events are fast and short-lived, so the timeframe for therapeutic interventions is greatly reduced.

Neonatal damage in rats can disrupt the neuroendocrine system, present low levels of circulating glucocorticoids, altering the hypothalamic-pituitary-adrenal (HPA) axis and can cause abdominal obesity. Increased basal corticosterone levels increase pulse rate, and may result in hypertension. The resulting alterations may also affect the monoaminergic system in regions such as striatum, prefrontal cortex and hippocampus and cause depletion of serotonin, norepinephrine and dopamine. These conditions are associated with depressive symptoms (Flandreu et al., 2012; Krishnan et al., 2008).

Dopamine decreases as a function of decreased tyrosine hydroxylase activity in the motor cortex and hippocampus and may reduced anxiety in animal models (Perrin et al., 2004; Hoeger et al., 2000). In perinatal asphyxia, the literature reports. Furthermore, hypoglycemia results from this insult which has been reported in relation to the change in structure and remodeling of synapses and altered neural plasticity. Synaptic modifications related to the efficacy of neuronal transmission, such as those of long-term potential, are common in this condition (Jourdane et al., 2002).

The brain, because it is high metabolic tissue requires adequate supply of glucose and oxygen to maintain its normal functions. This critical requirement is compromised during biochemical changes generated by anoxic injury, leading to

excitotoxicity and influx of Ca2 + ions, resulting in overproduction of nitric oxide (NO), and other reactive oxygen species (ROS) within the mitochondria (Marschitz et al., 2014). The depletion of ATP by anoxia or hypoxia has important consequences, since it implies a failure in the functioning of sodium-potassium ionic pumps, activation of free radical formation (Dell'Anna et al., 1997), changes in the phosphorylation state of Different enzymes and structural proteins. Failure in ionic pumps causes ionic passage through the membrane below the concentration gradients and promotes consequent depolarization with lethal consequences and massive loss of neurotransmitters such as glutamate, aspartate and dopamine into the extracellular space in a toxic amount (Lofton et al. , 1989).

Stages of Brain Development in Rodents and Humans

The main event in the formation of CNS in all vertebrates is the formation of a specialized fold of ectodermal tissue called the neural tube, from which the spinal column and the brain subsequently differentiate. Neural tube formation occurs approximately midway through gestation in gestational day (GD) 10.5-11 and 9.95 in rats. In humans, this event occurs earlier during prenatal development, between GD 24 and 28 (3-4 weeks), in a gestational period of 266-280 days (40Weeks) (Rice et al., 2000).

Critical Period of Injury and Cell Proliferation

The critical period corresponds to the one in which there is greater susceptibility of the neurons and the glia to damages, this comprises the phase of the proliferation and cellular migration, which is considerable in the last trimester of gestation of the human brains and in rats occurs in the week post (Bayer et al., 1993). During development, in the neonatal mammalian brain, the layer of glutamatergic and gamma-aminobutyric acid (GABA), or GABAergic neurons, between the immature cerebral cortex and the region of the white matter below the neural plate is the source of new neurons (Kostovic et al., 1989). The cortical neurogenesis in the rat begins in GD 9.5 and is completed around the PND (Rice et al., 2000) whereas in humans, there is

predominance during gestation, which can continue until 2.5 years of age (Hovda et al., 2003).

In the second week of gestational development of rats and in the first month of gestation in humans, specific areas of the CNS begin to form with neurogenesis and cell migration in the brain, then follow the sequence of developmental processes that includes proliferation, migration, differentiation, onset of synapses, apoptosis and myelination. During the latter part of pregnancy and the first postnatal month, oligodendrocytes should develop their myelin sheath around axons. The maturation of axons and the appearance of synapses in humans occurs during the third trimester of the first year after birth (Mason et al., 1984).

Previous studies in neonatal anoxia, from this laboratory, have demonstrated the detrimental effects of this stimulus, both in glial and neural populations Of the hippocampus, depending on the region and its development period (Allemandi, 2011). In neuronal populations of the hippocampus (Amom's horn and dentate gyrus) a longitudinal effect of this stimulus was observed at 2, 14, 21 and 60 days after birth, with evidence of cell death and changes in organelles consistent with degenerative processes. There was also a decrease in neurogenesis and changes in hippocampal volume in the anoxic animal compared to the control animal (Takada et al., 2015b). These results were obtained by different histological techniques (TUNEL, Fluorojade B), immunohistochemistry, transmission electron microscopy, stereology and magnetic resonance imaging. Also, cognitive deficits were found in the acquisition and performance of spatial and reference memory by Morris water maze. Takada et al., 2006), as well as an increase in anxiety in anoxic animals compared to controls, with high cross-mite larvae in P90 (Takada et al., 2015a).

Sex differences in the developing nervous system

The sexual dimorphism of the differences in neuronal organization existing between the brain of man and woman stem from several biological factors, which are subject to important hormonal regulation. Sex hormones influence the modeling of hemispheric differences to cognitive function (Witelson et al., 1976).

Differences related to brain injury, unilateral related to intelligence, presented ipsilateral neurological association in the injured male brain, whereas in the female brain the cognitive deficit is less asymmetrical (Inglis, 1981).

The sexual differentiation of the male nervous system depends on the determinant gene of the testes located on the Y chromosome (SRY gene), which is responsible for the development of the gonads and is regulated by the gonadal hormone. This hormone has regulatory effects on nerve tissue, depending on the stage of development (Andersson M et al., 1986). That Gene is expressed specifically in neurons expressing the enzyme tyrosine hydroxylase of the mesencephalic black substance. In an experimental study with male rats, suppression of the SRY gene results in motor deficit, although the number of neurons remains the same (Dewing P., 2006), which suggests the involvement of this gene with dopaminergic neurons of the male nigroestrial system . Female and male brain cells exhibit differences in the expression patterns of other genes that are specific to the developing brain, which determine specific functions and abilities for each genus (McCarthy, 2011).

Gender differences have been reported in locomotor activity (Bucci et al., 1995). Male rats perform better on tasks that require spatial abilities compared to females (Roof et al., 1993). However, interestingly, the male brain is more vulnerable to aggression than females in the critical period of development (Venderhus et al., 2010). Circulating testosterone in the newborn male, by not binding to alpha fetoprotein, can enter the nervous system through the blood-brain barrier and be converted into estrogen. The estrogenic organizing action occurs early, already between days 16 and 19 post-conception, immediately prior to the critical period of neural development, where it exerts irreversible effects and thus constitutes a substrate for gender variations. This data coupled with the high OBRb receptor expression in the thyroid of animals with restricted calorie and protein diet suggests inhibition of thyroid hormone release and a relation with altered somatic and developmental parameters (Valenzuela et al., 2014).

In the cellular genesis the gender differences appeared in the hippocampal volume and in the behavior as revealed by a study with neonatal hypoxia-ischemia

model in rats. In this work, a significant increase in neurogenesis was demonstrated in females in relation to males (Waddell et al., 2015).

Hence our hypothesis is that rodent´s pups (30 hour of age), subjected to neonatal anoxia tend have white and gray matter injury in hippocampus and thining of corpus callosum, ventroposteolateral thalamus nucleus, and substantia nigra compact dorsal altering motor and behavior along with nociceptive pathway of the pain.

II. JUSTIFICATION

Due to the clinical relevance of neonatal anoxia, the Neuroscience Laboratory adapted and developed the rodent anoxic animal model previously cited, validated by Takada et al., (2011). This model allowed to observe cytological alterations that indicated apoptosis, necrosis, autophagy and excitotoxicity in hippocampal cells later in adult rats, as well as memory deficits (Takada et al., 2015a). It also detected reduced hippocampal volume and decreased neurogenesis (Takada et al., 2015b). The early assessment of the severity of an acute brain injury, secondary to oxygen deprivation, provides an extremely useful basis for prevention and therapy in neonatology clinics (Naithani et al., 2010).

In agreement with the mentioned research, it has shown in humans that the ongoing changes in white matter brain, its matter microstructural changes, between 8 and 18 years of age, revealed by increased fractional anisotropy in the left frontal lobe, was positively correlated with the development of working memory capacity, while an increase in the left temporal lobe was associated with improvements in reading ability (Nagy et al., 2004). In addition, the different rates of myelination along the corpus callosum extent, highest during childhood (Keshavan et al., 2002), was also shown by fractional anisotropy (Lebel et al., 2012). These changes are speculated to relate to faster and more effective communication between brain regions, and reflect the increasing cognitive capacity seen during adolescence (Lenroot and Giedd, 2006) that might be disrupted by the neonatal anoxic insult with gender differences.

Therefore, aiming to explore the causes of the altered development observed in adolescent male and females, after neonatal anoxia (Kumar et al., 2017) in relation

to the onset of ontogenetic reflexes and histological differences in the M1 and S1HL cortical layers, this study aims to explore how the neonatal anoxia affects sensory motor development in consideration to the related histological structure of substantia nigra compact dorsal, corpus callosum, as well as cognition in relation to the hippocampal structure. To check impact on behavior and cognition we check different field of hippocampus and anoxia insult can influence the cell density in particular regions.Lastly we wanted to check impact on nociceptive pathway of pain and impact on ventro posterolateral thalamus nucleus.

III. OBJECTIVES

In order to contribute to the understanding of the sequelae of this injury and with the proposition of strategies and procedures to avoid or minimize its negative impacts, later in life, since it constitutes a worldwide clinical problem, the Neuroscience Lab team (ICB-USP), is making an effort in characterizing neonatal anoxia model, this study aims to explore its effects on the sensory motor development and behaviour in male and female Wistar rats.

1. Specific Objectives

1.1 To evaluate, the impact of neonatal anoxia on development of Sensory- motor abilities in male and female wistar rats along with the histological structure of Substantia Nigra Compact Dorsal (SNCD) and corpus callosum (cc).

1.2 To evaluate, the impact of neonatal anoxia on behavioral, cognitive and histological structure of hippocampus following neonatal anoxia in male and female wistar rats differences at adolescent age .

1.3 To evaluate, the impact of neonatal anoxia on mechanical pain histological structure of ventral posterolateral thalamus (VPL) nucleus.

Since the aim of the research is to approach both development and behaviour aspects of neonatal anoxia effects, this thesis is divided into three chapters to facilitate readers understanding and keep the focus as follows:

Chapter 1- Sex differences in neurological and motor development after neonatal anoxia in wistar rats.

Chapter 2- Sex difference in reference memory, anxiety and histological damage after neonatal anoxia in wistar rats.

Chapter 3-Sex differences in hypersensitivity to mechanical allodynia in wistar rats.

2. Material & Methods

1. Animals

All experimental procedures complied with the Brazilian Guidelines for the Care and Use of Animals in Education or Scientific Research Activities formulated by the National Council of Animal Experimentation Control (DBCA − CONCEA, 2016) based on National Institutes of Health guide for the care and use of Laboratory animals and were approved by the Ethics Committee of the Institute of Biomedical Sciences of the University of São Paulo (CEUA Protocol No 199/139f, book 2).

2. Animals and treatment

Male and Female Wistar rats and their offspring (n =64) from Animal Resource of the Institute of Biomedical Sciences (São Paulo, Brazil) were kept under temperature-controlled environment (22±2°C) and strict light–dark cycle conditions (lights-on at 7:00), with food and water ad libitum. The litters were randomly assigned

to the anoxia or the control treatments such that each of the resulting groups was composed of subjects coming from at least 4 different litters (to avoid litter effects).

3. Anoxia

Anoxia will be induced in 30-h-old rats (P2) in the female and male groups, using the procedures and system previously adapted and validated (Takada et al. 2011). In short, subjects will be submitted to continuous flow (3 L/min) of 100% nitrogen (N2) for 25 min at 36.5ºC and 101.7 KPa in a non-hermetic chamber (Anoxia treatment – A group). After that, around 5 min will be waited for the animal´s recovery, confirmed by the animal's skin coloring, breathing and active movement, then they will be returned to their parents. Control is subject to open chamber, and we have four set of groups CM (Male Control); AM (Anoxia Male); CF (Control Female) and AF (Anoxia Female).

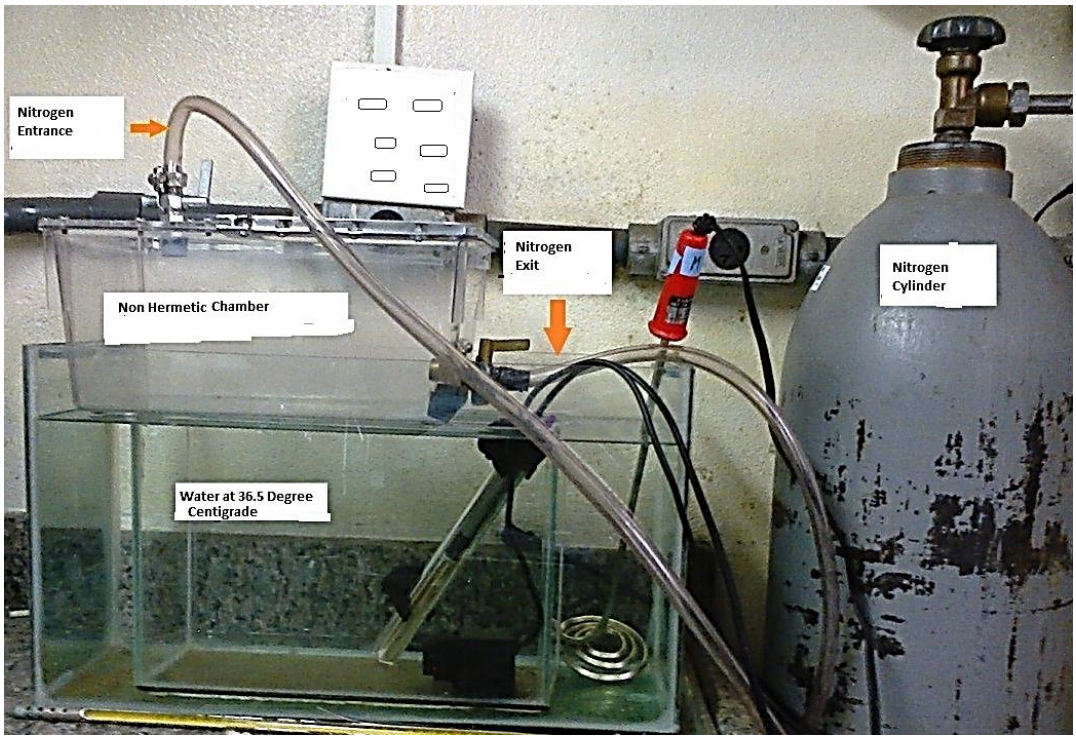


Figure 1. Model of Anoxia system has chamber composed of semi-hermetic housing with opening for nitrogen inlet and outlet; immersed in thermostat-heated water bath at 36 ± 1ºC; Nitrogen cylinder, where the nitrogen is stored and from where it is drained into the chamber; thermostat and heating resistance (Takada et al., 2011).

4. Formation of the groups

Briefly, eight couples of males and females rats (Rattus norvegicus, Wister strain), were used from the animal facility of the Physiology Department, Biosciences, University of São Paulo (USP) and were put together and pregnancy was observed by end of next 21 days The male anoxia (AM) and anoxia female (AF) groups were submitted to neonatal anoxia in P2 (weight between 6 and 8 g) for 25 minutes. The CM and CF groups underwent the same conditions, but with no change in atmospheric composition.

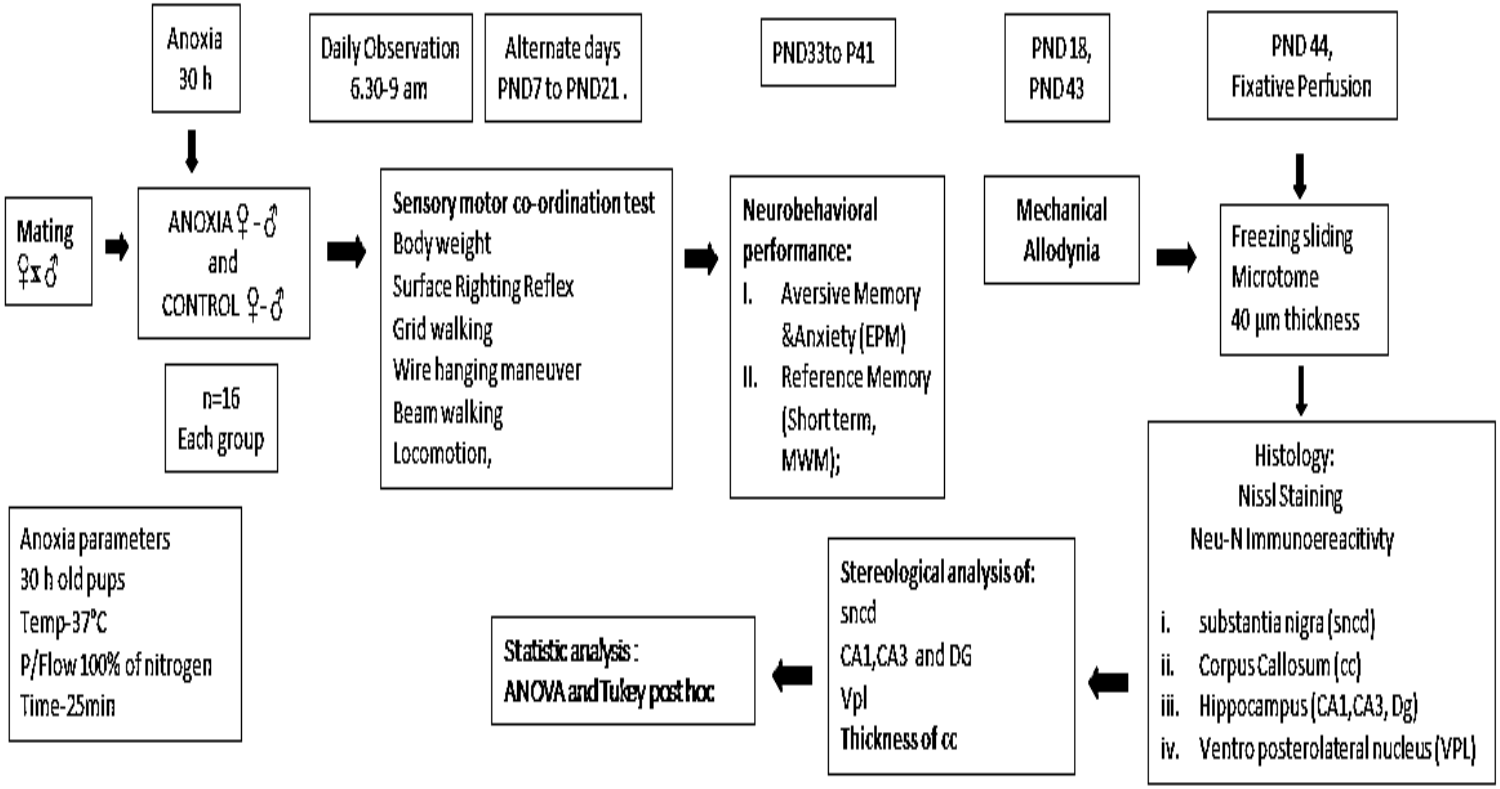


Figure 2. Experimental Design

Sex differences in neurological and motor development after neonatal anoxia in Wistar rats

Abstract

The aim of the present study was to evaluate how neonatal anoxia affects the complex early somatic, neurological and motor development analysing also if there are sex differences. The used model of neonatal anoxia is based on exposure of 100% nitrogen when pups are 30 hours old (6-8g). In the present study we observed that neonatal anoxia has affected the neurological and motor development of the suckling pups. Anoxia male has fast catch up with weight gain even after suckling period and female showing significantly less in weight gain. In locomotion anoxia male has been seen more active than control and anoxia female. In wire hanging anoxia animals show less latency than the control ones, also in grid walking they have covered less grid and have made more foot faults than control. In beam walking anoxia animals covered less steps and made more slips in the later part of suckling period. Time to perform surface righting was significantly longer during the first few weeks in anoxic pups. This has altered physical development (body weight) and neurobehavioral performance, and also demonstrates brain injury as shown in substantia nigra compact dorsal (sncd) and thinning of corpus callosum seen in anoxia male more prominently. The present data also indicates that the vulnerability of brain damage and retardation in neurobehavioral performance is gender dependent, i.e. male rats are affected the most. In previous research, this lab found alteration in somatic development, sensorimotor alterations, early neurological reflexes as well as the density of cells in motor and sensorimotor cerebral cortex of adolescent rats submitted to neonatal anoxia with sex differences. Quantification of cells in motor cortex and primary sensory hind limb and forelimb regions in anoxic group, along with sex differences, as compared to control groups.

Key words: Perinatal asphyxia, Neurological reflex, Motor development, Sex difference, Substantia Nigra Compact Dorsal, corpus callosum.

4.1 Introduction

The important hormonal regulation, are attributed to sexual dimorphism, linked to the differences in neuronal organization existing between the brain of man and woman stem from a number of biological factors.

The modeling of hemispheric differences to human cognitive function is based on influence of the sexual hormones (Witelson et al., 1976). Differences in human sexuality due to brain damage, unilateral related to intelligence, presented ipsilateral neurological association in the injured male brain, whereas in the female brain the cognitive deficit is less asymmetrical (Inglis et al., 1981).

The sexual differentiation of the male nervous system depends on the determinant gene of the testes located on the Y chromosome (SRY gene), which is responsible for the development of the gonads and is regulated by the gonadal hormone. This hormone has regulatory effects on nerve tissue, depending on the stage of development (Andersson et al., 1986). This gene is expressed specifically in neurons expressing the enzyme tyrosine hydroxylase of the mesencephalic black substance. In an experimental study with male rats, suppression of the SRY gene results in motor deficit, although the number of neurons remains the same (Dewing et al., 2006), suggesting the involvement of this gene with dopaminergic neurons of the nigroestrial system in sex male. The brain cells of women and men present differences in the expression patterns of other genes that are specific to the developing brain, which determine specific functions and abilities for each genetic pattern (McCarthy et al., 2009).

Somatosensory cortex

All the sensory input from the body are received by somatosensory cortex. Neurons from somatosensory cortex, send all their information for processing skin, pain, visual, or auditory stimuli. Serial connection of identifiable groups of neurons forms functional paths, and each group processes more specific information and different pathways run through the spinal cord, brain stem, and into the cortex imparting to the sensations. The following diagram (Fig.3) shows that skin sends sensations through neurons to the brain for processing and the way afferent sensory information pathway processing sets.

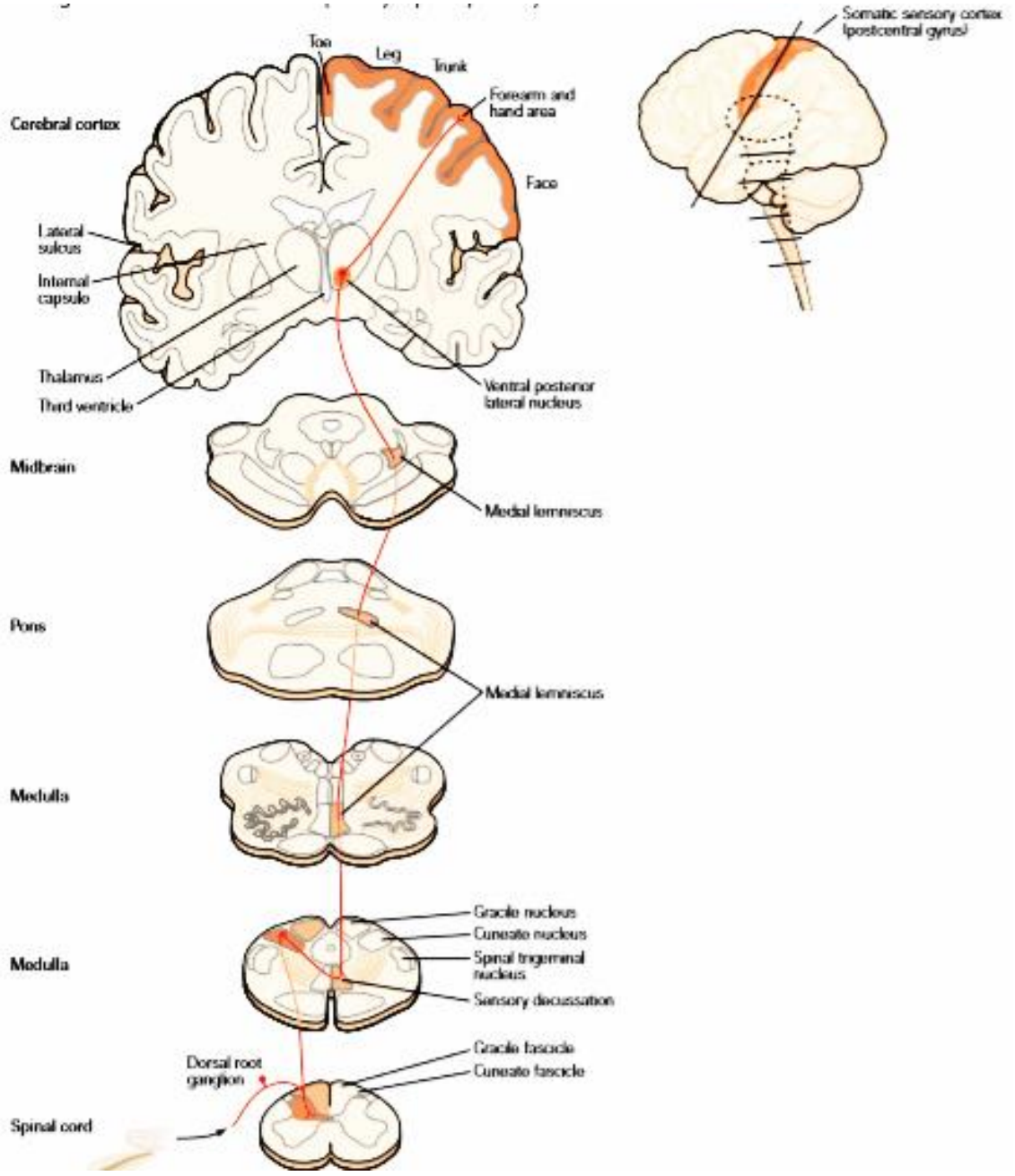


Figure 3. Afferent sensory pathway. Carry signals from mechanoreceptor 1´afferent terminate and form synaptic connection with 2nd order neurons in the dorsal column nuclei within the medulla. 2nd order cross medulla and continue to the thalamus via medial lemniscus pathway. After thalamic processing, 3rd order neuron project to the primary somatosensory cortex. The medial lemniscus is a major afferent pathway for somatosensory information in Humans.Kandel et al., 2005. In rats they have sensory receptors and primary afferent neurons, ascending spinal pathways , somatosensory nuclei in the brainstem and thalamus and somatosensory region of the cortex (Paxinos 2004),

Somatosensory cortex is excited by the information that enters the central nervous system through the dorsal root ganglion cells. Fibers that relay information from different parts of the body maintain an orderly relationship to each other and form a neural map of the body surface in their pattern of termination at each synaptic relay and their axons project to the primary somatosensory cortex (Figure 3). The rest of the thalamus participate in motor functions, transmitting information from the

cerebellum and basal ganglia to the motor regions of the frontal lobe. The cortical connections somatosensory information is used in motor control, eye–hand coordination is seen in higher hierarchy.

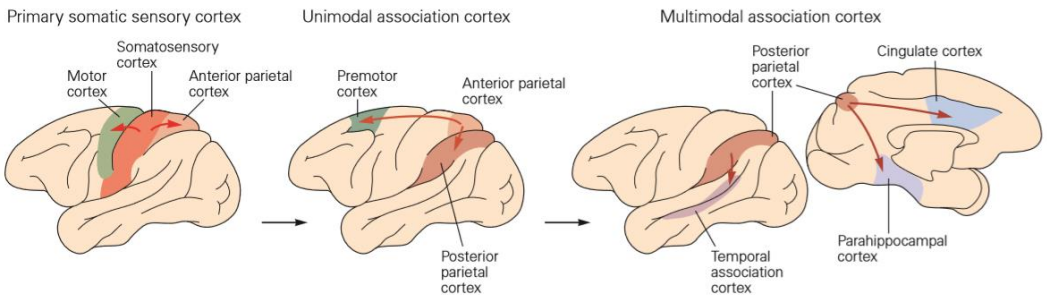


Figure 4. Primary somatosensory cortex. The processing of sensory information in the cerebral cortex begins with primary sensory areas, continues in unimodal association areas, and is completed in multimodal association areas in Human brain. Kandel et al., 2005 . In rat neural bases of multisensory interactions in cortex have focused on specialized higher-order multisensory association areas (Paxinos 2004).

As the primary somatosensory cortex projects to the motor area in the frontal lobe and to the somatosensory association area in the parietal cortex and are in communication with sensory motor cortex.

The axons of neurons in layer V of the primary motor cortex project through the corticospinal tract to the ventral horn of the spinal cord. In the Figure 3 axons descend through the subcortical white matter, the internal capsule, and the cerebral peduncle in the midbrain.

The cerebellum receives somatosensory information directly from primary afferents originating in the spinal cord as well as from corticospinal axons descending information from the neocortex. It enables motor control systems to adapt motor commands to the changing condition of the musculature.

The basal ganglia are a collection of subcortical nuclei (Figure 3) that receive direct projections from much of the neocortex, including sensory, motor, and premotor areas, and those parts of association cortex that are important for motivation, cognition, and emotion. The output nuclei of the basal ganglia send signals to regions of the thalamus that project to the cerebral cortex. (Kandel book 4th edition 2005)

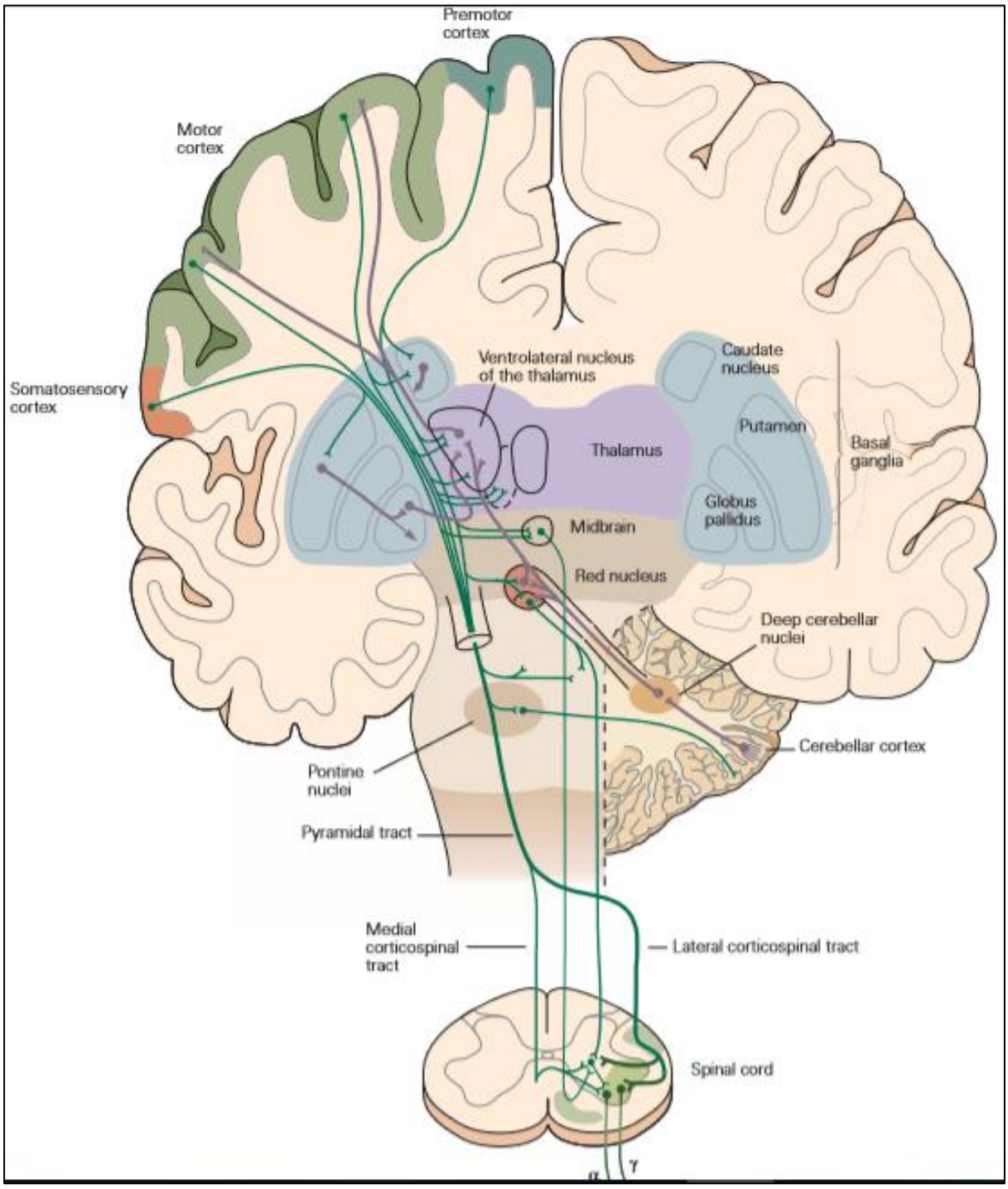


Figure 5. Output nuclei of basal ganglia. Voluntary movement requires coordination of all components of the motor system. The principal components are the motor cortex, basal ganglia, thalamus, midbrain, cerebellum, and spinal cord. The principal descending projections are shown in green; feedback projections and local connections are shown in purple. All of this processing is incorporated in the inputs to the motor neurons of the ventral horn of the spinal cord, innervates muscles and commences movement. Kandel et al., 2005 . In rat brain voluntary movement is associated with ascending pathways from the diencephalon to the hippocampus and neocortex. The motor cortices are activated during skilled voluntary movements with forelimbs (Paxinos 2004).

Impact of Oxygen deprivation

Complications derived from premature birth account for 29% of global neonatal deaths yearly and around 3% of total disability during the lifespan (Lawn et al., 2010; Howson et al., 2013). Premature newborns have a high incidence of neonatal brain injury (Gopagondanahalli et al., 2016) linked to subcortical white and gray matter lesions, impaired structural connectivity (Volpe et al., 2011; Salmaso et al., 2014) which cause lifelong neurodevelopment disturbances (Robinson, 2005; Allin et al., 2008; Delobel-Ayoub et al., 2009; Pyhälä, 2012; Breeman et al., 2015;Hübneretal.,2015;Thomasonetal.,2017).

Pathologies such as cerebral palsy(CP),developmental delay,attention deficit and hyperactivity disorder (ADHD) learning deficits is contributed by neonatal hypoxia-ischemia (HI) (Fatemi et al., 2009; Volpe, 2009a; Phillips et al., 2013). The model commonly used is unilateral carotid ligation followed by a period of hypoxic exposure (Levine, 1960; Rice et al., 1981) leading to deficits in motor coordination (Lubics et al., 2005), anxiety-related behavior and cognitive impairment (Kumar et al., 2018) in early and late development, due to lesions in hippocampus, striatum and cortex (Arteni et al., 2010; Sanches et al., 2015).

Also, studies using HI at postnatal day 7 have shown that cell death occurs in brain regions that are not directly affected by ischemia, such as the cerebellum (Joyal et al., 1996; Kim et al., 2004; Northington et al., 2011) suggesting that neuronal connectivity may play a role in neurodegeneration following HI to the immature brain. The HI model performed at postnatal day 3 mimics the lesion observed in very preterm infants brains(Sizonenko et al., 2003; Sanches et al., 2013; Ginetet al., 2016). HI in the very immature rat brain causes disruption in cell metabolism, development and in cortical cytoarchitecture (Sizonenko et al., 2008; Van de Looij et al., 2011; Misumi et al., 2016), alters the myelination pattern and leads to behavioral impairments (Huang et al., 2009; Sanches et al., 2015; Misumi et al., 2016). Our lab has also seen alteration in metabolism with anoxia rats subjected to neonatal anoxia (Cruz et al., 2019)

Normal structural and functional development of the cerebral cortex and its connectivity are critically dependent on appropriate neuro axonal activation. In the normal mature brain, numerous neural circuits, mostly closed loop, connect a

spectrum of motor and non-motor regions of the cerebral cortex with the cerebellum. Development and consolidation of infrastructure critical for these circuits begin during late fetal and early postnatal life. Cerebellar lesion is a previously under-recognized form of prematurity-related brain injury (Limperopoulos et al., 2005; Messerschmidt et al., 2008) occurring most commonly among infants born before 28-wk gestation, thus before the onset of the third trimester (Limperopoulos et al., 2005), that also might affect the uninjured contralateral cerebral hemisphere (Limperopoulos et al., 2005a). The regional specificity of such crossed cerebello-cerebral diaschisis has not been studied in the immature developing brain, and the regional specificity of these remote growth effects in the cerebrum after prematurity-related cerebellar injury remains unknown.

The cerebellar granule cells continue to divide and migrate after birth, until the end of the first year of life in the human (Rakic et al., 1970). Apoptosis is frequently observed in this cell population, consistent with a previous observation of widespread apoptosis in the granule cell layer following birth asphyxia in humans (Larroche et al., 1968). Similarly, the inner layer of granule cells in the hippocampal dentate fascia are late to mature (Wasterlain et al., 1990). In the cerebral neocortex the earliest migrating cells reside in the deeper cortical layers while the latest differentiated cells to arrive occupy the superficial layers. The oxygen deprivation tend to favor apoptosis in the inner layer of the dentate fascia, the superficial layers (II and III) of the cerebral cortex while necrosis occurs in the deeper layers (IV, V and VI).

Due to various injury linked to early oxygen deprivation, maturation of neurological reflexes and motor coordination can be an indicator to postnatal development. The appearance of certain reflexes and motor performance is influenced by oxygen deprivation in early hours of life (Kiss et al., 2005;2007; Lubics et al., 2005; Reglodi et al., 2003). These shortcomings can be early short term neurological deficits which can be linked to longterm functional deficit like reference memory impairement (Ten et al., 2003).

In our previous work (Kumar et al., 2017) results of stereology show that the neonatal anoxic insult was able to reduce the content of neurons in the motor cortex and sensory motor regions especially in the region of the hindlimbs and forelimbs

there was a significant difference of group and gender. Anoxia female had shown reduced number of neurons in this region than males. Studies show that, in addition to apoptosis in the motor cortex in mass, the decrease may cause epilepsy and cause neurological deficits and motor deficits including spacticity (Jansen et al., 1997). These deficits are associated with degeneration of the cortical and subcortical areas of the brain.

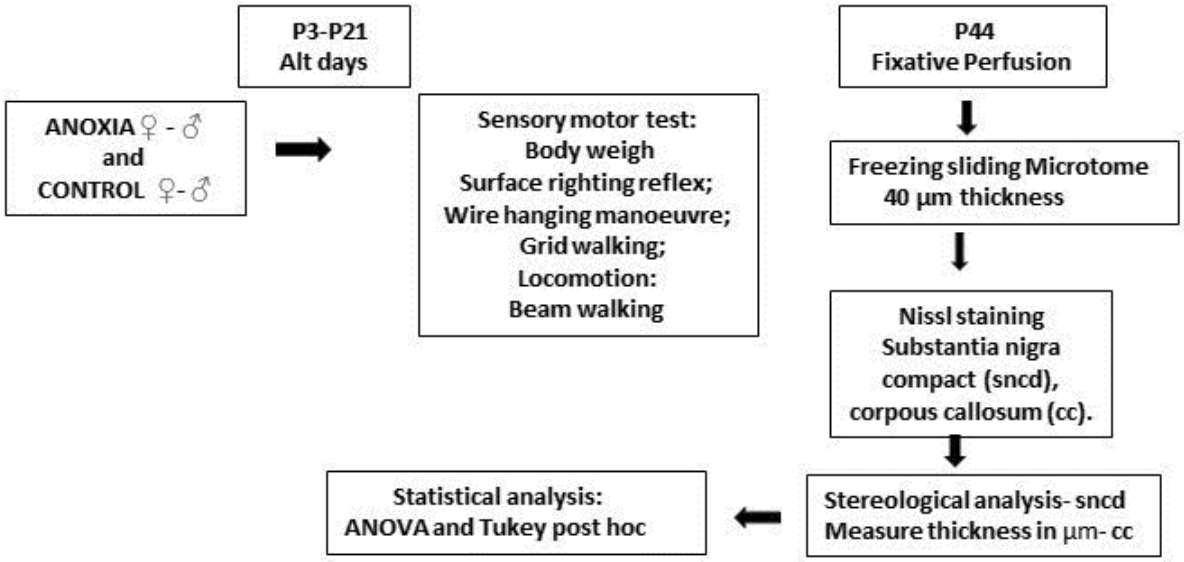


Figure 6. Experimental design for motor test

Material and method

Has been described on page 22 with detailed experimental design. Animals go through battery of neurological & motor development as described above in the design.

4.3. Analysis of neurological and motor development

4.3.1 Somatic growth (Body weight)

It was assessed by daily measuring of body weight using electronic scale On alternate days starting from P3 until P21.

4.3.2 Surface righting reflex (Neurological reflex)

This test is a reflection of subcortical maturation, it evaluates the time, in ms, the animal takes to turn over on all four feet and touch the platform, after being

placed on their backs. The cut-off time was 60 s. (Altman & Sudarshan, 1975; Hermans et al., 1992). Newborn pups can right themselves within 1 min observation period, but with the maturity of the animal style of righting and speed changes provides aid in turning over. Individuated movements of the forelimbs in support of the righting response are not manifest until days 6 and 7 Data will be collected from PND3 until 21 on every alternate days.

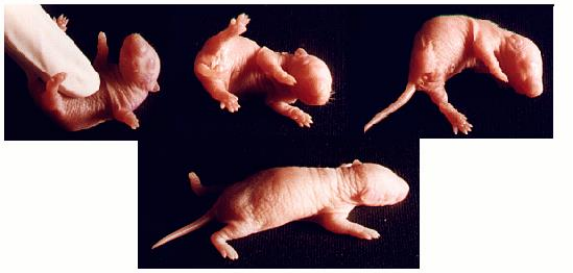


Figure 7. Surface righting reflex, showing four stages in milliseconds to turn over to prone position and place all four paws in contact with the surface.

4.3.3 Grid-walking and foot-fault test:

Rats were placed on a stainless steel grid floor (60 × 60 cm with a mesh size of 2.5 × 2.5 cm) elevated 1 m above the floor. For a 1-minute observation period, the total number of steps was counted. The number of footfault errors, when the animals misplaced a forelimb or hindlimb that it fell through the grid, was also recorded during a 1-minute period. (Markgraf et al 1992; Rogers et al., 1997). Data will be collected from PND3 until 21 on every alternate day.

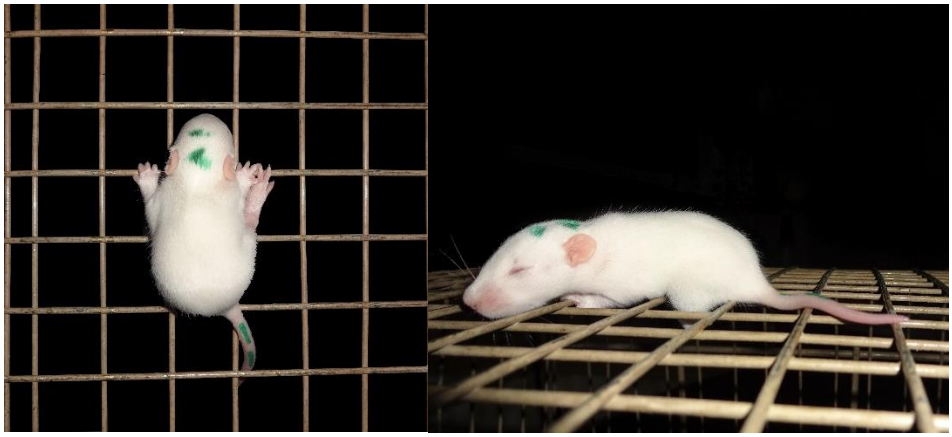


Figure 8. Grid walking showing number of grids covered and number of foot fault errors (misplaced a forelimb or hindlimb) walking on a grid

4.3.4 Wire hanging maneuver (Hind Limb Support when suspended)

This maneuver tests neuromuscular and locomotor development. Pups suspended by their forelimbs from a horizontal rod (55 cm long, 2mm thick and 35 cm high from the bench between two poles) tend to support themselves with their hind limbs, preventing them from falling and aiding in progression along the rod. A foam pad placed at base served as protection for the falling pups. The cut-off time was 240 s. (Altman & Sudarshan, 1975; Hermans et al., 1992). Datas will be collected from PND3 until 21 on every alternate days.

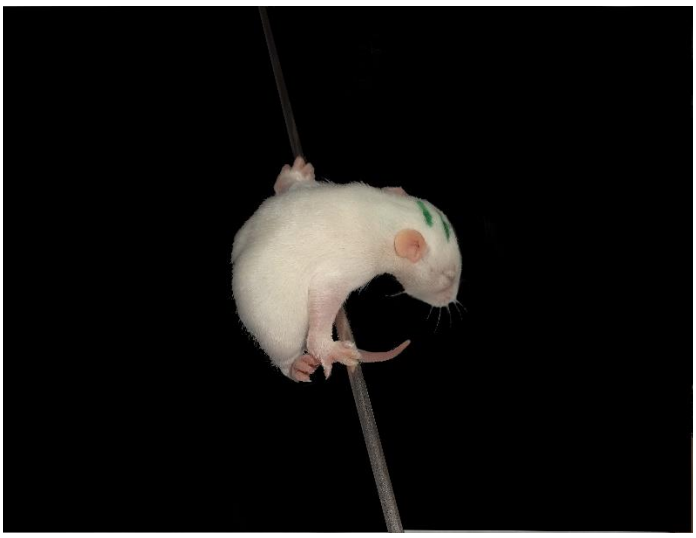


Figure 9. Wire hanging manoeuvre showing latency and also capability of bringing the rear paws to a metal wire

4.3.5 Locomotor activity

This test measures the activity and habituation response of animals on placement in a novel environment. Locomotor activity was measured by using the Video Tracking System SMART-2000 (San Diego Instruments, Inc., San Diego, CA, USA). Pups were placed in the activity chambers in a quiet room with dimmed light. The total distance travelled by the animal was recorded during a 5-min testing period. (Hermans et al., 1992; Tien et al., 2003). Data will be collected from PND3 until 21 on every alternate days.

Figure 10. Locomotor activity showing distance covered by animal while walking on open tunnel.

4.3.6 Tissue processing

Rats in the adolescent period (at postnatal day 44) were deeply anesthetized with a lethal dose of ketamine and xylazine (100 and 7 mg/kg, respectively by intraperitoneal injection and perfused transcardially with 0.9% saline followed by 4% formaldehyde in 0.1M PBS, pH 7.4, at 4 °C. Their brains were dissected and subjected to overnight post-fixation in the latter fixative solution, followed by immersion in a cryoprotective solution composed of 0.1M PBS and 20% sucrose for 48 h. The brains were then frozen in a solution of water-soluble glycols and resin (Tissue-Tek® O.C.T. ™ Compound, Sakura, Chuo-Ku, Tokyo, Japan) and stored at −80 °C until sectioning in a freezing sliding microtome (SM 2000R, Leica Bio systems, Wetzlar, Germany), in serial sections of 40μm thick.

Statistical analysis

Data were assessed for normality and homogeneity of variance to determine whether to use parametric or non-parametric statistical tests. All statistical analyses were performed using STATISTICA software version 7 (Tulsa, Oklahoma: Stat Soft Inc.). For statistical analysis, data related to the somatic growth were analyzed by two-way repeated-measures ANOVA, considering group and sex as between-subjects factors, Bon-Ferroni post hoc test was used. Somatic and motor development were analyzed by factorial ANOVA considering group and sex as between-subjects factors (Bon-Ferroni post-hoc). For the graph representation we used GraphPad Prism software

4.3.7. Results

The analysis revealed that probably a delay on somatic and motor development events, are reflected in anoxic animals performance when compared to control.

A) Body weight

Although pups with the same average birth weight were used in P2 (6-8 g), body weights were significantly lower than in control animals starting from just one day after the anoxic insult, but gradually anoxia male started gaining more than other groups. Going through datas it shows AM started weighing significantly more from PND 11 onwards until end of suckling period, and at same period females were gaining less than rest of the group. In control, difference between male and female is not as high as in anoxia, where males are weighing higher than females in both groups. Significant difference were spotted between gender in control at PND 9. Significant difference between CF and AF was seen from PND 13, and 21. At PND21 significant difference was seen also between CM and AM.

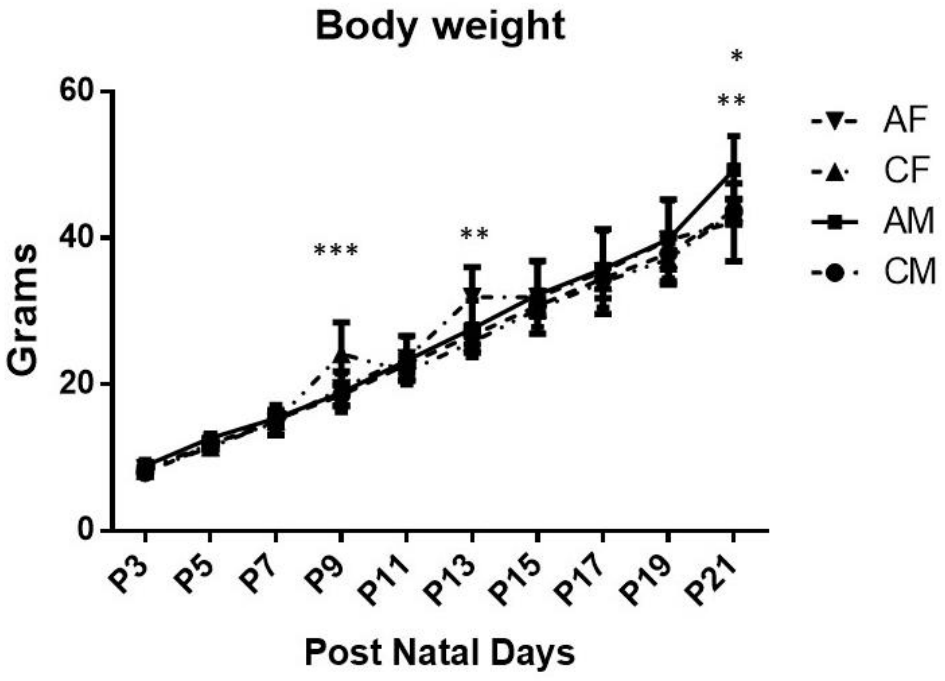


Figure 11. Neonatal anoxia has induced retarded wright gain in females
Evaluated from P3 to 21, significant difference was detected between stimuli (anoxia vs controls) and sexes (males vs females). CM#CF at PND 9, CF#AF at PND 13,21 &CM#AM at PND21. Data presented in mean ± SD. Two-way ANOVA test, Bonferroni post-test. $p < 0.05$. $n = 16$ in each group.

B) **Surface righting reflex (Neurological reflex)**

Righting reflex was performed in a significantly longer time by anoxic animals throughout the observation period. Recovery from this impairement could be seen from PND 15, there was less difference between group. Statistical difference in was seen in male between both groups at PND 3, 5(P<0.001), and there was no gender difference in anoxic animals. In control there was significant difference at PND 5(p<0.001) .

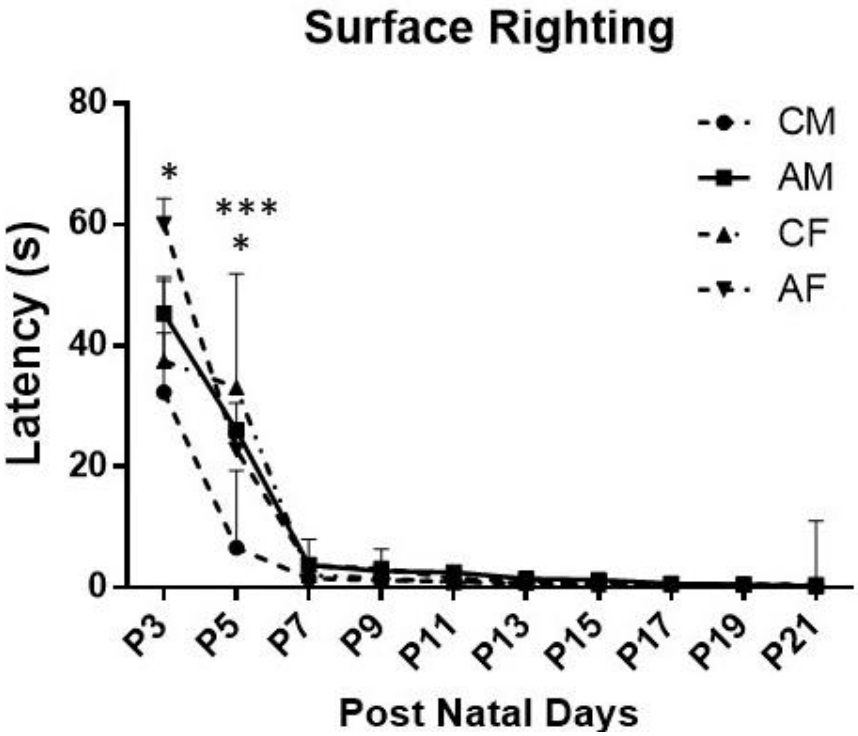


Figure 12. Neonatal anoxia has elongated mean latency time in righting reflex
Evaluated from P3 to 21, significant difference was detected between stimuli (anoxia vs controls) and sexes (males vs females). CM#AM at PND 3,5; CM#CF at PND 5 Data presented in mean ± SD. Two-way ANOVA test, Bonferroni post-test. p <0.05. n = 14 in each group.

C) **Wire Hanging maneuver**-

The wire hanging ability of the rat increased with age. Here we did not found any statistical difference in group factor P>0.05 but we could find statistical difference in time factor p<0.0001 as expected because development milestone will have an affect on their performance and also we found statistical difference between the interaction of the factors time and group. Significant difference with males at PND9 (p>0.01), PND17 (p>0.001) in both groups was seen. In females,

we found statistical difference only at PND17 ($p<0.001$). In between anoxia group there was no statistical difference between sexes, while in control we saw statistical difference at PND 17 ($p<0.05$), males showed less latency for this particularability.

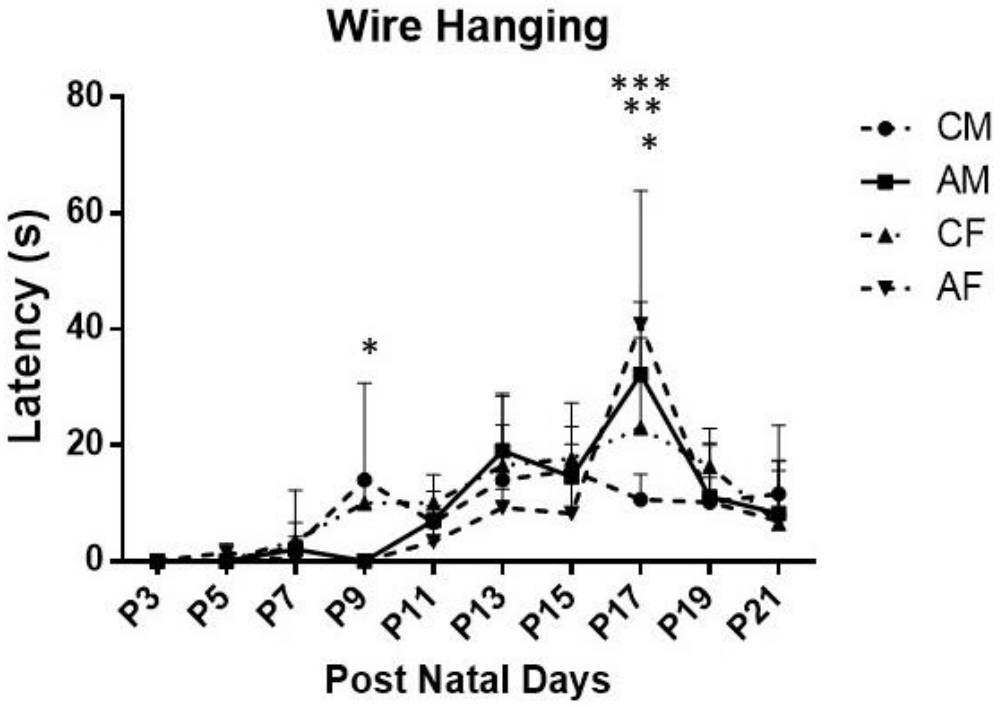


Figure 13. Neonatal anoxia has induced reduction of mean latency time in wire hanging maneuver. Evaluated from P3 to 21, significant difference was detected between stimuli (anoxia vs controls) and sexes (males vs females. CM#AM at PND 9, 17; CF#AF at PND17; CM#CF at PND 17. Data presented in mean ± SD. Two-way ANOVA test, Bonferroni post-test. $p < 0.05$. n = 16 in each group.

D) Grid walking- foot faults-

In the grid-walking test, anoxic animals had significantly less total number of steps at 2 and 3 weeks of age. In the same test, the number of foot. However, while normal pups made the same number of faults with both forelimbs, anoxic animals had a higher number of mistakes with the forelimb than with the hindlimbs.

Statistical difference was seen in male in both groups only at PND 19 ($p<0.001$). For the females, it was seen only at PND 21 ($P<0.001$). Anoxia has shown poor performance compared to control. While going through raw data we see anoxia male doing worst performance from PND 9 until PND 21 when compared to Control,

contrarily anoxia female has not shown this discrepancy. When comparing Anoxia, we found no statistical difference in between genders (P<0.05). In control we found statistical difference between genders at PND 19 (P<0.05) and PND 21 (p<0.01)

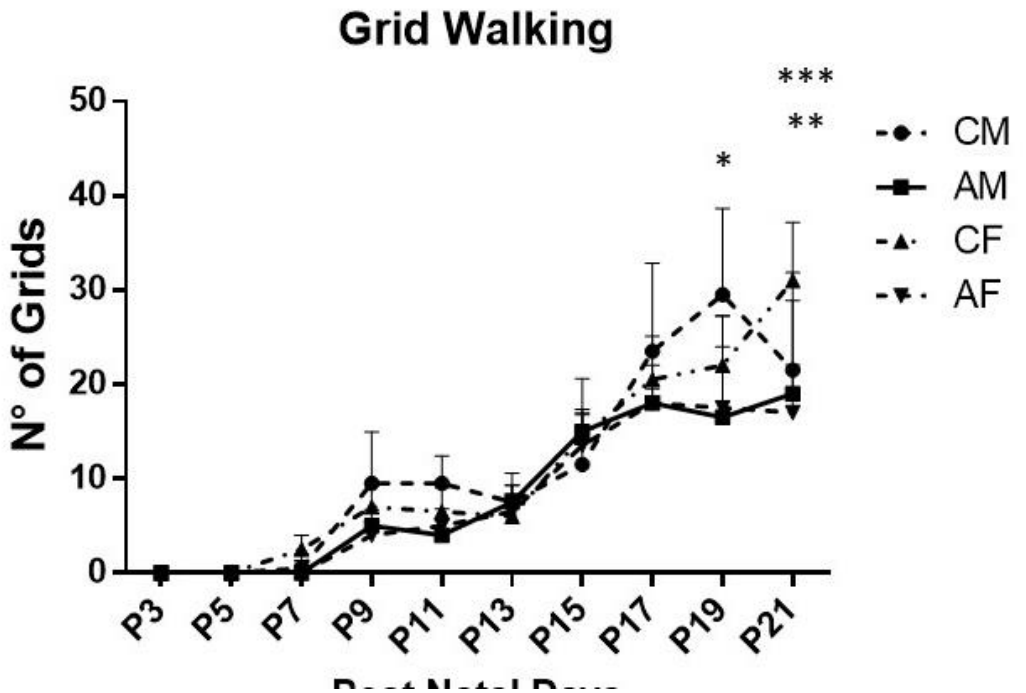


Figure 14. Neonatal anoxia has reduced total number of steps on grids. Evaluated from P3 to 21, significant difference was detected between stimuli (anoxia vs controls) and sexes (males vs females. CM#AM at PND 19; CF#AF at PND 21; CM#CF at PND 19Data presented in mean ± SD. Two-way ANOVA test, Bonferroni post-test. p <0.05. n = 16 in each group.

For this parameter we have found statistical difference in (Time, Group, Interaction), most precisely after using bonnferroni test we could see difference in male in both groups at P9 (p<0.001), P11 (p<0.05), P17 (P<0.001). In females we found difference at P9, and P15 (p<0.05). In anoxia and control group, we didn´t found any statistical difference (p<0.05) between genders.

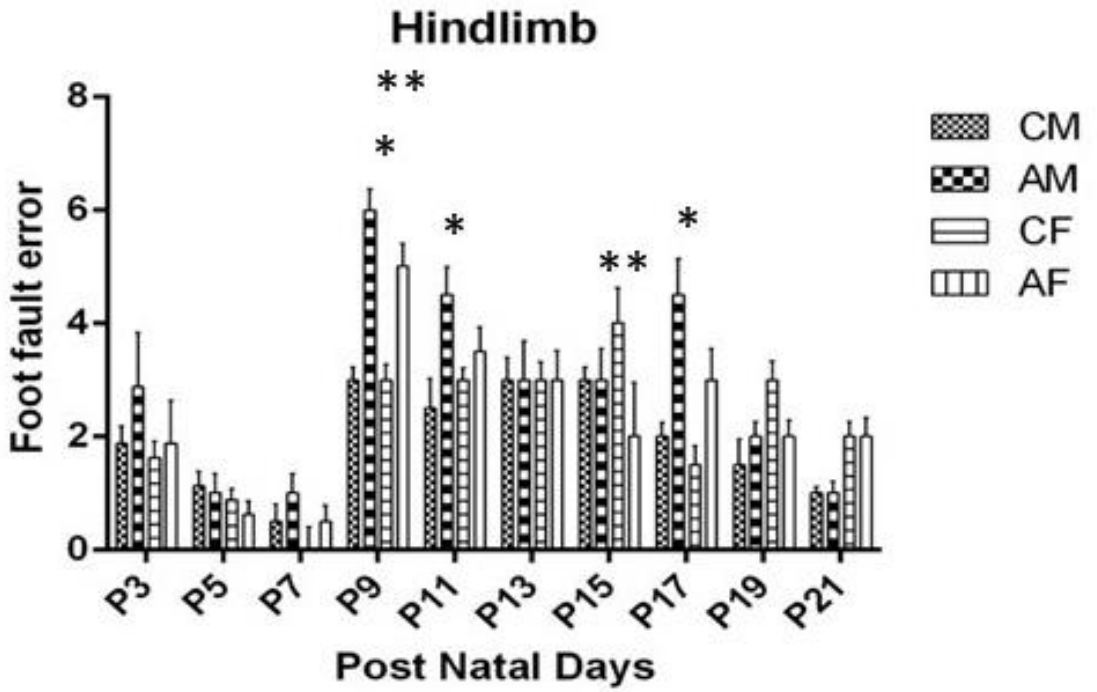


Figure 15. Neonatal anoxia has increased hindlimb errors. Evaluated from P3 to 21, significant difference was detected between stimuli (anoxia vs controls) and sexes (males vs femalesData presented in mean ± SD. CM#AM at PND 9, 11, 17, CF#AF at PND 9,15 Two-way ANOVA test, Bonferroni post-test. $p < 0.05$. $n = 16$ in each group.

Forelimb error

For this parameter we have found statistical difference in (Time, Group, Interaction), most precisely after using bonnferroni test we saw statistical difference between AM#CM at P9, P11 ($p<0.001$), AF#CF at PND 9 and PND 17 ($p<0.001$), AM#AF at PND 9 and PND 17 ($p<0.001$), CM#CF at PND 11 ($p<0.05$)

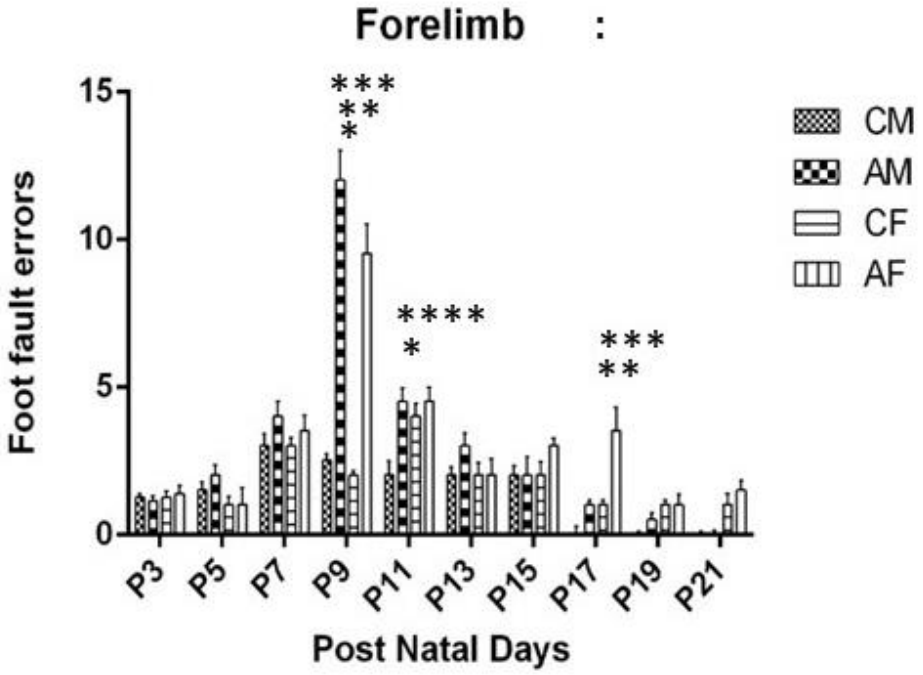


Figure 16. Neonatal anoxia has influenced forelimb error. Evaluated from P3 to 21, significant difference was detected between stimuli (anoxia vs controls) and sexes (males vs females). Data presented in mean ± SD. Statistical difference between AM#CM at P9, P11 ($p<0.001$), AF#CF at p9 and P17 ($p<0.001$), AM#AF at p9 and P17 ($p<0.001$), CM#CF at P11 ($p<0.05$)Two-way ANOVA test, Bonferroni post-test. $p <0.05$. n = 16 in each group.

E) Locomotion

Locomotor activity as measured as distance covered by individual rat in fixed time span was increased from PND7, but got a kick from PND 13 in all groups. Am was being hyperactive from PND15 till 21 as compared to control. There was significant difference between gender in Anoxia at PND 17, 21 and in same time frame had significant difference between male in both the groups.

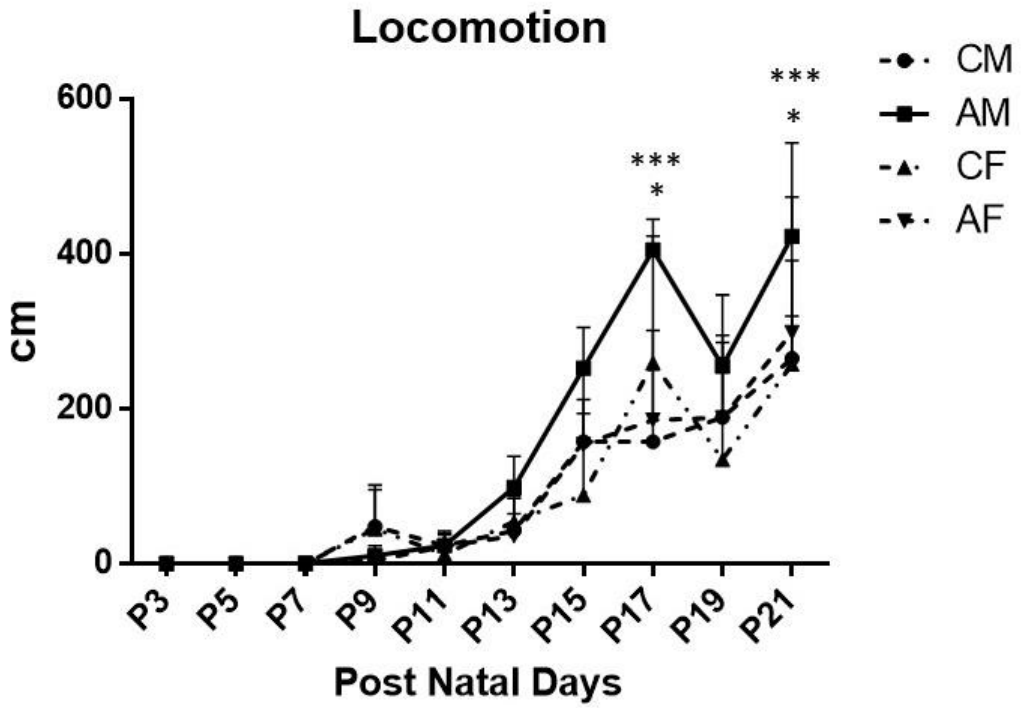


Figure 17. Neonatal anoxia has increased locomotor activity Evaluated from P3 to 21, significant difference was detected between stimuli (anoxia vs controls) and sexes (males vs femalesData presented in mean ± SD. Statistical difference between CM#AM at PND 17, 21; AM#AF at PND 17,21 ($p<0.05$)Two-way ANOVA test, Bonferroni post-test. $p < 0.05$. n = 16 in each group.

F) Histological evaluation by Nissl staining

i) Substantia Nigra Compact Dorsal

Histological evaluation along with stereology, quantitative analysis, reveals that Neonatal anoxia has reduced the number of cells in both sexes with similar reduction in both, male and female Figure 7A. Nissl stained coronal sections illustrates the impact of anoxia in rats. Since anoxia group present less amount of cells in SNCD figure 7B. Stereological estimates of Substantia nigra compact dorsal the number of Nissl stained neurons in the at PND 44 of male (M) and female (F) rats exposed to either neonatal anoxia (A) or control (C) treatment. Results were analyzed using two-way ANOVA, with Treatments and Sex as between-subjects factors. Tukey-Kramer post hoc test was used. * represents difference between groups cm#am,cf#af ($p < 0.05$).

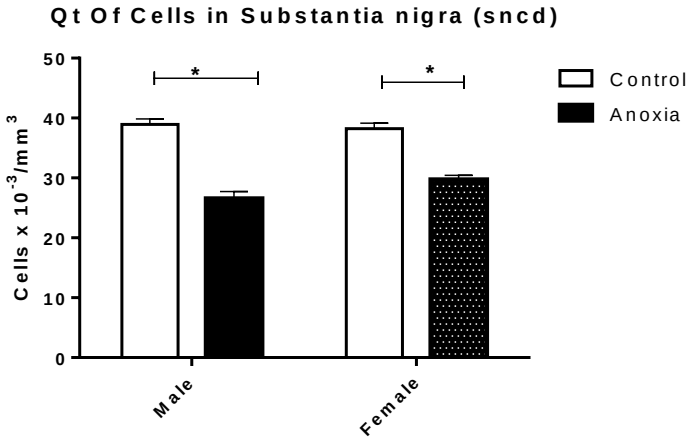


Figure 18. Quantitive analysis reveals that neonatal anoxia has induced brain injury as seen in nissl stained coronal sections with cell damage at SNCD area in P44 rat brains. Two-way ANOVA, post hoc test p< 0,05; n= 5 CM# AM, was significant but between female or sex no difference was seen.

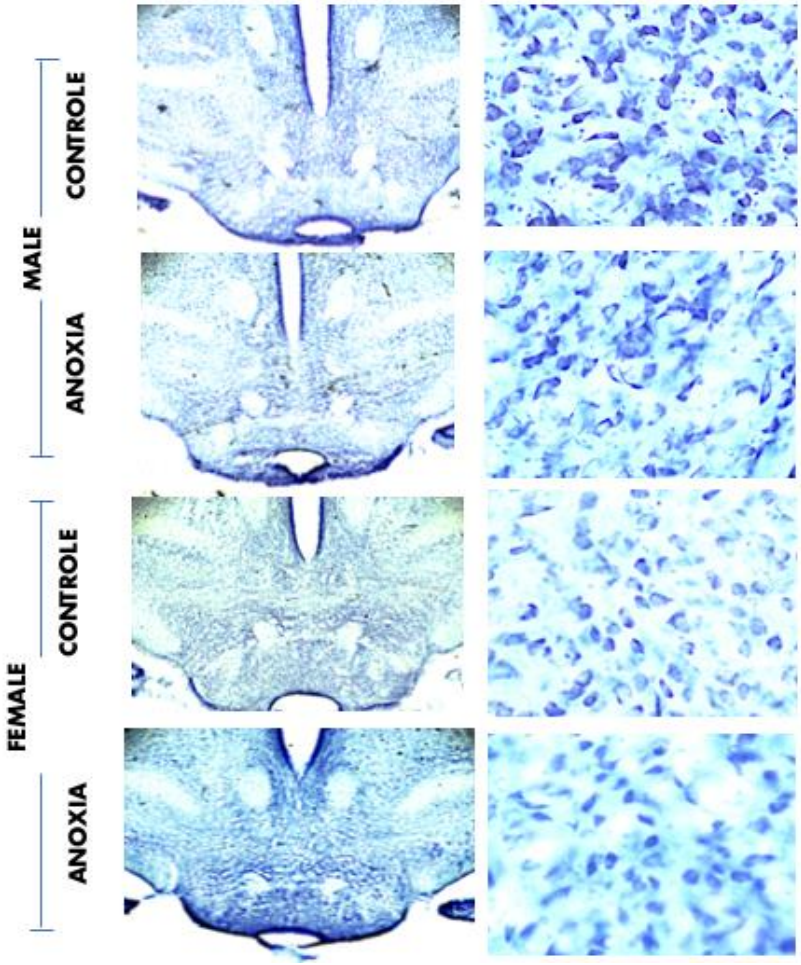


Figure 19. Representative photomicrographs of Nissl Stained coronal sections of SNCD, shows the impact of anoxia on cell number in SNCD male and female. Bregmas from -4.80 to -5.8

ii) Corpus callosum– thickness

Histological evaluation of the thickness of corpus callosum, along with Image J counting in Nissl stained sections, revealed that neonatal anoxia has reduced the thickness of corpus callosum Figure 7A. Results were analyzed using two-way ANOVA, with Treatments and Sex as between-subjects factors. Tukey-Kramer post hoc test was used. * represents difference between cm and am ($p < 0.05$),.Nissl stained coronal sections illustrates the impact of anoxia in male rats since this group presented figure 8A Illustrates the hippocampus at bregma: …

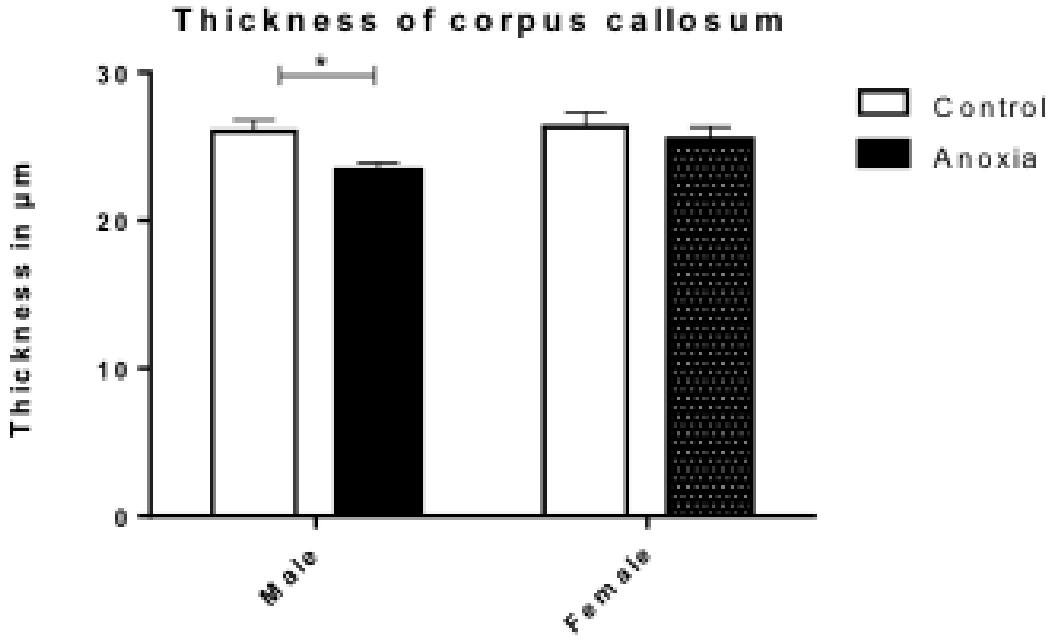


Figure 20. Image J data reveals that neonatal anoxia has induced brain injury as seen in nissl stained coronal sections with cthinning of corpus callosum (cc) area in P44 rat brains. Two-way ANOVA, post hoc test $p< 0.05$; n= 5 CM# AM, was significant but between female or sex no difference was seen.

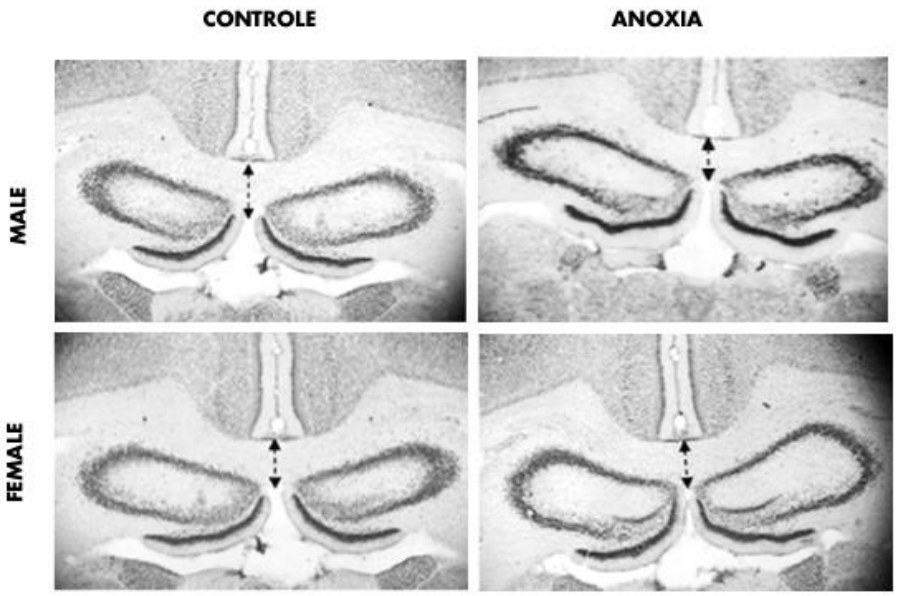


Figure 21. Histological evaluation reveals that neonatal anoxia has induced brain injury as seen in nissl stained coronal sections with thining of corpus callosum of anoxia male (cc) area in P44 rat brains.

Discussion

In the present study we showed that oxygen deprivation at 30 hour of age disturbs neurobehavioral development as shown by delayed performance of neurological reflexes, and retarded development of motor coordination and also in the body growth parameters. Anoxic animals were more active at 2 and 6 weeks of age.

The rat model of neonatal oxygen deprivation has always shown an extensive cerebral atrophy, but in contrast to human neonates, rats do not show gross functional deficits: they move like normal animals and do not display obvious postural and locomotor abnormalities (Jansen et al., 1996). However, there are several functional tests that show short- and long-term deficits after exposure to early hours of oxygen deprivation. Animals with severe cortical damages can show recovery in some deficits (Barth et al., 1980; Felt et al., 2002), while others can be detected only in adult life. Several studies have reported that oxygen deprived animals display long-term deficits in motor coordination tasks such as rotarod test. Impaired performance has been

described also in a staircase test, which measures skilled forelimb use (Tomimatsu et al., 2002).

In accordance with other reports, we observed that animals subjected to anoxia have retarded neurological reflex as shown by the significantly delay in latency to perform righting reflex. Unlike current study, other study have shown weight loss, resulting in higher mortality as it has been observed by Wagner et al. 2012, who reported that weight loss was so severe that pups needed artificial feeding other than mums feeding to grow. Our previous results (Kumar et al., 2017) show that anoxic animals perform worst in negative geotaxis, and currently animals are delayed in latency of righting reflexes as measured by the reflex times due to sensory motor delay in neurological reflexes. At later stages, differences between oxygen deprived and normal rats disappeared, which indicates that anoxic pups showed a considerable degree of recovery.

Several tests for evaluating motor coordination following neonatal oxygen deprivation have been described. However, there are contradictions between results obtained by different investigators. According to our observations, one of the most reliable tests was the forelimb and hindlimb footfault test, since anoxic animals made more mistakes throughout the whole observation period. The impaired use of the contralateral forelimb assessed by staircase test has also been reported (Tomimatsu et al., 2002). The finding that the forelimbs are more affected than the hindlimbs might be explained by the size of the injury in the area of cortical representation for the hindlimbs. This is in accordance with results reported for adult rats after unilateral middle cerebral artery occlusion, where the behavioral signs were less damaged in the hindlimbs (Reglodi et al., 2002).

. Wire hanging test insensitive to show differences between normal and anoxic rats. In adult rats subjected to neonatal oxygen deprivation they show no difference with control (Aronowski et al., 1996), (Borlongan et al., 1995), (Markgraf et al., 1992), (Reglodi et al ., 2003), (Rogers et al., 1997), (Van der Staay et al., 1996), our study shows that in spite of brain damage, these tests are less sensitive in neonatal rats.

The present rsults, thus confirm those of Balduini et al.2000 who examined motor coordination (wire hanging tests) and found that they show no difference between control and anoxic rats. While adult rats being subjected to neonatal oxygen deprivation show a certain degree of recovery in sensorimotor deficits (Reglodi et al., 2003), neonatal rats recover significantly better. This may be explained by the higher level of plasticity of the neonatal brain (Balduini et al., 2000), which can involve compensatory changes also in the contralateral hemisphere. Several reports document the plasticity of neonatal brain that attenuate the functional deficits after different types of cerebral injuries (Barth et al., 1990), (Huttenlocher et al ., 1989), (Kartje-Tillotson et al., 1985), (Reinoso et al., 1989).

Regarding locomotor activity, both hyper- and hypoactivity have been described following neonatal oxygen deprivation (Antier et al., 1998), (Balduini et al., 2000). In rats exposed to anoxia, postweaning transitory hyperactivity with increased sniffing, rearing and ambulation has been described (Iuvone et al., 1996). Balduini et al.(2000; 2001) found hyperactivity at 3 weeks of age, but no differences in spontaneous or in open-field activity at 90 days. In contrast, Antier at al. 1998 found hypoactivity in adult rats who underwent neonatal hypoxia/ischemia.

The present data observations reveal that rats subjected to anoxia are hyperactive at 2 weeks of age, when normal pups hardly move at all. This may also explain why anoxic animals spent more time at the walls at 2 weeks of age: a natural reflex of the pups is to quickly find the wall where they feel more safe. Since normal pups hardly move at all, several of them stayed in the center where they were originally placed and did not move to the wall at all, while anoxic animals moved more and found the wall more quickly. Since the difference in this thigmostactic behavior disappeared at later stages, it was probably due to higher activity level in anoxic pups rather than increased anxiety at 2 weeks, although this latter possibility cannot be excluded either. There is a significant increase in locomotion at 3 weeks of age also in normal pups (Reglodi et al., 2003), which may explain why no significant differences were found between control and hypoxic animals at this age. However, while normal rats did not show further increase in any activity sign, anoxic animals were more active at 6 weeks of age as shown by the number of rearings, the distance travelled, ambulation time and their speed when handling for cognitive test.

Although the immature nervous system is capable of considerable compensatory reorganization following injury, most studies have described severe cerebral atrophy after oxygen deprivation (Andine et al., 1990), (Jansen et al., 1996; 1997,Tuor et al., 1995). Grafe et al. (1994) have observed neuronal injury ranging from focal neuronal death to massive infarction.

Prematurity also affects cerebellar development even in the absence of detectable brain injury (Limperopoulos, 2005b). Neonates suffering from perinatal HI exhibit neurological disorders, learning disabilities, hyperactivity, visual impairments and other limitations that compromise their life quality (Aarnoudse-Moens et al., 2009; Volpe, 2009a). Preterm babies suffering unilateral cerebral injuries indicate cerebellar damage in the contralateral hemisphere (Limperopoulos, 2005a). In rat model following neonatal HI at PND7, mild to marked locomotor abnormalities were reported, including shorter intervals for falling from rotarod, impairments in beam walking (McQuillen and Ferriero, 2004) and delayed motor abilities (Lubics et al., 2005). Less investigation has been done for HI at PND7 (Ikeda et al., 2001; McQuillen and Ferriero, 2004; Lubics et al., 2005; Lu et al., 2014), studies using HI at PND3 have shown delays in neurological reflex maturation that lead to motor deficits weeks after the insult near adulthood (Misumi et al., 2016; Durán-Carabali et al., 2017; Sanches et al., 2017).

In the present study, after the stimulus , anoxia male showed behavior hyperactivity as seen in locomotion and accompanied by very mild motor dysfunction at developing period. The deficits in motor coordination and locomotion tasks associated with cortical damage have been reported shortly after neonatal HI (Ten et al., 2003), e.g., a disorganization of oligodendrocyte development in the sensorimotorcortex(Misumietal.,2016). In this study we see impact of the insult on substantia nigra compact dorsal and thinning of corpus callosum and it has been shown in behavior test (Kumar et al., 2019).

These kind of hyperactivity can be contributed to cellular abnormalities and leading to mild motor dysfunction observed in HI rats at adulthood. This can be due to

disturbance in neuron-glial interaction due to myelin loss that was evidenced by altered membrane phospholipids and confirmed by reduced myelination,; and an imbalance in glutamatergic neurotransmission with increases in both glutamine and glutamate. In this study we saw thining of cc which can be contributed by demyelination and males are more vulnerable leading for higher percentage in motor disability.

In our behavior study we see anoxia males having higher velocity but couldnot complete the memory task (Kumar et al., 2019) at adolescence which can be due to myelin loss. Furthermore, our results indicate that the neuronal loss and hypomyelination due to the insult has affected male rat mostly.

In the current study hyperactivity (in locomotion test) is represented by anoxia and male rats are vulnerable. This is a sign of attention deficit hyperactivity which is a clinical sign (Biederman et al., 2005) and the locomotor disorder can be due to damage to dopaminergic cells in substantia nigra (Fig.9) as seen by Bakos et al., 2004. In our histological evaluation of nissl stained sections anoxia shows more damage of cells. The reduction in anxiety by anoxia (Kumar et al., 2019) can be due to thinning of corpus callosum. In our qualitative analysis of cortex we see thinning of corpus callosum in anoxia as shown in Fig.10. Our lab is doing further investigation by checking demyelination in this particular region. This similarity in pattern of damage was also seen by Del Bigio et al., 2003 in brain injured rat model of hydrocephalus.

4.9 Conclusion

The results of this study show that the appearance of neurologic reflexes and motor performances are strongly affected by neonatal anxic insult in rats. At early stages, most motor coordination tests also serve as a useful tool to show differences between normal and anoxic pups, however, anoxic animals show excellent recovery in most of these tests by later ages, except for the footfault test. We see that anoxic can perform all test but they make lot of errors, high number of foot fault error was seen in them. Anoxia female showed impaired somatic growth as her weight gain was less than the rest of the groups. Anoxia male was seen hyperactive in locomotion compared to female and control males. Anoxia male started weighing more than rest of the groups from PND 11 and AF was significantly low than control female from PND,

In early days anoxic pups were delayed in surface righting reflex but later there was no difference between groups that can show plasticity of rat brain. Anoxia pups had less hanging ability when compared to control and no gender difference was seen in anoxia or control. Anoxia has shown retarded performance in grid walking, and had more hindlimb error and forelimb error. In locomotion anoxia male were covering longer distances after PND 15, but females did not show same pattern.

Sex differences in reference memory, anxiety like behavior and neuronal morpholgy after neonatal anoxia.

Abstract

Neonatal anoxia produces long term brain injury which not only persists, but may also be linked with neurobehavioural deficits in adolescents. The model of neonatal anoxia proposed in this research is based on exposure of 100% nitrogen when pups are 30 hours old (6-8g). Previous work of this lab showed that this exposure to anoxia specifically modifies the behavior of male and female rats, which showed impairment in spatial memory and alterations in anxiety-like behavior. These results provides critical information about brain response to injury during adolescence as related to cognitive impairments manifested clinically in humans prematurely born. The aim of the present study is to evaluate how neonatal anoxia affects sex differences in reference memory, anxiety like behavior and neuronal morpholgy. In morris water maze tests, latency time and path length were longer in the anoxia than in controls animals. These parameters also were prolonged in anoxic males compared with females ($p < 0.05$). In elevated plus maze, anoxic female spent more time in the open arm than male indicating less anxiety. The histological analysis of the different hippocampal fields demonstrated a significant difference between anoxic and control male rats in CA1 and dentate Gyrus subfields and also between male and female anoxic rats. In conclusion, our data revealed that neonatal anoxia have affected reference memory and anxiety, male rats are more vulnerable. Anoxic males indicates severe memory impairment and more histological damage than female rats. However, neonatal anoxia promoted more decreased anxiety-like behavior in adolescent female rats than in males.

Keywords: oxygen deprivation, hippocampus, anxiety, spatial memory, adolescence.

5.1 Introduction

The brain goes through complex changes in late childhood and adolescence and sex difference is evident that biological factors like hormones such as testosterone and estrogen have a play. The presence of sexual dimorphism in the hippocampal formation has long been recognized. Differences between male and female rats have been detected with respect to the number of dentate granule cells and branching patterns of dentate granule and hippocampal pyramidal cell dendrites. The volume of

the mossy fiber system was found to be smaller in females. Because the number of dentate granule cells is smaller in females than in males, the increased numerical density of synapses may be thought of as a compensatory mechanism to equalize the number of synaptic contacts between dentate granule and CA3 pyramidal cells in both sexes (Madeira et al., 1991).

Sexual differences were observed in the dendritic trees of both dentate granule and hippocampal CA3 pyramidal cells (Juraska et al. 1985, 1989), in the ability of axon sprouting of hippocampal afferents (Morse et al. 1986) and, finally, in the number of dentate granule cells which in rodents are more numerous in males than in females (Wimer and Wimer 1985; Madeira et al. 1988b, 1990). Conversely, sex-related numerical differences were not demonstrated in CA3 pyramidal cells (Madeira et al. 1990).

Hippocampus

The entorhinal cortex send multimodal sensory and spatial to the hippocampus. The pyramidal neurons in the CA1 region, which project back to the entorhinal cortex and to the subiculum, another medial temporal lobe structure are the major output of the Hippocampus. The profound memory loss exhibited by patients with lesions has marked the critical importance of CA1 neurons in learning and memory is seen in the. Information from the entorhinal cortex reaches CA1 neurons along two excitatory pathways, one direct pathway and one indirect. Together these inputs are termed the perforant pathways. The direct pathway has its origins in neurons of layer III of the entorhinal cortex. The axons of these neurons form synapses on the very distal apical dendrites of CA1 neurons (such perforant projections are also called the temporoammonic pathway). In the indirect pathway information from neurons of layer II of the entorhinal cortex reaches CA1 neurons through the trisynaptic pathway. In the initial leg of this pathway the axons of layer II neurons project through the perforant pathway to the granule cells of the dentate gyrus (an area considered part of the hippocampus). The granule cell axons project in the mossy fiber pathway to excite the pyramidal cells in the CA3 region of the hippocampus. Finally, the CA3 axons project through the Schaffer collateral pathway to make excitatory synapses on more proximal regions of CA1 pyramidal cell dendrites (Figure 21). The fact that CA1

pyramidal neurons receive cortical information through two pathways has led to the view that CA1 neurons compare information in the indirect circuit with sensory input from the direct pathway. Lesion studies indicate that both direct and indirect inputs to CA1 may be necessary for normal learning and memory. Lesions of the indirect Schaffer collateral pathway limit the ability of mice to perform a complex spatial learning and memory task, although some form of spatial learning remains intact. Lesions of the direct pathway to CA1 do not appear to alter initial formation of memory, but inhibit the ability of an animal to store those initial memories as long-term memory, a process termed consolidation. Genetic inactivation of the direct path also interferes with episodic memory, in which an animal must learn about the temporal relation between two or more events. (Kandel book 4th edition 2005)

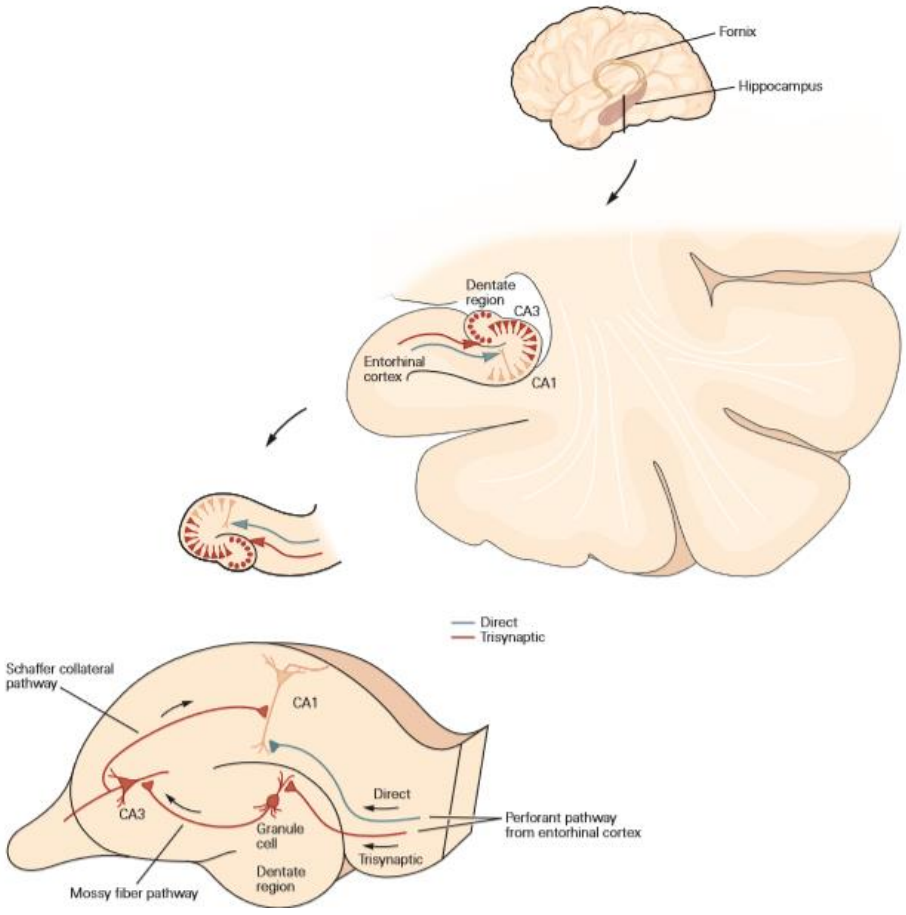


Figure 22. Direct pathway for memory. Information arrives in the hippocampus from entorhinal cortex through the perforant pathways, which provide both direct and indirect input to CA1 pyramidal neurons, the major output neurons of the hippocampus. (Arrows denote the direction of impulse flow.) In the indirect trisynaptic pathway neurons in layer II of entorhinal cortex send their axons through the perforant path to make excitatory synapses onto the granule cells of the dentate gyrus. The granule cells project through the mossy fiber pathway and make excitatory synapses with the pyramidal cells in area CA3 of the hippocampus. The CA3 cells excite the pyramidal cells in CA1 by means of the Schaffer collateral pathway. In the direct pathway neurons in layer III of entorhinal cortex project through the perforant path to make excitatory synapses on the distal dendrites of CA1 pyramidal neurons without intervening synapses (Kandel et al., 2005)

Hippocampal cell death, reduced hippocampal volume, decreased adult neurogenesis and spatial memory (Takada et al., 2011; 2014, 2015) have been reported. The stimulus applied in the critical period of the brain development promoted also complex damages; necrosis has been reported as the main type of cell death, immediately after the hypoxic or anoxic exposure followed by apoptosis (Northington et al., 2001, Fan et al., 2005). The cell death may lead to alterated somatic and sensorimotor development of the offspring which might lead to persistent consequences in adult life. (Takada et al, 2014, 2015; Chaves-Valdez et al., 2011, Vasconcelos et al., 2017, Kumar et al., 2017) as well as cognitive impairments in rodents which might persist in adult life (Takada et al., 2015; Galeano et al., 2014). However, few studies focused on the effects of neonatal hypoxia or anoxia in postnatal development and adolescent rodents considering gender differences (Adriani et al., 2006) including somatic and neurological development in relation to their controls, with gender differences (Kumar et al., 2017).

An issue of particular relevance is the sex-related vulnerability to brain injury in both human and rodents. Severity of brain injury following OD appear to be sex dependent, with the male neonates being more susceptible to the effects of anoxic insult, resulting in more severe neurological outcomes (motor and spatial memory) and poor recovery, as compared to females with similar brain injuries (Waddell et al., 2016; Huang et al., 2016; Roof & Hall., 2000; Seigel et al., 2010).

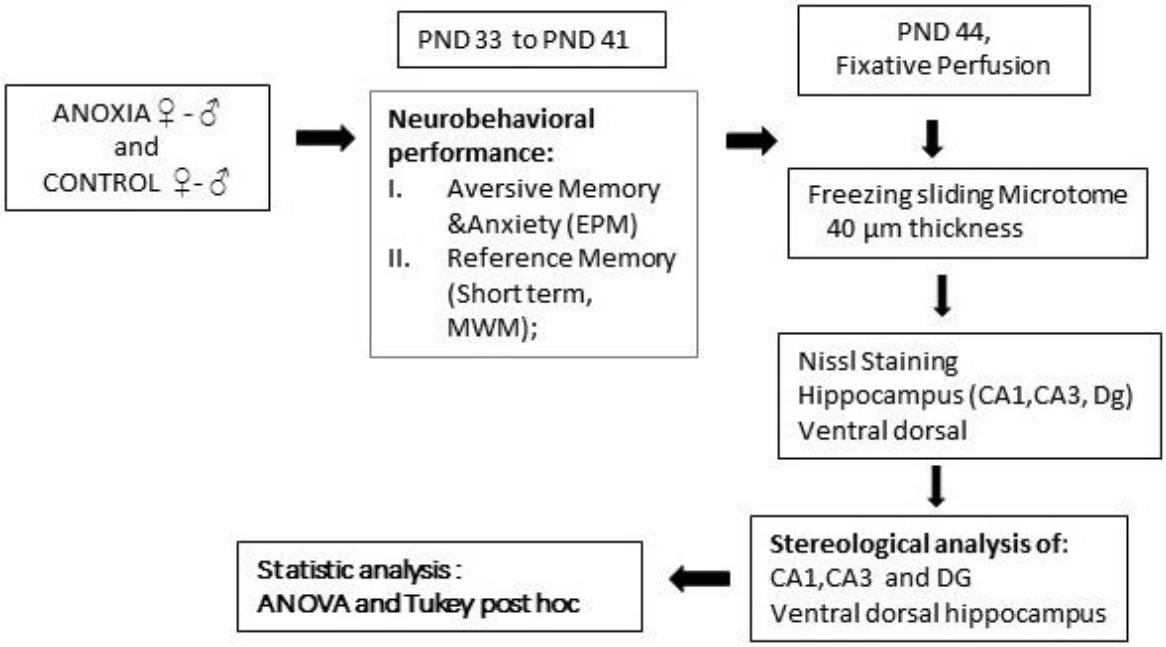


Figure 23. Experimental design for neurobehavioral test

Material and method

Has been described on page 22 with detailed experimental design. Animals go through Neurobehavioral performance as described above in the design.

5.2.3 Anxiety and reference memory tests

Thirty-three-day old rats submitted to neonatal anoxia were tested for anxiety in the elevated plus maze. The Morris water maze was used to assess spatial memory. For all tasks, the male rats were tested before females and apparatus were cleaned between each animal. Experiments were conducted during the light cycle.

A) Elevated plus-maze (EPM) test

The EPM test was performed on PND 33 (n = 14 in each group at each time). The EPM apparatus consisted of two open arms (50 x 10 x 1cm) and two enclosed arms (50 x 10 x 40 cm) originating from a common central platform (10 x 10 cm) to form a plus shape. The entire apparatus was elevated to a height of 50 cm above the floor. A video camera and illumination lamps were mounted on the ceiling. We adopted a test-retest paradigm as described in Takada et al., 2016, in which in the beginning of the test, the rat was placed on the central platform with its head facing the closed arm and they were allowed to freely explore the maze for 5 min. After an interval (5 min), they were exposed again to the same maze. The arm entry was defined as all four paws into an open or closed arm. The following parameters were estimated: the total time each animal spent in various sections of the maze (open arms, center, or closed arms) and the number of entries. The results are reported as time spent in the open arm and percentage of time spent in the open arm (OAT%, corresponding to the time spent in open arms divided by the sum of time spent in both closed and open arms).

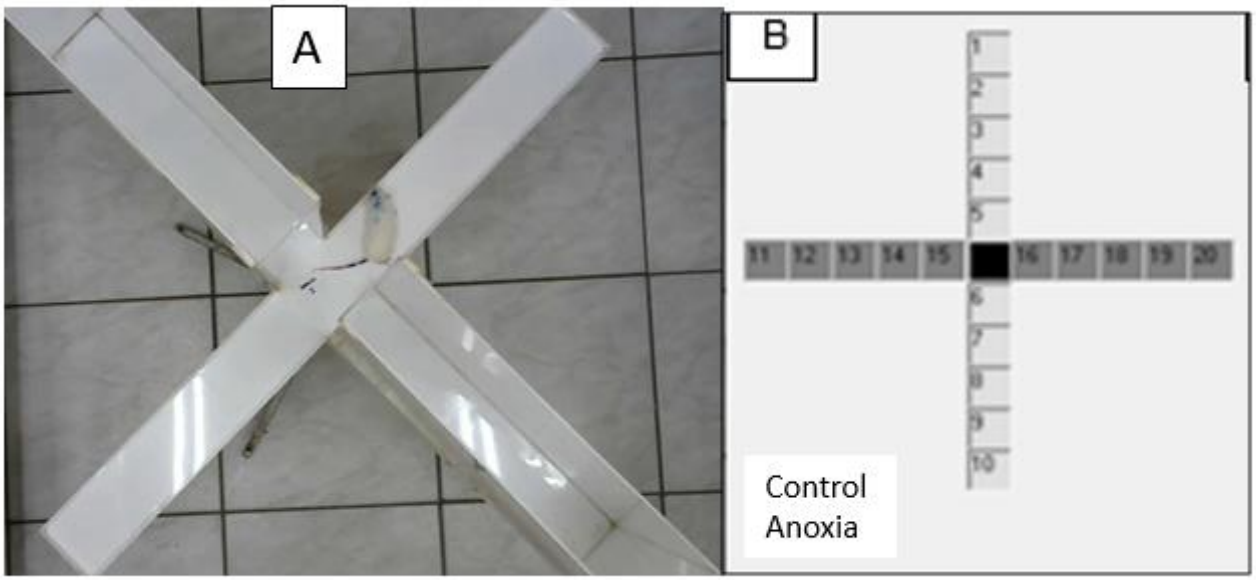


Figure 24. Elevated Plus maze apparatus A. Top View of EPM. B. Design of the LCE segmented in the X-Plo-Rat program 3.3. Source-Kumar 2017

B) Morris water maze apparatus and reference memory test

The Morris' water maze apparatus and the employed procedures were similar to those described by Motta-Teixeira (2016). The pool is a round, black fiberglass tank, 200 cm in diameter, 50 cm in height, filled to a depth of 25 cm with water (26 °C ± 1). A movable circular plastic platform 9 cm in diameter, mounted on a plastic column, is placed in the center of one of the quadrants of the pool, about 2 cm below the water surface. For descriptive data analyses, an area measuring 30 cm in diameter, concentric to the platform, named critical counter, was defined.

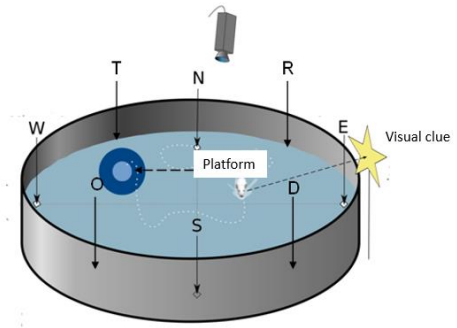


Figure 25. Representative scheme of the MWM, used for conducting the tests. The gray lines represent the virtual divisions of the quadrants. The circular area represented in dark blue, concentric to the platform area, represents the critical counter. The arrows with letters W, T, N, R, E, D, S, O indicate the insertion points of the animal in the water. Source-Kumar 2017

The frequency of entrances within the critical counter and the percentage of time spent in it relative to the time spent in the remaining pool areas provided specific indexes of spatial location. The water maze was located within a 3.13 × 3.5 m room with several salient cues hanging on the walls.

Each trial consisted of placing the rat near the border of the pool (in one of the variable starting locations) and allowing it to swim until the platform be located. If the rat did not find the platform within 120 seconds, the animal was then manually guided to the platform, where it remained for a 20 seconds period. The trials were recorded using a computerized video-tracking system (EthovisionPro, Noldus Information Technologies, Wageningen, Netherlands) which allowed to measure the latency, the path length and the time spent within and the frequency across the critical

counter. Animals will be tested along three trials per session, one session per day. The platform located in one quadrant called critical quadrant and animal will be introduced to the pool at different starting locations and the sequence will be randomly picked. Reference memory will be acquired by parameters: decreases of latency and path length, and by the percentage of time spent within the critical counter where the platform was located in the previous session.

5.2.4 Processing of biological material

The animals were deeply anesthetized with an intraperitoneal injection of acepromazine followed by ketamine and xylazine (7.5:1/kg) followed by transcardial perfusion with 0.9% saline solution and then by 4% paraformaldehyde in 0.1 M PBS, pH 7.4, at 4°C. The brains were dissected and overnight postfixed with this last fixative, followed by immersion in a cryoprotectant solution composed of 0.1 M PBS and 20% sucrose for 48 hours. The brains were then frozen in a solution of water-soluble glycols and resin (Tissue-Tek® O.C.T. ™ Compound, Sakura, Chuo-Ku, Tokyo, Japan) and stored at -80°C until sectioning in a cryostat (SM 2000, Leica Biosystems , Wetzlar, Germany) and then cut into sections of 40 µm-thick.

5.2.5 Histological procedures: Nissl staining

The sections were mounted on gelatinized slides and dried for 24 hours in an oven at 40°C, after which they were stained by the Nissl method with Cresyl Violet to facilitate the visualization of the Nissl corpuscles of the neuron bodies and thus enable counting of the cells in the hippocampal region. The staining process was consisted of immersing the sections first in two solutions of Xylol. They were then immersed in alcoholic solutions with decreasing concentrations (100%, 100%, 95%, 70%, 50%), then washed in distilled water for 2 minutes for rehydration. Subsequently, the sections were immersed in the Cresil Violet dye for 4 minutes and then in distilled water for 30 seconds for 50% alcohol and 70% for 30 seconds each, and if necessary, subjected to the alcohol differentiation process 70% with 1% of acetic acid for 5 seconds. After this step, the sections were dehydrated with 95% alcohol, 100% alcohol, and

diaphanized with xylol for 1 minute in each solution, mounted with permount or DPX and covered with coverslips.

5.5 Stereological evaluations

For stereological quantification of hippocampal cells (neurons and astrocytes) four sections were used per animal (n=5/group), and one section every 320 µm was analyzed, unilaterally. The hippocampal sections used corresponded to bregma -2.30 mm to –4.30 mm, (Atlas Paxinos& Watson, 1997). The sections stained with cresyl violet allowed the delimitation of structures under optical microscope coupled to a stereology system consisting of a motorized platinum (LudlElectronicProducts, Hawthorne, NY), capture camera (Nikon Instruments Inc., Melville, NY) and software StereoInvestigator (MBF Biosciences, Williston, VT). For the delimitation of the hippocampal structures, the 4x objective was used. The delimitation of the CA1, CA3, and DG regions was performed in order to standardize the regions at the different levels along the rostrocaudal axis as previously described by Takada (Takada et al., 2015a).

5.5.1 Statistical analysis

Data were assessed for normality and homogeneity of variance to determine whether to use parametric or non-parametric statistical tests. All statistical analyses were performed using STATISTICA software version 7 (Tulsa, Oklahoma: Stat Soft Inc.). For statistical analysis, scores of the water maze memory test and elevated plus maze were analyzed using a repeated-measures ANOVA using group as the between-subjects factor and sessions, sex and trials as within-subjects factors. Tukey-Kramer as post hoc test was used. The histological data were analyzed by two-way ANOVA, considering the groups as between-subject factor and the hippocampal subfields (CA1, CA3, DG) as within-subject factors region (using Tukey-Kramer as post-hoc). Differences were considered significant when $p < 0.05$.

5.6 Results

5.6.1 Anxiety test results by Elevated Plus Maze

The effects of neonatal anoxia on elevated plus maze with adolescent rats, P33, were evaluated by the time spend in the open and closed arms, using the concept of test-restests (sessions 1 and 2). The data (mean ± SEM) were analyzed by repeated measures ANOVA, using as subjects the groups and the arms (open and closed), post-hoc Tukey-Kramer test at $p < 0.05$. It was possible to observe significant differences between the first and second sessions, Test and Retest, of the control and anoxia groups in males and females and between genders. Evaluation of different parameters are shown in Figure 5.

a) Time spend in enclosed arms

The animals of the groups Test CM and Retest (respectively 167.09 ± 17.46 and 238.48 ± 15.153); Test and Retest AM (respectively 134.69 ± 12.21 and 183.05 ± 16.09) presented significant differences both in the sessions and between the sessions. The females group in the control and anoxia condition in the Test and Retest sessions did not present CF differences (respectively 181.91 ± 10.01 and 194.68 ± 18.97) FA (respectively 132.21 ± 14.93 and 143.66 ± 16.16), but there was a significant difference between control and anoxia. The test and retest sessions were significantly different regardless of gender and the stimulus at $p < 0.001$. These results indicate that the animals remained longer in the open arms in the Retest session in all groups. The anoxia decreased the length of stay in the open arms in both sexes in relation to the respective controls.

b) Time spend in open arms

Male animals presented significant differences between the control and anoxia groups in the CM test (87.05 ± 14.30) and Reteste CM (33.83 ± 12.06); AM test (120.40 ± 10.61) and Reteste AM (77.57 ± 13.19). In the group of females, the CF Test (74.41 ± 8.73), Reteste CF (67.77 ± 14.58) and AF Test (124.73 ± 13.47) Retest AF (119.13 ± 14.87) presented a significant difference, $p < 0.001$. These results indicate that the animals in the Retest sessions remained less time in the open brackets compared to the Test session in all groups. The anoxia increased the length of stay in the open

arms in both sexes in relation to the respective control in both the Test and Retest sessions.

c) Number of entries in the Central Quadrant.

The animals presented significant differences between the Test and Retest groups, independent of the group and gender p <0.004. CM test (45.34 ± 5.13) Retest CM (28.40 ± 4.38); AM Test (44.91 ± 3.90) Retest AM (39.37 ± 4.27) and CF Test (43.68 ± 3.34) Retest CF (37.54 ± 5.10); AF test (43.06 ± 5.5) Retest AF (37.20 ± 3.39) independent of group and gender (Plate IV - Figure C). The anoxia increased the number of entries in the central quadrant in both the Test and Retest sessions relative to the respective control. The females of the control group had a greater number of entries than the males.

d) Time spend in the central quadrant

The animals presented significant differences between the Test and Retest groups, independently of the experimental group (p = 0.041). CM test (39.78 ± 3.43) Retest CM (35.78 ± 4.5); AM test (55.851 ± 5.00) Retest AM (53.21 ± 9.31); CF test (59.57 ± 5.98) Retest CF (44.5 ± 7.08); AF test (61.71 ± 6.73) Retest AF (63.85 ± 8.32).

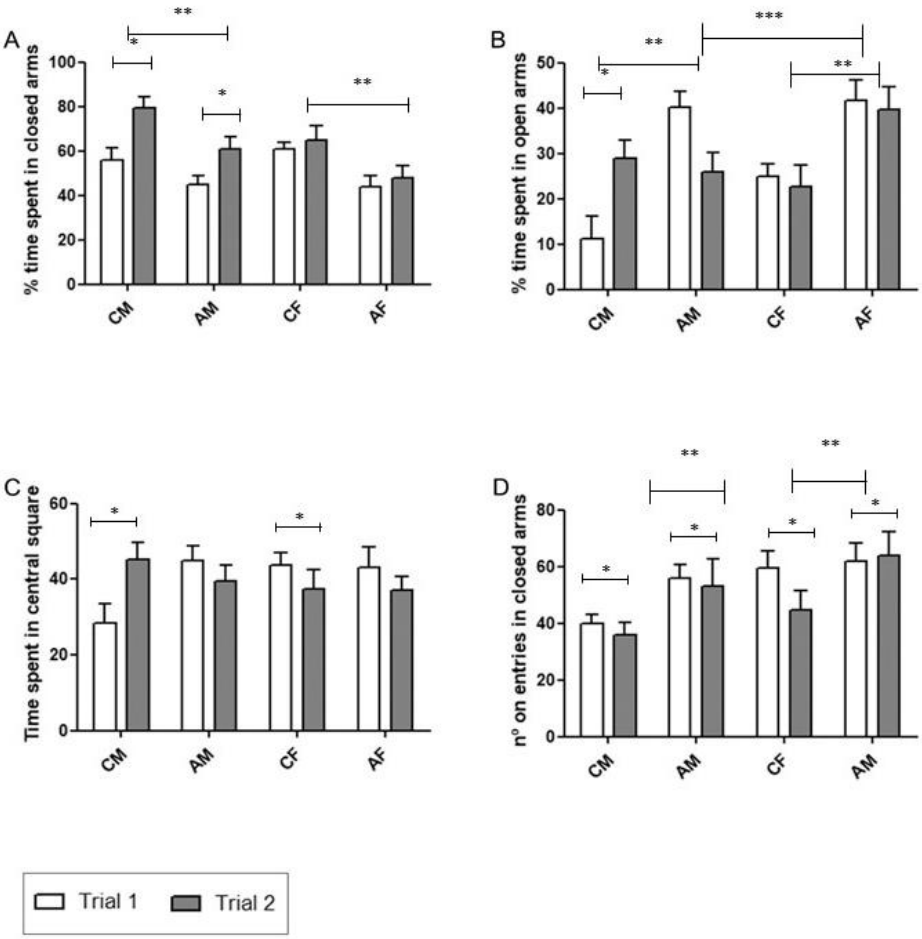


Figure 26. Elevated plus maze navigation at PND 33. (A) Time spent in enclosed arms. (B) Time spent in open arms. (C)Number of entries in central quadrant. (D)Time spend in central quadrant. Data are expressed as mean ± S.E.M. n=16 in each group. Results were analyzed by two-way repeated-measures ANOVA, considering group and sex as between subjects factors. Tukey-Kramer post hoc test was used ($p < 0.05$).

5.6.2 Reference memory test shown by morris water maze

The results below represent the performance evaluations in the MWMh with 3 evaluations during six days, which were summarized in each parameter indicated (Figure.6 A to D) with the respective statistical evaluations.

A) Latency (S)

In the latency to reach the platform, there was effect of the session (mean latency of all animals decreased throughout the sessions) and the attempt (mean latency of all animals declined throughout the trials), indicating that the animals in the

control groups and anoxia and females behaved as expected, i.e. improving performance with trials and sessions.

B) Percentage of Time in the Critical Counter

Measurements of percent and dwell time in the previous quadrant and critical counter, i.e. the quadrant and counter where the platform was presented in the previous day session, were included in order to evaluate the memory on the location of the platform 24 h later, as well as the behavioral flexibility of CM animal groups; CF; AM; AF to interrupt the search of the location where the platform was previously and search for the new location.

C) Percentage of time in the Critical Quadrant

The percentages of frequency and dwell time in the quadrant and critical counter of the previous day, ie, the quadrant and the counter where the platform was presented in the previous day session, were included in order to evaluate the memory on the location of the platform 24 h after the test, and the behavioral flexibility of the CM animal; CF; AM; AF to stop performing the search of the location where the platform was previously and search for the new location.

D) Pathlength

In the distance covered, there was effect of the session (the average distance of all the animals decreased during the sessions) and of the attempt (the average distance of all the animals decreased during the trials), indicating that CM animals; CF; AM and AF behaved as In the reference memory test, it is expected that in the first sessions all animals exhibit similar measures, since none of them has the necessary information to orient themselves successfully in the pool. Of course, throughout the experiment sessions the animals will acquire platform location information and gradually improve their performance. However, when there is some impairment of the areas involved in learning and spatial orientation, it is possible to distinguish larger scores both in the latency within the pool and in the path travelled by the animals.

E) Swimming speed

It is calculated by dividing path length with latency for each animal. Anoxia male animals swan faster than control animals. Graph is not shown for this parameter.($F_{1,340}$ = 7.60, p = 0.006).

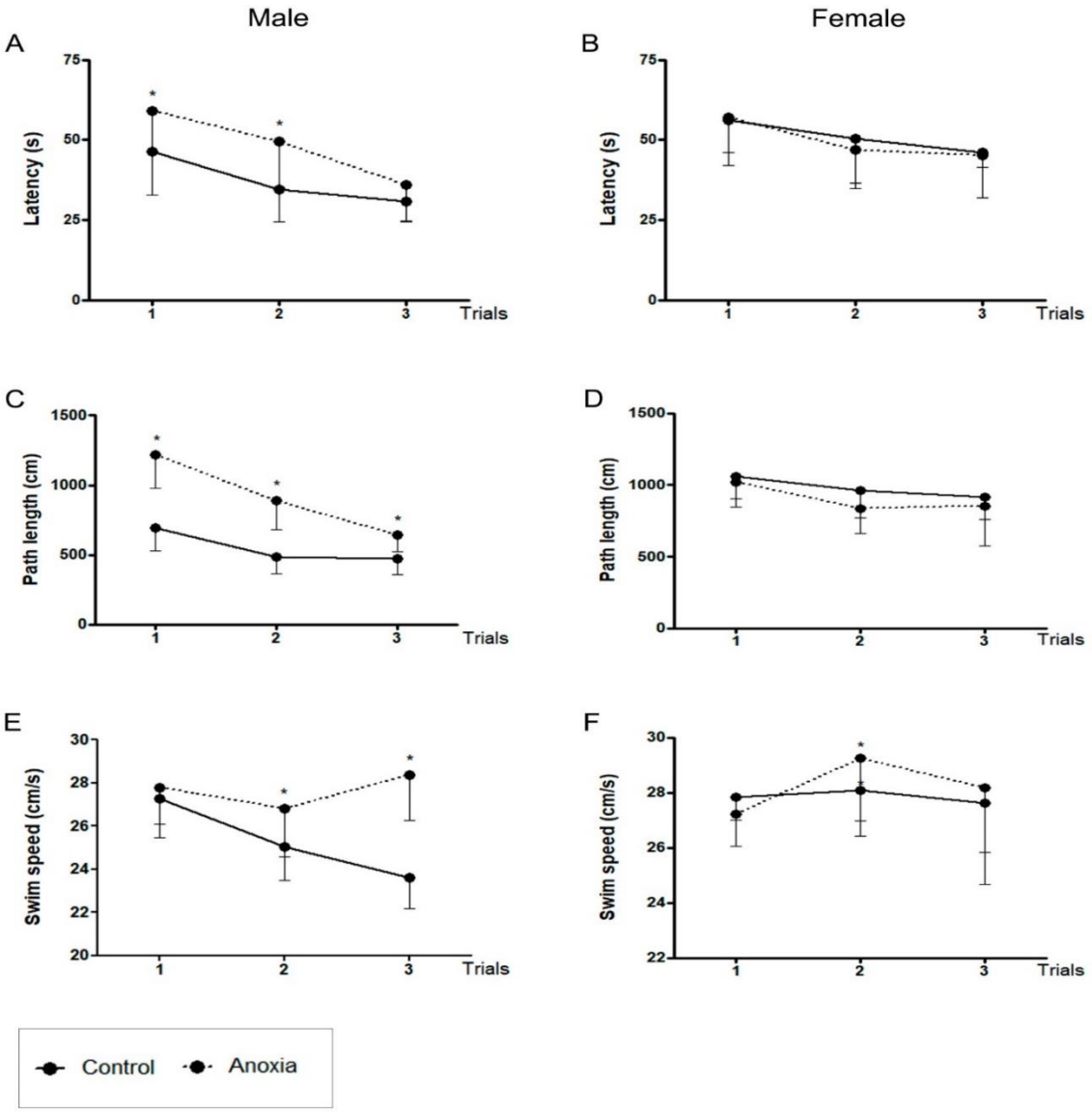
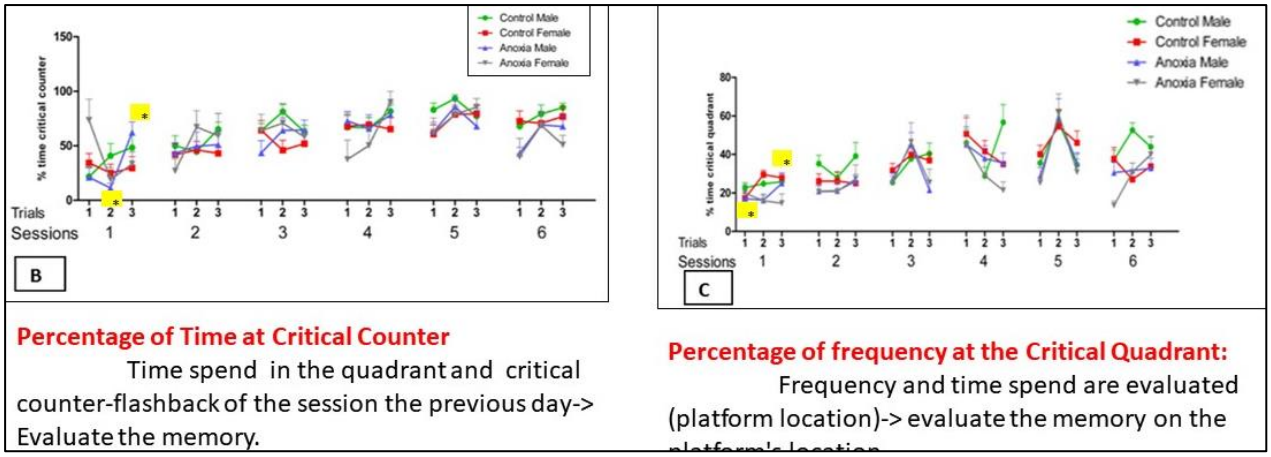


Figure.27. Comparison of the mean scores of the animals in 3 trials, 6 training sessions in the parameters evaluated in the Morris water maze of male and female rats of the control and anoxia groups. Statistical evaluation with repeated measures ANOVA and Tukey-Kramer post-hoc test, p≤0.05. The values represent M + SD, n = 16 in each group:

A. Latency in seconds. Effect of the F group (3.609) = 6.39;
B. Percentage of time in critical counter. Effect of the group [F (3.609) = 13.06;
C. Percentage of time in the critical quadrant. Effect of the group [F (3.609) = 7.11
D. Path length in centimeters. Effect of the group [F (3.609) = 4.04.



Percentage of Time at Critical Counter

Time spend in the quadrant and critical counter-flashback of the session the previous day-> Evaluate the memory.

Percentage of frequency at the Critical Quadrant:

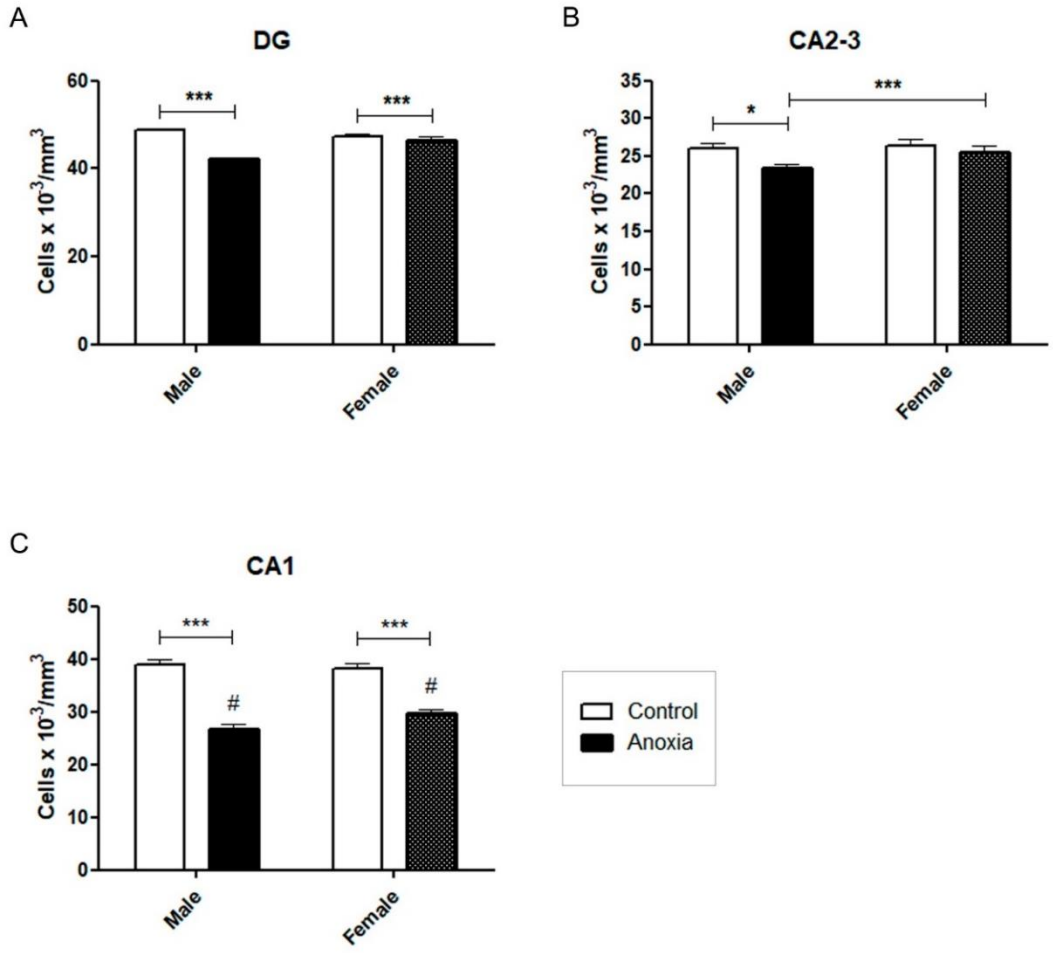
Frequency and time spend are evaluated (platform location)-> evaluate the memory on the platform's location

C) Quantitative analysis of Hippocampus

Using morphological criteria (Konopaske et al., 2007), neurons were distinguished from glia on the basis of size, the presence of euchromatin in the nucleus, a clearly visible nucleolus and surrounding cytoplasm. Glial cells marked by presence of heterochromatin in the nucleus and lack of cytoplasm The number of hippocampal neurons significantly differed in Anoxic animals in relation to control animals. (Figure. 3). Specifically, the average numbers of CA1 neurons were Male (20.54 ± 1.18) and Female(29.74 ± 0.69); respectively, in the Anoxic group and male (38.93 ± 0.94) and female (38.24 ± 0.89), respectively, in the CTL group. Moreover, the Anoxic male animals exhibited markedly fewer neurons than the control male animals (Tukey Pos-hoc test, $p < 0.05$).In the CA3 region, two-way ANOVA revealed interaction Group^Sex p =0.03, and sex differences ($F_{1,16} = 36.407$, $p = 0.00002$): AM (42.06 ± 0.42); CM(48.68 ± 0.30); AF (46.46 ± 0.74); CF (47.21 ± 0.82). In relation to dentate gyrus, two-way ANOVA revealed group effect ($F_{1,16} = 23.15$, $p = 0.00019$), sex differences ($F_{1,16} = 5.7689$, $p = 0,02881$) and interaction Group*Sex ($F_{1,16} = 23.150$, $p = 0.0019$) (AM (42.06 ± 0.42); CM (48.68 ± 0.30); AF (46.46 ± 0.74); CF (47.21 ± 0.82)). As seen in Figure. 3, the anoxic male animals were more susceptible to injury in DG and CA1 regions.

C.1) Anatomical results

Quantitative analysis was carried out only on the Cresyl violet-stained sections. Differences in the cell density between anoxic and control samples were seen in the CA1 field & DG hippocampal fields and anoxic male suffered more than female. In this field, the mean cell density in control animals showed results of 39.28 + 4.11 cells/mm 2 and of 25.6 + 3.58 cells/mm 2 in anoxic animals. This difference resulted of high statistical significance (Student's t-test, P < 0.001)



FIigura 28. Quantity of Nissl stained cells in different field of hippocampus at PND41, between bregma -1.30 mm to -2.12 mm (400 µm distant). (A) DG. (B) CA2-3 regions. (C) CA1 region. Data are expressed as mean ± S.E.M. n=5 in each group. Results were analysed by two-way ANOVA, considering the groups as between-subject factor and the cortex regions as within-subject factors. Tukey-Kramer post-hoc test was used. $p < 0.05$.

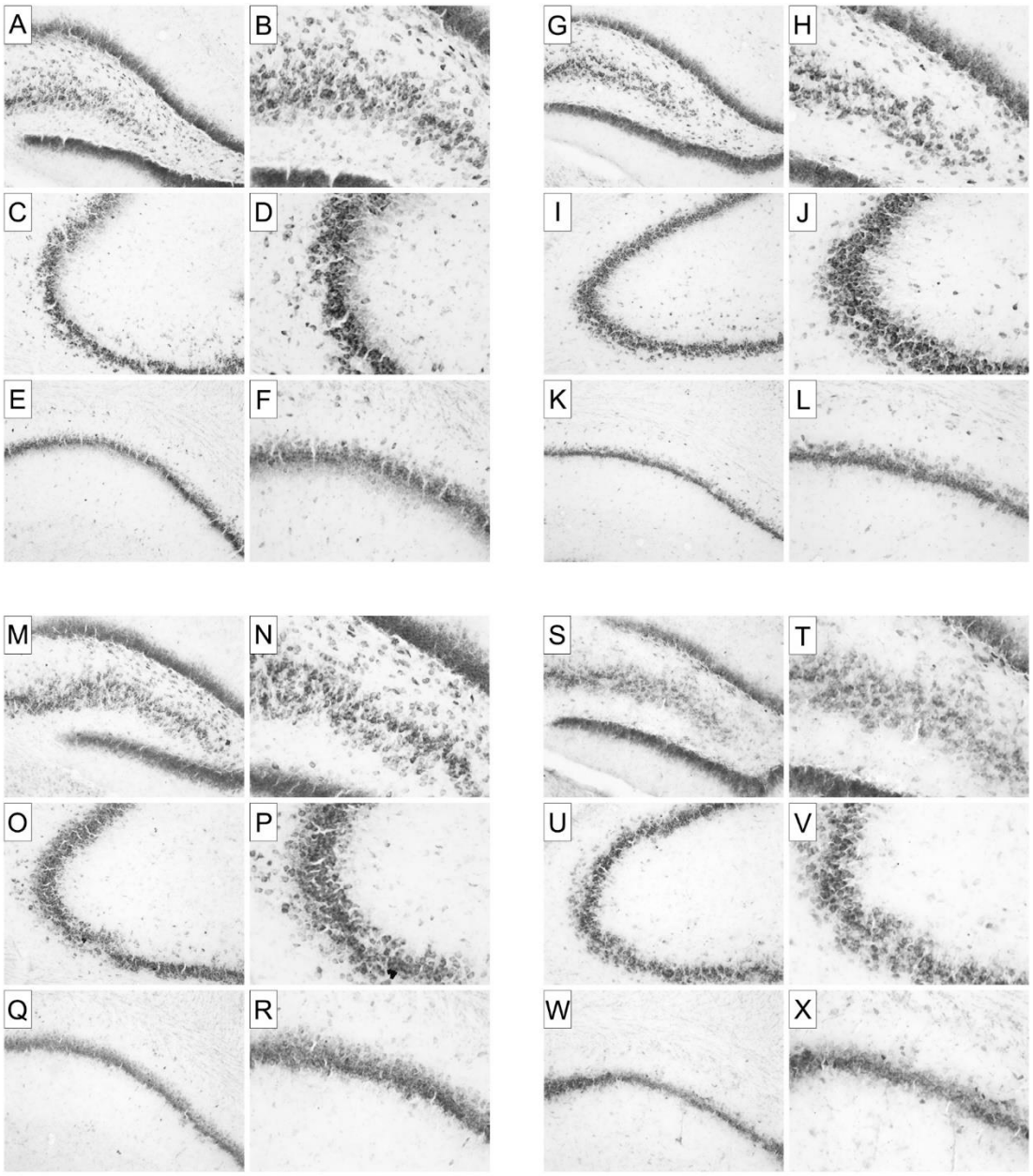


Figure 29. Representative microphotograph of coronal Nissl stained sections on hippocampus at PND44. (A-F) Control male. (G-L) Anoxia male. (M-R) Control female. (S-X) Anoxia female. (A, G, M and S) DG hilus at 10X. (B, H, N and T) DG hilus at 20X. (C, I, O and U) CA2-CA3 region at 20X (D, J, P and V) CA2-CA3 region at 10X. (E, K, Q and W) CA1 region at 20 X. (F, L, R and X) CA1 region at 10 X.

5.7 Discussion

Our results show that neonatal anoxia leads to reduced anxiety-like behavior and spatial memory deficits in adolescent rats. In the current study, sex-dependent responses to neonatal anoxia were observed such as that males were more susceptible. Male was the most affected, having worst performance in spatial memory tasks and larger histological damage in DG and CA1 region when assessed at adolescence .

It is also possible that the increase in the number of entrances and length of stay in the open and closed arms is indicative of differences in the extinction rate to aversive environments (Caputa et al. , 2005) which demonstrates the difference in behavior exhibited by the animal in the first and second test sessions. It is consistent to think that the animal will have the information acquired in the first session to move better in the second, and it can be inferred that the animal will tend to avoid the most aversive sites. As for the control groups of both genders, as expected, there was a decrease in the number of entrances and the percentage of time in the open arm, which shows that the rat, in fact, prefers closed (less aversive) sites to places where is exposed (more aversive) in the first session, although it can move freely through the EPM maze in both sessions (Winter et al., 2005).

The elevated plus maze evaluates the animal's length of stay in the closed and open arms, as well as the number of entrances in the central quadrant and the length of stay in this quadrant. In closed arms, it is considered a measure of locomotor activity, and length of stay in open arms is considered an indirect measure of anxiety, while the number of entrances and length of stay in the central square represents an expression of risk assessment (Darwish et al ., 2001).

Data from elevated plus maze indicates that the number of open arm entries and the percentage of time spent on the open arm was significantly higher in anoxic animals than in control animals, indicating that anoxia leads to reduced anxiety-like behaviors during adolescence (P33). Anoxic females have spend more time in open arms thus indicating a decreased anxiety-like behavior in relation to males. In accordance with our results reduction in HI-induced anxiety-like behavior has also

been reported in other animal models of neonatal cerebral hypoxia-ischemia (Fan et al., 2006; Caputa et al., 2005; Ming Yan et al., 2012).

Ming Yang et al (2012) showed that neonatal HI results in decreased anxiety-like behavior during the juvenile period of Sprague-Dawley rats showed that young rats with mild and severe perinatal hypoxia had low levels of anxiety compared to the control groups showing its anxiolytic effect in young rats (14-28 days). The same effect was observed by Sanches et al (2013), in this study rats subjected to HI on a postnatal day (7) showed an increased percentage of time in open arms and in the central quadrant of the EPM as compared to control groups at adulthood.

On the other hand, our group has shown that on PND 90 time spend in open arms was significantly lower and more time was spent on the closed arm and was significantly higher in Adult anoxia subjects than in controls (Takada et al., 2015), indicating an anxiogenic (anxiety causing) like effect of neonatal anoxia at PND 90.

A possible explanation is neonatal anoxia could induce an increase in exploration and hyperactivity, which increases the open: closed ratio at an early stage. It is of note that circadian phase and sex could influences anxiety-like behaviors. Verma et al. 2010 reported that, when tested during the dark phase, females increased anxiety-like behavior, whereas males showed higher anxiety scores in the light phase. Furthermore, the open / closed arm ratio was lower in the dark than the light for females, but not males. Our data corroborate the claim that anoxia regulates the anxiety/depression balance, regulating mood and emphasizing the importance of considering circadian influences in animal tests of depression and anxiety like behaviors (Verma et al., 2010).

Galeano et al. (2011) indicated deficiencies in spatial and operational reference memory in rats undergoing perinatal asphyxia, but no differences in anxiety-related behaviors were observed in 3-month-old rats. Contrary to this research, Hoeger et al. (2000) using the same model of perinatal asphyxia, observed that rats with long-term exposure to hypoxia (15 and 20 min of asphyxia) showed anxious behavior. Corroborating this study, rats in the elevated cross maze with long-term exposure to hypoxia (15 and 20 min asphyxiation) showed reduced anxiety-related behavior.

It is also possible that the increase in the number of entrances and length of stay in the open and closed arms of the elevated cross maze (Plank IV-Figures B and C) is indicative of differences in extinction rate to aversive environments (Caputa et al. , 2005) which demonstrates the difference in behavior exhibited by rats in the first and second session of the test. It is coherent to think that the rat will have the information acquired in the first session to move better in the second, and it can be inferred that the animal will tend to avoid the most aversive places to him. In the case of the control groups of both genders, as expected, there was a decrease in the number of entrances and the percentage of time in the open arm, which shows that the rat actually prefers closed (less aversive) places over places where it is exposed (more aversive) in the first session, although it can move freely through the maze in both sessions (Winter et al., 2005).

Investigations focussing on ventral hippocampus involvement in anxiety-related behaviors, shows lesions ended to be anxiolytic, increasing time spent in the anxiogenic open arms of the EPM (Kjelstrup et al., 2002). On the other hand investigations have also shown effects on anxiety-related behaviors, including altering the activity or level of neurogenesis in the dentate gyrus subregion (Kheirbek et al., 2013; Wu and Hen, 2014), altering basal amygdala inputs (Felix-Ortiz et al., 2013), and altering outputs to the lateral septum and medial prefrontal cortex (Kjaerby et al., 2016; Padilla-Coreano et al., 2016; Parfitt et al., 2017). Schumacher et al., 2016 has observed ventral hippocampus to be a criticial element of the neural circuitry and is linked with inhibitory control over approach tendencies under circumstances in which motivational conflict is experienced.

Reference and working memory impairments after neonatal HI and anoxia have been previously reported (Ikeda et al., 2001; Arteni et al., 2003, 2010; Huang et al., 2009: Takada et al., 2015, 2016). Present data show that anoxic males had the worst performance in the spatial reference memory.The increased path length to find the platform observed in the Anoxic male group during the test appears to be due to learning impairment since the swimming speed was higher in an anoxic group with rules out the motor damage that may influence the measurement of other variables.

One possible key is the neuroprotective effect of estrogen and progesterone found in females which can reduce intracranial pressure and cerebral perfusion pressure after injury (Shahrokhi et al., 2010).

Memory has been defined as the ability of animals to modulate their behavior according to previous experiences (Xavier, 1993). In practical terms, memory is the expression that learning has occurred. Thus, memory and learning processes are difficult to study separately. For the investigation of memory in rodents, different types of mazes have been used. Among these learning tasks, the water maze, introduced by Richard Morris. (1984), is the most widely used in studies on memory neurobiology (Garthe & Kempermann., 2013). This maze is extensively used in research into rodent spatial orientation capabilities, the retention of this information in the nervous system for varying periods of time, and the investigation of the neural mechanisms underlying these capabilities (Dong et al., 2013; Miyoshi et al., 2012; Xavier et al., 1999).

In the reference memory test, it is expected that in the first sessions all animals exhibit similar measurements, since none of them have the information necessary to successfully orientate themselves in the pool. Of course, throughout the experiment sessions the animals will acquire the platform location information and gradually improve their performance. However, when there is some impairment of the areas involved in learning and spatial orientation, it is possible to distinguish higher scores both in latency within the pool and in the path taken by animals.

In the spatial reference memory task, it was clear that there is a behavioral impairment in relation to the rats submitted to neonatal anoxia when compared to the control groups exhibiting a lower tendency in the learning rate and information retention. Previous studies have shown the relationship between neonatal anoxia impairment and deficits in memory acquisition and spatial learning (Buwalda et al., 1995; Dell 'Anna et al., 1991).

In 1999, Xavier et al. observed marked impairment in spatial tasks in animals subjected to selective lesion of the dentate gyrus cells. Even so, the animals could obtain different degrees of improvement during the training. It was suggested by the authors that this would be due to the use of other spatial orientation strategies by the injured animals, probably involving preponderant lane orientation or egocentric orientation, but not cognitive mapping, which would make it possible to improve over repetitive training. The use of other spatial strategies when it is only possible to be guided by distal or egocentric clues makes the search less efficient and this results in worse scores than those obtained by healthy animals. Additionally, several studies indicate that injured animals have some ability to improve (Shohan et al., 2003; Sallai, 2005; Tanhoffer, 2006).

In a recent survey by Takada et al. (2015) indicated the existence of differences between control rats and those submitted to neonatal anoxia when tested in the spatial reference memory task. It is noteworthy in this research that anoxic rats were unable to exhibit the typical pattern of decreasing latency scores, suggesting that these animals cannot retain spatial information for the period required to perform the task rapidly.

Regarding velocity (Figure 8 (C)), the results showed a clear difference between the AC group and the others. This difference was maintained throughout the tasks in the spatial reference memory, suggesting that the rats submitted to neonatal anoxia possibly had a greater motivation to perform the tasks. It is important to highlight that the ability to move quickly in the pool makes it possible to rule out that rats have motor impairments that may influence the measurement of other variables.

The data obtained in the percentage of time in the counter and in the critical quadrant are useful to show the learning rate of the animals in relation to the number of sessions and attempts developed in the task. As expected, all animals showed higher measurements in the percentage of time over the sessions (spatial reference memory).

All animals had the same ability to retain the information obtained during the three time intervals evaluated (ITI 10 minutes) and it is possible that this capacity will vary as a function of longer periods of time.

Cell densities in the CA1, CA3, and GD regions of hippocampal formation in the different groups in the Anoxia and Gender-Independent Control Group. The reading suggests that neonatal oxygen deprivation found a progressive decrease in CA1 pyramidal layer cell density. Takada et al., 2015a concluded that neonatal anoxia promotes neural death in rat hippocampal subregions CA1 and CA3 24 hours after anoxic insult. She also looks at that adult animals undergoing neonatal anoxia have decreased neurogenesis in the hippocampal dentate gyrus.

We suggest that these spatial memory impairments are related to more cell loss in CA1 field of hippocampus and DG founded in male anoxic animals. The DG highly sensitive to oxygen deprivation plays a role in memory and mood regulation, and it is also a key area of the production of new neurons (neurogenesis) in the adult brain (Liam et al., 2013). There are several studies that have sought to link cognitive functions related to neurogenesis in rodents to human clinical conditions (for review Aimone et al., 2014). The DG cell loss induced by anoxia could lead to disturbances in the neurogenesis mechanisms that reflect on impairments in hippocampal-dependent tasks and emotional regulation effect (Sanghee et al., 2016). We have shown that adult rats who had experienced neonatal anoxia has hippocampal volume reduction and decreased neurogenesis rate (Takada et al., 2016) , we presume these cognitive deficits found in adolescent anoxic rats are due to alterations in new neurons production that might alter neuronal networks critical to spatial learning and memory and emotional balance, what remains to be studied in the future.

5.8 Conclusion

In summary, neonatal anoxia at 30 h age results not only in long-term neurological dysfunction like alteration in anxiety and memory impairement but also have induced damage to brain neurons including fields of CA1 and DG in anoxic males more than in females. The hyperactivity and poor performance of anoxic males can be contributed to the reduced number of cells in these areas by the anoxic stimulus.

Chapter 3

Neonatal anoxia and hypersensitivity to mechanical allodynia in Wistar rats.

Abstract

Chronic neuropathic pain, resulting from damage to the central or peripheral nervous system, is a prevalent and debilitating condition, affecting 7-18% of the population. Symptoms include spontaneous pain, dysaesthesia, paraesthesia, allodynia and hyperalgesia. The sensory reported symptoms are co-morbid with behavioural disabilities, such as insomnia and depression. The effect of neonatal anoxia on maturation of nociceptive pathways has been sparsely explored. Perinatal asphyxia produces long term deficits and represents a major problem in both neonatal and paediatric care. Through a neonatal anoxia model, based on exposure of 100% nitrogen when pups are 30 hours old (6-8g) this question was explored. At PND 18 and 43 they were subjected for pain test with von Frey monofilaments in the hind paws. The results revealed a significant reduction in the threshold of pain perception, around 50% in the animals exposed to anoxia, in relation to its control (those subjected to the same conditions but except for gas exchange, maintaining the environmental air flow). Therefore, the reflex of paw withdrawal in response to an innocuous stimulus, named mechanical allodynia, was unequivocally caused by the early long lasting effects of anoxia. It showed the presence of mechanical pain hypersensitivity, a feature of chronic neuropathic pain. These results might have come from the decreased number of cells in the ventral posterolateral nucleus (VPL) thalamic nucleus, which carries the sensory inputs from the body to the sensory cortex.

Key words: neonatal anoxia, mechanical allodynia, sex difference, thalamus (VPL), NeU-N, von Frey monofilament.

Introduction

The Somatosensory System - The ventral posterior nucleus, sometimes named the ventrobasal complex, is a wedge-shaped cell group located caudally in the

thalamus. Its lateral border abuts the internal capsule, and ventrally it borders on the external medullary lamina. The ventral posterior nucleus is composed of the laterally located VPL and the medially located ventral posteromedial nucleus (VPM). Although these nuclei have also been termed the ventralis caudalis externus and ventralis caudalis internus in humans, the more widely used and recognized terms VPL and VPM are used in this book. The VPL is separated from the VPM by fibers of the arcuate lamina. The ventral posterior nucleus (VPM and VPL) is supplied by thalamogeniculate branches of the posterior cerebral artery, and compromise of these vessels can result in loss of all tactile sensation over the contralateral body and head.

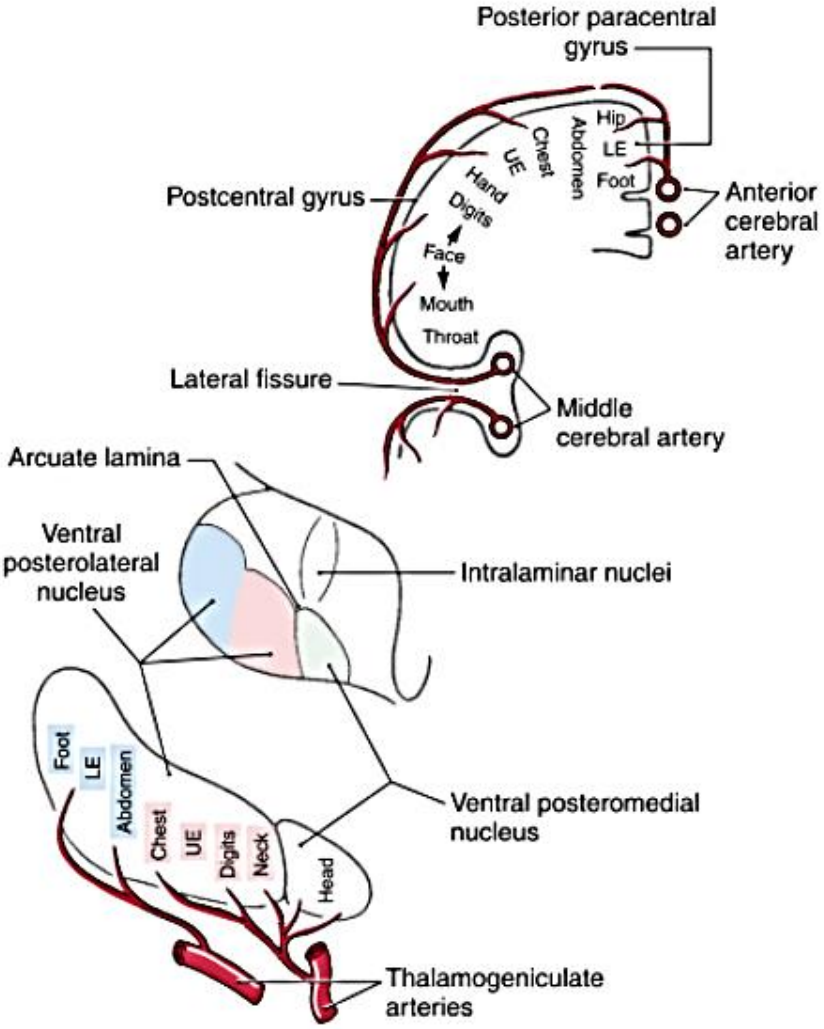


Figure 30. somatotopic organization. Blood supply and somatotopic organization of the body in the ventral posterolateral and posteromedial nuclei and in the primary somatosensory (SI) cortex. LE, lower extremity; UE, upper extremity. (Adapted from Fundamental neuroscience for basic and clinical application, Duane E.Haines. Third edition)

The VPL receives ascending input from the medial lemniscus, and input to the VPM is from the trigeminothalamic tracts. Within the VPL, medial lemniscal fibers from the contralateral cuneate nucleus terminate medial to those from the gracile nucleus (Talbot et al., 1991).

In neonatal injury engaging deeper or visceral tissue long-term pain hypersensitivity is associated with peripheral sensitization. Full thickness skin removal results in permanent changes in the innervation of the neonatally damaged region itself long after the wound has healed, leaving the area hyperinnervated by both myelinated A- and unmyelinated C-fibres (Reynolds & Fitzgerald, 1995; Beggs et al., 2012a). This hyperinnervation depends on the release of the neurotrophin NT-3 from the damaged region, which is highly up-regulated in the neonatal damaged area (Beggs et al., 2012a) combined with site-specific down-regulation of factors that normally inhibit axonal growth into the skin (Moss et al., 2005).

Brain injury in newborns can cause deficits in motor and sensory function. In most models of neonatal brain injury, thalamic damage often occurs (Volpe et al., 2009). Energy failure, free radical, cytokine, and excitatory amino acid release, and caspase-dependant cell death are known to contribute to injury in the neocortex, striatum, and periventricular white matter (McDonald et al., 1988; Barks and Silverstein,1992; Haganetal.,1996; Liuetal.,1996; Martinetal., 1997a, b; Back et al., 1998; Cheng et al., 1998). However, the degeneration of thalamus and other non-forebrain structures after hypoxia–ischemia are studied less frequently. Injury to somatosensory thalamus has been described in human newborns after hypoxia–ischemia (Barkovich, 1995; Roland et al., 1998) and may contribute to sensorimotor deficits in infants with perinatal brain injury and cerebral palsy. Sensorimotor deficits have been detected in neonatal rats subjected to the Rice–Vannucci model (Rice et al., 1981) of hypoxic–ischemic injury (Bona et al., 1997). A few detailed neuropathological studies of animal models have revealed injury to the developing thalamus after neonatal hypoxia–ischemia (Towfighi et al., 1991.) where 7 day old rat go undergo occlusion of common carotid arteries and are placed in chamber with 8% oxygen and 92% nitrogen for period of 30min to 3hour. They worked only on male rats and then investigated the impact of injury on the developing brain.

Chronic neuropathic pain, resulting from damage to the central or peripheral nervous system, is a prevalent and debilitating condition, affecting 7-18% of the population. Symptoms include spontaneous (tingling, burning, electric-shock like) pain, dysaesthesia, paraesthesia, allodynia (pain resulting from normally non-painful stimuli) and hyperalgesia (an increased response to painful stimuli). The sensory symptoms are co-morbid with behavioural disabilities, such as insomnia and depression.

The effects of neonatal anoxia on maturation of nociceptive pathways has been sparsely explored. To investigate whether this treatment alters nociceptive behavior this study has been designed.

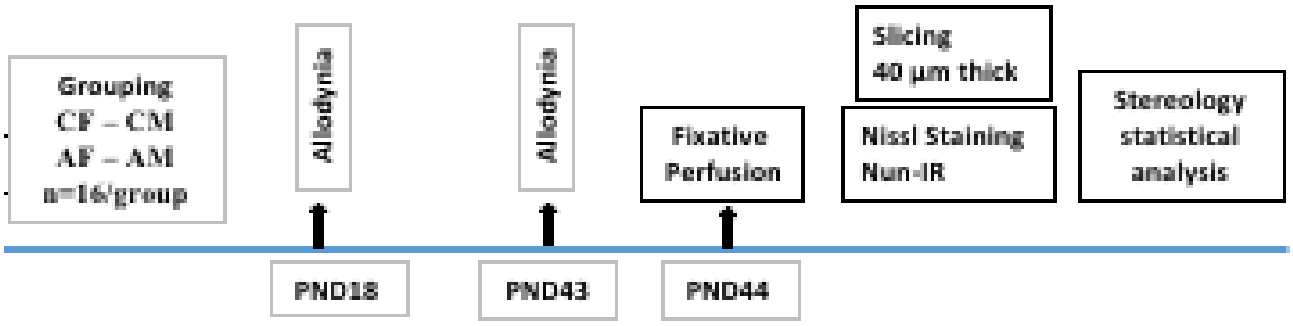


Figure 31. Experimental design

Material and method

Has been described on page 22 with detailed experimental design. Animals go through Allodynia test as described above in the above design.

Nociceptive stimulation, mechanical allodynia

After acclimation in the laboratory environment for 30 min, the rats were individually placed in acrylic boxes with an open base and a lid containing small openings. The acrylic box stands on a grid, measuring 1 meter long by 45 cm wide, with space between the 1 cm wires. The grid is fixed to the wall to allow access to the

legs of these animals. The mechanical stimulation was applied to the plantar surface of the hind paw for 10 s with a series of ten von Frey monofilaments respectively: 3.61 (0.407g); 3.84 (0.692g); 4.08 (1.202g); 4.17 (1.479g); 4.31 (2.041g); 4.56 (3.630g); 4.74 (5.495g); 4.93 (8.511g); 5.07(11.749g) and 5.18 (15.136g). Abrupt withdrawal or flinching of the hind paw were regarded as a positive response, the filament capable of inducing paw withdrawal, twice consecutively, was considered as the force in grams required to induce the response named and quantified as mechanical allodynia (100% response) (Chaplan *et al.*, 1994).

Mechanical allodynia, that is a painful sensation caused by innocuous stimuli like light touch, is assessed by the paw withdrawal threshold (PWT) measured with von Frey filaments (Aesthesiometer Semmes-Weinstein, Stoelting, Wood Dale, IL, USA). Its determination was evaluated by the response to pressure stimulus applied to both the hind paws of the rat (Chaplan *et al*., 1994).

Histological procedures

The animals were deeply anesthetized by an intraperitoneal injection of xylazine, the transcardiac perfusion was performed with 0.9% saline solution followed by 4% formaldehyde in 0.1 M PBS, pH 7.4, at 4°C. The brains were dissected and overnight post fixed with this last fixative, then immersed in a cryoprotectant solution (0.1 M PBS and 20% sucrose) for 48 hours. The brains were then frozen in a solution of water and resin soluble glycols (Tissue-Tek® OCT ™ Compound, Sakura, Chuo-Ku, Tokyo, Japan) and stored at -80ºC until sectioned into coronal sections of 40 µm thick, in an sliding criomicrotome, 5 series of 5 compartments, for further histological processing.

-Nissl staining

The sections of one series, from Bregma -2.12 mm to -3.14 mm region of the thalamic ventral post lateral nuclei, were stained by the Nissl method with Cresyl violet according to Kumar et al. (2017). Then, they were mounted on gelatinized slides and dried for 24 hours at 40°C.

- NeuN immunoreactivity (Neu-IR)

Sections from one series from the same thalamic region were incubated for 24 h with the primary anti-NeuN antibody (rabbit 1.100, MAB377, Merck Millipore, lot 1991263) in PBS 0,1 M + Triton solution. Then, by incubation in biotinylated goat anti-rabbit igG secondary (1:200 Vector laboratories, lot: Y0515), followed by the ABC Vector kit and DAB (diaminobenzidine) revelation of the antibodies complex and mounted in gelatinized slides, coversliped with DPX.

- **Stereological Quantification of VPL**

For stereological quantification of VPL Nissl stained cells, four sections between Bregma's 2.12 mm to -3.14 mm of the rat brain Atlas (Paxinos and Watson, 2004), per animal (n=5 per Group) were unilaterally analysed. For that, it was used an optical microscope (Nikon – E1000) coupled to a system for stereology, consisting of a motorized platinum (Ludl Electronic Products, Hawthorne, NY), capture camera (Nikon Instruments Inc., Melville, NY) and software Stereo Investigator (MBF Biosciences, Williston, VT). The delimitation of the VPL, structures were captured with 4x objectives, also photographs with 10x objective were taken for illustrative purpose. The researcher was blind to the experimental group being analysed.

- Image J Counting

Image J, software (NIH, Bethesda, MD, USA), was used to evaluate the number of neurons immunoreactives to NeuN (NeuN-IR) in coronal sections of the VPL, from the bregma -3.00 mm to -3.24 mm, in each group.

Results

Neonatal anoxia impact in rats nociception was observed by a induced pain hypersensitivity. There was significant reduction in paw withdrawal threshold to mechanical stimuli compared to the control. No sex difference was observed in this nociceptive response in both, control and experimental groups. However, different sex effects were observed histologically in the number of total cells and number of neurons at the VPL nucleus.

The mechanical nociceptive stimulus performed by von Frey monofilament in the adolescent rats hindlimb, right and left paws, revealed that neonatal anoxia provoked a significant reduction of the pain threshold, about 50% , in all the evaluated groups, without sex difference among them nor between the right and left paws, as demonstrated in figure 2.

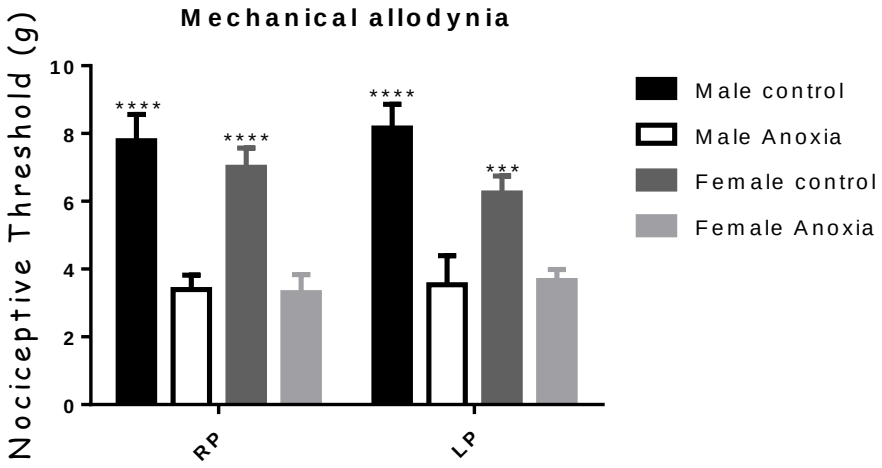


Figure 32. Nociceptive threshold of hindlimbs in Wistar rats submitted to neonatal anoxia at PND 43. RP (right paw) - **** $p \leq 0.0001$ when comparing the groups Male and Female control with Male and Female anoxia; LP (left paw) - **** $p \leq 0.0001$ when comparing Male control with Male and Female anoxia and ***$p \leq 0.0003$ when comparing Female control with Male and Female anoxia, n=5 in each group. Between the right and left sides there were no statistical difference.

Evaluation of nociception threshold at the weaning period, PND18, with stimulus under the same parameters did not show significant differences among the groups nor between left and right paws, figure 31.

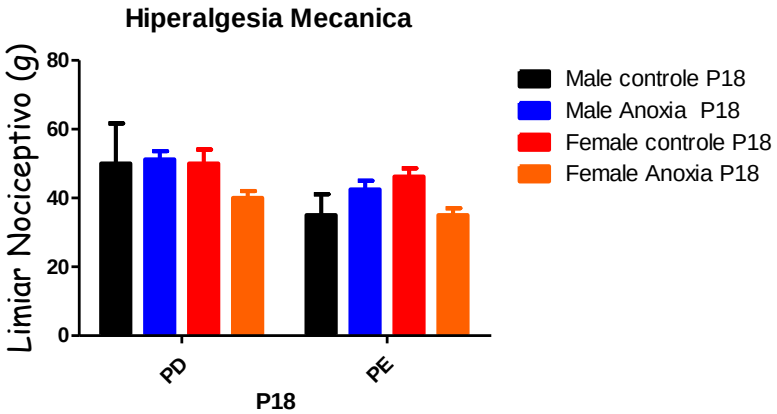


Figure 33. Nociceptive threshold of hindlimbs in Wistar rats submitted to neonatal anoxia at PND 18. No statistical difference was detected when comparing the groups or the right and left paws, n=5 in each group. Between the right and left sides there was no statistical differences .

Stereological procedures in the Nissl stained coronal sections of the VPL, from Bregma -2.12 to 3.14, demonstrated that neonatal anoxia in males caused a reduction in the cell number of the PND 43 male anoxic rats in relation to its control at $p < 0.05$, two-Way ANOVA statistical analysis and Tukey-Kramer post hoc test, n =5 per group. No difference was detected between sex or between female groups as depicted in figure 4.

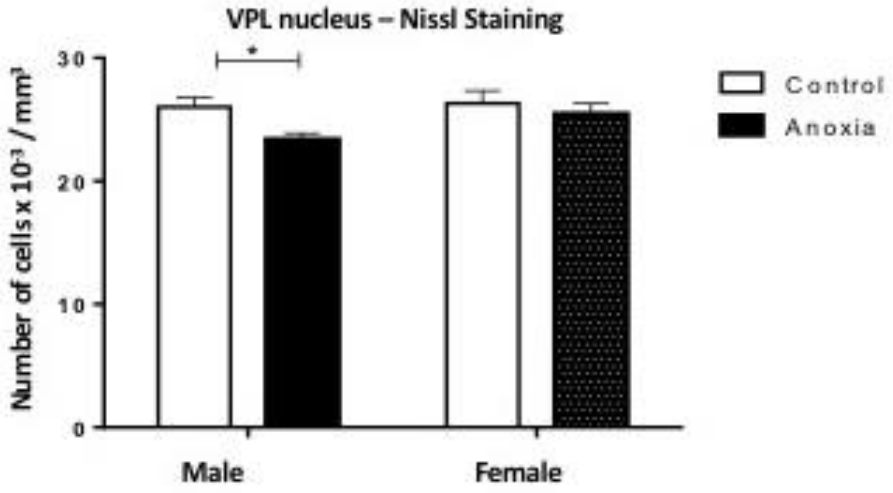


Figure 34. Effects of neonatal anoxia on cell number of the VPL nucleus. Data are expressed as mean ± SEM, stereology of Nissl stained coronal sections of male and female Wistar rats at PND 44. Bregma from -2.12 mm to -3.14. Two-way ANOVA, considered the groups as between-subject factor (Tukey-Kramer post-hoc test), n =5 per group, *p < 0.05.

The evaluation of the number of neurons was made by NeuN immunoreactivity analysis in VPL nucleus at the same regions of figure 2. It revealed sex difference among the groups, females presented more neurons than male rats and also anoxia female showed less neurons than its control as depicts figure 5. $F(1, 15) = 6,422$, $p = 0,02$). Two-way ANOVA, Tukey-Kramer post hoc, n = 5 per group, *p < 0.05.

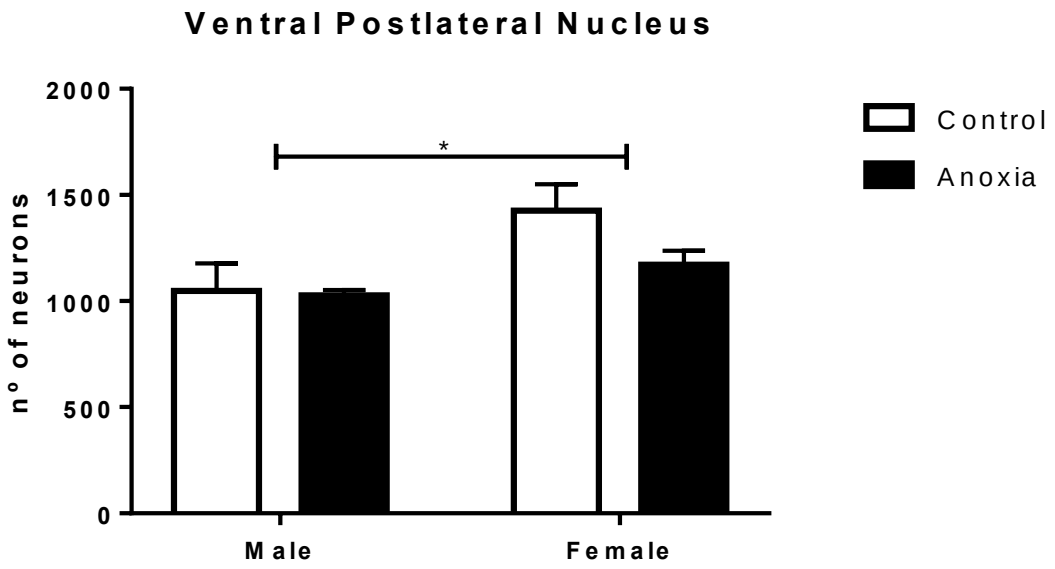


Figure 35. NeuN-IR, Effects of neonatal anoxia on neurons number in the VPL nuclei, Image J counting in coronal sections of male and female Wistar rats brain in control and anoxia groups, at PND 44. Data are expressed as mean ± SEM. Bregma from -2.12 mm to -3.14. Sex differences between sex, regardless the stimulus F (1, 15) = 6,422, p = 0,02). (Two-way ANOVA, Tukey-Kramer post hoc, n = 5 per group, *p < 0.05.

The results shown in graphics 4 and 5 are illustrated in the photomicrographs of figure 6, where images from VPL (4x) are compared with images of Nissl staining and NeuN-IR (10x). This figure depicts that neonatal anoxia damage was prominent in males through both used histological techniques, which were confirmed by stereology and image J counting.

Thalamic ventral post lateral thalamic nucleus (VPL)

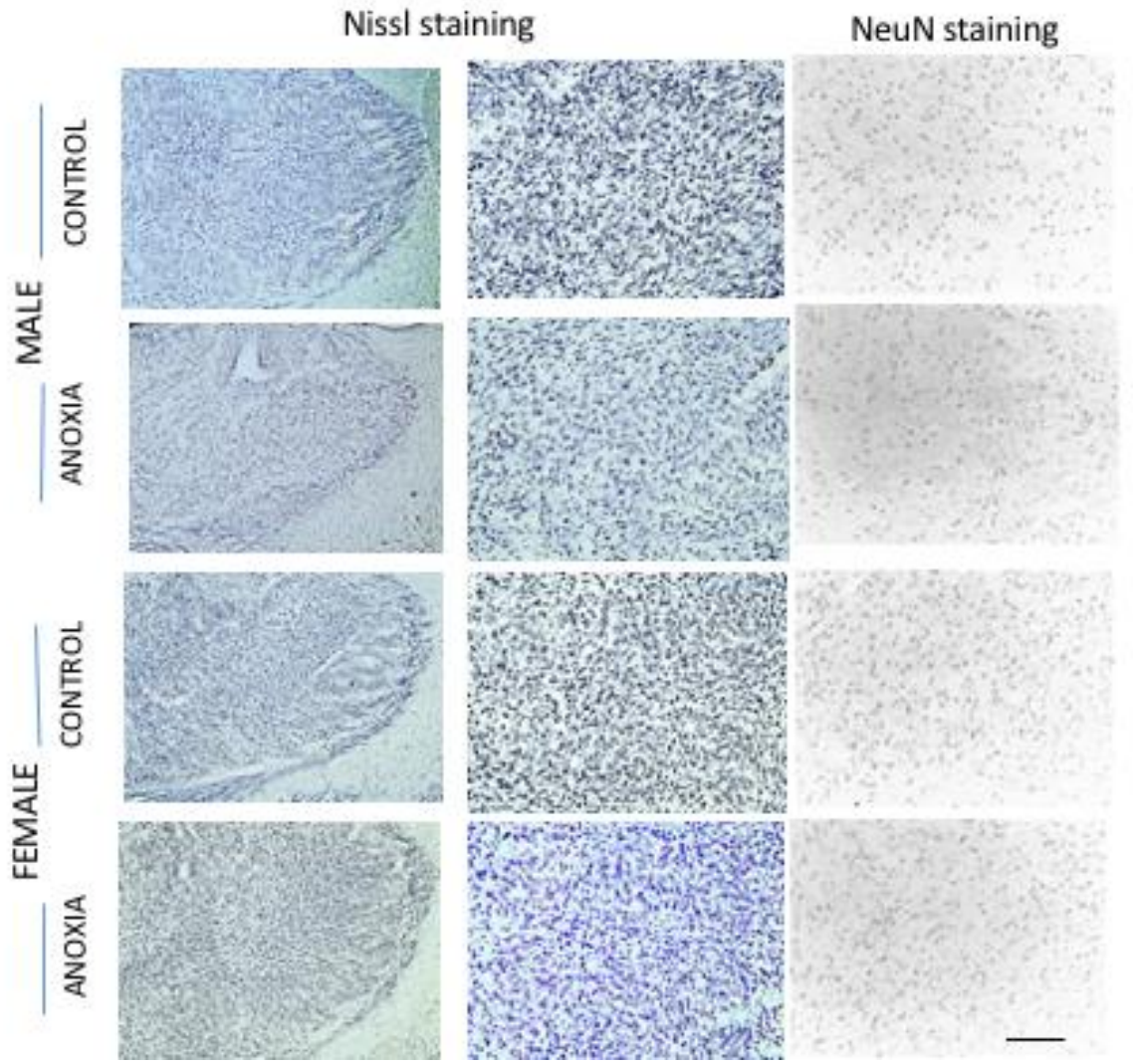


Figure 34. Representative photomicrograph of the VPL nucleus Nissl stained (columns 1 and 2) and NeuN-IR, in coronal sections of P44 male and female Wistar rats, control and anoxia groups. Bregma level from -2.56 mm to -3.14 mm (Paxinos & Watson Atlas, 2013). The image illustrates the effects of neonatal anoxia as shown in graphics 3 and 4. Bar 100 μm.

Discussion

Research on neonatal anoxia has shown its long lasting deleterious impact on morphofunctional, behavioral and cognitive aspects later in life. Through an animal model it was possible to demonstrate them in adult male rats (Takada et al., 2011). It was shown histological hippocampal alteration, at cellular and subcellular, level linked to cell death as well as cognitive impairments (Takada et al., 2015a). Reduction in

hippocampal volume associated with reduction in neurogenesis which might have caused disruption in working memory (Takada et al., 2015b).

There was less developmental and sex difference studies in rat model of brain injury in literature. Hence, the purpose of this research: to investigate the developmental parameters and eventual histological damages in different regions of the brain related to motor-sensory and cognitive development. So far, deficits in physical and sensory motor parameters of the weaning period were demonstrated. Alterations were seen in somatic evolution along with the onset of neurological parameters like; palmar grasp, auditory startle, negative geotaxis among others. Neonatal anoxia revealed sex differences in this battery of tests (Kumar et al., 2017). It has shown that males were more anxious than females and also, that their performance was worst in spatial memory test (Kumar et al., 2018). Histological damage was evident respectively on sensory motor cortex and in the different fields of hippocampus on that circumstances.

Would anoxia interfere with the maturation of nociceptive pathways, a subject sparsely approached but, with relevant role in clinical and educational development? The present research, conducted on male and female rats, showed that neonatal anoxia caused a significant reduction in mechanical withdrawal threshold, around 50% of the control response, indicating hypersensitivity in chronic neuropathic pain at adolescence but without sex differences. The pain test with von Frey monofilament, for mechanical allodynia evaluation, a painful sensation caused by innocuous stimuli like light touch (Chaplan et al., 1994; ….Marucia?), has been extensively used in the most studies of pathways related to pain and injury to the nervous system. Interestingly, the same stimulus could not evoke the mechanical pain response ate early age (PND 18 figure 3) although, the animal already was able to positively respond to reflexes like as cliff avoidance, negative geotaxis and acoustic startle reflex (Kumar et al., 2017). Probably the developmental nociceptive pathways at that age was still maturing (Schwaller and Fitzgerald, 2014)

The pain pathway involves many stations in the nervous system, from peripheric to central regions, and one must consider that this anoxia model, in spite of being noninvasive, it is also global and so as it is performed at the age when rats development corresponds to a human fetus of 32 weeks so, it simulates the condition

of the pre-term neonates exposed to anoxic conditions, a clinical worldwide problem (Takada et al., 2011). In this way, the anoxia impact reaches the peripheric elements of the sensorimotor system as well as the central ones in the critical period of development, when an injury in early life has great impact than in later life (Walker, 2013). Therefore it is an age sensible model, and decreased neurogenesis and cell death were higher in anoxic animals at adulthood (Takada 2015a).

Probably this hindlimb hypersensitivity have interfered in the results observed for sensorimotor development, since the anoxia had delayed the majority of palmar grasp reflex, for example (Kumar et al., 2017), also studies in course from this lab, has shown increased errors in hindlimb performance of anoxic animals in the grid walking test than the control group.

As thalamus is a key structure in the nociceptive pathway and also in motor coordination, it is also necessaire for the sensorimotor reflexes at both sensory and motor inputs to and from cortical areas. Neonatal anoxia, already, showed decreased cell density in the primary motor cortex and sensory hindlimb regions (Kumar et al., 2017).

Thalamic damage after hypoxia–ischemia in newborns has long been recognized and is particularly important in infants with extrapyramidal cerebral palsy (Malamud, 1950; Volpe, 1995; Roland et al., 1998). Human neuroimaging and neuropathological studies revealed that the thalamus is among the selectively vulnerable brain regions in the human newborn (Yokochi et al., 1991; Barkovich, 1995; Roland et al., 1998).

However, the degeneration of thalamus and other nonforebrain structures after hypoxia–ischemia is studied less frequently. Injury to somatosensory thalamus has been described in human newborns after hypoxia–ischemia (Barkovich, 1995; Roland et al., 1998) and may contribute to sensorimotor deficits in infants with perinatal brain injury and cerebral palsy. Sensorimotor deficits have been detected in neonatal rats subjected to the Rice–Vannucci model (Rice et al., 1981) of hypoxic–ischemic injury (Bona et al., 1997). A few detailed neuropathological studies of animal models have revealed injury to the developing thalamus after neonatal hypoxia–ischemia (Towfighi et al., 1991). In addition, it has been demonstrated that injury to the thalamus occurs in a delayed manner and exhibits prominent structural features of apoptosis when

compared with the early necrotic cell death seen in the forebrain after hypoxia–ischemia (Northington et al., 2001).

In oxygen deprivation model several investigators have shown behavioural disabilities, such as disrupted social interactions, sleep disturbances, depressive-like and anxiety-like behavior. The related neurological reflexes would be affected by the decreased cell amount in these areas by the anoxic stimulus as shown previously in motor and sensory primary cortices (Kumar et al., 2017).

Projections of nociceptive ascending neurons of the dorsal horn end in the ventral posterior nuclei of the thalamus, mainly the ventral posterior lateral (VPL) nucleus and then are carried to the somatosensory cortex with involvement of various motor circuits including connections of cerebellum and basal ganglia (Renato et al., 2007).

Neonatal brain injury is followed by the long-term enhancement of pain activity maintained by the neuroimmune system. This kinfd of injury is linked with glial activation in the peripheral and central nervous systems (Scholz & Woolf, 2007) and microglial activation in specific regions of dorsal horn circuitry play a key role in central sensitization and hyperalgesia, through the release of cytokines and growth factors which excite nociceptive dorsal horn neurons (Trang et al., 2011). Strong C-fibre input into the adult spinal dorsal horn is sufficient to activate microglia and increase nociceptive reflexes (Hathway et al., 2009b). In this study we see hypersensitivity to pain anoxia and male suffering more in ventral posterolateral nucleus.

it has been suggested the long-lasting changes in pain behaviour that follow early exposure to noxious stimuli may involve alterations in the stress HPA axis (Sternberg et al., 2005). While most studies make every attempt to control for handling and maternal separation, noxious stimuli are inherent stressors. Infant stress is a strong indicator of short- and long-term alterations in brain function (Meaney et al., 1988; Papaioannou et al., 2002; Moriceau et al., 2009) although the developmental periods at which the human brain is sensitive to environmental stressors are not known (Pryce, 2008; Pechtel & Pizzagalli, 2011). Early life stress alone (with no noxious stimulation), such as those that target dam–pup interactions and feeding and nesting behaviours, can alter pain behaviour in later life. Neonatal maternal separation

reduces sensitivity to noxious heat stimuli in adulthood compared with neonatally handled adults (Weaver et al., 2007) and induces visceral hypersensitivity in adult rats (Coutinho et al., 2002; Gosselin et al., 2010). Early life stress from a paucity of nesting material significantly prolongs muscle hyperalgesia following prostaglandin administration in the adult and increases the excitability of mature nociceptors innervating the muscle (Green et al. 2011). Restriction of dam and litter nesting material also increases plasma levels of the pro-inflammatory cytokine interleukin-6 in adulthood (Alvarez et al., 2013).

The mechanisms of the early life stress effect on pain pathways are not known but are probably mediated by the HPA axis, which in adults can directly influence the neurophysiological mechanisms underlying the perception of pain via brainstem descending pain control pathways (Butler & Finn, 2009). The HPA axis in rodents is relatively insensitive to noxious stimulation during the first 2 weeks of life, as long as maternal care is maintained (Levine, 1994, 2002; Lupien et al., 2009), but in preterm infants there is good evidence that intensive care procedures cause increases in plasma and salivary cortisol levels and in heart rate variability (Faye et al., 2010; Davis et al., 2011) that may be altered over longer periods (Grunau et al., 2010). It is not known whether this stress response directly influences the development of pain pathways in humans, but the immaturity of brainstem descending pain control pathways at birth (Hathway et al., 2009a, 2012) suggests that it could be highly modified by increased HPA activity.

The situation is complicated by the fact that development of the HPA axis itself is susceptible to a wide range of environmental, endocrine and immune stressors, some of which are also likely to be activated following neonatal injury. Important, too, is the other major stress axis, the sympathoadrenal system and its major mediator, the catecholamines, which have been shown to have a role in inducing and maintaining stress-induced enhancement of mechanical hyperalgesia in adults (Khasar et al., 2009).

The significant hypersensitivity of anoxic males to the mechanical pain compared to control, stereology confirmed this result by anoxic male showing significantly less cells when compared to females.

In conclusion, this study demonstrates that for same degree of anoxia insult male and female has affected thalamic circuit function in the Pain, as a submodality of somatic sensation. So, further investigation on sex differences and mechanical pain is required to enhance the understanding of the involved mechanisms of neonatal anoxia. Eventually, this study may be important for development of appropriately timed and targeted therapies for the protection of the neonatal brain and rescue of neurons after global neonatal anoxia.

VI.General Discussion

Delayed performance in neurobehavioral tests, motor coordination, impairment at cognition and spatial memory as well as a decrease in threshold for mechanical pain, were associated with morphological changes in neural tissue in this study. The current investigation complements previous data on neurological reflexes and somatic development at the suckling period (Kumar et al., 2017), which has relevant implications on clinical and educational fields. Moreover, a valuable contribution to understand this period of development is the approach of early sex difference, which has been sparsely considered.

There is a considerable bulk of studies on hypoxia in rodents however, many different models were used concerning the kind of stimulus (duration, age and the animal itself) which might be or not invasive and focal (damage to specific brain areas). The diversity of approaches has produced contradictory results in some parameters, which should be analysed under their peculiar conditions (Cruz-Ochoa, 2019). This research used a global and non-invasive model, performed at birth (30 h), an age that is comparable to human pre-term newborn (Semple et al., 2013, Takada et al., 2011) and so, it has clinical implications.

Neonatal anoxia differently affects body energy metabolism according to sex and age (Cruz-Ochoa 2019). This stimulus affects male and female differently, anoxia male weighs more than control and female weighs less than control. This pattern is observed since the early days confirming previous study (Kumar, 2017). In spite of this increased weight, anoxia male covered significant more distance in locomotion test. A result to be considered in relation to factors like neuroendocrine alterations in the hypothalamic adrenal axis; reduction of glucocorticoid increasing corticosteroids level and induction of impairment in the monoaminergic system (Flandreau et al., 2012, Krishnam et al., 2008). These alterations has been linked to hyperactive behavior in anoxia males (Kumar et al., 2019, Lubics et al., 2005, Nyakas et al. 1996).

However, both hyper- and hypoactivity were described following neonatal oxygen deprivation (Antier et al., 1998), (Balduini et al., 2000). Rats exposed to anoxia, presented postweaning transitory hyperactivity with increased sniffing, rearing and ambulation (Luvone et al., 1996). Balduini et al. (2000; 2001) found hyperactivity at 3 weeks of age, but no differences in spontaneous or in open-field activity at PND90. In contrast, hypoactivity was described in adult rats who underwent neonatal hypoxia/ischemia (Antier et al. 1998).

Several tests to evaluate motor coordination following neonatal oxygen deprivation have been described (Fan et al., 2005; 2006; Balduini et al., 2001). The battery test, used here, was chosen in order to access the eventual anoxia deficits in the suckling period, based on age and kind of injury on sensorimotor system.

The observed sensory motor alterations and the related hyperactivity gets support in the reduced number of cells in the substantia nigra compact dorsal here observed in anoxia, at both male and female. In addition, low level of dopamine in rat brain in association with injury in SNCD, previously described, confirms that locomotor disorders and reduced anxiety are associated (Boksa et al., 2004). A relationship that has been reinforced by the relation between altered HPA axis and monoaminergic systems functioning (Flandreu et al., 2012; Krishnan et al., 2008).

Oligodendrocytes immature stage vulnerability to hypoxic–ischaemic damage and haemorrhage might partly explain the impairment in pathways fibers (Back et al., 2001). Therefore, thinning of corpus callosum in anoxia male in addition to the hippocampal CA1 deficits in cell density (Kumar et al., 2019) favors the anxiety hyperactive behavior and the sensory motor impairments, which were more prominent in anoxia males.

Nevertheless, in contrast to human neonates, rats usually do not show gross functional deficits: they move like normal animals and do not display obvious postural and locomotor abnormalities (Jansen et al., 1996). However, there are several functional tests that actually show short- and long-term deficits after exposure to early oxygen deprivation, like the ones this research used. Animals with severe cortical damages can show recovery in some deficits (Felt et al., 2002), while others can be

detected only in adult life, having in mind that neonatal anoxia has long lasting effects. In early development certain reflexes and motor performance is influenced (Kiss et al., 2005; 2007; Lubics et al., 2005; Reglodi et al., 2003). Moreover, they can be linked to long-term functional deficit like memory and anxiety impairment (Kumar et al., 2019; Ten et al., 2003).

In neurobehavioral test this stimulus was able to reduce anxiety-like behavior and spatial memory deficits in adolescent rats. In the current study, sex-dependent responses to neonatal anoxia were observed as females showed alteration in anxiety than males and in spatial memory males gave retarded performance. Considering effect of injury on hippocampus, males showed more damages in DG and CA1 region when assessed at adolescence.

Data from elevated plus maze indicates that the number of open arm entries and the percentage of time spent on the open arm was significantly higher in anoxic animals than in control animals, indicating that anoxia leads to reduce the anxiety-like behaviors during adolescence. Anoxic females did not show differences in the %OAT and OAT during the rest session, thus indicating a decreased anxiety-like behavior in relation to males. Also anoxia female had shown higher exploratory behavior by spending more time in central quadrant. In accordance with our results reduction in HI-induced anxiety-like behavior has also been reported in other animal models of neonatal cerebral hypoxia-ischemia (Fan et al., 2006; Caputa et al., 2005; Ming Yan et al., 2012).

Ming Yang et al (2012) showed that neonatal HI results in decreased anxiety-like behavior, during the juvenile period of Sprague-Dawley rats, they showed that young rats with mild and severe perinatal hypoxia had low levels of anxiety compared to the control groups showing its anxiolytic effect in young rats (14-28 days). The same effect was observed by Sanches et al (2013), in this study rats subjected to HI on a postnatal day (7) showed an increased percentage of time in open arms and in the central quadrant of the EPM as compared to control groups at adulthood. Anoxia rats at early age were less anxious, however this behavior seems to be age dependent since at PND 90 they showed higher anxiety (Takada et al, (2016).

In this study, spatial memory impairments were related to more cell loss in CA1 field of hippocampus and DG by the anoxic male. The DG high sensitivity to oxygen deprivation, plays a role in memory and mood regulation, its relevance relays also because it is a key area of neurogenesis, even in the adult brain (Liam et al., 2013). Some research has related cognitive functions to neurogenesis in rodents and human clinical condition (Aimone et al., 2014).

The DG cell loss induced by anoxia could lead to disturbances in the neurogenesis mechanisms that reflect on impairments in hippocampal-dependent tasks and emotional regulation effect (Sanghee et al., 2016). Since loss of cells in dentate gyrus can leave animals with less strategy, we can see this tendency in anoxic males since, in spite of their swimming velocity be high, they cannot reach the destination. Lesions of this region, can disrupt performance of spatial task as animals shows less flexibility in applying strategy to perform the task (Xavier et al., 1999).

Hippocampal volume reduction and decreased neurogenesis rate after neonatal anoxia linked to cognitive deficits found in adolescent anoxic were observed (Takada et al., 2016. Adolescent anoxia female did all training well and didn't show impairement in performace of spatial memory task when compared to males (Kumar et al., 2019).

Relevant physiological impairment of neonatal anoxia, is the significant reduction in mechanical withdrawal threshold (around 50%) in relation to the control response. Then, reflecting hypersensitivity in chronic neuropathic pain at adolescence but, without sex differences. The pain test with von Frey monofilament, for mechanical allodynia evaluation, has been used in most studies of the pathways related to pain and injury to the nervous system (Chaplan et al., 1994; Chacur et al., 2018; 2019). Interestingly, the same stimulus could not evoke the mechanical pain response at early age (PND 18 figure 2) although, the animal already was able to positively respond to reflexes like cliff avoidance, negative geotaxis and acoustic startle reflex (Kumar et al., 2017). Probably the developmental nociceptive pathways at that age were still maturing (Schwaller and Fitzgerald, 2014) or the stimulus conditions were not able to evoke the right response may due to the alterations induced by neonatal anoxia (Souza et al. 2014).

The pain pathway comprises many stations in the nervous system, from periphery to central regions, and one must consider that this anoxia model, in spite of being noninvasive, it is also global and so, as it is performed at the age when rats development corresponds to a human fetus of 32 weeks, it simulates the condition of the pre-term neonates exposed to anoxic conditions, a clinical worldwide problem (Takada et al., 2011). In this way, the anoxia impact reaches the peripheric elements of the sensorimotor system as well as the central ones in the critical period of development, when an injury in early life has great impact than in later life (Walker, 2013). Therefore, it is an age sensible model, as was observed by decreased neurogenesis and higher cell death in anoxic animals at adulthood (Takada 2015a).

Probably, this hindlimb hypersensitivity had interfered in the results observed for sensorimotor development in addition to physical alterations, since the anoxia had delayed the majority of neurological reflexes, for example palmar grasp (Kumar et al., 2017). The human newborn developing brain injury has demonstrated to be highly responsive to pinching and noxious stimulation (Slater et al., 2010b; Fabrizi et al., 2011; Cornelissen et al., 2013).

As thalamus is a key structure in the nociceptive pathway and also in motor coordination, it is also necessary for the sensorimotor reflexes at both sensory and motor inputs to and from cortical areas. Neonatal anoxia, already, showed decreased cell density in the primary motor cortex and sensory hindlimb regions (Kumar et al., 2017).

This research revealed that anoxia male presents less Nissl stained cells in relation to female. However, NeuN immunoreactivity was significantly higher in females, what again shows that male was more susceptible to the stimulus. Probably the Nissl results are due to an altered glial population. In agreement, previous reports discuss the relation of these prosencephalic areas to nociception (Davis et al., 1998). Thalamic damage after hypoxia–ischemia in newborns has long been recognized and is particularly important in infants with extrapyramidal cerebral palsy (Malamud, 1950; Volpe, 1995; Roland et al., 1998). Human neuroimaging and neuropathological studies revealed that the thalamus is among the selectively vulnerable brain regions in the human newborn (Yokochi et al., 1991; Barkovich, 1995; Roland et al., 1998).

Injury to somatosensory thalamus has been described in human newborns after hypoxia–ischemia (Barkovich, 1995; Roland et al., 1998) and may contribute to observed sensorimotor deficits in infants with perinatal brain injury and cerebral palsy. In rodents, the invasive hypoxic–ischemic model showed sensorimotor deficits in neonatal rats (Rice et al., 1981; Bona et al., 1997). As well as injury in the developing thalamus (Towfighi et al., 1991). Thalamus showed delayed effects and exhibited prominent structural features of apoptosis when compared with the early necrotic cell death as seen in the forebrain after hypoxia–ischemia (Northington et al., 2001).

In oxygen deprivation models, several investigators have shown behavioral disabilities, such as disrupted social interactions, sleep disturbances, depressive- and anxiety-like behaviors (Li et al., 2015). Together with the altered neurological reflexes these behavioral data might have suffered the influence of the observed hyperalgesia beyond the decreased cell amount found in VPL or as shown previously in motor and sensory primary cortices (Kumar et al., 2017, 2018;).

The hypersentivity was equally observed at adolescence in both sexes after neonatal anoxia. In association to sex difference, we saw altered quantification of the cells at the VPL nucleus. These results reinforce the relevance of scientific and clinical approaches to consider both the sexes. In addition, it makes available a validated model to explore the mechanisms underlying the neonatal anoxia and possible strategies to minimize its effects.

This study validates the used neonatal anoxia model as an efficient tool to explore this clinical condition and so propose procedures and strategies to minimize its impact. In addition, it reinforces the relevance to consider the sensory motor disabilities early in life, and the sex differences, that due to neonatal anoxia nature, provoking long lasting impairments, impacts behaviour and cognition later in life along with pain perception.

VII. General conclusion

Our findings demonstrate that for same degree of insult male rat deficits were different from female in somatic and motor development. Also, in reference memory and anxiety test anoxia has impaired the output and we see differences in male and female performances. In nociceptive perception of pain anoxia is hypersensitive and histologically male shows more damage in Ventroposterolateral nucleus. Histologically SNCD,cc, hippocampus,VPL was affected and male was vulnerable.

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