ANA PAULA NASCIMENTO DE LIMA

Stress in adolescence: Hypothalamic-pituitary-adrenal axis dysfunctions and the long-term effects on behavior, neurochemistry and immune response in rodents

São Paulo

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Thesis submitted to the Postgraduate Program in Experimental and Comparative Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

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Prof. Dr. Cristina de Oliveira Massoco Salles Gomes,

São Paulo

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CERTIFICADO

Certificamos que o Projeto intitulado "ESTRESSE DE INSTABILIDADE SOCIAL: EFEITOS DE LONGO-PRAZO NO COMPORTAMENTO DE CAMUNDONGOS JOVENS", protocolado sob o CEUA nº 9297300915, sob a responsabilidade de **Cristina de Oliveira Massoco Salles Gomes** *e equipe; Cristiane Morais* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei 11.794, de 8 de outubro de 2008, com o Decreto 6.899, de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMZV) em reunião de 02/03/2016.

We certify that the proposal "SOCIAL INSTABILITY STRESS: LONG LASTING EFFECTS IN BEHAVIOR OF JUVENILE MICE", utilizing 80 Isogenics mice (80 males), protocol number CEUA 9297300915, under the responsibility of **Cristina de Oliveira Massoco Salles Gomes** *and team; Cristiane Morais* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes (or teaching) - it's in accordance with Law 11.794, of October 8 2008, Decree 6899, of July 15, 2009, with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of São Paulo (CEUA/FMZV) in the meeting of 03/02/2016.

Vigência da Proposta: de 11/2015 a 11/2017 Área: Fisiologia

Procedência:	Biotério do Departamento de Patologia da FMVZ USP						
Espécie:	Camundongos isogênicos	sexo:	Machos	idade:	21 dias	N:	80
Linhagem:	Balb/c			Peso:	15g		

Resumo: A adolescência é considerada um dos períodos críticos de desenvolvimento e de grande relevância para a saúde de um indivíduo quando adulto. Eventos estressores ou traumáticos durante este período estão associados a inúmeras alterações no desenvolvimento e na plasticidade do sistema nervoso central bem como o neuroendócrino. O estresse por instabilidade social é um modelo de estresse que provoca alterações nos comportamentos social e sexual de ratos. Entretanto, ainda não há estudos que investiguem alterações comportamentais em camundongos. Logo, o presente estudo busca avaliar os impactos na vida adulta de um estresse por instabilidade social na adolescência sobre o comportamento de camundongos. Para isso, serão utilizados camundongos Balb/c machos com 30 dias de vida que serão submetidos ao modelo de estresse por instabilidade social (10 dias).

São Paulo, 02 de março de 2016

Profa. Dra. Denise Tabacchi Fantoni
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretaria Executiva da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Animal Care Committee (ACC)
Chair –Jim Willwerth, PhD 905.688.5550 ext 5477
Clinical Veterinarians – Dr. Alistair Ker
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: August 15, 2017

Dear Dr. McCormick and Ms. Lima,

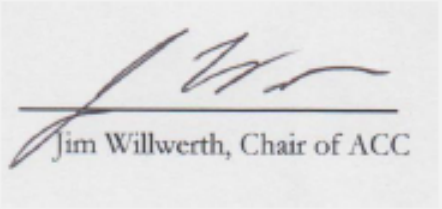
The Animal Care Committee has approved your "Animal Use Protocol (AUP)" entitled:

The effects of acute lipopolysaccharide challenge on Hypothalamic-Pituitary-Adrenal (HPA) function in females and male Long-Evans rats after social instability stress in adolescence.

This approval expires in one year on the last day of the month.
The number for this project is **AUP # 17 – 07 – 02.**
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 128 male & 128 female Long Evans rats

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.



THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING (PAM).
Arrangements for PAM must be made,
for one or more components of your study,
when animals are ordered.
N.B. Dr. Ker is here most Wednesday afternoons at 2:30.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.

EVALUATION FORM

Author: DE LIMA, Ana Paula Nascimento

Title: **Stress in adolescence:** Hypothalamic-pituitary-adrenal -axis dysfunctions and the long-term effects on behavior, neurochemistry and immune response in rodents

Thesis submitted to the Postgraduate Program in Experimental and Comparative Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

Date: _____/_____/_____

Committee Members

Prof. Dr._____
Institution:_____ Decision:_____

Prof. Dr._____
Institution:_____ Decision:_____

Prof. Dr._____
Institution:_____ Decision:_____

Prof. Dr._____
Institution:_____ Decision:_____

Prof. Dr._____
Institution:_____ Decision:_____

To all women who struggle against the male chauvinism and sexism of every single day in science and in life.

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Oh! And thank you for one more person, I mean, a cat. Thank you Atum for the purr and bites while I was writing the thesis.

“Viver não cabe no Lattes”
Autor desconhecido

ABSTRACT

DE LIMA, Ana Paula Nascimento. **Stress in adolescence:** Hypothalamic-pituitary-adrenal - axis dysfunctions on behavior, neurochemistry and immune response in rodents. [**Estresse na adolescência:** Disfunções no eixo hipotálamo-hipófise-adrenal e os efeitos de longo-prazo no comportamento, na neuroquímica e na resposta imune em roedores.] 2018. 92f. Thesis (PhD in Science) – School of Veterinary Medicine and Zootechnics, University of Sao Paulo, Sao Paulo, 2018.

Adolescence is considered one of the critical periods of development and of great relevance to the health of an individual as an adult. Stressor or traumatic events during this period are associated with numerous changes in the development and plasticity of the neuroimmuoendocrine system predisposing the individual to psychiatric disorders. However, studies that investigate neuroimmuno changes related to adolescence are still scarce in the literature. Therefore, the present study sought to evaluate the adult life impacts of two models of stress in adolescence on immune, neurochemical and behavioral parameters in rodents. The studies into this thesis present important results for understanding the HPA axis and how stressors during adolescence modulate the mechanisms involved in the neuroimmuno-endocrine and behavioral response in adult life. Briefly, our results show that stressful situations, during the developmental period of adolescence, homotypic or heterotypic models in a short term (10 -15 days), can be associated with long-lasting changes in neural pathways, behavior and immune parameters, eliciting a potential neuroimmuno-endocrine and behavioral vulnerability for the development of disorders related to stress response in adulthood and also with a greater opportunity to behavioral and physiological flexibility to respond to different environmental and social contexts.

Keywords: Neuroimmunomodulation. Stress. Adolescence

RESUMO

DE LIMA, Ana Paula Nascimento. **Estresse na adolescência:** Disfunções no eixo hipotálamo-hipófise-adrenal e os efeitos de longo-prazo no comportamento, na neuroquímica e na resposta imune em roedores. [**Stress in adolescence:** hypothalamic-pituitary-adrenal axis dysfunctions on behavior, neurochemistry and immune response in rodents.] 2018. 92f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

A adolescência é considerada um dos períodos críticos de desenvolvimento e de grande relevância para a saúde de um indivíduo quando adulto. Eventos estressores ou traumáticos durante este período estão associados a inúmeras alterações no desenvolvimento e na plasticidade do sistema neuroimunoendócrino predispondo o indivíduo a transtornos psiquiátricos. Entretanto, ainda são escassos na literatura os estudos que investigam as alterações neuroimunes relacionadas a adolescência. Logo, o presente estudo buscou avaliar os impactos na vida adulta de dois modelos de estresse na adolescência sobre parâmetros imunes, neuroquímicos e comportamentais em roedores. Os estudos presentes nesta tese apresentam resultados importantes para a compreensão do eixo HPA e de como estímulos estressores durante a adolescência modulam os mecanismos envolvidos na resposta neuroimuno-endócrina e comportamental na vida adulta. Em resumo, nossos resultados mostram que eventos estressores, durante o período da adolescência, sejam modelos homotípicos ou heterotípicos de curta duração (10 – 15 dias), estão associados com alterações a longo-prazo em padrões neurais, parâmetros comportamentais e imunes, levando a uma potencial vulnerabilidade neuroimune-endócrina para o desenvolvimento de transtornos relacionados a resposta ao estresse na vida adulta e também uma maior oportunidade de flexibilidade comportamental e fisiológica ao responder a diferentes ambientes e contextos sociais.

Palavras-chave: Neuroimunomodulação. Estresse. Adolescência

LIST OF FIGURES

SUMMARY

INTRODUCTION

Adolescence is the transition phase between childhood and adulthood. It is during this developmental stage that a great gain of strength, occurs in immune function and in cognitive abilities (CRONE and DAHL, 2012; EILAND and ROMEO, 2013). However, it is also when the individual presents a physiological and psychological vulnerability (ANDERSEN 2003; CRONE and DAHL, 2012). For example, psychiatric comorbidities, such as anxiety, depression, schizophrenia, and drug-related disorders of abuse increase during adolescence (SPEAR, 2000; ANDERSEN, 2003).

Rodents, more specifically mice and rats, are currently the most widely used animal species in biomedical research, including studies that investigate adolescence and the vulnerability of this transition phase. Therefore, this thesis comprises four different studies that comprise the neuroimmune-behavioral effects of a stress in the adolescence using rats and mice as experimental subjects. A brief review of the literature on adolescence in rodents follows.

1. ADOLESCENCE IN RODENTS

The gestation of the rodent has an averages period of 21 days. After birth, the puppy is dependent on parental care (for protection, warmth and breast milk) for a period ranging from the 14th to the 21st day of life. This phase is known as pre-weaning or neonatal.

After weaning, the animal is still considered an infant. Although the concept of puberty is unrelated to the concept of adolescence (best discussed below), this stage is known as prepubertal.

In rats, adolescence begins between the 30th and the 35th of life adolescence begins. As discussed previously, the terms adolescence and puberty were often treated as synonyms. However, current literature distinguishes and defines the two terms separately:

> Puberty is a distinct event in the development marked by substantial hormonal and somatic changes, occurring earlier in females than in males. Adolescence, however, is a term commonly used for the developmental stage that begins with puberty and ends with sexual

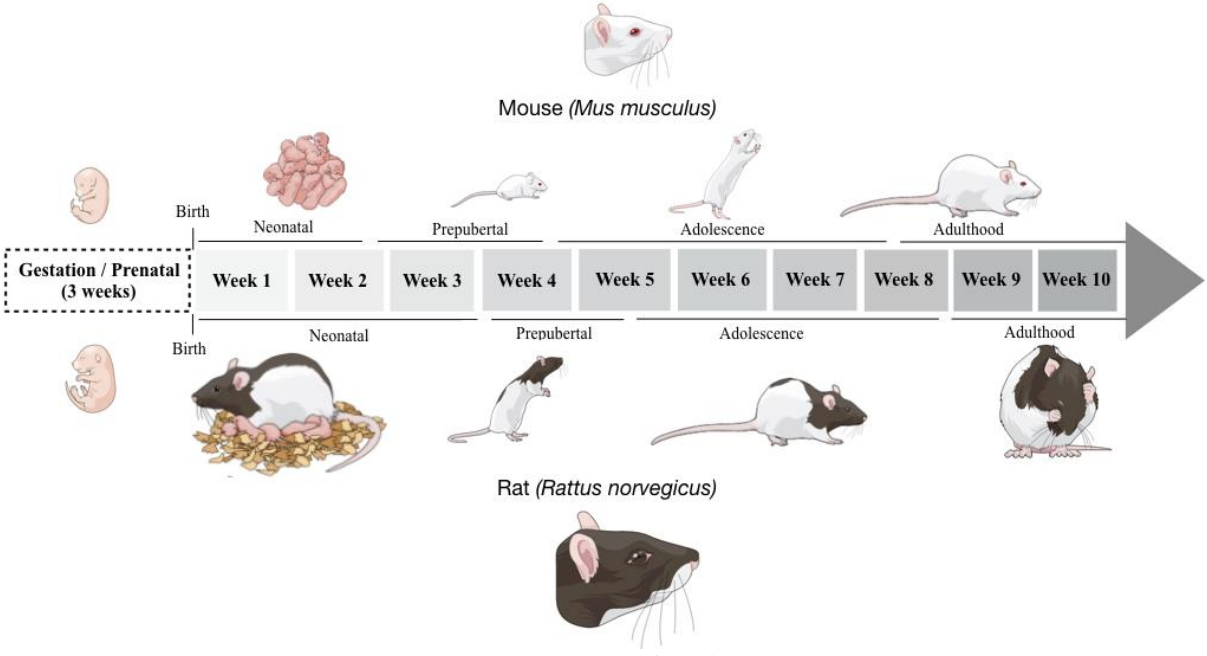
maturation and neurobehavioral characteristics compatible with the adult individual of the species (Sisk and Foster, 2004).

The specific age that covers adolescence in rat males and females is not entirely clear. However, between 30 and 60 days both sexes undergo behavioral and neurobiological transformations like those observed in other species during adolescence, including humans. In other words, it is at this stage that social behaviors, including play, mating attempts and aggressions, as well as the volume of brain gray and white matter, change to adult patterns (SPEAR, 2000; ROMEO and MCEWEN, 2006; EILAND and ROMEO, 2013)

In the case of mice, the period considered as adolescence is even more controversial. The highest rate of CNS development occurs during the first 30 days after conception, that is, from conception to PND 12 (FINLAY and DARLINGTON, 1995). Puberty occurs between PND25 and PND 40 and body growth only ends at PND 50. It is therefore reasonable to consider that the mouse is only considered adult at 50 days of age (BANDLOWSKI, 2014).

Figure 1 illustrates the life cycle of mouse and rat in weeks and its different phases, which also corresponds to the rodent life cycle according to the most recent revision on the subject (see EILAND and ROMEO, 2013).

Figure 1 – Life of a mouse and a rat in weeks and its different phases



Source: LIMA (2018).

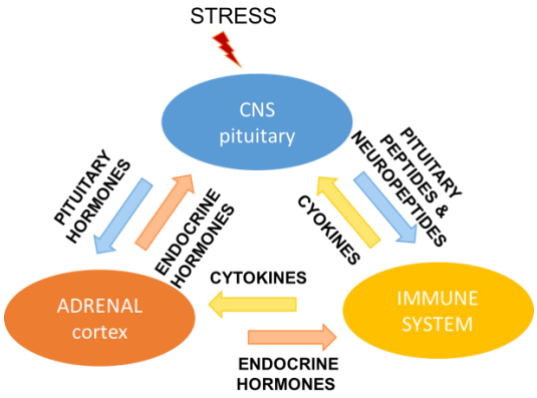
2. HYPOTHALAMIC-PITUITARY ADRENAL AXIS: AN IMPORTANT NEUROIMMUNE-ENDOCRINE CIRCUIT IN STRESS RESPONSE

The hypothalamic-pituitary-adrenal (HPA) axis is one of the most important neuroendocrine-immunomodulation circuits in stress response (Fig. 2). A stressful stimulus activates the HPA axis by different circuits: sympathetic, parasympathetic and limbic circuits. These three circuits activate the paraventricular nucleus (PVN) of the hypothalamus to release corticotropin-releasing hormone (CRH) and vasopressin (AVP). These neuropeptides go to the hypothalamus-pituitary portal system and binding to the corticotrophs in the anterior pituitary. This binding of neuropeptides-corticotrophs causes the processing of pro-opiomelanocortin (POMC) into adrenocorticotrophic hormone (ACTH), which is systemically released and sensitizes the adrenal cortex to release glucocorticoids (KOLBER et al., 2008).

These hormones, including the corticosterone (CORT), play a key role in the negative feedback decreasing HPA axis activation. However, the release and systemic action of glucocorticoids, due to a stress response, is not limited to HPA axis regulation, but it is much broader and more complex. These hormones bind to glucocorticoids receptors (GRs), have effects on metabolism, fat deposition, bone metabolism, and regulating diverse cellular functions, such as development, homeostasis, metabolism and cognition (RAMAMOORTHY and CIDLOWSKI, 2016). Furthermore, immune cells, such as, T cells, NK cells, macrophages, dendritic cells and B cells, have GRs. Thus, glucocorticoids, also cause effects on immune cells and regulates the immune response.

Finally, the third sphere of this regulatory circuit involves the immune system and its modulators. There are innumerable cell types, receptors, hormones and bio-compounds that play any immune-related function, including the cytokines released that have a specific effect on the interactions and communications between cells and in the neuroimmune-endocrine circuit. These small proteins of different types secreted by immune and non-immune cells can markedly affect neurotransmission within regulatory brain circuits for emotion responses and induce hormonal changes similar to those observed following stressor exposure (GADEK-MICHALSKA et al., 2013).

Figure 2 – Schematic illustration of the neuroimmune-endocrine circuit in stress response



Source: Lima (2018)

3. NEUROIMMUNE-ENDOCRINE AND BEHAVIOR DYSFUNCTIONS AFTER ADOLESCENT STRESS IN RODENTS

According to current literature, stressors or traumatic events during the adolescence are associated with several psychiatric disorders as related to anxiety or depression and cognitive impairments. Moreover, dysregulation of the neuroimmune-endocrine system is one of the fundamental biological mechanisms that underlie psychiatric disorders. As already mentioned, in a regular and healthy stress response, the GC's negative feedback decreased the HPA axis activation. However, chronic stress can induce inhibition of this feedback. Indeed, hyperactivity of the HPA axis is one of the most reliable biological findings in patients suffering from psychiatric disorders (KOLBER et al., 2008).

In the last 10 years, the number of studies they aimed to investigate the interaction between stress during adolescence and neuroimmunoendocrine and behavioral disorders in rodents increased. There is already evidence after a social or variable stress, both male rats and mice, shows an increase in the behavior of the anxious-like behavior (MCCORMICK et al., 2008; GIOVANOLI et al., 2013; CARUSO et al., 2018) . In addition, other studies had shown an emotional arousal after an unpredictable and social-environmental stress and a cognitive inflexibility after social defeat stress (XU et al., 2018).

The social defeat model is a model of effort induction widely used in studies with adult rodents and can be used as a model of adolescent bullying. In this respect, an interesting study investigated the role of the MAX transcription factor during adolescence, demonstrating that males after a social defeat stress for 21 days (PND 30 - PND51) presented a depressive

phenotype, represented by a decrease in sucrose preference and an increase in immobility in forced swim test (RESENDE et al., 2016).

Another model of stress induction, the social instability model, also found in adolescence studies, already demonstrates an increase in aggressive behavior (CUMMING et al., 2014) and an impairment in spatial memory (Green and Mccormick, 2013), in social interaction (GREEN et al., 2013; HODGES et al., 2017) and in social recognition performance (Hodges et al., 2017) in rats; and a passive adaptation to the response to stress in mice (DE LIMA and MASSOCO, 2017).

In order to correlate with behavioral changes due to stress, some studies have also shown that neurotransmitters such as dopamine, serotonin and noradrenaline are decreased in some areas of the CNS, such as, hypothalamus, hippocampus and cortex (DE LIMA et al., 2017; PAGE et al., 2018). In addition, Hodges et al. (2017) demonstrated an increase in oxytocin receptor binding, which plays an important role in social behavioral.

Although the most of investigations in the area focuses on studies with males, there is an increase interest in females and in comparing both sexes. As well as males, it is known that stressed females in adolescence tend to present an increase in anxiety-like behavior. In addition, recently Ganguly et al. (2018) demonstrated that males as females exhibited increased expression of IBA-1 after maternal separation followed by food restricted in adolescence, suggesting that of early-life stress could affect microglial responsivity to subsequent challenges. IBA-1 that is a microglia/macrophage-specific protein which plays an important role in the actin-bundling activity and participates in membrane ruffling and phagocytosis in activated microglia. (OHSAWA et al., 2004)

4. JUSTIFICATION, HYPOTHESES AND AIMS

It has been demonstrated that adolescents have greater stress hormone response than adult individuals (ROMEO, 2010; MCCORMICK AND MATHEWS, 2007) and their developing CNS is much more responsive to stress hormones compared to adults (GIEDD AND RAPOPORT, 2010). Therefore adolescence becomes an especially important phase for attempt to clarify the role of stress in neuroimmunomodulation, long-term dysfunctions on the HPA axis, and the susceptibility and development of psychiatric disorders..

Thus, our hypothesis are:

- Stress during adolescence would be related to long-term monoaminergic and behaviors dysfunctions in male mice.
- Stress during adolescence would be related to depressive-like and anxiety-like behaviors in adulthood of male mice.
- Stress during adolescence would change immune response in adulthood of male mice after an acute LPS challenge.
- Male and female stressed rats during adolescence would show different sickness behavior and corticosterone profile after an acute LPS challenge.

Considering the above-mentioned state of the art and hypothesis, the main aim is to investigate the effects of stress during adolescence in adult rodents on behavioral and immune responsiveness, and the specific aims are:

- To clarify the regulatory effects of adolescent stress in brain monoamine activity, as well as possible behavioral changes in male mice.
- To test the long-term behavioral effects of short-term social instability stress in male mice.
- To investigate the effects in adulthood of a socio-environmental stress model during adolescence on sickness behavior, TNF-α plasmatic levels and splenic immune cell populations frequency after an acute LPS challenge, seeking an overview of the immune status after an immune stressor of an adult mouse stressed during adolescence.
- To better understand the mechanisms for age and sex differences in communication of the HPA axis and the immune system.

5. DESCRIPTION AND SUMMARY OF CHAPTERS

In this section, we present a summary of each chapter.

5.1 Chapter 1

The first chapter gave rise to an article published in the scientific journal Behavioral Brain Research in 2017. It addresses the long-term effects on the behavior and neurochemistry of mice subjected to a socio-environmental stress model during adolescence. In this study, we determined how stress during adolescence affects behavior and neurochemistry in adulthood. Using an unpredictable paradigm (2 stressors per day for 10 days) in Balb/c mice, behavioral, hormonal, and neurochemical changes were identified 20 days after the cessation of treatment. Adolescent stress increased motor activity, emotional arousal and vigilance, together with a reduction in anxiety, and affected recognition memory. Furthermore, there was decreased serotonergic activity on hippocampus, hypothalamus and cortex, decreased noradrenergic activity on hippocampus and hypothalamus, and increased dopamine turnover in cortex. These data suggest behavioral phenotypes associated with emotional arousal, but not depression, emerge after cessation of stress and remain in adulthood. Social-environmental stress can induce marked and long-lasting changes in HPA resulting from monoaminergic neurotransmission, mainly 5-HT activity.

5.2 Chapter 2

The second chapter gave rise to the short communication published in the International Journal on the Biology of Stress in 2017. This study reports that short-term social instability stress (SIS) in adolescence increases passive-coping in adulthood in male mice. Short-term SIS decreased the latency of immobility and increased frequency and time of immobility in tail suspension test. These findings support the hypothesis that adolescent stress can induce a passive adaptation to stress in adulthood, even if it is a short period of stress.

5.3 Chapter 3

Previous work from our group (see Chapter 1) has shown that social-environmental stress during adolescence in mice affects their neurochemistry and behavior in adulthood. Therefore, in this chapter, we hypothesized that a stressed adolescent would be more sensitive to a single LPS-challenge in adulthood. We found that stressed animals have a prolonged sickness behavior after LPS injection and showed a decreased in populations of splenic NK

cells and T cells while showing an increase in monocytes of spleen. The results suggest that socio-environmental stressors during adolescence can induce marked and long-lasting changes in LPS-induced TNF-α released, affecting the sickness behavior and the peripheral immune cells population dynamic

5.4 Chapter 4

The forth chapter was the result of a one-year internship in the laboratory of Dr. Cheryl McCormick of Brock University in Canada. The chapter does not show all the results obtained during the stage and the project. There are some experiments to be performed and some results to be analyzed. In this chapter, we investigated how adolescent social instability stress (SS; from postnatal day [P] 30-45, daily 1-hour isolation + new cage partner) influenced responding to an immune challenge either soon after SS or several weeks later, compared with non-stress controls (CTL) in Long Evans male and female rats. At either P46 or P70, rats were injected with a low dose of lipopolysaccharide (LPS 0.1 mg/kg) or vehicle (saline). Sickness behaviour and plasma corticosterone concentrations were determined at 1, 2, 4, 6, and 24 h after injection. The main results (all $p < 0.05$), in brief, showed that males showed sickness behaviour earlier and for longer than did females after LPS, and SS males showed more sickness behaviour than did CTL males at P46. Among LPS-treated, CTL females had higher corticosterone concentrations at 1 hr and 6 hr than did SS rats, whereas SS had higher corticosterone concentrations at 2 hr than did CTL rats. In males, SS rats had higher corticosterone concentrations than CTL rats irrespective of treatment. Saline-treated rats showed no sickness behaviour and had lower corticosterone concentrations than did LPS-treated rats. These results indicate that stress in adolescence can lead to sex-specific, long-lasting, altered responses to an immune challenge. Thus, stress in adolescence may influence health outcomes in adulthood.

REFERENCES

BANDLOWSKI, A. Defining Adulthood. Available at: < **https://neuinfo.org/about/nifblog/500** > Access in: June 23th 2018.

CARUSO, M. J. et al. Adolescent Social Stress Increases Anxiety-like Behavior and Alters Synaptic Transmission, Without Influencing Nicotine Responses, in a Sex-Dependent Manner. **Neuroscience,** v. 373, p. 182-198, 2018.

CRONE, E. A.; DAHL, R.E. Understanding adolescence as a period of social-affective engagement and goal flexibility. **Nat Rec Neurosci**, v. 13, n. 9, p.636-550, 2012.

CUMMING, M. J.; THOMPSON, M. A.; MCCORMICK, C. M. Adolescent social instability stress increases aggression in a food competition task in adult male Long-Evans rats. **Dev Psychobiol,** v. 56, n. 7, p. 1575-88, 2014.

EILAND, L.; ROMEO, R.D. Stress and the developing adolescent brain. **Neuroscience**, v. 249, p 162-71, 2013.

FINLAY, BL; DARLINGTON, RB. Linked regularities in the development and evolution of mammalian brain. **Science New Series**, v. 268 (5217), Jun 1995.

GADEK-MICHALSKA, A.; TADEUSZ, J.; RACHWALSKA, P.; BUGAJSKI, J. Cytokines, prostaglandins and nitric oxide in the regulation of stress-response systems. **Pharmacol Rep**, v. 65, n. 6, p. 1655-62, 2013.

GANGULY, P. et al. Adolescent food restriction in rats alters prefrontal cortex microglia in an experience-dependent manner. Stress, v. 21, n. 2, p. 162-168, Mar 2018.

GIEDD, J.N., RAPOPORT, J.L. Structural MRI of pediatric brain development: what have we learned and where are we going? **Neuron**, v. 67, p. 728-734, 2010.

GREEN, M. R.; BARNES, B.; MCCORMICK, C. M. Social instability stress in adolescence increases anxiety and reduces social interactions in adulthood in male Long-Evans rats. **Dev Psychobiol,** v. 55, n. 8, p. 849-59, Dec 2013.

GREEN, M. R.; MCCORMICK, C. M. Effects of social instability stress in adolescence on long-term, not short-term, spatial memory performance. **Behav Brain Res,** v. 256, p. 165-71, Nov 1 2013.

HODGES, T. E. et al. Social instability stress in adolescent male rats reduces social interaction and social recognition performance and increases oxytocin receptor binding. **Neuroscience,** v. 359, p. 172-182, Sep 17 2017.

KOLBER, B. J.; WIECZOREK, L.; MUGLIA, L. J. HPA axis dysregulation and behavioral analysis of mouse mutants with altered GR or MR function. **Stress**, v. 11, n. 5, p. 321-38, 2008.

MCCORMICK, C.M., MATHEWS, I.Z. HPA function in adolescence: role of sex hormones in its regulation and the enduring consequences of exposure to stressors. **Pharmacol. Biochem. Behav.**, v. 86, p. 220-233, 2007

MCCORMICK, C. M.; SMITH, C.; MATHEWS, I. Z. Effects of chronic social stress in adolescence on anxiety and neuroendocrine response to mild stress in male and female rats. **Behav Brain Res,** v. 187, n. 2, p. 228-38, Mar 2008.

OHSAWA, K.; IMAI, Y; KOHSAKA S. Microglia/macrophage-specific protein Iba1 binds to fimbrin and enhances its actin-bundling activity. **J Neurochem**. v. 88(4), p. 844-56, Feb 2004.

PAGE, C. et al. Npas4 deficiency interacts with adolescent stress to disrupt prefrontal GABAergic maturation and adult cognitive flexibility. **Genes Brain Behav**, Jan 2018.

RESENDE, L. S. et al. Social stress in adolescents induces depression and brain-region-specific modulation of the transcription factor MAX. **Transl Psychiatry,** v. 6, n. 10, p. e914, Oct 11 2016.

ROMEO, R. D.; MCEWEN, B. S. Stress and the adolescent brain. **ANN N Y Acad**, v. 1094, p. 202-14, 2006.

RAMAMOORTHY, S.; CIDLOWSKI, J. A. Corticosteroids: Mechanisms of Action in Health and Disease. **Rheum Dis Clin North Am**, p. 15-31, 2016

SISK, C. L.; FOSTER, D. L. The neural basis of puberty and adolescence. **Nat Neurosci**, v. 7, n. 10, p.1040-7, 2004.

SPEAR, L.P. The adolescent brain and age-related behavioral manifestations. **Neurosci Biobehav Rev**, v. 24, n. 4, p. 417-63, 2000.

XU, H. et al. Effects of adolescent social stress and antidepressant treatment on cognitive inflexibility and Bdnf epigenetic modifications in the mPFC of adult mice. **Psychoneuroendocrinology,** v. 88, p. 92-101, Feb 2018.

CHAPTER 1: LONG-LASTING MONOAMINERGIC AND BEHAVIORAL DYSFUNCTIONS IN A MICE MODEL OF SOCIAL ENVIRONMENTAL STRESS DURING ADOLESCENCE[1]

1. INTRODUCTION

The experiences in perinatal phase are assimilated by the central nervous system (CNS) and contribute to the formation of innervation patterns. However, it is during adolescence that there is a neuronal reorganization in several regions of the mammalian CNS (ANDERSEN, 2003), similar to that seen in humans (GIEDDI et al, 1999) other primates (LIDOW et al, 1991; ROSENBERG et al, 1995) and rodents (TEICHER et al, 1995).

It is during this rearrangement of synapses and receptors that changes in the neurotransmitter pathways occurs (BARKS et al, 1988; SEEMAN et al 1987) and a decreased synaptic estimated at 40%(RAKIC, 1991), which can cause important adaptive functional changes as the development of abstract reasoning (BAIRD et al 1999; SPEAR, 2000) and maturation of motor, cognitive and emotional skills. Regarding mammalian development strategy, adolescents respond to stress differently than during any other stage of life (GUNNAR et al, 2009). In addition, the current literature shows that stress response to adverse social-environmental experiences can be associated with lasting changes in behavioral, neurobiology and neuroendocrinology levels (AVITAL et al 2006; BUWALDA et al 2005; KOOLHAAS et al 1997; MCCORMICK & MATHEWS, 2010; MCKITTRICK et al, 2000; ROMEO & MCEWEN, 2006), predisposing animals to the development of psychopathology such as depression and anxiety disorders in adulthood (ANDERSEN, 2003; HEIM & NEMEROFF, 2001; MCCORMICK & GREEN, 2013; MCCORMICK & MATHEWS, 2010; WATT et al, 2009).

In fact, recent studies show that rodents subjected to stress inducing models during adolescence are more anxious and less motivated, symptoms similar to those seen in humans with depression or anxiety disorders (JACOBSON-PICK & RICHTER-LEVIN, 2010; POST et al, 2014). In addition, situations of fear and ill-treatment during adolescence in humans

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increase the risk of aggressive behavior and impairment of social interaction in adulthood (CASPI et al, 2002; JONSON-REID et al, 2010; PEREPLETCHIKOVA & KAUFMAN, 2010), as well in other species (CORDERO et al, 2012; MARQUEZ et al, 2013; VEENEMA & NEYMANN, 2009; VEENIT et al, 2013; WOMMACK & DELVILLE, 2003). Many studies have evaluated the alterations in monoaminergic activity and behavior in adult rodents subjected to stress (BHUTANI, 2009).

Unpredictable stress models are useful tools for the study of these dysregulations that are closely related with affective and emotional disorders. However, in adolescent rodents, long-term effects of unpredictable stress are still poorly characterized. Thus, the aim of this study was to clarify the regulatory effects of adolescent stress in brain monoamine activity, as well as possible behavioral changes.

2. METHODS

2.1. Animals

Forty-four male Balb/c mice (4 weeks; 20 ± 2 g) from the Department of Pathology (School of Veterinary Medicine, University of São Paulo) were used. Animals were housed in groups of five per cage in a controlled environment (22 ± 2 ◦C temperature and 55–65% humidity) and in artificially lighted rooms on a well-defined 12 h light/dark cycle (lights on at 6:00 am). Food and water were provided ad libitum throughout the experiment except during stress sessions and behavioral testing. Mice were acclimated to the new environment for a week before experimental procedures. The experiments were performed in accordance with the guidelines of the Bioethical Committee on Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of São Paulo, Brazil (protocol no 4485180614), which are similar to those of the National Research Council, USA. All animals were weighed every three days and the weight gain was calculated.

2.2. Model of unpredictable stress in adolescence

Mice were randomly split into two groups: control animals (C) and stress-exposed animals (S). The stressed group were submitted to an unpredictable stress model and subjected to a random application time and pattern of stressful situations for 10 days with adaptations of protocol of Cox et al. (2011) as described in Table 1. The control group remained in its home-cage (5 animals per cage) throughout the experimental protocol and left undisturbed. All stressors of unpredictable stress are described in Supplementary material (Table 1).

Table 1 - Schedule of stressors for the unpredictable stress group

Day	Time of 1st Stressor	1st Stressor	Time of 2nd Stressor	2nd Stressor
1	8:00 AM	Exchange of residents	18:00 PM	Wet Bedding (24 h)
2	10:00 AM	Cold (30 min)	17:00 PM	Restraint (1 h)
3	9:00 AM	Resident/intruder (5 min)	16:30 PM	Forced Swimming (4 min)
4	6:00 AM	Slanted cage (24 h)	18:00 PM	Wet bedding (24 h)
5	8:30 AM	Exchange of residents	19:00 PM	Dirty bedding (24 h)
6	9:00 AM	Water deprivation (24 h)	15:30 PM	Cold (30 min)
7	11:00 AM	Restraint (2 h)	18:00 PM	Resident/intruder (5 min)
8	9:30 AM	Forced Swimming (4 min)	17:30 PM	Slanted cage (24 h)
9	8:00 AM	Exchange of residents	18:00 PM	Water deprivation (24 h)
10	7:00 AM	No bedding (6 h)	16:30 PM	Restraint (1 h)

Source: LIMA (2018)

2.3. Behavioral assessment

Behavioral tests were performed for evaluated changes in activity, anxiety, depression, learning and memory. All behavioral experiments were always performed during the evening (2:00 to 4:00 PM), in order to minimize possible interference from changes in the circadian rhythm. Before each session, the apparatus was cleaned with a 5% solution of alcohol in water. This procedure has been used in order to eliminate olfactory cues of the previous animal, and thus avoid potential interference with the behavior of animals evaluated. Twelve animals of each group were subjected to the open field test (OF), light-dark test and tail suspension test (TST), with a 24 h interval between each test. The other twenty-two animals were subjected to the novel object recognition test.

2.3.1. Open field test (OF)

Individual mice were placed in a circular arena (50 × 50 cm) and allowed to explore for 5 min while being recorded overhead (HALL, 1934). Total distance traveled (cm), frequency and time (s) in center and peripheral zone were analyzed with the use of Ethovision® XT-7 (Noldus Information Technology®, Leesburg, VA, USA). One day following the last day of stress protocol, all animals of both groups were submitted in open field test to evaluate the short-term effects in general locomotor activity. Twenty days later, the animals were in adulthood, new tests were carried to evaluate the long-term effects of stress in OF.

2.3.2. Light-dark box test

A box (45 cm × 20 cm) was used with an open white chamber connected by a square door (5 cm × 5 cm) to another covered black chamber (CRAWLEY AND GOODWIN, 1980). The black chamber occupied one-third of the box. Each mouse was placed in the center of the white chamber and recorded for 5 min Video recordings were later analyzed with Ethovision® and the time latency for the first entry in dark chamber, the time spent in each chamber, as well as number of transitions between them, was noted. To control for odor cues, the box was thoroughly cleaned with 5% ethanol, dried, and ventilated for a few minutes between mice.

2.3.3. Tail suspension test (TST)

This test was performed essentially as described (Steru et al, 1985). Briefly, immobility was measured during 6 min of session while the mice were suspended by the midpoint of the tail, 40 cm above the surface. Time spent immobile, frequency of immobility and latency to the first immobility on this test were used to measure behavioral despair. The test was assessed by a blinded observer.

2.3.4. Novel object recognition test.

This task is based on the innate tendency of rodents to differentially explore novel objects over familiar ones. This test was performed for three days. On the first day, mice were placed into an OF apparatus just for habituation. On second day, 24 h later, in the training trial, the animals were presented with a pair of identical objects until they had explored the objects for 20 s in a 5-min period. Finally, on the third day in the testing trial, one of the familiar objects was changed for another object and the animals were left in the OF for 5 min. Exploration of the objects is considered as any investigative behavior (head orientation or sniffing). The exploration time for the familiar object (FO) or the novel object (NO) during the test phase was recorded and the time spent with the novel object was calculated in percentage. To control for odor cues, the OF arena and the objects were thoroughly cleaned with 5% ethanol, dried, and ventilated for a few minutes between mice. The test was assessed by a blinded observer.

2.4. Adrenal weight

Right adrenals of animals were collected after euthanasia. The relative adrenal weight (organ weight/body weight) was calculated.

2.5. Corticosterone (CORT)

After sixty-five days of life mice were rapidly decapitated. Trunk blood was collected between 8:00 and 10:00 a.m. to avoid possible effects of circadian variations on serum corticosterone levels. Samples were stored at −80 ◦C until biochemical testing. Corticosterone serum levels were determined by the enzyme linked-immunosorbent assay (ELISA) method using a commercial CORT enzyme immunoassay kit (ARBOR ASSAYS®). All samples were analyzed in duplicate and were run in just one assay. The results were expressed in ng/mL, with sensitivity set at 18.6 ng/mL and the limit of detection set at 16.9 ng/mL.

2.6. Monoamines and their metabolites levels

At postnatal day (PND65), mice from both groups were decapitated. The brains were dissected on dry ice and prepared as previously described (FELICIO et al, 1996). Briefly, the prefrontal cortex, hypothalamus, and hippocampus were weighed and stored at −80 ◦C until neurochemical analyses were carried out. Following sample collection, perchloric acid was added to the tissues, which were then homogenized by sonication for immediate determination of the monoamine levels. Dopamine (DA) and its metabolites 3,4- dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), serotonin (5-HT) and its metabolite 5-hydroxyindolacetic acid (5- HIAA) and noradrenaline (NOR) its metabolite vanillylmandelic acid (VMA). Concentrations were measured by HPLC (Shimadzu, model 6A) using a C-18 column (Supelco®, Sigma), electrochemical detector (Shimadzu, model 6A), sample injector (15 and 20 ml valve) and an integrator (Shimadzu, model 6A Chromatopac). Each sample was run for 18 min. The detection limit was 0.2 ng for all of the analyses.

2.7. Data analysis

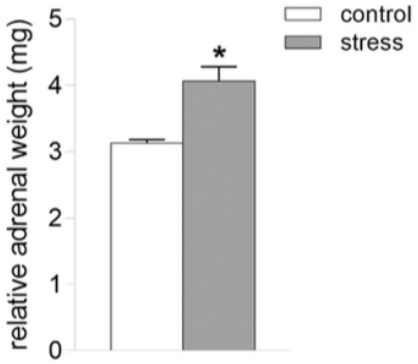
The data were analyzed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). OF test data were analyzed by repeated measures variance ANOVA with treatment and time as factors and Sidaks multiple comparisons test was used to compare data between groups. Other behavioral data, adrenal relative weight, corticosterone and monoamine concentrations data were analyzed by Student's t-tests. Statistical significance was set at $p < 0.05$ with 95% confidence interval. Data are reported as the mean ± SD.

3. RESULTS

3.1. Adolescent unpredictable stress increased adrenal relative weight

Fig. 3 shows that adrenal relative weight of stressed animals group was statistically increased when compared with control group (C = 3.13 mg ± 0.21; S = 4.06 mg ± 0.05; t = 4.25, df = 10; $p < 0.05$).

Figure 3- Effects of stress in relative adrenal weight (mg)

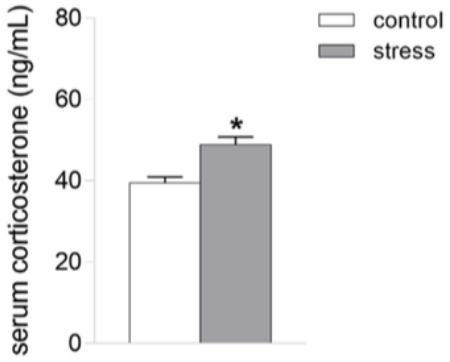


These data were analyzed by Student's t-test (*$p < 0.05$).

3.2. Adolescent unpredictable stress alters the reactivity of the HPA axis in adulthood.

Stressed animals showed higher levels of serum corticosterone compared to control animals (C = 39.43 ng/mL ± 1.50; S = 48.81 ng/mL ± 1.96; t = 3.79, df = 14; $p < 0.05$) (Fig. 4).

Figure 4 - Corticosterone serum evaluation. Stressed mice had increased levels of serum corticosterone (ng/mL).



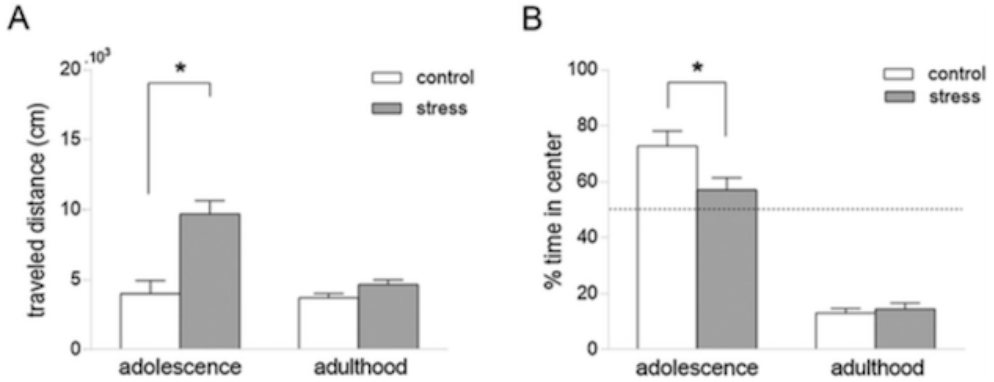
These data were analyzed by Student's t-test (*$p < 0.05$).

3.3. Adolescent unpredictable stress alters behavioral response in adulthood.

3.3.1. Open field test

On total traveled distance, there was a statistically significant effect on period of life [F (1,8) = 17; $p < 0.05$], treatment [F (1,8) = 25; $p < 0.05$], with interaction between both factors [F (1,8) = 6.6; $p < 0.05$]. Sidak's multiple comparisons test revealed stress increases total traveled distance in adolescence ($p < 0.05$) (Fig. 5A). Furthermore, as seen in Fig. 5B, on percentage of time in center, there was a significant effect on period of life [F (1,8) = 192; $p < 0.05$], treatment [F (1,8) = 5.9; $p < 0.05$], with interaction between both factors [F (1,8) = 5.9; $p < 0.05$]. Sidak's multiple comparisons test revealed stress increases total traveled distance in adolescence ($p < 0.05$).

Figure 5 - Behavioral assessment. Traveled distance (cm) (A) and percentage of time spent in center (B) of OF.



These data were analyzed by two-way ANOVA followed by Sidak's multiple comparisons test (*$p < 0.05$).

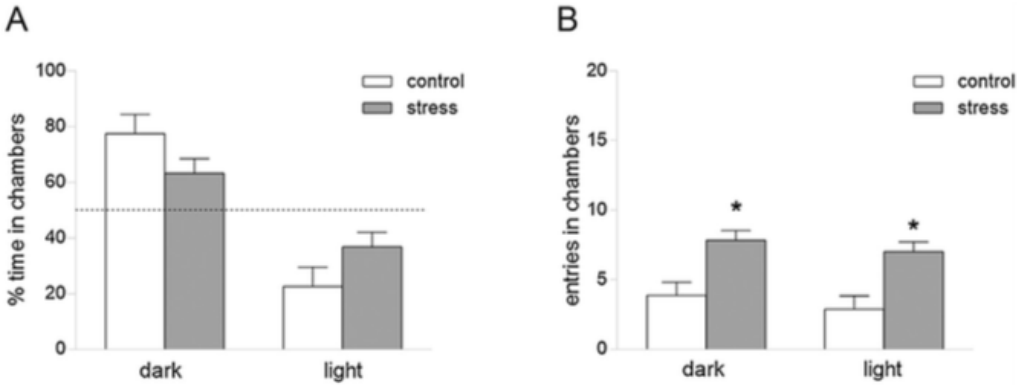
3.3.2. Light-dark box test

According to Fig. 6A. no difference between groups was observed in percentage of time in dark and in light chambers. However, according to Fig. 6B, stressed group presented an increase in the number of entries in dark (C = 3.89 ± 0.95; S = 7.83 ± 0.71; t = 3.41, df = 19; $p < 0.05$) and in light (C = 2.89 ± 0.94; S = 7.00 ± 0.73; t = 3.49, df = 19; $p < 0.05$) chambers of the box test.

3.3.3. Tail suspension test

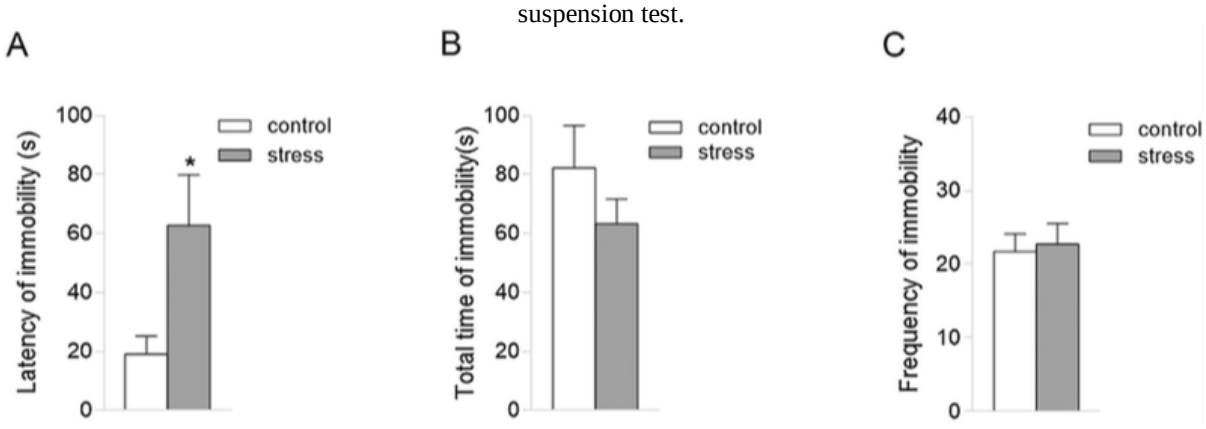
As illustrated in Fig. 7, stressed group presented an increased in latency of immobility (C = 19.10s ± 6.14; S = 62.80s ± 17.07; t = 2.40, df = 18; $p < 0.05$), but there were no differences in time and frequency of immobility when compared with control group.

Figure 6 - Behavioral assessment. Percentage of time spent in dark and light chambers (A) and frequency of entries in both chambers (B) of dark/light box.



These data were analyzed by Student's t-test (*$p < 0.05$).

Figure 7 - Behavioral assessment. Latency (s) (A), total time (s) (B) and frequency (C) of immobility in tail suspension test.



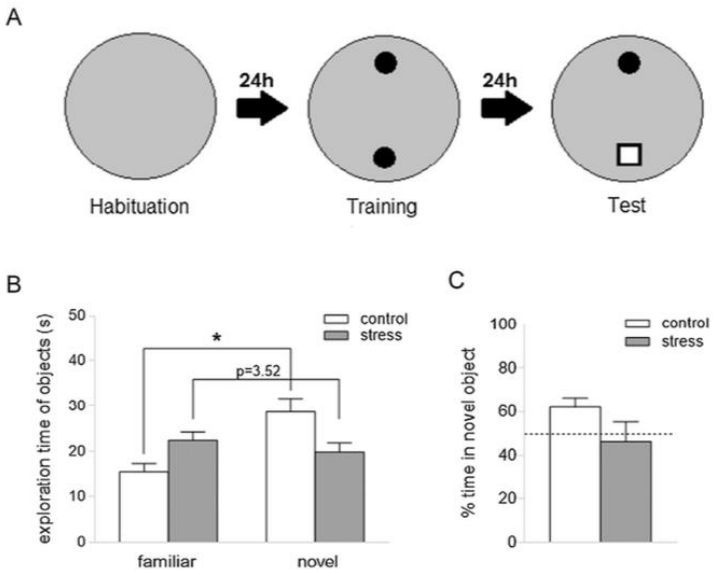
These data were analyzed by Student's t-test (*$p < 0.05$).

3.3.4. Novel object recognition test

According to Fig. 8 in the test phase, there were no differences in time of exploration between the FO and NO objects by stressed group (FO = 22.48s ± 1.82; NO = 19.93s ± 1.97; t = 0.95; df = 20; p = 0.352), while control group explored the novel object more (FO = 15.62s

± 1.84; NO = 28.78s ± 2.99; t = 3.73; df = 20; p < 0.05). However, there were no significant differences in the percentage of time in novel object between the control and stressed groups.

Figure 8 - Behavioral assessment. Schematic representation of novel object recognition test (A). Total object exploration time of familiar and novel objects (B). Percentage of time of novel object exploration in the test trail (C).



These data were analyzed by Student's t-test (*p < 0.05).

3.4. Adolescent unpredictable stress alters concentrations of monoamines and their metabolites in adulthood**.**

3.4.1. Serotonin

There was a significant decrease in cortical 5-HT (C = 1.49 ng/mg ± 0.14; S = 1.09 ± 0.12 ng/mg; t = 2.17, df = 20; p < 0.05) (Fig. 9A) and a significant increase in turnover of cortical 5-HT in stressed group (C = 0.46 ± 0.02; S = 0.81 ± 0.16; t = 2.10, df = 20; p < 0.05) (Fig. 9B). Furthermore, stressed group presented a significant decrease in 5-HT (C = 1153 ng/mg ± 120.5, S = 85 ng/mg 5 ± 69.37; t = 2.14, df = 16; p < 0.05) and in 5HIAA (C = 1471 ng/mg ± 119.7; S = 1030 ng/mg ± 77.0; t = 2.72, df = 16; p < 0.05) (Fig. 9C) in hippocampus. Also, as seen in other structures, at hypothalamus, stressed group presented a significant decrease in 5-HT (C = 2381 ng/mg ± 328.9; S = 1563 ng/mg ± 193.1; t = 2.52; df = 17 p < 0.05)

and in 5HIAA (C = 1842 ng/mg ± 260.8; S = 1207 ng/mg ± 103.2; t = 2.26, df = 20; $p < 0.05$) (Fig. 2.7E).

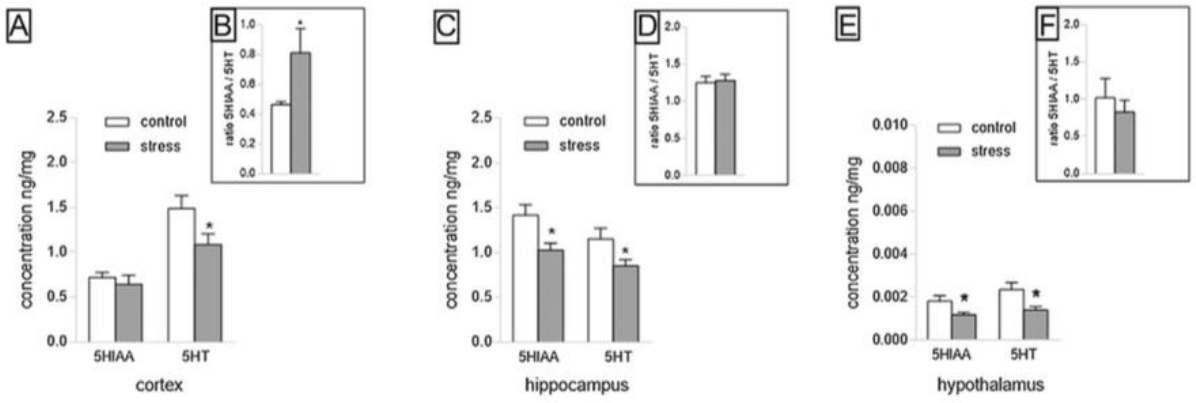
3.4.2. Noradrenaline

There was a significant decrease of NOR (C = 0.0068 ng/mg ± 0.0002; S = 0.0051 ± 0.0005 ng/mg; t = 2.20, df = 14; $p < 0.05$) in hippocampus (Fig. 10C) and hypothalamus (C = 0.0025 ng/mg ±0.0003; S = 0.0017 ng/mg ± 0.0001; t = 2.99, df = 14; $p < 0.05$) in stressed group when compared to control group (Fig. 10E). Furthermore, there was a significant decrease in metabolite VMA in hippocampus (C = 0.0031 ng/mg ± 0.0004; S = 0.0020 ng/mg ± 0.0002; t = 2.32, df = 14; $p < 0.05$) (Fig. 10C).

3.4.3. Dopamine

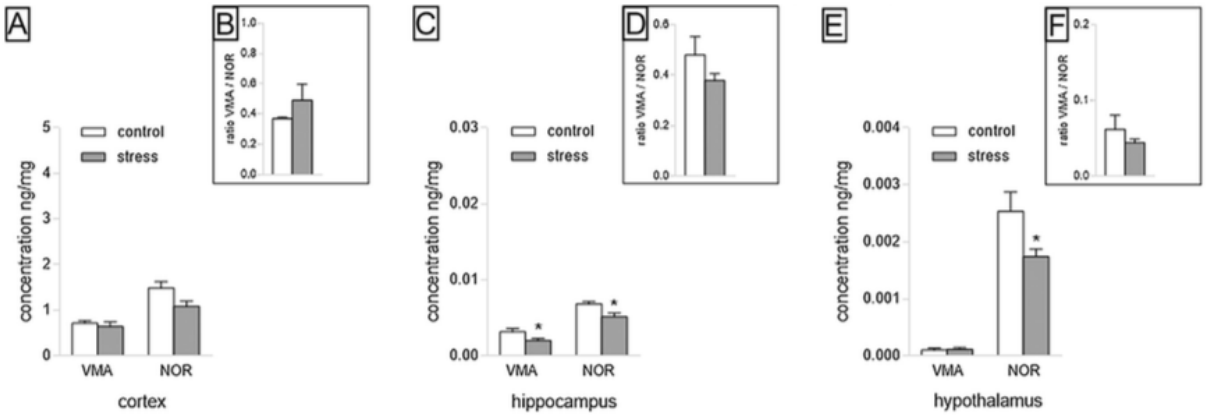
In cortex, dopamine concentrations between groups were not different, but the turnover of dopamine in stressed group is significant higher than control group (C = 0.5634 ng/mg ± 0.01181; S = 2.309 ng/mg ± 0.77668; t = 2.25, df = 14; $p < 0.05$) (Fig. 11B). Furthermore, there was a significant decrease in metabolite HVA in hypothalamus (C = 0.1373 ng/mg ± 0.01306; S = 0.09295 ng/mg ± 0.009152; t = 2.82, df = 19; $p < 0.05$) (Fig. 11E).

Figure 9 - Neurochemistry assessment. Concentrations of 5HIAA and 5HT (A, C and E) and turnover of 5HT (B, D and F) in cortex, hippocampus and hypothalamus.



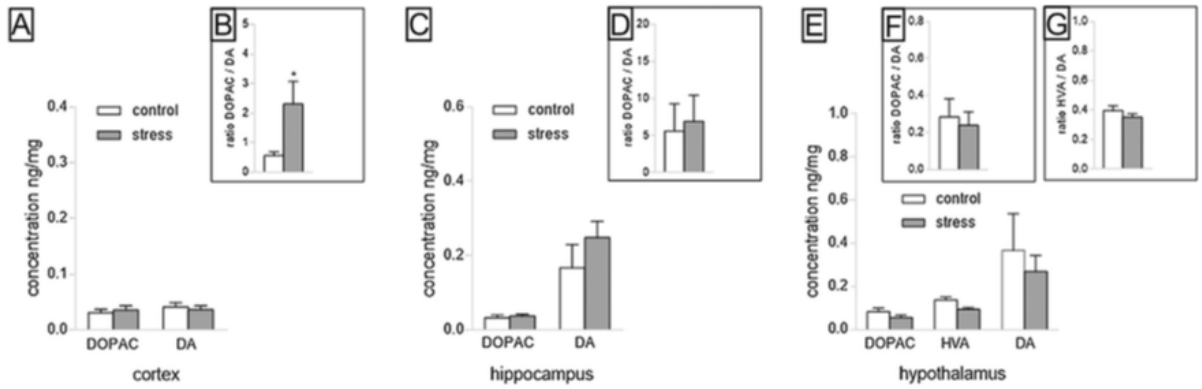
These data were analyzed by Student's t-test (*$p < 0.05$).

Figure 10 - Neurochemistry assessment. Concentrations of VMA and NOR (A, C and E) and turnover of NOR (B, D and F) in cortex, hippocampus and hypothalamus.



Legend: These data were analyzed by Student's t-test (*p < 0.05).

Figure 11 - Neurochemistry assessment. Concentrations of DOPAC, DA in cortex (A) and hippocampus (C). Concentrations of DOPAC, HVA and DA in hypothalamus (E). Turnover of DA (B, D, F and G) in cortex, hippocampus and hypothalamus.



These data were analyzed by Student's t-test (*p < 0.05).

4. DISCUSSION

Previous studies investigated how early stress affects adult rodents. Tzanoulinou et al. (2014) investigated in mice the impacts of a repetitive peripubertal stress in three different ways: with exposure to stress throughout adolescence; only during the pre-pubertal stage, and only at puberty. They observed behavioral and neurobiological effects only on the group exposed to stress throughout adolescence. Zhang et al. (2014), with a chronic unpredictable stress model (31 days), observed increase in anxiety-like behaviors and changes in monoaminergic activity in limbic regions of adult male rats. Our findings corroborate the

literature, since our unpredictable stress model during adolescence changed neurochemistry, behavior and affected HPA axis. When we compare our model with the above, we can strengthen the findings that stress in adolescence can alter the behavioral responses related to stress and anxiety in adulthood. Moreover, only 10 days of unpredictable stress was enough to observe behavioral and neurobiological changes. Thus, it seems that the unpredictability of stress generates a lower resistance to stress effects when compared to a repetitive stress model.

Unpredictable stress, when chronic (at least 21 days), is used to model depression. To evaluate the possible interference of depressive behavior as helplessness in our relatively short protocol of stress (only 10 days), we performed the TST. The results showed that mice subjected to our unpredictable stress did not exhibit depressive-like behaviors. However, the increase in latency of immobility, such as a mild trend to decrease total time of immobility of stressed animals, could be interpreted as an excessive emotional arousal and a high vigilance, similar to that reported by Boulle et al. (2014). Thus, we can suggest that short-term unpredictable stress, although not a model type-depressive behavior induction, changes long-lasting emotional behaviors.

Evaluating the central monoaminergic activity at the neurochemical level has great importance to correlate behavioral response with neurobiological changes. Brain monoamines are involved in behavioral modulation and stressors activate the central monoaminergic system (MALYSZKO et al, 1994; NISHI & AZMITIA, 1996; PARIS et al, 1987). Previous studies with adult rodents have shown that both long and short stress protocols indicate changes in monoamines in several brain areas (ADELL, 1997; AMAT, 1998; RASHEED et al, 2008; VANCASSEL, 2008), such as frontal cortex, hippocampus, striatum and amygdala.

These findings are reinforced by studies that observed an increase in the types 5HT1A receptors (LI et al, 2012), 5HT2A (CAHIR et al, 2007) and -adrenergic (BASSO et al 1993; PAPP et al 2002), suggesting a compensatory mechanism for reduction of these monoamines. Furthermore, there is strong evidence that catecholaminergic neurons are involved in the activation of the HPA axis, the final pathway of the response to stress (HERMAN & CULLIMAN, 1997).

In adolescent, as well as in adult mice, it has been shown that stressed animals present serotonin and noradrenaline downregulation that persist into adulthood (WONG et al, 2015). Our results corroborate with the literature and suggest that stressful situations in this period of life not only cause a change in central monoaminergic activity, but also affects the long-term capacity of compensatory mechanisms.

This diversity of types, along with a complex distribution in the CNS, highlights the important role of 5-HT in several key functions modulating in the brain, including sensory processing, cognitive control, emotion regulation, autonomic control and motor activity. In addition, modification of various aspects of serotonergic neurotransmission may lead to vulnerability and the development of depression, anxiety disorders and others psychiatric disorders (JANS et al, 2016). Although there is no longer doubt about the importance of serotonin in emotion regulation, the relationship between both is quite controversial. Emotion includes several behavioral aspects, including the anxiety that can be expressed through different ways such as hyperactivity and risk behavior. In our results, adult animals, stressed during adolescence, showed alterations in serotonin activity in all areas assessed as well in behavioral tests. The animals tested during adolescence, 24 h after the paradigm of stress, presented an increase in motor activity and decrease of anxiety in OF test such as observed in other studies (BOULLE et al 2014; WILNER 2005). In adulthood, the increase in alertness (flight response) and an emotional arousal seen on the light-dark box test reinforced our results in relation to stress causing long-lasting behavioral changes.

Depending on its type and location in the synapse and the specific area of the brain, 5-HT receptors play different roles in the regulation of anxiety (HANDLEY, 1993). The pre and postsynaptic 5HT1A receptors are important modulators of emotionality and there is evidence that patients with anxiety disorders have altered function of these receptors. In the cortex, specifically, the serotonergic 5HT2A receptors have an important role in controlling anxiety [69]. In this way, we suggest that the unpredictable causes changes in serotonergic activity at the level of receptors which reflects on the availability of 5HT, as well as on the behavioral response.

Serotonin also plays an important role in cognitive processes, including learning and memory. Although a causal relationship between serotonin and impaired memory is not well established, we cannot rule out the function of this neurotransmitter in these processes, since its deficiency in many brain disorders affects cognition. Fernandez et al. (FERNANDEZ et al, 2016) showed that serotonergic projections of the raphe nucleus by inhibitory control of 5HT1A receptors regulate memory and hippocampal synaptic plasticity, thus suggesting that hyposerotonergia affects hippocampal-dependent memory.

Stress in adulthood not only promotes emotional memories related to the traumatic event, but they are also associated with changes in the hippocampus that may impair the formation of emotional memories (MCKITTRICK, et al, 2000). Recently, we have seen that chronic stress in adolescence increases anxiety as well as fear impairs learning in adulthood

(CHABY et al, 2015; NOVICK et al, 2016). Both studies emphasize the importance of context when considering the effects of long-term stress and they suggest that stress affects learning through fear of injury in emotional memory.

Although the impact of stress on emotional memories is well studied, little is known about the influence of emotional state in the formation of non-emotional memories. Therefore, since our stress model promoted a decrease in serotonin activity affecting anxiety and motor activity, we sought to determine if that could also promote changes in learning and memory. Therefore, we chose the object recognition test, which does not require extra motivation, rewards or punishments (NEMANIC et al,2004). Furthermore, the object recognition task does not generate stress while offering robust results of non-emotional recognition memory (BOWMAN et al, 2003; MARAS et al, 2014).

Our results showed that stressed animals not only showed a clear change in the emotional state in adulthood, but also demonstrated impaired formation of non-emotional memory hippocampus-dependent. Therefore, our stress model not only affects emotional behavior, it also seems to affect, to a lesser degree, performance in non-emotional cognitive tasks such as object recognition.

In contrast, in an evolutionary perspective, according to Coppens et al. (2012), different individuals within the same gender across species adopt different behavioral strategies to cope with stress within a population. Therefore, we suggest that stress during adolescence could contribute to the appearance of a new phenotype in a population of isogenic mice, such as Balb/c mice. That is, the unpredictable stress model seems to be related to development of a different phenotype observed in the control group, which, in turn, generates a distinct physiological response that generates a behavioral strategy that is also different. However, we cannot affirm that this new phenotype is more or less adaptive to the phenotype of the control group. For example, our results showed an apparent deficit in novel object recognition test of the stressed group, but, on the other hand, the emotional arousal in the other behavioral tests (open field, light-dark box and tail suspension test) could be interpreted as a proactive coping of stressed animals and, thus, a phenotype more adaptive in specific situations.

Adolescence is characterized as a period of high plasticity and behavioral flexibility (CRONE & DAHL, 2012). Behavioral flexibility is adaptive, since it facilitates the integration of new social and environmental contexts. A possible mechanism for the impairment in cognition produced by stress is the HPA axis dysfunction seen in our results. HPA axis activated for a prolonged period during adolescence increases glucocorticoid levels during this period

and probably is associated with changes in hippocampus, and, therefore, will cause an impairment in learning mechanism and memory of those animals in adulthood.

Stress models during adolescence, as seen in adulthood, vary according to the duration and the types of stressors (e.g. physical, social, predation). The excess of glucocorticoid production that occurs during exposure to stress during adolescence induces long-term changes in the brain, such as changes in glucocorticoid receptors and neuronal density and structural changes. A better understanding of what underlies the long-term effects of stress during adolescence on adult behavior can be obtained by factors investigating mechanisms related to the regulation of glucocorticoids, such as the proportion of free and bound corticosterone the linker globulin corticosteroids, as well as investigating the receptors for glucocorticoids and mineralocorticoids.

Animals exposed to stressful events during adolescence present higher circulating levels of corticosterone and when exposed to stressful events also during adulthood these levels remain high (BAZAK et al, 2009; WONG et al, 2015). In fact, chronic social stress in mice in early life promotes permanent changes in the circadian rhythm of glucocorticoids (STERLEMANN et al, 2008). In humans, it has been seen that students who have gone through situations of early loss in life have different cortisol levels compared to students who did not experience loss in similar situations (MEINLSCHMIDT et al, 2005). Our results here showed that stress in adolescent mice resulted in an increase of corticosterone levels and an increase in relative weight of adrenal gland in adulthood. This upregulation in HPA axis supports the long-term behavioral changes obtained in stressed group.

Our results discussed in conjunction with previous study findings suggest that unpredictability of stress during adolescence not only compromises the HPA axis to respond to a stressor stimulus properly in adulthood, but also causes alterations in the CNS. In turn, this results in long-lasting changes in neural pathways and behavior, eliciting the potential neuroendocrine and behavioral vulnerability for the development of disorders related to stress response in adulthood.

The idea of a critical window during the developmental period of adolescence may be a plausible explanation for the permanent effects of behavioral and neurochemical regulation in mice undergoing social-environmental stress, as seen previously with other models of stress. However, this critical window should not be understood only as a vulnerable stage but also an opportunity. Stressful situations, then, can not only be related to a future psychiatric disorder but can also be associated with greater behavioral and physiological flexibility to respond to

different environmental and social contexts in the future (MCCORMICK & MATHEWS, 2010; ROMEO, 2015).

Future studies are required to better understand how this stress model acts and interacts with factors such as gender and environmental enrichment and with more naturalistic environments.

REFERENCES

ADELL, A.; CASANOVAS, J. M.; ARTIGAS, F. Comparative study in the rat of the actions of different types of stress on the release of 5-HT in raphe nuclei and forebrain areas. **Neuropharmacology,** v. 36, n. 4-5, p. 735-41, 1997 Apr-May 1997.

AMAT, J. et al. Escapable and inescapable stress differentially alter extracellular levels of 5-HT in the basolateral amygdala of the rat. **Brain Res,** v. 812, n. 1-2, p. 113-20,

ANDERSEN, S. L. Trajectories of brain development: point of vulnerability or window of opportunity? **Neurosci Biobehav Rev,** v. 27, n. 1-2, p. 3-18, 2003 Jan-Mar 2003.

AVITAL, A. et al. Effects of early-life stress on behavior and neurosteroid levels in the rat hypothalamus and entorhinal cortex. **Brain Res Bull,** v. 68, n. 6, p. 419-24, Feb 2006.

BAIRD, A. A. et al. Functional magnetic resonance imaging of facial affect recognition in children and adolescents. **J Am Acad Child Adolesc Psychiatry,** v. 38, n. 2, p. 195-9, Feb 1999.

BARKS, J. D. et al. Glutamate recognition sites in human fetal brain. **Neurosci Lett,** v. 84, n. 2, p. 131-6, Jan 1988.

BASSO, A. M. et al. Seven-day variable-stress regime alters cortical beta-adrenoceptor binding and immunologic responses: reversal by imipramine. **Pharmacol Biochem Behav,** v. 45, n. 3, p. 665-72, Jul 1993.

BAZAK, N. et al. Pre-pubertal stress exposure affects adult behavioral response in association with changes in circulating corticosterone and brain-derived neurotrophic factor. **Psychoneuroendocrinology,** v. 34, n. 6, p. 844-58, Jul 2009.

BHUTANI, M. K.; BISHNOI, M.; KULKARNI, S. K. Anti-depressant like effect of curcumin and its combination with piperine in unpredictable chronic stress-induced behavioral, biochemical and neurochemical changes. **Pharmacol Biochem Behav,** v. 92, n. 1, p. 39-43, Mar 2009.

BOULLE, F. et al. Hippocampal and behavioral dysfunctions in a mouse model of environmental stress: normalization by agomelatine. **Transl Psychiatry,** v. 4, p. e485, 2014.

BOWMAN, R. E.; BECK, K. D.; LUINE, V. N. Chronic stress effects on memory: sex differences in performance and monoaminergic activity. **Horm Behav,** v. 43, n. 1, p. 48-59, Jan 2003.

BUWALDA, B. et al. Long-term effects of social stress on brain and behavior: a focus on hippocampal functioning. **Neurosci Biobehav Rev,** v. 29, n. 1, p. 83-97, Feb 2005.

CAHIR, M. et al. Acute and chronic tryptophan depletion differentially regulate central 5-HT1A and 5-HT 2A receptor binding in the rat. **Psychopharmacology (Berl),** v. 190, n. 4, p. 497-506, Mar 2007.

CASPI, A. et al. Role of genotype in the cycle of violence in maltreated children. **Science,** v. 297, n. 5582, p. 851-4, Aug 2002.

CHABY, L. E. et al. Chronic Stress During Adolescence Impairs and Improves Learning and Memory in Adulthood. **Front Behav Neurosci,** v. 9, p. 327, 2015.

CHAOULOFF, F. Physiopharmacological interactions between stress hormones and central serotonergic systems. **Brain Res Brain Res Rev,** v. 18, n. 1, p. 1-32, 1993 Jan-Apr 1993.

COPPENS, C. M. et al. Impulsivity and aggressive behavior in Roman high and low avoidance rats: baseline differences and adolescent social stress induced changes. **Physiol Behav,** v. 105, n. 5, p. 1156-60, Mar 2012.

CORDERO, M. I. et al. Evidence for biological roots in the transgenerational transmission of intimate partner violence. **Transl Psychiatry,** v. 2, p. e106, 2012.

COX, B. M. et al. Neurochemical, hormonal, and behavioral effects of chronic unpredictable stress in the rat. **Behav Brain Res,** v. 220, n. 1, p. 106-11, Jun 2011.

CRAWLEY, J.; GOODWIN, F. K. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. **Pharmacol Biochem Behav,** v. 13, n. 2, p. 167-70, Aug 1980.

CRONE, E. A.; DAHL, R. E. Understanding adolescence as a period of social-affective engagement and goal flexibility. **Nat Rev Neurosci,** v. 13, n. 9, p. 636-50, Sep 2012.

DAVID, I. et al. [Depression and response: impact of personality and cognition]. **Psychiatr Prax,** v. 36, n. 3, p. 132-6, Apr 2009.

EILAND, L.; ROMEO, R. D. Stress and the developing adolescent brain. **Neuroscience,** v. 249, p. 162-71, Sep 2013. ISSN 1873-7544.

FELICIO, L. F. et al. Reproductive experience increases striatal and hypothalamic dopamine levels in pregnant rats. **Brain Res Bull,** v. 40, n. 4, p. 253-6, 1996.

FERNANDEZ, S. P. et al. Constitutive and Acquired Serotonin Deficiency Alters Memory and Hippocampal Synaptic Plasticity. **Neuropsychopharmacology**, Jul 2016a.

GIEDD, J. N. et al. Brain development during childhood and adolescence: a longitudinal MRI study. **Nat Neurosci,** v. 2, n. 10, p. 861-3, Oct 1999.

GUNNAR, M. R. et al. Developmental changes in hypothalamus-pituitary-adrenal activity over the transition to adolescence: normative changes and associations with puberty. **Dev Psychopathol,** v. 21, n. 1, p. 69-85, 2009.

HALL, C. Emotional behavior in the rat. I. Defecation and unination as measures of individual differences in emotionality. **J Comp Psychol,** v. 18, p. 15, 1934.

HANDLEY, S. L. et al. Multiple serotonin mechanisms in animal models of anxiety: environmental, emotional and cognitive factors. **Behav Brain Res,** v. 58, n. 1-2, p. 203-10, Dec 1993.

HEIM, C.; NEMEROFF, C. B. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. **Biol Psychiatry,** v. 49, n. 12, p. 1023-39, Jun 2001.

HERMAN, J. P.; CULLINAN, W. E. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. **Trends Neurosci,** v. 20, n. 2, p. 78-84, Feb 1997.

JACOBSON-PICK, S.; RICHTER-LEVIN, G. Differential impact of juvenile stress and corticosterone in juvenility and in adulthood, in male and female rats. **Behav Brain Res,** v. 214, n. 2, p. 268-76, Dec 2010.

JANS, L. A. et al. Serotonergic vulnerability and depression: assumptions, experimental evidence and implications. **Mol Psychiatry,** v. 12, n. 6, p. 522-43, Jun 2007.

JOHNSON-REID, M; PRESNALL, N; DRAKE, B; FOX, L; BIERUT, L; REICH, W; KANE, P; TODD RD; CONSTANTIN JN. Effects of Child Maltreatment and Inherited Liability on Antisocial Development: An Official Records Study. **Journal of the American Academy of Child & Adolescent Psychiatry,** v. 49, n. 4, p. 321-332, 2010.

KOOLHAAS, J. M. et al. The temporal dynamics of the stress response. **Neurosci Biobehav Rev,** v. 21, n. 6, p. 775-82, Nov 1997.

LI, J., TANG, Y. & CAI, D. IKKβ/NF-κB disrupts adult hypothalamic neural stem cells to mediate a neurodegenerative mechanism of dietary obesity and pre-diabetes. **Nature Cell Biol.**, p. 999-1012, 2012.

LIDOW, M. S.; GOLDMAN-RAKIC, P. S.; RAKIC, P. Synchronized overproduction of neurotransmitter receptors in diverse regions of the primate cerebral cortex. **Proc Natl Acad Sci U S A,** v. 88, n. 22, p. 10218-21, Nov 1991.

MALYSZKO J, URANO T, TAKADA Y, TAKADA A. Serotonergic systems in brain and blood under stress and tranylcypromine treatment in rats. **Brain Res Bull**, v. 35, n. 1, p. 9-13, 1994.

MCCORMICK, C. M.; GREEN, M. R. From the stressed adolescent to the anxious and depressed adult: investigations in rodent models. **Neuroscience,** v. 249, p. 242-57, Sep 2013.

MCCORMICK, C. M.; MATHEWS, I. Z. Adolescent development, hypothalamic-pituitary-adrenal function, and programming of adult learning and memory. **Prog Neuropsychopharmacol Biol Psychiatry,** v. 34, n. 5, p. 756-65, Jun 2010.

MCKITTRICK, C. R. et al. Chronic social stress reduces dendritic arbors in CA3 of hippocampus and decreases binding to serotonin transporter sites. **Synapse,** v. 36, n. 2, p. 85-94, May 2000.

MEINLSCHMIDT, G.; HEIM, C. Decreased cortisol awakening response after early loss experience. **Psychoneuroendocrinology,** v. 30, n. 6, p. 568-76, Jul 2005.

MÁRQUEZ, C. et al. Peripuberty stress leads to abnormal aggression, altered amygdala and orbitofrontal reactivity and increased prefrontal MAOA gene expression. **Transl Psychiatry,** v. 3, p. e216, 2013.

NEMANIC, S.; ALVARADO, M. C.; BACHEVALIER, J. The hippocampal/parahippocampal regions and recognition memory: insights from visual paired comparison versus object-delayed nonmatching in monkeys. **J Neurosci,** v. 24, n. 8, p. 2013-26, Feb 2004.

NISHI, M.; AZMITIA, E. C. 5-HT1A receptor expression is modulated by corticosteroid receptor agonists in primary rat hippocampal culture. **Brain Res,** v. 722, n. 1-2, p. 190-4, May 1996.

NOVICK, A. M. et al. Adolescent social defeat alters N-methyl-D-aspartic acid receptor expression and impairs fear learning in adulthood. **Behav Brain Res,** v. 304, p. 51-9, May 2016.

PAPP, M. et al. Behavioural and biochemical studies of citalopram and WAY 100635 in rat chronic mild stress model. **Pharmacol Biochem Behav,** v. 72, n. 1-2, p. 465-74, May 2002.

PARIS, J.; BROWN, R.; NOWLIS, D. Long-term follow-up of borderline patients in a general hospital. **Compr Psychiatry,** v. 28, n. 6, p. 530-5, 1987 Nov-Dec 1987.

PEREPLETCHIKOVA, F.; KAUFMAN, J. Emotional and behavioral sequelae of childhood maltreatment. **Curr Opin Pediatr,** v. 22, n. 5, p. 610-5, Oct 2010.

POST, R. J. et al. Effects of juvenile exposure to predator odor on adolescent and adult anxiety and pain nociception. **Physiol Behav,** v. 131C, p. 57-61, Apr 2014.

POTHION, S. et al. Strain differences in sucrose preference and in the consequences of unpredictable chronic mild stress. **Behav Brain Res,** v. 155, n. 1, p. 135-46, Nov 2004.

RAKIC, P. Development of the primate cerebral cortex. **Child and adolescent psychiatry**, 1991.

ROMEO, R. D. Adolescence and the sculpting of the stress response. **Psychoneuroendocrinology,** v. 61, p. 18, Nov 2015.

ROMEO, R. D.; MCEWEN, B. S. Stress and the adolescent brain. **Ann N Y Acad Sci,** v. 1094, p. 202-14, Dec 2006.

ROSENBERG, D. R.; LEWIS, D. A. Postnatal maturation of the dopaminergic innervation of monkey prefrontal and motor cortices: a tyrosine hydroxylase immunohistochemical analysis. **J Comp Neurol,** v. 358, n. 3, p. 383-400, Jul 1995.

SEEMAN, P. et al. Human brain dopamine receptors in children and aging adults. **Synapse,** v. 1, n. 5, p. 399-404, 1987.

SPEAR, L. P. The adolescent brain and age-related behavioral manifestations. **Neurosci Biobehav Rev,** v. 24, n. 4, p. 417-63, Jun 2000a.

STERLEMANN, V. et al. Long-term behavioral and neuroendocrine alterations following chronic social stress in mice: implications for stress-related disorders. **Horm Behav,** v. 53, n. 2, p. 386-94, Feb 2008.

STERU, L. et al. The tail suspension test: a new method for screening antidepressants in mice. **Psychopharmacology (Berl),** v. 85, n. 3, p. 367-70, 1985.

TEICHER, M. H.; ANDERSEN, S. L.; HOSTETTER, J. C. Evidence for dopamine receptor pruning between adolescence and adulthood in striatum but not nucleus accumbens. **Brain Res Dev Brain Res,** v. 89, n. 2, p. 167-72, Nov 1995.

TORRES, I. L. et al. Effects of chronic restraint stress on feeding behavior and on monoamine levels in different brain structures in rats. **Neurochem Res,** v. 27, n. 6, p. 519-25, Jun 2002.

TZANOULINOU, S. et al. Peripubertal stress-induced behavioral changes are associated with altered expression of genes involved in excitation and inhibition in the amygdala. **Transl Psychiatry,** v. 4, p. e410, 2014.

VANCASSEL, S. et al. n-3 polyunsaturated fatty acid supplementation reverses stress-induced modifications on brain monoamine levels in mice. **J Lipid Res,** v. 49, n. 2, p. 340-8, Feb 2008.

VEENEMA, A. H.; NEUMANN, I. D. Maternal separation enhances offensive play-fighting, basal corticosterone and hypothalamic vasopressin mRNA expression in juvenile male rats. **Psychoneuroendocrinology,** v. 34, n. 3, p. 463-7, Apr 2009.

VEENIT, V. et al. Increased corticosterone in peripubertal rats leads to long-lasting alterations in social exploration and aggression. **Front Behav Neurosci,** v. 7, p. 26, 2013.

WATT, M. J. et al. Adolescent male rats exposed to social defeat exhibit altered anxiety behavior and limbic monoamines as adults. **Behav Neurosci,** v. 123, n. 3, p. 564-76, Jun 2009.

WILLNER, P. Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. **Neuropsychobiology,** v. 52, n. 2, p. 90-110, 2005.

WOMMACK, J. C.; DELVILLE, Y. Repeated social stress and the development of agonistic behavior: individual differences in coping responses in male golden hamsters. **Physiol Behav,** v. 80, n. 2-3, p. 303-8, Nov 2003.

WONG, P. et al. Early life environmental and pharmacological stressors result in persistent dysregulations of the serotonergic system. **Front Behav Neurosci,** v. 9, p. 94, 2015.

ZHANG, L. et al. Effects of curcumin on chronic, unpredictable, mild, stress-induced depressive-like behaviour and structural plasticity in the lateral amygdala of rats. **Int J Neuropsychopharmacol,** v. 17, n. 5, p. 793-806, May 2014.

ZOLOTAREV, Y. A. et al. Anxiolytic activity of the neuroprotective peptide HLDF-6 and its effects on brain neurotransmitter systems in BALB/c and C57BL/6 mice. **J Psychopharmacol**, Jul 2016.

CHAPTER 2: PASSIVE ADAPTATION TO STRESS IN ADULTHOOD AFTER SHORT-TERM SOCIAL INSTABILITY STRESS DURING ADOLESCENCE IN MICE[2]

1. INTRODUCTION

Early life stress plays a putative role in the development of mood and anxiety disorders (LUPIEN et al., 2009). Bullying and subordination are ethologically relevant social stressors that are prevalent in adolescents. Bullying during adolescence can cause long-lasting behavioral and neural consequences that closely connect with depression in adulthood (NEWMAN et al., 2005). In rodent studies, social stress models have been a useful tool to evaluate how adolescent stress can induce a set of depressive-like and anxiety-like behaviors in adulthood.

In laboratory settings, social stress models have been shown to display differences in behavioral tests commonly used in neuroscience. The social instability stress (SIS) model is a model of social instability combined with daily confinement isolation. This combined model not only depends on the social novelty component but also on the repeated elevation in corticosterone evoked by the confinement that other social protocols have failed to show. In adolescent rats, SIS can cause anxiety-like and depression-like behaviors in adulthood (MCCORMICK and GREEN, 2013; MCCORMICK et al., 2008). However, in mice, although they are animals with a strong social hierarchy and widely used in biomedical research, this model is still little used.

Furthermore, the few studies that used the SIS model involved longer periods of time that extended into adulthood (7-weeks protocol) (SCHMIDT et al., 2010; SCHMIDT et al., 2008; STERLEMANN et al., 2008). Thus, in this study, we tested the long-term behavioral effects of short-term social instability stress (only 10 days) in Balb/c male mice.

2. METHODS

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Experimental subjects were 16 male Balb/c mice (4-weeks-old) from the Department of Pathology (School of Veterinary Medicine, University of São Paulo) kept in a controlled environment (22 ± 2 ℃ temperature and 55–65% humidity, lights on at 6:00 AM and off at 6:00 PM).

Mice were randomly split into two groups: control animals (C) and stress-exposed animals (S). The SIS procedure was as described previously (reviewed in MCCORMICK and GREEN, 2013) and involved isolation for 1 h in a plastic container (2100 cm^3), daily from PND 30 to 40 (mid adolescence). Isolation occurred at a variable time each day during the light phase to reduce habituation to the procedure. After each daily isolation, SIS males were pair-housed with a new cage partner that was also undergoing the stress procedure until the 7th day of stress, and for the last three days of stress each SIS male was pair-housed with new cage partner that participated in another later study. After the final isolation on PND 40, stressed males were returned to their original cage partner.

20 days after stress, with animals already in adulthood, tests were performed to evaluate changes in anxiety-like and depression-like behaviors. All behavioral experiments were always performed three days in a row, during the evening (2:00 to 4:00 PM), in order to minimize possible interference from changes in the circadian rhythm.

For the open field test, mice were placed in circular arena (50 × 50 cm) and allowed to explore for 5 min while being recorded overhead (HALL, 1934). Total distance traveled (cm), and time (s) in center zone were analyzed with the use of Ethovision® XT-7 (Noldus Information Technology®, Leesburg, VA, USA) detected by nose-point tracking.

For the light-dark box test, a box (45 cm × 20 cm) was used with an open, white chamber, connected by a square door (5 cm × 5 cm) to another covered and dark chamber (CRAWLEY and GOODWIN, 1980). The dark chamber occupied one-third of the box. Each mouse was placed in the center of the white chamber and recorded for 5 min. Video recordings were later analyzed with Ethovision® and the frequency of risk assessment (number of times that the animal explores the light chamber with two hind paws in the dark chamber), as well as the time spent in each chamber were noted.

Finally, the tail suspension was performed essentially as described (STERU et al., 1985). Briefly, immobility was scored during a 6-min session while the mouse was suspended by the midpoint of the tail, 40 cm above the surface. Total time of immobility, frequency of immobility and latency to the first immobility on this test were used to measure behavioral despair.

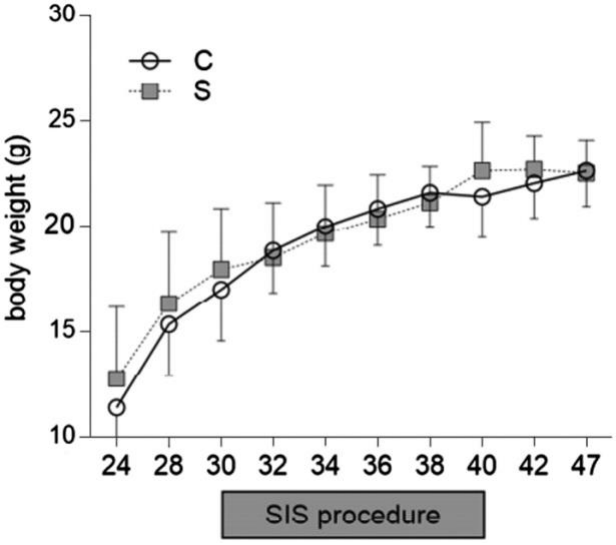
All experiments were performed in accordance with the guidelines of the Bioethical Committee on Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of Sao Paulo, Brazil (protocol no 4485180614), which are similar to those of the National Research Council, USA.

Body weight data were analyzed using analysis of variance (ANOVA) followed by using analysis of variance by Sidak's test. All behavioral data followed a normal distribution and were analyzed by Student's t-test. Statistical significance was set at $p < 0.05$ with 95% confidence interval. Data are reported as the mean ± SEM.

3. RESULTS

SIS did not affect body weight compared to control [F (1, 28) = 1.721; p = 0.1907, Fig. 12]. All subjects appeared healthy before, during and after stress procedure. Thus, short-term SIS do not affect the body weight.

Figure 12 - Mean (±SEM) weight at postnatal day (PND) ages before, during and after the social instability stress (SIS) procedure for S and control (C) mice.

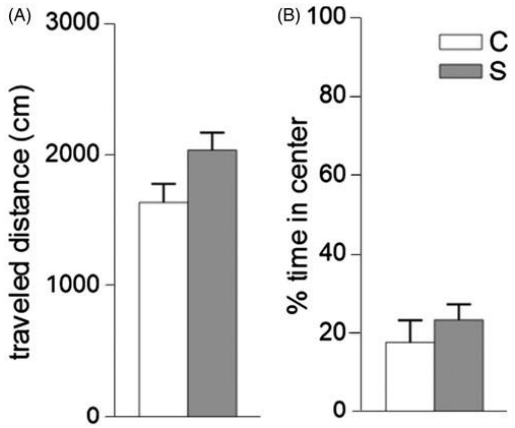


Source: (LIMA & MASSOCO, 2017).

Results from the open field indicated not effect of stress on motor activity and on anxiety-like behavior. More in detail, SIS group did not show changes in traveled distance [t = 1.947, df = 14; p = 0.0718, Fig.13A] and did not decrease the time in center [t = 0.8521, df = 14; p = 0.4096, Fig. 13B] compared with control group. In this way, we can affirm that short-

term SIS does not affect the locomotor activity and does not seem to affect anxiety-like behavior.

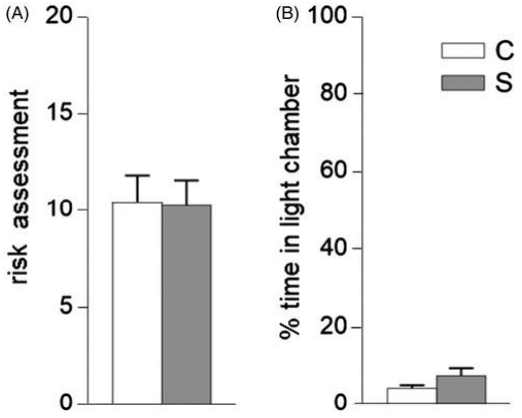
Figure 13 - Mean (±SEM) of traveled distance and percentage of time spent in center zone of open field test for S and C mice.



Source: (LIMA & MASSOCO, 2017).

In the light-dark box test, as in the open field, stressed group did not show differences in anxiety parameters compared with control group. Both groups demonstrated similar frequency in the risk assessment [t = 0.09293, df = 14; p = 0.9274, Fig. 14A] and in the percentage of time in light chamber [t = 1.458, df = 14; p = 0.1686, Fig. 14B]. Thus, we confirm that short-term SIS did not affect anxiety-like behavior.

Figure 14 - Mean (±SEM) of frequency in risk analysis and percentage of time spent in light chambers on light-dark box test.

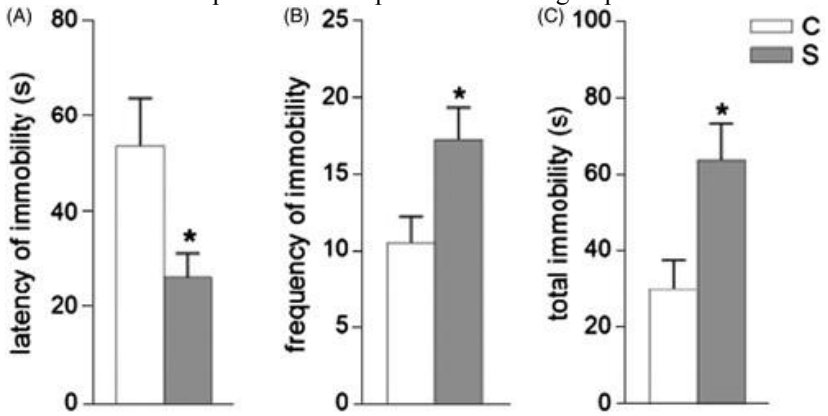


Source: (LIMA & MASSOCO, 2017).

In the tail suspension test, stressed group were characterized by a decrease in latency of immobility [t = 2.446, df = 14; p = 0.0282, Fig. 15A], and by an increase in frequency [t = 2.449, df = 14; p = 0.0281, Fig. 15B] and in total time [t = 2.800, df = 14; p = 0.0161, Fig. 15C]

of immobility. Therefore, this test indicated consistent results in relation to depressive-like behavior.

Figure 15 - Mean (±SEM) of latency, total time and frequency of immobility in tail suspension test.
* p < .05 for comparison between groups.



Source: (LIMA & MASSOCO, 2017).

4. DISCUSSION

In adult rats, social defeat, widely used as a social stress model, have been suggested as a model of depression (KOOLHAAS, 1990; WILLNER, 1995) and in adolescent rats, SIS can cause anxiety and depression behaviors in adulthood (MCCORMICK and GREEN, 2013; MCCORMICK et al., 2008). However, in mice, social stress models have been shown contradictory results. While Keeney and Hogg (1999) have reported depressive behavior as a consequence of social stress, other authors have reported that social defeat is responsible for increased anxiety-like but not depressive-like behavior assessed in the tail suspension test (KINSEY et al., 2007; SLATTERY et al., 2012). In addition, Schmidt et al. (2010) have been reported that a chronic (21 days) social instability stress is capable of inducing depression and anxiety-like behaviors in young mice and can be used as an animal model of depression–anxiety co-occurrence (SCHMIDT et al., 2010).

This current study was designed to evaluate whether a short-term SIS in adolescent mice could affect behavior in adulthood. Our results consistently showed that, in comparison with non-stressed mice, short-term SIS induced an adaptive stress-coping response in TST. However, different from chronic social instability stress, 10 days of stress, did not induce any anxiety-like behavior. The duration of stress, only 10 days, seems to be a marked factor in the long-term effects of stress in adolescence. Indeed, these results are consistent with those of

unpredictable stress in which chronic (at least 21 days) produces depression–anxiety co-occurrence long-term effects, while 10 days of the same protocol, in adolescence, were sufficient to produce anxiety-like behaviors but not depressive-like behaviors in adulthood (DE LIMA et al., 2017).

To study and better understand depression through animal models, behavioral studies alone are not enough. Thus, in the current study, we do not intend to diagnose depression in stressed mice or to establish a direct causal link between stress and depression-like behavior. The TST, in the current study, aimed to evaluate the stress-coping in adulthood after short-term social instability stress during adolescence. Kloet and Molendijk (2016) suggest that TST or forced swimming test are useful to research the mechanism of coping and adaptation that contributes to understanding the mechanism of passive coping with an apparently inescapable/uncontrollable situation. The immobility in TST has been reported as indicators of an adaptive stress-coping response (KLOET and MOLENDIJK 2016; SADLER and BAILEY, 2016). In addition, an increase of immobility in the TST has also been interpreted as a defense mechanism in response to the aversive nature of the behavioral tests (BOULLE et al., 2014).

Our results showed that short-term SIS in adolescent mice resulted in a passive coping with stress during adulthood given by an increase of immobility in TST. In this way, we may suggest that the confinement and isolation for 1 hour and the daily exchange of cage partners evokes a persistent elevation of corticosterone during adolescence, which in turn promotes permanent changes in the circadian rhythm of glucocorticoids.

Therefore, we may suggest that a short-term SIS exposure in adolescent mice reflect in stress coping strategies that have effects on stress challenges in adulthood. Finally, this short stress protocol is capable of being applied at a specific stage of development and may be useful for a better understanding about the relationships between stress in adolescence and stress-coping in adulthood.

REFERENCES

BOULLE, F., MASSART, R., STRAGIER, E., PAÏZANIS E., ZAIDAN L., MARDAY, S., GABRIEL C., MOCAER, E., MONGEAU R., AND LANFUMEY L. Hippocampal and behavioral dysfunctions in a mouse model of environmental stress: normalization by agomelatine. **Translational Psychiatry**, v. 4, e485, 2014

CRAWLEY, J.; GOODWIN, F. K. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. **Pharmacol Biochem Behav,** v. 13, n. 2, p. 167-70, Aug 1980.

DE LIMA, A. P., SANDINI, T. M., REIS-SILVA, T. M., MASSOCO, C. O. Long-lasting monoaminergic and behavioral dysfunctions in a mice model of socio-environmental stress during adolescence. **Behav Brain Res**, v. 317, p. 132-140, 2017.

HALL, C. Emotional behavior in the rat. I. Defecation and unination as measures of individual differences in emotionality. **J Comp Psychol**, v. 18, p. 15, 1934.

KEENEY, A. J., HOGG, S. Behavioural consequences of repeated social defeat in the mouse: preliminary evaluation of a potential animal model of depression. **Behav Pharmacol**, v. 10, p. 753-64, 1999.

KINSEY, S. G., BAILEY, M. T., SHERIDAN, J. F., PADGETT, D. A., AVITSUR, R. Repeated social defeat causes increased anxiety-like behavior and alters splenocyte function in C57BL/6 and CD-1 mice. **Brain Behav Immun**, v. 21, p. 458-66, 2007.

KOOLHAAS, JM, KEMPERMAN, HP., BOHUS, C., VANDENHOOFDAKKER, B, BEERSMA, RH. Single Social Defeat in Male-Rats Induces a Gradual but Long Lasting Behavioral Change - a Model of Depression. **Neurosci Res Commun**, p. 35-41, 1990.

KRISHNAN, V., NESTLER, E. J. Animal models of depression: molecular perspectives: **Curr Top Behav Neurosci**, v. 7, p. 121-47, 2011.

LUPIEN, S. J., MCEWEN, B. S. GUNNAR, M. R. AND HEIM, C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. **Nat Rev Neurosci**, v. 10, p. 434-45, 2009.

MCCORMICK, C. M., GREEN, M. R. From the stressed adolescent to the anxious and depressed adult: investigations in rodent models. **Neuroscience**, v. 249, p. 242-57, 2013.

MCCORMICK, C. M., SMITH, C., MATHEWS I. Z. Effects of chronic social stress in adolescence on anxiety and neuroendocrine response to mild stress in male and female rats: **Behav Brain Res**, v. 187, p. 228-38, 2008.

NEWMAN, M. L., HOLDEN, G. W., DELVILLE, Y. Isolation and the stress of being bullied. **J Adolesc**, v. 28, p. 343-57, 2005.

SCHMIDT, M. V., SCHARF, S. H., STERLEMANN, V. GANEA, K. LIEBL, C., HOLSBOER, F., MÜLLER M. B. High susceptibility to chronic social stress is associated with a depression-like phenotype: **Psychoneuroendocrinology**, v. 35, p. 635-43, 2010.

SCHMIDT, M. V., STERLEMANN, V, MÜLLER. M. B. Chronic stress and individual vulnerability. **Ann N Y Acad Sci**, v. 1148, p. 174-83, 2008.

SLATTERY, D. A., USCHOLD, N., MAGONI, M., BÄR, J., POPOLI, M., NEUMANN, I. D., REBER, S.O. Behavioural consequences of two chronic psychosocial stress paradigms: anxiety without depression. **Psychoneuroendocrinology**, v. 37, p. 702-14, 2012.

STERLEMANN, V., GANEA, LIEBL, K., C., HARBICH, D., ALAM, S., HOLSBOER, F., MÜLLER, M. B., SCHMIDT, M. V. Long-term behavioral and neuroendocrine alterations following chronic social stress in mice: implications for stress-related disorders. **Horm Behav,** v. 53, p. 386-94, 2008.

STERU, L., CHERMAT, R, THIERRY, B, SIMON, P. The tail suspension test: a new method for screening antidepressants in mice. **Psychopharmacology (Berl)**, v. 85, p. 367-70, 1985.

WILLNER, P. Animal models of depression: validity and applications. ADV BIOCHEM PSYCHOPHARMACOL, v. 49, p. 19-41., 1995.

CHAPTER 3: SOCIAL-ENVIRONMENTAL STRESS IN ADOLESCENCE SENSITIZES PERIPHERAL INFLAMMATORY AND SICKNESS BEHAVIOR RESPONSES TO LIPOPOLYSACCHARIDE IN ADULT MALE MICE.

1. INTRODUCTION

Stress can have divergent effects on immune function in adulthood. On the one hand, it can suppress, via high levels of glucocorticoids, responses to infection inhibiting cytokine production and release (BERKENBOSCH et al., 1991; FANTUZZI et al., 1995; GOUJON et al., 1995). On the other hand, stress can enhance some functional aspects of immune function (DHABHAR e MCEWEN, 1996; DEAK et al., 1999), increasing LPS-induced cytokine response in the periphery and brain (Johnson et al., 2002) and also exacerbate inflammatory diseases as a result of immune activation (MEI-TAL et al., 1970; O'CONNOR et al., 2003).

It is well known that stress modulates immune response via a variety of mechanisms, including by hypothalamus-pituitary-adrenal (HPA) axis. In adolescents, the HPA axis is not fully developed and does not adult-like until early adulthood (MCCORMICK and MATHEWS, 2007). In addition, in rodents, it is also during this period that the establishment of immune memory begins (KINCADE, 1981; LANDRETH, 2002) which may render the adolescent especially vulnerable to stressors and immune challenges.

The bacterial endotoxin lipopolysaccharide (LPS), isolated from the cell wall of Gram negative bacteria, induces an increase in plasma corticosterone and is believed to activate the HPA axis by inducing the release of cytokines (DANTZER and KELLEY, 2007). One of these cytokines, the tumor necrosis factor-α (TNF-α) is produced very early in inflammation, stimulates sickness behavior, and is one of the first proinflammatory cytokines released after LPS challenge followed later by IL-1 and IL-6 (HAWIGER, 2001).

Previous work by our group has shown that social-environmental stress, a heterotypic stress model, during adolescence can have a long-term effect on HPA axis in adulthood and suggests that high baseline corticosterone (CORT) levels can be involved in neurochemistry changes and behavioral impairments (DE LIMA et al., 2017). Because of these longstanding effects, we proposed to investigate the effects in adulthood of a socio-environmental stress model during adolescence on sickness behavior, TNF-α plasmatic levels and splenic immune cell populations after an acute LPS challenge, seeking an overview of the immune status after

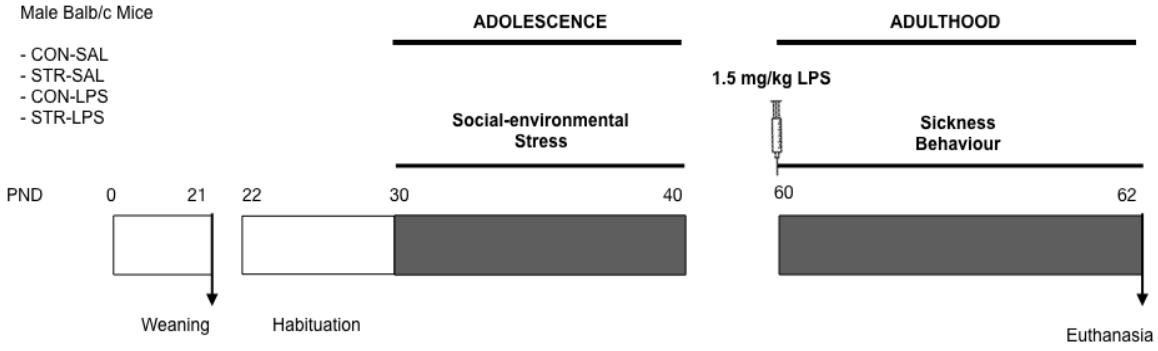
an immune stressor of an adult mouse stressed during adolescence. The results show that stress paradigm increased LPS-induced sickness behavior, plasma levels of TNF-α, and splenic monocytes population and decreased splenic natural killer (NK) cells and T cell populations.

2. METHODS

2.1. Subjects

Adult male (N = 33) Balb/c mice, aged 3 weeks, were obtained from the Department of Pathology (School of Veterinary Medicine, University of São Paulo), São Paulo, Brazil. Mice were housed five per cage and maintained in environmentally controlled conditions on a 12:12-h light/dark cycle (lights off at 6:00 p.m.) with food and water available ad libitum. Animals were allowed to acclimate to their new surroundings for 1 week before initiation of any experimental procedure. All procedures were approved by the Bioethical Committee on Care and Use of Laboratory Animal Resources of School of Veterinary Medicine (Protocol No. 4485180614) of the University of São Paulo. Figure 16 illustrates the timeline of experimental procedures.

Figure 16 - Timeline of experimental procedures



From PND 30 to PND 40, the stressed groups were subjected to unpredictable stress and after 20 days, at PND 60 the LPS groups received a LPS injection. Sickness behavior was measured for 48 hours at 6 different time points (after 4, 8, 24, 28, 32 and 48 h after injection). At PND 62 the animals were euthanized and blood and spleen were collected.

2.2. Social-environmental stress procedure

The social-environmental stress procedure was previously described elsewhere (DE LIMA et al., 2017). Briefly, during adolescence, mice were subjected to a random pattern of unpredictable and stressful situations twice daily for 10 days from PND 30 to PND 40. The control group remained in its home cage throughout the experimental protocol.

2.3. LPS challenge

Lipopolysaccharide (LPS) (from Escherichia coli serotype O127:B8) was diluted in sterile saline (0.2 mg/ml) prior to injection. At PND 60, LPS groups received an intraperitoneally (i.p.) at a dose of 1.5 mg/kg. This dosage was chosen because it caused mild sickness for approximately 48 h (ISMAEL and BLAUSTEIN, 2013). The control groups received an equal volume i.p. injection of vehicle (sterile saline).

2.4. Measuring sickness symptoms

Sickness monitoring consisted of looking for the presence of 4 symptoms (huddling, piloerection, ptosis, and lethargy) at 4, 8, 24, 28, 32 and 48 h after LPS or saline treatment. At each time point, mice were assigned a score between 0 and 4, depending on the number of symptoms displayed. Sickness score was assessed by a blinded observer and stopped at 48 h because there were usually no observable sickness symptoms after this point.

2.5. Blood and tissue collection

Forty-eight hours after the LPS challenge, mice were rapidly decapitated and blood samples collected in EDTA-treated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ,

USA) and stored at 4 ℃ until analysis. The spleen from each mouse was removed and transferred to tubes containing phosphate buffered saline (PBS) to obtain cell suspensions.

2.6. TNF-α plasmatic levels

TNF-α plasmatic levels were quantified, from blood samples collected, using commercial enzyme-linked immunosorbent assay (ELISA) kit (Becton Dickinson, Franklin Lakes, NJ, USA).

2.7. Isolation of splenocytes

Splenocyte suspensions were obtained by processing the tissue in 5 ml cold PBS for 60 s in a paddle blender. Red blood cells were removed using Ammonium-Chloride-Potassium (ACK) Lysing Buffer (0.16 M NH4Cl, 10 mM KHCO3, 0.13 mM EDTA, pH 7.2). Cells were then washed in PBS, filtered through a 70-μm nylon cell strainer, resuspended in PBS and cell concentrations were determined.

2.8. Phenotyping of splenocytes by flow cytometry

Total cell counts were counted and a single cell suspension (1×10^6 cells) of each animal was incubated for 30 min at 4°C in the dark with antibodies against CD3 PerCP (clone 145-2c11), CD4 APC (clone RM 4-5) and CD8 PE (clone Ly2;53-6-7) to assess splenic T cell populations, with antibodies against CD45 FITC (clone 30F-11)/CD49 PE (clone rmC5-4) to assess splenic monocytes population, and finally with antibodies against CD45 FITC (clone 30F-11)/CD49b APC (clone DX5) to assess NK cells. All antibodies (BD Biosciences Pharmingen, San Diego, CA, USA) were diluted 1:100 in PBS (phosphate-buffered saline). The cells were washed once, and the fluorescence was acquired using a BD FACSCalibur™ flow

cytometer (Becton Dickinson). The analysis was done using Cell Quest Pro (Becton Dickson Immunocytometry Systems, San Jose, CA, USA) software from 10,000 events in each sample.

2.9. Data analysis

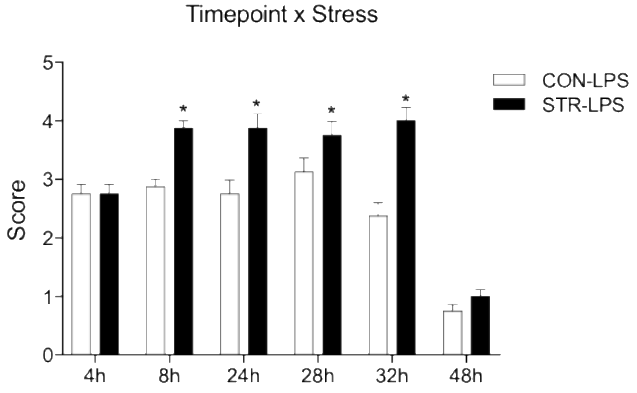
Data are expressed as mean ± SE (standard error). Statistical analysis consisted of between group, repeated measure analysis of variance (ANOVA), and multivariate analysis of variance (MANOVA) when appropriate. Analyses were performed using SPSS version 24 (IBM Corp, USA). An alpha level of $p < 0.05$ (two-tailed) was used to determine statistical significance.

3. RESULTS

3.1. Sickness behavior

Figure 17 illustrates sickness behavior results. Control groups (CON-SAL and STR LPS) almost did not show sickness behaviour, and thus ANOVA (Timepoint X Stress) was conducted for the LPS-treated mice only. The interaction was significant ($F_{4,332} = 3.52$, $p = 0.008$). Because of the large main effect of stress factor ($p < 0.0001$). In brief, STR-LPS showed sickness behaviour earlier and for longer than did CON-LPS group.

Figure 17 - Effects of stress during adolescence on LPS-induced sickness behavior in adult mice.

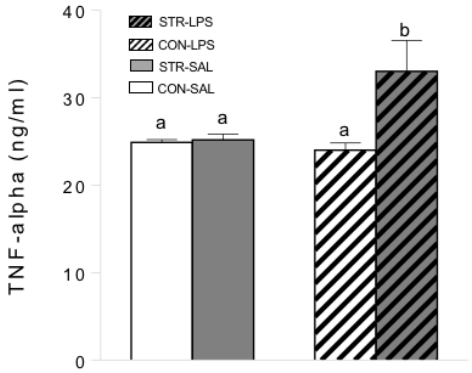


Data are expressed as median ± SE *p < 0.05 vs Control-LPS.

3.2. TNF-α plasma levels

Two-way ANOVA showed an interaction between both factors [(F (1,16) = 24.52; p < 0.05], a LPS-treatment effect (LPS vs saline) [(F (3,21) = 15.30; p < 0.05] and a stress effect [(F (1,16) = 27.76; p < 0.05]. Stress-LPS group had significantly higher TNF-α plasmatic levels than other groups (p < 0.05) (Fig. 18).

Figure 18 - Effects of stress during adolescence on TNF-α plasma levels after LPS challenge in adult mice.

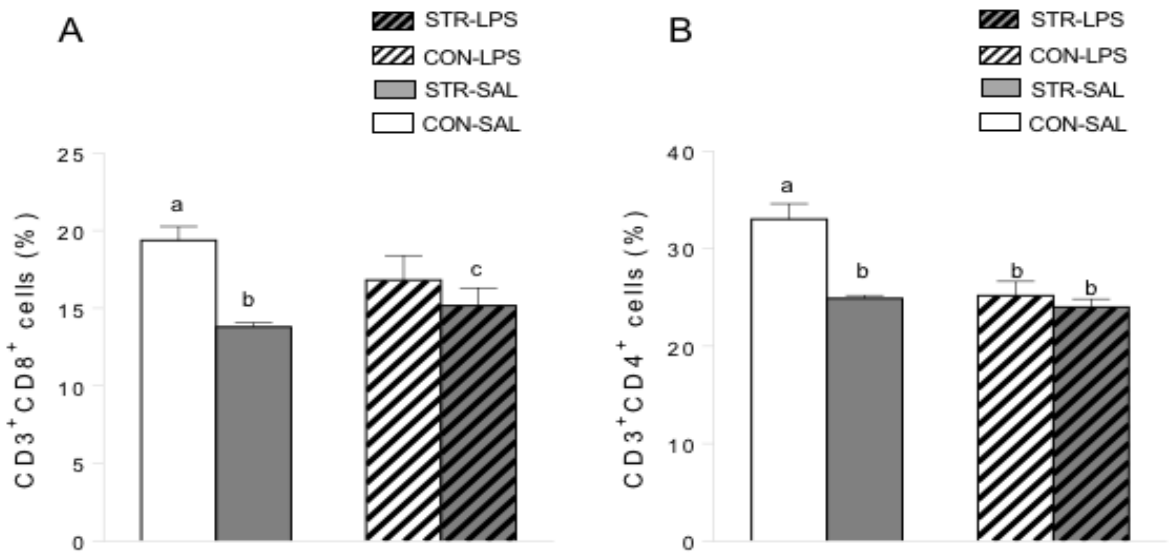


Data are expressed as mean ± SE, * p<0.05; different letters for statistically different groups.

3.3. Splenic T cells

When analyzing the population of T CD4 lymphocytes, two-way ANOVA indicated stress effect (stress vs control) [(F (1,16) = 28 p < 0.05]; LPS-treatment effect (LPS vs saline) [(F (1,16) = 25 p < 0.05] and interaction between both factors [(F (1,16) = 15 p < 0.05]. The multiple comparisons test revealed differences between the control-saline group and other groups. Figure 3 shows that the population of T CD4 decreased in LPS-treated groups (control-LPS and stress-LPS) and in the stress-saline group compared to control-saline group. When analyzing the population of T CD8 lymphocytes, two-way ANOVA revealed a stress effect (stress vs control) [(F (1,16) =32.17; p < 0.05], an interaction between both factors [(F (1,16) = 9.535; p < 0.05]. Figure 19 illustrates the statistical difference in the percentage of T CD8 cells among control-saline, stress-saline and stress-LPS groups when compared to each other.

Figure 19 - Effects of stress during adolescence on percentage of splenic lymphocytes T CD4+ and CD8+ after LPS challenge in adult mice.

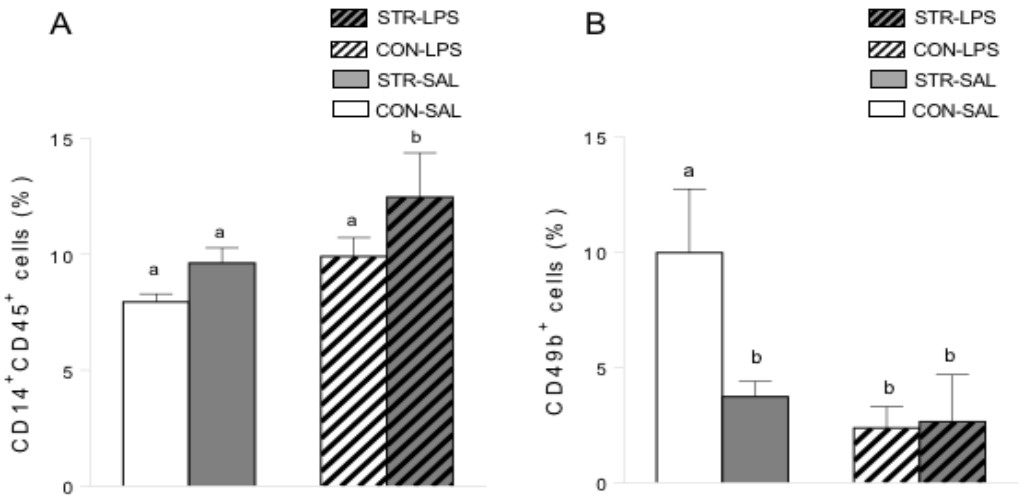


Data are expressed as mean ± SE; different letters for statistically different groups.

3.4. Splenic monocytes and NK cells

Two-way ANOVA showed an interaction between both factors LPS and stress [(F (1,16) = 6,012; $p < 0,05$], besides the LPS-treatment affect (LPS vs saline) [(F (1,16) = 38,54; $p < 0,05$] and the stress effect (control vs stress) [(F (1,16) = 6.012 $p < 0,05$]. On the multiple comparison test, the stress-LPS group presented an increase in percentage of monocytes when compared to other groups (Figure 20). Still on Fig. 5, in relation to NK cell population, there was interaction between both factors analyzed [(F (1,20) = 6,678; $p < 0,05$], with a significant effect of stress [(F (1,20) = 5,597; $p < 0,05$] and LPS [(F (1,20) = 11,86; $p < 0,05$]. In addition, in the multiple comparisons test, both the stress-saline group and the two LPS-treated groups showed a significant decrease in the percentage of NK cells when compared to control-saline group.

Figure 20 - Effects of stress during adolescence on percentage of splenic monocytes (CD14+CD45+) and splenic NK cells (CD49b+) after LPS challenge in adult mice.



Data are expressed as mean ± SE; different letters for statistically different groups.

4. DISCUSSION

The social-environmental stress model is a heterotypic stress regimen that prevents habituation and promotes increased glucocorticoids secretion. In addition, even for only 10 days during adolescence, this stress-induction model can affect the HPA function in adulthood, resulting in long-term increase in basal corticosterone levels (DE LIMA et al, 2017). Given the importance of modulatory effects of the glucocorticoids, the mechanisms of stress related to immune response should be better understood.

In our results, after the activation of the LPS immune response, we found altered periphery immune parameters, as well as prolonged sickness behavior in those animals that were stressed during adolescence. It is possible that these responses to the challenge with LPS seen in the stressed group are related to a dysfunction of the HPA axis. That is, high concentrations of corticosterone influence long-term immune responses induced by LPS. In fact, the low-dose single LPS i.p. injection alone increases plasmatic corticosterone levels in rodents as shown by Herman and Cullinan (1997). Thus, this study reinforces the idea that heterotypic chronic stress in adolescence, that prevents the habituation axis, had been related to dysfunction of HPA axis in adulthood, promoting increased basal glucocorticoid secretion which, in turn, affects long-term LPS-induced immune response.

Smith et al (2016) indicate that chronic variable stress in adult male rats promotes a tonic suppression of CNS immune response to LPS challenge. The bacterial endotoxin LPS i.p.

injection is a classic well-established model of peripheral innate immune response activation (ALLEN et al., 2012). It stimulates a wide variety of cell types such as phagocytic, including macrophages, and endothelial cells. The lipopolysaccharide-binding protein (LBP) binds to LPS and interacts with CD14, an essential membrane receptor for LPS-mediated cells. CD14 is a glycoprotein expressed only by monocytes and macrophages, and all other myeloid cell types (perhaps except for neutrophils and other non-myeloid cells), do not express this protein and are, therefore CD14 negative (JERSMANN, 2005). As required, monocytes or macrophages shed CD14 to facilitate LPS signaling for all other cells in conjunction with LBP. In our data, we observed an increase in CD14+CD45+ subset splenic cells only in STR-LPS. As we have not seen any difference in the CON-LPS group, it is reasonable to think that after 48 h of LPS injection, the CON-LPS group is already returning to baseline physiological conditions compared to both saline groups. Indeed, these results seen in this splenic subset population have the same pattern seen in the results of sickness behavior and peripheral TNF-α levels (discussed below), suggesting that chronic stress in adolescence affects the dynamics of immune response in adulthood.

Since this study is directly related to stress, we chose to evaluate sickness behavior by quantification of signs of sickness behavior, which is non-invasive and a practical and reliable method. Furthermore, it allowed the observation of sickness behavior directly related to TNF-α released induced by LPS. Sickness is defined as a stereotypical behavioral response to infection or injury (TIZARD, 2008) and the sources responsible to start these behavioral responses are not the neurons, but the cell sentinels of the innate immune system, such as dendritic cells, macrophages and mast cells (TIZARD, 2008). These changes in general modify physiological processes to promote disease resistance and recovery (JOHNSON et al., 2002).

In our data, the stressed animals after LPS injection (STR-LPS group) presented a slower recovery compared to non-stressed group (CON-LPS). In addition, TNF-α are still increased 48 h after challenge. Thus, it is important to consider an increase in the sickness behavior of this group due to the persistence of high levels of TNF-α, since in CON-LPS group, the levels of this cytokine already remain at baseline levels after 48 h of LPS challenge.

In addition, in light of an ethological perspective and according to SHAKHAR et al. (2007) that discussed altruism (HAMILTON, 1964; FOSTER et al., 2006) of the sickness behavior, animal species vary in the degree of intergroup relatedness based on their life history and social animal species adopt different sickness behavioral strategies to cope with a sick or immune challenge within a population. As we suggest in our previous work, stress during adolescence could contribute to the appearance of a new phenotype in a population of isogenic

mice, such as Balb/c mice, and here we reinforced and expanded this idea. That is, that social-environmental stress during adolescence seems to be related to development of different immunological strategies, which in turn, generate a distinct response to a LPS challenge in adulthood, both in sickness behavior and peripheral response.

Stefanski (2001) investigated the consequences of social stress on immunological measurements focused on T cells and NK cell subsets of dominant and submissive male rats. After stress, the submissive rats showed a decrease in peripheral TCD4, TCD8 and NK populations. The current results corroborate these data, since the STR-SAL group showed a decrease in the populations of CD4+, CD8+ and NK cells in the spleen compared to CON-SAL. The spleen is the largest secondary lymphoid organ and plays an essential role in the detection and removal of circulating pathogens. Thus, these results together with evidence in current literature, seem to suggest that stress in adolescence affects the dynamics and migration pattern of spleen immune cells in adult life interfering with the defense, resistance and recovery process in adulthood (SHAKHAR et al., 2007).

While TNF-α and other cytokines have classically been regarded as the major signaling molecules of the immune system, they are also, in many cases, potent neuromodulators (TIZARD, 2008). Moreover, cytokine response to LPS is inhibited if the LPS is administered during or immediately after stress, when glucocorticoid levels are high (GOUJON et al., 1995). Thus, in the present study, LPS, administered 20 days after the last day of stress, interferes with TNF-α. In this way, we can infer that corticosterone levels still high in the adulthood of the stressed adolescent animal act in the response to LPS as well as sensitize the cytokines response to LPS, in this case, specifically TNF- α in a long term.

Heterotypic chronic stress in adult rodents has peripheral action, such as a decrease in blood T cells (BASSO et al., 1993), and central immunosuppressive actions, such as the reduction of microglial activation in the prefrontal cortex (SMITH et al., 2016) and microglial apoptosis in the hippocampus (KREISEL et al., 2014). These current results together had shown that, in adolescent mice, heterotypic chronic stress (only 10 days), by persistent high corticosterone concentrations, also affects the immunomodulation mechanisms and has great relevance to immune response in adulthood.

Finally, these neuro-immune investigations provided new evidence that exposure of mice to socio-environmental stressors during adolescence can induce marked and long-lasting changes in LPS-induced TNF-α release, that can affect sickness behavior and peripheral immune cell population dynamic. Thus, the present results may help to better understand how a stressful adolescence can sensitize inflammatory reactions to an immune challenge in

adulthood. In addition, this stress model can be a useful tool for stress in adolescence studies, as well as long-term harmful effects of stress in individual life, including for immune parameters.

REFERENCES

ALLEN, R. G. et al. Stressor-Induced Increase in Microbicidal Activity of Splenic Macrophages Is Dependent upon Peroxynitrite Production. 2012-10-01 2012.

ANDERSEN, S. L. Trajectories of brain development: point of vulnerability or window of opportunity? **Neurosci Biobehav Rev,** v. 27, n. 1-2, p. 3-18, 2003 Jan-Mar 2003.

BASSO, A. M. et al. Seven-day variable-stress regime alters cortical beta-adrenoceptor binding and immunologic responses: reversal by imipramine. **Pharmacol Biochem Behav,** v. 45, n. 3, p. 665-72, Jul 1993.

BERKENBOSCH, F.; WOLVERS, D. A.; DERIJK, R. Neuroendocrine and immunological mechanisms in stress-induced immunomodulation. **J Steroid Biochem Mol Biol,** v. 40, n. 4-6, p. 639-47, 1991.

CARUSO, M. J. et al. Adolescent Social Stress Increases Anxiety-like Behavior and Alters Synaptic Transmission, Without Influencing Nicotine Responses, in a Sex-Dependent Manner. **Neuroscience,** v. 373, p. 182-198, Jan 2018.

CRONE, E. A.; DAHL, R. E. Understanding adolescence as a period of social-affective engagement and goal flexibility. **Nat Rev Neurosci,** v. 13, n. 9, p. 636-50, Sep 2012.

CUMMING, M. J.; THOMPSON, M. A.; MCCORMICK, C. M. Adolescent social instability stress increases aggression in a food competition task in adult male Long-Evans rats. **Dev Psychobiol,** v. 56, n. 7, p. 1575-88, Nov 2014.

DANTZER, R.; KELLEY, K. W. Twenty Years of Research on Cytokine-Induced Sickness Behavior. **Brain Behav Immun,** v. 21, n. 2, p. 153-60, Feb 2007.

DE LIMA, A. P. et al. Long-lasting monoaminergic and behavioral dysfunctions in a mice model of socio-environmental stress during adolescence. **Behav Brain Res,** v. 317, p. 132-140, Jan 2017.

DE LIMA, A. P. N.; MASSOCO, C. O. Passive adaptation to stress in adulthood after short-term social instability stress during adolescence in mice. **Stress,** v. 20, n. 3, p. 329-332, May 2017.

DEAK, T. et al. Long-term changes in mineralocorticoid and glucocorticoid receptor occupancy following exposure to an acute stressor. **Brain Res,** v. 847, n. 2, p. 211-20, Nov 1999.

DHABHAR, F. S.; MCEWEN, B. S. Stress-induced enhancement of antigen-specific cell-mediated immunity. **J Immunol,** v. 156, n. 7, p. 2608-15, Apr 1996.

EILAND, L.; ROMEO, R. D. Stress and the developing adolescent brain. **Neuroscience,** v. 249, p. 162-71, Sep 2013.

FANTUZZI, G. et al. Role of the hypothalamus-pituitary-adrenal axis in the regulation of TNF production in mice. Effect of stress and inhibition of endogenous glucocorticoids. **J Immunol,** v. 155, n. 7, p. 3552-5, Oct 1995.

FOSTER, K. R.; WENSELEERS, T.; RATNIEKS, F. L. Kin selection is the key to altruism. **Trends Ecol Evol,** v. 21, n. 2, p. 57-60, Feb 2006.

GADEK-MICHALSKA, A. et al. Cytokines, prostaglandins and nitric oxide in the regulation of stress-response systems. **Pharmacol Rep,** v. 65, n. 6, p. 1655-62, 2013.

GANGULY, P. et al. Adolescent food restriction in rats alters prefrontal cortex microglia in an experience-dependent manner. **Stress,** v. 21, n. 2, p. 162-168, Mar 2018.

GIOVANOLI, S. et al. Stress in puberty unmasks latent neuropathological consequences of prenatal immune activation in mice. **Science,** v. 339, n. 6123, p. 1095-9, Mar 2013.

GOUJON, E. et al. Stress downregulates lipopolysaccharide-induced expression of proinflammatory cytokines in the spleen, pituitary, and brain of mice. **Brain Behav Immun,** v. 9, n. 4, p. 292-303, Dec 1995.

GREEN, M. R.; BARNES, B.; MCCORMICK, C. M. Social instability stress in adolescence increases anxiety and reduces social interactions in adulthood in male Long-Evans rats. **Dev Psychobiol,** v. 55, n. 8, p. 849-59, Dec 2013.

GREEN, M. R.; MCCORMICK, C. M. Effects of social instability stress in adolescence on long-term, not short-term, spatial memory performance. **Behav Brain Res,** v. 256, p. 165-71, Nov 1 2013.

HAMILTON, W. D. The genetical evolution of social behaviour. II. **J Theor Biol,** v. 7, n. 1, p. 17-52, Jul 1964.

HAWIGER, J. Innate immunity and inflammation: a transcriptional paradigm. **Immunol Res,** v. 23, n. 2-3, p. 99-109, 2001.

HODGES, T. E. et al. Social instability stress in adolescent male rats reduces social interaction and social recognition performance and increases oxytocin receptor binding. **Neuroscience,** v. 359, p. 172-182, Sep 17 2017.

JERSMANN, H. P. Time to abandon dogma: CD14 is expressed by non-myeloid lineage cells. **Immunol Cell Biol,** v. 83, n. 5, p. 462-7, Oct 2005.

JOHNSON, J. D. et al. Prior stressor exposure sensitizes LPS-induced cytokine production. **Brain Behav Immun,** v. 16, n. 4, p. 461-76, Aug 2002.

KINCADE, P. W. **Formation of lymphocytes in fetal and adult life** Adv Immunol 31**:** 177 – 245 p. 1981.

KOLBER, B. J.; WIECZOREK, L.; MUGLIA, L. J. HPA axis dysregulation and behavioral analysis of mouse mutants with altered GR or MR function. **Stress,** v. 11, n. 5, p. 321-38, 2008.

KREISEL, T. et al. Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis. **Mol Psychiatry,** v. 19, n. 6, p. 699-709, Jun 2014.

LANDRETH, K. S. Critical windows in development of the rodent immune system. **Hum Exp Toxicol,** v. 21, n. 9-10, p. 493-8, 2002 Sep-Oct 2002.

MCCORMICK, C. M.; MATHEWS, I. Z. HPA function in adolescence: role of sex hormones in its regulation and the enduring consequences of exposure to stressors. **Pharmacol Biochem Behav,** v. 86, n. 2, p. 220-33, Feb 2007.

MCCORMICK, C. M.; SMITH, C.; MATHEWS, I. Z. Effects of chronic social stress in adolescence on anxiety and neuroendocrine response to mild stress in male and female rats. **Behav Brain Res,** v. 187, n. 2, p. 228-38, Mar 2008.

MEI-TAL, V.; MEYEROWITZ, S.; ENGEL, G. L. The role of psychological process in a somatic disorder: multiple sclerosis. 1. The emotional setting of illness onset and exacerbation. **Psychosom Med,** v. 32, n. 1, p. 67-86, 1970 Jan-Feb 1970.

O'CONNOR, K. A. et al. Peripheral and central proinflammatory cytokine response to a severe acute stressor. **Brain Res,** v. 991, n. 1-2, p. 123-32, Nov 2003.

PAGE, C. E. et al. Npas4 deficiency interacts with adolescent stress to disrupt prefrontal GABAergic maturation and adult cognitive flexibility. **Genes Brain Behav**, Jan 2018.

RAMAMOORTHY, S.; CIDLOWSKI, J. A. Corticosteroids: Mechanisms of Action in Health and Disease. **Rheum Dis Clin North Am,** v. 42, n. 1, p. 15-31, vii, Feb 2016.

RESENDE, L. S. et al. Social stress in adolescents induces depression and brain-region-specific modulation of the transcription factor MAX. **Transl Psychiatry,** v. 6, n. 10, p. e914, Oct 11 2016.

ROMEO, R. D.; MCEWEN, B. S. Stress and the adolescent brain. **Ann N Y Acad Sci,** v. 1094, p. 202-14, Dec 2006.

SHAKHAR, G. et al. Amelioration of operation-induced suppression of marginating pulmonary NK activity using poly IC: a potential approach to reduce postoperative metastasis. **Ann Surg Oncol,** v. 14, n. 2, p. 841-52, Feb 2007.

SISK, C. L.; FOSTER, D. L. The neural basis of puberty and adolescence. **Nat Neurosci,** v. 7, n. 10, p. 1040-7, Oct 2004.

SMITH, B. L. et al. Divergent effects of repeated restraint versus chronic variable stress on prefrontal cortical immune status after LPS injection. **Brain Behav Immun,** v. 57, p. 263-270, Oct 2016.

SPEAR, L. P. The adolescent brain and age-related behavioral manifestations. **Neurosci Biobehav Rev,** v. 24, n. 4, p. 417-63, Jun 2000.

STEFANSKI, V. Social stress in laboratory rats: behavior, immune function, and tumor metastasis. **Physiol Behav,** v. 73, n. 3, p. 385-91, Jun 2001.

TIZARD, I. Sickness behavior, its mechanisms and significance. **Anim Health Res Rev,** v. 9, n. 1, p. 87-99, Jun 2008.

XU, H. et al. Effects of adolescent social stress and antidepressant treatment on cognitive inflexibility and Bdnf epigenetic modifications in the mPFC of adult mice. **Psychoneuroendocrinology,** v. 88, p. 92-101, Feb 2018.

CHAPTER 4: THE EFFECTS OF ACUTE LIPOPOLYSACCHARIDE CHALLENGE ON HYPOTHALAMIC-PITUITARY-ADRENAL FUNCTION IN FEMALE AND MALE LONG-EVANS RATS AFTER SOCIAL INSTABILITY STRESS IN ADOLESCENCE.

1. INTRODUCTION

The puberty is a transition for non-reproductive to reproductive status and has a great flux of gonadal hormones that lead an extensive remodeling (SCHULZ et al., 2009) and reorganizing of a central nervous system (LEVITT, 2003). The adolescence that begins with puberty and ends with sexual maturation (SISK and FOSTER, 2004) is also a period sensitive to stressors and other environmental influences, which can increase susceptibility to cognitive or neuropsychiatric disorders (PATTON and
VINER, 2007).

The social instability (SS) model is already well established in the literature regarding effects on social behavior and HPA axis in male rats. This model had showed SS rats have an increase in anxiety-like behavior (GREEN et al., 2013) and deficits in mating behavior (GREEN and MCCORMICK et al, 2013), as well in social interactions with an unfamiliar peer (GREEN et al., 2013). The SS stress model avoids habituation of the HPA axis, at the moment that the isolated confinement of 1 hour active to the stress, being the increase of corticosterone and still activated at the moment of the pairing with the unknown partner. This procedure for 15 consecutive days thus causes a hyperactivation of the HPA axis that leads us to think of other effects on the neuroendocrine-immune circuit modulating the stress response. About the effects on female rats, short and long-term immune response as well as long-term effects on the HPA axis had not yet been investigated.

Although incomplete, once the present study is still ongoing, a discussion related to sickness behavior and plasma levels of corticosterone can be performed. So far, data have shown differences between male and female adolescents stressed for 15 days both when challenged by LPS 24 hours after stress or when challenged by LPS in adulthood

2. METHODS

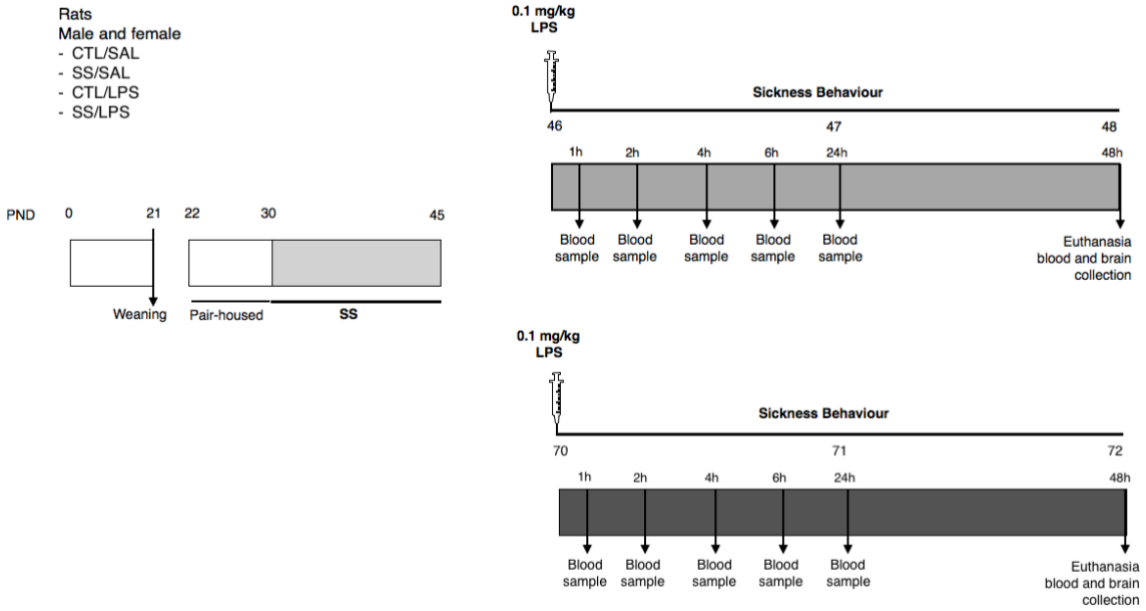
2.1. Subjects

Male (N = 80) and female (N = 80) Long-Evans rats were obtained from Charles River, St. Constant, Quebec, at 22 days of age. Rats were housed in pairs and maintained under a 12 h light–dark cycle (lights on at 07:00 h) with food and water available *ad libitum*. Male and female rats were randomly placed into one of two groups: unhandled control (CTL) or social instability stress (SS). All procedures were carried was approved by the Brock University Institutional Animal Care Committee (ACC) and was carried out in compliance with the Canadian Council of Animal Care guidelines.

2.2. Stress protocol

From 30 to 45 days of age all rats were randomly assigned to the social instability stress (SS, n = 40) group or to the non-stressed control (CTL, n = 40) group. The SS procedure was as described previously (reviewed in HODGES et al, 2017). Briefly, on postnatal day (P) 30, SS rats were isolated in a 12 cm × 10 cm ventilated plastic container in a room separate from the colony for 1 h each day until P45. Immediately after isolation each day, SS rats were returned to the animal colony and housed in a new cage with a new cage partner that had also undergone the 1 h isolation. The SS procedure was conducted at various times during the lights on phase of the light–dark cycle to minimize habituation to the procedure. On PND 45, after the final isolation, SS rats remained with the same cage partner and were left undisturbed except for cage maintenance or test procedures. CTL rats remained undisturbed in their home cages except for cage maintenance from time of arrival at the colony until the test procedures. (see Fig. 21 for experimental design and timeline of procedures).

Figure 21 - Experimental design and timeline of procedures.



2.3. LPS challenge

Lipopolysaccharide (LPS) (from *Escherichia coli* serotype O111:B4) was diluted in sterile saline (0.1 mg / ml) before injection. At 46 days of age, half of the SS group received a single intraperitoneal LPS (0.1 mg/kg) and were assigned to the stress/LPS (SS/LPS, n=12) group, and half of the CTL group also received a single intraperitoneal LPS (0.1 mg/kg) and were assigned to the control/LPS (CTL/LPS, n=12). The other half of SS and CTL groups received a vehicle (sterile saline) injection and were assigned to stress/saline (SS/SAL, n=8) group and control/saline (CTL/SAL, n=8) group respectively. The same follow as described above to animals at 70 days of age.

2.4. Sickness behavior

Sickness monitoring consisted in looking for the presence of 4 symptoms (huddling, piloerection, ptosis, and lethargy) at 1h, 2h, 4h, 6h, 24h and 48h after LPS or saline treatment (KOLMOGOROVA et al, 2017). At each time point, rats were assigned a score between 0 and 4, depending on the number of symptoms displayed. Sickness checks stopped at 48h because there were no observable sickness symptoms at this point in any group. Rats in the LPS groups that showed no sickness behavior at any time point nor any significant increase in corticosterone

across time points were removed from analysis as missed or mistaken injections, which resulted in SS/LPS adolescent males group (n=10), CTL/LPS adult females group (n=11) and SS/LPS adult females (n=10).

2.5. Blood collection

Blood samples were collected in un-anaesthetized rats from a tail nick. The blood collection intervals were: 0.5, 2, 4, 6, 24 and 48 hours after LPS-challenge. Sample collection required one small scissors nick on the end of tail and the rapid collection (in less than 3 min) of a drop of blood (MILOT et al, 2012) on Whatman blood stain cards (Sigma-Aldrich, Canada). This method provides reliable CORT values and requires a minimal amount of blood from the animal minimizing associated stress (MILOT et al., 2012). Once collected, samples were allowed to dry at room temperature overnight and then stored at -80^{o}C until ELISA procedure.

2.6. Brain collection

Brain were collected from rats at the time of decapitation. Brains were immediately sliced on dry ice with a razor blade, rapidly the slices were placed on slides and frozen and each slide was stored at -80 °C until processing. Slices of each animal was used for Western blot experiments. The dorsal hippocampus (-3.12 mm from bregma), ventral hippocampus (-4.56 mm from bregma), and medial prefrontal cortex (+3.24 mm from bregma) were dissected out from the left hemisphere of each sample, and each dissected brain region was placed into a separate microcentrifuge tube on dry ice.

2.7. Corticosterone (CORT) Immunoassay

Sample Whatman cards were thawed from -80^{o}C and CORT levels from blood droplets were determined in duplicates using a commercially available ELISA kit (Corticosterone EIA kit, Enzo Life Sciences, Cat. No. ADI-901-097). Briefly, a 3mm diameter circle of each group

drop sample (n=16 per group per blood collection interval) was punched from the blood stain cards using a McGill Handheld Gem Punch and placed in labelled tubes containing 280 µl of a solution consisting of assay buffer diluted in dH2O at 1:10 concentration. The tubes were covered with parafilm and shaken on the Belly Dancer® Laboratory shaker for 24h at RT prior to the ELISA procedure. On the next day, 214.5 µl of each sample and 5.5 µl of steroid displacement reagent (SDR) were mixed in labelled aliquots and vortexed. Standards and samples were prepared in 96-well plates as recommended by the manufacturer. CORT concentrations were determined in Biotek Synergy plate reader.

2.8. Tissue collection for microbiome

After euthanasia of each animal, the intestinal distal colon was located and cut longitudinally 0.25 cm over under the feces. Once the colon was open, the feces were collected in a sterile tube while the colon was gently washed. After, feces and the colon were flash frozen and storage at -80°C.

2.9. Statistical analysis

Statistical analysis consisted of between group, repeated measure analysis of variance (ANOVA), and multivariate analysis of variance (MANOVA) when appropriate. Analyses were performed using SPSS version 24 (IBM Corp, USA). An alpha level of $P < 0.05$ (two-tailed) was used to determine statistical significance. Post hoc analyses include F tests for simple effects, t-tests, and LSD, when appropriate.

3. RESULTS

3.1. Weight

Weight gain from the day of injection (pre-injection) to three days later (post-injection) were analyzed. Because of the sex and age differences were as expected, Stress Group X Treatment ANOVAs were conducted for these groups separately on weight gain from the day

of injection (pre-injection) to three days later (post-injection). For all Age and Sex groups, LPS-treated gained less weight than did saline-treated rats (all $p < 0.01$), and there was no main effect of Stress Group (all $p > 0.16$) or interaction (all $p > 0.30$) for any of these groups (data not shown).

3.2. Sickness Behavior

Figure 22 illustrates the sickness behavior results. No saline-treated rat showed sickness behaviour, and thus a mixed-factor ANOVA (Sex X Age X Stress Group X Timepoint) was conducted for the LPS-treated rats only. The four-way interaction was significant ($F_{4,332} = 3.52$, $p = 0.008$). Because of the large main effect of Sex ($p < 0.0001$), separate Stress Group X Timepoint X Age analyses were conducted for males and females. In brief, males showed sickness behaviour earlier and for longer than did females, independent of age.

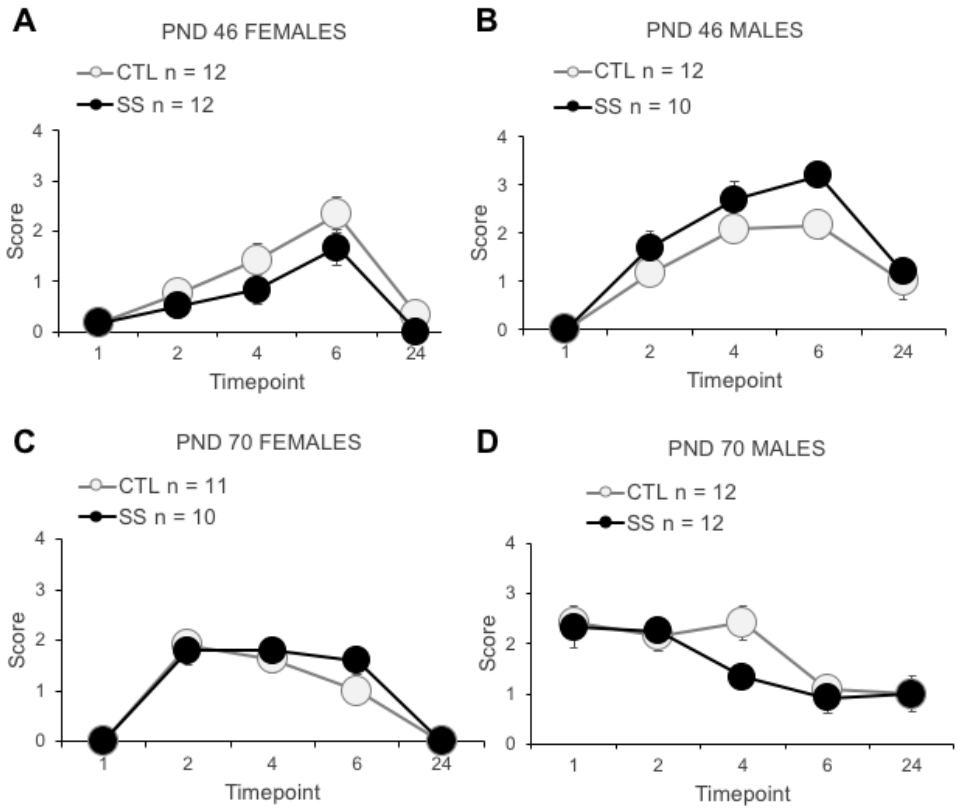
For PND 46 (Fig. 22A) and PND 70 (Fig. 22C) females, there was no, or almost no sickness behaviour at the 1 h and 24 hr timepoints, and thus only the 2, 4, and 6 hr times were included in the analysis. The interaction of Age and Timepoint ($F_{2,84} = 30.00$, $p < 0.001$) and the interaction of Stress Group and Age ($F_{1,42} = 3.52$, $p = 0.007$) were significant (3-way interaction, $p = 0.147$). Post hoc t-tests indicated that PND 46 females had higher sickness scores than PND 70 females at 2 hr, whereas PND 70 females had higher sickness scores than PND 46 females at 6 hr ($p < 0.0001$). For PND 46 females, sickness behaviour increased between 2 hr and 4 hr ($p = 0.001$) and between 4 hr and 6 hr ($p < 0.001$).

For PND 70 females, there was no change in sickness behaviour 2 hr and 4 hr ($p = 0.576$) and between 4 hr and 6 hr ($p = 0.083$). CTL and SS females did not differ in sickness behaviour at either PND 46 ($p = 0.221$) or PND 70 ($p = 0.378$), although among SS rats, PND 70 females had higher sickness scores than did PND 46 females ($p = 0.041$), a difference that was not evident in CTL females ($p = 0.964$) (Fig. 23).

For PND 46 males (Fig. 22B), there was no, or almost no sickness behaviour at the 1 hr timepoint, whereas for PND 70 males (Fig. 22D), sickness behaviour was observed at all time points. Thus, separate analyses were conducted for the two age groups. For PND 46 males, the analysis was conducted on the 2, 4, 6, and 24 hr timepoints. The main effect of Timepoint was significant ($F_{3,60} = 15.731$, $p < 0.001$), and SS rats had higher sickness scores than did CTL rats ($p = 0.024$; interaction, $p = 0.494$) (Fig. 23). Sickness behaviour increased from 1 to 2 ($p < 0.001$) and from 2 to 4 hr ($p = 0.001$) and did not change from 4 to 6 hr ($p = 0.367$) and decreased from 6 to 24 hr ($p < 0.001$). For PND 70 males, all timepoints were included in the analysis.

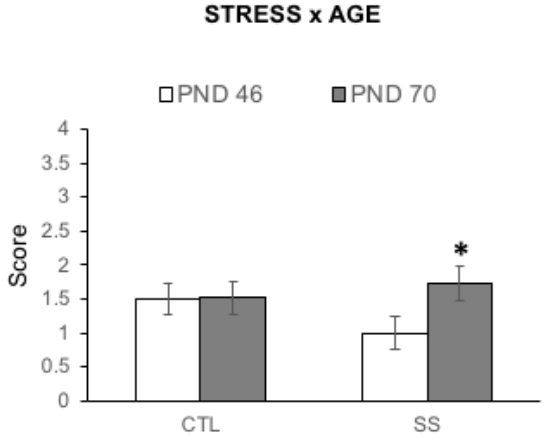
The main effect of Timepoint was significant ($F_{4,88} = 15.92$, $p < 0.001$) (Fig 24B). There was no effect of Stress Group ($p = 0.381$) or interaction ($p = 0.090$). Peak sickness behaviour was evident at 1 hr, and did not change from 1 to 2 ($p = 0.295$) or from 2 to 4 hr ($p = 0.088$), and declined from 4 to 6 hr ($p = 0.001$), and did not differ between 6 and 24 hr ($p = 1.00$) (Fig 24C).

Figure 22 – Stress x Age interaction in the score of sickness behavior after LPS challenge of stressed and control adolescent and adult rats.



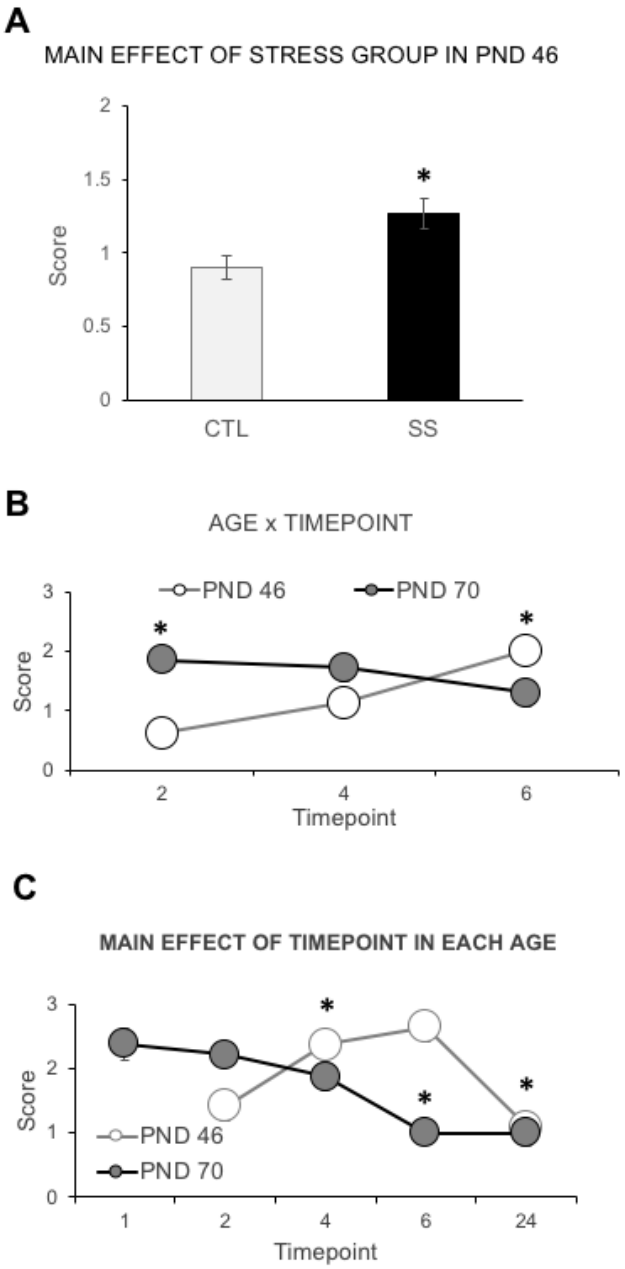
Data are expressed as mean ± SE.

Figure 23 – Stress x Age interaction in the score of sickness behavior after LPS challenge of stressed and control adolescent and adult female rats.



Data are expressed as mean ± SE.

Figure 24 – Sickness behavior of adolescent and adult male rats. Main effect of stress group in adolescent male rats (A). Age x Timepoint interaction (B) and main effect of timepoint in each age in the score of sickness behavior after LPS challenge of stressed and control adolescent and adult rats.



Data are expressed as mean ± SE.

3.3. Corticosterone concentrations

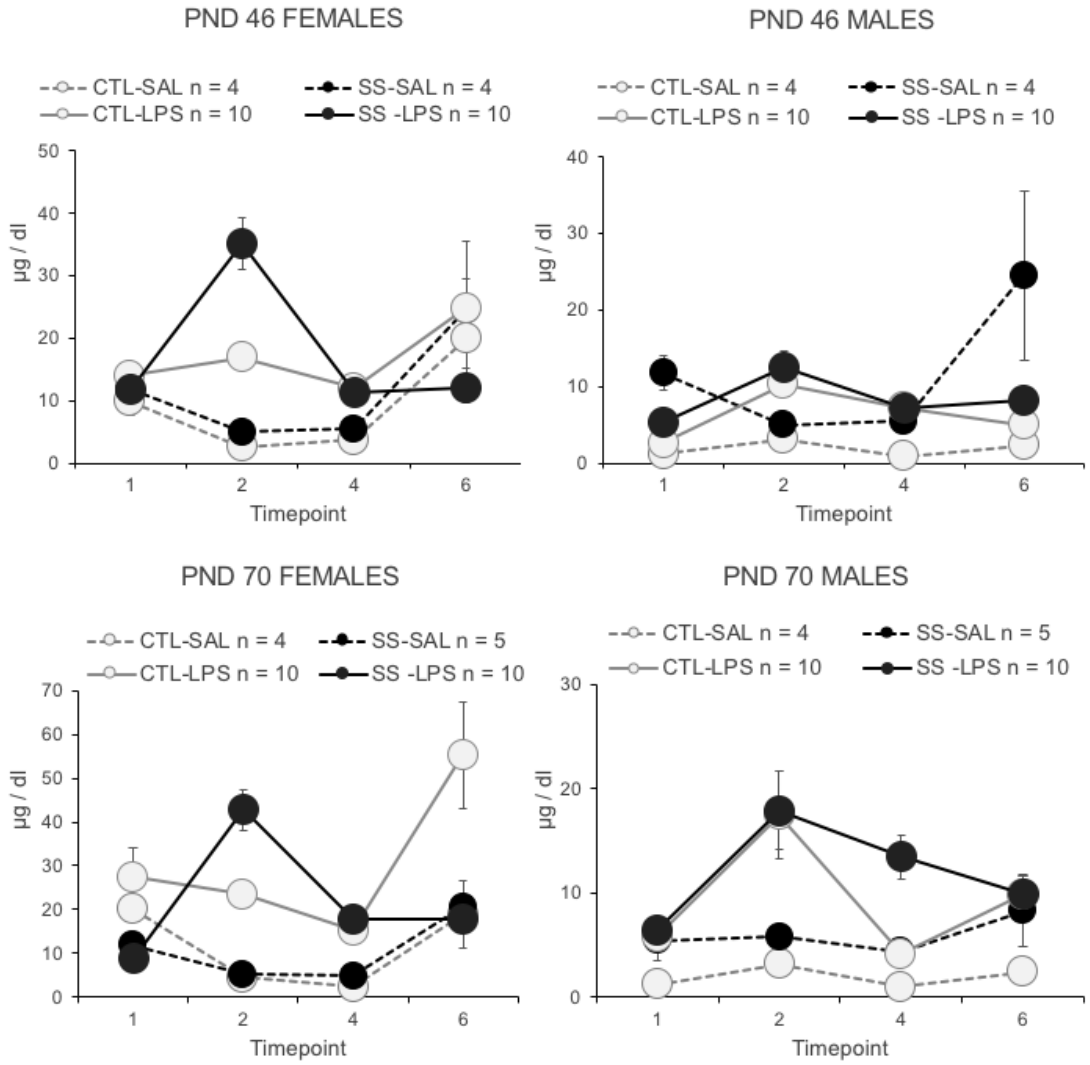
Figure 25 illustrates the corticosterone concentrations results. A mixed-factor ANOVA (Sex X Age X Stress Group X Treatment X Timepoint) on corticosterone concentrations

indicated significant interactions of Sex X Stress Group X Treatment X Timepoint ($F_{3,294}$ = 5.64, p = 0.001), which obviated any other main effects of, or interactions among, those factors. No other four-way interaction was significant (all p < 0.20), nor was the five-way interaction (p = 0.753). For the remaining factor of Age, the main effect (p = 0.001) and the interaction of Treatment X Age (p = 0.010) were significant, whereby PND 70 rats had higher corticosterone concentrations than did PND 46 rats in the LPS-treated rats, and there was no age difference for saline-treated rats.

To interpret the 4-way interaction, separate post hoc ANOVAs (Stress Group X Treatment X Timepoint) were conducted for females and males. For females, the 3-way interaction was significant ($F_{3,159}$ = 6.87, p < 0.001) (Fig. 26), and, based on the higher corticosterone concentrations in the LPS-treated than in the saline-treated (p < 0.0001), t-tests were conducted for the Stress groups at each timepoint for the Treatment groups separately. Saline-treated SS and CTL females did not differ at any timepoint (all p > 0.05). Among LPS-treated, CTL females had higher corticosterone concentrations at 1 hr (p = 0.012) and 6 hr (p < 0.002), whereas SS had higher corticosterone concentrations at 2 hr (p < 0.001).

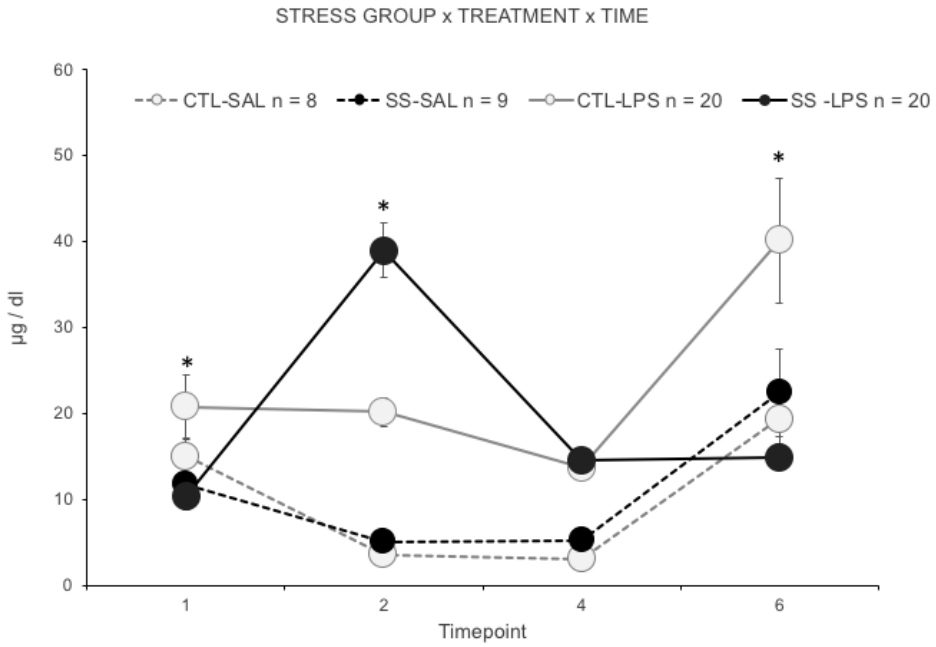
For males, the main effect of Stress Group was significant ($F_{1,53}$ = 11.04, p = 0.002), with SS rats having higher corticosterone concentrations than CTL rats. (Fig. 27A) The interaction of Treatment and Timepoint was significant ($F_{3,156}$ = 5.65, p = 0.001). LPS-treated males had higher corticosterone concentrations than did saline-treated males at 2 (p < 0.001) and 4 hr (p = 0.001) (Fig. 27B).

Figure 25 – Plasma corticosterone concentrations of adolescent and adult female, and adolescent and adult male stressed rats after LPS injection.



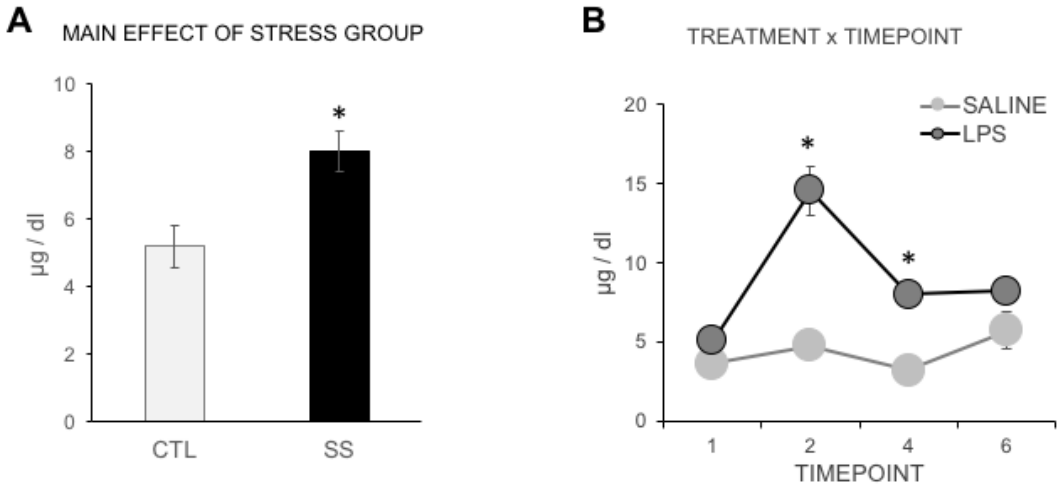
Data are expressed as mean ± SE.

Figure 26 – Plasma corticosterone concentrations of female rats. Stress x treatment x time interaction in female rats.



Data are expressed as mean ± SE.

Figure 27 – Plasma corticosterone concentrations of male rats. Main effect of stress group (A) and the interaction of treatment x timepoint (B) in male rats.



Data are expressed as mean ± SE.

4. DISCUSSION

Adults rats lost weight after LPS challenge, while adolescence gained weight.

Overall, adult rats lost weight following LPS exposure, while adolescence gained weight, although LPS-adolescent weight gain was significantly less than the saline group. The weight-loss resistance seen in adolescence is easily explained by the growth adolescents undergo during this time. As well, LPS adult males show greater weight loss in comparison to other groups. Since adult males typically have greater sickness responses, it is likely that the adult males are experiencing more hypothermia and reduced appetite, and thus eating less.

Males showed greater sickness behaviour than did females after LPS challenge.

As mentioned previously, males showed significantly greater sickness behaviours when compared to females. Indeed, in mice, it has already seen that males displayed more sickness than females (CAI et al., 2016). These effects as suggested by Cai et al. (2016) due to complex interactions between the hypothalamus-pituitary-gonadal (HPG) and hypothalamus-pituitary-adrenal (HPA) axes, since the immune-suppressive properties of androgens, such that males show reduced activity of phagocytic immune cells, with enhanced pro-inflammatory cytokine release, that is correlated with increased sickness behaviours.

Adolescents showed less sickness behaviour than did adults after LPS challenge.

In relation to age, adolescence show reduced sickness behaviours compared to adults. This is consistent with previous research, which has shown that in males, adults show greater sickness behaviours and a larger drop in body temperature following LPS when compared to pubertal mice (CAI et al., 2016). The reduced sickness behaviours seen in adolescence are likely due to the increasing HPG activation throughout puberty. The reduced HPG activity in adolescents (when compared to adults) will consequentially have reduced regulation of the immune system. In males, reduced HPG regulation of the immune system would reduce the cytokine release, and thus the sickness behaviours seen in males.

Stressed females show significantly increased sickness behaviours in adult females, when compared to adolescence.

The presence of a stress effect in adult females (but not adult males) suggests a long-term change in mechanisms underlying the HPA-immune axis response, that is sex specific. This finding is contradictory to some previous research, where chronic adolescent stress exacerbates sickness behaviours in males, but not females (YEE and PRENDERGAST, 2010). Other study has shown that adult females display higher cytokine levels than pubertal females, due to the higher stress reactivity in adult females compared to adolescent females (ISMAIL et al., 2013). The controversy results could be due to the different type of chronic stress.

In males, the SS effect was only seen in adolescence

As well as in females, we showed in our results the same once already demonstrated in mice in the literature, that adolescents and adults respond differently to LPS (CAI et al., 2016). In males, reduced HPG regulation of the immune system would reduce the cytokine release, and thus the sickness behaviours seen in males. In addition, we have shown that social instability stress model seems to affect, in short but not long term, the response to an immune stressor stimulus confirming that stress due to social instability actually acts on the modulation of the HPA axis and consequently is able to affect on a short term in sickness behavior of LPS-challenged males.

Females showed higher CORT levels when compared to male rats

As expected, females show significantly higher CORT levels when compared to males. This is a well-established sex differences, due to the different proportions of gonadal hormones. As well, adults showed higher CORT responses than adolescents. It has been previously shown that corticosterone release is greater and more prolonged after injections of LPS in adult when compared to adolescents (LEAH et al., 2013). Males showed main effects of stress and LPS, such that each condition independently enhanced the CORT response. As well, there was no significant age effect. Both the main effects support previous research that social instability stress and LPS can enhance corticosterone release, through different mechanisms of HPA activation (MCCORMICK et al., 2018). Interestingly, previous research has shown that following an acute LPS challenge, adult male mice show higher CORT responses compared to adolescents (CAI et al., 2016). As well, MCCORMICK et al (2015) has shown that social instability stress procedures enhance corticosterone release in adolescence when compared to adulthood. However, other studies have shown no effect of chronic stress, or a mitigate

(reduced) corticosterone response to an acute stressor in Sprague-Dawley and Long-Evans rats (MCCORMICK et al., 2004; KABBAJ et al., 2002).

Stressed LPS-treated female rats had a higher corticosterone profile than adolescent females.

According to the literature, adolescent females present higher levels of CORT than adult females (LUO et al., 2013). Our data corroborate with the literature all LPS-treated males and females displayed higher serum corticosterone concentrations than saline controls two hours after treatment (GIRARD-JOYAL et al., 2015) and provides further evidence that the adolescent HPA responds differently from the HPA axis of an adult.

The enhanced CORT response seen in SS-LPS adolescent females was still present in SS females injected in adulthood, weeks after the stress procedure had ended. Alongside this, stressed adult females show enhanced sickness behaviours when compared to adolescent females. Thus, stress during adolescent alters the developmental trajectory of the CORT response in a way that is conserved into adulthood. Notably, McCormick studies suggest that adolescent females exposed to social instability stress procedure do not differ in their HPA responsivity when compared to their stressed adult female counterparts (GREEN et al., 2018; MCCORMICK et al., 2017). The results suggest that systems interacting with the HPA response are being morphologically altered, thus indirectly effecting the CORT response.

For example, previous study has suggested that chronic stress suppresses the HPG axis in females (ISMAIL, 2011). Adolescent female CD1 mice exposed to shipping stress in adolescence showed reduced estrogen receptor alpha (ER-a) expression in brain regions such as medial preoptic area (MPOA), and nuclei within the hypothalamus. The result is reduced behavioural and physiological responsivity to estrogen and progesterone, thus altering the regulation of systems. ER-a receptors are key modulators of the immune system. Peripheral immune challenges (through LPS) can induce alterations in ER-a receptors on immune cells (LANDI et al., 1982; LU et al., 1999). In females, reduction of ER-a receptors would increase expression of cytokines, and reduce activity of macrophages (ISMAIL et al, 2011). Increase in production of cytokines (such as IL-16) would correspondingly increase activation of the HPA, and the CORT response following an injection with LPS, which would explain in our results the increase of CORT in adult females.

The long-term effect of chronic stress in female rats appears to be beneficial and harmful at the same time.

Previous research has suggested that estradiol is neuro-protective to chronic stressors, such that females have reduced central inflammation when compared to males. The enhanced peripheral immune reactivity is theorized to be an adaptive physiological mechanism with immuno-protective properties (DHABHAR, 2014). Thus, it is suggested that the enhanced CORT response seen in SS-LPS females is adaptive to reduce the impact of an acute immune challenge in the brain. However, females have an enhanced risk of autoimmune disorders (VOSKUHL, 2011) Autoimmune disorders are associated with shifts in the ratio of cytokines (pro:anti), and immune cell types (YANG et al, 2009; OSNES et al, 2013). Previous research has already shown that repeated HPA activation and morphological changes in regulation (through ER-a and GR) can shift the balance to a higher pro-inflammatory marker ratio (such as IL-6). Thus, stress during a time of developmental plasticity, such as adolescence, could theoretically predispose females to developing autoimmune disorders. Therefore, while females may be protective centrally, chronic stress may predispose females to developing peripheral immune disorders, such as autoimmune disorders.

The data from the microbiota and the brain will bring new discussions and conclusions regarding the differences between males and females and the effects of stress at the different moments of the LPS challenge. But for now, this study so far identified important age and sex-specific effects of an acute immune challenge. As well, we have identified that long-term effects of social instability stress during adolescent are sex-specific. The response seen on corticosterone to LPS in adult female rats is probably due to morphological changes that indirectly interact with the HPA axis, via the HPG axis, and the immune system. Further studies are needed to investigate the relationship and effects of a stress in adolescence on HPA axis regulation systems, as well as morphological changes within the immune system and the HPG axis that induce long-term changes in adult female rats.

REFERENCES

BOURKE, CH; HARRELL, CS; NEIGH, GN. Stress-Induced Sex Differences: Adaptations Mediated by the Glucocorticoid Receptor. **Horm Behav**. v. 62(3), p. 210–218, 2012.

CAI KC, VAN MIL S, MURRAY E, MALLET JF, MATAR C, ISMAIL N. Age and sex differences in immune response following LPS treatment in mice. **Brain Behav Immun**. v. 58, p. 327-337, 2016.

DHABHAR FS. Effects of stress on immune function: the good, the bad, and the beautiful. **Immunol Res.**v. 58(2-3), p. 193-210, 2014.

GIRARD-JOYAL, O.; FARAGHER, A.; BRADLEY, K.; KANE, L.; HRYCYK, L.; ISMAIL, N. Age and sex differences in c-Fos expression and serum corticosterone concentration following LPS treatment. **Neuroscience**. v. 305, p. 293-301, 2015.

GREEN, M. R.; BARNES, B.; MCCORMICK, C. M. Social instability stress in adolescence increases anxiety and reduces social interactions in adulthood in male Long-Evans rats. **Dev Psychobiol,** v. 55, n. 8, p. 849-59, 2013.

GREEN, M. R.; MARCOLIN, M. L.; MCCORMICK, C. M. The effects of ovarian hormones on stressor-induced hormonal responses, glucocorticoid receptor expression and translocation, and genes related to receptor signaling in adult female rats. **Stress**. v. 21(2), p. 90-100, 2018.

GREEN, M. R.; MCCORMICK, C. M. Effects of social instability stress in adolescence on long-term, not short-term, spatial memory performance. **Behav Brain Res,** v. 256, p. 165-71, 2013.

HODGES, T. E. et al. Social instability stress in adolescent male rats reduces social interaction and social recognition performance and increases oxytocin receptor binding. **Neuroscience,**v. 359, p. 172-182, Sep 17 2017.

ISMAIL, N.; GARAS, P.; BLAUSTEIN, J. D. Long-term effects of pubertal stressors on female sexual receptivity and estrogen receptor-α expression in CD-1 female mice. **Horm Behav**. v. 59 (4), p. 565-71, 2011.

KABBAJ M, ISGOR C, WATSON SJ, AKIL H. Stress during adolescence alters behavioral sensitization to amphetamine. **Neuroscience**. v.113(2), p.395-400, 2002.

KOLMOGOROVA, D., MURRAY, E., ISMAIL, N. Monitoring pathogen-induced sickness in mice and rats. **Current Protocols in Mouse Biology**, v.7, p. 65–76, 2017.

LANDI MS, KREIDER JW, LANG CM, BULLOCK LP. Effects of shipping on the immune function in mice. **Am. J. Vet. Res**. v.43, p. 1654–1657, 1982.

LEAH, MP; SEAN, DK; CONSTANCE, SH; GRETCHEN, NN. Sex differences in the effects of adolescent stress on adult brain inflammatory markers in rats. Brain Behav Immun. v. 30, p. 88-94, 2013.

LEVITT, P. Structural and functional maturation of the developing primate brain. **J. Pediatr**. v. 143 (4), p.S35-45, 2003.

LU ZW, HAYLEY S, RAVINDRAN AV, MERALI Z, ANISMAN H. Influence of psychosocial, psychogenic and neurogenic stressors on several aspects of immune functioning in mice. **Stress.** v. 3(1), p. 55–70, 1999.

LUO J., WANG T., LIANG S., HU X., LI W., JIN F. Experimental gastritis leads to anxiety- and depression-like behaviors in female but not male rats. **Behavioral and Brain Functions**. v. 9, 2013,.

MCCORMICK, C. M.; CAMERON, N. M.; THOMPSON, M. A.; CUMMING, M. J.; HODGES, T. E.; LANGETT, M. The sexual preference of female rats is influenced by males' adolescent social stress history and social status. **Horm Behav**. v. 89, p. 30-37, 2016.

MILOT, MR; JAMES, JS; MERALI, Z; PLAMONDON H. A refines blood collection method for quantifying corticosterone. **Lab Anim**, v. 41(3), p.77-83, 2012.

OSNES L. T., NAKKEN B., BODOLAY E., SZODORAY P. Assessment of intracellular cytokines and regulatory cells in patients with autoimmune diseases and primary immunodeficiencies—Novel tool for diagnostics and patient follow-up. **Autoimmunity Reviews**. v.12(10), p.967–971, 2013.

PATTON, GC; VINER, R. Pubertal transitions in health. **Lancet**. v. 369 (9567), p. 1130-9, 2007.

SCHULZ, K. M.; MOLENDA-FIGUEIRA, H. A.; SISK, C. L. Back to the future: The organizational-activational hypothesis adapted to puberty and adolescence. **Horm Behav**. v. 55(5), p. 597-604, 2009.

SISK, C. L.; FOSTER, D. L. The neural basis of puberty and adolescence. **Nat Neurosci**, v. 7, n. 10, p.1040-7, 2004.

VOSKUHL, R. Sex differences in autoimmune diseases. **Biol Sex Differ**. v. 2 (1), 2011.

YANG J, CHU Y, YANG X, GAO D, ZHU L, YANG X, WAN L, LI M. Th17 and natural treg cell population dynamics in systemic lupus erythematosus. **Arthritis and Rheumatism**. v. 60(5), p. 1472–1483, 2009.

YEE, JR; PRENDERGAST, BJ. Sex-specific social regulation of inflammatory responses and sickness behaviors. **Brain Bhav Immun**. v. 24 (6), p. 942-51, 2010.

CONCLUSION

This thesis, composed of four distinct chapters, investigated the effects of stress during adolescence, using animal models, mice and rats, focusing on HPA axis, neuroimmune-endocrine modulation, and behavioral aspects.

Although it did not focus on a single model of stress induction or on a single animal species, the thesis addressed the subject of adolescent stress in rodents by different aspects and concentrating in long-term effects (in adulthood), of a stress still during adolescence.

Directly and summarily, as the main conclusions of this thesis, which are best discussed in each chapter are:

The social-environmental stress during adolescence in male mice

- compromises the HPA axis to respond to a stressor stimulus properly in adulthood;
- causes alterations in the CNS;
- and also, can induce marked and long-lasting changes in LPS-induced TNF-α release, that can affect sickness behavior and peripheral immune cell population dynamic.

The social instability stress during adolescence in male mice

- results in a passive coping with stress during adulthood;
- replicates results, already demonstrated in rats, and reinforces that confinement and isolation for 1 hour and the exchange of cage partners daily evoke a persistent elevation of corticosterone during adolescence, which in turn promotes permanent changes in the circadian rhythm of the glucocorticoids;
- reflects in stress coping strategies that have effects on stress challenges in adulthood.

And as expected, males and females, adolescents, and adult rats respond differently to an acute LPS treatment, and the social instability stress model affects the immune response of males, especially in the short term, but does not appear to affect females in the same way.

Therefore, stressful situations, during the developmental period of adolescence, homotypic or heterotypic models in a short term (10 – 15 days), can be associated with long-lasting changes in neural pathways, behavior and immune parameters, eliciting a potential

neuroimmuno-endocrine and behavioral vulnerability for the development of disorders related to stress response in adulthood and also with a greater opportunity to behavioral and physiological flexibility to respond to different environmental and social contexts.

ANNEXE

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Title: Long-lasting monoaminergic and behavioral dysfunctions in a mice model of socio-environmental stress during adolescence

Author: A.P.N. de Lima,T.M. Sandini,T.M. Reis-Silva,C.O. Massoco

Publication: Behavioural Brain Research

Publisher: Elsevier

Date: 15 January 2017

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Title: Passive adaptation to stress in adulthood after short-term social instability stress during adolescence in mice

Author: A. P. N. de Lima, C. O. Massoco

Publication: STRESS: THE INTERNATIONAL JOURNAL ON THE BIOLOGY OF STRESS

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São Paulo, 2 de abril de 2018

Eu, Cristina de Oliveira Massoco Salles Gomes, CPF 167643398-80 co-autor do artigo "Long-lasting monoaminergic and behavioral dysfunctions in a mice model of socio-environmental stress during adolescence.", publicado em 2017 pela revista:"Behavioral Brain Research.", declaro estar de acordo com a inclusão e publicação do acima referido artigo na Tese de Doutorado intitulada "Hypothalamic-pituitary-adrenal axis dysfunctions and the long-term effects on behavior, neurochemistry and immune response in rodents" de autoria de Ana Paula Nascimento de Lima, aluna de Doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Cristina de Oliveira Massoco Salles Gomes

São Paulo, 2 de abril de 2018

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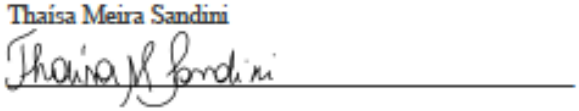
Atenciosamente,

Thaísa Meira Sandini



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Atenciosamente,

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Atenciosamente,

Cristina de Oliveira Massoco Salles Gomes