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NILTON JOSÉ DA SILVA FILHO

Effects of early life stress on the innervation of the tongue: an experimental study in rats

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Orientador: Prof. Assoc. Daniel Galera Bernabé

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“Todas as vitórias ocultam uma abdicação.”
BEAUVOIR, S.

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RESUMO

Evidências crescentes mostram que várias formas de estresse estão associadas a alterações no sistema nervoso. No entanto, não foi analisado como o estresse afeta a inervação da língua. Para examinar se o estresse precoce altera a inervação da língua de ratos, vinte ratos machos Wistar foram divididos em 2 grupos (n=10): Controle – ratos não expostos ao EPV por SM; SM – ratos expostos a EPV por SM. O protocolo de SM consistiu em separar as ninhadas da mãe por um período de 3 horas por 21 dias consecutivos após o nascimento. Após completar sete meses de idade, todos os ratos foram eutanasiados. Foram realizadas reações imunohistoquímicas para S100 e TH. As análises para o S100 revelaram um aumento na densidade dos nervos na região subepitelial e média, enquanto que na região média e inferior houve um aumento no número de estruturas neurais. Já na análise para o TH, apesar de uma tendência no aumento da densidade dos nervos, não houve significância estatística. Também não observamos diferenças no número de estruturas neurais simpáticas. Não houve diferença estatística no diâmetro dos nervos para ambos marcadores. Nós demonstramos pela primeira vez que ratos submetidos ao estresse precoce por separação materna apresentaram maior densidade de nervos na região subepiteligal da língua e também um número maior de estruturas neurais na região média e inferior da língua (S100). Também mostramos que ratos submetidos à SM tiveram um aumento na densidade de nervos na região média da língua. O número e a densidade das fibras nervosas simpáticas não foram afetados. O EPV também induziu uma atrofia do baço e glandulas adrenais. Nossos resultados indicam que o estresse precoce de vida por separação materna induziu mudanças na estrutura neural das línguas de ratos adultos.

Palavras-chave: Estresse psicológico. Inervação. Privação materna. Língua.

Silva Filho NJ. Effects of early life stress on the innervation of the tongue: an experimental study in rats [dissertation]. Araçatuba: São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil; 2023.

ABSTRACT

Increasing evidence has shown that various forms of stress are associated with changes in the nervous system. However, it has not been analyzed how stress affects the tongue innervation. To examine whether early life stress alters the innervation of the tongue of rats, twenty male Wistar rats were divided into 2 groups (n=10): Control – rats not subjected to ELS by MS; MS – rats subjected to ELS by MS. The MS protocol consisted of separating the litters from the dam for a period of 3 hours for 21 days consecutively after birth. After reaching seven months old, all the rats were euthanized. Immunohistochemical reactions for S100 and TH were performed. The analysis for S100 revealed an increase in nerve density in the subepithelial and median areas, whereas in the median and inferior areas there were an increase in neural structures. On the analysis for TH staining, despite a trend toward an increase in nerve density, there was no statistical significance. We also did not observe differences in the number of sympathetic neural structures. There were no differences regarding nerve diameter for both markers. Here, we demonstrated for the first time that rats subjected to early life stress by maternal separation displayed higher nerve fibers density in the subepithelial area of the tongues and also had higher number of neural structures in the median and inferior areas of the tongues. We also showed that MS rats had an increase in nerve density in the median area of the tongues. The number and nerve density of sympathetic nerve fibers were not affected. The ELS also induced an atrophy of the spleen and adrenal glands. Our results indicate that early life stress by maternal separation induced changes in the neural structure of the tongues of adult rats.

Keywords: Psychological stress. Innervation. Maternal deprivation. Tongue.

LISTA DE FIGURAS

respectively **(J and K)** Comparison of nerve diameter in the control and MS groups, respectively. (original magnification 400x). Student *t* test; Bars represent the mean ± SEM. *p<0.05.

Figure 6. Effects of ELS on depression-like behavior and body, spleen, and adrenal gland weight and correlation of innervation of the tongue and depression-like behavior. (A) Student *t* test showed that there were no differences in body weight variation between the groups. **(B)** Student *t* test showed that rats subjected to ELS exhibited an atrophy of the spleen. **(C)** Student *t* test showed that rats subjected to ELS had an atrophy of the adrenal glands. **(D)** Student *t* test showed no differences in depression-like behavior in MS rats compared to control group. **(E and F)** Pearson correlation test did not find correlation between immobility time and nerve density in the subepithelial area of the tongue of control **(E)** and MS rats **(F)**. **(G and H)** Pearson correlation test did not find correlation between immobility time and nerve density in the median area of the tongue of control **(G)** and MS rats **(H)**. **(I and J)** Pearson correlation test did not find correlation between immobility time and number of nerves in the median area of the tongue of control **(I)** and MS rats **(J)**. **(K and L)** Pearson correlation test did not find correlation between immobility time and nerve diameter in the median area of the tongue of control **(K)** and MS rats **(L)**. **(M and N)** Pearson correlation test did not find correlation between immobility time and nerve density in the inferior area of the tongue of control **(M)** and MS rats **(N)**. **(O and P)** Pearson correlation test did not find correlation between immobility time and number of nerves in the inferior area of the tongue of control **(L)** and MS rats **(M)**. **(Q and R)** Pearson correlation teste did not find correlation between immobility time and nerve diameter in the inferior area of the tongue of control **(Q)** and MS rats **(R)**. Correlation between immobility time and nerve density in the subepithelial area of the tongue. Student *t* test; Bars represent the mean ± SEM. *p<0.05. Pearson correlation test (r).

LISTA DE ABREVIATURAS

µm	Micrometers
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
DPB	Day post-birth
ELS	Early life stress
FST	Forced swimming test
GAP-43	Growth associated protein 43
H&E	Hematoxylin & eosin
HPA	Hypothalamic-pituitary-adrenal
MS	Maternal separation
NE	Norepinephrine
PNS	Peripheral nervous system
SEM	Standard error of mean
SNS	Sympathetic nervous system
TH	Tyrosine hydroxylase

SUMÁRIO

Effects of early life stress on the innervation of the tongue: an experimental study in rats*

Nilton José da Silva Filho[a], Giseli Mitsuy Kayahara[a,b], Emmanuelly Lemos de Souza[a], Renata Alfena Zago[a], Marcelo Macedo Crivelini[b], Vitor Bonetti Valente[a,b], Glauco Issamu Miyahara[a,b], Daniel Galera Bernabé[a,b]*

Affiliations

[a]Psychoneuroimmunology Laboratory, Psychosomatic Research Center, Oral Oncology Center, São Paulo State University (Unesp), School of Dentistry, 1193 José Bonifácio St, SP 15050-015, Araçatuba, São Paulo, Brazil

[b]Department of Diagnostic and Surgery, São Paulo State University (Unesp), School of Dentistry, 1193 José Bonifácio St, SP 15050-015, Araçatuba, São Paulo, Brazil

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***Corresponding author:**

Associate professor; Psychoneuroimmunology Laboratory, Psychosomatic Research Center, Oral Oncology Center, São Paulo State University (Unesp), School of Dentistry, 1193 José Bonifácio St, SP 15050-015, Araçatuba, São Paulo, Brazil **Telephone numbers**: +55 18 36363268 / +55 18 36363275

E-mail: daniel.bernabe@unesp.br

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INTRODUCTION

1 INTRODUCTION

Increasing evidence has shown that stressful psychological conditions in childhood may have long-lasting effects in life (Bolton et al., 2017; Garcia-Laguna et al., 2021; Krizanova et al., 2016). Stress triggers the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) (Antoni et al., 2006; Reiche et al., 2004; Valente et al., 2018). The activation of this neuroendocrine system, as a response to the stressor stimulus, promotes an increase in the secretion of the stress-related hormones cortisol and catecholamines (e.g. norepinephrine and epinephrine) from adrenal glands and SNS (Figueira et al., 2022).

Experiences of chronic stress in early childhood, often also called as early life stress (ELS), have persistent and pervasive consequences on the body, by increasing the risk of developing psychiatric, cardiovascular, endocrine, and auto-immune diseases (Eriksson et al., 2014; Smith et al., 2017; Smith et al., 2020). Maternal separation (MS) has been extensively used as an animal model to investigate the impact of ELS on the neuroendocrine system (Marco et al., 2013; Nakamura et al., 2011). Evidence shows that chronic stress induced by MS during postnatal period is damaging to the development of the brain and endocrine systems. It may result in an allostatic overload, characterized by modified development of the immune, endocrine, and nervous systems (Danese et al., 2012). These effects culminate in extended activation of physiological stress response and negative psychological and behavioral outcomes which may persist into adulthood (Gogberashvili, 2007; Smith et al., 2020; Sterley et al., 2013).

Early stress events have been associated with the development of mood disorders, such as depression, anxiety, and anhedonia in adulthood (Goodwill et al., 2019; Herbison et al., 2017). Depression is a multifactorial disorder that comprises

heterogeneous symptomatology and its precise etiology remains poorly understood (Menard; Hodes; Russo, 2016). In rodents, depressive-like behavior can be investigated by several methods, such as forced swim test (FST), sucrose preference test, and tail suspension test (Belovicova et al., 2017).

Previous studies have shown the wide range of effect that stress may have on the organism. For example, stress can exacerbate bone marrow resorption (Bertolini Botelho et al., 2022), increase inflammatory responses (Minhoto et al., 2021), modify the distribution patterns of T-cell subpopulations (Dominguez-Gerpe and Rey-Mendez, 2001), and affect the brain region by decreasing dendritic spine density (Murmu et al., 2006). It has also been shown that stress leads to thymus involution (Clarke and Kendall, 1994), enhances sympathetic innervation of lymph nodes and induces neuronal plasticity (Peters et al., 2005; Sloan et al., 2007).

The inter-relation between central nervous system and neuro-immune response may be disrupted by the increase of stress-related substances (NE, epinephrine and cortisol) in response to a stressor, whereas the peripheral nervous system (PNS) communicates with the immune system according to the sympathetic/parasympathetic nerve fibers of the respective organ (e.g. spleen, thymus) (Al-Shalan et al., 2019; Wang and Chiou, 2004). For example, catecholamines secreted during stress bind to β-adrenergic receptors and modulates immune responses (Tang et al., 2013). Moreover, other mediators such as brain-derived neurotrophic factor (BDNF) and Neurotrophin 3, widely present in the CNS, are modulated by stress and expressed in several tissues, including the tongue (Nosrat et al., 1996; Oakley and Witt, 2004; Valente et al., 2018).

It is well known that the mammalian tongue exhibits significant morphological variations (Abayomi et al., 2009). Most research has focused on the analysis of structural and functional characteristics of the tongue, with emphasis on muscle and

papillae morphology (Davydova et al., 2017; McClung and Goldberg, 2000). McClung and Goldberg (2000) using Sihler's technique illustrated the detailed entrance of lingual branch of the trigeminal nerve (V), the hypoglossal (XII) and glossopharyngeal nerves (IX) within the body of the tongue of rats. In addition, Dotson et al., (2012) showed that the chorda tympani (CT), a branch of the facial nerve (VII), mediates taste and other modalities and sends efferent fibers to salivary glands and other structures in the rat tongue (Hellekant, 1971). The anatomical location of the posterior region of the oral cavity and its mucosal-associated lymphoid tissues represents the front line of defense against potential threats (Weihe et al., 1999). The posterior one-third of the tongue of the rat is mainly innervated by sensory fibers provided by the glossopharyngeal nerve but also receives sympathetic nerve fibers, and some neuropeptides, and it is highly likely that these structures under stress secrete neurotransmitters in the microenvironment (Altschuler et al., 1989; Fitzgerald, 2009; Madden et al., 1995; Wang and Chiou, 2004). Interestingly, some studies of our team associating chronic stress and a chemically induced oral carcinogenesis model have reported that the posterior region of the rat tongue is the predominant for occurrence of tumors (Figueira, 2018; Verza et al., 2020). However, no studies have documented the structural changes of the tongue innervation after a period of chronic stress.

Based on these considerations and the effect of chronic stress in the neuroendocrine system, we have investigated whether early life stress by maternal separation alters the innervation in the posterior region of the tongue of adult rats. Secondly, we evaluated whether tongue innervation is correlated with depression-like behavior in rats exposed or not to ELS.

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Animals and experimental conditions

All experimental protocols involving animals were approved by the Animal Welfare Committee at the Araçatuba Dental School (São Paulo State University – Unesp, Araçatuba, São Paulo, Brazil) (Protocol number: 0183-2022) and were performed according to the guideline of principles for the use of animals in the laboratory. Twenty male Wistar rats (*Rattus norvegicus*) were group-housed (4 per cage) (25.9 x 47.6 x 20.9 cm, polypropylene), under standardized conditions (25 ± 2ºC), 12h light/dark cycle, and received food (Purina®, Paulínia – SP, Brazil) and drinking water *ad libitum* throughout the experimental period.

2.2 Experimental design

To assess whether ELS affects the tongue innervation in adulthood, the study was conducted with two experimental groups: 1) Control group – 10 male Wistar rats not subjected to ELS by maternal separation and 2) MS group – 10 male Wistar rats subjected to ELS by maternal separation. The parental generation was mated in our facilities (one male Wistar rat with two females) for 10 days and then the female rats were isolated, and the day of birth was rigorously controlled. After the birth of offspring, the experiments were divided into two phases. In phase 1, the animals from MS group were subjected to ELS for 21 days post-birth (DPB). All rats were weaned and separated by gender on 22nd day post birth (DPB). Only the male rats of each group were used for the later phases of the experiment. When they reached DPB90, all rats were weighed weekly until euthanasia. In phase 2, after reaching 7 months of age, all animals were subjected to forced swimming test (FST) for evaluation of depression-like behavior. A week later rats were euthanized by decapitation and tongue, spleen,

and adrenal glands were extracted for histological analysis and immunohistochemical reactions.

2.3 Early life stress by maternal separation

ELS was induced by MS. First day post birth (DPB1), the litters were randomly divided between the Control and MS groups. Litters from MS group were separated from the dam for 3 hours/day (08h00 – 11h00), for 21 days (DPB1 – DPB21) (Figueira, 2018; Matthews; Wilkinson; Robbins, 1996). Pups were then moved to a clean cage in another room with a thermal blanket at 32ªC to prevent hypothermia. After the 3-hour separation period, litters were placed back to their dam's cage. The control rats were not disturbed throughout this period. Litters were weaned on DPN22, and the rats were housed in four per cage until they reached 7 months of age when they were euthanized by decapitation.

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2.4 Assessment of depression-like behavior

To evaluate whether ELS induces depression-like behavior, the rats were subjected to the Forced Swim Test (FST) when they were 7 months old. FST was performed as described by Porsolt et al., (1977). Firstly, in a pre-test, rats were forced to swim for 15 minutes in a plastic cylinder (25 cm in height x 12 cm in diameter) filled with water for 15 minutes. After 24 hours, animals were again placed into the cylinder filled with water and forced to swim for 6 minutes at the same pre-test conditions. FST was recorded by a camera positioned in front of the apparatus. To determine the depression-like behavior, immobility time of each rat was analyzed using the ANY-maze software

(Stoelting Co., Wood Dale, IL, USA). Immobility was defined as absence of escape-oriented behaviors (Anyan & Amir, 2018; Deng et al., 2022; Kayahara et al., 2020).

2.5 Body, spleen, and adrenal gland weight

To evaluate whether ELS would induce body weight variation of MS rats compared to control, initial body weight in later adolescence (DPB90) and final body weight (when they were seven months old) were recorded. After euthanasia, the weight variation of the rats from the experimental groups was calculated in grams. Spleen and adrenal glands were collected, weighted, and values were adjusted for the final body weight of each animal.

2.6 Histological analysis

After euthanasia, tongues were longitudinally sectioned and fixed in 10% buffered formaldehyde solution (Merck, Darmstadt, Germany) for 48 hours. The tissues were alcohol dehydrated and paraffin embedded. Histological sections (4 μm of thickness) were obtained and stained with hematoxylin and eosin (H&E, Merck). All tongues from control and MS rats were analyzed to confirm the absence of morphological alterations on the tissue. Histological analysis was performed by a researcher blinded to the experimental groups.

2.7 Selection of tongue region

The anatomical location of the posterior region of the tongue, with its innervation and mucosal-associated lymphoid tissues, represents the front line of defense against potential threats (Weihe et al., 1999). Additionally, in vivo studies using chemically induced oral carcinogenesis from our laboratory and others have shown that the posterior one-third region of the tongue is the most predominantly affected location area for the development of oral tumors (Figueira, 2018; Kanojia and Vaidya, 2006; Verza et al., 2020). On this regard, we first defined the posterior region of the tongues as the spot of analysis. Then, at a low magnification (10x), we selected the subepithelial (Fig. 1C), median (Fig. 1D), and inferior (Fig. 1E) areas of the tongue as the regions of interest.

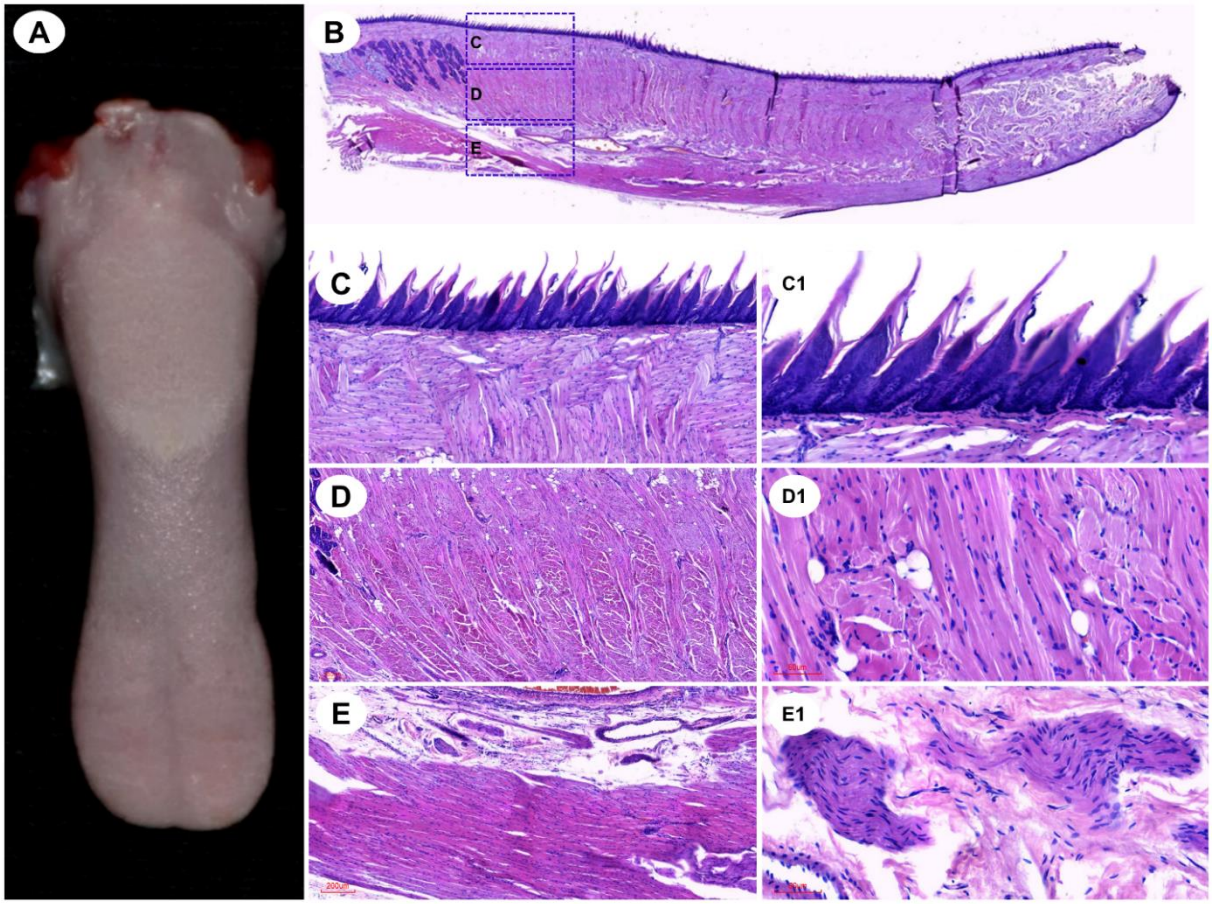

Figure 1. Clinical and histological images of the rat tongue. (A) Clinical image of the rat tongue. **(B)** Histological image of the rat tongue stained in (H&E) demonstrating the three selected areas of study. **(C and C1)** Subepithelial area of the rat tongue in 10x and 20x magnification, respectively. **(D and D1)** Median area of the rat tongue in 10x and 20x magnification, respectively. **(E and E1)** Inferior area of the rat tongue in 10x and 20x magnification, respectively.

2.8 Immunohistochemistry

In order to evaluate the effects of ELS on the innervation of the rat's tongues, we conducted immunohistochemistry assays. S100, a sensitive and reliable nerve marker, was used as a nonspecific innervation marker, while tyrosine hydroxylase (TH) was used as a sympathetic nerve fiber marker. Histological sections of the tongues of all experimental groups were deparaffinized and rehydrated to immunohistochemistry (IHC) analysis. Heat-induced epitope retrieval was performed by 10mM citrate buffer, pH 6.0, at 55ºC for 20 minutes. Blockade of endogenous peroxidase activity was achieved by 3% H_2O_2 for 15 minutes. The slides were washed with PBS solution (pH 7.4). Sections were then incubated with primary antibody anti-S100 (dilution 1:10,000, polyclonal, Dako) for 2 hours, and anti-TH (dilution 1:2,000, monoclonal, Abcam) at 4º overnight. Then, the sections were incubated with Histofine antibody polymer conjugated with horseradish peroxidase (Nichirei Biosciences, Tokyo, Japan) for 40 minutes. The slides were washed with PBS buffer (pH 7.4) and a chromogenic substrate (3,3',5,5'-tetramethylbenzidine) was incubated for 5 minutes. Reactions were stopped in deionized water. Harris's hematoxylin was used to perform counter-staining for 15 seconds. The histological sections were dehydrated and covered with coverslip for microscopic examination. The experiments were carried out with positive and negative controls. All slides were scanned using the slides scanner MoticEasyScan One (Kowloon, Hong Kong) The analyzes were performed by a researcher blinded to the experimental groups.

2.8.1 Nerve fibers density

The number of positive nerve fibers for S-100 and TH was determined on immunostained specimens and counted manually in the subepithelial, median, and inferior areas of the posterior region of the tongue for the MS and control groups. We analyzed the total immunopositive areas in 5 fields. The density was defined by the following formula: total immunopositive area (μm)/5 (number of fields analyzed) (Sigorski et al., 2021). All these analyses were performed using ImageJ software.

2.8.2 Measurement of nerve fibers and nerve diameter

For quantification of S100 and TH staining, all slides were digitized at 400x magnification using EasyScanOne scanner (Motic Asia). The number of nerve fibers was counted manually in five fields at 400x magnification (Albo et al., 2011; Zhang et al., 2016). Five fields of each area were evaluated. Any immunopositive structure taking the shape of a line, dot, or a linear format was considered and counted (Sigorski et al., 2021). The diameter of counted neural structures was measured from largest dimension of the nerve section (largest dimension (μm)/number of nerves) (Albo et al., 2011; D'Silva et al., 2023). Due to the diffuse pattern of innervation of the subepithelial area of the tongue, we were not able to measure the number of nerve fibers nor the nerve diameter in this area. All these analyses were performed using ImageJ software.

2.9 Statistical analyses

GraphPad prism 8.02 software (GraphPad Software Inc., San Diego, CA, USA) was used to perform the statistical analysis. Data were tested for normality using the Shapiro-Wilk test. Student's *t* test evaluated whether ELS affected the neural density, number of neural structures and nerve diameter. Student's *t*-test was also used to determine differences between the control and MS groups concerning the depression-like behavior. Pearson correlation test was used to assess the association between depression-like behavior and innervation of the tongue. Data are shown as mean ± standard error of mean (SEM). Statistical significance was considered at a *p* value less than 0.05 ($p<0.05$) for all tests.

RESULTS

3 RESULTS

3.1 ELS by maternal separation increases tongue non-specific innervation in adulthood.

All longitudinally sectioned tongues of rats from control and MS groups were immunostained with S-100 antibody to assess whether MS would affect global innervation of posterior region of the tongue. Our results showed that rats subjected to ELS by MS had an increase in number of nerve fibers in the subepithelial, median and inferior areas of the tongues (Fig. 2). When we evaluated the subepithelial area, MS rats had an increase of nerve density (684.1±82.47 μm) compared to control group (332.5±130.4 μm) (p=0,0389) (Fig 2A;E-F). In the median area of the tongue, MS group showed increased neural density and higher number of neural structures (1639±231.5 μm; 45.11±5.579 respectively) than control rats (519.0±49.17; 31.22±2.707 respectively) (p=0.003) (Fig 2B;G-H; Fig. 2C;I-J). Differences were not found between groups concerning the diameter of the nerves in median area of the tongue (MS, 72.92±9.019 μm vs Control, 60.60±6.249 μm) (p>0.05) (Fig. 2D;K-L). In the inferior area of the tongues, MS rats did not have differences in nerve density and diameter of nerve fibers (6316 ± 1107 μm; 72.92±9.019 μm respectively) when compared to those from control group (4863 ± 862.3 μm; 60.60±6.249 μm respectively) (p>0.05) (Fig. 3A;D-E; 3C;H-I). However, MS animals had a greater number of neural structures (21.50±1.946) than control rats (14.25±1.677) (p=0,0136) (Fig. 3B;F-G).

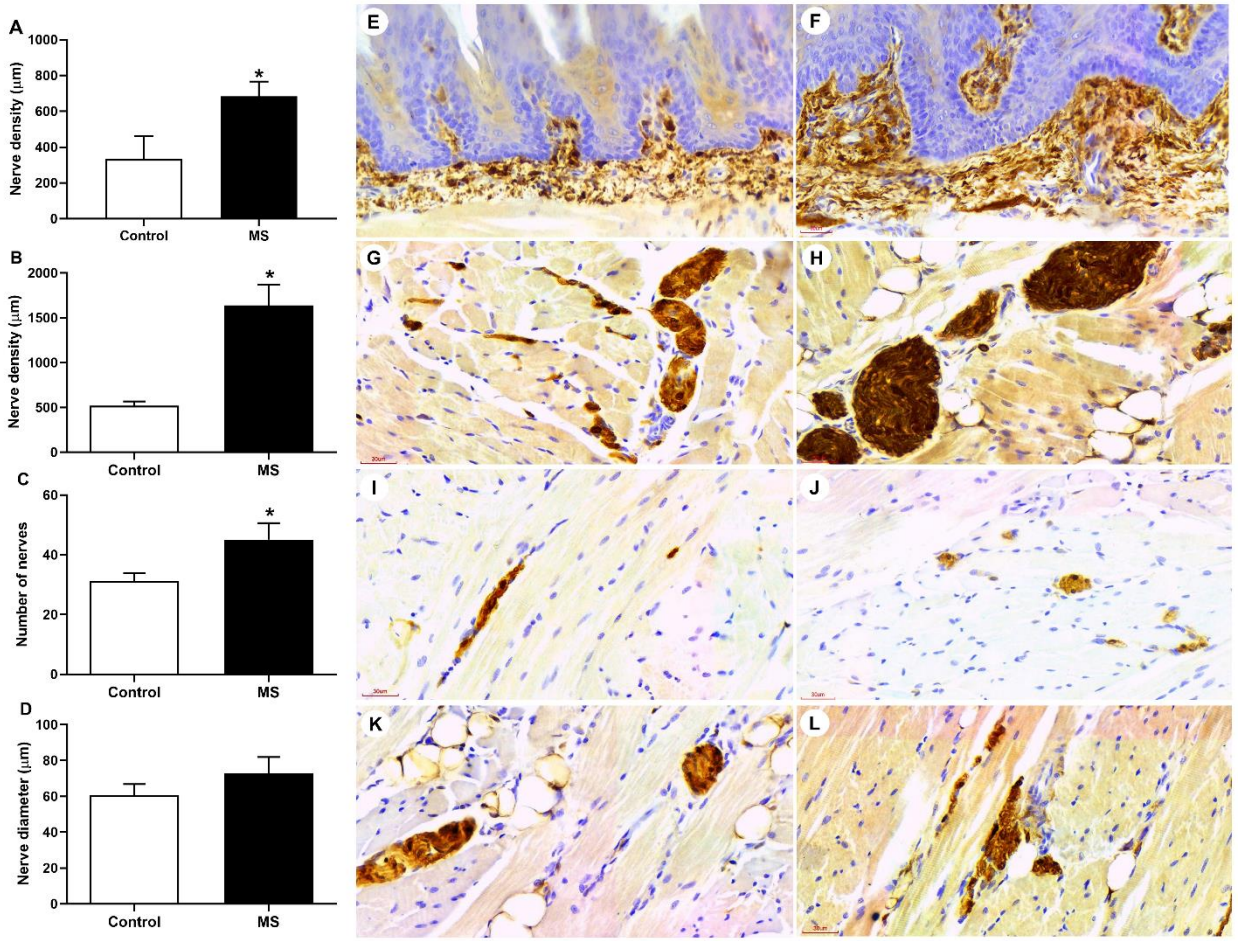

Figure 2. Effects of ELS on the nonspecific innervation of the subepithelial and median areas of the tongue. (A) Student's *t* test revealed that rats subjected to MS had an increase in nerve density in the subepithelial area of the tongue compared to control group. **(B)** Student's *t* test revealed that rats subjected to MS also had an increase in nerve density in the median area of the tongue. **(C)** Student's *t* test revealed that MS rats had an increase in neural structures compared to control group. Subepithelial area of the tongue of control group. **(D)** Student's *t* test revealed that there was no difference in nerve diameter between the groups. **(E and F)** Comparison of nerve density in the subepithelial area of the rat tongue in control and MS rats, respectively. **(G and H)** Median area of the tongue of control and MS rats, respectively, showing significant differences in nerve density. **(I and J)** Median area of the tongue of control and MS group, showing differences in number of nerve fibers. **(K and L)** Nerve fibers showing no difference in regard to nerve diameter in the control and MS groups, respectively. (original magnification 400x). Student *t* test; Bars represent the mean ± SEM. *p<0.05.

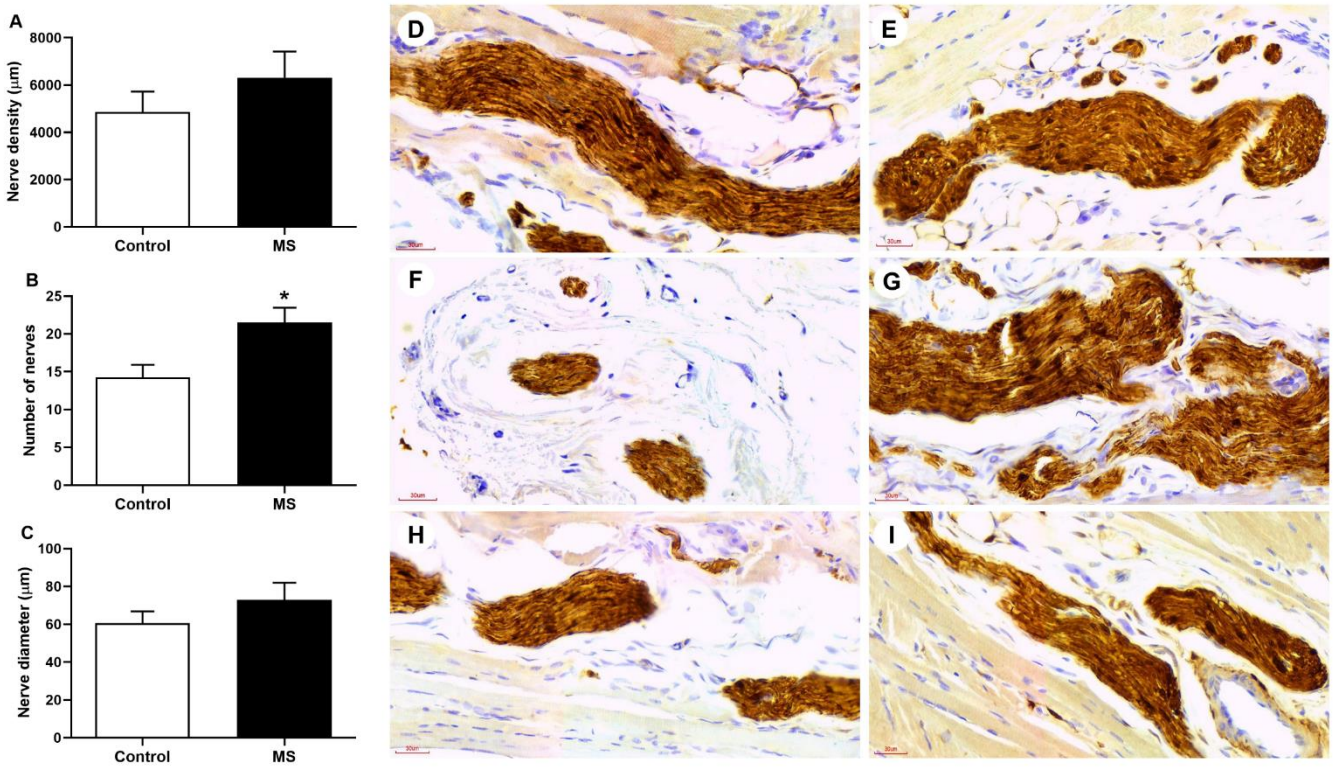

Figure 3. Effects of ELS on the nonspecific innervation of the inferior area of the tongue. (A) Student's *t* test revealed that rats submitted to MS did not exhibit differences in nerve density in the inferior area of the tongue compared to control group. **(B)** Student's *t* test showed an increase in neural structures in the inferior area of the tongue of MS group compared to control. **(C)** Student's *t* test revealed that there was no difference in nerve diameter between the groups. **(D and E)** Inferior area of the rat tongue in control and MS rats showing no differences in regard to nerve density, respectively. **(F and G)** Comparison of the number of nerve fibers in the inferior area of the tongue of control and MS groups, respectively. **(H and I)** Comparison of nerve diameter in the control and MS groups, respectively. (original magnification 400x). Student *t* test; Bars represent the mean ± SEM. *p<0.05.

3.2 Effects of ELS on sympathetic innervation of the tongue in adulthood.

To evaluate whether ELS affects the sympathetic innervation, all tongues were immunostained with anti-TH antibody. Our results showed that there was no immunoexpression of TH in the subepithelial area of the tongues (Fig. 4D-E). When we evaluated the neural density in the median area of the tongues, the MS rats displayed an increased neural density (115.8±53.06 μm) compared to control (5.249±2.969 μm) but this result did not reach statistical significance (p=0.0563) (Fig. 4A;F-G). There were also no significant differences in the number of sympathetic neural structures between MS rats (0.7500±0.3660) and controls (1.750±0.9590) (p>0.05) (Fig. 4B;H-I). MS rats had an increase in nerve diameter in the median area of the tongue (3.040±1.242 μm) compared to control group (0.4307±0.2785 μm) but without reaching statistical significance (p>0.05) (Fig. 4C;J-K). There were no differences in nerve density and number of neural structures in the inferior area of the tongues between MS rats (580.3± 538.3 μm; 1.900±1.278 respectively) and control rats (395.5± 273.6 μm; 1.700±0.9185) (p>0.05). (Fig. 5A;D-E; Fig. 5B;F-G). Differences were not found between groups concerning the diameter of the nerves in the inferior area of the tongue (MS, 6.044±4.863 μm *vs* Control, 8.949±4.624 μm) (p>0.05) (Fig.5C;H-I).

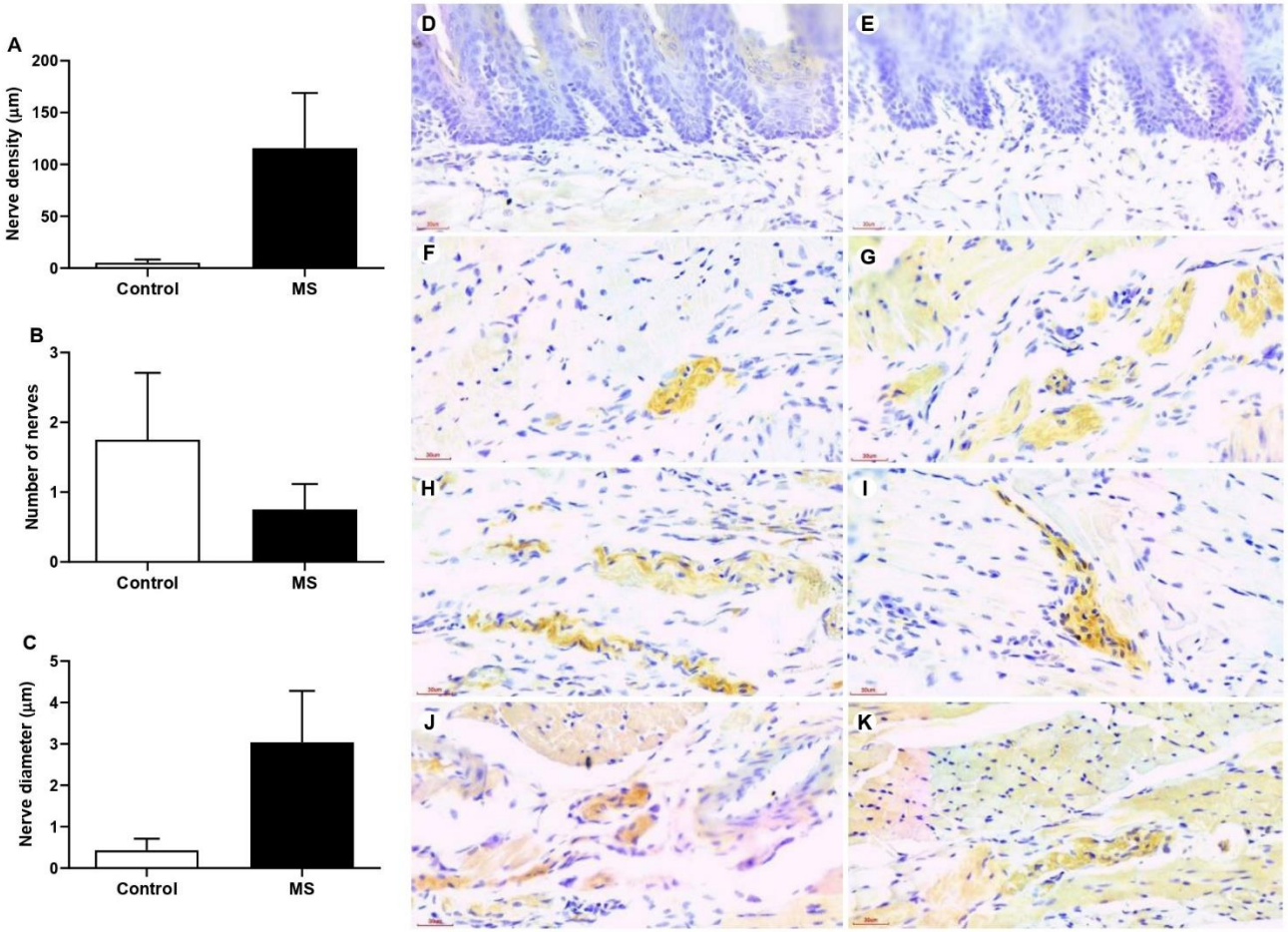

Figure. 4. Effects of ELS on the sympathetic innervation of the subepithelial and median areas of the tongue. (A) Student's *t* test revealed that rats submitted to MS did not exhibit differences in sympathetic nerve density in the median area of the tongue compared to control group **(B)** Student's *t* test showed no statistical difference regarding number of neural structures between the groups **(C)** Student's *t* test revealed that there was no difference in the nerve diameter between the groups. **(D and E)** Subepithelial area of the tongue showing no TH immunoexpression in control and MS rats, respectively. **(F and G)** Comparison of nerve density in the median area of the tongue of control and MS groups, respectively **(H and I)** Comparison of the number of TH-stained nerve fibers in the median area of the tongue of control and MS groups, respectively **(J and K)** Comparison of nerve diameter in the control and MS groups, respectively. (original magnification 400x). Student *t* test; Bars represent the mean ± SEM. *p<0.05.

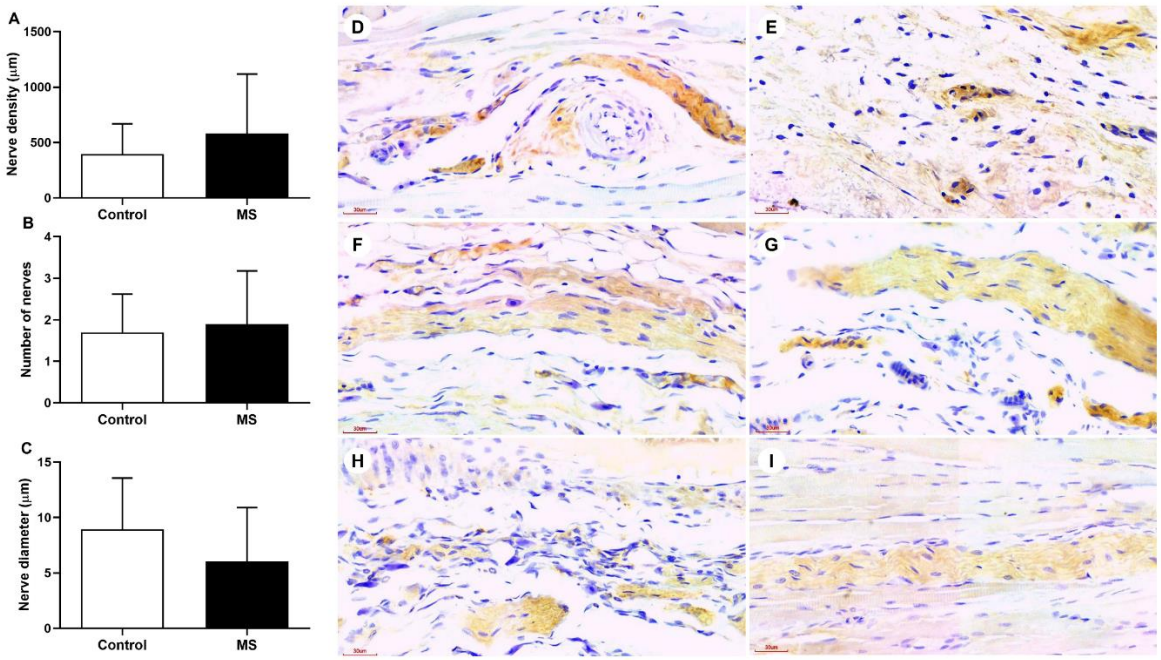

Figure. 5. Effects of ELS on the sympathetic innervation of the inferior area of the tongue. (A) Student's t test showed no differences in nerve density between the groups in the inferior area of the tongues. (B) Student's t test showed no statistical difference regarding number of neural structures stained with TH between the groups (C) Student's t test showed that there was no difference in the nerve diameter between the groups. (D and E) Comparison of nerve density in the inferior area of the tongue of control and MS groups, respectively (F and G) Comparison of the number of TH stained nerve fibers in the inferior area of the tongue of control and MS groups, respectively (H and I) Comparison of nerve diameter in the control and MS groups, respectively. (original magnification 400x). Student t test; Bars represent the mean ± SEM. *<0.05.

3.3 Effects of ELS on the depression-like behavior and body, spleen and adrenal gland weight

We assessed the rat's body weight from DPB90 until they were seven months old. There was no difference in body weight variation between MS (115.6±9.221 g) and control rats (92.30±10.92 g) ($p>0.05$) (Fig. 6A). Rats subjected to ELS exhibited an atrophy of the spleen (0.8996±0.04498 g) when compared to control rats (1.145±0.05677 g) ($p=0.0009$) (Fig. 6B). MS rats also displayed an atrophy of the adrenal glands (0.0001001±7.782e-006 g) compared to control group (0.0001300±6.099e-006 g) ($p=0.0090$) (Fig. 6C). In order to evaluate differences in depression-like behavior between both groups, all rats were subjected to FST. Student *t* test showed no differences in the immobility time from rats exposed to ELS (186.8±18.15 sec) compared to control group (161.8±16.03 sec) (p=0,3161) (Fig. 6D).

4. Association between tongue innervation and depression-like behavior

Pearson correlation coefficient was applied for analyzing association between depression-like behavior and innervation of the tongue. There was no association between immobility time and the variables analyzed in the study (nerve density, number of nerves, and nerve diameter) (Fig. 6E-R).

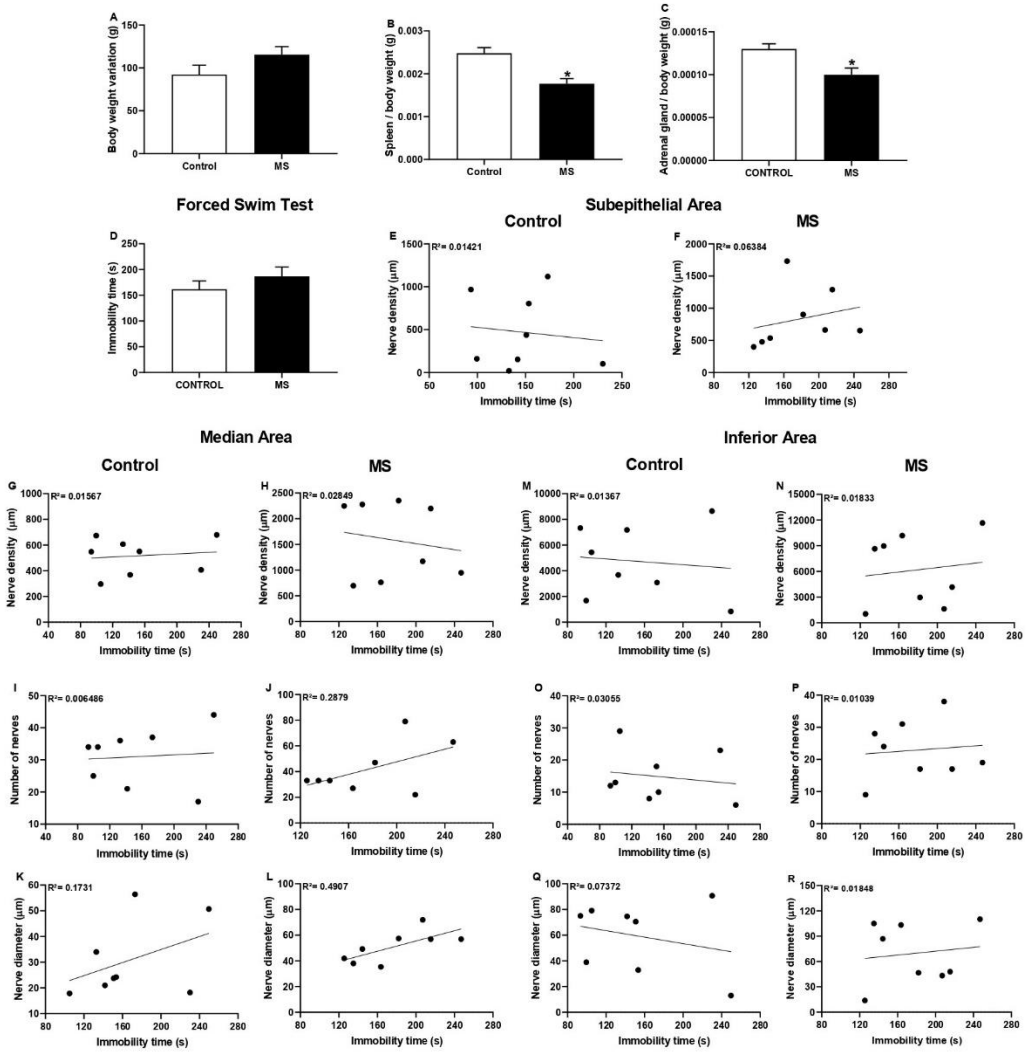

Figure 6. Effects of ELS on depression-like behavior and body, spleen, and adrenal gland weight and correlation of innervation of the tongue and depression-like behavior. (A) Student *t* test showed that there were no differences in body weight variation between the groups. **(B)** Student *t* test showed that rats subjected to ELS exhibited an atrophy of the spleen. **(C)** Student *t* test showed that rats subjected to ELS had an atrophy of the adrenal glands. **(D)** Student *t* test showed no differences in depression-like behavior in MS rats compared to control group. **(E and F)** Pearson correlation test did not find correlation between immobility time and nerve density in the subepithelial area of the tongue of control **(E)** and MS rats **(F)**. **(G and H)** Pearson correlation test did not find correlation between immobility time and nerve density in the median area of the tongue of control **(G)** and MS rats **(H)**. **(I and J)** Pearson correlation test did not find correlation between immobility time and number of nerves in the median area of the tongue of control **(I)** and MS rats **(J)**. **(K and L)** Pearson correlation test did not find correlation between immobility time and nerve diameter in the median area of the tongue of control **(K)** and MS rats **(L)**. **(M and N)** Pearson correlation test did not find correlation between immobility time and nerve density in the inferior area of the tongue of control **(M)** and MS rats **(N)**. **(O and P)** Pearson correlation test did not find correlation between immobility time and number of nerves in the inferior area of the tongue of control **(L)** and MS rats **(M)**. **(Q and R)** Pearson correlation teste did not find correlation between immobility time and nerve diameter in the inferior area of the tongue of control **(Q)** and MS rats **(R)**. Correlation between immobility time and nerve density in the subepithelial area of the tongue. Student *t* test; Bars represent the mean ± SEM. *p<0.05. Pearson correlation test (r).

DISCUSSION

4. DISCUSSION

In the present study we showed for the first time an association of early life stress and innervation of the tongue in adulthood. We evaluated the effects of ELS on the tongue innervation of adult rats using a well-established model of ELS, which protocol is carried out during a critical period of development of the litters (Marco et al., 2013). Our results showed that stress induces an increase in the innervation of the tongue of rats. Nerve density in the subepithelial and median area of the tongues were increased, and number of nerve fibers were also increased in the median and inferior areas of the tongues, indicating the ELS effect on nerve modulation.

When stressful events occur in critical periods of development (perinatal phase and childhood), they can affect psychological and behavioral functions, including growth, metabolism, reproduction, inflammatory and neuro-immune responses in adulthood (Nishi et al., 2013; Silberman et al., 2016). Several variations of ELS by MS are used worldwide, with the number of days and time of procedure varying according to each laboratory protocol (Arborelius and Eklund, 2007). Regardless of variations, ELS by MS leads to a long-lasting hyperactivity of the HPA axis and it can impair the neuroendocrine system (Aisa et al., 2008; Elwenspoek et al., 2017). Recently, a growing body of data has reinforced the idea of the central regulation of the neuro-immune system via activation of SNS (Stavropoulos, Sarantopoulos and Liverezas, 2020). For a period, the nervous system was thought to remain rather static shortly after birth (Peters et al., 2005). Contemporary studies have shown that the process of continuous adaptive growth, modification, and reorganization of nerve fibers, described as neuronal plasticity, may also happen under physiological conditions (Peters et al., 2005). For example, a recent study reported that ELS accelerates age-induced effects

on neurogenesis in both dorsal and ventral hippocampus, with negative consequences in metabolic and behavioral processes. Additionally, stress may be also linked to plasticity of innervation and neurotrophins expression (Nawa and Takei, 2001; Peters et al., 2005; Rage et al., 2002).

Neuronal plasticity of adult peripheral innervation was reported by Fantini and Johansson (1992) in human skin by immunoexpression of growth associated protein 43 (Gap-43). An in vivo study suggested a remodeling process of nerve fibers of the rat lower lip (Verzé et al., 1999). Peters et al., (2005) observed a rapid increase in the number of Gap-43+ nerve fibers 24 hours after a single exposure to sonic stress, with a mild decline 48 hours post stress. In contrast to this transient effect of the stressor used, ELS can promote neurobiological changes that may persist to adulthood (Garcia-Laguna et al., 2021). This is in accordance with our results. We demonstrated for the first time that a chronic stress exposure early in life induced an alteration of nerve density that persisted to adulthood. This brings support to the concept that early adverse experiences can induce long-lasting effects on the orofacial structures.

Studies investigating the effects of ELS on tongue innervation are scarce. In our results, the most interesting finding was that the density of nerve fibers labeled with S100 antibody was significantly increased in the subepithelial and median areas of the posterior region of the tongues in adult stressed rats. Concomitantly, we also found an increase in number of nerve fibers in the median and inferior areas of the tongue of the MS group. Recently, a study demonstrated that ELS increased bone innervation in mice with altered bone remodeling process (Wuertz-Kozak et al., 2020). In addition, Romeo et al., (2001) demonstrated that the rat glossopharyngeal afferent fibers appear to be a neural route by which neuro-immune systems of the posterior oral cavity conveys information to the brain and bring out CNS manifestations of acute response.

In our study, we showed that MS rats in adulthood displayed a widespread innervation in the subepithelial area of the tongue compared to control group. This location is highly innervated by the chorda tympani and glossopharyngeal nerves (Geran et al., 2004; Romeo et al., 2001). Therefore, it may be assumed that nerve profile can be modulated by chronic stress. This finding is of great interest because it indicates a long-lasting effect of this adaptive response. In contrast with the increase in nerve density in both subepithelial and median areas, there were no significant differences in this regard in the inferior area of the tongue between the groups. We did not find differences in nerve diameter either. Its noteworthy that this area receives the hypoglossal nerve and the lingual branch of the trigeminal nerve that also enters the ventral aspect of the tongue to innervate the body of the tongue (McClung and Goldberg, 2000). We hypothesize that the intensity of our MS protocol was not strong enough to induce changes in these nerve trunks.

Previous studies revealed the presence of many noradrenergic fibers in the rat's tongue with a similar pattern distributed among the organs (Wang and Chiou, 2004). In our study, there was no immunopositivity for sympathetic fibers in the subepithelial area of the tongues, which indicates that this location is predominantly innervated by sensory fibers (Altschuler et al., 1989). Surprisingly, the density of sympathetic nerve fibers in adult stressed rats was not affected by MS, although its distribution pattern tended to augment. We also did not find differences regarding sympathetic nerve diameter between the groups; however, a trend towards increasing in nerve diameter and nerve density could be observed. We consider the small sample size as one of the possible interfering factors. Sloan et al., (2007) observed that social stress enhanced the density of catecholaminergic neural fibers in lymph nodes and that this alteration can significantly impact the physiological function of the affected organ, linking this

effect to an increased transcription of NGF. On the other hand, other study showed that forced swimming stress reduced sympathetic nerve fibers on rat thymus (Zivkovic et al., 2004). In our study, we did not evaluate the expression of any neurotransmitters.

Some studies have established that ELS negatively affects behavioral and social-emotional development. However, pre-clinical studies investigating the effect of ELS on body weight in adulthood display conflicting results (de Lima et al., 2020; Peña et al., 2019). In our study, no differences were seen between the groups regarding body weight. In counterpart, some studies reported an initial weight loss and weight normalization in senility (de Lima et al., 2020; Goodwill et al., 2019). On the other hand, others reported weight gain (Peña et al., 2019; Ruiz et al., 2018). In accordance with our study, Gareau et al., (2006) revealed that no significant deficiencies in body weight were found in MS *versus* control rats, indicating that the MS rats were receiving adequate nutrition and growth process was not affected. Nevertheless, other study using a MS protocol like the present one did not show any significant deficits in body weight gain in rats separated from their mothers early in life (Wu and Groat, 2006). The investigation of the effects of ELS on eating behavior and metabolic health in pre-clinical models has shown several gaps and conflicting results that may be associated not only with the complexity of the subject but also with the variety of protocol applied worldwide (Colleluori et al., 2022). Chronic stress has a known effect on some organs including adrenal gland and the spleen (Thornton et al., 2021). Most studies report that ELS induces a hypertrophy of the adrenal glands (Ulrich-lai et al., 2006). On contrary, in our results the stressed animals had an atrophy of the adrenal glands. Similar findings were reported by Slotten et al., (2006) and Alario et al., (1987). In our study, although ELS by MS did not result in significant changes in body weight of animals, ELS promoted an atrophy in spleen. In accordance with our results, Xiao et al., (2014)

showed that ELS caused a reduction in spleen mass in mice. In contradiction, Thornton et al., (2021) and Weiss (2008) reported that maternal separated rats had larger spleens, in contrary to previous results of ELS response. An increase in spleen weight in stressed rats compared to control group would indicate that ELS increased immune activity, as increased spleen mass is associated with a better immune response to fight disease, therefore these results do not appear to be consistent with previous ELS studies (Gutman & Nemeroff, 2002; Maccari et al., 2014). Furthermore, these results indicate a link between the CNS and the immune system and how ELS can modulate the immune function (Zivkovic et al., 2004).

The development of mental health disorders has been associated with early life adverse experiences (Lai et al., 2011). Depressive-like behavior appears as one of the main consequences of chronic stress in pre-clinical models (Lai et al., 2011). In the current study, maternal separation did not affect the depressive-like behavior of rats, represented in the FST by the time spent immobile. Similar to our findings, Weiss (2008) found no differences regarding depressive-like behavior in MS rats. In other pre-clinical studies, ELS rats had increased immobility time than control group (Martisova et al., 2012; Ruiz et al., 2018; Thornton et al., 2021). A previous study revealed that ELS increased immobility time of 3-months old maternal separated rats (Martisova et al., 2012). In accordance with this result, Réus et al., (2017) also reported that MS rats spent more time immobile than control group on DPB60. More recently, Deal et al., (2022) showed that social isolation as early life stressor decreased immobility time in adolescent rats. A study from our laboratory showed that social isolation stress did not affect the depressive-like behavior of adult cancer rats (Verza et al., 2021). In parallel, we did not evaluate the rats at the adolescence period for depressive-like behavior, which we consider a limitation of the study. All in all, it

appears that ELS may accelerate depressive-like behavior in young animals (Lajud et al., 2012; Marais et al., 2008; Vargas et al., 2016); however, more studies are needed exploring its effects on old adult rats (Ruiz et al., 2018).

In summary, previous studies in mice skin and mammalians lymph nodes revealed that an altered innervation due to stress exposure was associated with aggravation of the disease (Peters et al., 2005; Sloan et al., 2008). Our results showed for the first time that early life stress modulates the tongue innervation of rats. This neuro-immune interaction may provide an altered microenviroment that could be more susceptible to the development of diseases. Further investigations assessing the stress-induced altered innervation are needed to help clarify the implications of this event.

CONCLUSION

5 CONCLUSION

This study provides the first evidence that ELS alters the innervation of the tongue in a pre-clinical model. In view of our findings, further studies are needed to explore the implications involved in the association of ELS and tongue innervation.

Conflicts of interest: The authors have no conflicts of interest to disclose.

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ANEXOS

ANEXO A - Normas de Publicação

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ANEXO B - PARECER CEUA (FOA-UNESP)

UNIVERSIDADE ESTADUAL PAULISTA
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CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Avaliação da influência do estresse crônico sobre o padrão de invervação da língua de ratos com e sem indução carcinogênica"**, Processo FOA nº 0183-2022, sob responsabilidade de Daniel Galera Bernabé apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 28 de Março de 2022.

VALIDADE DESTE CERTIFICADO: 07 de Julho de 2023.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 07 de Agosto de 2023.

CERTIFICATE

We certify that the study entitled **"Evaluation of the influence of chronic stress over the innervation pattern of the tongue of rats with and without carcinogenic induction"**, Protocol FOA nº 0183-2022, under the supervision of Daniel Galera Bernabé presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on March 28, 2022.

VALIDITY OF THIS CERTIFICATE: July 07, 2023.
DATE OF SUBMISSION OF THE FINAL REPORT: August 07, 2023.

Prof. Dr. João Carlos Callera
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
Rua José Bonifácio, 1193 – Vila Mendonça - CEP: 16015-050 – ARAÇATUBA – SP
Fone (18) 3636-3234 Email CEUA: ceua.foa@unesp.br