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INFLUÊNCIA DA PRIVAÇÃO DO SONO E DO ESTRESSE EMOCIONAL NA DOR MIOFASCIAL EM MÚSCULOS MASTIGATÓRIOS DE RATOS

FORTALEZA

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Morfofuncionais da Faculdade de Medicina da Universidade Federal do Ceará, como parte dos requisitos para obtenção do título de Mestre em Ciências Morfofuncionais. Área de concentração: Neurociência.

Orientadora: Profa. Dra. Mariana Lima Vale

Coorientadora: Profa. Dra. Delane Viana Gondim

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Dissertação de Mestrado (Qualificação) apresentada à coordenação do Programa de Pós-Graduação em Ciências Morfofuncionais da Faculdade de Medicina da Universidade Federal do Ceará, como requisito parcial para obtenção do título de Mestre em Ciências Morfofuncionais.

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RESUMO

A dor miofascial mastigatória é uma das causas mais comuns de dor musculoesquelética na região orofacial. Fatores psicossociais, como o estresse e os distúrbios do sono podem influenciar negativamente na dor miofascial orofacial. O papel dos distúrbios do sono na neurobiologia da dor miofascial nos músculos mastigatórios ainda não foi investigado até o momento e nem a associação entre estresse emocional e distúrbios do sono. Assim, o objetivo deste estudo foi investigar a influência da privação de sono e do estresse emocional na sensibilidade nociceptiva dos músculos mastigatórios em ratos. Para isso, quarenta ratos Wistar machos foram submetidos ao modelo de estresse emocional na Caixa de Comunicação e à privação do sono paradoxal através do método das plataformas múltiplas modificado. Os animais foram divididos em quatro grupos experimentais: controle; estresse emocional (EE); privação de sono (PS); e estresse emocional associado à privação do sono (EEPS). Testes nociceptivos mecânicos, teste de campo aberto e teste do labirinto em Y foram realizados para investigar a hiperalgesia nos músculos masseter e temporal, as atividades locomotora e exploratória e a memória de trabalho, respectivamente. A expressão de c-Fos foi avaliada no gânglio trigeminal (GT), no subnúcleo caudal do trato espinhal do trigêmeo (Sp5C), no tálamo (TÁL) e na substância cinzenta periaquedutal (PAG). A presença de vacúolos citoplasmáticos nas glândulas adrenais foi analisada. Os grupos EE, PS e EEPS apresentaram hiperalgesia mecânica nos músculos mastigatórios e o grupo EEPS mostrou uma maior resposta hiperalgésica. Houve aumento da expressão de c-Fos em GT, Sp5c e PAG nos grupos EE, PS e EEPS. No TÁL, apenas os grupos EE e EEPS apresentaram aumento da expressão de c-Fos, mas isso não ocorreu no grupo PS. Além disso, houve aumento das atividades exploratória e locomotora, redução da memória de trabalho nos grupos EE, PS e EEPS, além de aumento da concentração de vacúolos citoplasmáticos nas glândulas adrenais. Dessa forma, apesar das limitações deste estudo, pode-se concluir que o estresse emocional e a privação do sono causaram hiperalgesia nos músculos mastigatórios com envolvimento da via nociceptiva trigeminal por meio da expressão de c-Fos em várias regiões, incluindo áreas responsáveis pela modulação descendente da dor. A associação do estresse com a privação do sono exacerbou a sensibilidade à dor e provocou comportamentos de ansiedade e alterações nas glândulas adrenais, provavelmente relacionadas a alterações neuroendócrinas induzidas por estresse.

Palavras-chave: Privação do Sono. Angústia Psicológica. Dor Facial. Síndromes da Dor Miofascial.

ABSTRACT

Masticatory myofascial pain is one of the most common causes of musculoskeletal pain in the orofacial region. Psychosocial factors, such as stress and sleep disorders, can negatively influence orofacial myofascial pain. The role of sleep disturbances in myofascial pain in masticatory muscles has not yet been investigated to date and nor the association between emotional stress and sleep disorders. Thus, the purpose of the present study was to investigate the influence of sleep deprivation and emotional stress on masticatory muscles nociceptive sensitivity in rats. For this, forty male Wistar rats were submitted to emotional stress model in Communication Box and paradoxical sleep deprivation in multiple platforms method. Animals were divided into four groups: control; emotional stress (ES); sleep deprivation (SD); and emotional stress associated with sleep deprivation (ESSD). Mechanical nociceptive tests, Open-field test and Y-maze test were performed to investigate the hyperalgesia in masseter and temporalis muscles, the locomotory and exploratory activities and the working memory, respectively. c-Fos expression was assessed in trigeminal ganglia (TG), spinal trigeminal nucleus caudalis (Sp5C), thalamus (THA) and periaqueductal gray (PAG). The presence of cytoplasmic vacuoles in adrenal glands were analyzed. The ES, SD and ESSD groups showed mechanical hyperalgesia in the masticatory muscles and the ESSD group showed a higher hyperalgesic response. There was an increase in c-Fos expression in TG, Sp5c and PAG in the ES, SD and ESSD groups. In THA, only the ES and ESSD groups showed an increase of c-Fos expression, but this did not occur in the SD group. In addition, there were an increase in exploratory and locomotor activities, a decrease in working memory and an increase in the concentration of cytoplasmic vacuoles in the adrenal glands in ES, SD and ESSD groups. In conclusion, emotional stress and sleep deprivation caused hyperalgesia in the masticatory muscles with involvement of the trigeminal nociceptive pathway through the expression of c-Fos in various regions, including descending pain modulatory brain area. The association of emotional stress with sleep deprivation exacerbated the pain sensitivity and provoked anxiety-like behaviors and changes in adrenal glands, probably related to stress-induced neuroendocrine changes.

Keywords: Sleep Deprivation. Psychological Distress. Facial Pain. Myofascial Pain Syndromes.

SUMÁRIO

1. INTRODUÇÃO GERAL

1.1. Dor orofacial

Dor orofacial é um termo global que abrange uma variedade de condições dolorosas na face e na cavidade oral, como dor musculoesquelética, dor neurovascular e dor neuropática. É um grupo complexo e multifatorial de condições que, na maioria das vezes, requerem um tratamento multidisciplinar (CRANDALL, 2018).

A dor miofascial mastigatória é uma das causas mais comuns de dor musculoesquelética na região orofacial, sendo caracterizada por uma sensação dolorosa referida nos músculos mastigatórios que geralmente envolve limitação na abertura da boca por conta da dor. Isto pode comprometer funções básicas do sistema estomatognático, como a mastigação e a fala (CUMMINGS; BALDRY, 2007; DIRAÇOĞLU et al., 2016). A Classificação Internacional de Dor Orofacial utiliza o termo dor miofascial orofacial como um rótulo abrangente que envolve as disfunções dolorosas relacionadas com os músculos da região orofacial, entre os quais tem-se os músculos mastigatórios ("International Classification of Orofacial Pain, 1st edition (ICOP).", 2020). Um critério-chave para o diagnóstico de dor muscular é que os pacientes relatem dor nos testes de provocação (palpação padronizada) e/ou dor durante a abertura da mandíbula (SCHIFFMAN et al., 2014).

A dor miofascial mastigatória é uma condição muscular dolorosa regional caracterizada pela presença de faixas musculares tensas palpáveis, onde é possível a identificação de pontos intensamente dolorosos nos músculos da mastigação, principalmente os músculos masseter e temporal (FERNÁNDEZ-DE-LAS-PENAS; SVENSSON, 2016; FIAMENGUI et al., 2013; KUĆ; SZAREJKO; SIERPIŃSKA, 2019). Esta condição tem sido apontada como um fator considerável de busca por tratamento, pois influencia negativamente na qualidade de vida (KAPOS et al., 2020).

1.2. Via nociceptiva trigeminal

A condução das informações nociceptivas da região orofacial envolve uma série de componentes do sistema nervoso. Os nervos trigêmeos, quinto par de nervos cranianos, são responsáveis pela inervação sensitiva dos músculos da mastigação. Eventos químicos, físicos

ou biológicos podem causar despolarização de terminações nervosas livres no nervo trigêmeo. A partir de então, o impulso nervoso é conduzido por fibras nervosas mielinizadas ou amielinizadas em direção ao gânglio trigeminal, local onde se encontram os corpos dos neurônios pseudounipolares responsáveis pela condução da informação nociceptiva da região orofacial (LAVIGNE; SESSLE, 2016; SESSLE, 1986; TAKEMURA et al., 2006). A primeira sinapse da via nociceptiva trigeminal relacionada com os músculos mastigatórios ocorre no subnúcleo caudal do trato espinhal do trigêmeo (Sp5c) (DALLEL et al., 2003; HALL; GUYTON, 2011; HARPER; SCHREPF; CLAUW, 2016). A partir daí, a segunda sinapse ocorre no tálamo. Essa estrutura possui núcleos mediais, relacionados aos aspectos afetivos da dor e núcleos laterais, relacionados aos componentes sensitivos-discriminativos. A ativação da via trigeminal provoca ativação principalmente nos núcleos laterais do tálamo, mais especificamente no núcleo ventroposteromedial (VPM) e no núcleo ventroposterolateral (VPL). Desses núcleos talâmicos, as informações podem seguir para as áreas somestésicas do córtex (ROY et al., 2009; WANG et al., 2009; WILSON; UHELSKI; FUCHS, 2008).

Além disso, a dor crônica é comumente modulada por sistemas supressores e facilitadores que compõem uma via modulatória descendente. A substância cinzenta periaquedutal (PAG) é um importante centro que participa da modulação do sistema descendente de controle da dor. Esta região geralmente é ativada por meio da via nociceptiva, que, por sua vez, ativa a via inibitória descendente da dor (SAMINENI et al., 2017). Essas regiões do sistema nervoso que participam da modulação descendente da dor também fazem parte de circuitos neuronais que conduzem informações neurofisiológicas referentes a outros processos do corpo, como a regulação do sono, do humor, da memória, do comportamento, do estresse, entre outros (BRANDÃO; LOVICK, 2019; DELLA VALLE; MOHAMMADMIRZAEI; KNOX, 2019; MOKHTAR; SINGH, 2020; WEBER et al., 2018).

1.3. Estresse emocional e sua influência na dor orofacial

O estresse tem se tornado um problema recorrente na sociedade, tornando-se uma das principais razões para procura por serviços médicos. Caracteriza-se como uma resposta fisiológica e psicológica a estímulos nocivos e mudanças ambientais que induzem reações adaptativas no organismo controladas por processos neuro-hormonais, a fim de manter a

integridade fisiológica (GLAROS; MARSZALEK; WILLIAMS, 2016; KUBO; IINUMA; CHEN, 2015; ULRICH-LAI; HERMAN, 2009; ZHANG et al., 2011).

Durante as reações aos mais diversos tipos de estresse, ocorre um aumento da ativação do eixo neuroendócrino (hipotálamo-hipófise-adrenal) com aumento da circulação de cortisol sistêmico, além de diversas respostas neurovegetativas (GENERAAL et al., 2016; HALL; GUYTON, 2011; KOKO et al., 2004; LENT, 2009). Dentre os tipos de estresse, o emocional vem ganhando notoriedade e é apontado como um fator que pode influenciar no aparecimento e manutenção de dores na região da face (DUBROVSKY et al., 2017; HAYTHORNTHWAITE, 2010; YAP et al., 2002).

Estudos clínicos têm evidenciado que o estresse é um fator preditor para o surgimento de dor orofacial, possibilitando o entendimento da influência de fatores psicossociais interagindo com outras variáveis para o aparecimento e manutenção da dor na região da face (DIRAÇOĞLU et al., 2016; FILLINGIM et al., 2013). Além disso, indivíduos que convivem com dor na região da face apresentam maiores níveis de ansiedade, depressão e catastrofização: fatores intimamente correlacionados com o estresse emocional (HEO; PARK; PYO, 2018; WILLASSEN et al., 2020).

Estudos experimentais já demonstraram que o estresse psicológico foi capaz de reduzir o limiar nociceptivo dos músculos mastigatórios e, após a remoção do estímulo estressor, houve aumento do limiar nociceptivo, evidenciando que a presença do fator que gera o estresse psicológico está diretamente relacionado à dor nos músculos da mastigação (SHIMIZU et al., 2020; ZHANG et al., 2011).

Estudos conduzidos por nosso grupo evidenciaram que o estresse emocional induzido através do modelo da caixa da comunicação em ratos foi capaz de provocar aumento da imunoexpressão de c-Fos em gânglio trigeminal (GT) e subnúcleo caudal do trato espinhal do trigêmeo (Sp5c) (ALVES, 2016; PONTE, 2017; SILVEIRA, 2015). O c-Fos é um marcador de ativação neuronal e neuroplasticidade e sua expressão é geralmente baixa em condições basais. Na presença de estímulos nociceptivos, geralmente ocorre um aumento na expressão desse marcador (BULLITT et al., 1992; KOVÁCS, 2008; PEREIRA et al., 2018, 2019). O aumento da expressão de c-Fos induzido pelo estresse emocional aconteceu tanto em ratos quanto em ratas, mas o comportamento nociceptivo avaliado pelo teste de hiperalgesia mecânica foi mais intenso em ratas, evidenciando que fatores neuro-hormonais relacionados ao sexo podem influenciar na modulação da dor na região dos músculos mastigatórios (PONTE, 2017). Além

disso, sabe-se que o estresse emocional pode provocar a ativação de sistemas modulatórios descendentes da dor, como sistema endocanabinoide, através do aumento da imunomarcação de receptores CB1 e CB2 em GT e Sp5C (ALVES, 2016; SILVEIRA, 2015).

1.4. Privação do sono

O sono é constituído por dois estados biológicos conhecidos como sono REM (do inglês, *Rapid Eye Moviments*) e sono NREM (do inglês, *Non Rapid Eye Moviments*). Durante um ciclo de sono, é possível observar uma alternância de fases entre o sono REM e NREM. Durante a noite, este ciclo do sono REM/NREM é repetido de 4 a 6 vezes durante a noite (KIM et al., 2019; LENT, 2009). O sono REM também é conhecido como sono paradoxal. Nesta fase do sono, ocorre intensa atividade cerebral, com aumento expressivo da síntese proteica no tecido cerebral, com liberação de agentes neurotróficos (LIMA et al., 2019; MACHADO et al., 2004).

A remoção parcial ou supressão do sono em um organismo é conhecida como privação de sono. Alterações no padrão de sono podem provocar diversas consequências, como alterações no processamento cognitivo na responsividade atencional, além de prejuízo na memória, aumento da irritabilidade, alterações metabólicas, endócrinas, imunológicas, diminuição da libido e maior susceptibilidade à dor (BONNET; ARAND, 2003; DANIELE et al., 2017; MEIRA E CRUZ et al., 2019; SCHMITTER et al., 2015). Os distúrbios que acometem o sono possuem fisiopatologias distintas, mas todas prejudicam a arquitetura do sono e associam-se com a redução das horas de sono (CIPRIANI et al., 2015). Atualmente conhecem-se sete categorias de distúrbios do sono classificados pela Classificação Internacional de Distúrbios do Sono (SATEIA, 2014).

O sono tem funções importantes para todos os órgãos do corpo e a privação de sono pode levar a distúrbios que causam danos irreparáveis (LIMA et al., 2014). Durante a privação do sono, a alternância dos ciclos do sono REM/NREM é desregulada, impactando negativamente na reparação das funções específicas de cada fase do sono. Para que ocorram os mecanismos de homeostase, é necessário que o tempo de sono e a transição entre as fases aconteça de maneira regulada (MACHADO; SUCHECKI; TUFIK, 2005; PIRES et al., 2016).

A privação do sono pode levar a respostas neuro-hormonais sistêmicas. Estudos evidenciam que a privação do sono paradoxal (sono REM) foi capaz de provocar elevação dos

níveis de hormônios esteroides (MACHADO; TUFIK; SUCHECKI, 2013) e ativar o eixo (hipotálamo-hipófise-adrenal), provocando aumento dos níveis de hormônio liberador de corticotrofina (CRH) que prejudica o sono. O CRH tem como função a liberação do hormônio adrenocorticotrófico (ACTH) (MACHADO; TUFIK; SUCHECKI, 2010). O ACTH é o principal responsável pela produção de esteroides no córtex das glândulas adrenais e pode causar alterações morfológicas, como hiperplasia no nessa região (ULRICH-LAI et al., 2006; ULRICH-LAI; HERMAN, 2009). Além disso, a privação do sono pode provocar consequências graves para as funções nervosas nos centros superiores, induzindo, por exemplo, uma hipersensibilidade nos receptores de dopamina em várias áreas encefálicas (TUFIK; LINDSEY; CARLINI, 1978).

Estudos mostram que a privação do sono provoca retardo no processo de regeneração muscular, sendo acompanhada da presença de infiltrado inflamatório e alterações na organização das fibras musculares. A recuperação do sono após a privação não foi suficiente para normalizar a regeneração muscular, evidenciando que os efeitos negativos da privação do sono são permanentes (MÔNICO-NETO et al., 2017; RAPHAEL et al., 2013).

1.5. Relação entre dor orofacial, estresse emocional e privação do sono

Condições psicológicas, como estresse emocional, e alterações na qualidade do sono podem influenciar diretamente na exacerbação dos sintomas da dor orofacial. Normalmente, fatores biopsicossociais estão relacionados à somatização e incapacidades funcionais (MEIRA E CRUZ et al., 2019; SIDEBOTTOM; PATEL; AMIN, 2013; YAP et al., 2002; YENG; KAZIYAMA; TEIXEIRA, 2001).

Estudos recentes têm mostrado uma relação bidirecional entre a privação de sono e a presença de dor. Assim, a presença de dor pode prejudicar o sono, e o sono curto ou perturbado, por sua vez, pode ser capaz de reduzir os limiares de dor, aumentando a dor espontânea (AFOLALU; RAMLEE; TANG, 2018; FINAN; GOODIN; SMITH, 2013; HAACK et al., 2020). O sono é essencial para preservar e manter a saúde. A dor, principalmente a dor crônica, está comumente associada a distúrbios do sono em indivíduos com dor miofascial mastigatória (LAVIGNE; SESSLE, 2016). A privação do sono pode provocar deterioração de circuitos centrais que participam da modulação da dor, contribuindo, assim, para a vulnerabilidade à dor

crônica, gerando prejuízo nos processos de habituação e sensibilização à dor (FROHNHOFEN, 2018; HAACK et al., 2020; SIMPSON et al., 2018).

Um estudo recente demonstrou que a privação do sono REM foi capaz de alterar o limiar nociceptivo em modelo experimental de alodinia na região da face e estimular a ativação neuronal em áreas cerebrais relacionadas com a condução de informações nociceptivas, sugerindo que houve aumento na transmissão nociceptiva na via trigeminal (KIM et al., 2019). Alguns autores apontaram que distúrbios do sono tem relação com neuroinflamação, hiperexcitabilidade neuronal e ativação da glia no sistema nervoso central (NIJS et al., 2017, 2018; NIJS; VAN HOUDENHOVE; OOSTENDORP, 2010).

No tratamento de pacientes com dor crônica, normalmente os distúrbios do sono são deixados numa posição secundária ou não são abordados (CHEATLE et al., 2016). Considerar a influência da dor nos vários aspectos do sono e entender seu impacto nos distúrbios da dor orofacial pode ajudar no desenvolvimento de uma abordagem de gerenciamento prudente e eficiente (KLASSER; ALMOZNINO; FORTUNA, 2018).

2. PROPOSIÇÃO

Com base em achados clínicos, sabe-se que fatores psicossociais, como estresse e distúrbios do sono influenciam na exacerbação de sintomas dolorosos. Estas condições têm relação com somatização, catastrofização e incapacidades funcionais. Além disso, ambas estão relacionadas com mecanismos de neuroinflamação e hiperexcitabilidade neuronal.

Na rotina de avaliação e tratamento da dor miofascial mastigatória, a avaliação dos distúrbios do sono muitas vezes não é considerada, apesar do esforço de estudiosos da área em implementá-la. Existem evidências experimentais que relacionam os distúrbios do sono à dor inflamatória ou neuropática na região orofacial. Entretanto, não há estudos experimentais que relacionem os distúrbios do sono à dor miofascial mastigatória.

Apesar de ser objeto de vários estudos recentes, ainda há pouco conhecimento científico sobre os mecanismos neuroquímicos subjacentes à relação entre sono, estresse e dor orofacial. A compreensão desses mecanismos neurobiológicos é importante para estimular o desenvolvimento de novas abordagens terapêuticas que possam controlar ou aliviar efetivamente a dor orofacial por meio de vias compartilhadas que podem modular o sono e o estresse.

Dessa forma, o objetivo do presente estudo foi investigar a influência da privação de sono, do estresse emocional e da associação entre privação do sono e estresse emocional na dor miofascial nos músculos mastigatórios e se estes estímulos provocam ativação da via nociceptiva trigeminal (gânglio trigeminal, subnúcleo caudal do trigêmeo – Sp5c –, tálamo e substância cinzenta periaquedutal – PAG) em ratos. Além disso, buscou-se avaliar se o estresse emocional e a privação do sono provocam alterações comportamentais nas atividades locomotora e exploratória, na memória de trabalho e alterações na glândula adrenal em ratos.

3. DESENVOLVIMENTO

Por se tratar de pesquisa envolvendo animais, o projeto de pesquisa referente a esta dissertação foi submetido à apreciação da Comissão de Ética em Pesquisa Animal (CEPA) da Universidade Federal do Ceará, tendo sido aprovado sob número de protocolo 8694160418 (ANEXO A).

Esta dissertação de mestrado baseia-se no aditivo ao regimento interno do Programa de Pós-Graduação em Ciências Morfofuncionais da Universidade Federal do Ceará, que regulamenta o formato alternativo para dissertações de mestrado e teses de doutorado. Este capítulo consta de uma cópia do artigo científico de autoria do candidato, redigido de acordo com as normas da revista científica escolhida para publicação ("Journal of Sleep Research").

Artigo Científico

SLEEP DEPRIVATION AND EMOTIONAL STRESS INDUCE MASTICATORY MUSCLES HYPERALGESIA AND INCREASE c-FOS EXPRESSION IN TRIGEMINAL PAIN PATHWAY IN RATS

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ABBREVIATIONS

ACTH, Adrenocorticotropic hormone; BSA, Bovine serum albumin; CNS, Central Nervous System; ES, Emotional Stress; EFS, Electrically foot-shocked (stress-emitting animals); ESSD, Emotional Stress and Sleep Deprivation; PAG, Periaqueductal gray; PFA, Paraformaldehyde;

SD, Sleep Deprivation; Sp5c, spinal trigeminal subnucleus caudalis; THA, thalamus; TG, trigeminal ganglia;

ABSTRACT

The role of sleep disturbances in myofascial pain in masticatory muscles has not yet been investigated to date and nor has the association between emotional stress and sleep disorders. Thus, the purpose of the present study was to investigate the influence of sleep deprivation and emotional stress on masticatory muscles nociceptive sensitivity in rats, through nociceptive behavior, anxiety-like behavior and c-Fos expression in trigeminal pain pathway. For this, forty male Wistar rats were submitted to an emotional stress model in the Communication Box and paradoxical sleep deprivation in multiple platforms method. Rats were divided into 4 groups: control, emotional stress (ES), sleep deprivation (SD) and ES associated with SD (ESSD). Mechanical nociceptive tests, Open-field test and Y-maze test were performed to investigate the hyperalgesia in the masseter and temporalis muscles, the locomotory and exploratory activities and the working memory, respectively. c-Fos expression was assessed in the trigeminal ganglia (TG), spinal trigeminal nucleus caudalis (Sp5C), thalamus (THA) and periaqueductal gray (PAG). The presence of cytoplasmic vacuoles in the adrenal glands were analyzed. SD and ES increased mechanical hyperalgesia in masticatory muscles and c-Fos expression in TG, Sp5c and PAG. ES also caused increased c-Fos expression in THA, but this did not occur with SD. ESSD showed a higher hyperalgesic response in masticatory muscles. In addition, SD, ES and ESSD increased exploratory and locomotory activities, decreased working memory and provoked a higher concentration of cytoplasmic vacuoles in adrenal glands. In conclusion, SD and ES caused hyperalgesia in masticatory muscles with trigeminal nociceptive pathway involvement through c-Fos expression in various regions, including descending pain modulatory brain area. ESSD exacerbated the pain sensitivity and provoked anxiety-like behavior, work memory deficit and changes in adrenal glands, probably related to stress-induced neuroendocrine changes.

Keywords: Sleep Deprivation. Emotional Stress. Orofacial Pain. Myofascial Pain Syndrome.

INTRODUCTION

Masticatory myofascial pain is one of the most common causes of musculoskeletal pain in the orofacial region. It is characterized by a painful sensation referred to in masticatory muscles, which usually involves limitation of the opening of the mouth (Cummings & Baldry, 2007; Fernández-de-las-Penas & Svensson, 2016; Rossetti et al., 2008). It is a regionalized painful muscle condition characterized by the occurrence of palpable tense muscle bands, identifying intensely painful points. Psychological conditions, such as stress, and changes in quality of sleep can directly influence in the exacerbation of the symptoms of this type of pain, related to somatization and functional disabilities (Meira e Cruz et al., 2019; Sidebottom et al., 2013; Yap et al., 2002).

Stress has become a recurring problem in society, becoming one of the main reasons for seeking medical services. It is characterized as a physiological and psychological response to harmful stimuli and environmental changes that induce adaptive reactions controlled by neurohormonal processes, in order to maintain the physiological integrity of the organism (Koko et al., 2004). During reactions to stress, there is an increase in the activation of the (hypothalamus-pituitary-adrenal) neuroendocrine axis with increased circulation of systemic cortisol, in addition to several neurovegetative responses. Among the types of stress, the emotional one has gained notoriety and is identified as one of the causes of orofacial pain (Kubo et al., 2015; Ulrich-Lai & Herman, 2009).

Recent studies have shown a bidirectional relationship between sleep deficiency and the presence of pain. Thus, pain can disrupt sleep, and short or disturbed sleep, in turn, reduces pain thresholds, increasing spontaneous pain (Afolalu et al., 2018; Finan et al., 2013; Haack et al., 2020). The better knowledge of pain influence on the various aspects of sleep and the understanding of its impact on orofacial pain disorders, can contribute in developing a prudent and efficient management approach (Klasser et al., 2018). Sleep is essential for preserving and maintaining health. Recent studies have also demonstrated that pain, particularly chronic pain, is commonly associated with sleep disorders (Lavigne & Sessle, 2016).

Although being the subject of several recent studies, there is still little scientific knowledge about neurochemical mechanisms underlying the relationship between sleep, stress and orofacial pain. Understanding these neurobiological mechanisms is important to stimulate

the development of new therapeutic approaches that can effectively control or relieve orofacial pain through shared pathways that can modulate sleep and stress.

Zhang et al (2011) have demonstrated that emotional stress provokes masticatory muscles hyperalgesia in rats (Zhang et al., 2011). However, the role of sleep disturbances in myofascial pain in the orofacial region has not yet been investigated to date and nor the association between emotional stress and sleep disorders. Thus, the purpose of the present study was to investigate the influence of sleep deprivation and emotional stress on the masticatory muscles' nociceptive sensitivity in rats, through nociceptive behavior, anxiety-like behavior and c-Fos expression in trigeminal pain pathway.

MATERIALS AND METHODS

Animals

This study was conducted according to the ethical principles of the Universal Declaration of Animal Rights by the United Nations Educational, Scientific and Cultural Organization and after review and was approved by the Ethic Committee on Animal Use of the Federal University of Ceará (protocol 8694160418). This report followed the guidelines established by the ARRIVE statement (Kilkenny et al., 2010; Percie du Sert et al., 2020).

Forty adult male Wistar rats (Rattus norvegicus, albinus), weighing 200-250 g, obtained from the Central Animal Facility (Federal University of Ceará), were used for experimental protocols. The animals were maintained in microisolator cages under controlled temperature (22 ± 2 °C), in a silent room under a 12h/12h light/dark cycle, with access to solid food and water *ad libitum*.

The animals were randomly allocated in 5 experimental groups (n=8): 1) Control: animals not submitted to emotional stress or sleep deprivation; 2) Emotional stress (ES): animals subjected to emotional stress for 14 consecutive days in communication box; 3) Sleep deprivation (SD): animals subjected to sleep deprivation (24 hours) on 1st, 4th, 8th and 11th experimental days. 4) Emotional stress and sleep deprivation association (ESSD): animals subjected to emotional stress for 14 consecutive days in communication box and sleep

deprivation (24 hours) on 1st, 4th, 8th and 11th experimental days; 5) Electrically foot-shocked (EFS): stress-emitting animals (these animals were not analyzed in our study; these animals were used as part of the experimentally induced emotional stress in the Communication Box model) (Rosales et al., 2002).

Experimental protocol

The experimental protocol lasted 14 days. Five days before the induction of emotional stress and sleep deprivation, the animals were adapted to manipulation, tests and experimental models. A schematic diagram can be observed in **Figure 1**.

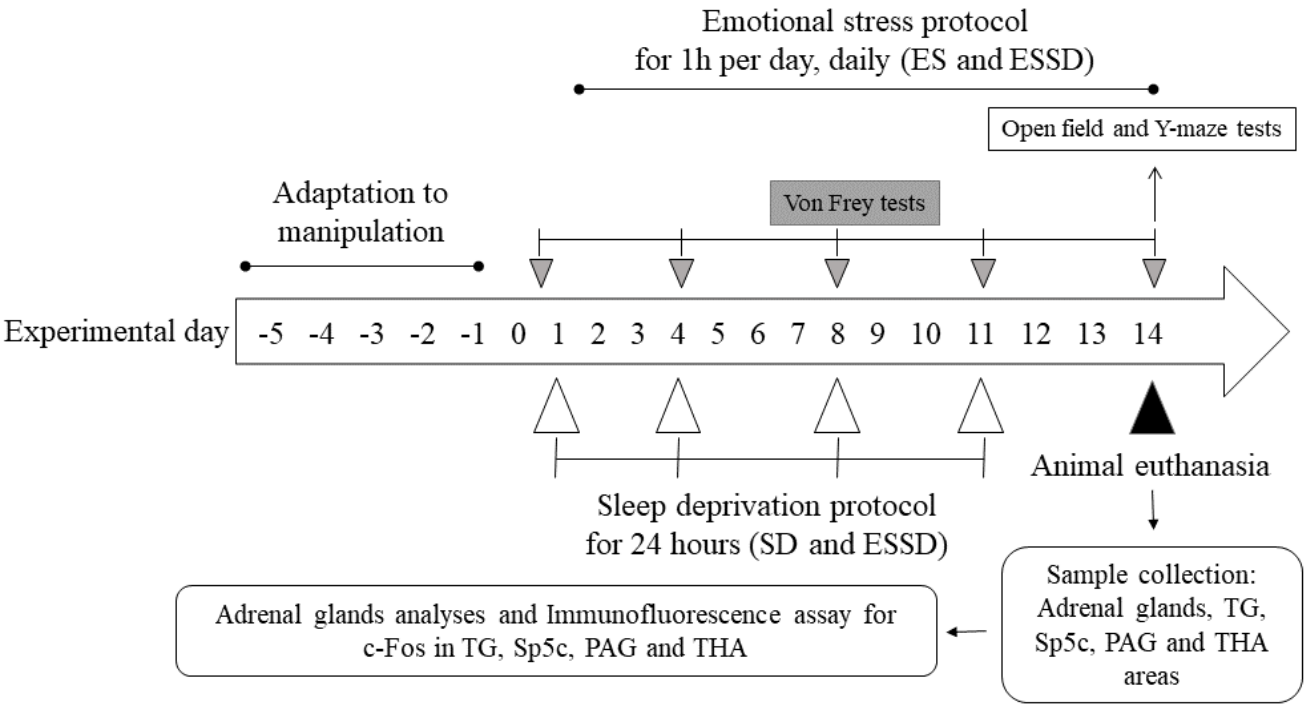

Fig. 1. Schematic diagram of the experimental protocol. Abbreviations: Control, animals not submitted to emotional stress or sleep deprivation; ES, animals subjected to emotional stress; SD, animals subjected to sleep deprivation; ESSD, animals subjected to emotional stress associated to sleep deprivation; TG, trigeminal ganglia; Sp5c, spinal trigeminal nucleus caudalis; PAG, periaqueductal gray; THA, thalamus.

Emotional Stress Model

The communication box was used as an instrument to induce emotional stress, which was an adapted method (Rosales et al., 2002). The box consists of 16 compartments (16 × 16 cm) separated by transparent acrylic walls with small holes, preventing the physical contact between adjoining animals, but allowing visual, auditory and olfactory sensations. The compartments were equipped with a grid floor of stainless-steel rods (5 mm in diameter and 1.3 cm placed apart). An electro-stimulator (Model NKL EL-608, Brusque, Santa Catarina, Brazil) of direct current (1 Hz) was connected to the floor of the steel rods to generate an electric current of 40 V, producing an electric foot-shock for 10 seconds with an interval of 60 seconds. The floors of the eight compartments were covered by plastic shields to evade electric foot-shock and served as a non-shock compartment for the study animals (ES and ESSD groups). Initially, all the animals were confined in the compartments of the communication box for 1 hour, without any electric foot-shock, for 5 days in order to adapt them to the environment. Emotional stress was induced for 14 consecutive days (ES and ESSD groups), where the animals were placed in the same compartment of the adaptation period. The EFS group received electric foot-shock for 1 hour (10s shock with 60s intervals) for 14 consecutive days, daily, at 11:00 AM. The animals were handled by the same researcher. The ES and ESSD groups allocated in the non-shock compartment were exposed to emotional stimuli from neighboring animals, such as vocalizations, urine or feces smell and jump response. Therefore, ES and ESSD rats were considered in a state of fear or anxiety. The control and SD groups were also confined in the non-shock compartment, but without any electric foot-shock. The EFS group was only used for stress emission and was not evaluated in any parameter.

Paradoxical Sleep Deprivation Model

Paradoxical sleep deprivation was induced by adaptation of the multiple platform method developed for rats (Machado et al., 2004). The animals of SD and ESSD groups were organized in subgroups of four animals and were placed in water tanks (50 cm × 60 cm × 20 cm) containing 8 platforms (6 cm in diameter), surrounded by water up to 1 cm beneath the surface, for 24 hours in 1^{st}, 4^{th}, 8^{th} and 11^{th} experimental days. The control and ES groups were also

confined in the tanks, but without water. Time to initiation of SD protocol was around Zeitgeber Time (ZT) 6 and ES was also conducted at daytime, under conditions of darkness, from 11:00 AM to 01:00 PM (ZT 6-7). The animals were handled by the same researcher.

Evaluation of the mechanical hyperalgesia in masseter and temporalis muscles

The mechanical nociceptive threshold of the animals was assessed using an electronic Von Frey (Insight, Ribeirão Preto, SP, Brazil). For the test, the animals were placed into individual plastic cages 30 min before beginning the assay. In the 5 days preceding the experimental period, the animals were adapted to cages and experimental procedures avoiding stress bias. The Von Frey transducer was applied perpendicularly to the center of masseter and temporalis muscles with a gradual increase in pressure. The stimulus was automatically discontinued after the head withdrawal, and the intensity of the applied pressure was recorded (Denadai-Souza et al., 2009; Zhang et al., 2011). Measurements were performed on 4^{th}, 8^{th}, 11^{th} and 14^{th} experimental days, half an hour after stimulus in the communication box. The baseline measurement was performed before emotional stress and sleep deprivation protocols.

Evaluation of locomotory and exploratory behavior

Spontaneous behaviors of crossing and rearing were evaluated with the open-field test. An acrylic arena apparatus with an inner dimension of 50×50 cm and a floor divided into nine equal squares was used. The test of each animal lasted 5 minutes. The locomotory activity was evaluated through the number of square crossings and the vertical exploratory activity was evaluated by counting the number of rearing behaviors, when the rat putted its weight on its hind legs, raise its forelimbs from the ground, and extended its head upwards. A careful cleaning of the apparatus was performed between each animal testing (Rabelo-da-Ponte et al., 2019).

Evaluation of working memory

Y-maze test was used to evaluate working memory. The animal was placed individually in the apparatus (three equal arms with 75.5cm × 34.5cm × 11.7cm, arranged at 120º from each other, forming a central triangle) for 8 minutes and its spontaneous movement was visually observed. Alternations were defined as entries in all three arms on consecutive occasions. The percentage of alternation was calculated as total of $(\frac{number\ of\ alternations}{(total\ arm\ entries-2)}) \times 100$. A careful cleaning of the apparatus was performed between each animal testing (Lima et al., 2019).

Immunofluorescence assay and analysis

For immunofluorescence assay, samples of trigeminal ganglia (TG), spinal trigeminal nucleus caudalis (Sp5C) [bregma interval: -10.20, -11.52], periaqueductal gray (PAG) [bregma interval: -5.42, -6.72] and thalamus (THA) [bregma interval: -3.24, -4.36] areas were harvested on the 14th experimental day (Defazio et al., 2015). For such, the rats were anesthetized with an intraperitoneal administration of ketamine (100 mg/kg) (König do Brasil Ltda, Mairinque, SP, Brazil) and xylazine (10 mg/kg) (König do Brasil Ltda, Mairinque, SP, Brazil). The intracardiac perfusion was then performed using 60 mL of sterile saline followed by 60 mL of 4% paraformaldehyde (PFA) (Sigma-Aldrich ®, St. Louis, MO, 17 USA) solutions. The tissues were removed and placed in 4% PFA for two hours, followed by a 2-day cryoprotection with 30% sucrose solution (Dinâmica Química Contemporânea Ltda, Rio de Janeiro, RJ, Brazil). The tissues were then embedded in Tissue Plus® O.C.T Compound (Fisher Healthcare, Houston, TX, EUA) and stored at -80 °C. The samples were sliced (a 10-µm thick) using a cryostat (Leica CM1850, Leica, 22 Wetzlar, Germany).

Slices were placed in Flex IHC microscope slides (Code K8020, DAKO, Agilent Technologies, Denmark) fixed in methanol (Vetec Química Fina Ltda., Duque de Caxias, RJ, Brazil), washed in phosphate buffered saline (PBS) and therefore antigenic recovery was performed with 0.1 M (pH 6.0) sodium citrate buffer at 95 °C. The tissue sections were incubated for 10 min with 0.1% Triton X-100 for nuclear membrane permeabilization. After blocking of unspecified sites with 0.3 M glycine/5% BSA solution for 30 min, slices were

incubated overnight at 2 – 8 °C with the rabbit anti-c-Fos antibody (Cell Signaling Technology®, Danvers, MA, USA), diluted 1:300 in BSA 5% solution plus Triton X-100 0.1%. After PBS washing, slides were incubated with donkey anti-rabbit IgG Alexa Fluor 568 (Invitrogen ®, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) secondary antibody, at a dilution of 1:400, for 1h and 30min. For neuronal labeling, the NeuN antibody conjugated to Alexa Fluor 488 (Merck Millipore®, Billerica, MA, USA) was used at a dilution of 1:100 for 2 hours. Nuclear labeling was done by DAPI (4,6'-diamidino-2-phenylindole; Invitrogen®, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). Slides were mounted with ProLong Gold Antifade Mountant (Invitrogen®, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA).

A laser scanning confocal microscope (Zeiss LSM 710, Carl Zeiss, Jena, Germany) was used for the acquisition of the photomicrographs with master gain and digital offset standardized for analysis. The photomicrographs were blindly analyzed through image software (Fiji ImageJ, National Institutes of Health, Washington, DC, USA). The "Cell Counter plugin" was used to count the cells labeled with the antibodies. The results were presented in percentage, calculated by the proportion of neurons marked with c-Fos in relation to neurons marked with NeuN.

Adrenal glands analyses

Adrenal glands were carefully separated from the fat layer and weighed on a high precision analytical balance (Mettler Toledo AL204) to compare the weight between the experimental groups.

Adrenal glands were fixed in 10% formaldehyde for 24 hours, then, placed in 70% alcohol for another 24h and processed for inclusion in paraffin. Serial sections (4 μm thickness) were obtained for hematoxylin and eosin staining. Slides were examined and photographed under an optical microscope (Leica DM 2000), with magnification of 1000x.

The zona fasciculata of adrenal cortex was observed, quantifying the area of the cytoplasmic vacuoles. For this, the software ImageJ (Fiji ImageJ, National Institutes of Health, Washington, DC, USA) was used and the upper and lower limits were previously standardized by the color threshold to define the selected and unselected pixels. The results were presented in percentage, calculated by comparing the cytoplasmic vacuoles area to total area.

Evaluation of body weight gain

The animals' body weight was measured before the protocols for inducing emotional stress and sleep deprivation (baseline), on the 7th and 14th days of the experimental protocol. The body weight gain was presented as a percentage relative to baseline body weight.

Statistical analyses

The results were presented as the mean ± SEM. The statistical difference in the behavioral tests was determined by the two-way ANOVA, followed by Tukey's test. One-way ANOVA, followed by Tukey's test, was used for other assays. The level significance was established at $P < 0.05$. For the analyses, the software GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA, USA) was used.

RESULTS

Sleep deprivation and emotional stress increases mechanical hyperalgesia in masticatory muscles

Figure 2 shows that emotional stress reduced the nociceptive threshold in masseter and temporalis muscles from the 8th day compared with the control group ($P < 0.05$). This threshold reduction remained until the 14th day of emotional stress induction. SD and ESSD groups showed earlier reduction of the nociceptive threshold in masseter (**Fig. 2A**) and temporalis muscles (**Fig. 2B**) from the 4th experimental day ($P < 0.05$). The sleep deprivation associated with emotional stress decreased further this nociceptive threshold in the masseter muscle (**Fig. 2A**) on the 8th day with significant differences from ES and SD groups ($P <0.05$). The decrease of nociceptive threshold of ESSD group in masseter muscle persisted until 14th day when compared to ES group ($P < 0.05$). In temporal muscle the association between emotional stress and sleep deprivation had no additive effect upon nociceptive threshold (**Fig. 2B**).

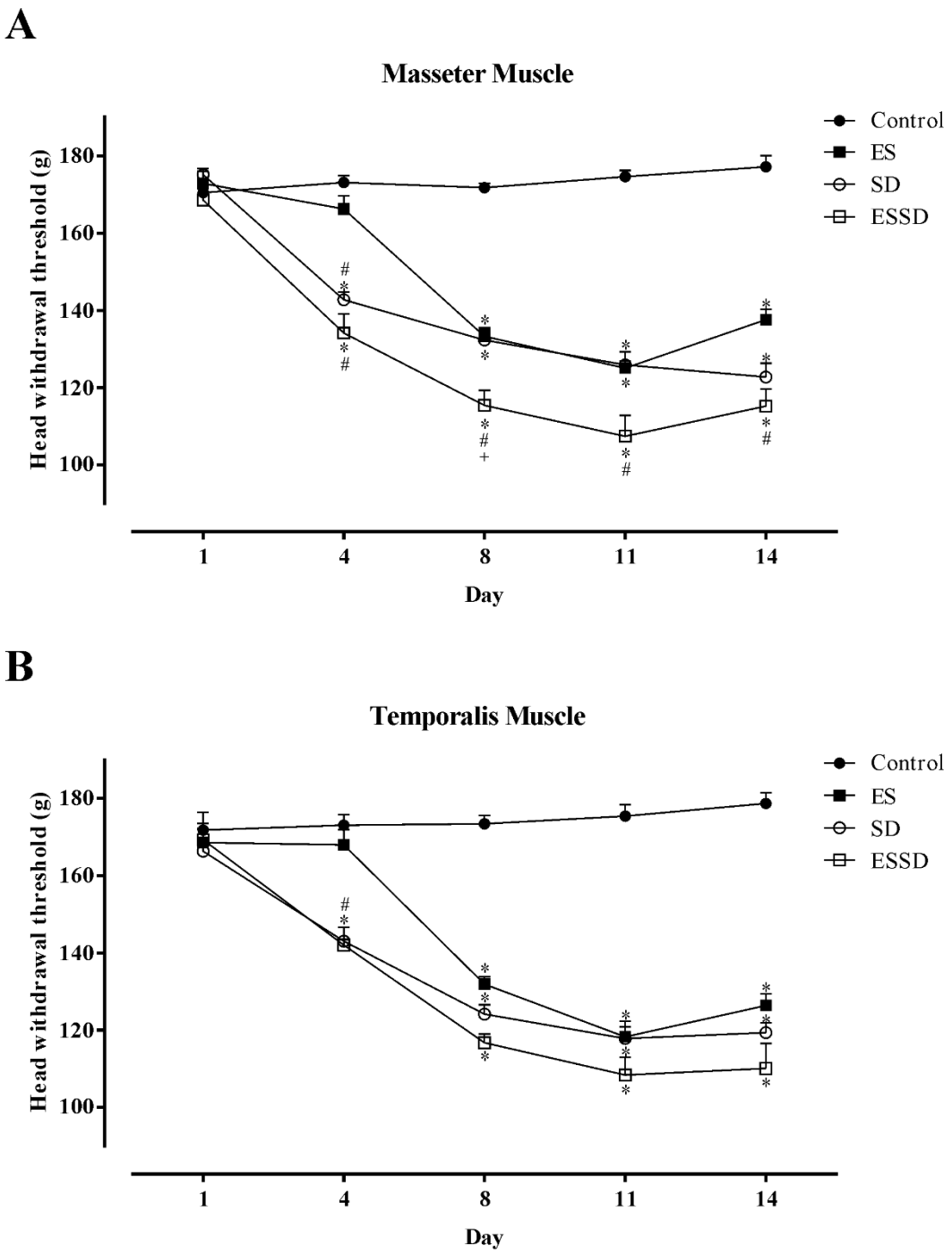

Fig. 2. Effect of sleep deprivation (SD), emotional stress (ES) and their association (ESSD) on nociceptive threshold of masseter and temporalis muscles in rats. The graphs represent the development of mechanical hyperalgesia in the region of the masseter **(A)** and temporalis **(B)** muscles in rats submitted to sleep deprivation (SD), emotional stress (ES) or their association (ESSD). The results are expressed as mean ± standard error of the mean (SEM) of the head withdrawal threshold in grams (g) and expresses an intergroup comparison (*P <0.05 versus control; #P <0.05 versus ES; $^{+}$P <0.05 versus SD; two-way ANOVA, Tukey post-test). Abbreviations: Control, animals not submitted to emotional stress or sleep deprivation; ES, animals subjected to emotional stress; SD, animals subjected to sleep deprivation; ESSD, animals subjected to emotional stress associated to sleep deprivation;

The intragroup assessment (**Fig. 3**) of head withdraw threshold kinetics showed a reduction in the masseter and temporalis nociceptive threshold of ES group on 8th, 11th and 14th days in comparison to 1st experimental day (up to 27.6% in the 11th day; P <0.05 versus 1st day) and in relation to 4th day (up to 24.8% in the 11th day; P <0.05 versus 4th day). Sleep deprivation decreased the nociceptive threshold from the 4th experimental day (*P < 0.05 versus 1st day) and decreased further in the 8th day (P < 0.05 versus 4th day). This decrease in nociceptive threshold was maintained until the last day of the experimental protocol (**Fig. 3**). The association between emotional stress and sleep deprivation decreased the nociceptive threshold from the 4th experimental day (P < 0.05 versus 1st day) and decreased further from 8th day (up to 36.26% in 11th day; #P < 0.05 versus 4th day).

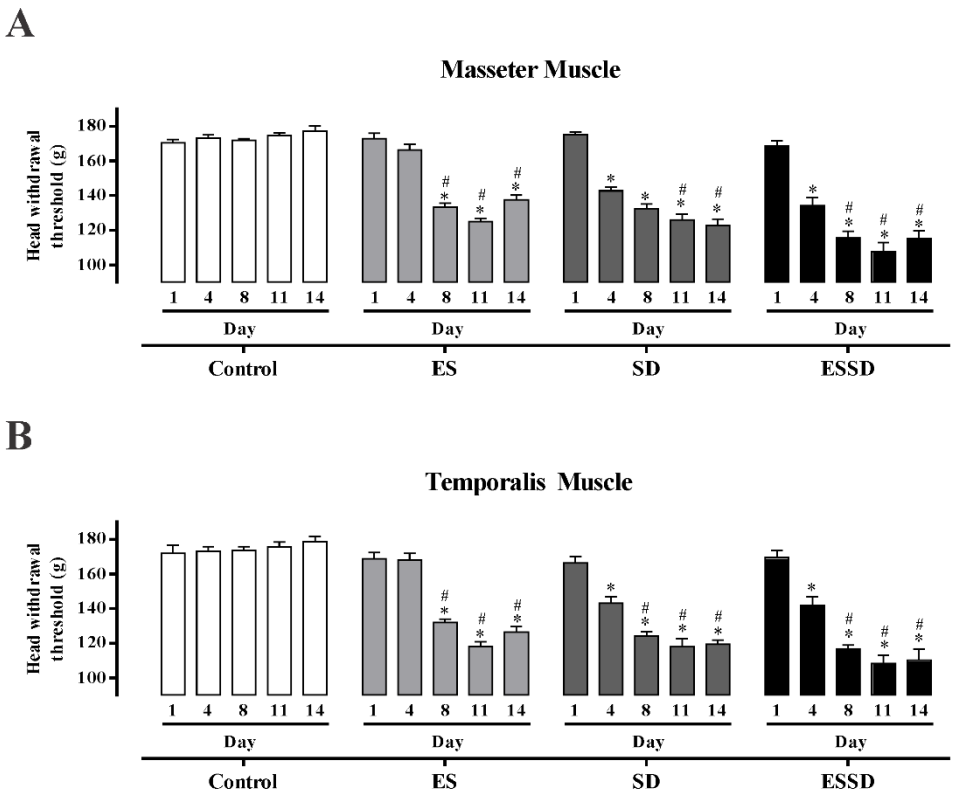

Fig. 3. Effect of sleep deprivation (SD), emotional stress (ES) and their association (ESSD) on nociceptive threshold of masseter and temporalis muscles in rats: analysis of intragroup temporal evolution. The graphs represent the development of mechanical hyperalgesia in the region of the masseter **(A)** and temporalis **(B)** muscles in rats submitted to sleep deprivation (SD), emotional stress (ES) or their association (ESSD). The results are expressed as mean ± standard error of the mean (SEM) of the head withdrawal threshold in grams (g) and express an intragroup comparison (* P <0.05 versus 1st day; #P <0.05 versus 4th day; two-way ANOVA, Tukey post-test). Abbreviations: Control, animals with no submitted to emotional stress or sleep deprivation; ES, animals subjected to emotional stress; SD, animals subjected to sleep deprivation; ESSD, animals subjected to emotional stress associated to sleep deprivation;

Sleep deprivation and emotional stress increases c-Fos expression in trigeminal ganglia (TG), spinal trigeminal nucleus caudalis (Sp5c), thalamus (THA) and periaquedutal gray (PAG)

Our results showed that emotional stress, sleep deprivation and the association of both increased the c-Fos expression in comparison with control group ($P < 0.05$) in neuronal cells (marked with NeuN) in TG (**Fig. 4**), Sp5c (**Fig. 5**) and PAG (**Fig. 8**).

Emotional stress or emotional stress associated with sleep deprivation caused an increase in c-Fos expression in the lateral THA (**Fig. 6**, $P < 0.05$ versus control), while sleep deprivation did not differ from the control group. In lateral THA, ESSD group showed a difference in relation to the SD group ($P < 0.05$ versus SD).

When evaluating the expression of c-Fos in medial THA, we observed that the groups subjected to emotional stress (ES and ESSD) showed an increase when compared to control (**Fig. 7**), $P < 0.05$ versus control), while sleep deprivation did not cause a significant increase.

Additionally, the c-Fos expression analysis in PAG showed that among the 3 groups, emotional stress induced a significant greater effect, when compared to sleep deprivation (**Fig. 8**, $P < 0.05$ versus ES).

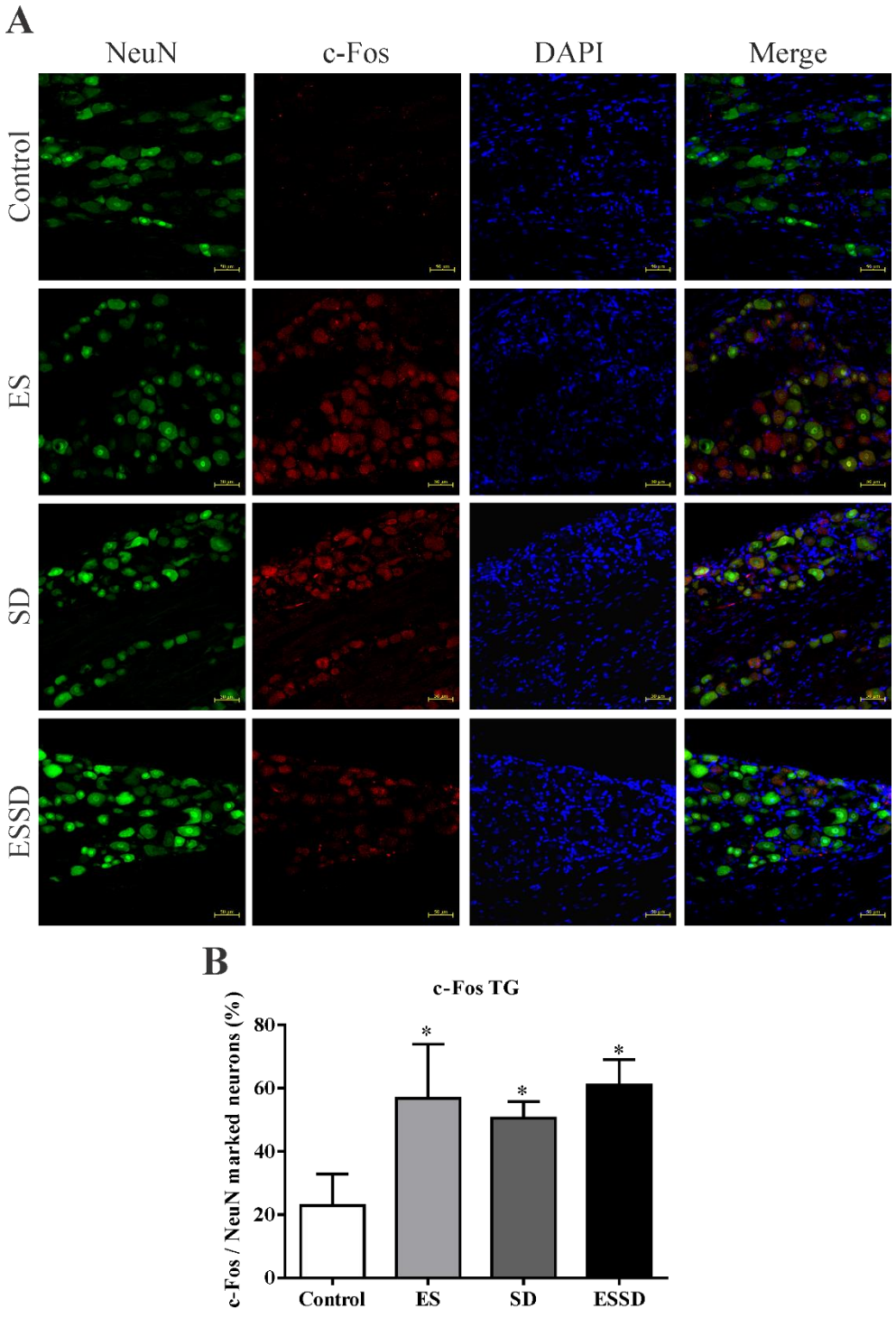

Fig. 4. Effect of sleep deprivation (SD), emotional stress (ES) and their association (ESSD) on c-Fos expression in trigeminal ganglia (TG). (A) Confocal photomicrography of trigeminal ganglia (TG), showing in green: NeuN (neuronal marker); red: c-Fos; blue: DAPI (nuclear marker); yellow: Merge. Magnification: 200x. Scale: 50 µm. **(B)** Percentage of c-Fos marked neurons relative to NeuN marked neurons. The results are expressed as mean ± standard error of mean (SEM) of c-Fos marked neurons (%) of 6 confocal images per group (*P < 0.05 versus control, one-way ANOVA, Tukey post-test). Abbreviations: Control, animals not submitted to emotional stress or sleep deprivation; ES, animals subjected to emotional stress; SD, animals subjected to sleep deprivation; ESSD, animals subjected to emotional stress associated to sleep deprivation; TG, trigeminal ganglia.

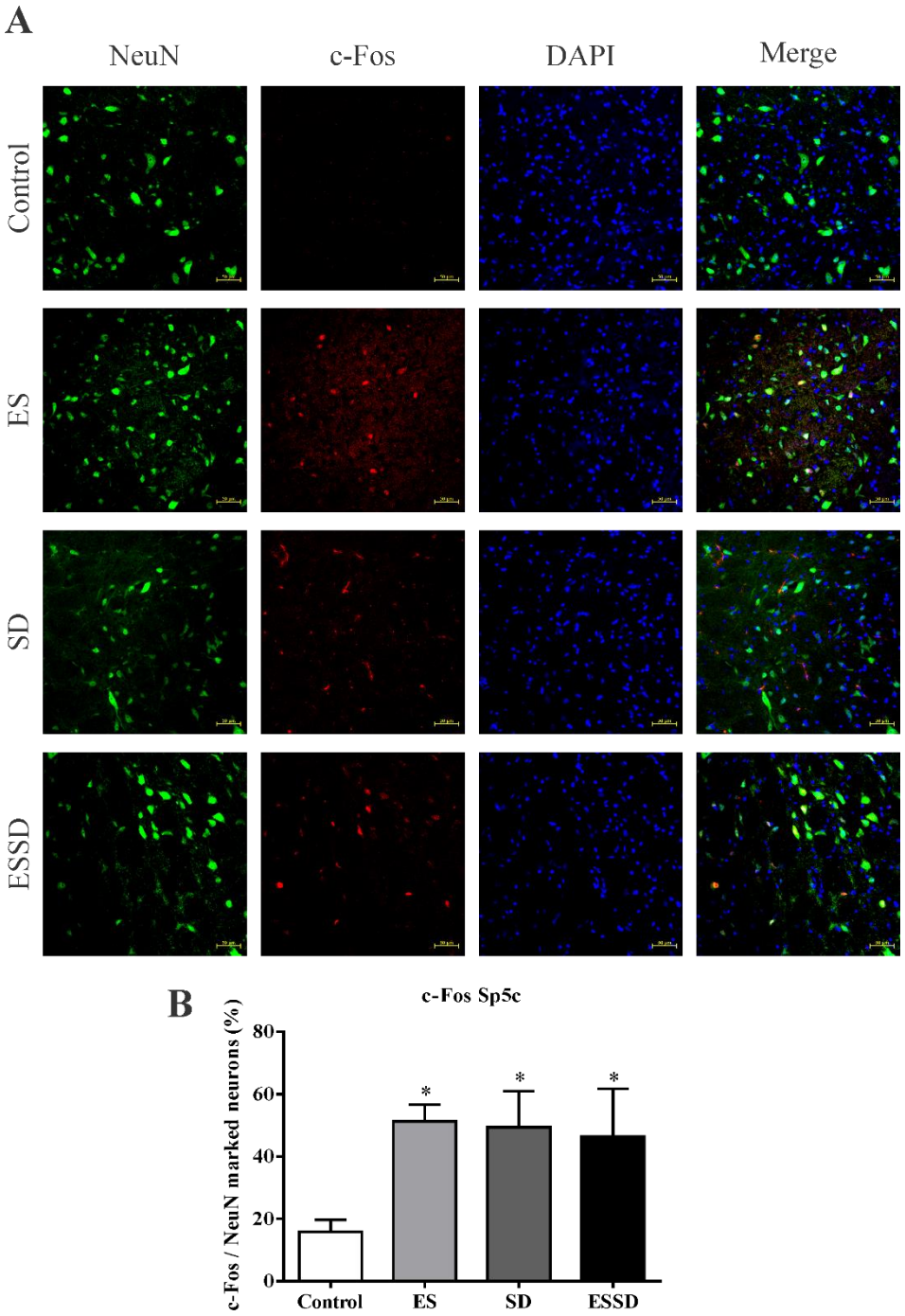

Fig. 5. Effect of sleep deprivation (SD), emotional stress (ES) and their association (ESSD) on c-Fos expression in spinal trigeminal nucleus caudalis (Sp5c). (A) Confocal photomicrography of spinal trigeminal nucleus caudalis (Sp5c), showing in green: NeuN (neuronal marker); red: c-Fos; blue: DAPI (nuclear marker); yellow: Merge. Magnification: 200x. Scale: 50 µm. **(B)** Percentage of c-Fos marked neurons relative to NeuN marked neurons. The results are expressed as mean ± standard error of mean (SEM) of c-Fos marked neurons (%) of 6 confocal images per group (*P < 0.05 versus control, one-way ANOVA, Tukey post-test). Abbreviations: Control, animals not submitted to emotional stress or sleep deprivation; ES, animals subjected to emotional stress; SD, animals subjected to sleep deprivation; ESSD, animals subjected to emotional stress associated to sleep deprivation; Sp5c, spinal trigeminal subnucleus caudalis.

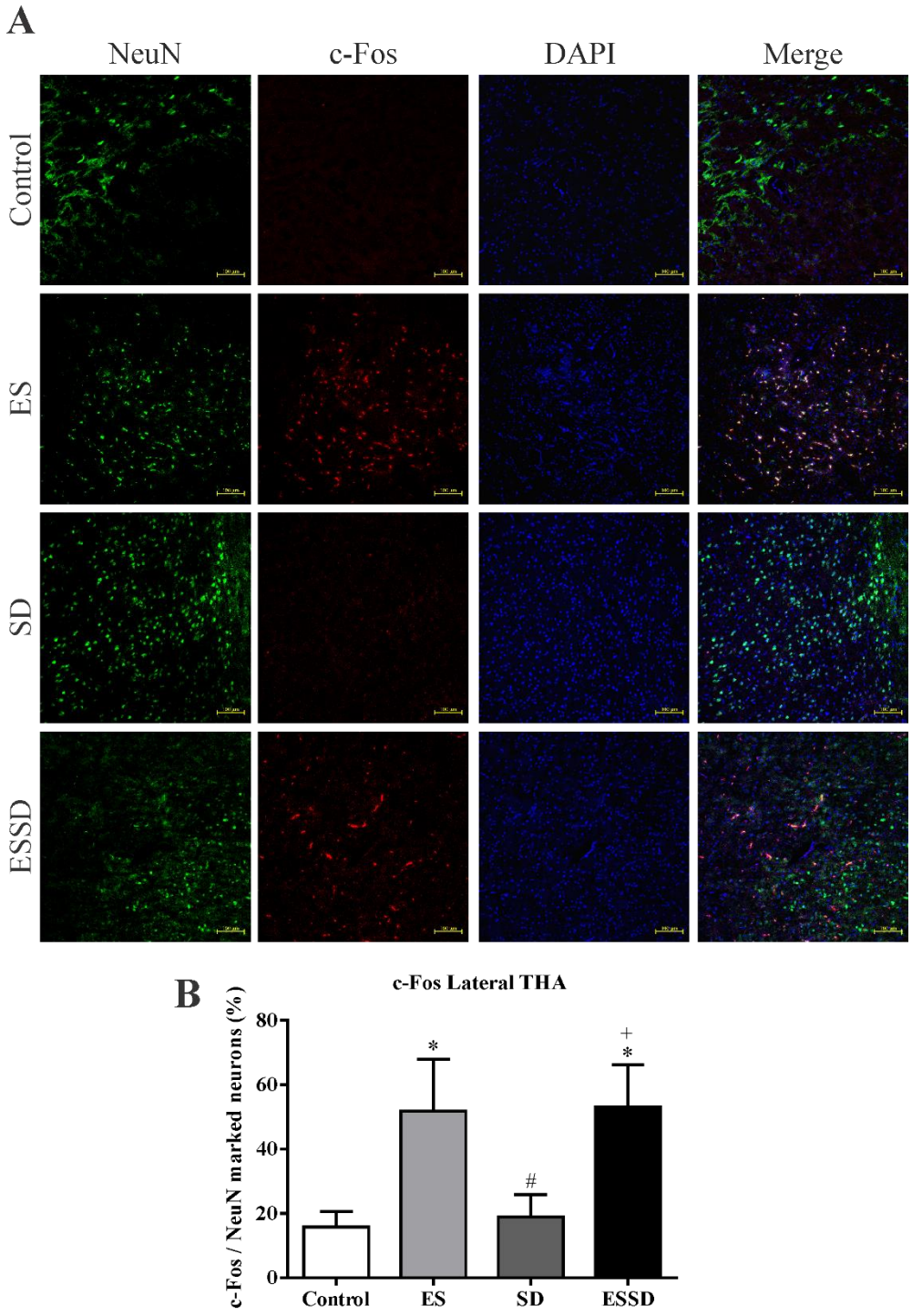

Fig. 6. Effect of sleep deprivation (SD), emotional stress (ES) and their association (ESSD) on c-Fos expression in lateral thalamus (lateral THA). Green: NeuN (neuronal marker); red: c-Fos; blue: DAPI (nuclear marker); yellow: Merge. Magnification: 200x. Scale: 100 µm. **(A)**. THA were harvested on the 14th experimental day. The quantification represents of the c-Fos marked neuron relative to NeuN marked neurons **(B)**. The results are expressed as mean ± standard error of mean (SEM) (*P < 0.05 versus control; #P < 0.05 versus ES; $^{+}$P < 0.05 versus SD; one-way ANOVA, Tukey post-test). Abbreviations: Control, animals with no submitted to emotional stress or sleep deprivation; ES, animals subjected to emotional stress; SD, animals subjected to sleep deprivation; ESSD, animals subjected to emotional stress associated to sleep deprivation; THA, thalamus.

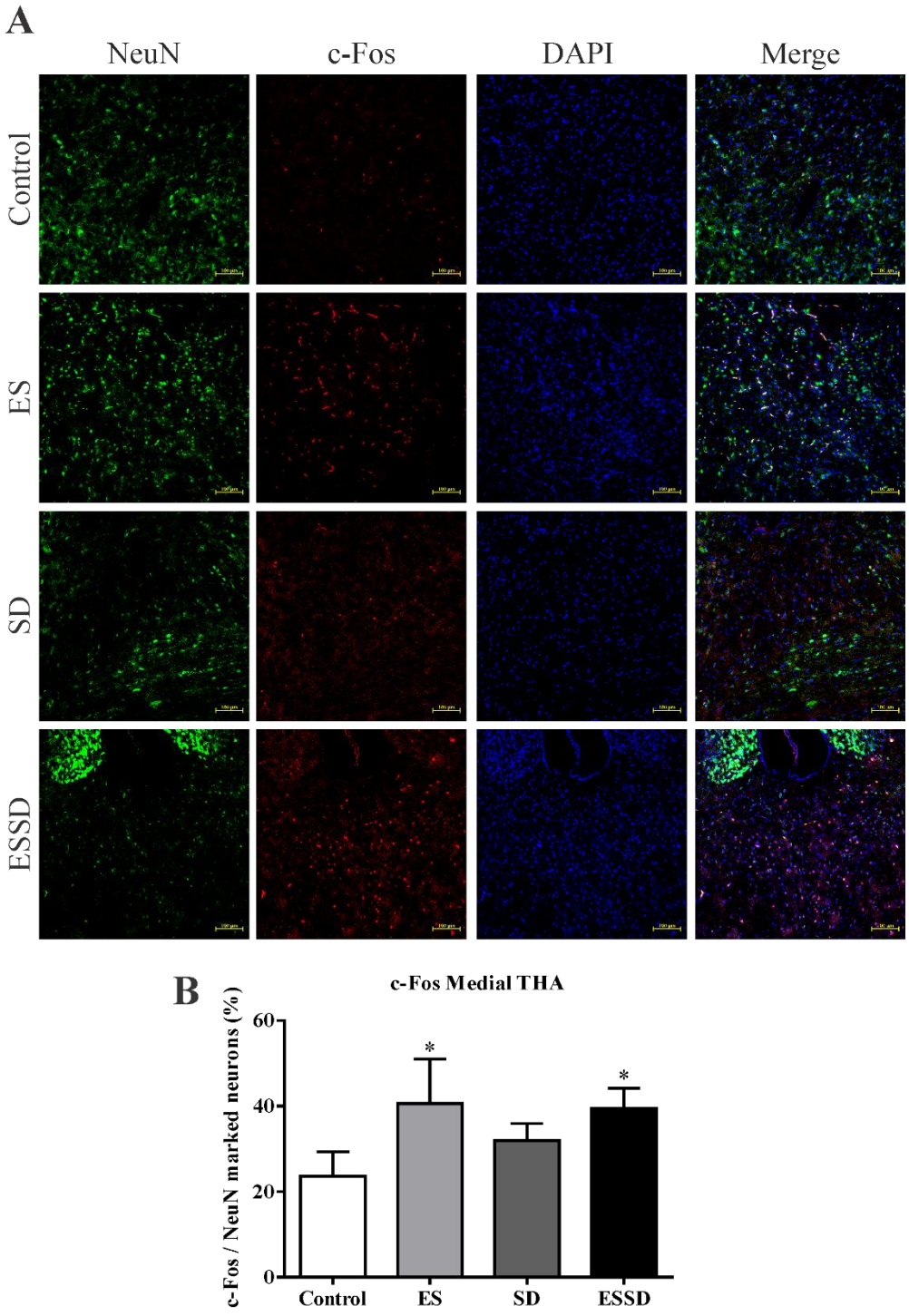

Fig. 7. Effect of sleep deprivation (SD), emotional stress (ES) and their association (ESSD) on c-Fos expression in medial thalamus (medial THA). Green: NeuN (neuronal marker); red: c-Fos; blue: DAPI (nuclear marker); yellow: Merge. Magnification: 200x. Scale: 100 µm. **(A)**. THA were harvested on the 14th experimental day. The quantification represents of the c-Fos marked neuron relative to NeuN marked neurons **(B)**. The results are expressed as mean ± standard error of mean (SEM) (*P < 0.05 versus control; one-way ANOVA, Tukey post-test). Abbreviations: Control, animals with no submitted to emotional stress or sleep deprivation; ES, animals subjected to emotional stress; SD, animals subjected to sleep deprivation; ESSD, animals subjected to emotional stress associated to sleep deprivation; THA, thalamus.

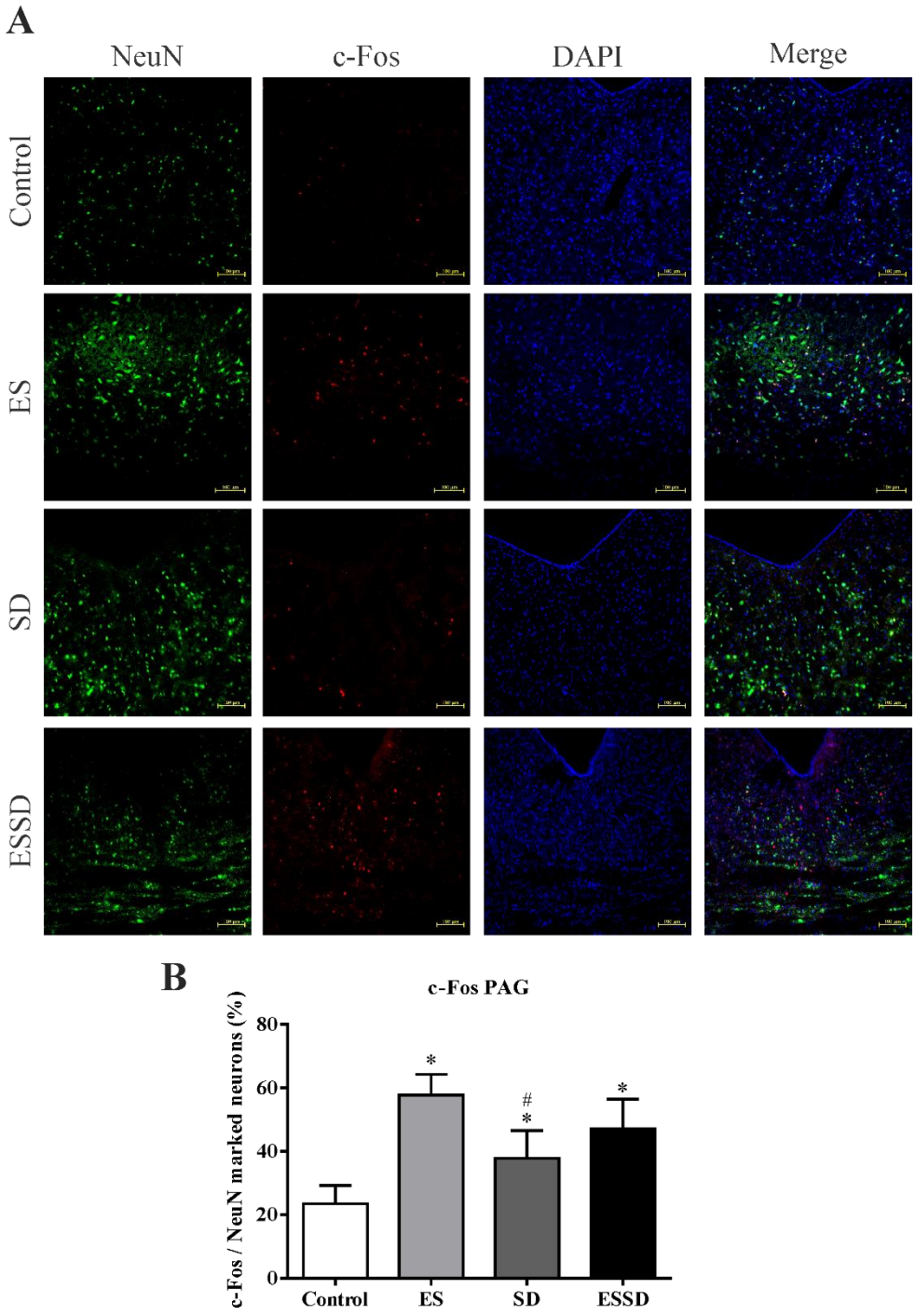

Fig. 8. Effect of sleep deprivation (SD), emotional stress (ES) and their association (ESSD) on c-Fos expression in periaqueductal gray (PAG). (A) Confocal photomicrography of trigeminal ganglia (TG), showing in green: NeuN (neuronal marker); red: c-Fos; blue: DAPI (nuclear marker); yellow: Merge. Magnification: 200x. Scale: 100 µm. **(B)** Percentage of c-Fos marked neurons relative to NeuN marked neurons. The results are expressed as mean ± standard error of mean (SEM) of c-Fos marked neurons (%) of 6 confocal images per group (*P < 0.05 versus control; #P < 0.05 versus ES; one-way ANOVA, Tukey post-test). Abbreviations: Control, animals not submitted to emotional stress or sleep deprivation; ES, animals subjected to emotional stress; SD, animals subjected to sleep deprivation; ESSD, animals subjected to emotional stress associated to sleep deprivation; PAG, periaqueductal gray.

Sleep deprivation and emotional stress increase exploratory and locomotor activity in rats

The Open Field Test showed that ES, SD and ESSD groups showed hyperlocomotion (increased number of crossings compared to control group: 55.9%, 58.5% and 77.6%, respectively; **Fig. 9A**), an increase in the vertical exploratory activity values (number of rearings; 55.9%, 121.2% and 133.6%, respectively; **Fig. 9B**) when compared to control group ($P < 0.05$). Sleep deprivation groups (SD e ESSD) showed a significant increase in the number of rearings, when compared to control group (up to 133.6%), and ES group (up to 49.8%) ($P < 0.05$ versus ES).

Sleep deprivation and emotional stress decrease the working memory in rats

The working memory evaluated by Y-maze test (**Fig. 9C**), showed that ES, SD and ESSD groups presented an increased up to 399.9% of incorrect alternations when compared to control group ($P < 0,05$ versus control).

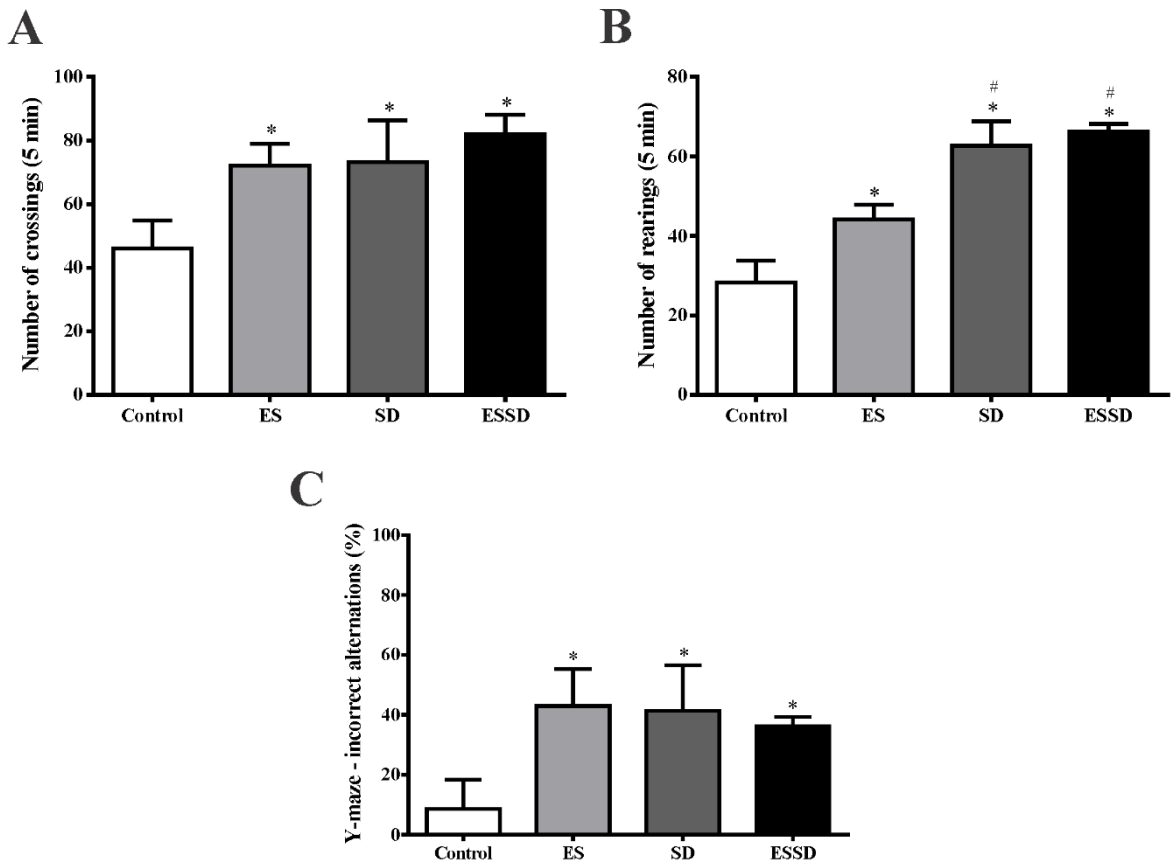

Fig. 9. Effect of sleep deprivation (SD), emotional stress (ES) and their association (ESSD) on locomotory/exploratory activities and on working memory in rats. The graphs represent the number of crossings **(A)** and rearings **(B)** behaviors evaluated per 5 minutes in Open Field test and the working memory expressed in percentage of incorrect alternations in Y-maze test **(C)**. The results are expressed as mean ± standard error of mean (SEM) of the number of crossings (A), rearings (B) and percentage of incorrect alternations (C) of 8 rats per group (*P < 0.05 versus control; one-way ANOVA, Tukey post-test). Abbreviations: Control, animals not submitted to emotional stress or sleep deprivation; ES, animals subjected to emotional stress; SD, animals subjected to sleep deprivation; ESSD, animals subjected to emotional stress associated to sleep deprivation.

Effects of sleep deprivation and emotional stress on adrenal glands and body weight gain

The cytoplasmic vacuoles analysis of adrenal glands cortex fasciculate zone showed different profiles between the ES, SD and ESSD groups when compared to the control group ($P < 0.05$; **Fig. 10**). These groups showed a higher concentration of cytoplasmic vacuoles (58.4%, 131.3% and 105.7%, respectively; $P < 0.05$ versus control), while the control group analysis showed a basal content of these vacuoles. Sleep deprivation groups (SD e ESSD) showed a significant increase in the percentage of cytoplasmic vacuoles area, when compared to ES group, with an increase of up to 46% in the SD group ($P < 0.05$ versus ES; **Fig. 10B**).

Emotional stress and sleep deprivation had no effect on adrenal glands gross weight. However, the combination of the two experimental models (ESSD group) increased in 33.45% the gross weight of these glands ($P < 0.05$, **Fig. 10C**). SD and ESSD groups showed an increase in adrenal glands weight relative to body weight (up to 31.47%) ($P < 0.05$, **Fig. 10D**).

To exclude the influence of body weight on the final weight of the adrenal glands, body weight of the rats was evaluated on the last experimental day (14th day). In the 14th experimental day, both SD and ESSD groups showed a decrease in gross body weight ($P < 0.05$, **Fig. 10E**). There were no statistical differences in the variation of the animals' body weight in the different experimental groups (**Fig. 10F**).

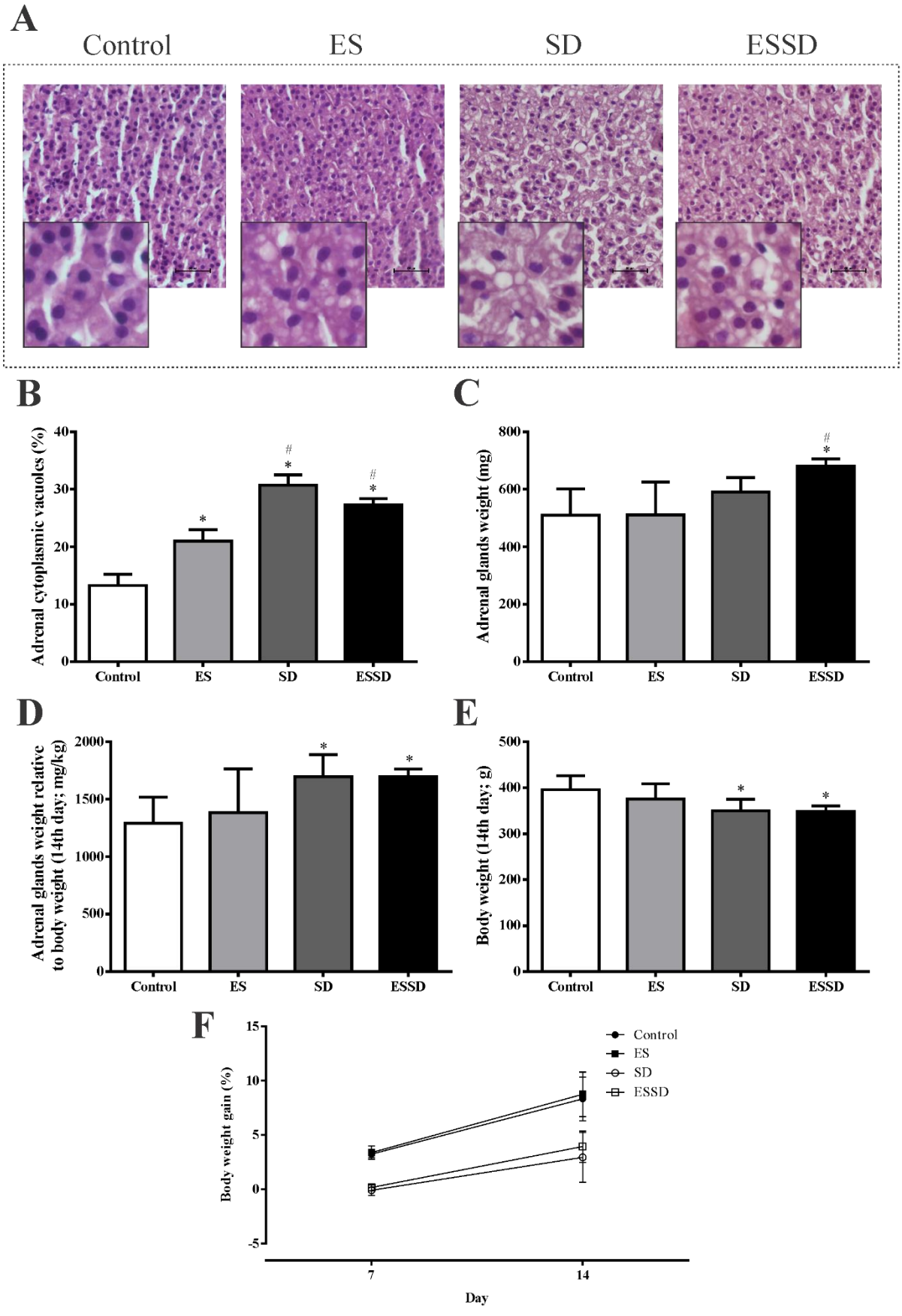
A
Control
ES
SD
ESSD
B
Adrenal cytoplasmic vacuoles (%)
0
10
20
30
40
Control
ES
SD
ESSD
C
Adrenal glands weight (mg)
0
200
400
600
800
Control
ES
SD
ESSD
D
Adrenal glands weight relative to body weight (14th day; mg/kg)
0
500
1000
1500
2000
Control
ES
SD
ESSD
E
Body weight (14th day; g)
0
100
200
300
400
500
Control
ES
SD
ESSD
F
Body weight gain (%)
-5
0
5
10
15
7
14
Day
Control
ES
SD
ESSD

Fig. 10. Effect of sleep deprivation (SD), emotional stress (ES) and their association (ESSD) on adrenal glands and on body weight gain. (A) photomicrography of hematoxylin and eosin staining showing the fasciculate zone of the adrenal gland cortex of rats subjected to emotional stress (ES), sleep deprivation (SD), the association between experimental models (ESSD) and animals not submitted to emotional stress or sleep deprivation (control); (B) percentage of the area corresponding to cytoplasmic vacuoles in the fasciculated zone of the adrenal gland cortex; (C) gross weight of the adrenal glands in milligrams; (D) adrenal glands weight relative to body weight in milligrams per kilograms; (E) body weight in 14th experimental day in grams; and (F) the percentage body weight gain relative to 1st experimental day. The results are expressed as mean ± standard error of mean (SEM) of the adrenal cytoplasmic area (B), adrenal weight (C), adrenal glands weight relative to body weight (D), body weight (E) of 8 rats per group (*P < 0.05 versus control; #P < 0.05 versus ES, one-way ANOVA, Tukey post-test). The results of body weight gain (F) are expressed as mean ± standard error of mean (SEM) of 8 rats per group (two-way ANOVA, Tukey post-test).

DISCUSSION

The present study investigated the effect of sleep deprivation, emotional stress and their association in the neuromodulation of orofacial pain in masticatory muscles.

Here, we found that emotional stress, paradoxical sleep deprivation and the association between these two experimental models caused a decrease in the orofacial nociceptive threshold in masseter and temporalis muscles in rats. In addition, according to this we found an increase in c-Fos expression in the trigeminal ganglia, spinal trigeminal nucleus caudalis (Sp5c), thalamus and PAG.

The emotional stress was induced using the communication box model, which is effective in producing psychological stress based on emotional communication between animals, without direct physical stress . This model is already widely used and known to induce nociceptive changes in the masticatory muscles (Rosales et al., 2002; Wu et al., 2013; Zhang et al., 2011).

Based on our results, we observed that emotional stress reduced the nociceptive threshold in the orofacial region, interpreted as a masticatory muscle hyperalgesia. Animals subjected to emotional stress showed a significant decrease in the head withdrawal threshold from the 8th day of exposure in the communication box, which confirms previous studies conducted by our group (Ponte, 2017; Silveira, 2015), corroborating with other authors (Zhang et al., 2011).

Clinical studies have sought to understand a relationship between stress, pain and muscle hyperactivity, reporting that patients with higher levels of stress have higher susceptibility to orofacial painful symptomatology (Bodéré et al., 2005; Dıraçoğlu et al., 2016; Fillingim et al., 2013; Klasser et al., 2018; Molina et al., 2003). Experimental studies confirm that the communication box satisfactorily produces an anxious-like behavior and that increased masticatory muscle activity in emotionally stressed animals seems to be involved with the reduction of the nociceptive threshold in the masticatory muscles (Chen et al., 2010; Qiang Li et al., 2013; Silveira, 2015; Zhang et al., 2011). These studies correlate stress and sensitivity to pain and mimic the clinical situation found in individuals with myofascial orofacial pain in masticatory muscles. Indeed, our data show that emotional stressed rats showed anxiety like behavior, observed by the open field test and Y-maze test.

In order to investigate the influence of sleep deprivation on masticatory muscles myofascial pain, we used the modified multi-platform model. In this model, each animal is kept on a platform surrounded by water for 24 hours (Machado et al., 2004). This model selectively deprives the animal of paradoxical sleep, causing stress associated with immobilization, isolation, falling into the water, etc., characterizing a chronic stressful condition. In this protocol, sleep deprivation is a component of generalized stress (Lima et al., 2019; Rabelo-da-Ponte et al., 2019). During the experimental procedures, sleep deprivation was performed four times, with intervals of at least 48 hours.

Sleep deprivation can be studied through several experimental models that vary from partial deprivation of specific phases of sleep to total sleep deprivation. There are more aggressive models that can be used to study brain damage, metabolic disorders, etc (Daniele et al., 2017; Lima et al., 2019; Machado et al., 2005; Pires et al., 2016). We had chosen the paradoxical sleep deprivation model because it is directly related to what happens in the clinic with individuals with masticatory myofascial pain. REM sleep is known to influence muscle repair, memory and cognition. In addition, in this stage of sleep, the maximum muscle relaxation is reported (Mônico-Neto et al., 2017; Peever, 2011). The literature shows that individuals with this type of pain also have changes in sleep architecture, with evidence of increased NREM stage and impaired REM sleep duration. In these individuals, the number of awakenings during sleep is common. Therefore, masticatory myofascial pain may be related to reduced sleep efficiency and sleep fragmentation (Boris Dubrovsky et al., 2014). Therefore, this is the best model to simulate what happens in patients with masticatory myofascial pain.

Our results demonstrated that sleep deprivation influenced the nociceptive threshold in masticatory muscles, causing hyperalgesia in the masseter and temporalis muscles. A single paradoxical sleep deprivation performed on the 1st day of the experimental protocol reduced the nociceptive threshold in masseter and temporalis. Emotional stress reduced the nociceptive threshold only after eight daily exposures in the communication box. Sleep deprivation amplified this effect of emotional stress on the 8th day, as it was seen when sleep deprivation was associated with emotional stress in the ESSD group.

Among people with chronic pain, sleep disorders are highly prevalent, being closely related to mechanisms of central sensitization, neuroinflammation, stress and anxiety (Nijs et al., 2018). Several works have shown that sleep and orofacial pain share a bidirectional relationship (Klasser et al., 2018; Rener-Sitar et al., 2016; Schmitter et al., 2015). Sleep disturbances are usually associated with chronic pain states, including those that occur in the orofacial region and are usually related to a state of hypervigilance (Lavigne & Sessle, 2016). In an experimental study, animals submitted to an inflammatory orofacial pain model showed a reduction in sleep efficiency and an increase in sleep latency, beside the number of awakenings (Schütz et al., 2003). Meira and Cruz et al (2019) reported in their study that 1 in each 3 people with orofacial pain had some sleep disorder (Meira e Cruz et al., 2019).

Smith et al (2009) showed that the presence of sleep disorders is associated with a reduction in the nociceptive threshold in the masseter muscle in individuals with masticatory myofascial pain (Smith et al., 2009). The reduction in muscle relaxation during sleep, characterized by increases in masticatory muscles electromyographic activity, seems to be related to mechanisms of induction and maintenance of orofacial pain (Raphael et al., 2013). Possibly, this relationship between sleep disorder and myofascial masticatory pain may be related to impaired REM sleep. Authors showed that paradoxical sleep deprivation induces inflammation, atrophy and myogenesis in the masticatory muscles of rats, through mechanisms related to an overloading of allogeneic substances production, such as TNF-alpha (Yujra et al., 2020). Sleep deprivation also causes a delay in the process of muscle regeneration, being accompanied by the presence of inflammatory infiltrate and changes in muscle fibers organization. This could explain our data concerning sleep deprivation, where a single protocol is sufficient to promote muscular hyperalgesia. In addition, other authors have shown that the recovery of sleep after deprivation is not enough to normalize muscle regeneration, suggesting that the negative effects of sleep deprivation are permanent (Mônico-Neto et al., 2017).

Studies show that sleep disturbances reported in patients with masticatory myofascial pain is better explained by psychosocial symptoms than by myofascial pain, warning that future investigations should take into account psychosocial symptoms when interpreting reports of poor sleep quality (Dubrovsky et al., 2017; Klasser et al., 2018). Almoznino et al (2017) showed that there is a reciprocal relationship between chronic orofacial pain and sleep disorders. The authors state that there is a vicious cycle where pain worsens the quality of sleep, further compromising the intensity of pain. Sleep disturbances and psychological stress symptoms are risk indicators in masticatory myofascial pain patients. Thus, stress can act as a factor that feeds the vicious cycle of pain and causes maintenance of this cycle, influencing, even in sleep disorders. Here we have a multifactorial relationship, where stress and sleep deprivation seem to feed back the vicious cycle of pain. This can contribute to the chronicity of pain that can be explained by central sensitization mechanisms (Almoznino et al., 2017; Costa et al., 2017; Lavigne & Sessle, 2016; Lei et al., 2016).

In this study we associated emotional stress and sleep deprivation to verify if both conditions together would exacerbate nociceptive behavior in the masticatory muscles. Our results showed that both models provoked hyperalgesia in masticatory muscles and the association between the two models intensified the hyperalgesia, mainly in the masseter muscle.

The expression of c-Fos, a marker of neuronal activation and neuroplasticity, is usually low under baseline conditions. During exposure to painful stimuli, there is usually an increase in the expression of this marker (Bullitt et al., 1992; Kovács, 2008; Pereira et al., 2019). Our results showed that emotional stress and sleep deprivation alone, or in association, increased expression of c-Fos the trigeminal ganglia, spinal trigeminal nucleus caudalis (Sp5c), showing the relationship between nociceptive behavioral and neuronal response in the nociceptive pathway caused by stress and sleep disturbance.

The activation of trigeminal nociceptive pathway begins by peripheral neuron stimulation, whose celular body lies on trigeminal ganglia. The first synapse of this nociceptive pathway occurs in spinal trigeminal nucleus caudalis (Sp5c), which is a sub-nucleus related to sensory information of the masseter and temporalis muscles (Dallel et al., 2003; Harper et al., 2016). In our study, the increase in c-Fos expression in TG and Sp5c shows that emotional stress, sleep deprivation and their association caused peripheral neuron activation and transmission to CNS. Subsequently, nociceptive information is conveyed to the THA. This structure has medial nuclei (related to the affective aspects of pain) and lateral nuclei (related

to the sensitive-discriminative components of pain). The activation of the trigeminal pathway causes activation mainly in the lateral nuclei, more specifically in the ventroposteromedial (VPM) and in the ventroposterolateral (VPL) nuclei (Wang et al., 2009; Wilson et al., 2008).

Interestingly, in our study, only emotional stress promoted significant increased expression of c-Fos in the thalamus. The sleep deprivation alone did not increase c-Fos in thalamus areas here investigated. This effect suggests that other thalamic areas could be involved. In fact, thalamus reticular nucleus is a key area that links sleep disorders to sensory cortical activity. As a source of NREM sleep, the reticular nucleus could be activated in sleep deprivation conditions, causing aberrant functioning of somatosensory cortex, as we see in central sensitizations conditions (Fernandez et al., 2018; Thankachan et al., 2019). The dysfunction of this thalamic area causes central mediated pain (Olivéras & Montagne-Clavel, 1994). Future investigations of specific thalamic nuclei, such as thalamic reticular nuclei, could advance in the understanding of the integration between sleep deprivation, emotional stress, motor and sensory informations.

In our study, we studied only the thalamic nuclei related to the transmission of pain in the trigeminal pathway. The thalamic reticular nucleus receives afferent input from the reticular formation and in turn projects to the other thalamic nuclei, regulating information flow through to the cortex (Mihailoff et al., 2018). The literature points out that the limbic system, under stressful conditions, causes activation of the hypothalamus which in turn, communicates with the reticular formation and activates the cranial nerves motor nuclei, such as the trigeminal nerve. Brain circuits related to the reticular formation are involved both with regulating mechanisms of sleep-wake cycle and with sensory stimuli filtering. Sleep deprivation additionally turns on the ascending activating reticular system, causing cranial nerve motor nuclei activation, inducing parafunctional muscular activity (Bourque & Kolta, 2001; Mascaro et al., 2009; Mogoseanu et al., 1993). The gamma efferent system is activated by this pathway which, in turn, stimulates the sensitive fibers of the skeletofusimotor neurons increasing muscular tone and reflex contractions (Ulrich-Lai & Herman, 2009; Wieckiewicz et al., 2014). Thus, the maintenance of the muscle contraction state seems to be related to the pathophysiology of myofascial pain, which can have a central origin (Jafri, 2014).

The chronic pain is commonly modulated by suppressor and facilitating systems, forming a descending modulatory pathway. PAG is an important center that activates the descending pain control system and is usually activated through the nociceptive pathway, which

activates the pain descending inhibitory pathway (Samineni et al., 2017). In our study, there was an increased c-Fos expression in PAG, suggesting a PAG activation in response to emotional stress and sleep deprivation. Descending pain-modulatory pathways originate from various cerebral structures involved in emotions. These regions are thought to affect nociception through their projections to several brainstem structures, including the PAG (Roy et al., 2009). Long-lasting neuroplastic changes in the PAG can persist after a single stress aversive stimulation (Brandão & Lovick, 2019). In addition, PAG also participates in sleep regulation. Studies show that the activation of neurons in PAG suppresses the initiation and maintenance of REM sleep (Weber et al., 2018). Emotional stress and sleep deprivation groups showed PAG activation, through c-Fos expression, suggesting the modulatory descending pathway to be active during these experimental conditions. Animals subjected only to sleep deprivation showed less PAG activation when compared to emotional stress group. This may have resulted in less descending modulation of pain and consequently less pain control as it was seen in mechanical hyperalgesia test. In fact, on the 14th day, pain sensitivity in emotionally stressed rats is lower than in rats subjected to sleep deprivation protocol. The lower PAG activity in the sleep deprivation group could be related to this higher and long lasting hyperalgesic effect. In addition, PAG is activated on demand in presence of stressor or injurious stimuli. The last induction of sleep deprivation was performed on the 11th day of the experimental protocol and euthanasia was performed on the 14th day. In our study, the induction of emotional stress occurred until the day of euthanasia. Thus, our experimental protocol did not allow us to evaluate the activation of PAG on the 11th day.

Pain is a symptom known to disrupt various aspects of normal physical and psychological life, including work, social activities and sleep. Studies have shown that depressive symptoms and psychological distress were predictive factors that negatively influenced the perception and the prognosis for improvement of patients with chronic orofacial pain (Penlington et al., 2020). Anxiety and depression can cause orofacial pain or orofacial pain can trigger some psychiatric disorders, such as anxiety and depression (Dıraçoğlu et al., 2016). Increased anxiety is a behavioral consequence commonly reported during sleep deprivation in humans. However, studies with rodents carried out so far have produced inconsistent results, failing to reproduce the same anxiety induced by sleep deprivation seen in clinical experiments. The use of animal models to assess the relationship between sleep deprivation lacks translational applicability (Pires et al., 2016).

Despite the inconsistencies in the literature, our results showed that animals subjected to emotional stress and sleep deprivation showed increased exploratory activity, with hyperlocomotion and an increase in the number of vertical surveys evaluated in the open field test. These results suggest that these animals have developed anxious behavior and that they are less afraid to explore new environments. It is interesting to highlight that the animals submitted to sleep deprivation had a significant increase in exploratory behavior. Conditioning by fear is an important survival mechanism, as is the ability to generalize learned responses to fear to stimuli similar to the original conditioned stimulus (Davidson et al., 2016). We understand that animals exposed to sleep deprivation developed a state of hypervigilance, as if all manipulation was to put them on the sleep deprivation platforms.

Sleep affects memory, learning, mood, behavior, immune responses, metabolism, hormone levels, the digestive process and many other physiological functions. Changes in sleep quality have been shown to produce deficits in memory consolidation and learning (Acosta, 2019). Cognitive impairment is commonly associated with pain. In an experimental study, rats submitted to an inflammatory model of orofacial pain showed impairment in spatial learning and memory (Kooshki et al., 2017). In our study, animals subjected to emotional stress and sleep deprivation showed an increase in the number of incorrect alternations in the Y-maze test, suggesting loss in working memory, in addition to the anxious and painful effects.

Stress can promote changes in adrenal glands. Studies who used chronic experimental models for stress induction showed an increase in the weight of the adrenal glands (Li et al., 2011; Ulrich-Lai et al., 2006; Ulrich-Lai & Herman, 2009). In our study, there were no differences in adrenal gland gross weight between animals submitted to emotional stress and sleep deprivation isolated, but the association of both models provoked a gain in gross adrenal weight. Sleep deprivation caused an increase in the weight of the adrenal glands relative to the animals' body weight. Although we have not investigated in this study, other authors show that chronic activation of the hypothalamic-pituitary-adrenal axis causes hyperplasia in the adrenal glands’ cortex. This trophic effect is caused by the pituitary hormone ACTH (Ulrich-Lai et al., 2006). Possibly, the sleep deprivation caused hyperplasia, which resulted in an increase in adrenal glands weight.

In addition, we found that emotional stress and sleep deprivation promoted an increase in the expression of cytoplasmic vacuoles in the adrenal gland zona fasciculata. The increase in the number of cytoplasmic vacuoles suggests an increase in the production of glucorticoids

(Nussdorfer et al., 1986; Yi et al., 1993). In our study, corticosterone measurement was not performed to check the plasmatic levels of this hormone. Thus, we limit ourselves to using an indirect measure of increased production of stress-related hormones.

Among the usual physiological responses to stress, one can mention inhibition of food intake and loss of body weight. In our study, emotional stress and sleep deprivation did not cause significant differences in weight gain, confirming the findings of other studies (Chen et al., 2010; Ishikawa et al., 1992; Rosales et al., 2002). On the other hand, some authors used protocols to induce stress for five weeks and observed decreased body weight (Qiang Li et al., 2013). Experimental models of chronic stress promote weight loss (Harris, 2015). In an analysis carried out on the last day of the experimental protocol, we found that sleep deprivation caused an increase in the animals' gross body weight.

In conclusion, this study found that sleep deprivation and emotional stress caused hyperalgesia in masticatory muscles with trigeminal nociceptive pathway involvement through c-Fos expression in various regions, including descending pain modulatory brain area. The association of these two experimental conditions exacerbated the pain sensitivity and, in addition, provoked anxiety-like behaviour, work memory deficit and changes in adrenal glands, probably related to stress-induced neuroendocrine changes.

Thus, correlating our data with common clinical findings, the results suggest that the investigation of the presence of sleep disorders and emotional stress in individuals with masticatory myofascial pain should be considered. Based on our results, sleep deprivation and emotional stress work as factors that, by themselves, can activate the trigeminal nociceptive pathway, causing pain/hyperalgesia in the masticatory muscles and that, when associated, cause exacerbation of this condition.

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4. CONCLUSÕES GERAIS

Dentro dos limites deste estudo, pode-se concluir que:

1. Privação do sono e estresse emocional provocam redução do limiar nociceptivo, ou hiperalgesia mecânica, nos músculos masseter e temporal e a associação das duas condições experimentais exacerbam esse efeito;

2. Privação do sono e estresse emocional promovem um aumento da expressão de c-Fos em gânglio trigeminal, em núcleo caudal do trigêmeo (Sp5c) e na substância cinzenta periaquedutal (PAG);

3. Estresse emocional promove um aumento da expressão de c-Fos nas regiões medial e lateral do tálamo, o que não ocorre com a privação de sono;

4. Privação do sono e estresse emocional causam aumento na atividade locomotora e exploratória e causam déficit na memória de trabalho em ratos;

5. Privação do sono e estresse emocional promoveram aumento do número de vacúolos citoplasmáticos nas glândulas adrenais em ratos e a associação das duas condições experimentais aumentaram o peso bruto das glândulas;

Dessa forma, correlacionando nossos dados com os achados clínicos comuns, os resultados sugerem que seja considerada a investigação da presença de distúrbios do sono e estresse emocional em indivíduos com dor miofascial de músculos mastigatórios. Com base nos nossos resultados, a privação do sono e estresse emocional funcionam como fatores que, por si só, podem ativar a via nociceptiva trigeminal, provocando dor/hiperalgesia dos músculos mastigatórios e que quando associados causam exacerbação dessa condição.

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ANEXO A – CERTIFICADO DE APROVAÇÃO NO COMITÊ DE ÉTICA

UNIVERSIDADE FEDERAL DO CEARÁ

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "INFLUÊNCIA DA PRIVAÇÃO DE SONO NO COMPORTAMENTO BRUXISMO-SÍMILE INDUZIDO POR ESTRESSE EMOCIONAL EM RATOS: ESTUDO MORFOFUNCIONAL DO SISTEMA DE CONTROLE MOTOR", protocolada sob o CEUA nº 8694160418 (ID 000199), sob a responsabilidade de **Mariana Lima Vale** *e equipe; Delane Viana Gondim; Bruno Wesley de Freitas Alves* - 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 - está 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, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal do Ceará (CEUA-UFC) na reunião de 26/04/2018.

We certify that the proposal "INFLUENCE OF SLEEP DEPRIVATION ON EMOTIONAL STRESS-INDUCED BRUX-LIKE BEHAVIOR IN RATS: MORPHOFUNCTIONAL STUDY OF MOTOR CONTROL SYSTEM", utilizing 120 Heterogenics rats (120 males), protocol number CEUA 8694160418 (ID 000199), under the responsibility of **Mariana Lima Vale** *and team; Delane Viana Gondim; Bruno Wesley de Freitas Alves* - 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 - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as 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 Federal University of Ceará (CEUA-UFC) in the meeting of 04/26/2018.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 06/2018 a 02/2020 Área: Departamento de Fisiologia E Farmacologia

Origem:	Biotério Central da UFC						
Espécie:	Ratos heterogênicos	sexo:	Machos	idade:	6 a 8 semanas	N:	120
Linhagem:	Wistar			Peso:	200 a 250 g		

Local do experimento: - Biotério do Núcleo de Pesquisa e Desenvolvimento de Medicamentos da Universidade Federal do Ceará; - Laboratório de Farmacologia da Inflamação e do Câncer do Núcleo de Pesquisa e Desenvolvimento de Medicamentos da Universidade Federal do Ceará;

Fortaleza, 27 de abril de 2018

Prof. Dr. Alexandre Havt Bindá
Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal do Ceará

Profa. Dra. Virginia Cláudia Carneiro Girão
Vice-Coordenadora da Comissão de Ética no Uso de Animais
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