UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

CARLOS EDUARDO DA SILVA NOSSA TUMA

INFLUÊNCIA DOS HORMÔNIOS SEXUAIS NA ANALGESIA INDUZIDA PELO ESTRESSE.

TESE DE DOUTORADO APRESENTADA A FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNICAMP PARA OBTENÇÃO DO TÍTULO DE DOUTOR EM ODONTOLOGIA, NA ÁREA DE FISIOLOGIA ORAL.

ORIENTADORA: PROFA. DRA. MARIA CECÍLIA FERRAZ DE ARRUDA VEIGA

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELO ALUNO, E ORIENTADA PELA PROFA. DRA. MARIA CECÍLIA FERRAZ DE ARRUDA VEIGA

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Assinatura do Orientador

PIRACICABA, 2011

FICHA CATALOGRÁFICA ELABORADA POR

GARDÊNIA BENOSSI – CRB8/8644 - BIBLIOTECA DA

FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNICAMP

T83i

Tuma, Carlos Eduardo da Silva Nossa, 1974-

Influência dos hormônios sexuais na analgesia induzida pelo estresse / Carlos Eduardo da Silva Nossa Tuma. -- Piracicaba, SP : [s.n.], 2011.

Orientador: Maria Cecília Ferraz de Arruda Veiga.

Tese (doutorado) - Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Estresse. 2. Articulação temporomandibular. 3. Estradiol. 4. Dor. 5. Analgesia. I. Veiga, Maria Cecília Ferraz de Arruda. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para a Biblioteca Digital

Título em Inglês: Influence of sex hormones in stress-induced analgesia
Palavras-chave em Inglês: Stress; Temporomandibular joint; Estradiol; Pain; Analgesia
Área de concentração: Fisiologia Oral
Titulação: Doutor em Odontologia

Banca examinadora:

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Data da defesa: 25-07-2011

Programa de Pós-Graduação: Odontologia

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 25 de Julho de 2011, considerou o candidato CARLOS EDUARDO DA SILVA NOSSA TUMA aprovado.

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DEDICO ESTE TRABALHO...

À minha mãe Ana Maria que aos 33 anos abdicou de quase tudo e dedicou-se a cuidar única e exclusivamente dos filhos. A senhora esteve presente em todas as etapas de minha educação e sempre apoiou de forma incondicional minhas escolhas. Se hoje sou uma pessoa melhor devo a você. Todas as palavras do mundo não conseguirão expressar minha eterna gratidão. Mãe essa conquista é nossa!

À memória de meu pai Geraldo José Tuma, que nos poucos anos de convivencia me demonstrou que carater e bondade são virtudes a serem almejadas e praticadas por toda a vida.

Aos meus sobrinhos Ananda e Pedro Henrique, amores verdadeiros e responsáveis pelos momentos de alegria mesmo após um dia inteiro de trabalho.

DEDICO ESTE TRABALHO...

À minha orientadora Maria Cecília Ferraz de Arruda Veiga por toda atenção, paciência e orientação transmitidas durante esses quatro anos.

Agradeço pelas orientações precisas nos momentos de dúvida, por desempenhar de forma brilhante e com extrema competencia o papel de mestre ajudando não só no meu desenvolvimento intelectual, mas tambem na formação do meu caráter.

A senhora é um exemplo para todos que querem seguir a carreira acadêmica pelo amor e dedicação à arte de ensinar. Sua paixão e dom inato na arte de transmitir o conhecimento de forma singular ajudaram sobremaneira na minha formação acadêmica e me fez querer ser um professor melhor. E muito mais do que isso, o exemplo de ser humano desprendido de vaidades pessoais, bondoso e de carater ilibado sempre serão referencia para que eu tente ser uma pessoa melhor.

"os professores ideais, são os que se fazem de pontes, que convidamos alunos a atravessarem edepois, tendo facilitado a travessia, desmoronam-se com prazer, encorajando-os a criarem suas próprias pontes."

Nikos Kazantzakis

AGRADECIMENTOS

À Deus pelo dom magnífico da vida.

À Universidade do Estado do Amazonas, na pessoa do magnífico Reitor Prof. Dr. José Aldemir de Oliveira pela oportunidade ímpar de proporcionar o aprimoramento de minha formação acadêmica, e desta forma qualificar o corpo docente para a melhora constante da qualidade da educação no Estado do Amazonas.

À Universidade Estadual de Campinas, na pessoa do seu Magnífico Reitor Prof. Dr. Fernando Ferreira Costa; à faculdade de Odontologia de Piracicaba na pessoa do seu diretor Prof. Dr. Jacks Jorge Junior, do coordenador geral da Pós-Graduação da FOP-UNICAMP Prof[a]. Dra. Renata Cunha Matheus Rodrigues Garcia, da coordenadora do programa de Pós-Graduação em Odontologiada FOP-UNICAMP Prof[a]. Dra. Cíntia Pereira Machado Tabchoury, por sua excelência acadêmica proporcionando uma formação de alta qualidade, me dando a oportunidade de dar importante passo na minha carreira acadêmica e científica.

À todos os professores da FOP-UNICAMP que se disponibilizaram a afastar-se momentaneamente de suas atividades acadêmicas e convívio familiar para irem a Manaus ministrar os módulos de concentração, e em especial às professoras Maria da Luz e Cíntia Pereira Machado Tabchoury.

À minha família que sempre me apoiou em todas as etapas de minha formação acadêmica, compreendendo e suportando minhas repetidas ausências.

Aos amigos do Dinter, Adelsilene, Andrea, Katllen, Lauramaris, Maria Eliana, Olavo, Tania e Vilma pelo convívio fraterno ao longo dos módulos e agradáveis momentos de descontração.

A professora Fernanda Klein Marcondes e à Rafaela Costa pela cessão do laboratório para realização dos experimentos de ansiedade, imprescindível para realização deste trabalho e pelas orientações no decorrer da pesquisa.

Aos companheiros de laboratório que sempre estiveram dispostos a auxiliar e que ajudaram a manter um ambiente de trabalho cordato e de ajuda mútua, em especial à Ana Paula que mesmo tendo que protelar um pouco seus experimentos nunca se furtou em me ajudar no manejo dos animais e em ensinar-me os procedimentos experimentais iniciais tão importantes para o desenvolvimento desta pesquisa.

Às professoras integrantes da banca examinadora de qualificação desta tese: Prof[a] Dra. Cinthia Pereira Machado Tabchoury, Prof[a] Dra. Fernanda Klein Marcondes e Prof[a] Dra. Maria Cristina Volpato, pela colaboração e sugestões fundamentais em nosso trabalho.

À Erika que esteve presente no início desta jornada e contribuiu com a formatação deste trabalho.

Ao colega Gustavo Gameiro que participou de forma irrestrita e foi de fundamental importância através de suas orientações precisas para elaboração deste trabalho.

Ao grande amigo Rodrigo Porto por todas as vezes que se prontificou em ajudar na tradução dos artigos.

Ao professor Fábio Mitsui, por toda dedicação e apoio tão necessários durante a realização do Dinter.

A todos os meus alunos que nestes 11 anos de docência foram a mola propulsora para que eu procurasse constantemente aprimorar meus conhecimentos técnico-científicos e desta forma proporcionar-lhes uma educação superior de melhor qualidade.

Ao técnico Carlos Alberto Feliciano por todo suporte técnico, paciência e essencial colaboração no manuseio e preparo dos animais.

As secretárias Eliete e Eliza sempre atenciosas e prestativas.

À FAPEAM pelo apoio financeiro para o desenvolvimento deste trabalho, na concessão da bolsa de doutorado e suporte financeiro para execução da pesquisa.

Aos ratos e outros animais utilizados nesta e em tantas outras pesquisas, que de maneira sublime contribuem para o progresso da ciência ajudando a reduzir o sofrimento humano.

"O respeito aos animais se dá a partir de atitudes éticas e tratamento digno de seres vivos e não somente mencionando-os nos resultados das pesquisas."

SBCAL – Sociedade Brasileira de Ciência de Animais de Laboratório

Meus sinceros agradecimentos.

"Se fui capaz de ver mais longe, é porque me apoiei em ombros de gigantes."

Isaac Newton

RESUMO

As disfunções temporomandibulares compreendem um grupo variável de condições que resultam em dor articular e/ou muscular e contribuem para o desenvolvimento de dores crônicas, sendo mais prevalente em mulheres em relação aos homens, principalmente durante o período reprodutivo. Envolvem os músculos da mastigação e a articulação temporomandibular (ATM), sendo geralmente associadas a processos inflamatórios, havendo ainda a participação dos hormônios gonadais nestas disfunções. O teste da formalina na ATM é um modelo animal válido para estudos de disfunções temporomandibulares. Os objetivos desta pesquisa foram avaliar 1) o efeito do estresse agudo de 15 minutos, 30 minutos e 1 hora sobre as respostas comportamentais nociceptivas após o teste da formalina na ATM de ratas em fase de estro (baixos níveis de estrógeno) e proestro (altos níveis de estrógeno) e ratos e ratas castrados com ou sem tratamento hormonal (17β-estradiol), 2) a participação do sistema opióide nas possíveis alterações nociceptivas induzidas por situações estressantes, e 3) a avaliação dos níveis sanguíneos de corticosterona e ansiedade após o estresse agudo. Os ratos foram castrados 60 dias após o nascimento, sendo o tratamento hormonal (17β-estradiol sigma® - E0756 – 5 mg em óleo mineral; s.c.; 50µg/Kg/day; as nove horas da manhã) ou a administração do veículo (óleo mineral), iniciados 21 dias após o procedimento cirúrgico, com duração de sete dias. Após o sétimo dia do tratamento hormonal ou administração do veículo, os animais foram submetidos a uma sessão de estresse agudo por imobilização durante uma hora e posteriormente: (1) mortos imediatamente para coleta de sangue e mensuração hormonal por radioimunoensaio; ou (2) submetidos ao teste do labirinto em cruz elevado para a avaliação da ansiedade; ou (3) submetidos ao teste da formalina na ATM para avaliação da nocicepção. Foi avaliado o papel do sistema opióide nas alterações nociceptivas induzidas pelo estresse, administrando-se nor-BNI- 200 µg/25µL (antagonista dos receptores kappa opioides) antes da avaliação da nocicepção. Para a análise estatística utilizou-se a análise de variância (ANOVA) seguida de testes específicos para comparação dos resultados finais, e o nível de significância estabelecido foi $p<0.05$. Todos os grupos submetidos ao estresse

agudo apresentaram aumento significativo da corticosterona plasmática. O estresse agudo induziu redução das respostas nociceptivas em fêmeas intactas na fase de proestro, efeito este que não se manifestou em ratos e em ratas ovariectomizadas. A analgesia induzida pelo estresse (SIA) foi parcialmente mediada pelo sistema opióide, uma vez que a administração de nor-BNI alterou a antinocicepção nas fêmeas, sendo mais efetiva em ratas na fase de proestro. Os ratos orquidectomizados apresentaram aumento das respostas nociceptivas (hiperalgesia), e a administração de estradiol restaurou a analgesia induzida por estresse em ratas ovariectomizadas, não afetando as respostas comportamentais nociceptivas em machos orquidectomizados. Concluiu-se que: 1) o estresse agudo causou analgesia em ratas intactas e ovariectomizadas com tratamento estrogênico; 2) O estresse agudo induziu um aumento nos níveis de ansiedade em ratas ovariectomizadas; 3) Os hormônios gonadais exercem influência significativa na nocicepção da ATM.

Palavras-chave: Estresse; Articulação temporomandibular; Formalina; Estradiol; dor; analgesia

ABSTRACT

Temporomandibular disorders comprehend a variable group of conditions which result in joint and/or muscle pain and contribute to the development of acute pains, being more prevalent in women than men, especially during the reproductive period. They involve the muscles of mastication and temporomandibular joint (ATM), being usually associated with inflammatory processes, ocurring the participation of gonadal hormones in these dysfunctions. The formalin test in the ATM is a valid animal model to study Temporomandibular dysfuntions. The aims of this research were to evaluate 1) the effect of acute stress of 15 minutes, 30 minutes and 1 hour over the nonciceptive behavioral responses after the formalin test in the ATM of female rats in estrus (low levels of estrogen) and proestrus (high levels of estrogen) phase and castrated female and male rats with or without hormonal manipulation (17β-estradiol), 2) to evaluate the role of opioid system in the possible nonciceptive alterations induced by stressful situations, and 3) the evaluation of the blood levels of corticosterone and anxiety after acute stress. The rats were were castrated 60 days after birth, being the hormonal replacement (17β-estradiol sigma® - E0756 – 5 mg in mineral oil; s.c.; 50μg/Kg/day; at nine AM) or the administration of the vehicle (mineral oil), initiated 21 days after the surgical procedure, for seven days. After the seventh day of hormonal treatment or administration of the vehicle, the animals were sumitted to a session of acute stress by restraint during one hour and afterwards: (1) were immediately killed for collection of blood and hormonal measurement by radioimmunoassay; or (2) were submitted to the elevated plus-maze for anxiety evaluation; or (3) submitted to the formalin test in the ATM for nociception evaluation. It was also evaluated the role of opioid system in nociceptive changes induced by stress, through the administration of nor-BNI- 200 μg/25μL (antagonist of the receptors kappa opioids) before the nociceptive evaluation. For statistical analysis, it was used the analysis of variance (ANOVA) followed by specific tests for the comparison of final results, the level of significance was set at $p<0.05$. All groups submitted to acute stress showed a significant increase in the plasma corticosterone. The acute stress induced the decrease of nociceptive

responses in intact females, an effect that was not expressed in orquidectomized and ovariectomized rats. The strees-induced analgesia (SIA) was partially mediated by opioid system, once the administration of nor-BNI changed the antinociception in the females, being more effective on female rats in proestrus phase. Orquidectomized male rats presented an increase of nociceptive responses (hyperalgesia), and the administration of estradiol restored the stress-induced analgesia in ovariectomized rats, not affecting the nociceptive behavioral responses in orquidectomized male rats. It has been concluded that: 1) acute stress caused analgesia in female intact rats in the proestrus phase and ovariectomized with estrogen treatment; 2) acute stress induced an increase in the levels of anxiety in ovariectomized female rats; 3) The gonadal hormones considerably influence the nociception of ATM.

Keywords: Stress; Ttemporomandibular Joint; Formalin; Estradiol; pain; analgesia

SUMÁRIO

INTRODUÇÃO

A dor é a principal razão para as pessoas procurarem os serviços de saúde. O conceito mais utilizado para definir dor é: "sensação desagradável e experiência emocional associada com atual ou possível dano tecidual, ou descrita nos termos de tal dano" (International Association for the Study of Pain, 1979). A dor é considerada uma forma de resposta somestésica onde receptores sensoriais específicos, os nociceptores, presentes na articulação temporomandibular (Aghabeigi, 1992), são sensibilizados por estímulos mecânicos, térmicos ou químicos. Além destes estímulos nocivos, modificações dos estados emocionais tanto em humanos (Barlow, Chorpita & Turovsky, 1996), quanto em animais (King *et al*., 1996), podem alterar significativamente as respostas nociceptivas.

Existem vários tipos de dores orofaciais que acometem o homem moderno, desde as dores de origem dentária até a nevralgia do trigêmeo. Dentre as condições dolorosas crônicas da região orofacial, a dor referida da ATM é uma das mais freqüentes (Adler, 1992) desordem que pode ser de origem dentária, muscular ou ainda estar associada a fatores psicossomáticos, tais como estresse e ansiedade. Uma avaliação mais detalhada das condições dolorosas da ATM demonstrou que existe uma maior prevalência de disfunções temporomandibulares (DTMs) em pacientes do gênero feminino, especialmente no período reprodutivo, em torno de 1,5 a 2 vezes maior do que em homens (Locker & Slade, 1988, LeResche, 1997; Shinal & Fillingim, 2007).

Vários autores têm demonstrado que a exposição a vários fatores estressores produz analgesia imediata em muitos testes de dor (Akil *et al*., 1976; Lewis, Cannon & Liebeskind, 1980; Calcagnetti & Holtzman, 1990; Lapo, Konarzewski & Sadowski, 2003). Pignatiello *et al*. (1989) observaram que o estresse prolongado pode inclusive causar analgesia. Contudo, em outros estudos foi verificado que em condições experimentais, o estresse crônico causou hiperalgesia em vez de analgesia. Por exemplo, ratos expostos a situações de estresse agudo e crônico por imobilização exibiram elevação e redução do período de latência do movimento rápido da cauda, respectivamente (Gamaro, 1998). Similarmente o

estresse agudo por imobilização reduziu a resposta de lamber o local de aplicação do agente nociceptor (analgesia) e aumentou a sensibilidade aos estímulos térmicos nos mesmos animais (hiperalgesia) (King *et al*., 2003). Estes resultados demonstram que os tipos de agentes estressores, intensidade, duração, bem como o modelo de nocicepção usado, influenciam não só na potência do efeito analgésico ou hiperalgésico, mas também nos mecanismos neuronais responsáveis por eles.

Um fator particularmente importante é o estado emocional induzido pelo estresse. Trabalhos recentes demonstraram que pacientes portadores de DTMs apresentaram aumento do estresse, depressão, ansiedade e somatização quando comparados com o grupo controle de pacientes saudáveis (Gatchel *et al*., 1996; Jones, Rollman & Brooke, 1997). Contudo os mecanismos responsáveis pela mudança das respostas nociceptivas induzidas pelo estresse moduladas por fatores psicológicos nas DTMs ainda não foram totalmente estabelecidos.

O teste da formalina vem sendo utilizado para avaliar o efeito do estresse sobre as respostas nociceptivas em diversos modelos experimentais com animais, tais como o estresse da natação em ratos (Carmody & Cooper, 1987; Vaccarino *et al*., 1992) e exposição do odor de gatos a ratos (Lester & Fanselow, 1985). Muitos destes modelos de mensuração da resposta nociceptiva foram obtidos através de estímulos superficiais, como o tail-flick (Gamaro *et al*., 1998), hot-plate (King *et al*., 2003) e injeção de formalina na pata (Aloisi, Ceccarelli & Lupo, 1998). Contudo existem diferenças entre as condições de dores profundas daquelas obtidas através de estímulos superficiais (Mense, 1986; Sessle & Hu, 1990). Muitas das dores orofaciais profundas, tais como as DTMs, são associadas a manifestações de dores expansivas e reflexas (Sessle, 2002). Contudo, os mecanismos de resposta nociceptiva ainda são pouco compreendidos, em parte por existirem poucos modelos experimentais para investigar esta condição (Roveroni *et al*., 2001).

A formalina é comumente utilizada em experimentos comportamentais com animais. O teste da formalina foi descrito originalmente por Dubuisson & Dennis em 1977 e consistia na injeção subcutânea de formalina na pata traseira de ratos produzindo uma

resposta nociceptiva bifásica sensível a várias classes de drogas analgésicas (Hunskaar & Hole, 1987; Coderre *et al*., 1990; Rosland *et al*., 1990; Taylor *et al*., 1995). O teste da formalina foi adaptado por Clavelou *et al*. (1989) para os tecidos superficiais da região orofacial. Neste teste a formalina era injetada no lábio superior de ratos, produzindo também uma resposta nociceptiva bifásica, além do animal apresentar o comportamento de coçar a região da aplicação do nociceptor.

Em trabalhos anteriores, onde foi estudado o efeito do estresse por imobilização sobre as respostas nociceptivas induzidas pelo teste da formalina na ATM, verificaram que o estresse agudo (1 hora) e crônico (40 dias – 1 hora) aumentaram os níveis sanguíneos de corticosterona (Gameiro *et al*., 2005), e que o estresse agudo (15 minutos, 30 minutos, 1 hora), sub-crônico (3 dias – 1 hora) e crônico (40 dias – 1 hora), aumentaram os níveis de ansiedade dos animais, confirmado pelo tempo de menor permanência nos braços abertos no labirinto de cruz elevado, quando comparados, ao grupo controle (Gameiro *et al*., 2006). Em ratos machos, apesar dos diversos protocolos de estresse (agudo, sub-crônico e crônico) alterarem significativamente os níveis hormonais e de ansiedade, apenas os animais estressados cronicamente apresentaram aumento da reposta nociceptiva (hiperalgesia) ao serem submetidos ao teste da formalina na ATM, além disso, no grupo de animais cronicamente estressados observou-se redução no efeito analgésico da morfina, demonstrando alteração do sistema opióide (Gameiro *et al*., 2005 e 2006), já os machos submetidos ao estresse agudo não apresentaram analgesia. Fischer *et al*., 2008 demonstraram que fêmeas em estro (com baixos níveis hormonais) eram mais sensíveis ao teste da formalina na ATM do que machos, e fêmeas em proestro (com altos níveis hormonais).

Ainda não está bem elucidada a participação dos hormônios sexuais na modulação das respostas nociceptivas diante de situações de estresse dos diversos processos dolorosos da ATM. Estudos eletrofisiológicos demonstraram que a ativação de receptores estrogênicos de membrana diminui a atividade da fibra nociceptiva primária pela modulação de canais iônicos (Lee *et al.* 2002; Claban & Micevych, 2005) o que sugere um

efeito antinociceptivo periférico do estrógeno. Desta forma, pode ser de relevância avaliar as prováveis diferenças entre os sexos na modulação da nocicepção induzida pelo estresse.

PROPOSIÇÃO

- Verificar o efeito do estresse agudo de 1 hora sobre as respostas comportamentais nociceptivas induzidas pelo teste da formalina na ATM de ratas na fase de estro e de proestro, de ratos orquidectomizados e de ratas ovariectomizadas.

- Avaliar a participação do sistema opióide nas possíveis alterações nociceptivas induzidas por situações estressantes.

- Avaliar a relação entre o protocolo de estresse agudo, níveis de ansiedade, e os níveis sanguíneos de corticosterona em machos e fêmeas castrados.

- Comparar as respostas comportamentais nociceptivas, níveis de ansiedade e concentrações sanguíneas de corticosterona entre ratos e ratas castrados com e sem reposição estrogênica.

O presente estudo foi realizado em formato alternativo, conforme deliberação da Comissão Central de Pós-graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP) nº 001/98.

CAPÍTULO 1

The effects of acute restraint stress on nociceptive responses evoked by the injection of formalin in the TMJ of female rats

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Artigo publicado no periodico *Stress*

ABSTRACT

The temporomandibular joint (TMJ) formalin test was used to evaluate the effects of acute restraint stress on the nociceptive behavioral responses of female rats during proestrus and estrus phases of the estrous cycle. Animals were subjected to one session of restraint stress

(15 min, 30 min or 1 h). They were then either immediately killed to allow the collection of blood for hormonal radioimmunoassay determinations, or subjected to the TMJ formalin test to evaluate nociception. All stress protocols significantly raised the levels of corticosterone. The performances of rats subjected to 15 and 30 min of restraint were similar to those of control animals, whereas rats that were stressed for 1 h showed a decrease in nociceptive responses, both during proestrus and estrus phases. The stress-induced analgesia was higher in the proestrus phase. To evaluate the role of kappa-opiate receptors, the selective receptor κ-opioid antagonist nor-BNI (200 µg or saline) was injected into the TMJ 24 hours prior to the 1 h stress period and the TMJ formalin test. The local administration of nor-BNI partially reversed the stress-induced analgesia during the proestrus phase. These findings suggest that 1) acute stress for 1 h can produce analgesia both during proestrus and estrus phases; this effect is greater during the proestrus phase; 2) κ-opioid receptor activation is involved in the stress-induced analgesia observed in the proestrus phase.

Keywords: Analgesia; Estrous cycle; Formalin test; Rats; Stress; Temporomandibular joint

INTRODUCTION

Temporomandibular dysfunctions are pain conditions of the masticatory muscles and temporomandibular joint (TMJ) (Denucci et al. 1996, Dworkin and LeResche 1992) in which spreading of the pain to adjacent regions of the head and neck is a frequent occurrence (Bereiter 2001). The prevalence, severity, and duration of these conditions are greater in women than in men (Fischer et al. 2007, LeResche 1997). This disparity between the genders might be attributed to a pronociceptive effect of ovarian hormones on TMJ pain modulation (Cairns et al. 2001, Craft et al. 2004, LeResche 1997). Using rats it was previously demonstrated that, in response to the injection of formalin into the TMJ, a significantly lower behavioral nociceptive response was obtained in males than in females (Clemente et al. 2004).

One possible explanation for the sexual dimorphism in the TMJ nociceptive responses is related to specific opioid mechanisms. As previously described, functional kappa-opioid receptors are located within the TMJ of rats. Although activation of these receptors suppresses formalin-induced TMJ nociceptive behavior in both males and females, the suppression is significantly higher in females (Clemente et al. 2004). When κ-opioid receptor agonists are used, the analgesic system in women has been found to be more sensitive than in men (Arthuri et al. 2005). It is possible that a male-related hormone, such as testosterone, interacts negatively with κ-opioid agonists; testosterone is present in both sexes, although the circulating testosterone levels in female subjects are typically about

10% of those observed in male subjects (Fischer et al. 2007). Alternatively, female-related hormones, such as progesterone or estrogen, may potentiate the action of κ-opioid (Gear et al. 1996).

Recently, it was demonstrated in rats that female sex hormones can modulate the function of the adrenal medulla; this resulted in a sexually dimorphic response in both the baseline mechanical nociceptive threshold and in epinephrine-induced hyperalgesia (Khasar et al. 2005). Considering that alterations in the activity of the endogenous opioid system underlie both the mechanisms that regulate the stress-induced changes in nociception (Amit and Galina 1988, Gear et al. 1996, Przewlocki et al. 1987, Yamada and Nabeshima 1995) and the hyperalgesia that is produced in rats in response to repeated (chronic) stress (Gameiro et al. 2005), we hypothesized that the differential activation of stress systems in male and female rats could be involved in the sexually dimorphic modulation of TMJ nociception.

Chronic generalized pain, which is characterized by a diffuse lowered pain threshold (such as fibromyalgia and irritable bowel syndromes), disproportionately affects women (Buskila 2001, Yunus 2002). In many cases, stress may precede or be comorbid with symptoms of generalized pain syndromes (Davis et al. 2001, Raphael et al. 2004). However, despite the fact that TMJ is more prevalent in women (LeResche 1997), little is known about the effect of female sex hormones in the orofacial region and the possible involvement of stress activation on TMJ nociception. As the nociceptive behavioral responses that are elicited by the injection of formalin into the TMJ are a valid and reliable model of deep orofacial pain (Roveroni et al. 2001), this study aimed to evaluate the effects of acute restraint stress on

the nociceptive behavioral responses induced by the TMJ formalin test in female rats during both the estrus and proestrus phases.

METHODS

1. Animals

This study was carried out in 3-month-old female rats obtained from Centro Multidisciplinar para Investigação Biológica (CEMIB), UNICAMP, Brazil. The rats were housed in groups of five and maintained in a temperature-controlled room (23 ± 1°C) with a 12/12 h light–dark cycle (lights on at 6:00 am), and food and water were available ad libitum. Animals were handled for at least 1 week prior to the experiments. This research was approved by the Committee on Animal Research of the University of Campinas (protocol 938-1) and conformed to IASP (International Association for the Study of Pain) guidelines for the study of pain in animals (Zimmermann 1983). Procedures were performed between 8:00 am and 1:00 pm.

2. Estrous Phase Determination

Proestrus and estrus phases were determined by daily microscopic examination of vaginal smears that were taken by gentle lavage between 8:00 and 10:00 am. Proestrus was identified by the predominance (>70%) of nucleated epithelial cells, and the occurrence of estrus was marked by the presence of anucleated cornified cells in rats with at least two consecutive regular 4-day cycles (Marcondes et al. 2002, Smith et al. 1975). These phases

were chosen because they represent phases of both high and low ovarian hormone levels (Butcher et al. 1974).

3. Stress Exposure

All animals were stressed by restraint for periods of 15 min, 30 min or 1 h for each exposure (Gameiro et al. 2006). Restraint was carried out by placing the animal in a plastic restraint device (adjustable in size depending on the animal's weight). The area of the tube could be adjusted individually and the tube was held firmly in place with adhesive tape. There was a 1 cm hole in the far end to allow breathing. The control group was not submitted to immobilization. The immobilization procedure was carried out in a separate quiet room between 9:00 and 11:00 am.

4. Hormonal Assay

The blood of proestrus and estrus female rats was collected after decapitation, which occurred either under basal conditions (within 30 s of removal from the home cage; also referred to as the 0 min time point) or at the end of each period of restraint (within 30 s of removal from the restrainer). Following decapitation, the blood was collected in tubes containing heparin, and then centrifuged at 2500 rpm, 4ºC for 10 min. The plasma was then collected and frozen at -20°C until use. Plasma corticosterone levels were determined by

radioimmunoassay (Anti-Corticosterone – C-8784, SIGMA) after plasma extraction with ethanol, as previously described (Castro et al. 1995).

5. Testing Procedure for TMJ Pain

The design of this study follows that used by (Roveroni et al. 2001). Testing sessions took place between 10:00 am and 12:00 am. in a quiet room maintained at 23 ± 1°C. Immediately after the period of stress, each animal was briefly anesthetized by inhaled halothane to allow the TMJ injection. The formalin solution was prepared from a commercially available (SIGMA) formalin stock (an aqueous solution of 37% formaldehyde). This solution was further diluted in 0.9% NaCl (saline) to a concentration of 1.5%. Rats received a 50 μl injection of formalin solution into the left TMJ region. The injections were performed via a 30-gauge needle introduced into the TMJ capsule. A cannula consisting of a polyethylene tube (30 cm) was connected to the needle and also to a Hamilton syringe (50 μl) that had previously been filled with formalin (1.5%). Following the TMJ injection, the rat was placed in a test chamber (30 x 30 x 30 cm mirrored-wood chamber with glass at the front side) and nociceptive behavioral responses, characterized by rubbing the orofacial region (amount of time in seconds) and flinching the head (number of head flinches), were quantified for 30 min (10 blocks of 3 min). Considering that the flinching of the head behavior followed a uniform pattern of 1 s in duration, each flinching was expressed as 1 s. The combination (sum) of both behaviors provides a better measure of pain intensity than any single behavior (Gameiro et al. 2003, Roveroni et al. 2001). To

confirm the TMJ injection site at postmortem, the animals were anesthetized at the end of each experiment and Evans blue dye (0.1%, 5 mg/ kg; SIGMA) was injected systemically (intracardially) as previously described (Haas et al. 1992). The formalin-induced plasma extravasation of the Evans blue dye bond to the plasma protein was then examined visually.

6. Assessing the role of the Opioid System on Stress-Induced Nociception

In the case of stressed animals that exhibited a significant reduction in nociception that indicated analgesia, the sensitivity of a specific opioid antagonist to the kappa receptor was tested. The kappa opioid antagonist nor-binaltorphimine (nor-BNI) (Binder et al. 2001) (200 μg/ 25 μl; SIGMA) was dissolved in saline. Because it has been reported that nor-BNI may not be selective for kappa opioid receptors until several hours after its administration (Schmidt et al. 2002), nor-BNI was administered into the left TMJ region one day (24 hours) prior to the experiment.

7. Statistical Analysis

Statistical analysis of the plasmatic corticosterone data was made using a two-way ANOVA based on Ranks. The sum of rubbing and flinching responses exhibited by each animal was calculated. The data were analyzed by a two-way ANOVA on Ranks followed by either Tukey or Student–Newman–Keuls post-hoc tests, as appropriate. All values are given as mean ± standard error of the mean (SEM). A level of 5% was taken as evidence of

statistical significance. All statistical analyses were performed using SIGMA STAT version 3.0 for Windows – licensed to the University of Campinas.

RESULTS

1. Effects of the Stress Procedures on Plasmatic Corticosterone

This experiment was carried out to define the efficacy of different acute restraint protocols (15 min, 30 min and 1 h) at inducing stress-like hormonal modifications. The corticosterone levels in female rats during proestrus and estrus phases of the estrous cycle are shown in Fig 1. Compared to the controls, there was a significant increase in the levels of plasmatic corticosterone after administration of the various stress protocols ($p < 0.001$, Student–Newman–Keuls). There was not a significant difference in the levels of corticosterone at the different phases of the estrous cycle ($p = 0.689$, two-way ANOVA).

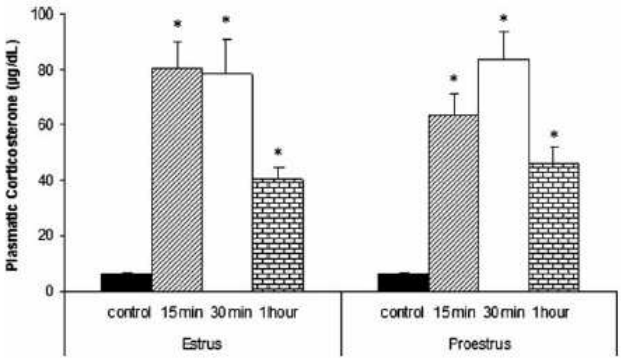

Fig 1. Basal and stress-induced plasmatic corticosterone levels after the 15 min, 30 min and 1 h stress procedures in the proestrus and estrus phases of the estrus cycle. Each column represents the mean, and error bars indicate the standard error of the mean (SEM). Number of animals was set as n = 8/group. (*) Indicates a significant difference when compared to control ($p < 0.001$, Student–Newman–Keuls).

2. Effect of Acute Stress on Nociceptive Behavioral Responses

Exposure to a single restraint session for either 15 min or 30 min did not affect the nociceptive responses evoked by injection of formalin (1.5%) into the TMJ of rats (Fig. 2). However, there was a significant difference between estrus (146.50 ± 6.4) *vs* proestrus (116.06 ± 2.0) in control (unstressed) rats ($p < 0.001$, two-way ANOVA).

Exposure to a single restraint session for a period of 1 h did have an affect on the nociceptive responses. An analgesic effect (i.e. a significant decrease in the nociceptive behavioral responses) was observed in the 1 h-stressed animals when compared to the control animals (Fig. 2). The nociceptive responses of the stressed (1 h) proestrus group (54.44 ± 7.7) were significantly lower than those of the estrus group (102.10 ± 9.4) ($F = 34.96$; $p < 0.001$, two-way ANOVA).

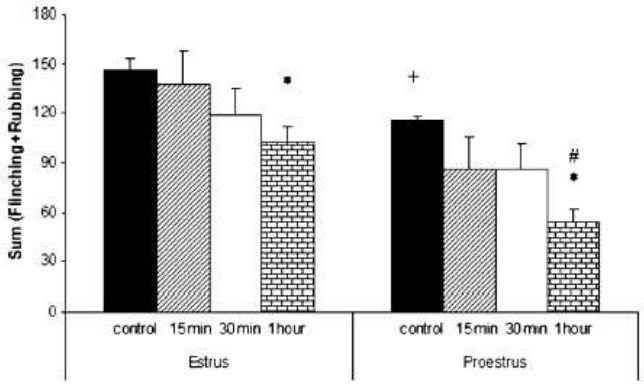

Fig. 2. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50 µl, 1.5%) previously submitted to 15 min, 30 min or 1 h of restraint (n = 6/group) or left undisturbed in their home cage (n = 6/group) during the estrus and proestrus phases. Each column represents the mean. Error bars indicate the SEM. (+) Significant difference between estrus vs proestrus in control rats. (*) Significant difference between the controls and stressed groups. ($^{\#}$) Significant difference at different phases (estrus and proestrus) in the stressed group ($p < 0.001$, two-way ANOVA).

3. Effect of Nor-BNI on Nociception in Rats Subjected to Acute Restraint Stress

The local pre-administration (24 h before the stress and TMJ formalin test) of nor-BNI partially reversed the stress-induced analgesia in proestrus females ($p < 0.009$, Tukey test). This effect was not observed in estrus females (Fig. 3).

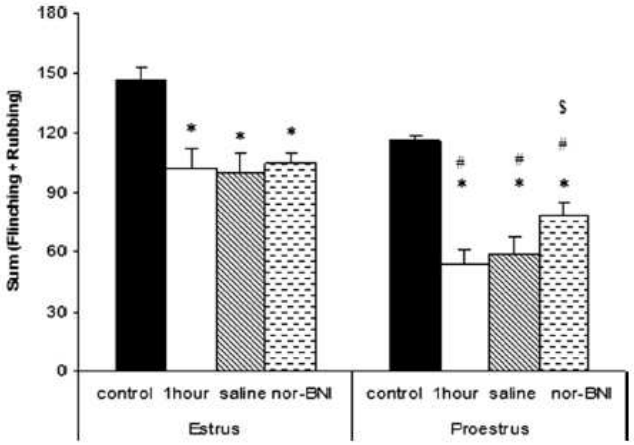

Fig. 3. Effects of control, stressed (1 h), saline (+ 1 h) and nor-BNI (+1 h) on formalin-treated animals (50 µl, 1.5%) in estrus and proestrus phases (n = 6/group). Each column represents the mean. Error bars indicate the SEM. (*) Indicates significant difference when compared to controls ($F = 15.42$; $p < 0.001$, two-way ANOVA). ($^{\#}$) Indicates significant difference when compared to different phases (estrus and proestrus) ($p < 0.001$, Tukey test). ($^{\$}$) Indicates significant difference when compared to stressed groups (nor-BNI and saline vs 1 h) ($p < 0.009$, Tukey test).

4. Effect of Nor-BNI on Nociception in Non-Stressed Rats

The occurrence of the proestrus phase affected the formalin-evoked (1.5%) nociceptive responses in rats that were not submitted to restraint stress. The decrease in the sum of nociceptive behaviors (flinching + rubbing) was statistically significant ($F = 6.91$; $p < 0.001$, two-way ANOVA, Fig. 4) when the proestrus females were compared to the estrus

females. However, the local pre-administration of nor-BNI did not affect the nociceptive responses in either estrus or proestrus females (Fig.4).

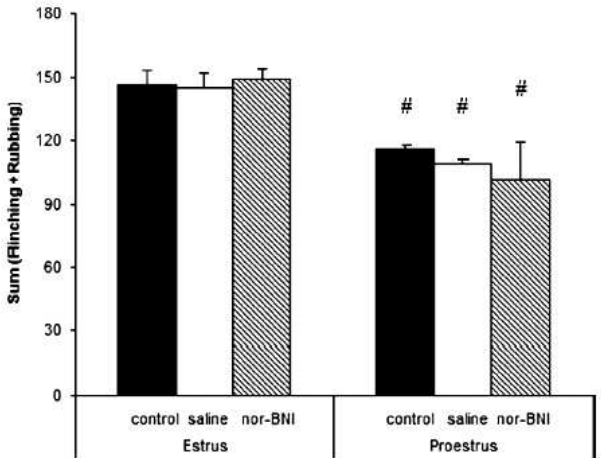

Fig. 4. Effects of nor-BNI and the control saline group on unstressed formalin-treated animals (50 µl, 1.5%) in estrus and proestrus phases (n = 6/group). Each column represents the mean. Error bars indicate the SEM. ($^{\#}$) Indicates significant difference compared with the estrus groups ($p < 0.001$, two-way ANOVA).

DISCUSSION

Numerous animal studies have demonstrated that pain-like behavior evoked by cutaneous (Martinez-Gomez et al. 1994), deep, or visceral tissue stimulation (Giamberardino et al. 1997, Kayser et al. 1996, Ness et al. 2001) varies throughout the estrous cycle. LeResche et al. (2003), using a human clinical assay, suggested that temporomandibular pain in women is highest at times of lowest estrogen. During the estrous cycle in animals, prolactin, LH and FSH remain low but increase throughout the afternoon of the proestrus phase. Estradiol levels begin to increase at metestrus, reaching peak levels during proestrus and returning to baseline at estrus. The secretion of progesterone also increases during metestrus and diestrus, but decreases afterwards. The progesterone levels then rise to reach a second peak towards the end of proestrus (Marcondes et al. 2002, Spornitz et al. 1999). Although studies in non–humans generally support the view that gonadal hormones influence nociception in females, the factors involved in mediating this effect are not well understood (Terner et al. 2005). Moreover, there are no experimental studies that examine the effects of stress on the modulation of nociceptive input from articular tissue in female rats during the estrus and proestrus phases.

A variety of environmental and/or stressful stimuli have been shown to elicit analgesia – a phenomenon often referred to as stress-induced analgesia (SIA) (Furuta et al. 2003, Gameiro et al. 2005, King et al. 2007, Watkins et al. 1982). For example, some evidence suggests that female rats are most sensitive to thermal nociceptive stimuli in proestrus

(Kayser et al. 1996, Vincler et al. 2001) when estrogen and progesterone levels peak. In contrast, others suggest that peak sensitivity occurs during estrus (Kayser et al. 1996, Martinez-Gomez et al. 1994, Stoffel et al. 2003) when estrogen and progesterone levels are relatively low. These discrepancies might, in part be related to the type of nociceptive stimulus used in the experiments. For example, lower nociceptive thresholds are typically observed in response to mechanical and electrical nociceptive stimuli during estrus (Kayser et al. 1996), whereas no differences in thresholds are seen across phases in response to chemical stimuli (Vincler et al. 2001) and higher thresholds are observed with visceral stimuli during estrus and proestrus (Bradshaw et al. 1999, Terner et al. 2005). In the present study, a single exposure (1 h) to restraint stress reduced the nociceptive behavioral responses evoked by nociceptive chemical stimulation (formalin 1.5%) of the female rat's TMJ in both estrus and proestrus phases; however, the stress-induced analgesia was higher in the proestrus phase. This finding is in accordance with those obtained in another study (Ryan and Maier 1988), in which female rats with high hormonal levels exhibited higher stress-induced analgesia in the tailshocks test.

The evidence which indicated that gonadal hormones influence pain sensitivity, came from studies that demonstrated a trend for higher pain thresholds and tolerance levels during the follicular phase in which estrogen levels peak (Riley et al. 1999, Terner et al. 2005). These results support the hypothesis suggested by other authors that elevated hormone levels are responsible for reduced pain sensitivity (Arthuri et al. 2005, Gupta et al. 2001, Liu and Gintzler 2000, Medina et al. 1993, Tall and Crisp 2004). The higher susceptibility to pain behavior in the present study was also observed in female rats with lower hormonal levels.

In the present study, the relationship between hormonal variations and nociceptive responses induced by stress was evaluated. A significant increase in plasma corticosterone levels was observed after acute (15 min, 30 min, 1 h) restraint stress sessions, although only the 1 h stress period was able to alter the nociception evoked by the TMJ formalin test. It is well established that not only the type of stressor, its intensity, and duration affects the stress-induced changes to pain modulation, but also the type of nociceptive model used has an effect as well (Gameiro et al. 2006). We have already demonstrated that restraint stress can release endogenous opioids (Gameiro et al. 2005), but this effect was not capable of inducing analgesia in male rats that were submitted to acute restraint stress before the TMJ formalin test. As female rats (in both estrus and proestrus phases) exhibited decreased nociceptive responses to the TMJ formalin test and this analgesic effect was higher in the proestrus females, the present results suggest that female sex hormones can modulate stress-induced analgesia. We suggest that female rats are more prone to exhibit stress-induced changes that are both hormonal and behavioral. The finding of higher levels of corticosterone levels after the stress procedures, when compared with those that we previously found in males, also supports this hypothesis.

Stress and opioid agents act throughout the neuraxis to modulate sensory, motor, autonomic, motivational and emotional responses to nociceptive stimulation (Hebb et al. 2005, Houshyar et al. 2001, King et al. 2007). Studies examining the involvement of exogenous opioids in the peripheral modulation of TMJ pain (Bakke et al. 1998, Cai et al. 2001), support the presence of peripheral opioid receptors in TMJ that may have a role in modulating nociceptive responses (Arthuri et al. 2005, Clemente et al. 2004). In normal

cycling females, morphine and buprenorphine (opioid agonist) were generally most potent in metestrus and proestrus and least potent in estrus (Terner et al. 2005). In the last experiment, we tested the nociceptive responses in control and single restrained (1 h) rats that were previously injected with nor-BNI (200 μg/ 25 μl) (opioid antagonist) in the TMJ formalin test. Our results demonstrated that the local pre-administration of nor-BNI reduced the stress-induced analgesia in proestrus females. The effect of nor-BNI in the estrus females was not significant; this indicated that higher hormonal levels can increase stress-induced analgesia via opioid mechanisms. Although the induction of hyperalgesia after several hours of nor-BNI administration has been reported (Schmidt et al. 2002), this finding was not observed in our study as the application of nor-BNI to unstressed rats did not evoke a hyperalgesic effect. This result is also supported by the findings of Arthuri et al., 2005.

The present data indicate that κ-receptors are in part involved in mediating stress-induced analgesia in female rats. The administration of nor-BNI before formalin to unstressed rats did not alter the nociceptive responses to the TMJ formalin test; this suggested that the κ-opioid antagonist selectivity of nor-BNI observed in the present results was related to the gonadal hormones.

In conclusion, acute restraint stress (1 h) could produce analgesia in proestrus and estrus female rats, but this effect was higher in the proestrus phase. Moreover, this study demonstrated that κ-opioid receptor activation is involved in the stress-induced analgesia that is observed during the proestrus phase. The data presented in this study could be of clinical value and might be important for understanding the neurobiological mechanisms

concerning temporomandibular disorders and the relationship among sex hormones, nociception, and stress.

ACKNOWLEDGMENTS

The authors thank Carlos Alberto Feliciano for technical assistance and Professor Margaret de Castro (FMRP-USP) for radioimmunoassay assistance. This work was supported by CNPq, Brazil.

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CAPÍTULO 2

Article

The influence of sex hormones on nociceptive responses evoked by the injection of formalin in the temporomandibular joint of stressed rats

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Artigo submetido à revista *Stress*

Abstract

The temporomandibular joint disorders (TMD) are characterized by pain in the masticatory muscles and temporomandibular joint (TMJ). These conditions are clearly affected by psychological stress and estrogen status. However, the biological basis for this relationship

is still unclear. The present study used the TMJ formalin test to evaluate the behavioral responses in castrated male and female rats, with and without estrogen replacement, after a session of restraint stress (1 h). The female rats exhibited lower nociceptive responses when compared to males, both in the control and stressed groups. The ovariectomized (OVX) female groups and orquidectomized (ORQ) male groups were hiperalgesic when compared to their respective SHAM groups. There was a reduction in the nociceptive responses in stressed SHAM females that received mineral oil and estrogen, but this stress-induced analgesia (SIA) was absent in the OVX females. In the OVX female rats, the estrogen replacement reduced the nociceptive responses in both control and stressed groups, and this antinociceptive effect was higher in the stressed group. The administration of estrogen did not affect the nociceptive responses of both control and stressed ORQ male rats. These findings suggest that gonadal hormones exert significant influences on TMJ nociception by both organizational and activational mechanisms; the former are exemplified by the antinociception action of estrogen and on its ability to evoke SIA only in females. The later are evidenced by the protective role of testosterone in male rats.

Keywords: Analgesia, estrogen, gonadectomy, temporomandibular disorders, temporomandibular joint, restraint stress.

Introduction

Pain is an extremely unpleasant and important sensation to man survival. It is caused by potentially harmful stimuli which cause reflex mechanisms of protection for the organism (Pazo 2003), being the main reason why patients search for medical or odontological care (Aghabeigi 1992; Shinal and Fillingim 2007). In the craniofacial region, some of the most common acute and chronic pain conditions occur in the face and mouth, such as, toothaches, migraine, headaches and temporomandibular disorders [TMD] (Aghabeigi 1992; Sessle 2001). The term TMD is used to describe a group of musculoskeletal conditions occurring in the temporomandibular region (LeResche 1997) and they are painful conditions characterized by inflammatory processes which may be caused by oclusal problems, hyperactivity and/or muscle parafunction and inflammatory processes of the temporomandibular joint (TMJ) or associated to psychosocial features, such as disability, depression and somatization (Locker and Slade 1988; Dworkin and LeResche 1992; Denucci et al. 1996). The biological psychosocial factors are more common in women than in men. Several lab and clinical researches suggest there may be sexual differences in the pain modulation. Despite contradictory results, most results point to increasing pain sensitivity in women compared to men. For instance, females exhibit greater sensitivity to experimentally-induced pain than males (Fillingim et al. 1999; Kuns et al. 2006) both in experimental studies as well as in clinical ones. Several factors influence on the difference of pain perception between sexes, such as biological (hormonal), social (developmental), and psychological (emotion) (Kuns et al. 2006).

Multiple neural mechanisms are involved in endogenous pain modulation and specific sexual aspects contribute to a greater sensitivity and higher prevalence of chronic pain in the female sex (Quiton and Greenspan 2007). The higher prevalence and severity of TMD in women may be linked to the presence and fluctuation of gonadal hormones (Gaumond et al. 2005; Fischer et al. 2008), which makes sense as there are connection sites for gonadal hormones widely distributed through areas of the central nervous system (CNS) involved in the transmission and inhibition of the pain (Gaumond et al. 2005) and the organizational effects which occur during the embryonic development may induce differences between

males and females in the CNS once the synaptic organization in the ventromedial hypothalamic nucleus differs between the sexes, and it is also known that the estrogens affect the anatomy and physiology of the rodent hippocampus (McEwen and Alves 1999; Aloisi and Bonifazi 2006).

It has been long known that stress may produce several hormonal and behavioral changes on individuals of both sexes (Aloisi et al. 1998; Gamaro et al. 1998; Torres et al. 2003). There are some evidences indicating that stress elicits antinociceptive effects, a phenomenon often referred to as stress-induced analgesia (SIA) (Lewis et al. 1980; Vacarino and Kastin 2001; King et al. 2003). The gonadal hormones are relevant modulators of the SIA. We have previously shown that female rats submitted to acute restraint stress for 1 hour presented a reduction in the nociceptive responses after the injection of formalin into the TMJ. This SIA was observed both in the proestrus and estrus phases, but the analgesic effect was higher in the proestrus phase (Botelho et al. 2010).

Considering that male rats did not exhibit a SIA in the TMJ formalin test (Gameiro et al. 2005, 2006), we hypothesized that the sex and the ovarian hormones play an important role in the differential activation of stress systems and in the sexually dimorphic modulation of TMJ nociception. Therefore, the aims of the current study were to evaluate the influence of sex, castration, and estrogen replacement on rats submitted to restraint stress and to the TMJ formalin test.

Methods

1. Animals

For the current study, 120 Wistar rats with 150 to 250 g of both genders (males and females in the proestrus phase), from Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório-CEMIB and kept in the biotherium of the Piracicaba Dental School, were used. The rats were held in plastic cages (5 per cage) containing wood shavings, in environment with luminosity control (clear /dark each 12hs; lights on at 6:00 am), humidity and temperature (23 ± 2^0C). They were fed with industrialized food and *ad libitum* water. The experiments were performed during the clear cycles between 09:00 am to 5:00 pm under silent conditions. All experimental procedures were previously submitted to the Ethics Committee for animal research of Universidade Estadual de Campinas (protocol 1555-1), and followed the guidelines proposed by the Ethics Committee for Research of the International Association for the Study of Pain in Conscious Animals (Zimmermann 1983).

2. Estrous Phase Determination

Proestrus and estrus phases were determined by daily microscopic examination of vaginal smears that were taken by gentle lavage between 8:00 and 10:00 am. Proestrus was identified by the predominance (>70%) of nucleated epithelial cells, and the occurrence of estrus was marked by the presence of anucleated cornified cells in rats with at least two consecutive regular 4-day cycles (Marcondes et al. 2002, Smith et al. 1975). These phases were chosen because they represent phases of both high and low ovarian hormone levels (Butcher et al. 1974).

3. Orchidectomy and Ovariectomy

The orchidectomy and ovariectomy (Gordon and Soliman 1994) were done in male and female rats at 60 days of age. The procedures were performed under induced anesthesia by intramuscular injection of ketamine (55mg/Kg) and xylazine (5.5mg/Kg). A subcutaneous injection of ketoprofen (5mg/Kg) was used for post-surgery analgesia (Roughan and Flecknell 2000). Sham operated animals were used as castration controls. The efficiency of the orchidectomy and ovariectomy was confirmed through the evaluation of the atrophy of the seminal vesicles/ prostrate and womb respectively, in the animals which have not received hormonal manipulation.

4. Hormonal manipulation

After castration procedures or surgical procedure (Sham), the hormonal treatment was performed by daily injection of 17β-estradiol (sigma® - E0756 – 5 mg) (in mineral oil; s.c.; 50μg/Kg/day; at 9:00 am) (Gordon and Soliman 1994) during seven days. Mineral oil (0.15 – 0.2 mL/day; at 9:00 am; was used for the control groups. The rats were castrated at the age of 60 days, and the hormonal replacement or the application of mineral oil started 21 days after the surgical procedure. On the seventh day, the hormonal injection with 17β-estradiol or mineral oil was done 1 hour before the respective experiments.

5. Acute stress protocol

The male and female rats were submitted to the acute stress protocol through immobilization during 1 hour (Gameiro et al., 2006; Botelho et al., 2010). The animal was put in a plastic tube ($25x7cm^2$) allowing adjustments to the size of the animal. In one end of the tube, it was made a 1-cm hole to favor the animal breathing during the experiment. In the control group, the animals were not submitted to stress sessions. The immobilization procedures were held between 10:00 am to 12:00 pm in a silent environment.

6. Testing procedure for TMJ pain

The formalin application sessions were held in day period, between 07:00 am to 12:00 pm in silent room with constant environment temperature of 23±2°C (Rosland, 1991). For the behavioral evaluation, an observation chamber was used (30 cm x 30cm x 30 cm) with mirror sides and base. Each animal was initially placed in a glass container (10 X 10 X 18 cm) containing one cotton ball soaked with 5 ml of halothane (CRISTÁLIA) until the rat falls over (takes around 10 sec). After anesthesia, 50 µl of formalin was used at 1.5% in the left TMJ with a 30G needle 30 connected to a Hamilton syringe (50 µl) through a P_{50} polyethylene tube. Right after the injection, the animal was placed in the observation chamber and the nociceptive behavioral responses characterized by the acts of itching the orofacial region (time in seconds) and quickly raising the head (number of movements) were quantified for 30 minutes (10 blocks of 3 minutes) assisted by a chronometer and a cell counter, respectively (Roveroni et al. 2001). The nociceptive behaviors were analyzed along in group by the addition of the period of time the animals presented the behavior of itching orofacial region (CO) plus the number of times they quickly raised the head (LC). For the sum of behaviors, it was determined that each act of quickly raising the head (LC) corresponded to 1 second, being those added and evaluated together (Roveroni et al. 2001). After the end of the experiments, the *post-mortem* confirmation of the site of formalin application was done through an edema indicator, caused by the plasma extravasation of the Evans blue dye, applied by an intracardiac injection (0.4 ml), in the concentration 1% (5 mg/Kg). As the Evans blue has the capacity of linking plasma proteins (Haas et al. 1992), it was possible to visually identify the site of application of the irritating agent.

7. Statistical analysis

The nociceptive behavioral responses were evaluated by analysis of variance (ANOVA), followed by Tukey test for multiple comparisons. All values are given as mean ± standard deviation (SD). For all tests, the significance level was set at $p < 0.05$. All statistical analyses were performed using SIGMA STAT version 3.0 for Windows.

Results

1. Effects of sex and restraint stress on nociceptive behavioral responses

The female rats exhibited lower nociceptive responses when compared to males, both in the control and in the stressed groups. The exposure to a single restraint session of 1h reduced the nociceptive responses evoked by formalin only in the female rats - ANOVA + Tukey [$F(3.23)=40.57$; $p<0.001$]. The male rats did not exhibit this stress induced analgesia (Figure 1).

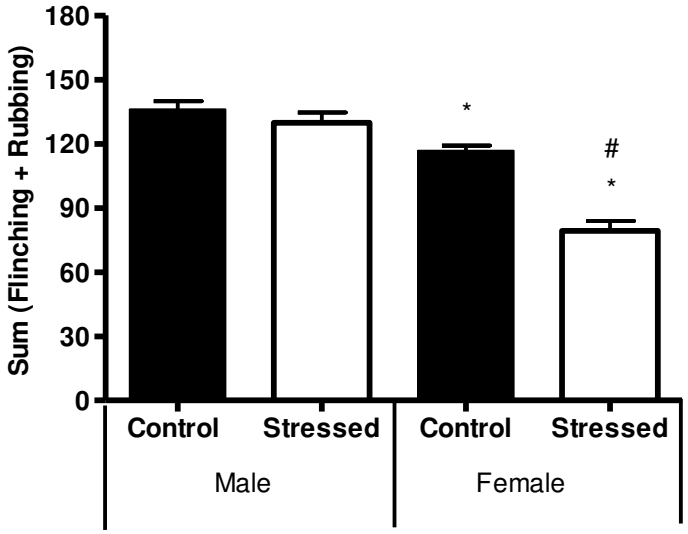


Figure 1. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50µL, 1.5%) previously submitted to acute stress or left undisturbed in their home cage in male and female rats (n=6/group). Each column represents the mean. Error bars indicate the SD. (*) Significant difference between male and female groups. (#) Significant difference between control and stressed females ($p<0.001$).

2. Effect of castration and acute stress on nociceptive behavioral responses in female rats.

The ovariectomized (OVX) female groups were hiperalgesic when compared to the SHAM groups. A statistically reduction in the nociceptive behavioral responses was observed only in the stressed SHAM group - ANOVA + Tukey [$F(3.23)=49.95$; $p<0.001$]. The nociceptive responses in the OVX groups (control vs. stressed) were similar, indicating the absence of stress-induced analgesia in the OVX females (Figure 2).

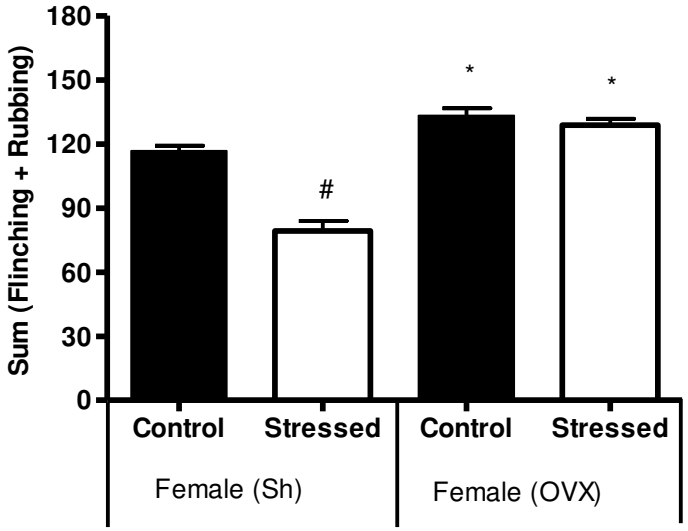


Figure 2. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50 µL, 1.5%) previously submitted to acute stress or left undisturbed in their home cage in SHAM and ovariectomized groups (n=6/group). Each column represents the mean. Error bars indicate the SD. (*) Significant difference between the SHAM and ovarictomized groups; (#) significant difference between the control and stressed SHAM groups ($p<0.001$). Sh: SHAM; OVX: ovariectomized

3. Effect of castration and acute stress on nociceptive behavioral responses in male rats.

The orquidectomy affected the nociceptive responses evoked by formalin 1.5% injected in TMJ of castrated rats when compared to the respective SHAM groups (Fig. 3). A statistically significant increase in the nociceptive behavioral responses was observed in both the control and stressed orquidectomized groups (hyperalgesia) - ANOVA + Tukey [$F(3.23)=13.91$; $p<0.001$]; there were no statistical differences ($p = 0.283$) between the control and stressed SHAM groups, nor between the control and stressed gonadectomized groups ($p = 0.396$) (Figure 3).

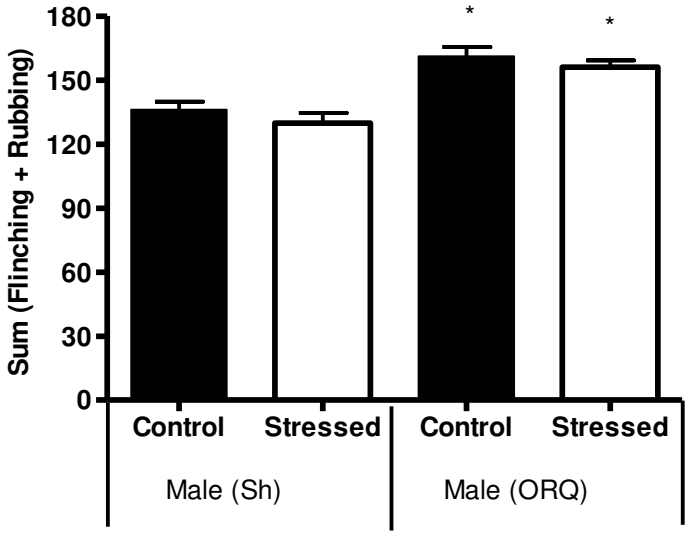
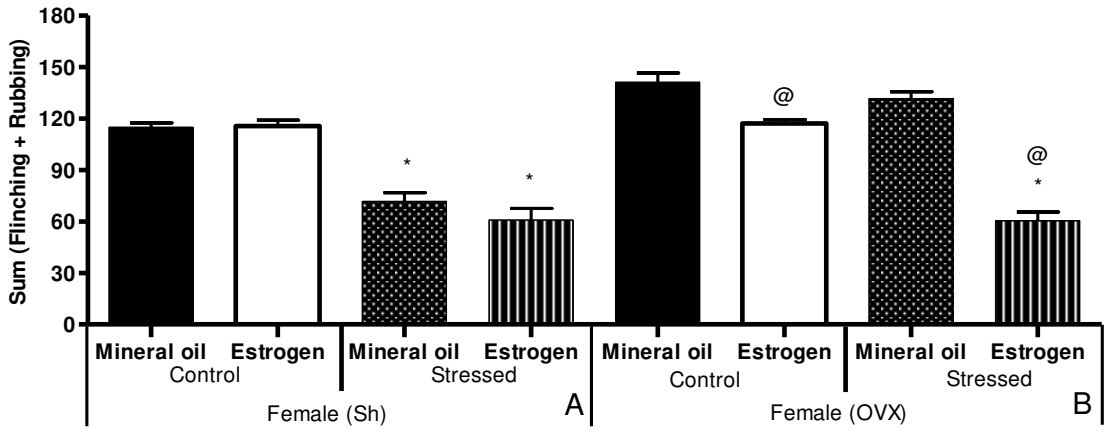


Figure 3. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50µL, 1.5%) previously submitted to acute stress or left undisturbed in their home cage in SHAM and orquidectomized male rats (n=6/group). Each column represents the mean. Error bars indicate the SD. (*) Significant difference between the orquidectomized and their respective SHAM groups. ($p < 0.001$). Sh: SHAM; ORQ: orquidectomized

4. Effects of hormonal manipulation and accute stress on nociceptive behavioral responses in female rats

The exposure to a single restraint session of 1h reduced the nociceptive responses evoked by 1.5% formalin injected in TMJ of female SHAM rats, both in the group which received mineral oil, as well as in the group with estrogen manipulation ($p<0.001$). The differences between mineral oil and estrogen were not significant in both stressed and control SHAM groups (Figure 4A). In the ovariectomized female rats, the estrogen replacement was able to reduce the nociceptive responses in both control and stressed rats, but this analgesic effect was higher in the stressed group - ANOVA + Tukey [$F(3.23)=69.27$; $p<0.001$] (Figure 4B).



Figures 4A and 4B. Sum of flinching and rubbing behaviors recorded in formalin-treated animal (50µL, 1.5%) previously submitted to acute stress or left undisturbed in their home cage in SHAM (4A) and ovariectomized (4B) female rats with mineral oil or estrogen manipulation (n=6/group). Each column represents the mean. Error bars indicate the SD. (*) Significant difference between stressed and their respective control groups. (@) significant difference between estrogen and mineral oil ($p < 0.001$). Sh: SHAM; OVX: ovariectomized

5. Effects of hormonal manipulation and acute stress on nociceptive behavioral responses in male rats

The estrogen administration was not able to alter the nociceptive responses evoked by formalin 1.5% injected in TMJ of both control and stressed rats - ANOVA + Tukey [F(3.23)=2.06; p=0.138] (Figure 5).

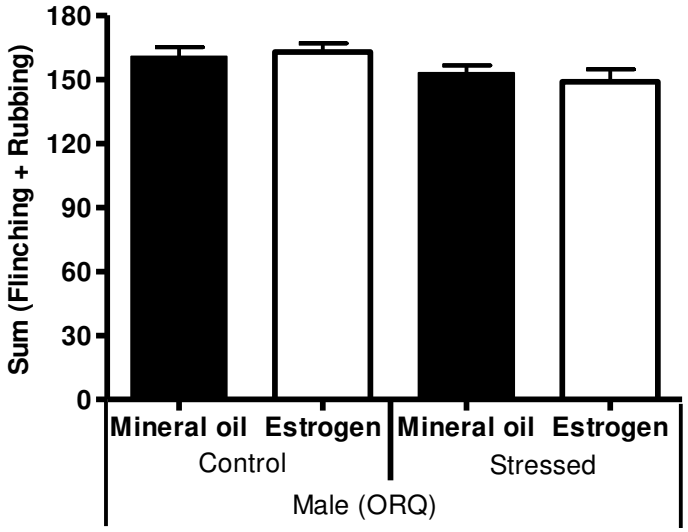


Figure 5. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50µL, 1.5%) previously submitted to acute stress or left undisturbed in their home cage in orquidectomized males manipulated with mineral oil or estrogen (n=6/group). Each column represents the mean. Error bars indicate the SD. No significant differences were found in nociceptive responses for controls vs. stressed groups (p = 0.138). Sh: SHAM; ORQ: orquidectomized

Discussion

The present study revealed significant sex differences in the nociceptive responses induced by the TMJ formalin test in rats, and also pointed to an important role of gonadal hormones in the modulation of stress-induced analgesia (SIA). Various studies in humans and animals supports that females are more sensitive to experimental pain than males (Cairns et al. 2002; Gaumond et al. 2002; Okamoto et al. 2003; Clemente et al. 2004). However, the role of ovarian hormones in TMJ nociception is still controversial. For example, LeResche et al. (2003), using a human clinical assay, suggested that temporomandibular pain in women is highest at times of lowest estrogen, a finding that agrees with data obtained with the TMJ formalin behavior model in female rats (Clemente et al. 2004; Botelho et al. 2010). Moreover, the lower TMJ pain observed during pregnancy in women (LeResche et al. 2005) and the lower formalin-induced TMJ nociception in pregnant rats (Arthuri et al. 2005) also indicate a protection role of female sex hormones in the TMJ nociception. On the other hand, there are also comparable animal studies using nociceptive agents other than formalin, indicating that estradiol appears to exacerbates peripheral pain sensitization by decreasing mechanical threshold or increasing response to noxious stimuli administered to the TMJ (Cairns et al. 2002; Okamoto et al. 2003; Wu et al. 2010). These discrepancies may result from several variables including nociceptive agent, dose, route of drug delivery, state of consciousness of the animal, estrous cycle stage, and species and strain of animals tested. Our results support an antinociceptive effect of female sex hormones in TMJ nociception, a finding corroborated by numerous animal studies that used formalin as the nociceptive agent in the TMJ behavioral model (Clemente et al. 2004; Arthuri et al. 2005; Fischer et al. 2008; Botelho et al. 2010). Although our findings suggest that ovarian hormones attenuate craniofacial pain, this suggestion does not explain clinical observations that TMD is more prevalent and severe in women than in men (Gonçalves et al. 2010). The higher prevalence of TMJ pain in women than in men may be due to a protective effect of testosterone (Fischer et al. 2007), or due to the physiological fluctuation in ovarian hormones serum levels during the reproductive cycle (LeResche et al. 2003; Fischer et al.

2008). In fact, TMD pain in woman is highest during low endogenous serum level of estrogen (LeResche et al.,2003).

The antinociceptive effect of the endogenous ovarian hormones was also evidenced by the results obtained in the ovariectomized (OVX) rats. The OVX animals showed enhanced nociceptive responses when compared to the sham groups. Another relevant finding of the present study was the absence of SIA in the OVX group. As previously shown, female rats usually exhibited a reduction in nociception after acute stress models (Botelho et al. 2010). This SIA was confirmed in the present research, in which the stressed females of the intact (not castrated) and sham groups exhibited a significant reduction in their nociceptive behaviors, when compared to their respective unstressed controls. However, the SIA was not observed in the OVX rats, indicating that the endogenous levels of ovarian hormones play an essential role for the occurrence of SIA. The mechanisms by which ovarian hormones activate the SIA are not well understood. Considering that an increasing number of studies indicates that the antinociceptive effect of gonadal hormones in the TMJ formalin nociceptive model is opioid mediated (Arthuri et al. 2005; Fischer et al. 2009; Botelho et al. 2010), we hypothesized that an interaction between gonadal hormones and endogenous opioid system could also be responsible for the occurrence and magnitude of SIA observed in female rats. We have previously shown that acute restraint stress (1 h) could produce analgesia in proestrus and estrus female rats, but this effect was higher in the proestrus phase. This effect was in part due to peripheral kappa opioid receptor activation, since the local pre-administration of nor-Binaltorphimine (selective receptor kappa opioid antagonist) partially reversed the SIA during the proestrus phase (Botelho et al. 2010). Another recent study has demonstrated that the activation of the kappa opioid receptor in the spinal cord of rats produces an estrogen-dependent attenuation of acute and persistent nociception, respectively induced in the tail and in the paw (Lawson et al. 2010). Taken together, these findings suggest an estrogen dependence of kappa opioid receptor effects in SIA.

In the evaluation of nociceptive behavior of male rats submitted to the TMJ formalin test, we observed a significant hyperalgesic effect of castration in the orquidectomized (ORQ)

group. This result could be due to the protective effect of testosterone in the development of TMJ pain (Fischer et al. 2007). The fact that the serum level of testosterone but not of estrogen and progesterone significantly decreased in gonadectomized male rats suggests that testosterone is the hormone underlying the decreased intact male rat's risk for development of TMJ pain (Fischer et al. 2007). Moreover, the present study showed that acute restraint stress was not able to reduce the nociceptive responses in any of the stressed male groups. We have already demonstrated that restraint stress in males can release endogenous opioids (Gameiro et al. 2005), but this effect was not capable of inducing analgesia in male rats that were submitted to acute restraint stress before the TMJ formalin test. As female rats (in both intact and sham groups) exhibited decreased nociceptive responses to the TMJ formalin test and this analgesic effect was higher in the proestrus females (Botelho et al. 2010), these findings reinforce the idea that female sex hormones are essential to evoke SIA in the TMJ formalin test.

The effects of estrogen replacement on the TMJ nociception were evident only in the castrated females. In the unstressed OVX, the estrogen replacement evoked an antinociceptive effect, whereas this hormonal manipulation was essential to the SIA observed in the stressed OVX. The antinociceptive action of estrogen replacement in OVX females have already been reported in studies using the complete Freund's adjuvant (Kramer and Bellinger, 2009) or formalin (Fischer et al. 2008, 2009) to induce TMJ nociception. These authors demonstrated that central opioid mechanisms mediate the antinociceptive effect of estradiol, a similar finding reported by a human study showing that women during a high estradiol state, compared with those during a low estradiol state, have lower pain scores (experimental masseter pain) because of a greater activation of central opioid system (Smith et al. 2006). This greater central opioid activation could also be responsible for the SIA dependent of estrogen, since hormonal replacement have reestablished the SIA in OVX rats, whereas the SIA was not observed in OVX rats treated with vehicle. On the other hand, the present results show that estrogen administration did not induce antinociception in female sham rats. It is important to point out that these rats were in the proestrus phase, in which the physiological levels of gonadal hormones are already high. Therefore, these results indicate that the hormonal administration in the sham

groups did not potentiate the antinociceptive effect of estrogen, suggesting that its maximum antinociceptive action might have been reached by the high physiological levels of the hormone during the proestrus phase. The study of Fischer et al. 2008 have used a protocol of estrogen administration similar to the present one, and they found that the antinociceptive action of exogenous estrogen (at supraphysiological dose) was similar to that observed in intact or sham-operated females in the proestrus phase of the estrous cycle, corroborating with the present results.

Finally, the estrogen administration in ORQ males did not have an antinociceptive action, both in the control and in the stressed groups. These results indicate an organizational effect of estrogen on both its antinociception action and on its ability to evoke SIA. Although fluctuations in endogenous estrogen that occur normally during the estrous cycle were shown to modulate the kappa opioid receptor-mediated antinociception as well as the kappa opioid receptor gene expression (Lawson et al. 2010), neither testosterone nor low levels of estrogen is responsible for the lack of kappa opioid receptor-mediated antinociceptive effects in the male. Interestingly, neither gonadectomy in the male nor administration of estradiol to ORQ males enhanced the expression of the kappa opioid receptor gene (Lawson et al. 2010), which supports our conclusions drawn from these behavioral studies. However, the activational antinociceptive effect of gonadal hormones in the TMJ should not be neglected. This is evidenced by the greater nociceptive response in females with lower than higher hormonal levels (Botelho et al. 2010), and by the protective role of testosterone observed in the present study.

In conclusion, the present study showed that gonadal hormones exert significant influences on TMJ nociception, and in its susceptibility to be modulated by acute stress. The organizational effects occurred during critical periods of the development and are exemplified by the absence of antinociception induced by estrogen or stress in male rats. The activational antinociceptive effects are clearly demonstrated by the protective role of testosterone in male rats.

Acknowledgements

The authors thank Carlos Alberto Feliciano for technical assistance. This work was supported by FAPEAM, Brazil.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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CAPÍTULO 3

Article

Effects of acute restraint stress on anxiety in gonadectomized rats.

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Abstract

Anxiety may be considered as an emotional anticipation of an aversive situation, difficult to predict and control, but it has high prevalence in the population. This condition seems to be affected by stress and gonadal hormones. The aim of present study was to evaluate the concentration of blood levels of corticosterone and the levels of anxiety in castrated rats with and without estrogen replacement tested in elevated plus-maze after acute restraint stress for 1 hour. Animals were initially submitted to acute stress (1 h) then they were

immediately killed to collect blood for hormonal radioimmunoassay determinations; or submitted to elevated plus-maze test. All stressed groups showed increase of plasmatic corticosterone levels. The acute stress caused no reduction in the number of entries in closed arms in orquidectomized male rats and ovariectomized female rats; ovariectomized female rats that received mineral oil replacement had a significant reduction in the percentage of entries into open arms, in the number of entries into the end of open arms and in total percentage of entries into open arms. The lack of difference in the number of closed-arm entries between the stressed (all groups) and control rats indicate that the locomotor activity was not influenced by acute stress procedure, we conclude that the anxiety levels of castrated male and female rats with estrogen replacement are not modulated by stress.

Keywords: Restraint stress, anxiety, elevated plus-maze test, corticosterone, gonadectomy, estrogen.

Introduction

Anxiety may be considered an emotional state which is subjectively experienced as unpleasant or threatening feeling, presenting some symptoms such as: changes in mood (e.g. apprehension and fear) and cognitions (e.g. thoughts of impending mishap) (Pratt 1992; McCormick et al. 2008). Usually, anxiety is characterized by physiological, environmental and behavioural changes such as palpitations, sweating and hypervigilance that commonly increase attention to pain in human subjects (Rhudy and Meagher 2000), and is a normal emotion, a response to threatening or stressful stimuli. However, it becomes a pathological alteration when the response to the perceived stimuli becomes overexagerated and irrational, interfering in the person's ability to function normally (Pratt 1992). Women usually suffer more of anxiety and also have a higher incidence of mood or affective disorders than men (Chada and Devauld 2005; Dalla et al. 2005; Shinal and Fliingim 2007; Liang et al. 2008; McCormick et a. 2008) and anxiety disorders are usually more persistent than mood disorders (Kessler 2007).

Animal models of anxiety have considerably contributed to the understanding of the neuroanatomical basis and neurobiological correlates of anxiety and clinical impact, favoring the development of novel diagnostic, therapeutic agents and efficient treatments (Rodgers et al. 1997; Landgraf 2003) as it is extremely difficult to evaluate the mechanisms involved in humans in the development of anxiety. Behavior is classified as conditioned or unconditioned responses to stimuli. However, the models involving unconditioned (e.g. spontaneous) behavior usually have a higher degree of ecological validity, presenting less susceptibility to confounds arising from interference with nociceptive mechanisms or learning/memory protocols (Pratt 1992; Rodgers et al. 1997).

Orofacial pain is a painful condition of the superficial and deep tissues of the craniofacial region. Whereas acute pain is often associated with anxiety, chronic pain is characterized by depression and is often accompanied by addictive/appetitive disease (e.g. substance abuse, eating disorders) (Gatchel et al. 1996). Temporomandibular disorders (TMDs) are musculoskeletal pain conditions commonly characterized by painful conditions in the

temporomandibular joint (TMJ) and/or muscles of mastication (Haas et al. 1992; LeResche et al. 2003). Some studies show that patients with TMD present higher levels of stress, anxiety, depression and somatization when compared to healthy controls (Gatchel et al. 1996; Jones et al. 1997). The treatments proposed to the clinical control of anxiety disorders are based on the use of serotonin (5-HT) reuptake blockers (Griebel et al. 1994) such as tricycle antidepressants and benzodiazepines, that produce their pharmacological effects through recognition site on the $GABA_A$ receptor (Pesold and Treit 1996). Stress exposure results in a myriad of responses, of which activation of the hypotalamic-pituitary-adrenal (HPA) axis may lead to an increase in energy expenditure, a decrease in feeding and appetite (Chotiwat and Harris 2006) and has been associated to the increase of anxiety and depression in humans and rats (Landgraf et al. 1999; Lund et al. 2005; Zuloaga et al. 2008).

One of the most used methods for screening drugs with ansiolytic potential as well as to study the neurobiology of anxiety in rodents is the elevated pluz-maze (EPM) (Pellow et al. 1985; Dawson and Tricklebank 1995; Weiss et al. 1998; Jones et al. 2002; Pereira et al. 2005). In the EPM, the rodent is allowed to explore an environment formed by four elevated arms, being two opened arms and two closed arms, and the animal behavior is driven by a conflict between the tendencies to explore the new environment. In order to avoid potencial dangers, this conflict is similar to human anxiety (Pereira et al. 2005). Several studies show a higher number of entries in the closed arms (Dawson and Tricklebank 1995; Weiss et al. 1998) showing that fear for open space is the anxiogenic stimulus in the EPM (Dawsson and Tricklebank 1995), and it is also generally assumed that ansiolytic drug-induced behavior increases in open arm exploration (Weiss et al. 1998) while anxiogenic agents cause the opposite (Pellow et al. 1985).

Considering that there are sexual variations in the anxiety disorders, and the levels of corticosterone are usually higher in animals submitted to stress, the aims of the present study is to evaluate the levels of anxiety in orquidectomized and ovariectomized rats with estrogen replacement, submitted to a single session of stress by restraint (1h) and to compare them to their respective control groups.

Methods

1. Animals

For the current study, 160 Wistar rats with 150 to 250 g of both genders (males and females in the proestrus phase), from Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratorio-CEMIB, and kept in the animal facilities of the Piracicaba Dental School, were used. The rats were held in plastic cages (5 per cage), containing wood shavings, in an environment with luminosity (clear /dark each 12hs; lights on at 6:00 am), humidity and temperature (23 $\pm$ 2^0C) controls. They were fed with industrialized food and *ad libitum* water. The experiments were performed during the clear cycles between 09:00 am and 5:00 pm, under silent conditions. All experimental procedures were previously submitted to the Ethics Committee for animal research of Universidade Estadual de Campinas (protocol 1555-1), and followed the guidelines proposed by the Ethics Committee for Research of the International Association for the Study of Pain in Conscious Animals (Zimmermann 1983).

2. Estrous Phase Determination

Proestrus and estrus phases were determined by daily microscopic examination of vaginal smears that were taken by gentle lavage between 8:00 and 10:00 am. Proestrus was identified by the predominance (>70%) of nucleated epithelial cells, and the occurrence of estrus was marked by the presence of anucleated cornified cells in rats with at least two consecutive regular 4-day cycles (Marcondes et al. 2002, Smith et al. 1975). These phases were chosen because they represent phases of both high and low ovarian hormone levels (Butcher et al. 1974).

3. Orchidectomy and Ovariectomy

The orchidectomy and ovariectomy (Gordon and Soliman 1994) were done in male and female rats at 60 days of age. The procedures were performed under induced anesthesia by intramuscular injection of ketamine (55mg/Kg) and xylazine (5.5mg/Kg). A subcutaneous injection of ketoprofen (5mg/Kg) was used for post-surgery analgesia (Roughan and Flecknell 2000). Sham operated animals were used as castration controls. The efficiency of the orchidectomy and ovariectomy was confirmed through the evaluation of the atrophy of the seminal vesicles/prostate and womb respectively, in the animals which have not received hormonal manipulation.

4. Hormonal manipulation

After castration or surgical procedure (Sham), some animals received hormonal treatment was performed by daily injection of 17β-estradiol (sigma® - E0756 – 5 mg) (in mineral oil; s.c.; 50μg/Kg/day; at 9:00 am) (Gordon and Soliman 1994), and others received vehicle (0.15 – 0.2 mL/day; at 9:00 am) during seven days. The rats were castrated at 60 days of age, and the hormonal treatment or the application of mineral oil started 21 days after the surgical procedure. On the seventh day, the hormonal injection with 17β-estradiol or vehicle was done 1 hour before the respective experiments.

5. Acute stress protocol

The male and female rats were submitted to the acute stress protocol through immobilization during 1 hour (Gameiro et al. 2006; Botelho et al. 2010). The animals were put in a plastic tube (25x7cm^2) allowing adjustments to the size of the animal. In one end of the tube, a 1-cm hole was made to favor the animal breathing during the experiment. In the control group, the animals were not submitted to stress sessions. The immobilization procedures were held between 10:00 am to 12:00 pm, in a silent environment.

6. Hormonal assays

In order to measure the corticosterone levels, the animals submitted to acute stress by imobilization, or the animals of the control group were immediately killed by decapitation for blood collection and hormonal measurement by radioimmunoassay (RIA) (Castro et al. 1995). The blood was collected in tubes with heparin and centrifuged at 2500 rpm for 15 min, at 4ºC and the serum kept at -20ºC until use. The interval between the stress protocol and manipulation until the sacrifice were kept standardized (about 30 sec.) for the different groups.

7. Evaluation of anxiety level

The elevated plus-maze test in elevated cross, an animal model that proved to be very efficient in the study of neurogenic mechanisms for anxiety, was used to evaluate the animals anxiety level (Pesold and Treit, 1996). The animals were put in the center of the maze, presenting two open arms (50 cm long *x* 10 cm wide) and two closed arms (50 cm long *x* 10 cm wide, with 40 cm high walls) that extended from a central platform elevated 50 cm above the floor (Marcondes et al. 2001). According to the protocol proposed by Cruz et al. (1994), each animal was submitted once to the test, being placed in central square facing an enclose arm and its behavior was recorded for 5 minutes. All tests were made after acute stress session (1 h); the controls were not previously stressed. Before the next rat was introduced, the maze was cleaned with a solution of 20% ethanol and dried. Next, without any knowledge about the group to which the animal belonged, the data were analyzed by the EthoVision® XT program (version 4, Noldus information technology 2006 – program licensed to Fernanda Klein Marcondes - blind study). The time of permanence and exploration of open arms and the number of entries in the open and closed arms were verified. After that, the results were converted in percentage of time of exploration of open arms, multiplying the time of permanence in the open arms and dividing by the time of observation (100 x time in open arms / total time of observation), classically considered as

anxiety level (Pellow et al. 1985). The greater permanence time in the open arms and in the end of open arms, evidenced a lower level of anxiety in the animal (Cruz et al. 1994).

8. Statistical analysis

Statistical analysis of plasma corticosterone data were made using the analysis of variance based on ranks (ANOVA-R) using SYSTAT 12 by Hearne Scientific Sofyware, Chicago:IL, USA. The comparison of anxiety-related behavior groups was made by analysis of variance (ANOVA). Studies on supposition were carried out prior to the application of the variance analysis, in order to validate the data grip to the conditions that lead the techinique application. The Tukey test (HSD) was chosen in the first place to compare the means, two by two, whenever significant effects were detected. The 5% significance level ($p<0.05$) was adopted and the analysis were calculated using the SAS system (version 9.2 for windows) by SAS institute Inc., Cary:NC, USA 2008.

Results

1. Effects of stress procedure on plasma corticosterone

The hormone levels in Sham, orquidectomized (ORQ) and ovariectomized (OVX) rats are shown in figure 1 (A – Sham; B – ORQ with mineral oil or estrogen and C – OVX with mineral oil or estrogen). There was a significant increase in plasma corticosterone levels after stress protocol when compared to control in all groups ($p<0.001$).

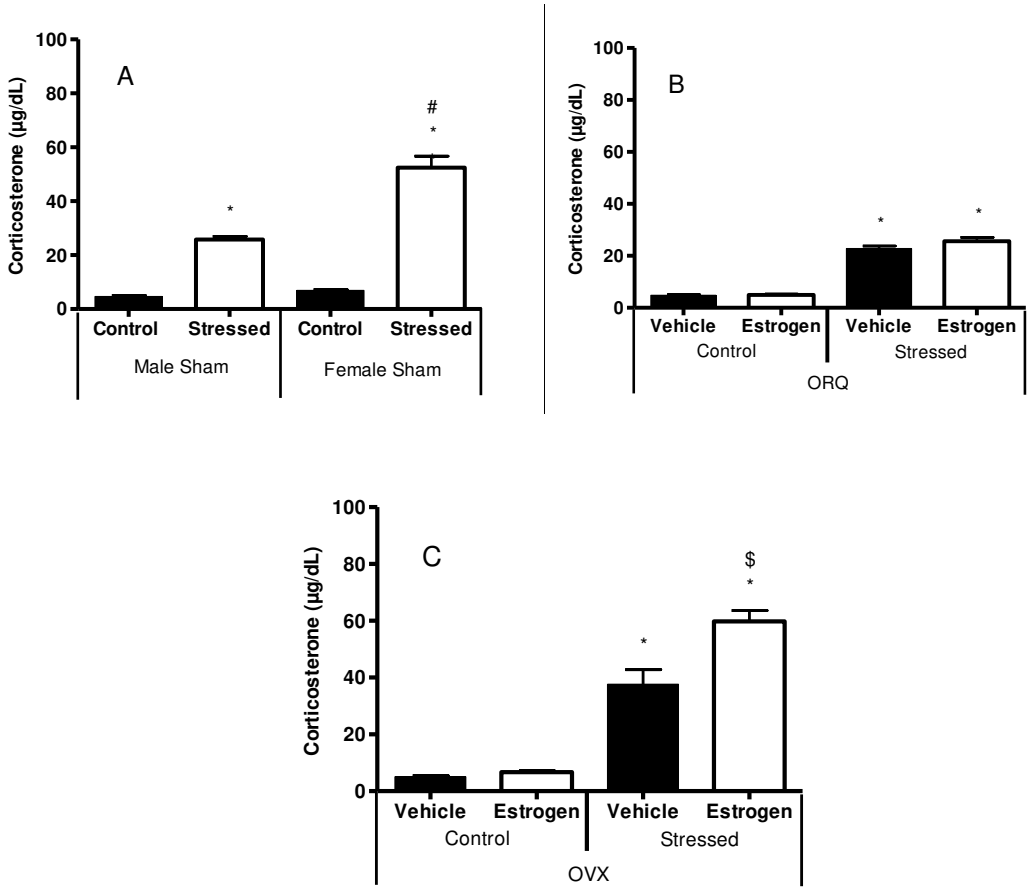

Figure 1. Basal and stressed-induced plasma corticosterone levels after acute stress in Sham, ORQ and OVX rats (A – Sham male and female groups; B – ORQ with vehicle or estrogen and C – OVX with vehicle or estrogen). Each column represents the mean and error bars indicate the standard error of the mean (S.E.M). Number of subjects was set as n = 8/group. (*) indicates significant difference when compared to controls. (#) indicates significant difference between male and female. ($) indicates significant difference of estrogen when compared with vehicle (ANOVA – $p<0.001$).

2. Effects of stress on the number of entries in closed arms (elevated plus-maze)

There were not significant differences on entries in the closed arms in the control group when compared to stressed group. In all groups, controls which received vehicle entered more times in the closed arms when compared to their stressed groups (figures 2A, 2B,2C and 2D), but the difference was not significant (p>0.05 – ANOVA-R).

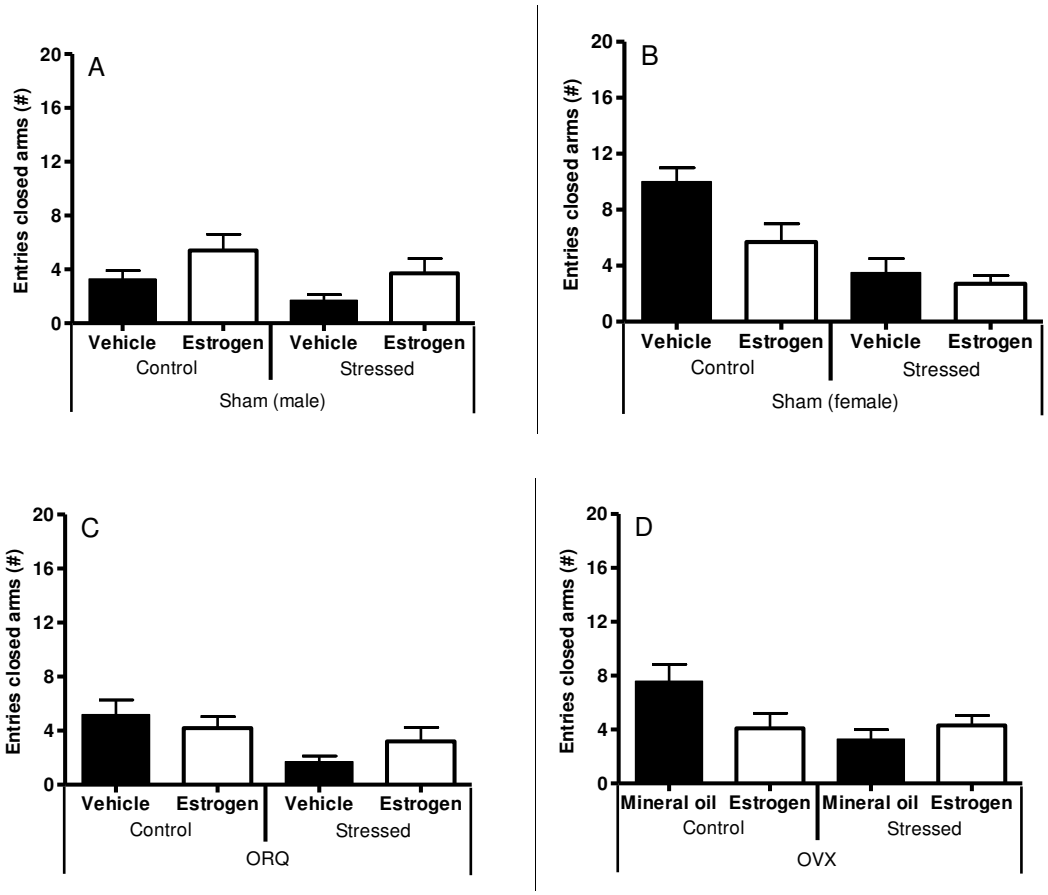

Figure 2. Number of entries in closed arms in male (A) and female (B) Sham, ORQ (C) and OVX (D) rats submitted to acute stress. Each column represents the mean. Error bars indicate the S.E.M. Number of subjects was set as n =10/group (p>0.05).

3. Effects of stress on the percentage of entries into the open arms (elevated plus-maze)

There were no significant differences between the stressed male Sham (Figure 3A) and female Sham (3B), both in animals that received estrogen as in those who received vehicle in the open arms entries when compared to the control ones; only the male orquidectomized rats (3C) and female ovariectomized females (3D) who had received vehicle presented a significant increase time in the open arms when compared to the stressed groups. This demonstrates a lower anxiety level in these animals when compared to the groups that received acute stress ($p<0.05$ – ANOVA-R).

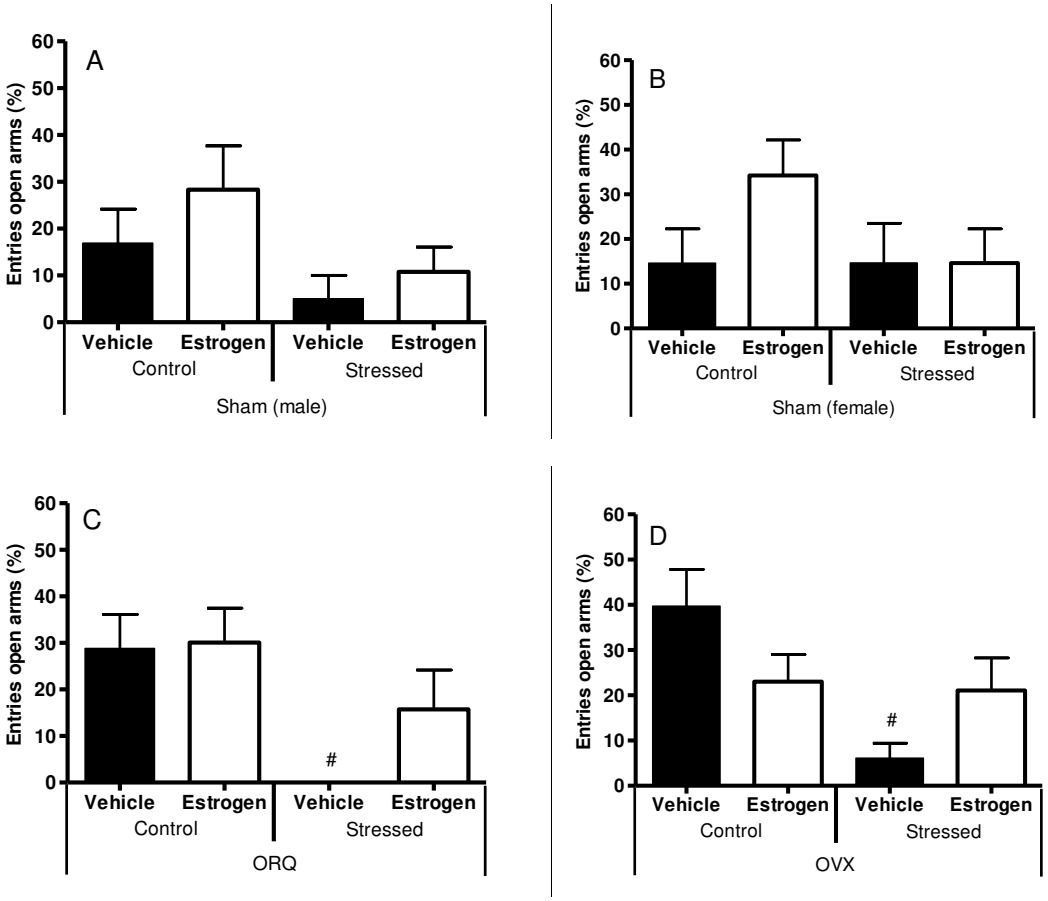

Figure 3. Percentage of entries into open arms in male (A) and female (B) Sham, ORQ (C) and OVX (D) rats that received acute stress. Each column represents the mean. Error bars indicate the S.E.M. Number of subjects was set as n =10/group. (#) indicate significant difference between vehicle in control and stressed groups ($p<0.05$).

4. Effects of stress in the number of entries into the end of open arms (elevated plus-maze)

Figure 4 shows a significant decrease in the number of entries into the end of open arms in the stressed female Sham (B) and ovariectomized female groups (D) that received vehicle, when compared to their respective controls ($p<0.05$ – ANOVA-R). There were no differences in the performance of Sham male rats and orquidectomized male rats, in stressed and control groups, as there were no difference between animals that received estrogen and vehicle.

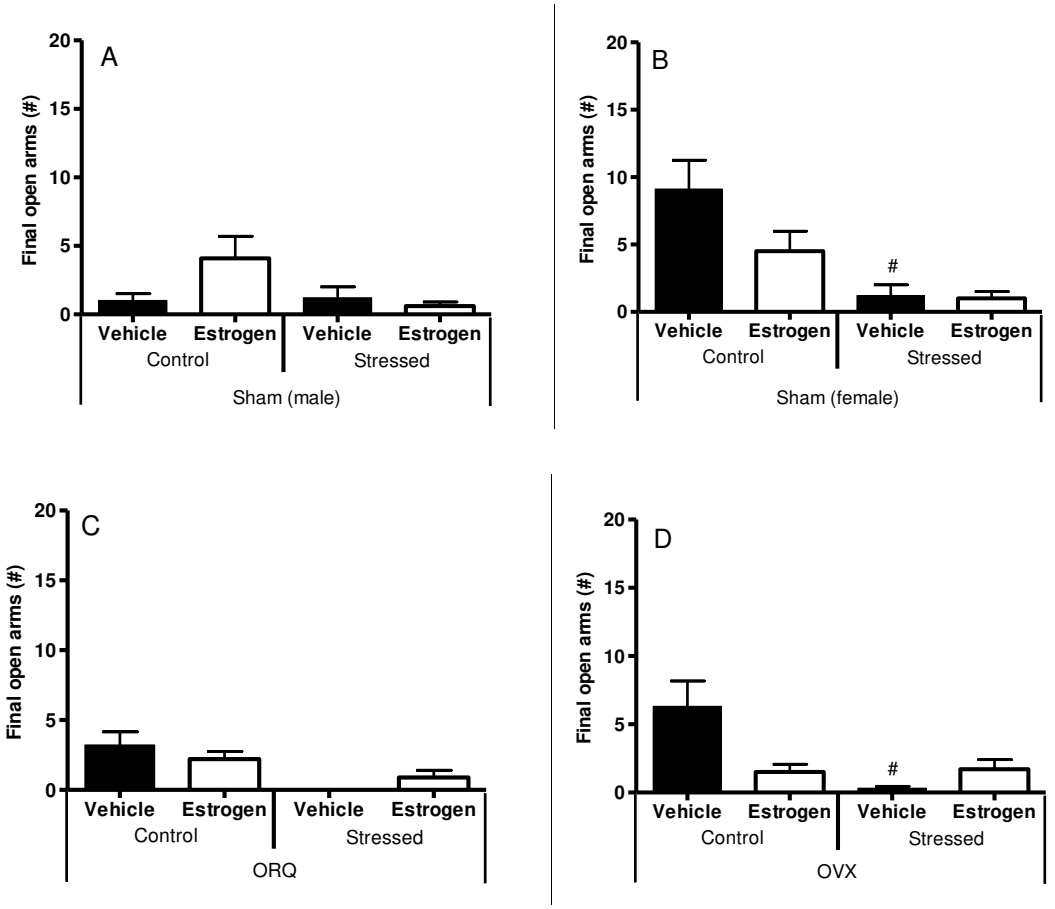

Figure 4. Effects of restraint stress (1 h) on the number of entries into the end of the open arms. Each column represents the mean. Error bars indicate the S.E.M. Number of subjects was set as n =10/group. A) male Sham; B) female Sham; C) orquidectomized male rats and D) ovariectomized female rats (#) indicate significant difference between control and their respective stressed groups with vehicle ($p<0.05$).

5. Effects of restraint stress (1 h) in the percentage of times into the open arms (elevated plus-maze)

The exposure to a single restraint session of 1h caused an increased response of anxiety behaviors in female rats which received vehicle ($p<0.05$). The differences between vehicle and estrogen were not significant in stressed Sham, ORQ and OVX groups (figures 5A, 5B, 5C and 5D). In control female Sham and ovariectomized female rats the vehicle was able to increase in the percentage of times into the open arms ($p<0.05$ – ANOVA-R) (figures 5B and 5D).

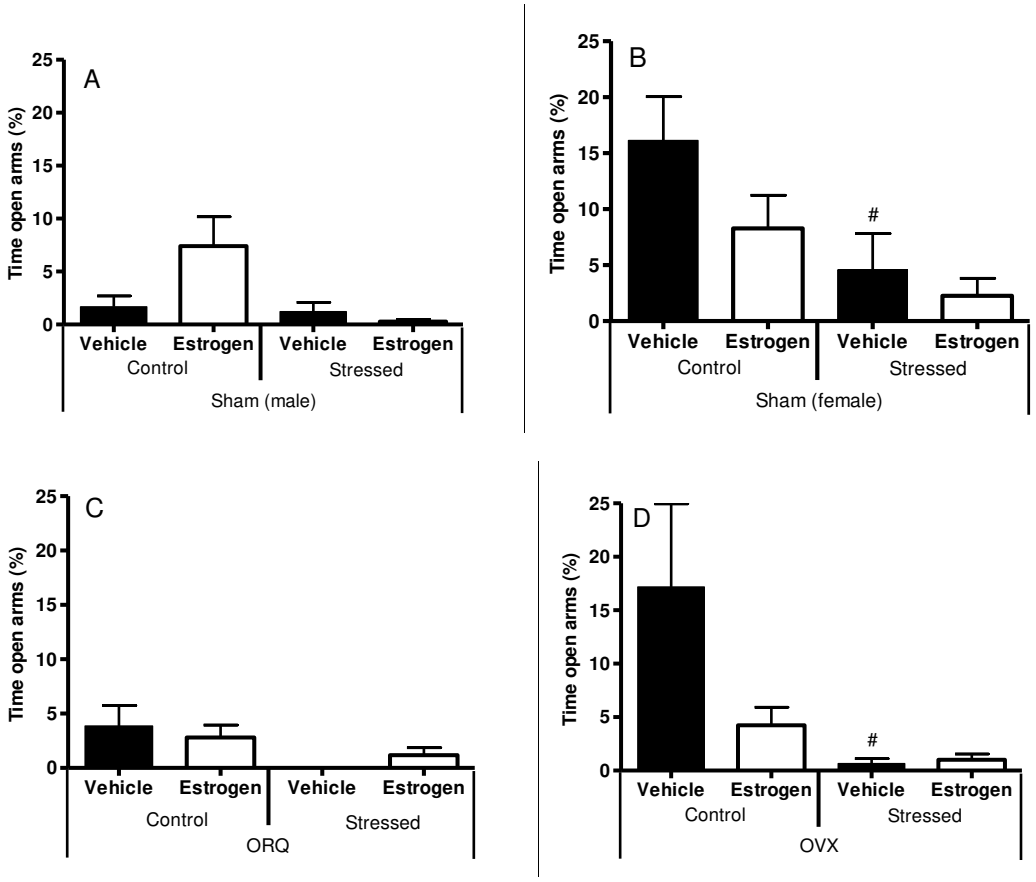

Figure 5. Effects of acute stress in the percentage of times into the open arms. Each column represents the mean. Error bars indicate the S.E.M. Number of subjects was set as n =10/group. A) male Sham; B) female Sham; C) orquidectomized male; D) ovariectomized female. (#) indicate significant difference between mineral vehicle in control when compared with their stressed groups ($p<0.05$).

Discussion

Some behavioral measures indicative of anxiety and stress (e.g. defecation and displacement activities) are correlated with reduction in social interaction. The measurement of adrenocorticotrophic hormone (ACTH) (Bogdanov and Yarushkina 2003) and corticosterone levels (Kelly et al. 1993) are methods usually used to indicate stress levels (File 1987; Bielajew 2003). In this study, we measured the corticosterone plasma to confirmed the efficacy of acute stress. A significant increase in plasma corticosterone was observed in stressed animals when compared to their respective control groups, indicating a deregulation of the HPA axis after exposure to stress (Frye and Lacey 2001, Chotiwat and Harris 2006). The female ones presented higher concentrations of corticosterone when compared to male ones. Similar results were found by Bowman et al. (2006) as both Sham females as well as the ovariectomized female rats of the stressed group which received estrogen presented a significant increase of corticosterone when compared to ones which received mineral oil. Several studies with animals showed differences between the sexes in normal HPA activity, pointing a significant increase in the secretion of corticosterone plasma in response to physical and psychological stressors in the females rather than in males (Figueiredo et al. 2002; Sliwowska et al. 2008). A previous study in our lab with females in estrus and proestrus phases demonstrated that the increase in corticosterone level was lower after chronic and sub-chronic stress when compared to acute protocols. Gameiro et al. (2006), found similar results with male rats, and postulated that hyperalgesia on chronic stress was the result of long-term effects evoked by persistent stress and anxiety. It appears that sex differences in HPA axis activity, that are established following gonadal steroid exposure during the organizational period, are maintained in adulthood when the activational influences of hormones on HPA activity are decreased, showing a direct effect of circulating gonadal steroids in brain.

The elevated plus-maze is, one of the most used model of evaluation of the anxiety levels in animals (Hogg 1996, Weiss et al. 1998). It is known that the measurement of anxiety may be influenced by the motor activity; we evaluated this parameter through the number of entries in the closed arms considering an index of locomotor activity (Cruz et al. 1994). In

all groups, there was no significant reduction in the number of entries in the closed arms in stressed animals that received vehicle or estrogen when compared to the control group. However, this lower number of entries suggests a higher anxiety level in the stressed animals. Marcondes et al. (2001) found no significant differences in the number of closed-arms entries among male rats, ovariectomized female rats, females in proestrus, estrus, metestrus, diestrus and diestrus + estradiol phases. In a non-published study of our laboratory, we observed that after several protocols of acute stress (15; 30 and 60 min) and sub-chronic protocols of stress (3 days – 1h/day) there was no significant difference when compared stressed to controls in females in proestrus and estrus phases, ocurring a significant increase in the number of entries into the closed arms only in chronically stressed rats (40 days – 1h/day) when compared to controls. Gameiro et al. 2006 in a study with male rats submitted to several acute, sub-chronic and chronic stress protocols acquired similar results, showing that the motor activity was not influenced by stress procedure, indicating that the anxiogenic effect after stress protocols was indeed related to anxiety but not to the locomotor activity of the rats.

Anxiety is inversely proportional to the percentage of time and time of permanence into the open arms by the rats. In the current work, we observed that stressed female ovariectomized rats which received mineral oil entered fewer times in the open arms and remained there less time than the animals of the control group. No significant differences were observed in stressed and control male and female Sham in the percentage of entries into the open arms. The estrogen replacement in castrated rats caused no significant changes in the number of entries into the end of open arms and in the percentage of permanence in the open arms between stressed and control groups, while stressed ovariectomized female rats that received vehicle entered fewer times and spent less time in the open arms of the plus-maze test than their control groups. Similar results were observed by Marcondes et al. (2001) who found no significant differences in the percentage of open arms entries between male and female in different estrous cycles phases.

Studying the effects of repeated restraint (2 h of restraint on each of 3 consecutive days) in rats, Chotiwat and Harris (2006) found a significant decrease in the number of entries into

open arms of these animals when compared to the control group. Male wistar rats treated with nandrolone decanoate showed significant decrease in the percentage of time spent in the open arms when compared to control and vehicle (propyle glycol) groups, with an increase of anxiety in sedentary rats (Rocha et al. 2007). In a study with male rats submitted to acute, sub-chronic and chronic restraint stress, Gameiro et al. (2006) observed a significant increase of anxiety in these animals (decreased percentage of time into the open arms and decreased percentage of entries on open arms) when compared to controls. Using a rat model of adolescent social stress, McCormick et al. (2008) found significant differences in the time of open arms and ratio of open arms entries in the females submitted to stress that spent more time on the open arm than control group. There were no significant differences between stressed and control male rats.

Once the anxiety levels in animal models can be measured by the percentage of and time in the open arms of the plus-maze and that the locomotor activity is directly related to the number of entries in the closed arms, we concluded that there were no significant differences between the Sham stressed animals and the castrated ones which received vehicle or estrogen in the locomotor activity when compared to their respective control groups; The Sham females and ovariectomized female rats that received vehicle submitted to acute stress showed higher level of anxiety than their respective control groups and, that both in the Sham rats as well as in the castrated ones, the protocol of acute stress significantly raised the levels of corticosterone, being the more significant increase found in stressed ovariectomized female rats that in the ones which received estrogen, which indicates the efficacy of stress protocol.

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CONCLUSÃO

De acordo com os resultados do presente trabalho, concluiu-se que:

- Estressse agudo (1 h) induz redução das respostas nociceptivas (analgesia) em fêmeas quando submetidas ao teste da formalina na ATM. Efeito este que não se manisfesta em machos e em ratas ovariectomizadas.

- A redução das respostas comportamentais nociceptivas (analgesia) induzida pelo estresse é significativamente maior em fêmeas na fase de proestro.

- A analgesia induzida pelo estresse é parcialmente mediada pelo sistema opióide.

- Ratos orquidectomizados apresentam aumento das respostas nociceptivas (hiperalgesia), evidenciando um possível efeito protetor da testosterona no comportamento nociceptivo.

- A administração de estrógeno restaura a analgesia induzida por estresse em ratas ovariectomizadas, e não afeta as respostas comportamentais nociceptivas em machos orquidectomizados, indicando um efeito possivelmente organizacional do estrógeno na ação antinociceptiva.

- Pelas diferenças sexuais apresentadas nas respostas nociceptivas induzidas pelo estresse agudo (1h), pode-se inferir a participação dos hormônios gonadais na modulação da analgesia induzida pelo estresse.

- O protocolo de estresse agudo (1h) induz aumento dos níveis de corticosterona plasmática em ambos os sexos, porém significativamente maior em fêmeas.

- O estresse agudo induz um aumento nos níveis de ansiedade em ratas ovariectomizadas.

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* De acordo com a norma da UNICAMP/FOP, baseada na norma do International Commitee of International Journal Editors – Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

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APÊNDICE

FIGURAS

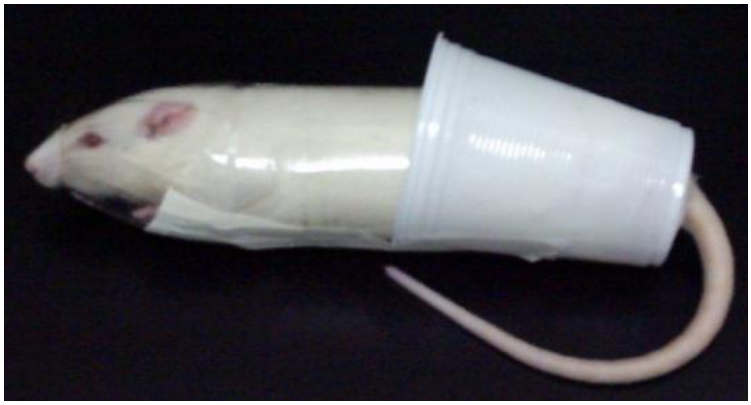

Figura 1 – Tubo plástico ajustável utilizado para realização do estresse por contenção.

Figura 2 – Local da punção para injeção de formalina (1,5 %) na ATM.

Figura 3 – Câmara de observação (30 cm^3) utilizada no registro dasrespostas nociceptivas.

Figura 4 – Contador de células e cronômetro utilizados para quantificação das respostas comportamentais nociceptivas.

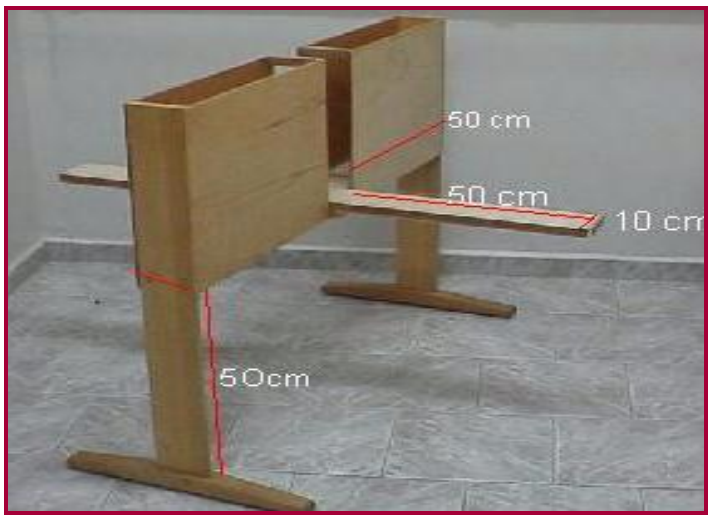

Figura 5 – Labirinto em cruz elevado utilizado para avaliação da ansiedade.

Figura 6 – Animal explorando o braço fechado durante teste no labirinto em cruz elevado.

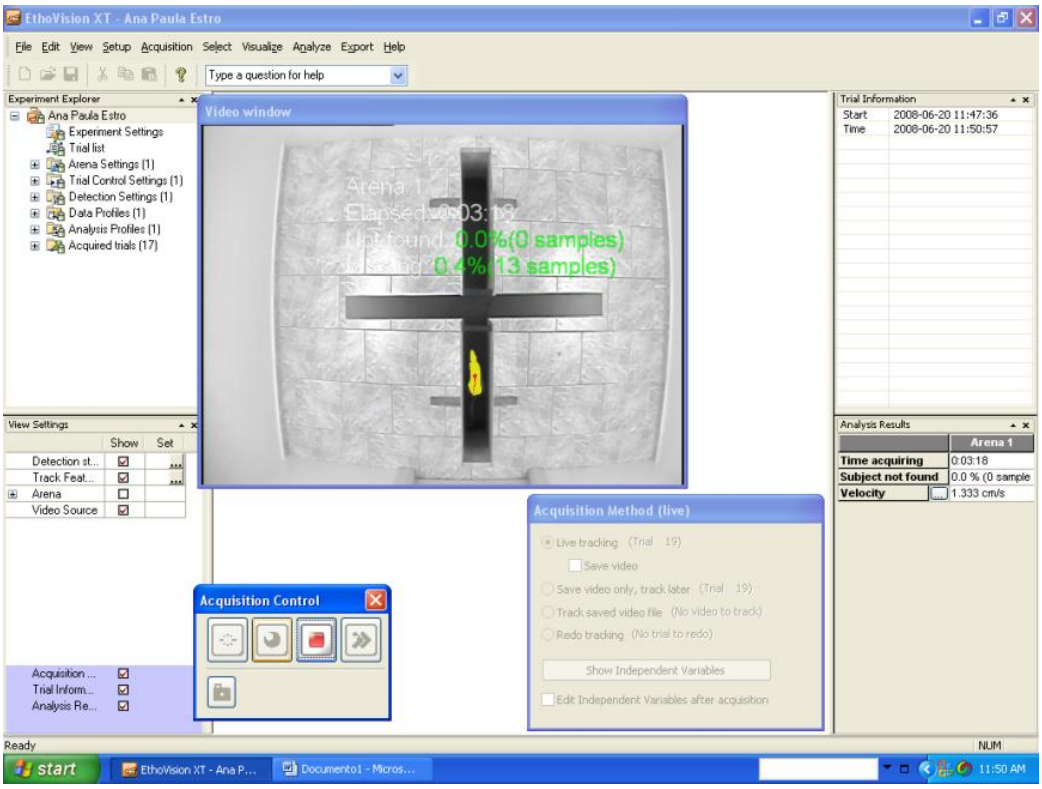

Figura 7 – Software EthoVision® XT, version 4, Noldus information technology 2006, durante a captação dos dados do teste do labirinto em cruz elevado (em amarelo, rato explorando labirinto).

ANEXO 1

Certificado do Comitê de Ética

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C E R T I F I C A D O

Certificamos que o Protocolo nº **1555-1**, sobre **"Influência dos hormônios sexuais na analgesia induzida pelo estresse"**, sob a responsabilidade de **Profa. Dra. Maria Cecília Ferraz de Arruda Veiga / Carlos Eduardo da Silva Nossa Tuma**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **30 de junho de 2008**.

C E R T I F I C A T E

We certify that the protocol nº **1555-1**, entitled **"Influence of sex hormones in stress-induced analgesia"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **June 30, 2008**.

Campinas, 30 de junho de 2008.

Profa. Dra. Ana Maria Garaldo
Presidente

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ANEXO 2

Confirmação de submissão do artigo (Capítulo 2)

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