UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

LUANA FISCHER

AVALIAÇÃO DA INFLUÊNCIA DOS HORMÔNIOS SEXUAIS NA NOCICEPÇÃO DA ARTICULAÇÃO TEMPOROMANDIBULAR DE RATOS E ESTUDO DOS MECANISMOS ENVOLVIDOS

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de Doutor em Odontologia, Área de Concentração em Fisiologia Oral.

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RESUMO

O objetivo deste estudo foi avaliar a influência dos hormônios sexuais na nocicepção da articulação temporomandibular (ATM) de ratos e os possíveis mecanismos envolvidos. A injeção de formalina na ATM em uma concentração (0,5%) que não induziu nocicepção em machos intactos, induziu em machos gonadectomizados e em fêmeas intactas, o que sugere que os níveis fisiológicos de testosterona diminuem o risco de machos desenvolverem nocicepção da ATM. A resposta nociceptiva induzida pela injeção de uma alta concentração de formalina (1,5%) na ATM de machos é significativamente menor que àquela induzida na ATM de fêmeas em diestro, fase do ciclo estral com baixos níveis de estrógeno, mas semelhante àquela induzida na ATM de fêmeas em proestro, fase do ciclo estral com altos níveis de estrógeno. Esse resultado sugere que a nocicepção da ATM, em fêmeas, é exacerbada durante a fase do ciclo estral em que os níveis de estrógeno estão baixos. A administração sistêmica de estrógeno ou progesterona em fêmeas gonadectomizadas e de testosterona em machos gonadectomizados reduz a resposta nociceptiva induzida pela injeção de formalina na ATM. A influência do sexo e dos hormônios ovarianos na nocicepção induzida pela injeção de formalina ou de glutamato na ATM foi exatamente a mesma, o que demonstra que o efeito antinociceptivo dos hormônios ovarianos na ATM não é estritamente relacionado a nocicepção induzida pela formalina. A semelhança entre estudos clínicos e os resultados obtidos utilizando estes dois agentes nociceptivos sugere que o modelo comportamental de nocicepção da ATM pode ser útil e confiável para estudar os mecanismos envolvidos no efeito antinociceptivo dos hormônios sexuais na ATM de ratos. A administração de drogas no líquido cefalorraquidiano da região de complexo sensorial trigeminal também é útil para o estudo desses mecanismos, mas o procedimento cirúrgico realizado para a implantação do cateter usado para a injeção pode afetar a expressão dos comportamentos relacionados a nocicepção orofacial. Portanto, a técnica que permite a injeção direta de drogas nessa região, sem a necessidade de procedimentos cirúrgicos contribui para o estudo dos mecanismos envolvidos no efeito antinociceptivo dos hormônios sexuais na ATM de ratos. A administração, por meio dessa técnica, do antagonista de receptores opióides naloxona no espaço subaracnóide da região do complexo sensorial trigeminal bloqueou o efeito antinociceptivo induzido pelos níveis

fisiológicos de estrógeno em fêmeas em proestro e pela administração sistêmica de estrógeno ou progesterona em fêmeas gonadectomizadas e de testosterona em machos o gonadectomizados. No entanto, a co-administração de naloxona e formalina na ATM bloqueou o efeito antinociceptivo da progesterona e da testosterona, mas não do estrógeno. Esses dados sugerem que mecanismos opióides centrais medeiam o efeito antinociceptivo do estrógeno, da progesterona e da testosterona, enquanto mecanismos opióides periféricos também medeiam o efeito antinociceptivo da progesterona e da testosterona. A administração local de estrógeno, conjugado ou não com a albumina plasmática, na ATM de fêmeas reduziu significativamente a nocicepção induzida pela formalina. Como o estrógeno conjugado com a albumina tem ação restrita a receptores de membrana, esse dado sugere que o estrógeno reduz a nocicepção através de uma ação periférica não genômica. O efeito antinociceptivo do estrógeno foi bloqueado pelo antagonista de receptores estrogênicos ICI 182 780 e pelos inibidores da óxido nítrico sintase, L-NNA, e da guanilato ciclase, ODQ, mas não pelo antagonista de receptores opióides, naloxona. Esse dado sugere que o efeito antinociceptivo periférico do estrógeno é mediado pela ativação da via do óxido nítrico/GMP cíclico. Juntos, os resultados desse estudo demonstram que os níveis fisiológicos de testosterona diminuem o risco de ratos desenvolverem nocicepção da ATM e os de estrógeno diminuem a nocicepção da ATM em ratas. Além disso, a nocicepção da ATM também é diminuída pela administração sistêmica de estrógeno ou progesterona em ratas e de testosterona em ratos. O efeito antinociceptivo dos hormônios sexuais é mediado por mecanismos opióides centrais, enquanto mecanismos opióides periféricos medeiam o efeito da progesterona e da testosterona, mas não do estrógeno. De fato, a administração de estrógeno na ATM reduz a nocicepção através de um mecanismo periférico não genômico, mediado pela ativação da via do óxido nítrico-GMPc, mas não pela ativação do sistema opióide periférico.

ABSTRACT

The aim of this study was to evaluate the effect of sex hormones on temporomandibular joint (TMJ) nociception in rats and the possible mechanisms underlying their effect. The TMJ injection of 0.5% formalin induced nociception in intact females and gonadectomized males, but not in intact males, suggesting that the physiological level of testosterone protect males by decreasing their probability to develop TMJ pain. A higher dose of formalin (1.5%) induced a nociceptive behavior response significantly higher in female rats during diestrus phase of the estrous cycle than in those during proestrus phase and male rats. Since estradiol serum level was higher in proestrus than in diestrus females, this finding suggests that during low estradiol level of the estrous cycle the TMJ nociception is increased in female rats. Systemic administration of estradiol or progesterone in gonadectomized females and of testosterone in gonadectomized males significantly decreased 1.5% formalin-induced TMJ nociception. The role of sex and ovarian hormones in formalin and glutamate-induced TMJ nociception was virtually the same, showing that the antinociceptive effect of ovarian hormones was not exclusively related to the nociception induced by formalin. The similarity between clinical studies and the present results, obtained by using two different nociceptive agents, suggests that the TMJ behavior model may be useful and reliable to study the mechanisms underling the antinociceptive effect of sex hormones in the TMJ. Drug delivery to the medullary cerebrospinal fluid is also useful to study these mechanisms, however, the surgical procedure for implantation of the catheter used for drug delivery may affect the expression of the nociceptive behaviors related to orofacial nociception. Therefore, the technique for direct drug delivery to the medullary cerebrospinal fluid, without catheter implantation, will contribute for the study of the mechanisms underling the antinociceptive effect of sex hormones in the TMJ. The administration, though this technique, of the opioid receptor antagonist naloxone in the medullary region blocked the antinociceptive effect of estradiol, progesterone and testosterone. However, the co-administration of naloxone with formalin into the TMJ blocked the antinociceptive effect of progesterone and testosterone, but not of estradiol. These findings suggest that central opioid mechanisms mediate the antinociceptive effect of estradiol, progesterone and testosterone, while peripheral opioid

mechanisms also mediated the antinociceptive effect of progesterone and testosterone. The local administration of estradiol, conjugated or not with the bovine serum albumin, significantly decreased formalin-induced TMJ nociception in female rats. Given that estradiol conjugated with bovine serum albumin is a membrane impermeable compound, these findings suggest that estradiol decreases TMJ nociception by a peripheral non-genomic mechanism. The antinociceptive effect of estradiol was blocked by an estrogen receptor antagonist and by a nitric oxide synthase and a guanilato cyclase inhibitors, but not by a opioid receptor antagonist. These findings suggest that estradiol decreases TMJ nociception in female rats through a peripheral activation of NO-cGMP signaling pathway. Taken together, the findings of this study suggest that the high physiological level of testosterone decreases the risk of male rats develop TMJ pain and that of estradiol decreases TMJ nociception in female rats. Furthermore, TMJ nociception was also decreased by systemic administration of estradiol or progesterone in female and of testosterone in male rats. The antinociceptive effect of sex hormones is mediated by central opioid mechanisms, while peripheral opioid mechanisms mediate the antinociceptive effect of progesterone and testosterone, but not of estradiol. In fact, the administration of estradiol in the TMJ decreases nociception by a peripheral non-genomic mechanism mediated by activation of the nitric oxide-cGMP signaling pathway, but not by opioid receptors.

SUMÁRIO

INTRODUÇÃO

Disfunções Temporomandibulares (DTMs) são condições dolorosas crônicas envolvendo os músculos da mastigação e/ou articulação temporomandibular (ATM) (Dworkin *et al.* 1990). Estudos epidemiológicos relatam que aproximadamente 50% da população apresenta sinais e/ou sintomas de DTM (Carlsson 1984) e que 8% a 15% desses indivíduos necessitam de tratamento (Lipton *et al.* 1993). A prevalência em mulheres é duas vezes maior que em homens e elas correspondem a 80% dos pacientes que procuram tratamento (Dworkin *et al.* 1990), pois a dor relacionada as DTMs é mais severa no sexo feminino (Carlsson and LeResche 1995).

A dor é definida como uma experiência multidimensional (Associação internacional para o estudo da dor, IASP) e como tal sofre influência de vários fatores biológicos e psicossociais. No entanto, a marcante diferença na resposta dolorosa clínica (Riley *et al.* 1998; Fillingim *et al.* 1999; Riley and Gilbert 2001; Aloisi 2003; Frot *et al.* 2004) e experimental (Cairns *et al.* 2001a; Vincler *et al.* 2001; Gaumond *et al.* 2002; Clemente *et al.* 2004; Smith *et al.* 2006) entre os sexos sugere que os hormônios sexuais estejam entre os principais fatores moduladores da dor. O adequado entendimento do efeito e dos mecanismos que medeiam a modulação da dor pelos hormônios sexuais é essencial para o desenvolvimento de futuras modalidades terapêuticas que, ao manejar a poderosa influência desses hormônios sobre a dor, alcancem um maior índice de sucesso.

A menor prevalência e severidade das DTMs e outras condições dolorosas no sexo masculino tem sido classicamente associada a um papel pró-nociceptivo dos hormônios ovarianos (LeResche 1997; Warren and Fried 2001; Craft *et al.* 2004; Cairns 2007). No entanto, estudos recentes, que vêm demonstrando um papel antinociceptivo desses hormônios, não suportam essa idéia (Ceccarelli *et al.* 2003; LeResche *et al.* 2003; Clemente *et al.* 2004; Gaumond *et al.* 2005; Smith *et al.* 2006; Fischer *et al. in press*). Provavelmente, o estudo da influência da testosterona, principal hormônio masculino, sobre os mecanismos nociceptivos contribuiria não só para solucionar parte das discrepâncias da literatura nessa área, mas também para o entendimento dos mecanismos responsáveis pela

menor prevalência e severidade da maioria das condições dolorosas crônicas no sexo masculino. De fato, os poucos estudos que investigaram a influência desse hormônio sobre a nocicepção apontam para um papel protetor da testosterona. Por exemplo, foi demonstrado que baixos níveis de testosterona estão associados ao desenvolvimento e a manutenção de algumas condições dolorosas (Morales *et al.* 1994) e que em ratos submetidos a estímulos nociceptivos repetitivos, a testosterona induz adaptação progressiva com diminuição das respostas nociceptivas (Aloisi *et al.* 2003). Embora os efeitos da testosterona sobre a dor da ATM não sejam conhecidos, é possível que ela contribua para a menor prevalência e/ou para a menor severidade das DTMs no sexo masculino.

Inúmeros estudos têm sido delineados para avaliar influência dos hormônios sexuais sobre os mecanismos nociceptivos. De uma forma geral, eles apontam para uma maior resposta dolorosa experimental no sexo feminino, tanto em humanos (Riley *et al.* 1998; Fillingim *et al.* 1999; Riley and Gilbert 2001; Cairns *et al.* 2002; Zubieta *et al.* 2002; Aloisi 2003) quanto em animais (Gordon and Soliman 1994; Cairns *et al.* 2001a; Gaumond *et al.* 2002; Okamoto *et al.* 2003; Clemente *et al.* 2004; Gaumond *et al.* 2005; Fischer *et al. in press*) mas a unanimidade de opiniões termina quando começa a discussão a respeito do papel dos hormônios ovarianos sobre os mecanismos nociceptivos. A razão para as discrepâncias na literatura não é conhecida, mas o grande número de estudos conflitantes sugere que a modulação dos mecanismos nociceptivos pelos hormônios ovarianos seja um processo complexo e dinâmico. De fato, a característica mais marcante da fisiologia sexual feminina é a flutuação hormonal ao longo do ciclo reprodutivo, pois períodos de altos níveis hormonais são seguidos por quedas bruscas desses níveis. Nesse contexto, foi demonstrado que a dor da ATM em mulheres é maior durante períodos de baixo nível hormonal do ciclo menstrual (LeResche *et al.* 2003). Esse estudo sugere que baixos níveis de hormônios ovarianos estão associados ao aumento da dor da ATM e é aparentemente contraditório a um estudo anterior, dos mesmos autores, que demonstrou que a reposição hormonal em mulheres aumenta o risco de desenvolver DTM (LeResche *et al.* 1997). Para justificar a aparente contradição, os autores sugeriram que o fator responsável por aumentar o risco de desenvolver DTM não é o uso de hormônios, mas sim a interrupção de seu uso, pois na maioria dos casos de reposição hormonal interrompe-se o uso dos hormônios por

sete dias para permitir o sangramento menstrual mensal (LeResche *et al.* 2003). A maior sensibilidade dolorosa da ATM em mulheres durante o período de baixo nível hormonal do ciclo menstrual (LeResche *et al.* 2003) é consistente com dados experimentais obtidos em nosso laboratório que demonstraram que a resposta nociceptiva comportamental induzida pela injeção de formalina na região da ATM de ratas durante uma fase de baixo nível hormonal do ciclo estral é significativamente maior que aquela induzida em ratas durante uma fase de alto nível hormonal (Clemente *et al.* 2004). Juntos, esse estudo experimental (Clemente *et al.* 2004) e o estudo clínico (LeResche *et al.* 2003) sugerem que a dor da ATM, no sexo feminino, é diminuída durante o período de alto nível hormonal do ciclo reprodutivo. No entanto, ainda não se sabe qual é o hormônio ovariano que, em altos níveis, diminui a dor da ATM, ou se a presença de ambos, estrógeno e progesterona, é necessária.

O uso experimental de formalina como agente nociceptivo é considerado um modelo altamente representativo da dor observada clinicamente em humanos (Tjolsen *et al.* 1992) e a semelhança entre resultados clínicos (LeResche *et al.* 2003) e experimentais (Clemente *et al.* 2004) sugere que o teste da formalina na ATM de ratos (Roveroni *et al.* 2001) é um bom modelo experimental para avaliar a influência e os mecanismos envolvidos no efeito dos hormônios sexuais na dor da ATM. A manipulação hormonal, através da gonadectomia e da administração de hormônios também é um procedimento experimental útil para estudar esses mecanismos porque permite avaliar separadamente o efeito de cada hormônio, bem como da depleção hormonal. Além disso, a administração de hormônios induz um nível sérico constante que evita as influências relacionadas à liberação cíclica em fêmeas e permite induzir um nível sérico mais elevado, facilitando a detecção dos possíveis mecanismos envolvidos no efeito hormonal.

A administração de drogas no líquido cefalorraquidiano na região do subnúcleo caudal trigeminal é um procedimento experimental essencial para o estudo dos mecanismos nociceptivos da região orofacial e requer a implantação cirúrgica de um cateter posicionado na região do subnúcleo caudal trigeminal. A cirurgia pode ser realizada na região dorsal do pescoço, e nesse caso o cateter é implantado diretamente sobre a região do subnúcleo caudal (Flores *et al.* 2001; Tambeli *et al.* 2001; Wang *et al.* 2002), ou pode ser realizada na

região lombar, o que requer que o cateter seja avançado, através da medula espinhal, até a região do subnúcleo caudal (Grabow and Dougherty 2001). Ambos procedimentos são trabalhosos e causam sofrimento ao animal que necessita de vários dias para se recuperar da cirurgia e muitas vezes apresenta seqüelas, como dano motor. O número de animais envolvidos no experimento é elevado, pois alguns morrem após a cirurgia e aqueles que apresentaram dano motor têm de ser descartados. Além disso, a cirurgia na região do pescoço, causa desinserção, fibrose e dor nos músculos do pescoço, essenciais para a expressão dos comportamentos nociceptivos relacionados a dor orofacial, como o "flinch" de cabeça e o ato de coçar da região orofacial. Dessa forma, o desenvolvimento de técnicas para a administração de drogas na região do subnúcleo caudal que otimizem o trabalho experimental e não interfiram nos comportamentos nociceptivos orofaciais contribuirá para o estudo dos mecanismos envolvidos no efeito dos hormônios sexuais na dor da ATM, uma vez que como mencionado, o teste comportamental da formalina na ATM é um bom modelo experimental para esse estudo, pois replica dados clínicos.

Inúmeros mecanismos poderiam mediar o efeito dos hormônios sexuais sobre a nocicepção da ATM. Receptores para esses hormônios estão amplamente distribuídos na região da ATM (Aufdemorte *et al.* 1986; Abubaker *et al.* 1993; Yamada *et al.* 2003), nas fibras nociceptivas periféricas (Keast and Gleeson 1998; Koenig *et al.* 2000; Puri *et al.* 2005) e em regiões do sistema nervoso central que reconhecidamente participam da transmissão e modulação da informação nociceptiva (McEwen 2001). O sistema opióide está entre os mais poderosos mecanismos endógenos para o controle da dor (Stein *et al.* 2003) e diversos estudos têm relacionado alguns efeitos dos hormônios sexuais à ativação do sistema opióide. Por exemplo, esses hormônios são conhecidos por aumentar a expressão dos peptídeos (Johansson *et al.* 1997; Amandusson *et al.* 1999; Foradori *et al.* 2005; Bernardi *et al.* 2006) e dos receptores opióides (Hammer and Bridges 1987; Petersen and LaFlamme 1997; Quinones-Jenab *et al.* 1997; Harris *et al.* 2004). Um estudo recente demonstrou que altos níveis de estrógeno em mulheres estão associados à diminuição da dor experimental induzida no músculo mastigatório masseter e ao aumento da atividade do sistema opióide endógeno (Smith *et al.* 2006). Em ratas, foi demonstrado que os altos níveis hormonais durante a gestação diminuem a nocicepção através da ativação do sistema

opióide endógeno no sistema nervoso central (Dawson-Basoa and Gintzler 1993) e na periferia (Arthuri *et al.* 2005). No entanto, ainda não se sabe se o efeito antinociceptivo induzido pelos hormônios sexuais na ATM é mediado pela ativação do sistema opióide endógeno, e caso seja, se essa ativação se dá no sistema nervoso central ou na periferia.

Independente dos mecanismos envolvidos no efeito antinociceptivo dos hormônios sexuais, esses hormônios induzem todos os seus efeitos através de dois modos de ação: genômico e não genômico (Simoncini and Genazzani 2003). Os mecanismos genômicos envolvem a ativação de receptores nucleares que controlam a expressão gênica, e por isso induzem seus efeitos dias a horas após sua ativação (Simoncini and Genazzani 2003). Os mecanismos não genômicos são mediados pela ativação de receptores localizados na membrana plasmática e medeiam efeitos rápidos, como a ativação de vias de segundos mensageiros (Moss *et al.* 1997; Kelly *et al.* 1999) e modulação de canais iônicos (Mermelstein *et al.* 1996; Chaban *et al.* 2003). Entre os hormônios sexuais, o mais estudado é sem dúvida o estrógeno, 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; Chaban and Micevych 2005), o que sugere um efeito antinociceptivo não genômico periférico do estrógeno. Entre as vias de segundos mensageiros ativadas pelos receptores estrogênicos de membrana está a via da L-arginna – óxido nítrico – GMPc (Caulin-Glaser *et al.* 1997; Lantin-Hermoso *et al.* 1997; Simoncini *et al.* 2004). Essa via envolve a síntese de óxido nítrico a partir do aminoácido L-arginina pela enzima óxido nítrico sintase. O óxido nítrico formado ativa a enzima guanilato ciclase que converte a guanosina trifosfato (GTP) em guanosina monofosfato cíclico (GMPc). O GMPc, um segundo mensageiro com inúmeras funções fisiológicas (Lucas *et al.* 2000), é o produto final dessa via e o aumento de seus níveis periféricos tem sido associado a um efeito antinociceptivo (Durate *et al.* 1990; Qian *et al.* 1996; Cunha *et al.* 1999; Sachs *et al.* 2004; Almeida and Duarte 2007). Um dos mecanismos responsáveis pelo aumento dos níveis periféricos de GMPc é a ativação de mecanismos opióides periféricos (Granados-Soto *et al.* 1997; Pol 2007), que também parece estar envolvida no efeito antinociceptivo induzido, na ATM de ratas, pelos altos níveis hormonais da gestação. Dessa forma, é possível que o estrógeno reduza a nocicepção induzida pela injeção de formalina na ATM

de ratos por meio de um mecanismo não-genômico periférico, e que esse mecanismo seja mediado pela ativação da via L-arginna – óxido nítrico – GMPc e do sistema opióide.

Diante do exposto, o objetivo deste estudo foi avaliar a influência dos hormônios sexuais na nocicepção da ATM de ratos e os possíveis mecanismos envolvidos. Para tanto nós (1) Avaliamos o efeito da testosterona na nocicepção da ATM, verificando se ela reduz o risco de desenvolver nocicepção na ATM e se ela reduz a nocicepção da ATM já instalada; (2) Avaliamos o efeito do sexo e dos hormônios ovarianos na nocicepção da ATM, comparando a resposta nociceptiva de machos e fêmeas em fases de alto e baixo nível hormonal do ciclo estral e verificando o efeito da depleção dos hormônios ovarianos pela ovariectomia e de sua administração concomitante ou isolada; (3) Desenvolvemos uma técnica de administração de drogas na região de subnúcleo caudal trigeminal que melhora as condições experimentais e facilita o estudo comportamental dos mecanismos envolvidos no efeito dos hormônios sexuais sobre a nocicepção da ATM; (4) Avaliamos o envolvimento do sistema opióide endógeno central e periférico no efeito dos hormônios sexuais sobre a nocicepção da ATM; (5) Avaliamos o efeito periférico do estrógeno sobre a nocicepção da ATM, verificando se ele afeta a nocicepção por meio de um mecanismo periférico não genômico e se esse mecanismo depende da ativação da via do Óxido nítrico – GMPc e de receptores opióides.

Conforme deliberação da Comissão Central de Pós-graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP) nº 001/98, o presente estudo está apresentado em formato alternativo, contendo como capítulos artigos publicados ou submetidos a publicação em periódicos internacionais.

CAPÍTULOS

Capítulo 1**- The protective role of testosterone in the development of temporomandibular joint pain**. Artigo publicado no periódico The Journal of Pain, 2007 May;8(5):437-42.

Capítulo 2- **The effect of sex and ovarian hormones on temporomandibular joint nociception in rats.** Artigo aceito para publicação no periódico The Journal of Pain.

Capítulo 3- **A novel method for subarachnoid drug delivery in the medullary region of rats.** Artigo publicado no periódico Neuroscience Methods, 2005 Oct 30;148(2):108-12.

Capítulo 4- **The role of endogenous opioid system in sex hormones-induced TMJ antinociception.** Artigo submetido para publicação no periódico Neuroscience Letters.

Capítulo 5- **Peripheral mechanisms involved in estradiol -induced TMJ antinociception in female rats.** Artigo submetido para publicação no periódico Neuroscience.

Capítulo 1

The protective role of testosterone in the development of temporomandibular joint pain

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Original Article

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Abstract

The lower prevalence of many pain conditions, including temporomandibular dysfunctions in men than in women, has not as yet been clarified. The aim of this study was to investigate the effect of testosterone on the risk of developing temporomandibular joint (TMJ) pain and on acute persistent TMJ pain. The TMJ formalin test was used as experimental assay in the rat. Intra-TMJ 0.5% formalin induced a significant nociceptive behavior in naïve females and gonadectomized males, but not in naïve males, suggesting that naïve males have a lower risk of developing TMJ pain. The finding that the serum level of testosterone but not of estrogen and progesterone significantly decreased in gonadectomized males, suggests that testosterone is the hormone underlying the decreased naïve male's risk of developing TMJ pain. The magnitude of the nociceptive behaviors induced by intra-TMJ 1.5% formalin was similar in gonadectomized and naïve males. Therefore, in contrast to the protective role of testosterone in TMJ pain development, testosterone, at physiological serum levels, does not appear to modulate acute persistent TMJ pain induced by the TMJ injection of 1.5% formalin. At a supraphysiological serum level, however, testosterone significantly attenuated 1.5% formalin-induced nociception in males, but not in females. This antinociceptive effect was not mediated by estrogen derived from testosterone aromatization, because estrogen administration did not affect 1.5% formalin-induced TMJ nociception in gonadectomized males.

Perspective

The present findings not only help to explain the lower prevalence of TMJ pain in males versus females but also show that testosterone reduces TMJ pain at supraphysiological serum levels.

Introduction

Temporomandibular dysfunctions are pain conditions of the masticatory muscles and temporomandibular joint (TMJ) (Dworkin and LeResche 1992; Denucci *et al.* 1996) with greater prevalence, severity and duration in women than in men (LeResche 1997). We have previously demonstrated that the injection of formalin into the rat's temporomandibular joint induces a behavioral nociceptive response significantly lower in male than in female rats (Clemente *et al.* 2004). Similar results were also obtained by the TMJ injection of other algogenic agents (Bereiter 2001; Cairns *et al.* 2001b; Okamoto *et al.* 2003). The cause of this lower sensitivity to experimentally-induced TMJ nociception in males than in females as well as the lower prevalence of many pain conditions (Unruh 1996) including TMJ pain (LeResche 1997) in men than in women, has not as yet been clarified. Although it might be attributed to a pronociceptive effect of ovarian hormones, (LeResche 1997; Cairns *et al.* 2001b; Craft *et al.* 2004) an antinociceptive effect of ovarian hormones has also been reported in animal (Clemente *et al.* 2004) and human (LeResche *et al.* 2003) studies. The aim of this study was to investigate if testosterone protects males by decreasing their risk of developing TMJ pain or by decreasing TMJ pain. Given that testosterone has been used in a wide range of therapeutic approaches other than hormone replacement therapy (Basaria *et al.* 2001) and also widely used by healthy individuals to enhance athletic performance and appearance (Brown 2005). we also investigated the effect of a supraphysiological dose of testosterone on TMJ pain. The TMJ formalin test (Roveroni *et al.* 2001) was used as experimental assay in the rat.

Material and Methods

Animals

This study was carried out in 200-300g male and female Wistar rats housed (five per cage) in a temperature-controlled room (23 ± 1°C) on a 12:12 light cycle, with food and water available *ad libitum*. The experiments were approved by the Committee on Animal Research of the University of Campinas and are in accordance with IASP guidelines for the study of pain in animals (Zimmermann 1983). Naïve, gonadectomized (Gx) and gonadectomized with sex hormone administered rats were used in experiments.

Gonadectomy

Three-week-old female rats were ovariectomized through bilateral upper flank incisions (Waynforth and Flecknell 1992b). The ovarian bundles were tied off with 4-O silk sutures and the ovaries removed. The fascia and the skin were closed with 5-O silk sutures. Three-week-old male rats were castrated through a single scrotal incision (Waynforth and Flecknell 1992b). The testicular bundles were ligated with 4-O silk sutures before removing the testes, and the skin closed with 5-O silk sutures. These procedures were carried out under anesthesia induced by an intramuscular injection of a mixture of ketamine (55mg/Kg) and xylazine (5.5mg/Kg). Gx rats were used in experiments 5–6 weeks after surgery. The efficacy of gonadectomy was verified in males by measuring testosterone serum level and in females by the observation of vaginal smears during seven days and by *post mortem* examination of uterine atrophy.

Hormonal manipulation

Testosterone propionate (1mg) (Campos *et al.* 2003) or 17β-estradiol (estrogen, 50μg/kg) (Gordon and Soliman 1996) was daily injected for seven days. At the seventh day, hormone injection was performed 1 hour prior to the TMJ injection of formalin. Hormones were obtained from Sigma Chemicals, St Louis, Missouri, USA and dissolved in propyleneglycol.

Nociceptive assay

Behavior test was performed during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23ºC (Rosland 1991). Before the experiments, each animal was manipulated for 7 days to be habituated to the experimental manipulation. On the day of the experiment, each animal was individually placed in a test chamber (30 x 30 x 30 cm mirrored-wood chamber with a glass at the front side) for a 15 min habituation period to minimize stress. Animals were briefly anesthetized by inhalation of halothane to allow the TMJ injection of 30µl of formalin or its vehicle (0.9% NaCl). Formalin solutions were prepared from commercially (Sigma) available stock formalin (an aqueous solution of 37% of formaldehyde) further diluted in 0.9% NaCl (saline) to concentrations of 0.5%, 1% or 1.5%. Each animal regained consciousness approximately 30 seconds after discontinuing the anesthetic and was returned to the test chamber for counting nociceptive responses during a 45-min observation period. The nociceptive response score was defined as the cumulative total number of seconds that the animal spent rubbing the orofacial region asymmetrically with the ipsilateral fore or hind paw plus the number of head flinches counted during the observation period as previously described (Roveroni *et al.* 2001). From a theoretical perspective, the occurrence of a given behavior is expressed as the proportion of time that the behavior occupies. Since head flinches followed a uniform pattern of 1s of duration, each flinch was expressed as 1s (Roveroni *et al.* 2001). The recording time was divided into 9 blocks of 5 minutes. Rats did not have access to food or water during the test and each animal was used once. At the conclusion of the experiment (45 minutes after TMJ formalin injection) animals were anesthetized by an intraperitoneal injection of a mixture of urethane (1g/kg) and α-chloralose (50mg/kg) and a cardiac puncture was performed to allow blood collection (to measure hormonal serum level), and the injection of Evans blue dye (1%), in order to visualize formalin-induced TMJ plasma extravasation upon *post-mortem* examination (Haas *et al.* 1992). This latter procedure allowed confirmation that the TMJ injection was restricted to the immediate TMJ region (Roveroni *et al.* 2001). Testosterone, estrogen and progesterone serum levels were determined by radioimmunoassay using hormone specific kits from Diagnostics System Laboratories, Inc.

Formalin (0.5%, 1% or 1.5%) or its vehicle (0.9% NaCl) was injected into the TMJ of naïve male rats.

Study design

The effect of the injection of increasing concentrations of formalin 0.0% (n = 6), 0.5% (n = 11), 1.0% (n = 6) and 1.5% (n = 6) into the TMJ of naïve males was first determined. Formalin (0.5% and 1.5%) data from naïve males were used for subsequent comparisons. To evaluate if male sex hormones, at physiological levels, protect males by decreasing their risk of developing TMJ pain, formalin at a concentration (0.5%) that did not induce nociception in naïve males was injected into the TMJ of Gx males (n = 6). To compare the risk of developing TMJ pain between males and females, 0.0% or 0.5% formalin was injected into the TMJ of naïve females (n = 6 per group). To evaluate if male sex hormones, at physiological serum levels, reduce persistent acute TMJ nociception, formalin at a concentration (1.5%) that induced nociception in naïve males was injected into the TMJ of Gx males (n = 6). To ensure that the response observed in Gx males is due to androgen, rather than to a non-androgen hormone (estrogen and progesterone) deficit, testosterone, estrogen and progesterone serum levels were measured in gonadectomized and naïve males (n = 6 per group). To evaluate the effect of supraphysiological serum levels of testosterone on TMJ nociception, 1.5% formalin was injected into the TMJ of Gx males (n = 7) and females (n = 6) pre-treated with testosterone or with its vehicle (n = 6 and 5, respectively). To evaluate if testosterone's effect in males could be mediated by an indirect action of estrogen derived from testosterone aromatization, 1.5% formalin was injected into the TMJ of Gx males pre-treated with estrogen (n = 7). Sham-operated and hormone's vehicle pre-treated Gx rats received 1.5% formalin into the TMJ and were used as controls (n=6 per group).

Statistics

To determine if there were significant differences ($p < 0.05$) between treatment groups presented in Figs. 1 (0, 0.5, 1.0 or 1.5% TMJ formalin in naïve males), 2A (0 or 0.5% TMJ formalin in naïve males and 0.5% TMJ formalin in Gx males), 3 (0 or 1.5%

TMJ formalin in naïve males and 1.5% TMJ formalin in Gx males) and 4A (1.5% TMJ formalin in Gx males plus testosterone, estrogen or its vehicle), one-way ANOVA was performed. If there was a significant between-subjects main effect of treatment group following one-way ANOVA, post-hoc contrasts, using the Tukey test, were performed to determine the basis of the significant difference. Data from Figs. 2B (0 or 0.5 TMJ formalin in naïve females) and 4B (1.5% TMJ formalin in Gx females plus testosterone or its vehicle), were analyzed by t test. The t test was also applied to detect significant differences in formalin-induced TMJ nociception between sham operated and naïve animals, between Gx animals with or without hormone's vehicle pre-treatment and to detect significant differences in testosterone, estrogen or progesterone serum level between naïve and Gx animals. Data from sham operated animals were not plotted in figures.

Results

The TMJ injection of formalin induced a significant dose-dependent nociceptive response in naïve males (Fig. 1). The dose of 0.5% formalin did not induce TMJ nociception in naïve males, but it did in Gx males (Fig. 2A) and naïve females (Fig. 2B), suggesting that naïve males rats have a lower risk of developing TMJ nociception than females and Gx males. Testosterone (2.12 $\pm$ 0.60 ng/ml), but not estrogen (35.00 $\pm$ 4.97 pg/ml) and progesterone (8.00 $\pm$ 0.97 ng/ml) serum level significantly decreased after gonadectomy (0.16 $\pm$ 0.06 ng/ml, 26.50 $\pm$ 2.56 pg/ml and 10.00 $\pm$ 1.63 ng/ml, respectively), suggesting that the protective effect observed in naïve males is due to testosterone rather than to a non-androgen hormone action.

The TMJ injection of 1.5% formalin induced similar nociceptive response in Gx and naïve males (Fig. 3), suggesting that testosterone, at physiological serum levels, does not attenuate TMJ nociception.

Exogenous testosterone administration significantly decreased TMJ 1.5% formalin-induced nociception in Gx males (Fig. 4A), but not in Gx females (Fig. 4B). This antinociceptive effect in males was induced by a supraphysiological serum level of testosterone (124.93 $\pm$ 40.58 ng/ml), and not by estrogen derived from testosterone aromatization, since exogenously administered estrogen did not affect formalin-induced TMJ nociception in Gx male rats. TMJ injection of formalin's vehicle (0.9% NaCl) induced similar nociceptive response in all experimental groups (Tukey's test, $p>0.05$, data not shown).

TMJ 1.5% formalin-induced nociception in sham-operated males (252.2 $\pm$ 23.9) and sham-operated females (417.6 $\pm$ 19.6) was similar to that of naïve males (281.4 $\pm$ 25.5) and naïve females (432.8 $\pm$ 24.5), respectively. Furthermore, TMJ 1.5% formalin-induced nociception in hormone's vehicle pre-treated Gx males (243.4 $\pm$ 40.8) and females (387.2 $\pm$ 21.9) was similar to that of Gx males (287.1 $\pm$ 30.7) and females (416.6 $\pm$ 20.3). Therefore, neither gonadectomy nor hormone's vehicle (propyleneglycol) pre-treatment affected TMJ formalin-induced nociception.

Discussion

In this study, we have shown that testosterone protects males by decreasing their risk of developing TMJ pain. Specifically, we have found that injection of formalin at a concentration (0.5%) that did not induce TMJ nociception in naïve males, induced a nociceptive behavior in Gx males and naïve females (Fig. 2A and B). Our findings suggest that the protective effect observed in naïve males is due to testosterone, since the serum level of testosterone, but not estrogen and progesterone significantly decreased after gonadectomy. Although testosterone is present in both sexes, the sex-specificity of its protective effect in males is not surprising, since the circulating testosterone levels in females are typically about 10% of those observed in males (Evans 2004). The protective effect of testosterone in male's nociceptive system has been supported by some clinical studies demonstrating that its deficit can contribute to the development and maintenance of some pain conditions (Morales *et al.* 1994). Although testosterone, at physiological serum levels, protects males from developing TMJ pain, it does not attenuate acute persistent TMJ pain, as shown by the lack of effect of gonadectomy on 1.5% formalin-induced TMJ nociception (Fig. 3). This finding is consistent with those of previous studies evaluating nociception in other body regions and reporting that nociception in males is not affected by gonadectomy (Ali *et al.* 1995; Cicero *et al.* 1996). In contrast to physiological serum levels, supraphysiological serum levels of testosterone significantly decreased TMJ nociception in Gx males (Fig. 4A), which is consistent with some studies showing that testosterone or other androgens, at increased serum levels, decreases pain in men (Isaacs *et al.* 1972; Wu and Weng 1993; Heintjes *et al.* 2004) and animals (Gaumond *et al.* 2005). Although most of the effects induced by testosterone are mediated by estrogen derived from testosterone aromatization, (Lombardi *et al.* 2001) our finding that estrogen pre-treatment did not affect TMJ nociception in males suggests that the antinociceptive effect of testosterone is mediated by the activation of androgen receptors rather than estrogen receptors. This antinociceptive effect of testosterone in males is sex-specific, as shown by the lack of effect of testosterone administration in Gx females. Given that testosterone plays important, but distinct roles in development and differentiation of male and female organ systems (Forest

1983), the antinociceptive effect of testosterone at increased serum levels probably depends on previous action of this hormone during development and maturation of the nervous system. In fact, it has been demonstrated that sex-specific responses to sex hormones are dependent on their action during critical periods of development (Heil 1999) and can be changed by post-natal gonadectomy and hormone manipulation (Heil 1999). Apparent discrepancies between our findings that testosterone does not affect *TMJ* nociception in females and the findings that it decreases formalin-induced *paw* nociception in females (Aloisi *et al.* 2004) may be due to the different body region used for formalin injection. According to that, it has been previously demonstrated that the effect of sex hormones on formalin-induced nociception in females is not the same in the orofacial region and in the paw of the same animal (Pajot *et al.* 2003). This effect could be explained by differential expression of proteins involved in receptor structures and/or in intracellular enzymatic activities or in primary afferent neurons (Pajot *et al.* 2003).

In summary, we showed that testosterone protects males by decreasing their probability of developing TMJ pain. This finding may help explain the lower prevalence and severity of many pain conditions (Unruh 1996) including TMDs (LeResche 1997) in males than in females. Understanding the mechanisms behind the protective effect of testosterone on the development of TMJ nociception will be very useful to establish more successful treatments. Further studies are needed to evaluate a potential therapeutic use of testosterone in some persistent TMD pain conditions.

References

Ali BH, Sharif SI, Elkadi A. Sex differences and the effect of gonadectomy on morphine-induced antinociception and dependence in rats and mice. Clin Exp Pharmacol Physiol 1995;22(5):342-344.

Aloisi AM, Ceccarelli I, Fiorenzani P, De Padova AM, Massafra C. Testosterone affects formalin-induced responses differently in male and female rats. Neurosci Lett 2004;361(1-3):262-264.

Basaria S, Wahlstrom JT, Dobs AS. Clinical review 138: Anabolic-androgenic steroid therapy in the treatment of chronic diseases. J Clin Endocrinol Metab 2001;86(11):5108-5117.

Bereiter DA. Sex differences in brainstem neural activation after injury to the TMJ region. Cells Tissues Organs 2001;169(3):226-237.

Brown JT. Anabolic steroids: what should the emergency physician know? Emerg Med Clin North Am 2005;23(3):815-826, ix-x.

Cairns BE, Sessle BJ, Hu JW. Temporomandibular-evoked jaw muscle reflex: role of brain stem NMDA and non-NMDA receptors. Neuroreport 2001;12(9):1875-1878.

Campos M, Morais Pde L, Pupo AS. Effects of castration and of testosterone replacement on alpha(1)-adrenoceptor subtypes in the rat vas deferens. Eur J Pharmacol 2003;471(2):149-155.

Cicero TJ, Nock B, Meyer ER. Gender-related differences in the antinociceptive properties of morphine. J Pharmacol Exp Ther 1996;279(2):767-773.

Clemente JT, Parada CA, Veiga MC, Gear RW, Tambeli CH. Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint. Neurosci Lett 2004;372(3):250-255.

Craft RM, Mogil JS, Aloisi AM. Sex differences in pain and analgesia: the role of gonadal hormones. Eur J Pain 2004;8(5):397-411.

Denucci DJ, Dionne RA, Dubner R. Identifying a neurobiologic basis for drug therapy in TMDs. J Am Dent Assoc 1996;127(5):581-593.

Dworkin SF, LeResche L. Research diagnostic criteria for temporomandibular disorders: review, criteria, examinations and specifications, critique. J Craniomandib Disord 1992;6(4):301-355.

Evans NA. Current concepts in anabolic-androgenic steroids. Am J Sports Med 2004;32(2):534-542.

Forest MG. Role of androgens in fetal and pubertal development. Horm Res 1983;18(1-3):69-83.

Gaumond I, Arsenault P, Marchand S. Specificity of female and male sex hormones on excitatory and inhibitory phases of formalin-induced nociceptive responses. Brain Res 2005;1052(1):105-111.

Gordon FT, Soliman MR. The effects of estradiol and progesterone on pain sensitivity and brain opioid receptors in ovariectomized rats. Horm Behav 1996;30(3):244-250.

Haas DA, Nakanishi O, MacMillan RE, Jordan RC, Hu JW. Development of an orofacial model of acute inflammation in the rat. Arch Oral Biol 1992;37(5):417-422.

Heil SH. Activational and organizational actions of gonadal hormones and the sex-specific effects of prolactin on food intake by rats. Dev Psychobiol 1999;35(1):61-67.

Heintjes E, Berger MY, Bierma-Zeinstra SM, Bernsen RM, Verhaar JA, Koes BW. Pharmacotherapy for patellofemoral pain syndrome. Cochrane Database Syst Rev 2004(3):CD003470.

Isaacs WA, Effiong CE, Ayeni O. Steroid treatment in the prevention of painful episodes in sickle-cell disease. Lancet 1972;1(7750):570-571.

LeResche L. Epidemiology of temporomandibular disorders: implications for the investigation of etiologic factors. Crit Rev Oral Biol Med 1997;8(3):291-305.

LeResche L, Mancl L, Sherman JJ, Gandara B, Dworkin SF. Changes in temporomandibular pain and other symptoms across the menstrual cycle. Pain 2003;106(3):253-261.

Lombardi G, Zarrilli S, Colao A, Paesano L, Di Somma C, Rossi F, De Rosa M. Estrogens and health in males. Mol Cell Endocrinol 2001;178(1-2):51-55.

Morales AJ, Nolan JJ, Nelson JC, Yen SS. Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age. J Clin Endocrinol Metab 1994;78(6):1360-1367.

Okamoto K, Hirata H, Takeshita S, Bereiter DA. Response properties of TMJ units in superficial laminae at the spinomedullary junction of female rats vary over the estrous cycle. J Neurophysiol 2003;89(3):1467-1477.

Pajot J, Ressot C, Ngom I, Woda A. Gonadectomy induces site-specific differences in nociception in rats. Pain 2003;104(1-2):367-373.

Rosland JH. The formalin test in mice: the influence of ambient temperature. Pain 1991;45(2):211-216.

Roveroni RC, Parada CA, Cecilia M, Veiga FA, Tambeli CH. Development of a behavioral model of TMJ pain in rats: the TMJ formalin test. Pain 2001;94(2):185-191.

Unruh AM. Gender variations in clinical pain experience. Pain 1996;65(2-3):123-167.

Waynforth H, Flecknell P. Experimental and surgical technique in the rat. Academic, London 1992.

Wu SZ, Weng XZ. Therapeutic effects of an androgenic preparation on myocardial ischemia and cardiac function in 62 elderly male coronary heart disease patients. Chin Med J (Engl) 1993;106(6):415-418.

Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16(2):109-110.

Legends

Figure 1- Dose-dependent nociceptive behavior induced by the injection of formalin into the TMJ region of male rats.

Injection of formalin into the TMJ region of male rats induced nociception from the concentration of 1%. The symbol "*" indicates a response significantly greater than that induced by 0% formalin (0.9% NaCl) and 0.5% formalin (Tukey test, $p < 0.05$). The symbol "+" indicates a response significantly greater than that induced by 1% formalin (Tukey test, $p < 0.05$). In this and subsequent figures, data are plotted as mean ± s.e.m. and group sample sizes are shown in parentheses; see Methods for additional details regarding data presentation and analysis.

Figure 2- Effect of testosterone on the development of TMJ nociception in male and female rats.

A- TMJ injection of 0.5% formalin induced nociceptive behavior in Gx, but not in naïve male rats. The symbol "*" indicates a response significantly greater than that of naïve male rats receiving 0.0% or 0.5% formalin into the TMJ region (Tukey test, $p < 0.05$). Data from naïve male rats are re-plotted from figure 1

B- TMJ injection of 0.5% formalin induced nociceptive behavior in naïve female rats. The symbol "*" indicates a response significantly greater than that of naïve female rats receiving 0.0% formalin into the TMJ region (t test, $p < 0.05$).

Abbreviation: Gx = gonadectomized.

Figure 3- Effect of gonadectomy on formalin-induced TMJ nociception in male rats.

TMJ injection of 1.5% formalin induced similar nociceptive behavior in Gx and naïve male rats. The symbol "*" indicates a response significantly greater than that of naïve male rats receiving 0% formalin (0.9% NaCl) into the TMJ region (Tukey test, $p < 0.05$).

Figure 4- Effect of pre-treatment with testosterone on formalin-induced TMJ nociception in male and female rats.

A- Testosterone but not estrogen significantly reduced 1.5% formalin-induced TMJ nociception in male rats. The symbol "*" indicates a nociceptive behavior significantly lower than that of Gx + V and Gx + E (Tukey test, $p < 0.05$).

B- Testosterone did not affect formalin-induced TMJ nociception in female rats. There was no significant difference between the response of Gx + V and Gx + T (t test, $p > 0.05$). Abbreviations: Gx = gonadectomized; Gx+V= gonadectomized plus vehicle (propyleneglycol) Gx + T = gonadectomized plus testosterone).

Figures

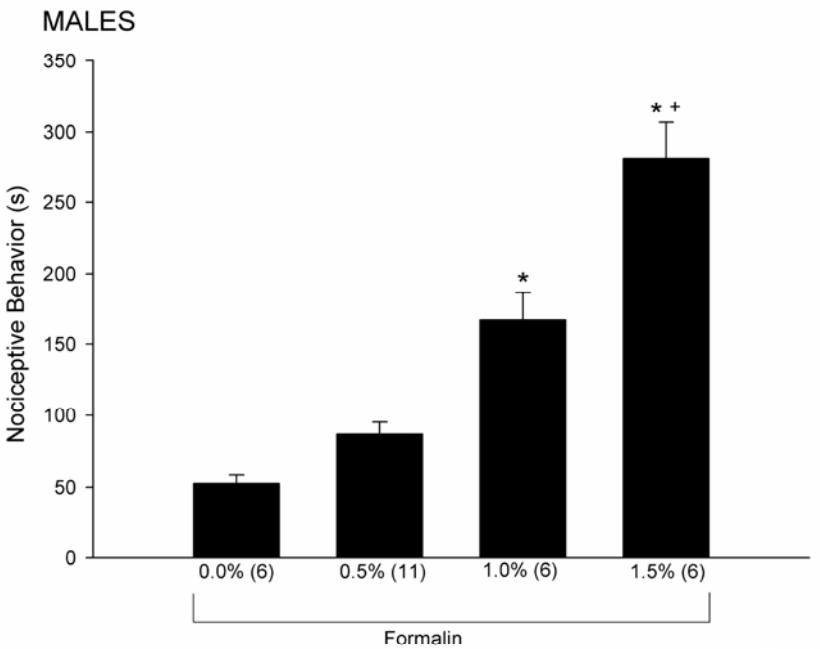
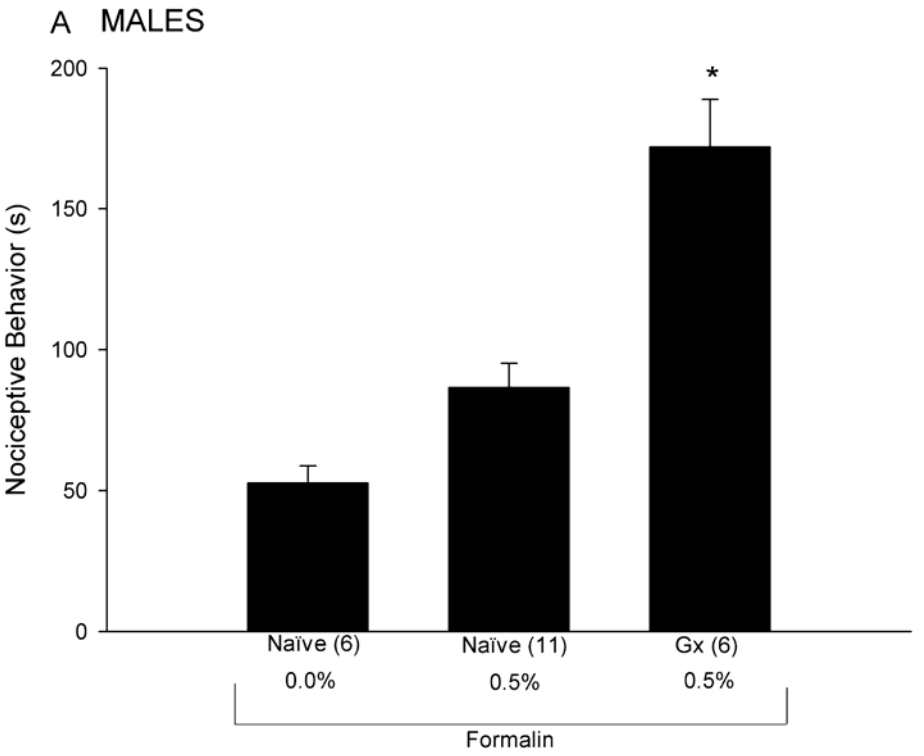


Fig 1


A MALES
Nociceptive Behavior (s)
200
150
100
50
0
*
Naïve (6)
0.0%
Naïve (11)
0.5%
Gx (6)
0.5%
Formalin

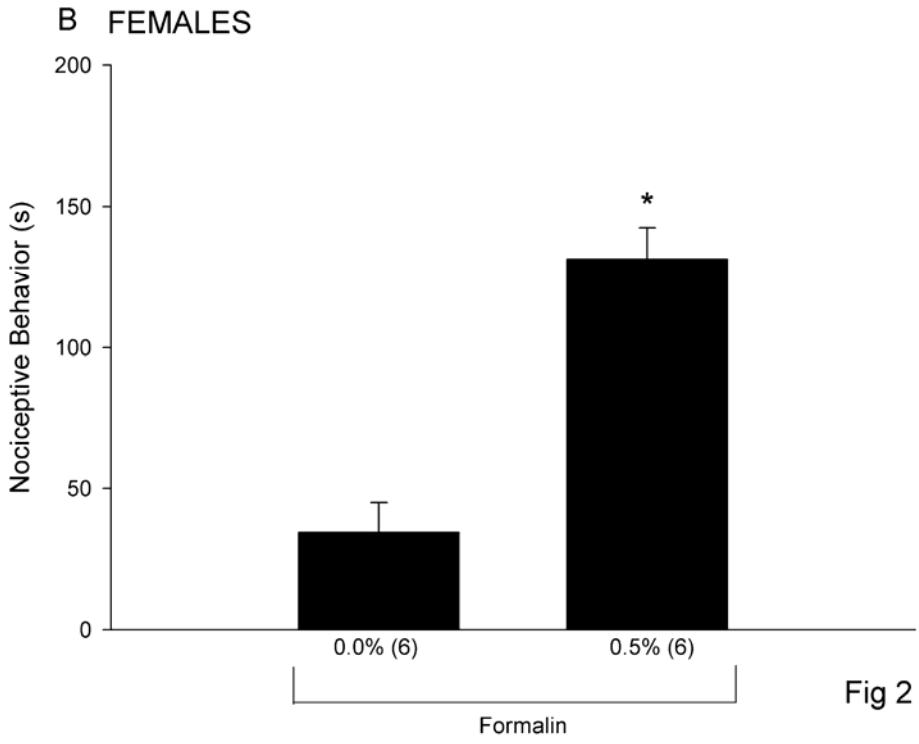

B FEMALES
Nociceptive Behavior (s)
200
150
100
50
0
*
0.0% (6)
0.5% (6)
Formalin

Fig 2

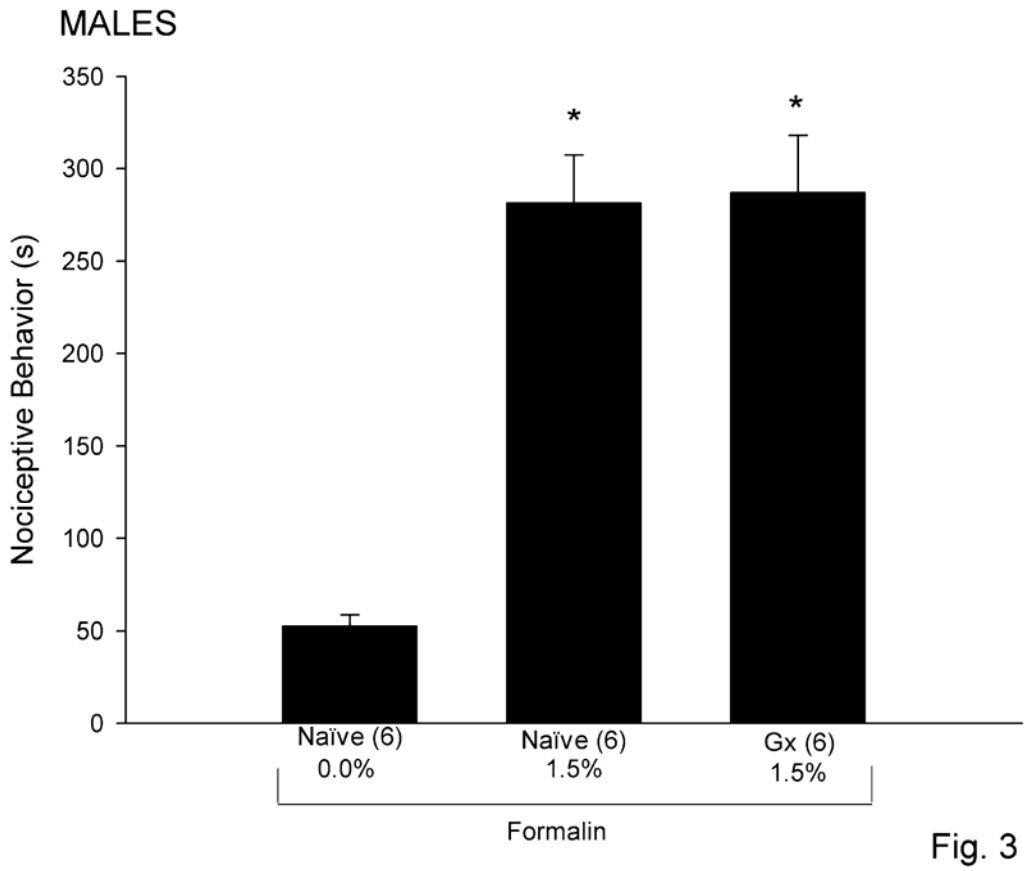
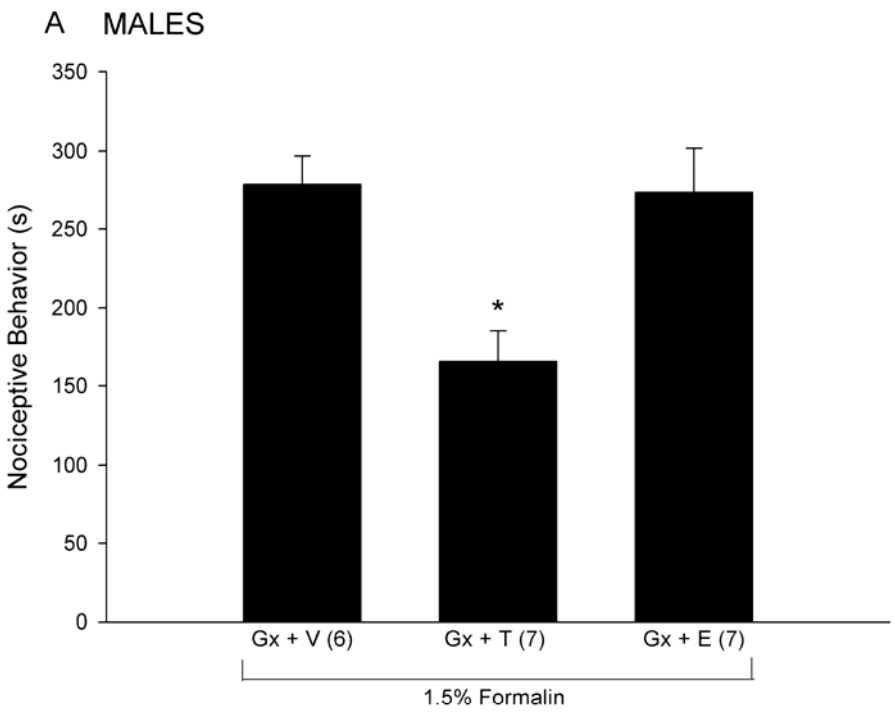

MALES
Nociceptive Behavior (s)
350
300
250
200
150
100
50
0
*
*
Naïve (6)
0.0%
Naïve (6)
1.5%
Gx (6)
1.5%
Formalin

Fig. 3


A MALES
Nociceptive Behavior (s)
350
300
250
200
150
100
50
0
*
Gx + V (6)
Gx + T (7)
Gx + E (7)
1.5% Formalin

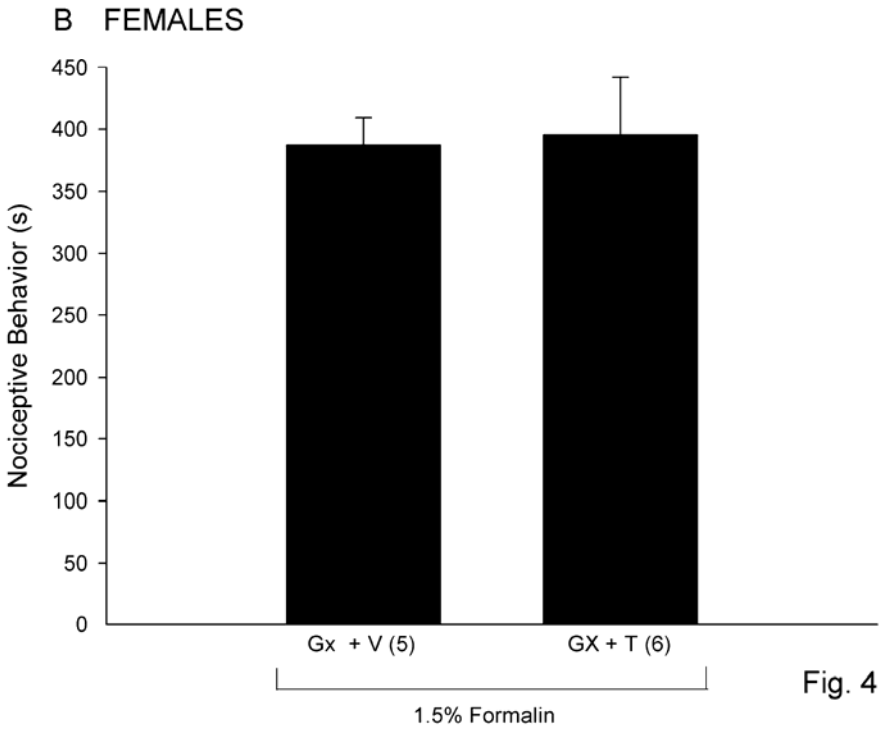

B FEMALES
Nociceptive Behavior (s)
450
400
350
300
250
200
150
100
50
0
Gx + V (5)
GX + T (6)
1.5% Formalin

Fig. 4

Capítulo 2

The effect of sex and ovarian hormones on temporomandibular joint nociception in rats

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Original Article

Keywords: Formalin; Glutamate; Estrogen; Progesterone; Temporomandibular joint pain; Sex differences

Abstract

The aim of this study was to investigate the influence of sex and ovarian hormones on formalin or glutamate-induced temporomandibular joint nociception in rats. The influence of sex and ovarian hormones on nociceptive behavior response induced by formalin or glutamate was virtually the same. The nociceptive behavior response of male rats was similar to that of female rats in the proestrus phase of the estrous cycle, but significantly lower than that of in the diestrus phase. Since estradiol but not progesterone serum level was significantly higher during proestrus than during diestrus, these data suggest that females with lower endogenous estradiol level have an exacerbation of temporomandibular joint nociception. The nociceptive behavior response of ovariectomized rats was similar to that of diestrus females and significantly greater than that of proestrus females. While the administration of estradiol or progesterone in ovariectomized females significantly reduced temporomandibular joint nociception to the level observed in proestrus females, the combination of both hormones did not increase the antinociceptive effect induced by each of them, suggesting that they decrease temporomandibular joint nociception in an independent way.

Perspective:

We reported that ovarian hormones have an antinociceptive effect on the temporomandibular joint formalin and glutamate nociceptive behavior models. Therefore, the greater prevalence and severity of temporomandibular joint pain in women of reproductive age may be a consequence of hormonal fluctuation during reproductive cycle, in that during low endogenous estradiol serum level temporomandibular joint pain sensitivity is increased enhancing the risk of females experiencing temporomandibular joint pain.

Introduction

Temporomandibular disorders (TMDs) are musculoskeletal pain conditions characterized by pain in the temporomandibular joint (TMJ) and/or the muscles of mastication. The higher prevalence and severity of TMD in women than in men (Carlsson and LeResche 1995) suggests that gonadal hormones may play a role in these pain conditions. We have recently proposed that this prevalence pattern may be a consequence of a protective effect of testosterone. Specifically, TMJ injection of 0.5% formalin induced a significant nociceptive behavior in naive female and gonadectomized male rats, but not in naive male rats, suggesting that testosterone decreases the risk of males developing TMJ pain (Fischer *et al.* 2007). Although evidences accumulated from experimental studies in humans (Cairns *et al.* 2001a), (Carlsson and LeResche 1995; Riley *et al.* 1998; Zubieta *et al.* 2002; Frot *et al.* 2004) and animals (Bereiter 2001; Cairns *et al.* 2002; Okamoto *et al.* 2003; Clemente *et al.* 2004) uniformly point that females are more sensitive to experimental pain than males, the role of ovarian hormones in TMJ nociception is still controversial. It has been demonstrated that TMJ pain is highest during lowest estradiol times of the menstrual cycle in women (LeResche *et al.* 2003), a finding that parallels our previous data obtained with the TMJ formalin behavior model in female rats (Clemente *et al.* 2004). The lower TMJ pain during pregnancy in women (LeResche *et al.* 2005) also parallels the lower formalin–induced TMJ nociception in pregnant rats (Arthuri *et al.* 2005). However, it has been reported in electrophysiological studies using nociceptive agents other than formalin that estradiol appears to increase TMJ nociception (Cairns *et al.* 2002; Okamoto *et al.* 2003; Flake *et al.* 2005). For example, reflex jaw muscle activity evoked by injection of glutamate into the TMJ is higher in ovariectomized female rats (OVX) receiving estradiol administration than in those receiving vehicle (Cairns *et al.* 2002). The discrepancy between these studies is unknown, but the use of different nociceptive agents and models could contribute to that.

The influence of sex and ovarian hormones on TMJ nociception has not yet been investigated in a behavior model of TMJ nociception other than the TMJ formalin behavior model. Therefore we standardized the TMJ glutamate behavior model and used this model to compare the influence of sex and ovarian hormones on the nociceptive

behavior response induced by formalin and glutamate. Although our previous findings suggest that ovarian hormones attenuate TMJ nociception in the TMJ formalin behavior model it is not known if estrogen, progesterone or the combination of both hormones is necessary to mediate this effect. To answer this question we also investigated the influence of ovariectomy and exogenous administration of estrogen, progesterone or the combination of both hormones on both the TMJ formalin and TMJ glutamate behavior model.

Material and methods

Animals

This study was carried out in 200 - 300g male (n= 71) and female (n= 140) Wistar rats and in female Sprague-Dawley (n= 21) rats. All animal experimental procedures and protocols were approved by the Committee on Animal Research of the University of Campinas and are in accordance with IASP guidelines for the study of pain in animals (Zimmermann 1983). The animals were maintained on a temperature-controlled room (± 23ºC) and were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle (lights on at 06:00 A.M.) with food and water available ad libitum.

Estrous phase determination

Estrous phase was determined by daily microscope examination of vaginal smears taken by gentle lavage, between 7 and 8 a.m. Estrous phase was confirmed before and immediately after each experiment to ensure that the rats remained in the same phase during the experiment. Proestrus phase and the initial phase of diestrus (first 4 h) were identified by the predominance (>70%) of nucleated epithelial cells and leukocytes, respectively (Marcondes *et al.* 2002) in rats with at least two consecutive regular 4-5 day cycles. These phases were chosen because they represent phases of high and low ovarian hormonal level, respectively (Butcher *et al.* 1974).

Gonadectomy

Ovariectomy (45 days old females, (Gordon and Soliman 1994)) was performed through bilateral upper flank incisions. The ovarian bundles were tied off with 4-O silk sutures and the ovaries removed. The fascia and the skin were closed with 4-O silk sutures (Waynforth and Flecknell 1992a). Sham operated animals underwent a surgical procedure similar to that of OVX animals, except that the ovaries were not removed. The procedures were carried out under anesthesia induced by an intramuscular injection of a mixture of ketamine (55mg/Kg) and xylazine (5.5 mg/Kg). A subcutaneous injection of

ketoprofen (5 mg/kg) was used for post-operative analgesia (Roughan and Flecknell 2000). OVX and sham-operated rats were used in experiments when they were three months of age. The efficacy of ovariectomy was confirmed by the absence of estrous cycle determined via vaginal smear during ten days and by *post mortem* examination of uterine atrophy in animals that did not receive hormones.

Hormonal Manipulation

Hormonal manipulation was performed by daily injection of 17β-estradiol (50μg/kg, (Gordon and Soliman 1994)) and/or progesterone (8mg/Kg) (He *et al.* 2004) during seven days. At the seventh day, hormone injection was performed 1 hour prior to the experiment. Hormones were obtained from Sigma Chemicals, St Louis, Missouri, USA and dissolved in propyleneglycol.

Hormonal Determination

Estradiol and progesterone serum levels were determined by radioimmunoassay using hormone specific kits (DSL – 4400 and DSL – 3400, respectively) from Diagnostics System Laboratories, Inc., Texas USA.

Drugs

Formalin solution was prepared from commercially (Sigma) available stock formalin (an aqueous solution of 37% of formaldehyde) further diluted in 0.9% NaCl to a concentration of 1.5%. Glutamate 1M (Cairns *et al.* 2002); NBQX (1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo f quinoxaline-7-sulfonamide disodium salt), AMPA/ Kainate receptor antagonist 100 and 500μg (Beirith *et al.* 2002); AP-5 (D-2-Amino-5-phosphonovaleric acid), NMDA receptor antagonist, 20 and 100 μg (Cairns *et al.* 1998) and QX-314 (Lidocaine N-ethyl bromide), quaternary hydrophilic lidocaine derived, 2%, were obtained from Sigma and dissolved in 0.9% NaCl.

TMJ Injections

The animals were briefly anesthetized by inhalation of halothane to allow the TMJ injection, each animal regained consciousness approximately 30 s after discontinuing the anesthetic. The TMJ injection was performed with a 30-gauge needle introduced into the TMJ at the moment of injection. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50μl) (Roveroni *et al.* 2001). At the conclusion of the behavior test, each animal was anesthetized by an intraperitoneal injection of a mixture of urethane (1g/kg) and α-chloralose (50mg/kg). The Evans blue dye (5 mg/kg) was injected systemically, 15 minutes later the animals were submitted to cardiac perfusion with normal saline. Since this dye binds to plasma protein, the correct site of injection was indicated by the observation that the plasma extravasation induced by the TMJ injection of formalin or glutamate was restricted to the TMJ region (Haas *et al.* 1992). In females submitted to the formalin test blood was collected from the heart, before injection of the dye, to allow estradiol and progesterone serum level determination.

Testing procedure for TMJ pain

Behavior test was performed during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at ± 23ºC (Rosland 1991). The nociceptive response was assessed by an observer blinded to the experimental manipulation. Before the experiments, each animal was manipulated for 7 days in the test room (handled for approximately one minute) to be habituated to the experimental manipulation. On the day of the experiment, each animal was individually placed in a test chamber (30 x 30 x 30 cm mirrored-wood chamber with a glass at the front side) for a 15 min habituation period to minimize stress. After TMJ injection the animal was returned to the test chamber for counting nociceptive responses. The nociceptive behavior characterized by rubbing the orofacial region and flinching the head was counted in blocks of 5 minutes for 45 (formalin) or 30 (glutamate) minutes. For each block of 5 min, the behavior characterized by rubbing the orofacial region was quantified by the amount of time that the animal exhibited it and the behavior characterized by flinching the head was quantified by its occurrence. Considering that the head flinching behavior follows an uniform pattern of 1 s in duration, each flinching was

counted as 1 s as previously described (Roveroni et. al., 2001). Rats did not have access to food or water during the test and each animal was used once.

Experimental Design

To standardize a behavior model to study glutamate-induced TMJ nociception, 0.9% NaCl (glutamate vehicle, 10µl, n = 4) or Glutamate (1M, 10µl (Cairns *et al.* 2002), n = 11) was firstly injected into the TMJ of male rats. We chose to use glutamate 1M because it is the dose necessary to observe sex differences in jaw muscle activity induced by injection of glutamate into the TMJ (Cairns *et al.* 2002). The nociceptive behavior responses characterized by flinching the head and rubbing the orofacial region were quantified for 30 minutes. To demonstrate the nociceptive character of the behavior response, the quaternary hydrophilic lidocaine derived QX-314 (2%, 10µl, n = 6) was co-administered with glutamate into the TMJ. To demonstrate that glutamate-induced TMJ nociception is mediated by activation of glutamate receptors, the AMPA/kainate and the NMDA receptor antagonists NBQX (100 and 500µg, 10µl, n = 6) and AP-5 (20 and 100µg, 10µl, n = 4 and 7, respectively), was co-administered with glutamate into the TMJ or administered into the contralateral TMJ (n = 4 and 6, respectively). In subsequent experiments, Formalin (1.5%, 30µl), Glutamate (1M, 10µl) or their vehicle (0.9%NaCl, 30µl and 10µl, respectively), was unilaterally injected into the TMJ of male and female rats. To evaluate the effect of sexual dimorphism on formalin or glutamate-induced TMJ nociception we compared the nociceptive behavior responses between males (n = 6 and 11, respectively) and females during diestrus (low hormonal level (Butcher *et al.* 1974), n = 6 and 9, respectively) and proestrus (high hormonal level (Butcher *et al.* 1974), n = 6 and 13, respectively) phases of the estrous cycle. To evaluate the effect of ovarian hormones depletion by ovariectomy on formalin or glutamate-induced TMJ nociception we compared the behavioral nociceptive response between OVX (n = 6 and 12, respectively), sham-operated diestrus (n = 6 and 6, respectively) and sham-operated proestrus (n = 6 and 12, respectively) females. To evaluate the differences in hormonal serum level between females, we measured the serum level of estradiol and progesterone in proestrus (n = 6), diestrus (n = 6), and OVX (n = 6) female rats with or without hormone administration. To

evaluate the effect of each ovarian hormone and the combination of both estradiol and progesterone on formalin-induced TMJ nociception, we compared the behavioral nociceptive response between OVX females receiving vehicle (n = 7), estradiol (n = 7), progesterone (n = 8) or the combination of both hormones (n = 5). Glutamate-induced TMJ nociception was also evaluated in OVX females receiving vehicle (n = 11), estradiol (n = 11) or progesterone (n = 9). Because many studies evaluating the role of estradiol in TMJ nociception have used Sprague–Dawley rather than Wistar rats, in this study we used Sprague–Dawley in addition to Wistar rats to evaluate glutamate-induced TMJ nociception in OVX rats receiving vehicle (n = 11) or estradiol (n = 10). This allowed us to evaluate if different strains of animals contributes to the discrepancies between studies evaluating the role of estradiol in TMJ nociception.

Statistical analysis

A two-way repeated-measures ANOVA with one between-subjects factor (i.e. treatment for groups showed in Figure 1A and gender or hormone for other groups compared) and one within-subjects factor (i.e. time) was used to determine if there were significant ($p \leq 0.05$) differences in nociceptive responses among the groups. For Figs. 1B-6, the area under the curve (AUC) was calculated for each treatment group by summing the behaviors recorded in each block of 5 min during the entire duration of the experiment. To determine if there were significant differences (p 0.05) between the treatment groups, one-way ANOVA using AUC as the dependent variable was performed. If there was a significant between-subjects main effect of treatment group, post-hoc contrasts, using the Student-Newman-Keuls method test, were performed to determine the basis of the significant difference. A t test was used to determine if there was a significant difference ($p< 0.05$) between groups showed in Fig. 6, between the administration of formalin and its vehicle, and between the administration of glutamate and its vehicle. Data are expressed in figures as means ± S.E.M.

Results

The TMJ injection of glutamate induced a behavior response significantly higher ($p<0.05$) than that induced by its vehicle and by the quaternary hydrophilic lidocaine derived QX-314, that lasted for 30 minutes. There is not a significant interaction ($p>0.05$) between treatment (i.e. TMJ injection of glutamate, vehicle (0.9% NaCl) or glutamate plus QX-314) and time (Fig. 1 A). Therefore, the effect of TMJ treatment does not depend on what level of time is present. The behavior response induced by TMJ glutamate was reversed by the co-administration of the quaternary hydrophilic lidocaine derived QX-314, confirming its nociceptive character. Glutamate-induced TMJ nociception was significantly reduced ($p<0.05$) by the co-administration of AMPA/Kainate or NMDA receptor antagonists, indicating that glutamate-induced TMJ nociception is mediated by activation of glutamate receptors (Fig. 1 B).

Injection of formalin or glutamate into the rat's TMJ induced a significant ($p<0.05$) group-dependent nociceptive behavior, which was not observed when vehicle (0.9% NaCl) was injected into the TMJ.

The influence of sex and ovarian hormones on the nociceptive behavior induced by formalin and glutamate was virtually the same. The nociceptive behavior induced by the TMJ injection of formalin (Fig. 2 A) or glutamate (Fig. 2 B) in male rats was similar to that of female rats in the proestrus phase of the estrous cycle and significantly lower ($p<0.05$) than that of diestrus females. There is not a significant interaction ($p>0.05$) between sex or estrous cycle phase and time in either the formalin or glutamate behavior model. Therefore, the effect of sex and estrous cycle phase does not depend on what level of time is present.

Formalin (Fig. 3 A) and glutamate (Fig. 3 B) induced TMJ nociception in OVX females was similar to that of sham-operated diestrus females and significantly greater ($p<0.05$) than that of sham-operated proestrus females.

Estradiol serum level was significantly higher ($p<0.05$) during proestrus than during diestrus (Fig. 4 A), while progesterone serum level was similar in these phases (Fig.

4 B). As expected, estradiol (Fig. 4 A) and progesterone (Fig. 4 B) serum level was significantly lower ($p<0.05$) in OVX than in proestrus and in diestrus females.

Estradiol or progesterone administration in OVX females significantly reduced ($p<0.05$) formalin (Fig. 5 A) and glutamate (Fig. 5 B) induced TMJ nociception. The combination of estradiol and progesterone did not increase the antinociceptive effect induced by each hormone by itself (Fig. 5 A) in the formalin test. Estradiol serum level in OVX females that received estradiol (mean ± S.E.M. 998.12 ± 96.69 pg/ml) and progesterone serum level in OVX females that received progesterone (81.10 ± 9.56 ng/ml) was significantly higher ($p<0.05$) than the estradiol and progesterone serum level of proestrus (68.28 ± 9,20 pg/ml and 33.0 ± 3.04 ng/ml, respectively) and diestrus (33,62 ± 2,63 pg/ml and 36,21 ± 2,56 ng/ml, respectively) females.

Estradiol administration in OVX Sprague-Dawley female rats also significantly decreased ($p<0.05$) glutamate-induced TMJ nociception (Fig. 6).

Discussion

In order to compare the influence of sex and ovarian hormones on TMJ nociception induced by glutamate or formalin we first standardized the TMJ glutamate behavior model. Like formalin (Roveroni *et al.* 2001), glutamate also induced nociceptive behaviors characterized by flinching the head and rubbing the orofacial region when injected into the TMJ.

The influence of sex and ovarian hormones on TMJ glutamate and TMJ formalin behavior models was virtually the same and it is supported by two important clinical findings about sex hormones modulation of TMJ pain: the higher severity of TMD pain in women than in men (Carlsson and LeResche 1995) and the lower TMJ pain in women during high estradiol times of the menstrual cycle (LeResche *et al.* 2003). The evidence is that TMJ formalin and TMJ glutamate-induced nociceptive behavior was significantly greater in diestrus female than in male and proestrus female rats. Since diestrus females showed similar progesterone but lower estradiol serum level than proestrus females, this finding suggests that females have an exacerbation of the TMJ nociception during low endogenous estradiol serum level. Additional support to the antinociceptive effect of estradiol on the TMJ of females was given by data from ovariectomized rats with or without estradiol administration. The nociceptive behavior induced by the TMJ injection of formalin or glutamate in OVX females was similar to that of diestrus females and significantly higher than that of proestrus females, while estradiol administration in OVX females significantly reduced TMJ nociception to the levels observed in proestrus females. Although progesterone administration in OVX females also decreased formalin and glutamate–induced TMJ nociception, the combination of both hormones did not enhance their antinociceptive effect in the formalin test, suggesting that the exogenous administration of each of these hormones decreases TMJ nociception in an independent way.

Our findings showing a greater formalin and glutamate-induced TMJ nociception in female than in male rats generally corroborate human studies showing that women experience more pain than men (Carlsson and LeResche 1995; Riley *et al.* 1998; Zubieta *et al.* 2002; Frot *et al.* 2004). For example, the injection of glutamate in the

masticatory muscle masseter induces significantly higher pain in women than in men (Cairns *et al.* 2001a). In another study that also evaluated experimental pain in the masseter muscle, but analyzed women during a high and a low estradiol state, the pain rates were significantly lower during the high estradiol state (Smith *et al.* 2006). This finding perfectly agrees not only with our present findings that diestrus females have higher TMJ nociception than proestrus females, but also with clinical studies showing greater TMJ pain during low-estradiol times in women (LeResche *et al.* 2003; LeResche *et al.* 2005). Although our findings together with clinical findings (LeResche *et al.* 2003; LeResche *et al.* 2005; Smith *et al.* 2006) 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 (Carlsson and LeResche 1995) and also more prevalent in women during reproductive age than in women outside this period (before menarche and after menopause) (Carlsson and LeResche 1995). The higher prevalence of TMJ pain in women than in men may be due to a protective effect of testosterone, as we have previously demonstrated (Fischer *et al.* 2007). Importantly, the mechanisms underlying the prevalence may not be the same to those underlying the severity of TMJ pain and in this study we have investigated the influence of sex and ovarian hormones in the severity and not in the prevalence of TMJ pain. Despite of that, women of reproductive age have physiological fluctuation in ovarian hormones serum levels, and we believe that this fluctuation might affect TMJ pain. According to that, it has been previously demonstrated that TMJ pain (LeResche *et al.* 2003) and migraine (Ashkenazi and Silberstein 2006) are exacerbated during rapid changes in hormonal serum level. Thus, we believe that during the reproductive cycle, the fluctuations of ovarian hormones increases pain sensitivity enhancing the risk of female experiencing TMJ pain

However, the literature regarding the role of ovarian hormones in TMJ pain is controversial. For example, data obtained by Fos-positive neurons (Bereiter 2001), single units recording of primary afferents (Flake *et al.* 2005) and neurons of the trigeminal subnucleus caudalis (Okamoto *et al.* 2003) in rats suggest that ovarian hormones increase TMJ nociception. Similarly, estradiol administration in OVX rats significantly increases jaw muscle activity evoked by injection of Glutamate into the TMJ (Cairns *et al.* 2002).

Although the discrepancy between the current study and these previous animal studies is unknown many factors could contribute to that. One possible factor is the use of different nociceptive agents. However, the similarity between the effect of sex and ovarian hormones on formalin and glutamate-induced TMJ nociception does not support this possibility, whereas it does not completely exclude it. Therefore, we are further investigating this possibility through the use of other nociceptive agents. Another possible factor is the use of different strain of rats since comparative studies among different strains of rats have demonstrated that genetic factors affect nociception in different nociceptive models (Benoliel *et al.* 2002a; Vendruscolo *et al.* 2004; Herradon *et al.* 2007). More importantly, the influence of sex and sex hormones in nociception may differ in different strains of rats (DeLeo and Rutkowski 2000; Vendruscolo *et al.* 2004). Thus, because the studies suggesting a pronociceptive effect of ovarian hormones in the TMJ (Bereiter 2001; Cairns *et al.* 2002; Okamoto *et al.* 2003; Flake *et al.* 2005) have used Sprague-Dawley rats while we have used Wistar rats, we have investigated if the discrepant results could be due to the use of different strains of rats. However, our finding that estradiol administration also decreased glutamate-induced TMJ nociception in Sprague-Dawley female rats does not support this possibility. Finally, another possible factor that could also contribute to the discrepancy between the current study and these previous animal studies is the state of consciousness of the animal. While these previous studies have used anesthetized rats to evaluate the role of ovarian hormones in TMJ nociception (Bereiter 2001; Cairns *et al.* 2002; Okamoto *et al.* 2003; Flake *et al.* 2005), we have used awaked rats. The evidence that support this latter possibility is that the use of different general anesthetics modifies the electrophysiological properties of neurons from areas of the central nervous system involved in pain transmission and modulation (Heym *et al.* 1984; Collins and Ren 1987; Kuroiwa *et al.* 1991; Oliveras *et al.* 1991; McGaraughty *et al.* 1995; Montagne-Clavel *et al.* 1995; Shaw *et al.* 2001). For example, animals tested first awaked and then anesthetized exhibit drastic changes in peripheral stimulus-evoked activity in dorsal raphe nucleus (Heym *et al.* 1984; Montagne-Clavel *et al.* 1995), ventromedial medulla (Oliveras *et al.* 1991; McGaraughty *et al.* 1995) and in somatosensory cortex (Shaw *et al.* 2001). Since ovarian hormones receptors are expressed in most of these regions (Simerly *et al.* 1990;

Voisin *et al.* 1997; Alves *et al.* 1998), the changes induced by general anesthetic in the physiological properties of these neurons might affect ovarian hormones modulation of nociception.

The protective effect of ovarian hormones on nociception is not limited to the TMJ. Many other studies evaluating formalin-induced nociception in the lip (Pajot *et al.* 2003) and in the paw (Ceccarelli *et al.* 2003; Gaumond *et al.* 2005; Kuba *et al.* 2006; Mannino *et al.* 2007) have demonstrated an antinociceptive effect of ovarian hormones. However, given that the effect of ovarian hormones on nociception may vary accordingly to the nociceptive model employed (Vincler *et al.* 2001) the results from animal studies evaluating nociception using different nociceptive models were not compared with the current results.

In summary, this study demonstrates that high physiological level of estradiol during the estrous cycle and exogenous administration of estradiol and progesterone in OVX female rats attenuate TMJ nociception. Based on that, we believe that the greater prevalence and severity of TMJ pain in women of reproductive age may be a consequence of estrogen fluctuation during reproductive cycle in that during low endogenous estrogen serum level TMJ pain sensitivity is increased enhancing the risk of females experiencing TMJ pain. The similarity between the current findings and previous clinical findings (LeResche *et al.* 2003), suggests that the TMJ nociceptive behavior model is a good model to study the mechanisms underlying hormonal modulation of clinical TMJ pain.

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References

Alves SE, Weiland NG, Hayashi S, McEwen BS. Immunocytochemical localization of nuclear estrogen receptors and progestin receptors within the rat dorsal raphe nucleus. The Journal of comparative neurology 1998;391(3):322-334.

Arthuri MT, Gameiro GH, Tambeli CH, de Arruda Veiga MC. Peripheral effect of a kappa opioid receptor antagonist on nociception evoked by formalin injected in TMJ of pregnant rats. Life Sci 2005;76(10):1177-1188.

Ashkenazi A, Silberstein SD. Hormone-related headache: pathophysiology and treatment. CNS drugs 2006;20(2):125-141.

Beirith A, Santos AR, Calixto JB. Mechanisms underlying the nociception and paw oedema caused by injection of glutamate into the mouse paw. Brain research 2002;924(2):219-228.

Benoliel R, Eliav E, Tal M. Strain-dependent modification of neuropathic pain behaviour in the rat hindpaw by a priming painful trigeminal nerve injury. Pain 2002;97(3):203-212.

Bereiter DA. Sex differences in brainstem neural activation after injury to the TMJ region. Cells Tissues Organs 2001;169(3):226-237.

Butcher RL, Collins WE, Fugo NW. Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat. Endocrinology 1974;94(6):1704-1708.

Cairns BE, Hu JW, Arendt-Nielsen L, Sessle BJ, Svensson P. Sex-related differences in human pain and rat afferent discharge evoked by injection of glutamate into the masseter muscle. Journal of neurophysiology 2001;86(2):782-791.

Cairns BE, Sessle BJ, Hu JW. Evidence that excitatory amino acid receptors within the temporomandibular joint region are involved in the reflex activation of the jaw muscles. J Neurosci 1998;18(19):8056-8064.

Cairns BE, Sim Y, Bereiter DA, Sessle BJ, Hu JW. Influence of sex on reflex jaw muscle activity evoked from the rat temporomandibular joint. Brain research 2002;957(2):338-344.

Carlsson G, LeResche L. Epidemiology of temporomandibular disorders. Seattle, WA 1995.

Ceccarelli I, Fiorenzani P, Massafra C, Aloisi AM. Long-term ovariectomy changes formalin-induced licking in female rats: the role of estrogens. Reprod Biol Endocrinol 2003;1(1):24.

Clemente JT, Parada CA, Veiga MC, Gear RW, Tambeli CH. Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint. Neuroscience letters 2004;372(3):250-255.

Collins JG, Ren K. WDR response profiles of spinal dorsal horn neurons may be unmasked by barbiturate anesthesia. Pain 1987;28(3):369-378.

DeLeo JA, Rutkowski MD. Gender differences in rat neuropathic pain sensitivity is dependent on strain. Neuroscience letters 2000;282(3):197-199.

Fischer L, Clemente JT, Tambeli CH. The protective role of testosterone in the development of temporomandibular joint pain. J Pain 2007;8(5):437-442.

Flake NM, Bonebreak DB, Gold MS. Estrogen and inflammation increase the excitability of rat temporomandibular joint afferent neurons. Journal of neurophysiology 2005;93(3):1585-1597.

Frot M, Feine JS, Bushnell MC. Sex differences in pain perception and anxiety. A psychophysical study with topical capsaicin. Pain 2004;108(3):230-236.

Gaumond I, Arsenault P, Marchand S. Specificity of female and male sex hormones on excitatory and inhibitory phases of formalin-induced nociceptive responses. Brain research 2005;1052(1):105-111.

Gordon FT, Soliman MR. Diurnal variation in the acute effects of estradiol and progesterone on beta-endorphin and Met-enkephalin levels in specific brain regions of ovariectomized rats. Pharmacology 1994;49(3):192-198.

Haas DA, Nakanishi O, MacMillan RE, Jordan RC, Hu JW. Development of an orofacial model of acute inflammation in the rat. Arch Oral Biol 1992;37(5):417-422.

He J, Evans CO, Hoffman SW, Oyesiku NM, Stein DG. Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury. Exp Neurol 2004;189(2):404-412.

Herradon G, Ezquerra L, Nguyen T, Wang C, Siso A, Franklin B, Dilorenzo L, Rossenfeld J, Alguacil LF, Silos-Santiago I. Changes in BDNF gene expression correlate with rat strain differences in neuropathic pain. Neuroscience letters 2007;420(3):273-276.

Heym J, Steinfels GF, Jacobs BL. Chloral hydrate anesthesia alters the responsiveness of central serotonergic neurons in the cat. Brain research 1984;291(1):63-72.

Kuba T, Wu HB, Nazarian A, Festa ED, Barr GA, Jenab S, Inturrisi CE, Quinones-Jenab V. Estradiol and progesterone differentially regulate formalin-induced nociception in ovariectomized female rats. Horm Behav 2006;49(4):441-449.

Kuroiwa T, Bonnekoh P, Hossmann KA. Therapeutic window of halothane anesthesia for reversal of delayed neuronal injury in gerbils: relationship to postischemic motor hyperactivity. Brain research 1991;563(1-2):33-38.

LeResche L, Mancl L, Sherman JJ, Gandara B, Dworkin SF. Changes in temporomandibular pain and other symptoms across the menstrual cycle. Pain 2003;106(3):253-261.

LeResche L, Sherman JJ, Huggins K, Saunders K, Mancl LA, Lentz G, Dworkin SF. Musculoskeletal orofacial pain and other signs and symptoms of

temporomandibular disorders during pregnancy: a prospective study. J Orofac Pain 2005;19(3):193-201.

Mannino CA, South SM, Quinones-Jenab V, Inturrisi CE. Estradiol replacement in ovariectomized rats is antihyperalgesic in the formalin test. J Pain 2007;8(4):334-342.

Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. Braz J Biol 2002;62(4A):609-614.

McGaraughty S, Reinis S, Tsoukatos J. A correlogram analysis of the activity in the rostral ventromedial medulla of awake rats and in rats anesthetized with ketamine or pentobarbital following the administration of morphine. Experimental brain research Experimentelle Hirnforschung 1995;106(2):283-290.

Montagne-Clavel J, Oliveras JL, Martin G. Single-unit recordings at dorsal raphe nucleus in the awake-anesthetized rat: spontaneous activity and responses to cutaneous innocuous and noxious stimulations. Pain 1995;60(3):303-310.

Okamoto K, Hirata H, Takeshita S, Bereiter DA. Response properties of TMJ units in superficial laminae at the spinomedullary junction of female rats vary over the estrous cycle. Journal of neurophysiology 2003;89(3):1467-1477.

Oliveras JL, Montagne-Clavel J, Martin G. Drastic changes of ventromedial medulla neuronal properties induced by barbiturate anesthesia. I. Comparison of the single-unit types in the same awake and pentobarbital-treated rats. Brain research 1991;563(1-2):241-250.

Pajot J, Ressot C, Ngom I, Woda A. Gonadectomy induces site-specific differences in nociception in rats. Pain 2003;104(1-2):367-373.

Riley JL, 3rd, Robinson ME, Wise EA, Myers CD, Fillingim RB. Sex differences in the perception of noxious experimental stimuli: a meta-analysis. Pain 1998;74(2-3):181-187.

Rosland JH. The formalin test in mice: the influence of ambient temperature. Pain 1991;45(2):211-216.

Roughan JV, Flecknell PA. Effects of surgery and analgesic administration on spontaneous behaviour in singly housed rats. Res Vet Sci 2000;69(3):283-288.

Roveroni RC, Parada CA, Cecilia M, Veiga FA, Tambeli CH. Development of a behavioral model of TMJ pain in rats: the TMJ formalin test. Pain 2001;94(2):185-191.

Shaw FZ, Chen RF, Yen CT. Dynamic changes of touch- and laser heat-evoked field potentials of primary somatosensory cortex in awake and pentobarbital-anesthetized rats. Brain research 2001;911(2):105-115.

Simerly RB, Chang C, Muramatsu M, Swanson LW. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. The Journal of comparative neurology 1990;294(1):76-95.

Smith YR, Stohler CS, Nichols TE, Bueller JA, Koeppe RA, Zubieta JK. Pronociceptive and antinociceptive effects of estradiol through endogenous opioid neurotransmission in women. J Neurosci 2006;26(21):5777-5785.

Vendruscolo LF, Pamplona FA, Takahashi RN. Strain and sex differences in the expression of nociceptive behavior and stress-induced analgesia in rats. Brain research 2004;1030(2):277-283.

Vincler M, Maixner W, Vierck CJ, Light AR. Estrous cycle modulation of nociceptive behaviors elicited by electrical stimulation and formalin. Pharmacology, biochemistry, and behavior 2001;69(3-4):315-324.

Voisin DL, Simonian SX, Herbison AE. Identification of estrogen receptor-containing neurons projecting to the rat supraoptic nucleus. Neuroscience 1997;78(1):215-228.

Waynforth H, Flecknell P. Experimental and surgical technique in the rat

Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16(2):109-110.

Zubieta JK, Smith YR, Bueller JA, Xu Y, Kilbourn MR, Jewett DM, Meyer CR, Koeppe RA, Stohler CS. mu-opioid receptor-mediated antinociceptive responses differ in men and women. J Neurosci 2002;22(12):5100-5107.

Figure Legends

Figure 1- Effect of the TMJ injection of Glutamate.

A- Time course of the nociceptive behavior induced by the TMJ injection of Glutamate. A repeated measures two way ANOVA showed a significant main effect of treatment group (F= 24,149, p□0.05), main effect of time (F=1,865, p<0.05), but not a significant group × time interaction (F= 0,942, p>0.05). SNK post hocs showed that the effect of glutamate was significantly different from that of vehicle and the glutamate plus the local anesthetic QX-314 (p < 0.05), which were not significantly different from each other (p>0.05).

B- Effect of glutamate receptor antagonists. The TMJ injection of glutamate induced a nociceptive behavior that was significantly reduced by the co-administration of the AMPA/Kainate (NBQX) and NMDA (AP-5) receptor antagonists. The symbol "+" indicates a response significantly higher than that induced by vehicle. The symbol "*" indicates that the co-administration of the glutamate receptor antagonists significantly reduced glutamate-induced TMJ nociception. In this and subsequent figures, data are plotted as mean ± s.e.m. and group sample sizes are shown in parentheses; see Methods for additional details regarding data presentation and analysis.

Figure 2- Effect of sex and estrous cycle on formalin and glutamate-induced TMJ nociception.

A- Formalin-induced TMJ nociception in males and proestrus and diestrus females. Formalin-induced TMJ nociception in males and proestrus females was similar to each other and significantly lower than that of diestrus females. The symbol "*" indicates a nociceptive behavior significantly greater than that induced by vehicle (0.9% NaCl) (p< 0.05, t test). The symbol "+" indicates a nociceptive behavior significantly greater than that of males and proestrus females.

B- Glutamate-induced TMJ nociception in males and proestrus and diestrus females. Glutamate-induced TMJ nociception in males and proestrus females was similar to each other and significantly lower than that of diestrus females. The symbol "*" indicates a nociceptive behavior significantly greater than that induced by vehicle (0.9% NaCl) ($p < 0.05$, t test). The symbol "+" indicates a nociceptive behavior significantly greater than that of males and proestrus females.

Figure 3- Effect of ovarian hormones depletion on formalin and glutamate-induced TMJ nociception.

A- Formalin-induced TMJ nociception in sham-operated proestrus, sham-operated diestrus and OVX females. Formalin-induced TMJ nociception in sham-operated diestrus and OVX females was similar to each other and significantly greater than that of sham-operated proestrus females. The symbol "*" indicates a nociceptive behavior significantly greater than that of sham-operated proestrus females.

B- Glutamate-induced TMJ nociception in sham-operated proestrus, sham-operated diestrus and OVX females. Glutamate-induced TMJ nociception in sham-operated diestrus and OVX females was similar to each other and significantly greater than that of sham-operated proestrus females. The symbol "*" indicates a nociceptive behavior significantly greater than that of sham-operated proestrus females.

Abbreviation: OVX = ovariectomized.

Figure 4- Estradiol and progesterone serum level in proestrus, diestrus and OVX females.

A- Estradiol serum level was significantly lower in diestrus and OVX females than in proestrus females. The symbol "*" indicates an estradiol serum level significantly lower than that of proestrus females. The symbol "+" indicates an estradiol serum level significantly lower than that of other groups.

B- Progesterone serum level was significantly lower in OVX females than in proestrus and diestrus females. The symbol "*" indicates a progesterone serum level significantly lower than that of other groups.

Abbreviation: OVX = ovariectomized

Figure 5- Effect of estradiol and progesterone administration on formalin and glutamate-induced TMJ nociception.

A- Administration of estradiol or progesterone or estradiol plus progesterone in OVX females significantly reduced formalin-induced TMJ nociception. The symbol "*" indicates a nociceptive behavior significantly lower than that of OVX females receiving vehicle administration.

B- Administration of estradiol or progesterone in OVX females significantly reduced glutamate-induced TMJ nociception. The symbol "*" indicates a nociceptive behavior significantly lower than that of OVX females receiving vehicle.

Abbreviations: OVX = ovariectomized; V = vehicle; E = estradiol; P = progesterone, E/P = estradiol plus progesterone.

Figure 6- Effect of estradiol administration in OVX Sprague-Dawley rats. Estradiol administration significantly reduced glutamate-induced TMJ nociception in OVX Sprague-dawley rats as indicated by the symbol "*".

Abbreviations: OVX = ovariectomized; E = estradiol.

Figures

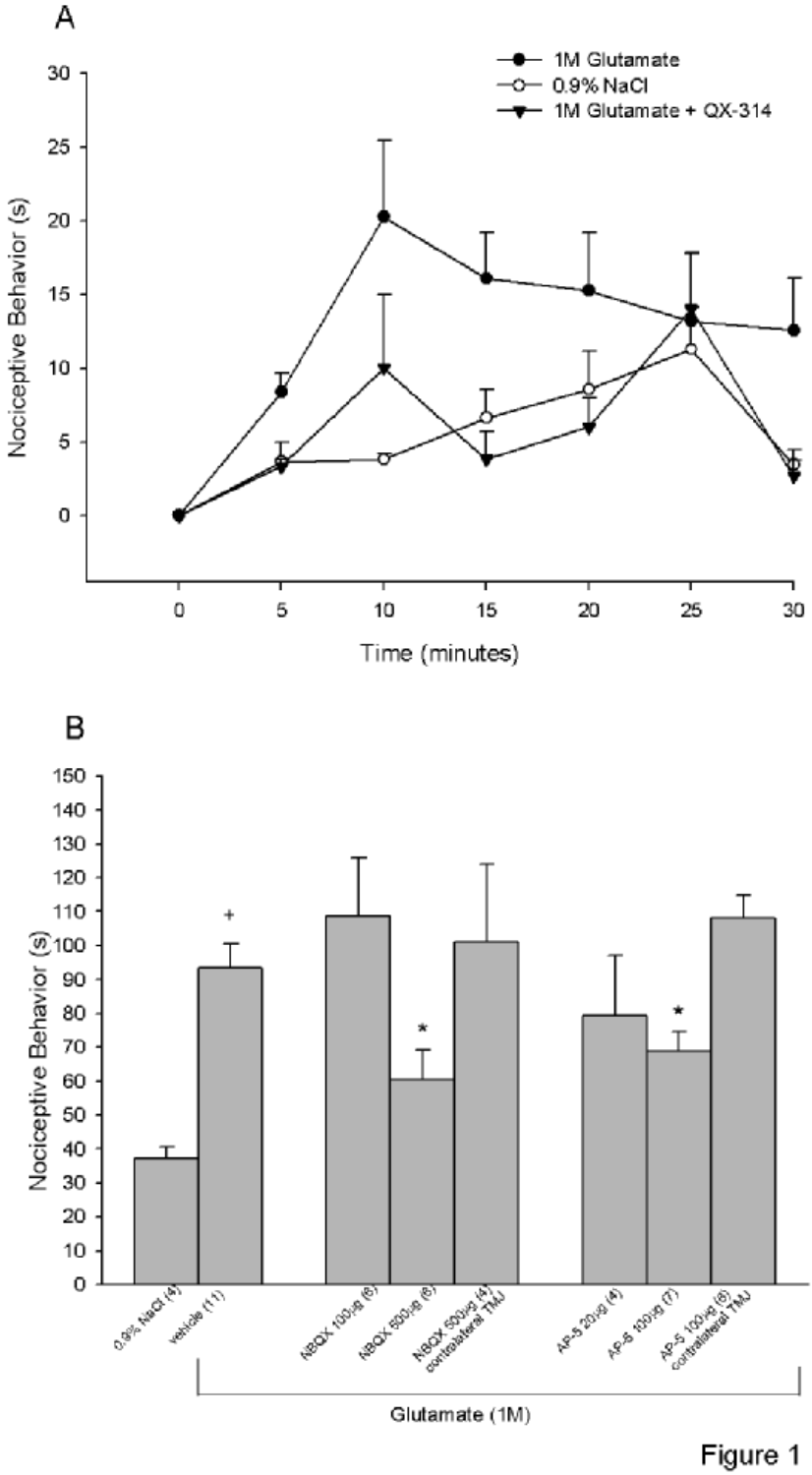
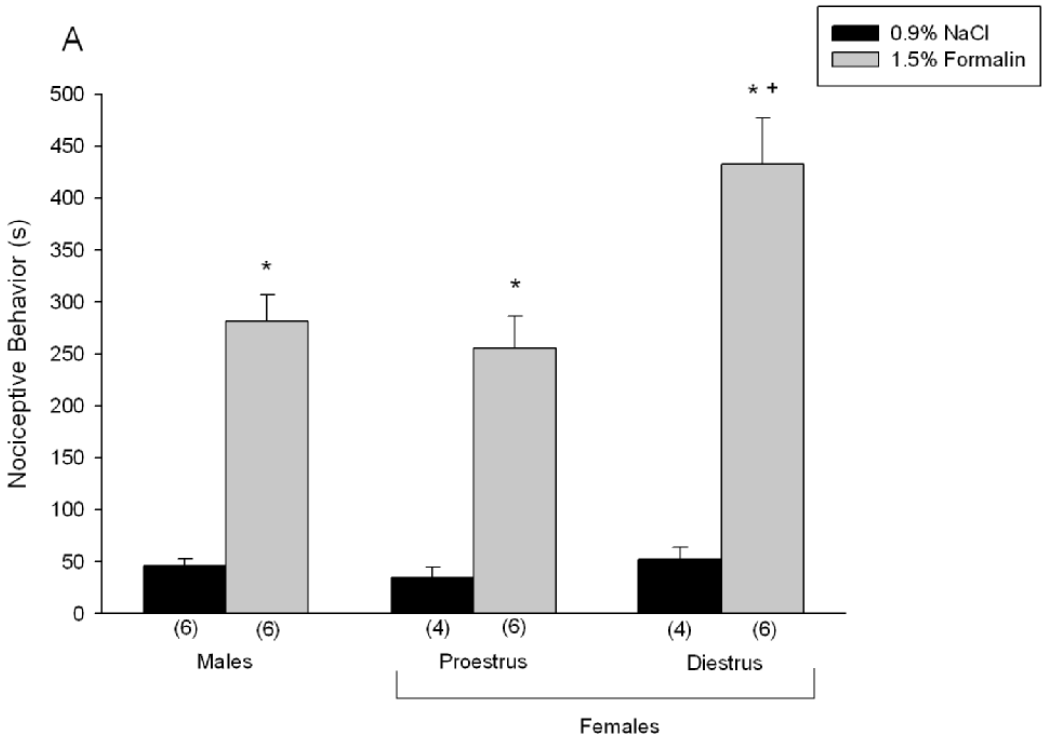


Figure 1


A
0.9% NaCl
1.5% Formalin
Nociceptive Behavior (s)
500
450
400
350
300
250
200
150
100
50
0
* +
*
*
(6) (6) (4) (6) (4) (6)
Males
Proestrus
Diestrus
Females

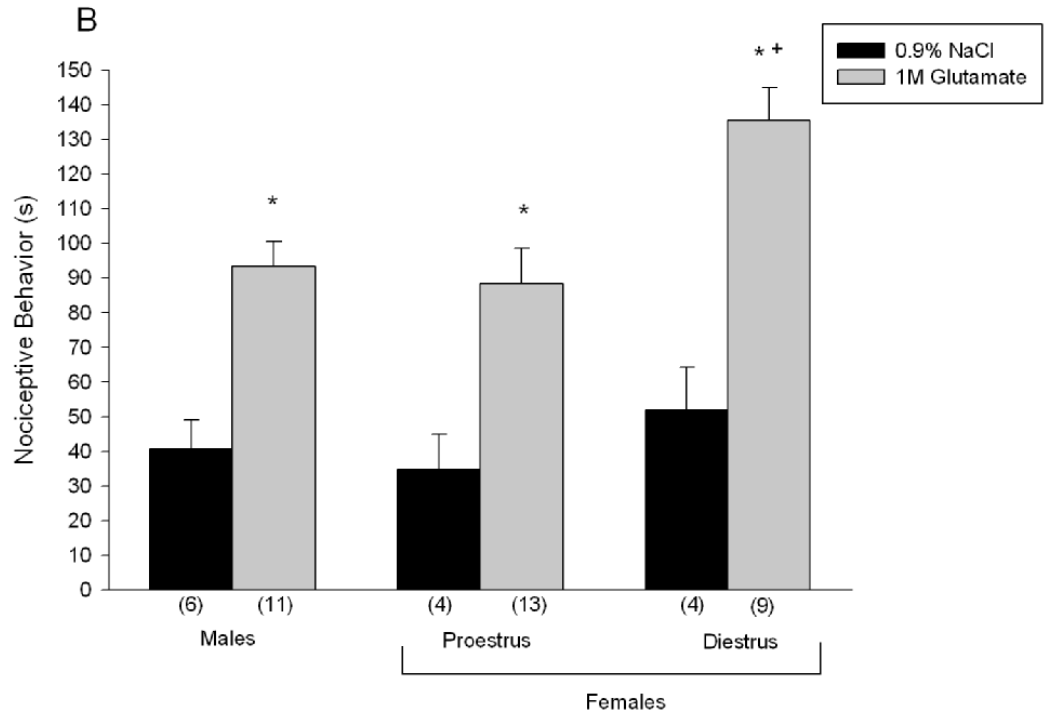

B
0.9% NaCl
1M Glutamate
Nociceptive Behavior (s)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
* +
*
*
(6) (11) (4) (13) (4) (9)
Males
Proestrus
Diestrus
Females

Figure 2

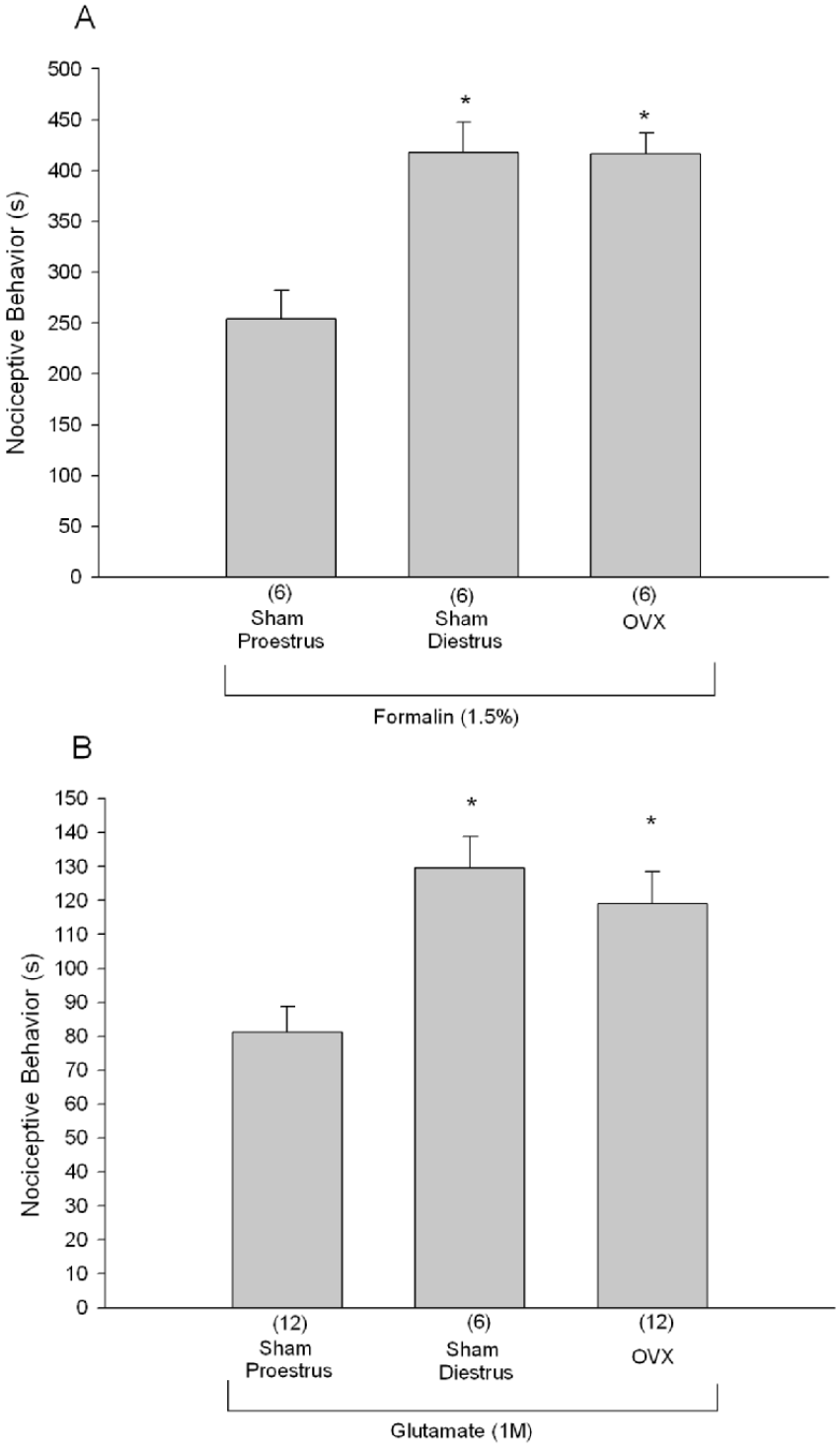
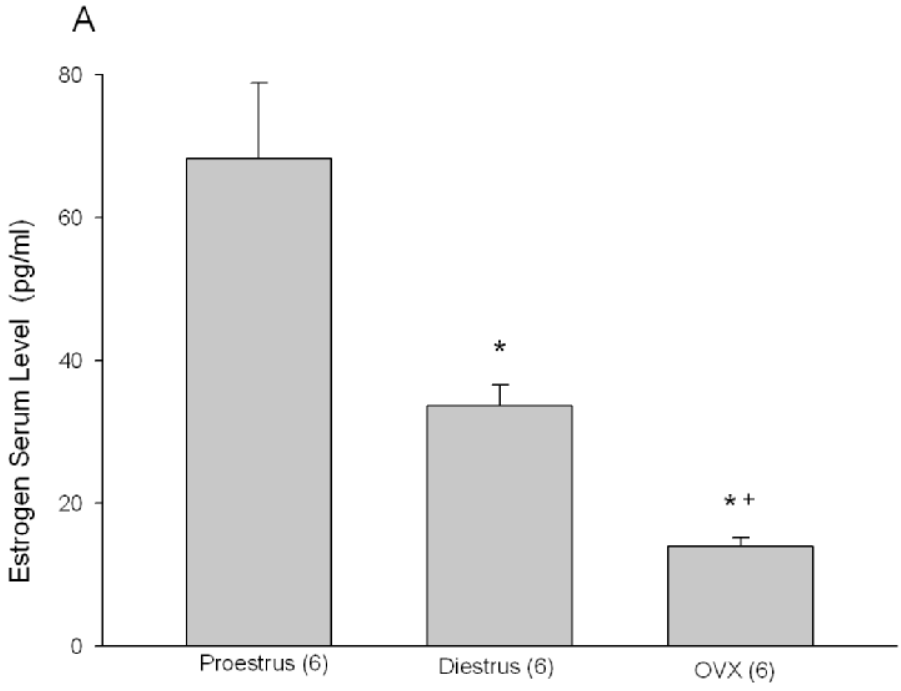

A
Nociceptive Behavior (s)
500
450
400
350
300
250
200
150
100
50
0
*
*
(6)
Sham
Proestrus
(6)
Sham
Diestrus
(6)
OVX
Formalin (1.5%)
B
Nociceptive Behavior (s)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
*
*
(12)
Sham
Proestrus
(6)
Sham
Diestrus
(12)
OVX
Glutamate (1M)

Figure 3


A
80
60
40
20
0
Estrogen Serum Level (pg/ml)
*
* +
Proestrus (6)
Diestrus (6)
OVX (6)

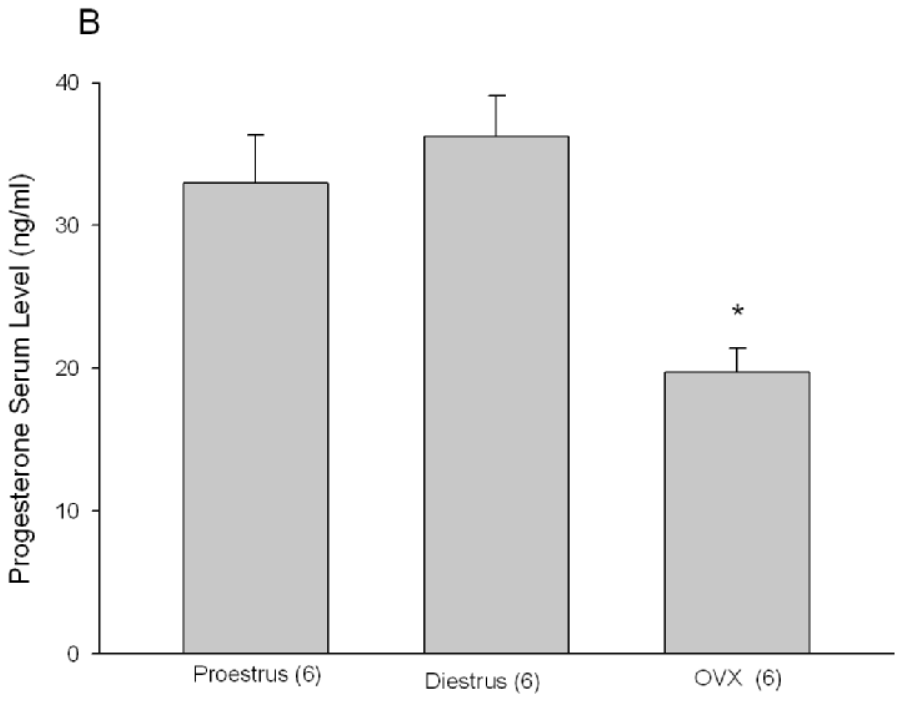

B
40
30
20
10
0
Progesterone Serum Level (ng/ml)
*
Proestrus (6)
Diestrus (6)
OVX (6)

Figure 4

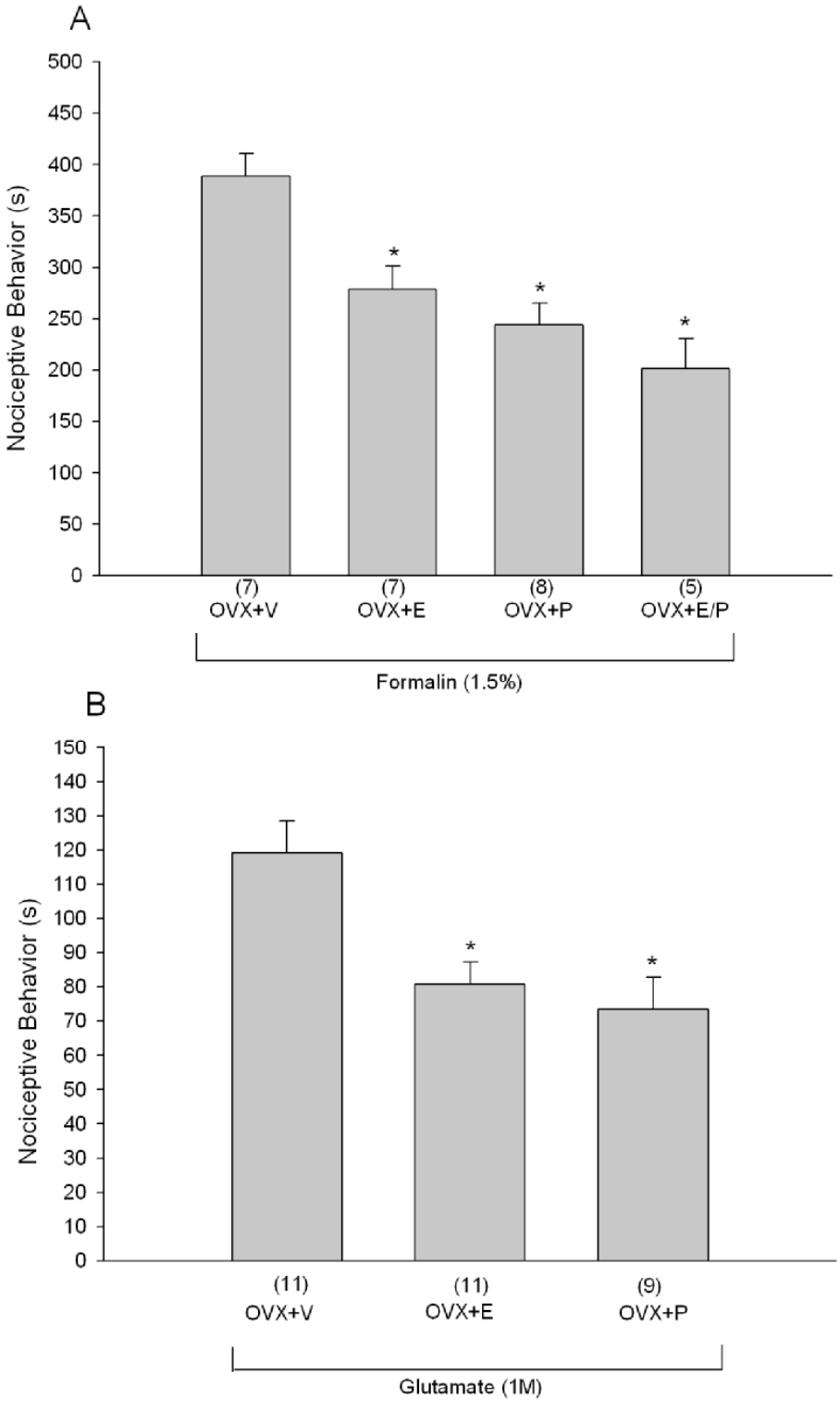

A
Nociceptive Behavior (s)
500
450
400
350
300
250
200
150
100
50
0
*
*
*
(7)
OVX+V
(7)
OVX+E
(8)
OVX+P
(5)
OVX+E/P
Formalin (1.5%)
B
Nociceptive Behavior (s)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
*
*
(11)
OVX+V
(11)
OVX+E
(9)
OVX+P
Glutamate (1M)

Figure 5

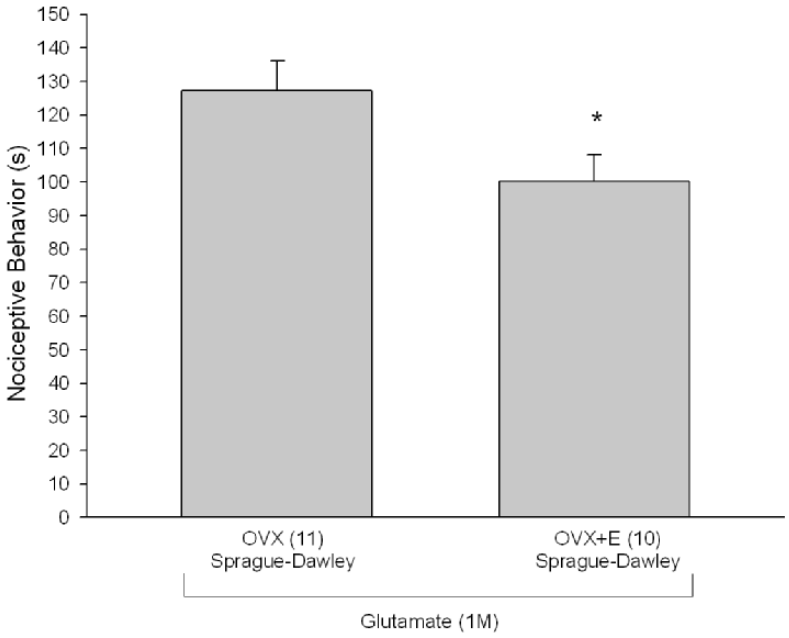

Nociceptive Behavior (s)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
*
OVX (11)
Sprague-Dawley
OVX+E (10)
Sprague-Dawley
Glutamate (1M)

Figure 6

Capítulo 3

A novel method for subarachnoid drug delivery in the medullary region of rats

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Original Article

Keywords: Formalin test; Testosterone; Estrogen; Temporomandibular joint pain; Sex differences

Abstract

This study describes a novel method for direct subarachnoid drug delivery to the medullary dorsal horn region of rats, without introducing a catheter. The reliability of the method was demonstrated by a pharmacological validation; that is, morphine administration to the medullary region blocked the nociceptive response to formalin injected in the temporomandibular joint (TMJ) region, an effect that was prevented by co-administration of naloxone.

The method proposed offers many advantages over the existing methods for medullary drug delivery with catheter implantation. It is easy to be employed, it does not induce any sign of motor impairment, and it does not require the neck surgery performed to implant a catheter in the medullary dorsal horn region. Therefore, it is an useful method for subarachnoid drug delivery in behavioral trigeminal pain studies, particularly when nociceptive behavioral measures that require normal neck muscle activity to occur, such as head withdraw or head flinch, are evaluated.

Introduction

The orofacial region is one of the most densely innervated areas of the body, which focuses common acute, chronic and referred pain (Sessle 2000). Drug administration to the medullary cerebrospinal fluid is an useful tool in the orofacial pain research, and is currently accomplished by a catheter implantation in the surroundings of trigeminal subnucleus caudalis, also known as medullary dorsal horn. This procedure is commonly performed through a surgical exposition of the dorsal surface of the neck and insertion of a catheter in the subarachnoid space through a slit in the atlanto-occipital membrane (Aigouy *et al.* 1992; Flores *et al.* 2001; Tambeli *et al.* 2001; Wang *et al.* 2002).

It is well known that the ability to correlate a behavioral measure with pain arising from an orofacial region in animal studies is essential in elucidating the underlying mechanisms of pathophysiology of orofacial pain syndromes and temporomandibular disorders. However, the surgical catheter implantation performed to study the effect of drugs delivered in the medullary dorsal horn region may affect some of the frequently used nociceptive behavioral measures that require normal neck muscle activity to occur, such as head withdraw in response to a local mechanical stimulus (Vos *et al.* 1994; Anderson and Rao 2001; Christensen *et al.* 2001; Imbe *et al.* 2001; Benoliel *et al.* 2002b; Ogawa *et al.* 2003), or head flinch induced by local chemical stimulation (Anderson and Rao 2001; Roveroni *et al.* 2001; Chidiac *et al.* 2002; Gameiro *et al.* 2003; Hartwig *et al.* 2003; Clemente *et al.* 2004). Therefore, the aim of this study was to develop a method for direct subarachnoid drug delivery to the medullary region that facilitates animal investigation of orofacial pain, and that can be combined with many orofacial pain models, particularly with those that use nociceptive behavior measures such as head flinch or head withdraw.

Methods

Animals

Experiments were performed on 250 – 320 g male Wistar rats housed (five per cage) in a temperature-controlled room (23 ± 1°C) on a 12:12 light cycle (lights on at 6 AM), with food and water available ad libitum. Animals were handled for at least one week prior to the experiments. Experimental protocols were approved by the Committee on Animal Research of the University of Campinas and conformed to IASP guidelines for the study of pain in animals (Zimmermann 1983).

General Procedures

Testing sessions took place during the light phase in a quiet room maintained at 23°C. Prior to the experiments, each animal was placed in the test chamber (30 x 30 x 30cm mirrored-wood chamber with a glass at the front side) for a 15-minutes habituation period.

Drugs

Formalin solutions were prepared from commercially (Sigma) available stock formalin (an aqueous solution of 37% of formaldehyde) further diluted in 0.9% NaCl (saline) to concentration of 1.5% (Roveroni *et al.* 2001). Morphine sulfate (Sigma) 3, 6 and 9 μg (Grabow and Dougherty 2001) and Naloxone hydrocloride (Sigma) 15μg (Danzebrink *et al.* 1995) were dissolved in saline.

Subarachnoid medullary injection

Rats were briefly anesthetized with halothane, and a small area of skin overlying the high cervical region was shaved with an electric razor. Animals were dorsally positioned, so that the sub occipital space could be easily found.

A 30-gauge needle connected to a 50μl Hamilton syringe by a polyethylene cannula was first inserted bellow the occipital bone up to 4mm, and slightly inclined in a cranial direction. The needle was advanced more 2mm to perforate the atlanto-occipital membrane and reach the medullary subaracnoid space (Figure 1). This technique allowed

direct drug delivery in the cerebrospinal fluid in the surroundings of trigeminal subnucleus caudalis. Total injection volume in all experiments was 10 µl. All injections were performed at rate of 1 µl/sec. Each animal regained consciousness approximately 30 seconds after discontinuing the anesthesia

Testing for correct site of subarachnoid injection

In preliminary experiments the injection procedure was tested by the administration of Evans blue dye (0.1%, 10µl) in 10 rats. Following the injection, the rats were euthanized by a lethal dose of halothane. Cervical laminectomy and occipital craniotomy were performed using blunt dissection techniques and the site of injection as well as dye spread was examined.

Testing procedure for temporomandibular joint pain

Animals were briefly anesthetized by inhalation of 4% halothane to allow the temporomandibular joint (TMJ) injection, which was performed with a 30-gauge needle connected to a 50µl Hamilton syringe. Injection volumes were 50µl in all cases. Each animal regained consciousness approximately 30 seconds after discontinuing the anesthesia and was returned to the test chamber for counting nociceptive responses during a 45-minutes observation period. The nociceptive response score was defined as the cumulative total number of seconds that the animal spent rubbing the orofacial region asymmetrically, with the ipsilateral fore or hind paw, plus the number of head flinches counted during the observation period, as previously described (Roveroni *et al.* 2001). From a theoretical perspective, the occurrence of a given behavior is expressed as the proportion of time that the behavior occupies. Since head flinches followed an uniform pattern of 1s of duration, each flinch was expressed as 1s (Roveroni *et al.* 2001). At the conclusion of the experiments, rats were anesthetized by inhalation of 4% halothane and maintained at halothane level of 1.5-2%. Evans blue dye (0.1%, 5mg/Kg) was then intravenously administered in order to visualize formalin-induced plasma extravasation. Ten minutes later, the animals were euthanized by halothane inhalation and a post-mortem examination of the injected TMJs was performed (Haas *et al.* 1992). This procedure also allowed

confirmation that the plasma extravasation induced by the TMJ injection at the correct site was restricted to the immediate TMJ region.

Motor Assessment

To verify whether the subarachnoid injection in the medullary region induces motor impairment, an extra set of experiments was performed using the rota-rod test. Rats were initially trained at a low velocity and the cut-off time was 120 seconds. After a subarachnoid injection of either saline of morphine (9μg) each animal was placed in the rota-rod for three measurements. Rats that received 9 □g of morphine were also tested for signs of catalepsy (loss of spontaneous mobility) by placing the forepaws of the rat on a horizontal bar 8 cm above the table surface ((Simon *et al.* 1970). Animals were considered cataleptic if they remained in position for longer than 10 s.

Pharmacological experiments

The reliability of the method for direct subarachnoid drug delivery in the medullary dorsal horn region was demonstrated by a pharmacological validation. Morphine (3, 6 or 9 μg) or saline was administered in the subarachnoid space of the medullary dorsal horn region, prior to the TMJ injection of formalin. To test for the reversal of the effect of morphine, naloxone (15μg) or saline was co-administered with morphine. The following subarachnoid treatments were applied in animals injected with formalin into the TMJ region: saline, morphine (3, 6 or 9 μg), morphine (9 μg) + naloxone (15 μg), and naloxone (15 μg). The injections were performed 10 min prior to the formalin TMJ injection.

Statistical analysis

The sum of the behavioral responses measured for 45 min was used for statistical analysis. Data with homogeneity of variance were analyzed by One-Way Analysis of Variance (ANOVA) and multiple post-hoc comparisons were performed using Tukey test. A probability level of $p < 0.05$ was considered statistically significant. Data are plotted in figures as mean ± S.E.M.

Results

Verification of the site of injection

A well defined blue mark was observed at the site of the needle penetration into the skin of the animals that received a subarachnoid injection of Evan's blue dye, but no staining was found in the neck muscles. The atlanto-occipital membrane was densely stained, and diffuse staining was evident along 8 or 10 mm in the cervical (Figure 2) and brain region.

Pharmacological validation

The magnitude of nociceptive behavior induced by the TMJ injection of formalin was compared to that of previous studies (Roveroni *et al.* 2001; Gameiro *et al.* 2003; Clemente *et al.* 2004). Morphine injection into subarachnoid space of the medullary region produced a dose-dependent suppression of the formalin-induced nociceptive behavior (Fig. 3 A). Co-administration of naloxone (15 μg) prevented the antinociceptive effect of morphine (9 μg), but had no effect by itself ($p<0.05$, Tukey test; Fig. 3 B).

Motor Assessment

The time of permanence in the rota-rod was 120 seconds (cut-off) for either saline or morphine injected rats, suggesting that subarachnoid injection does not induce motor impairment. In addition, no signs of catalepsy were observed after the subarachnoid injection of the highest concentration of morphine (9 □g) used in the present study.

Discussion

The present study shows a method for direct subarachnoid drug delivery in the medullary region of rats without introducing a catheter. The reliability of this method was demonstrated by a pharmacological validation. Similarly to previous studies in the paw (O'Connor and Abram 1994) and in the upper lip (Grabow and Dougherty 2001), morphine administration to the medullary region blocked the nociceptive response to formalin injected in the TMJ, an effect that was prevented by co-administration of naloxone. Because drugs injected by this method cannot be assumed to remain locally in the medullary region, the site of morphine action may include the medullary region as well as several regions of the central nervous system.

The results of the present study were not affected by the anesthetic procedure used to perform the subarachnoid injections, as demonstrated by the similar nociceptive response of animals exposed to two anesthetic procedures, one for the TMJ formalin injection and the other for the subarachnoid injection of saline (current study), and animals exposed only to one anesthetic procedure for the TMJ injection of formalin (our previous studies: Roveroni et al., 2001; Clemente et al, 2004). However, if more than one subarachnoid injection is necessary, experimental protocol testing is recommended.

A variety of methods for catheterization of the spinal subarachnoid space have been extensively used in behavior studies on the effects of drugs on spinal (Yaksh and Rudy 1976; LoPachin *et al.* 1981; Dib 1984; Martin *et al.* 1984; Gonzalez-Darder *et al.* 1989; Storkson *et al.* 1996; Jasmin and Ohara 2001) and trigeminal (Aigouy *et al.* 1992; Flores *et al.* 2001; Grabow and Dougherty 2001; Wang *et al.* 2002) nociceptive mechanisms in rats.

In general, the catheterization methods currently used to study trigeminal nociceptive mechanisms by delivering drugs into the medullary cerebrospinal fluid are moderately invasive to cranial or upper cervical tissue, which may lead to the sensitization of cervicotrigeminal convergent neurons (Hu *et al.* 1993). Theoretically, this condition may limit the application of these methods of drug delivery to the study of trigeminal receptor

pharmacology using animal behavioral models of nociception (Grabow and Dougherty 2001).

The method proposed in the present study offers many advantages over the existing methods for medullary drug delivery with catheter implantation. First, since drug is delivered without a catheter implantation the method does not induce the known tissue reactivity to chronically implanted catheter, like fibrosis and inflammatory response, which have been associated with a decrease in the efficacy of drugs administered through subarachnoid catheters (Coombs *et al.* 1993; Yaksh *et al.* 1995; Gurun *et al.* 1997). Second, for the same reason, it does not require that rats be housed singly, a situation that results in the appearance of a social isolation syndrome (Hatch *et al.* 1965), which may affect nociceptive responses and behavioral test reliability (Jasmin and Ohara 2001). Third, it is minimally invasive and may be combined with many models of orofacial pain (Vos *et al.* 1994; Clavelou *et al.* 1995; Roveroni *et al.* 2001; Chidiac *et al.* 2002; Hartwig *et al.* 2003; Ogawa *et al.* 2003), particularly with the orofacial behavioral pain models in which head flinch or head withdraw is evaluated (Vos *et al.* 1994; Anderson and Rao 2001; Christensen *et al.* 2001; Imbe *et al.* 2001; Roveroni *et al.* 2001; Benoliel *et al.* 2002a; Gameiro *et al.* 2003; Hartwig *et al.* 2003; Ogawa *et al.* 2003; Clemente *et al.* 2004). In contrast, the conventional catheterization methods for medullary drug delivery (Aigouy *et al.* 1992; Flores *et al.* 2001; Tambeli *et al.* 2001; Wang *et al.* 2002) require surgical exposition of neck muscles, and implies freeing these muscles from the occipital crest to allow the catheter implantation. This procedure may affect the function of these muscles, and consequently, the head flinch and head withdraw behavior. Although the method for medullary drug administration performed by advancing a lumbar spinal implanted catheter to the medullary region (Grabow and Dougherty, 2001) does not require surgical exposition of neck muscles, it has the limitations of the catheterization methods. Finally, the method proposed does not induce motor impairment as seen in some catheterized animals, and reduces animal suffering. Taken together, these features facilitate trigeminal pain studies.

In summary, this study reports a non-invasive method for subarachnoid drug delivery in the medullary dorsal region. The method is easy to be employed, reliable, with

no sign of motor impairment and very useful for behavioral trigeminal pain studies, particularly when head finch or head withdraw are evaluated.

Acknowledgements

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References

Aigouy L, Fondras JC, Pajot J, Schoeffler P, Woda A. Intrathecal midazolam versus intrathecal morphine in orofacial nociception: an experimental study in rats. Neurosci Lett 1992;139(1):97-99.

Anderson LC, Rao RD. Interleukin-6 and nerve growth factor levels in peripheral nerve and brainstem after trigeminal nerve injury in the rat. Arch Oral Biol 2001;46(7):633-640.

Benoliel R, Eliav E, Tal M. Strain-dependent modification of neuropathic pain behaviour in the rat hindpaw by a priming painful trigeminal nerve injury. Pain 2002a;97(3):203-212.

Benoliel R, Wilensky A, Tal M, Eliav E. Application of a pro-inflammatory agent to the orbital portion of the rat infraorbital nerve induces changes indicative of ongoing trigeminal pain. Pain 2002b;99(3):567-578.

Chidiac JJ, Rifai K, Hawwa NN, Massaad CA, Jurjus AR, Jabbur SJ, Saade NE. Nociceptive behaviour induced by dental application of irritants to rat incisors: a new model for tooth inflammatory pain. Eur J Pain 2002;6(1):55-67.

Christensen D, Gautron M, Guilbaud G, Kayser V. Effect of gabapentin and lamotrigine on mechanical allodynia-like behaviour in a rat model of trigeminal neuropathic pain. Pain 2001;93(2):147-153.

Clavelou P, Dallel R, Orliaguet T, Woda A, Raboisson P. The orofacial formalin test in rats: effects of different formalin concentrations. Pain 1995;62(3):295-301.

Clemente JT, Parada CA, Veiga MC, Gear RW, Tambeli CH. Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint. Neurosci Lett 2004;372(3):250-255.

Coombs DW, Colburn RW, McCarthy LE, DeLeo JA, Hoopes PJ, Twitchell BB. Neurotoxicology of chronic infusion of the ganglioside GM1 in the ewe: phase I. intrathecal administration. Anesth Analg 1993;77(3):507-515.

Danzebrink RM, Green SA, Gebhart GF. Spinal mu and delta, but not kappa, opioid-receptor agonists attenuate responses to noxious colorectal distension in the rat. Pain 1995;63(1):39-47.

Dib B. Intrathecal chronic catheterization in the rat. Pharmacol Biochem Behav 1984;20(1):45-48.

Flores CA, Wang XM, Zhang KM, Mokha SS. Orphanin FQ produces gender-specific modulation of trigeminal nociception: behavioral and electrophysiological observations. Neuroscience 2001;105(2):489-498.

Gameiro GH, Arthuri MT, Tambeli CH, de Arruda Veiga MC. Effects of ethanol on deep pain evoked by formalin injected in TMJ of rat. Life Sci 2003;73(26):3351-3361.

Gonzalez-Darder JM, Gomez-Cardenas E, Gil-Salu JL. [Microsurgical catheterization of the intrathecal space in the rat for chronic infusion of drugs]. Rev Esp Anestesiol Reanim 1989;36(3):153-156.

Grabow TS, Dougherty PM. Cervicomedullary intrathecal injection of morphine produces antinociception in the orofacial formalin test in the rat. Anesthesiology 2001;95(6):1427-1434.

Gurun MS, Leinbach R, Moore L, Lee CS, Owen MD, Eisenach JC. Studies on the safety of glucose and paraben-containing neostigmine for intrathecal administration. Anesth Analg 1997;85(2):317-323.

Haas DA, Nakanishi O, MacMillan RE, Jordan RC, Hu JW. Development of an orofacial model of acute inflammation in the rat. Arch Oral Biol 1992;37(5):417-422.

Hartwig AC, Mathias SI, Law AS, Gebhart GF. Characterization and opioid modulation of inflammatory temporomandibular joint pain in the rat. J Oral Maxillofac Surg 2003;61(11):1302-1309.

Hatch AM, Wiberg GS, Zawidzka Z, Cann M, Airth JM, Grice HC. Isolation syndrome in the rat. Toxicol Appl Pharmacol 1965;7(5):737-745.

Hu JW, Yu XM, Vernon H, Sessle BJ. Excitatory effects on neck and jaw muscle activity of inflammatory irritant applied to cervical paraspinal tissues. Pain 1993;55(2):243-250.

Imbe H, Iwata K, Zhou QQ, Zou S, Dubner R, Ren K. Orofacial deep and cutaneous tissue inflammation and trigeminal neuronal activation. Implications for persistent temporomandibular pain. Cells Tissues Organs 2001;169(3):238-247.

Jasmin L, Ohara PT. Long-term intrathecal catheterization in the rat. J Neurosci Methods 2001;110(1-2):81-89.

LoPachin RM, Rudy TA, Yaksh TL. An improved method for chronic catheterization of the rat spinal subarachnoid space. Physiol Behav 1981;27(3):559-561.

Martin H, Kocher L, Chery-Croze S. Chronic lumbar intrathecal catheterization in the rat with reduced-length spinal compression. Physiol Behav 1984;33(1):159-161.

O'Connor TC, Abram SE. Halothane enhances suppression of spinal sensitization by intrathecal morphine in the rat formalin test. Anesthesiology 1994;81(5):1277-1283.

Ogawa A, Ren K, Tsuboi Y, Morimoto T, Sato T, Iwata K. A new model of experimental parotitis in rats and its implication for trigeminal nociception. Exp Brain Res 2003;152(3):307-316.

Roveroni RC, Parada CA, Cecilia M, Veiga FA, Tambeli CH. Development of a behavioral model of TMJ pain in rats: the TMJ formalin test. Pain 2001;94(2):185-191.

Sessle BJ. Acute and chronic craniofacial pain: brainstem mechanisms of nociceptive transmission and neuroplasticity, and their clinical correlates. Crit Rev Oral Biol Med 2000;11(1):57-91.

Simon P, Malatray J, Boissier JR. Antagonism by amantadine of prochlorpemazine-induced catalepsy. J Pharm Pharmacol 1970;22(7):546-547.

Storkson RV, Kjorsvik A, Tjolsen A, Hole K. Lumbar catheterization of the spinal subarachnoid space in the rat. J Neurosci Methods 1996;65(2):167-172.

Tambeli CH, Seo K, Sessle BJ, Hu JW. Central mu opioid receptor mechanisms modulate mustard oil-evoked jaw muscle activity. Brain Res 2001;913(1):90-94.

Vos BP, Strassman AM, Maciewicz RJ. Behavioral evidence of trigeminal neuropathic pain following chronic constriction injury to the rat's infraorbital nerve. J Neurosci 1994;14(5 Pt 1):2708-2723.

Wang XM, Zhang ZJ, Bains R, Mokha SS. Effect of antisense knock-down of alpha(2a)- and alpha(2c)-adrenoceptors on the antinociceptive action of clonidine on trigeminal nociception in the rat. Pain 2002;98(1-2):27-35.

Yaksh TL, Grafe MR, Malkmus S, Rathbun ML, Eisenach JC. Studies on the safety of chronically administered intrathecal neostigmine methylsulfate in rats and dogs. Anesthesiology 1995;82(2):412-427.

Yaksh TL, Rudy TA. Chronic catheterization of the spinal subarachnoid space. Physiol Behav 1976;17(6):1031-1036.

Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16(2):109-110.

Figure Legends

Figure 1 - Schematic figure showing the site of injection. The needle is inserted closely to occipital bone up to 4mm and slightly inclined in a cranial direction. The needle is advanced more 2mm, to perforate the atlanto-occipital membrane and reach the medullary subaracnoid space.

Figure 2 - The subarachoid injection of Even Blue dye (panel B) was performed by introducing a needle the suboccipital space (arrow). Only the central nervous system was stained (compared with panel A). a: occipital bone; b: medullary region and c: cervical spinal cord.

Figure 3 - Pharmacological validation of the method for direct subarachnoid drug injection in the medullary dorsal horn region.

A: Formalin injection into the rat TMJ region induced nociceptive behavior. Medullary subaracnoid injection (s.i.) of morphine produced a dose-dependent suppression of the nociceptive response to TMJ formalin. The symbol "*" indicates significantly different ($p<0.05$, Tukey test) from saline (1^{rst} bar); the symbol "$^{\#}$" indicates significantly different ($p<0.05$, Tukey test) from 1.5% TMJ formalin + s.i. morphine (9 μg, last bar); the symbol "$^{+}$" indicates significantly different ($p<0.05$, Tukey test) from 1.5% TMJ formalin + s.i. vehicle (saline, 2nd bar). B: Co-administration of Naloxone (15 μg) with morphine (9 μg) blocked morphine-induced antinociception. Naloxone by itself did not affect formalin-induced nociception. The symbol "*" indicates significantly different ($p<0.05$, Tukey test) from other groups. Group sample size are shown in parentheses.

Figures

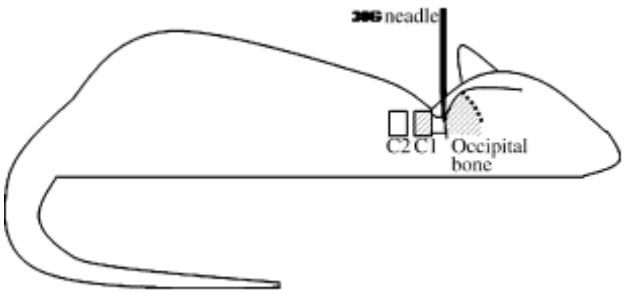

Figure 1

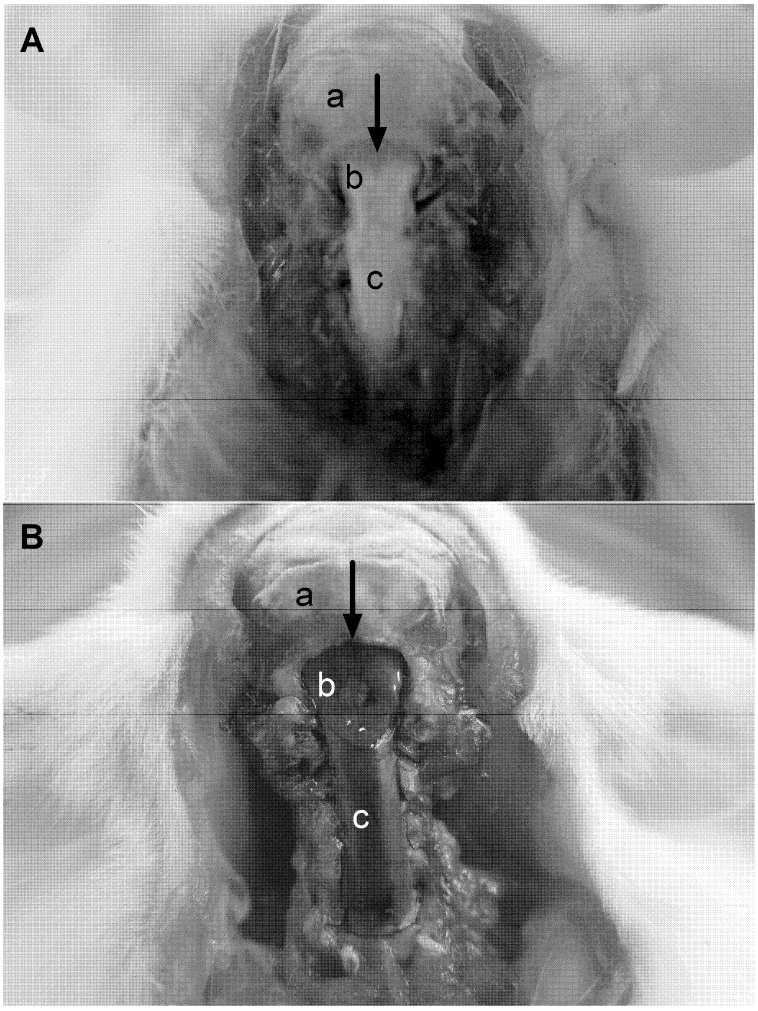

Figure 2

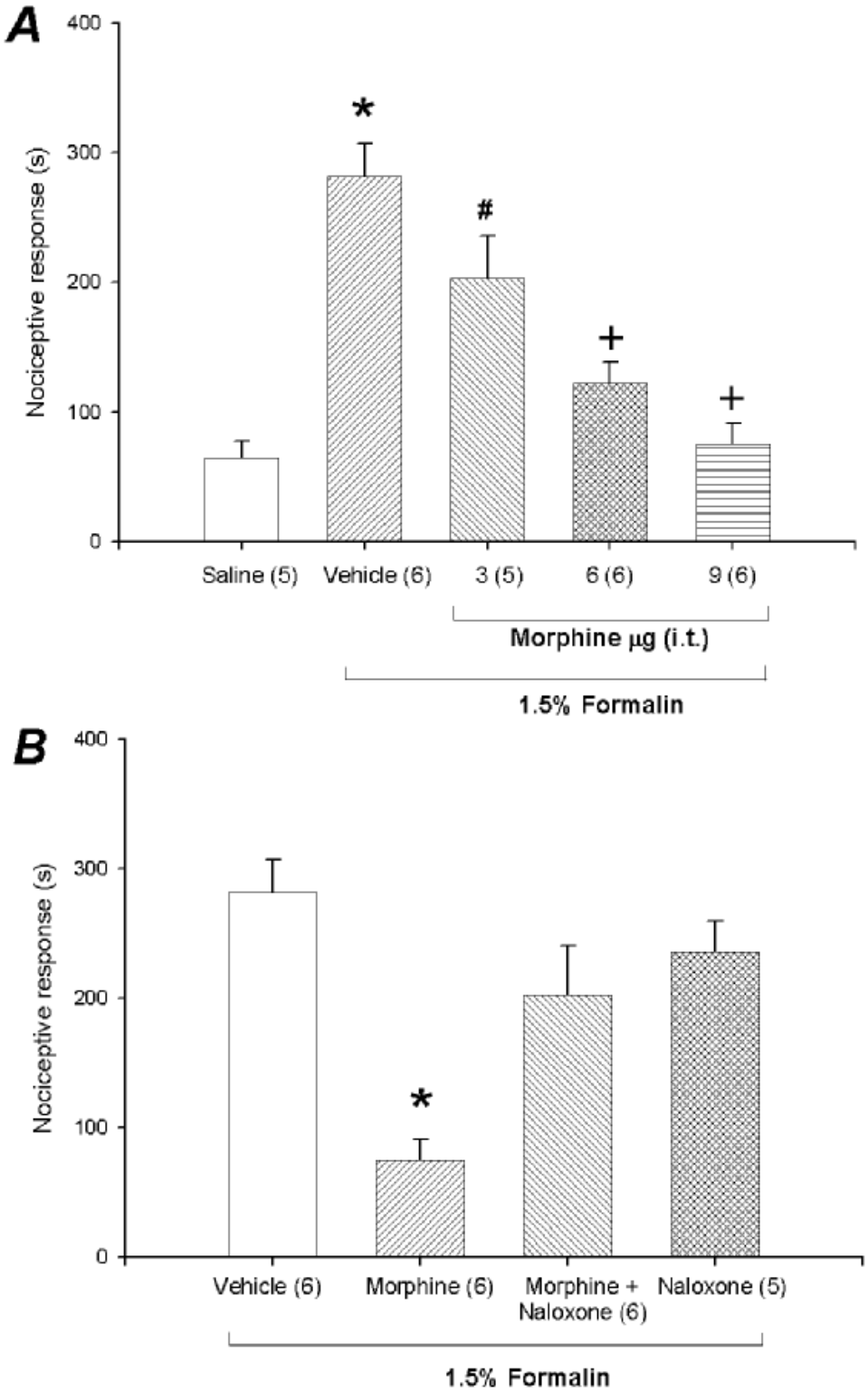
A
Nociceptive response (s)
400
300
200
100
0
*
#
+
+
Saline (5)
Vehicle (6)
3 (5)
6 (6)
9 (6)
Morphine µg (i.t.)
1.5% Formalin
B
Nociceptive response (s)
400
300
200
100
0
*
Vehicle (6)
Morphine (6)
Morphine + Naloxone (6)
Naloxone (5)
1.5% Formalin

Figure 3

Capítulo 4

The role of endogenous opioid system in sex hormones-induced TMJ antinociception

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Original Article

Keywords: Formalin test; Estradiol; Progesterone; Testosterone; Endogenous Opioid System; Temporomandibular joint pain

Abstract

We have recently demonstrated that the high estradiol level during the proestrus phase of the rat estrous cycle and that the administration of estradiol or progesterone in ovariectomized female and of testosterone in orchiectomized male rats significantly decreases formalin-induced temporomandibular joint nociception. One potential mechanism by which sex hormones may decrease temporomandibular joint nociception is by increasing opioid system activity in the central and/or peripheral nervous system. To test this hypothesis, we investigated whether the administration of the opioid receptor antagonist naloxone in the surrounding of trigeminal sensory complex, or in the TMJ region, reduces the antinociceptive effect of sex hormones in formalin-induced temporomandibular joint nociception. The antinociceptive effect induced by endogenous estradiol in proestrus females and by exogenous estradiol in ovariectomized females was blocked by the administration of naloxone in the surrounding of trigeminal sensory complex, but not in the temporomandibular joint. The antinociceptive effect induced by the administration of progesterone in ovariectomized females and of testosterone in orchiectomized males was blocked by the administration of naloxone in the surrounding of the trigeminal sensory complex and in the temporomandibular joint. These findings suggest that central and peripheral opioid mechanisms mediate the antinociceptive effect of progesterone and testosterone, while central opioid mechanisms mediate the antinociceptive effect of estradiol.

Introduction

Like many other chronic pain conditions (Unruh 1996), temporomandibular dysfunctions (TMDs) are less prevalent and severe in men than in women (Carlsson and LeResche 1995). This lower prevalence of TMD in men may result from a protective effect of testosterone. In this regard, we have demonstrated that the injection of 0.5% formalin in the temporomandibular joint (TMJ) induces a significant nociceptive behavior in naive female and gonadectomized male rats, but not in naive male rats, suggesting that testosterone decreases the risk of males developing TMJ pain (Fischer *et al.* 2007). The higher severity of TMD in women may be a consequence of hormonal fluctuation during reproductive cycle. In this regard, we have recently demonstrated that TMJ injection of 1.5% formalin induces a significantly higher nociceptive behavior in female rats during diestrus, a phase of the estrous cycle with lower estradiol level, than during proestrus, a phase with high estradiol level (Clemente *et al.* 2004; Fischer *et al. in press*). This finding parallels the higher TMJ pain in women during low estradiol times of the menstrual cycle (LeResche *et al.* 2003). The similarity between these studies suggests that the TMJ formalin model is a useful model to study the mechanisms underlying hormonal modulation of clinical TMJ pain. Hormone administration in gonadectomized animals is also useful to evaluate these mechanisms because allows to isolate the effect of each gonadal hormone, because induces a nonfluctuating hormonal level, and because allows to achieve a hormonal level that would maximizes the ability to detect the mechanisms underlying hormonal effect. Recently, we have showed that the administration of estradiol and progesterone in ovariectomized female (OVX) (Fischer *et al. in press*) and of testosterone in orchiectomized male (ORX) (Fischer *et al.* 2007) rats significantly decreases formalin-induced TMJ nociception. The mechanisms underlying the antinociceptive effect of gonadal hormones are presently unknown. However, a central or a peripheral induced increase in opioid system activity is a potential one. To test this hypothesis, we investigated whether the administration of the opioid receptor antagonist naloxone into the medullary subarachnoid space, or into the TMJ, reduces the antinociceptive effect induced by gonadal hormones. The TMJ formalin model was used as experimental assay in intact proestrus and

diestrus females and in gonadectomized males and females receiving vehicle or hormone administration.

Material and methods

Animals

This study was carried out in 200 - 300g male and female Wistar rats. All animal experimental procedures and protocols were approved by the Committee on Animal Research of the University of Campinas and are in accordance with IASP guidelines for the study of pain in animals (Zimmermann 1983). The animals were maintained on a temperature-controlled room (± 23ºC) and were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle with food and water available ad libitum.

Estrous phase determination

Estrous phase was determined by daily microscope examination of vaginal smears taken by gentle lavage, between 7 and 8 a.m. Estrous phase was confirmed before and immediately after each experiment to ensure that the rats remained in the same phase during the experiment. Proestrus phase and the initial phase of diestrus (first 4 h) were identified by the predominance (>70%) of nucleated epithelial cells and leukocytes, respectively (Butcher *et al.* 1974) in rats with at least two consecutive regular 4-5 day cycles. These phases were chosen because they represent phases of high and low ovarian hormonal level, respectively (Butcher *et al.* 1974).

Gonadectomy

Gonadectomy was performed in 45 days old animals (Gordon and Soliman 1994) under anesthesia induced by an intramuscular injection of a mixture of ketamine (55mg/Kg) and xylazine (5.5 mg/Kg). A subcutaneous injection of ketoprofen (5 mg/kg) was used for post-operative analgesia (Roughan and Flecknell 2000). Ovariectomy was performed through bilateral upper flank incisions. The ovarian bundles were ligated with 4-O silk sutures and removed, the fascia and the skin were sutured. Orchiectomy was performed through a single scrotal incision. The testicular bundles were ligated with 4-O silk sutures and removed and the skin was sutured. The efficacy of ovariectomy was verified by the absence of estrous cycle verified by observation of vaginal smears during

ten days and of orchiectomy was verified by *post mortem* examination of prostate and seminal vesicles atrophy. Sham operated animals underwent a surgical procedure similar to that of gonadectomized animals, except that the gonads were not removed. The animals were used in experiments 30-45 days after surgery.

Hormonal Manipulation

Hormonal administration was performed by daily injection of 17 β-estradiol (50μg/kg) (Gordon and Soliman 1994) and progesterone (8mg/Kg) (He *et al.* 2004) in females and of testosterone propionate (1mg) (Campos *et al.* 2003) in males, during seven days. At the seventh day, hormone injection was performed 1 hour prior to the TMJ injection of formalin. Hormones were purchased from Sigma Chemicals, St Louis, Missouri, USA and dissolved in propyleneglycol.

Drugs

Formalin solution was prepared from commercially available stock formalin (an aqueous solution of 37% of formaldehyde) further diluted in 0.9% NaCl to a concentration of 1.5%. Naloxone was dissolved in 0.9% NaCl and was injected in the subarachnoid medullary space, 15μg (Danzebrink *et al.* 1995), 30 μg or 60 μg or was co-administered, 10μg (Eisenberg *et al.* 1996) or 30 μg, with formalin in the TMJ. Formalin and naloxone were purchased from Sigma-Aldrich, St Louis, Missouri, USA.

Subarachnoid medullary injection

The injection of naloxone or its vehicle (0.9% NaCl) in the subarachnoid medullary space was performed as previously described (Fischer *et al.* 2005). Ten minutes before formalin injection into the TMJ, the rats were briefly anesthetized with halothane, and a small skin area overlying the high cervical region was shaved with an electric razor. Animals were dorsally positioned, so the suboccipital space could be easily found. A 30-gauge needle connected to a 50μl Hamilton syringe by a polyethylene cannula was first inserted below the occipital bone up to 2 mm, and slightly inclined in a cranial direction.

The needle was advanced more 2mm to perforate the atlanto-occipital membrane and reach the medullary subarachnoid space. Total injection volume in all experiments was 10µl. All injections were performed at a rate of 1µl/s.

TMJ Injections

The injection of formalin or its vehicle (0.9% NaCl) in the TMJ region was performed as previously described (Roveroni *et al.* 2001). The animals were briefly anesthetized by inhalation of halothane and a 30-gauge needle was introduced into the TMJ at the moment of injection. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50µl) (Roveroni *et al.* 2001). Each animal regained consciousness approximately 30 seconds after discontinuing the anesthetic. At the conclusion of the behavior test, each animal was anesthetized by an intraperitoneal injection of a mixture of urethane (1g/kg) and α-chloralose (50mg/kg). The Evans blue dye (5 mg/kg) was injected systemically and 15 minutes later the animals were perfused transcardially with saline (NaCl 0.9%). Since this dye binds to plasma protein, the correct site of injection was indicated by the observation that the plasma extravasation induced by the TMJ injection of formalin was restricted to the TMJ region (Roveroni *et al.* 2001).

Testing procedure for TMJ pain

Behavior test was performed during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at ± 23ºC. The nociceptive response was assessed by an observer blinded to the experimental manipulation. Before the experiments, each animal was manipulated for 7 days in the test room (handled for approximately one minute) to be habituated to the experimental manipulation. On the day of the experiment, each animal was individually placed in a test chamber (30 x 30 x 30 cm mirrored-wood chamber with a glass at the front side) for a 15 min habituation period to minimize stress. After TMJ injection, the animal was returned to the test chamber for counting nociceptive responses. The nociceptive behavior characterized by rubbing the orofacial region and flinching the head was counted in blocks of 5 minutes for 45 minutes. For each block of 5 min, the

behavior characterized by rubbing the orofacial region was quantified by the amount of time that the animal exhibited it and the behavior characterized by flinching the head was quantified by its occurrence. Considering that the head flinching behavior follows an uniform pattern of 1 s in duration, each flinching was counted as 1 s as previously described (Roveroni *et al.* 2001). Rats did not have access to food or water during the test and each animal was used once.

Statistical analysis

The area under the curve (AUC) over the entire duration of the experiment was calculated for each experimental group by summing the nociceptive behaviors induced by the TMJ injection of formalin. AUC was defined as the overall response and was used for statistical analyses. To determine if there were significant differences ($p < 0.05$) between the treatment groups in figures 1 - 4, one-way ANOVA using AUC as the dependent variable was performed followed by the Tukey post-hoc test. A t test ($p < 0.05$) was performed for comparisons between intact and sham-operated, between gonadectomized receiving or not vehicle, between contralateral injection of naloxone and 0.9% NaCl and between naloxone and 0.9% NaCl injection in the subarachnoid or in the TMJ region of animals receiving injection of 0.9% NaCl in the TMJ. Data are presented in figures as means ± S.E.M.

Results

The injection of naloxone in the medullary subarachnoid space or in the TMJ did not affect the normal behavior of the animals in any experimental group. This was evidenced by the similar behavior induced by the injection of 0.9% NaCl in the TMJ of animals receiving subarachnoid or TMJ injection of naloxone or 0.9% NaCl. (t- test, $p > 0.05$, data not shown).

The antinociceptive effect induced by high physiological level of estradiol in proestrus females or by estradiol administration in OVX females was reversed by the injection of naloxone in the surrounding of the trigeminal sensory complex (Fig 1 A and 2 A, respectively), but not in the TMJ region (Fig 1 B and 2 B, respectively).

The antinociceptive effect induced by progesterone administration in OVX females and testosterone administration in ORX males was reversed by the injection of naloxone in the surrounding of the trigeminal sensory complex (Fig 3 A and 4 A, respectively) and in the TMJ (Fig 3 B and 4 B, respectively).

The injection of naloxone in the contra-lateral TMJ did not affect formalin-induced TMJ nociception (t- test, $p > 0.05$), confirming the peripheral action of TMJ naloxone. This was assessed by the injection of formalin into the right TMJ and of naloxone or 0.9% NaCl into the left TMJ of proestrus females (303,11 $\pm$ 37,79 vs. 256,00 $\pm$ 29,97, respectively), OVX females receiving estrogen (351,19 $\pm$ 21,97 vs. 278,57 $\pm$ 22,14, respectively) or progesterone (294,02 $\pm$ 28,72 vs. 244,00 $\pm$ 21,10, respectively) and in ORX males receiving testosterone (218,25 $\pm$ 58,71 vs. 165,42 $\pm$ 19,74, respectively).

Formalin-induced TMJ nociception was similar (t- test, $p > 0.05$) between intact and sham-operated females in diestrus (432,50 $\pm$ 44,53 vs. 417,80 $\pm$ 29,65, respectively) and proestrus (256,00 $\pm$ 29,98 vs. 254,00 $\pm$ 27,95, respectively) phases of the estrus cycle and between intact and sham-operated males (281,50 $\pm$ 25,55 vs. 252,00 $\pm$ 33,92, respectively). This result demonstrates that the surgical procedure did not affect formalin-

induced TMJ nociceptive behavior. Formalin-induced TMJ nociception was similar (t- test, $p > 0.05$) between OVX females (388,86 $\pm$ 22,18 vs. 426,60 $\pm$ 21,75) and ORX males (243,40 $\pm$ 40,87 vs. 287,17 $\pm$ 30,69) receiving or not vehicle (propyleneglycol) administration, respectively. This result demonstrates that the vehicle administration did not affect formalin-induced TMJ nociception.

Discussion

This study demonstrates that sex hormones decrease formalin-induced TMJ nociception in male and female rats by increasing endogenous opioid system activity. This was evidenced by the blockade of the antinociceptive effect of sex hormones by the non-selective opioid receptor antagonist naloxone. The subarachnoid administration of naloxone in the surrounding of the trigeminal sensory complex blocked the antinociceptive effect induced by estradiol and progesterone in females and by testosterone in males. The administration of naloxone in the TMJ region also blocked the antinociceptive effect of progesterone and testosterone but not that of estradiol. These findings suggest that central and peripheral opioid mechanisms mediate the antinociceptive effect of progesterone and testosterone, while central opioid mechanisms mediate the antinociceptive effect of estradiol. The involvement of central opioid mechanisms in the antinociceptive effect induced by estradiol was demonstrated in intact proestrus females, who have high physiological level of estradiol and in OVX females receiving estradiol. This finding is in accordance with a recent human study showing that women during a high estradiol state have lower pain ratings associated with greater activation of central opioid system than women during a low estrogen state (Smith *et al.* 2006). However, it is important to point that while 15 μg of subarachnoid naloxone was enough to reverse the antinociceptive effect induced by estradiol administration in OVX females, a dose four times higher (60 μg) was necessary to induce the same effect in proestrus females. One possible explanation to this difference is that the antinociceptive effect induced by endogenous estradiol in proestrus females, but not by exogenous estradiol in OVX females, is preferentially mediated by δ and κ opioid receptor subtypes. Therefore, because naloxone preferentially blocks μ opioid receptor subtypes (Owen *et al.* 2000), higher doses might be necessary to block δ and κ opioid receptors. Consistent with this possibility, it was demonstrated that the efficacy of specific opioid receptor agonists is different in normally cycling and OVX females receiving estradiol (Stoffel *et al.* 2005).

Several lines of evidence have demonstrated that sex hormones interact with the opioid system in different brain areas involved in pain modulation (Amandusson *et al.*

1999; Flores *et al.* 2003; Smith *et al.* 2006). In this study, naloxone was injected in the surrounding of the trigeminal sensory complex, therefore, the trigeminal subnucleus caudalis, also knows as medullary dorsal horn, may be a candidate for the site of hormone-mediated opioid system activation. Subnucleus caudalis is known as a critical site for trigeminal pain modulation (Amandusson *et al.* 1996) and although no previous studies appear to have examined the presence androgen and progesterone receptors in this region, estrogen receptors are known to be present in opioid peptide-containing neurons in this region (Amandusson *et al.* 1996; Flores *et al.* 2003). However, other sites could also be involved, because naloxone can diffuse in the cerebrospinal fluid and block opioid receptors located in other areas of the central nervous system. The central mechanisms by which sex hormones modulate the opioid system to decrease TMJ nociception are presently unknown. However, the increase in the expression of opioid receptors or in the central release of endogenous opioids are potential ones, since sex hormones are known to increase the expression of endogenous opioids (Johansson *et al.* 1997; Amandusson *et al.* 1999; Bernardi *et al.* 2006) and their corresponding receptors (Petersen and LaFlamme 1997; Quinones-Jenab *et al.* 1997; Harris *et al.* 2004).

In contrast to central, peripheral opioid mechanisms do not mediate the antinociceptive effect of estradiol. Importantly, the lack of effect of the TMJ injection of naloxone in proestrus and OVX females receiving estradiol cannot be attributed to a low dose, since the lowest dose used (10 μg) is sufficient to block the effect of 1000 μg of morphine in the lip (Eisenberg *et al.* 1996). Furthermore, a dose higher than 30 μg cannot be used in the TMJ because it increases formalin-induced TMJ nociception by itself (60 μg, data not shown). The antinociceptive effect of progesterone and testosterone, in contrast to that of estradiol is also mediated by peripheral opioid mechanisms. Given that opioid receptors are expressed in the peripheral and in the central terminal of the primary sensory afferents (Stein *et al.* 2003), the injection of naloxone in the subarachnoid medullary space could block opioid receptors located in the central terminal of the primary afferent nociceptors. This possibility could explain why naloxone blocked the antinociceptive effect of progesterone and testosterone when injected in the subarachnoid or in the TMJ region.

One potential peripheral mechanism by which progesterone and testosterone decrease TMJ pain may be by increasing the rate of opioid receptor gene transcription in trigeminal ganglion, which results in increased opioid receptor expression in central and peripheral terminals of the primary afferent nociceptor. Although opioid receptors are present in trigeminal ganglion (Berg *et al.* 2007; Nunez *et al.* 2007), further studies are necessary to examine the presence of androgen and progesterone receptors in this ganglion. Another potential mechanism may be an increase in the release of opioids by inflammatory cells, since it is known that inflammatory cells release opioids (Stein *et al.* 2003) and express either progesterone (King *et al.* 1996) or testosterone receptors (Bebo *et al.* 1999).

In summary, this study showed that sex hormones decrease TMJ nociception by activating the endogenous opioid system. These findings suggest that the enhanced pain sensitivity during low hormonal states in women (LeResche *et al.* 2003; Smith *et al.* 2006) and animals (Clemente *et al.* 2004; Fischer *et al. in press*) may be mediated by a decrease in endogenous opioid activity during this period. This suggestion may help to explain the higher severity of some pain conditions (Unruh 1996), such as TMDs (Dworkin *et al.* 1990) in women than in men, that have no hormonal fluctuations. More studies are necessary to evaluate the mechanisms, pathways and opioid receptors subtypes involved in the antinociceptive effect of each sex hormone as well as the potential therapeutic interest of developing drugs that mimic or potentiate the effects of sex-hormones on opioid system.

Acknowledgements

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References

Amandusson A, Hallbeck M, Hallbeck AL, Hermanson O, Blomqvist A. Estrogen-induced alterations of spinal cord enkephalin gene expression. Pain 1999;83(2):243-248.

Amandusson A, Hermanson O, Blomqvist A. Colocalization of oestrogen receptor immunoreactivity and preproenkephalin mRNA expression to neurons in the superficial laminae of the spinal and medullary dorsal horn of rats. Eur J Neurosci 1996;8(11):2440-2445.

Bebo BF, Jr., Schuster JC, Vandenbark AA, Offner H. Androgens alter the cytokine profile and reduce encephalitogenicity of myelin-reactive T cells. J Immunol 1999;162(1):35-40.

Berg KA, Zardeneta G, Hargreaves KM, Clarke WP, Milam SB. Integrins regulate opioid receptor signaling in trigeminal ganglion neurons. Neuroscience 2007;144(3):889-897.

Bernardi F, Pluchino N, Pieri M, Begliuomini S, Lenzi E, Puccetti S, Casarosa E, Luisi M, Genazzani AR. Progesterone and medroxyprogesterone acetate effects on central and peripheral allopregnanolone and beta-endorphin levels. Neuroendocrinology 2006;83(5-6):348-359.

Butcher RL, Collins WE, Fugo NW. Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat. Endocrinology 1974;94(6):1704-1708.

Campos M, Morais Pde L, Pupo AS. Effects of castration and of testosterone replacement on alpha(1)-adrenoceptor subtypes in the rat vas deferens. Eur J Pharmacol 2003;471(2):149-155.

Carlsson G, LeResche L. Epidemiology of temporomandibular disorders. Seattle, WA 1995.

Clemente JT, Parada CA, Veiga MC, Gear RW, Tambeli CH. Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint. Neurosci Lett 2004;372(3):250-255.

Danzebrink RM, Green SA, Gebhart GF. Spinal mu and delta, but not kappa, opioid-receptor agonists attenuate responses to noxious colorectal distension in the rat. Pain 1995;63(1):39-47.

Dworkin SF, Huggins KH, LeResche L, Von Korff M, Howard J, Truelove E, Sommers E. Epidemiology of signs and symptoms in temporomandibular disorders: clinical signs in cases and controls. J Am Dent Assoc 1990;120(3):273-281.

Eisenberg E, Vos BP, Strassman AM. The peripheral antinociceptive effect of morphine in a rat model of facial pain. Neuroscience 1996;72(2):519-525.

Fischer L, Clemente JT, Tambeli CH. The protective role of testosterone in the development of temporomandibular joint pain. J Pain 2007;8(5):437-442.

Fischer L, Parada CA, Tambeli CH. A novel method for subarachnoid drug delivery in the medullary region of rats. J Neurosci Methods 2005;148(2):108-112.

Fischer L, Torres-Chávez KE, Clemente-Napimoga JT, Jorge D, Arsati F, Arruda Veiga MCF, Tambeli CH. The effect of sex and ovarian hormones on temporomandibular joint nociception in rats J pain *in press*.

Flores CA, Shughrue P, Petersen SL, Mokha SS. Sex-related differences in the distribution of opioid receptor-like 1 receptor mRNA and colocalization with estrogen receptor mRNA in neurons of the spinal trigeminal nucleus caudalis in the rat. Neuroscience 2003;118(3):769-778.

Gordon FT, Soliman MR. Diurnal variation in the acute effects of estradiol and progesterone on beta-endorphin and Met-enkephalin levels in specific brain regions of ovariectomized rats. Pharmacology 1994;49(3):192-198.

Harris JA, Chang PC, Drake CT. Kappa opioid receptors in rat spinal cord: sex-linked distribution differences. Neuroscience 2004;124(4):879-890.

He J, Evans CO, Hoffman SW, Oyesiku NM, Stein DG. Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury. Exp Neurol 2004;189(2):404-412.

Johansson P, Ray A, Zhou Q, Huang W, Karlsson K, Nyberg F. Anabolic androgenic steroids increase beta-endorphin levels in the ventral tegmental area in the male rat brain. Neurosci Res 1997;27(2):185-189.

King A, Gardner L, Loke YW. Evaluation of oestrogen and progesterone receptor expression in uterine mucosal lymphocytes. Human reproduction (Oxford, England) 1996;11(5):1079-1082.

LeResche L, Mancl L, Sherman JJ, Gandara B, Dworkin SF. Changes in temporomandibular pain and other symptoms across the menstrual cycle. Pain 2003;106(3):253-261.

Nunez S, Lee JS, Zhang Y, Bai G, Ro JY. Role of peripheral mu-opioid receptors in inflammatory orofacial muscle pain. Neuroscience 2007;146(3):1346-1354.

Owen MD, Unal CB, Callahan MF, Trivedi K, York C, Millington WR. Glycyl-glutamine inhibits the respiratory depression, but not the antinociception, produced by morphine. Am J Physiol Regul Integr Comp Physiol 2000;279(5):R1944-1948.

Petersen SL, LaFlamme KD. Progesterone increases levels of mu-opioid receptor mRNA in the preoptic area and arcuate nucleus of ovariectomized, estradiol-treated female rats. Brain Res Mol Brain Res 1997;52(1):32-37.

Quinones-Jenab V, Jenab S, Ogawa S, Inturrisi C, Pfaff DW. Estrogen regulation of mu-opioid receptor mRNA in the forebrain of female rats. Brain Res Mol Brain Res 1997;47(1-2):134-138.

Roughan JV, Flecknell PA. Effects of surgery and analgesic administration on spontaneous behaviour in singly housed rats. Res Vet Sci 2000;69(3):283-288.

Roveroni RC, Parada CA, Cecilia M, Veiga FA, Tambeli CH. Development of a behavioral model of TMJ pain in rats: the TMJ formalin test. Pain 2001;94(2):185-191.

Smith YR, Stohler CS, Nichols TE, Bueller JA, Koeppe RA, Zubieta JK. Pronociceptive and antinociceptive effects of estradiol through endogenous opioid neurotransmission in women. J Neurosci 2006;26(21):5777-5785.

Stein C, Schafer M, Machelska H. Attacking pain at its source: new perspectives on opioids. Nat Med 2003;9(8):1003-1008.

Stoffel EC, Ulibarri CM, Folk JE, Rice KC, Craft RM. Gonadal hormone modulation of mu, kappa, and delta opioid antinociception in male and female rats. J Pain 2005;6(4):261-274.

Unruh AM. Gender variations in clinical pain experience. Pain 1996;65(2-3):123-167.

Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16(2):109-110.

Figure Legends

Figure 1- Central opioid mechanisms in proestrus females.

A- Naloxone administration in the subarachnoid medullary space blocked the antinociceptive effect induced by high physiological level of estradiol in proestrus females. The symbol "*" indicates a nociceptive behavior significantly lower than that induced by TMJ injection of formalin in diestrus females. The symbol "+" indicates a nociceptive behavior significantly greater than that induced by injection of formalin in the TMJ of proestrus females receiving subarachnoid injection 0.9% NaCl or low doses (15 and 30μg) of naloxone, Tukey test, $p<0.05$, s.a. = subarachnoid

B- Naloxone co-administered with formalin in the TMJ did not affect the antinociceptive effect induced by high physiological level of estradiol in proestrus females. The symbol "*" indicates a nociceptive behavior significantly lower than that induced by TMJ injection of formalin in diestrus females. The symbol "+" indicates a nociceptive behavior significantly greater than that induced by injection of formalin co-administered with 0.9% NaCl or the lower dose of naloxone (10 μg) in proestrus females, Tukey test, $p<0.05$.

Figure 2- Central opioid mechanisms in OVX females receiving estradiol.

A- Naloxone administration in the subarachnoid medullary space blocked the antinociceptive effect induced by estradiol administration in OVX females. The symbol "*" indicates a nociceptive behavior significantly lower than that induced by TMJ injection of formalin in OVX+V females. The symbol "+" indicates a nociceptive behavior significantly greater than that induced by injection of formalin in the TMJ of OVX+E females receiving subarachnoid injection 0.9% NaCl, Tukey test, $p<0.05$, s.a. = subarachnoid; OVX+V = ovariectomized receiving vehicle; OVX+E = ovariectomized receiving estradiol.

B- Naloxone co-administered with formalin in the TMJ did not affect the antinociceptive effect induced by estradiol administration in OVX females. The symbol "*" indicates a nociceptive behavior significantly lower than that induced by TMJ injection of

formalin in OVX+V females. The symbol "+" indicates a nociceptive behavior significantly greater than that induced by TMJ injection of formalin co-administered with 0.9% NaCl or with the lower dose of naloxone (10 μg) in OVX+E females, Tukey test, $p<0.05$, s.a. = subarachnoid; OVX+V = ovariectomized receiving vehicle; OVX+E = ovariectomized receiving estradiol.

Figure 3- Central and peripheral opioid mechanisms in OVX females receiving progesterone.

A- Naloxone administration in the subarachnoid medullary space blocked the antinociceptive effect induced by progesterone administration in OVX females. The symbol "*" indicates a nociceptive behavior significantly lower than that induced by TMJ injection of formalin in OVX+V females. The symbol "+" indicates a nociceptive behavior significantly greater than that induced by injection of formalin in the TMJ of OVX+P females receiving subarachnoid injection 0.9% NaCl, Tukey test, $p<0.05$, s.a. = subarachnoid; OVX+V = ovariectomized receiving vehicle; OVX+P = ovariectomized receiving progesterone.

B- Naloxone co-administered with formalin in the TMJ blocked the antinociceptive effect induced by progesterone administration in OVX females. The symbol "*" indicates a nociceptive behavior significantly lower than that induced by TMJ injection of formalin in OVX+V females. The symbol "+" indicates a nociceptive behavior significantly greater than that induced by TMJ injection of formalin co-administered with 0.9% NaCl in OVX+P females, Tukey test, $p<0.05$, s.a. = subarachnoid; OVX+V = ovariectomized receiving vehicle; OVX+P = ovariectomized receiving progesterone.

Figure 4- Central and peripheral opioid mechanisms in ORX males receiving testosterone.

A- Naloxone administration in the subarachnoid medullary space blocked the antinociceptive effect induced by testosterone administration in ORX males. The symbol "*" indicates a nociceptive behavior significantly lower than that induced by TMJ injection of formalin in ORX+V males. The symbol "+" indicates a nociceptive behavior

significantly greater than that induced by injection of formalin in the TMJ of ORX+T males receiving subarachnoid injection 0.9% NaCl, Tukey test, $p<0.05$, s.a. = subarachnoid; ORX+V = orchiectomized receiving vehicle; ORX+T = orchiectomized receiving testosterone.

B- Naloxone co-administered with formalin in the TMJ blocked the antinociceptive effect induced by testosterone administration in ORX males. The symbol "*" indicates a nociceptive behavior significantly lower than that induced by TMJ injection of formalin in ORX+V males. The symbol "+" indicates a nociceptive behavior significantly greater than that induced by TMJ injection of formalin co-administered with 0.9% NaCl in ORX+T males, Tukey test, $p<0.05$, s.a. = subarachnoid; ORX+V = orchiectomized receiving vehicle; ORX+T = orchiectomized receiving testosterone.

Figures

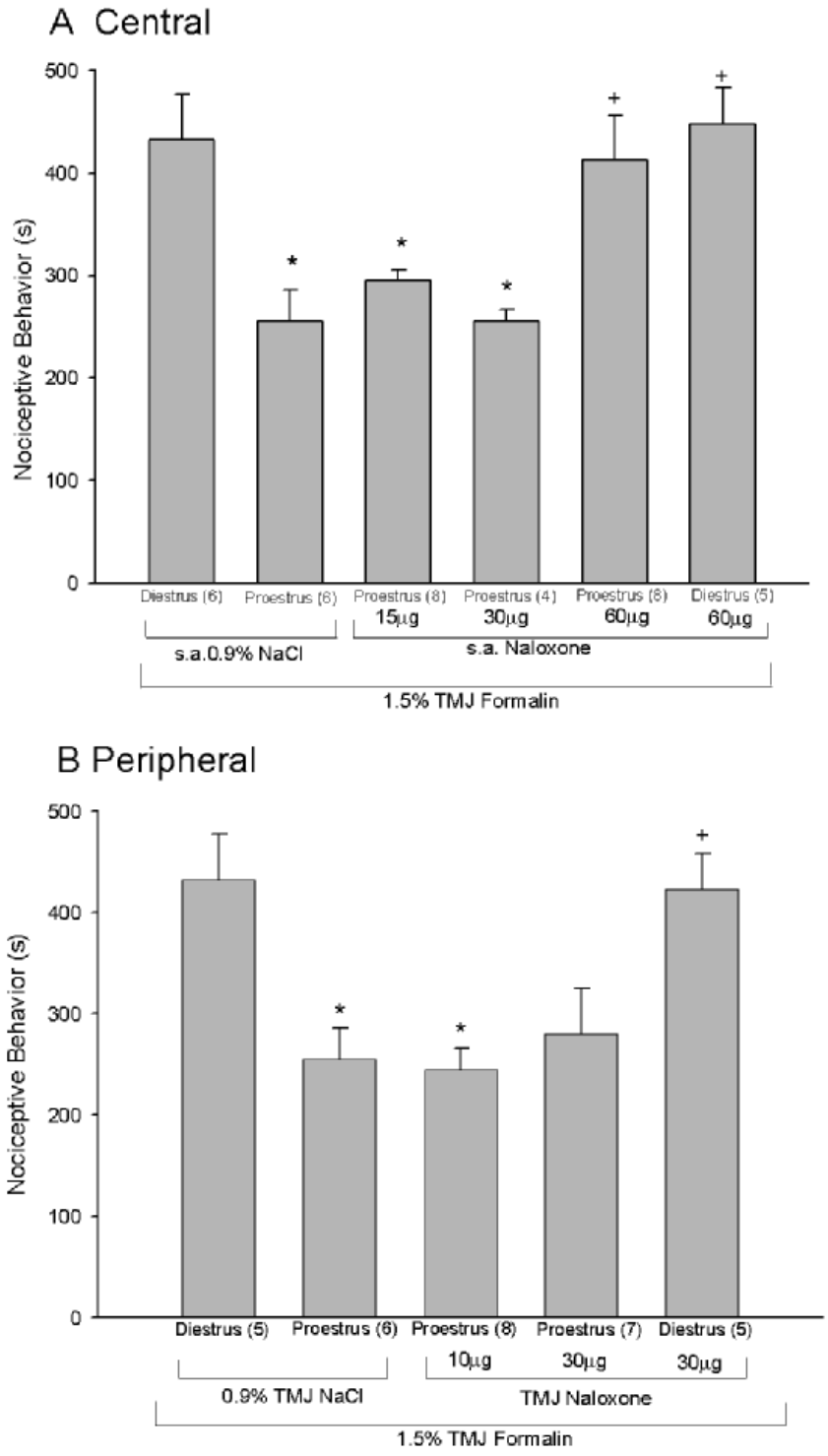


Figure 1

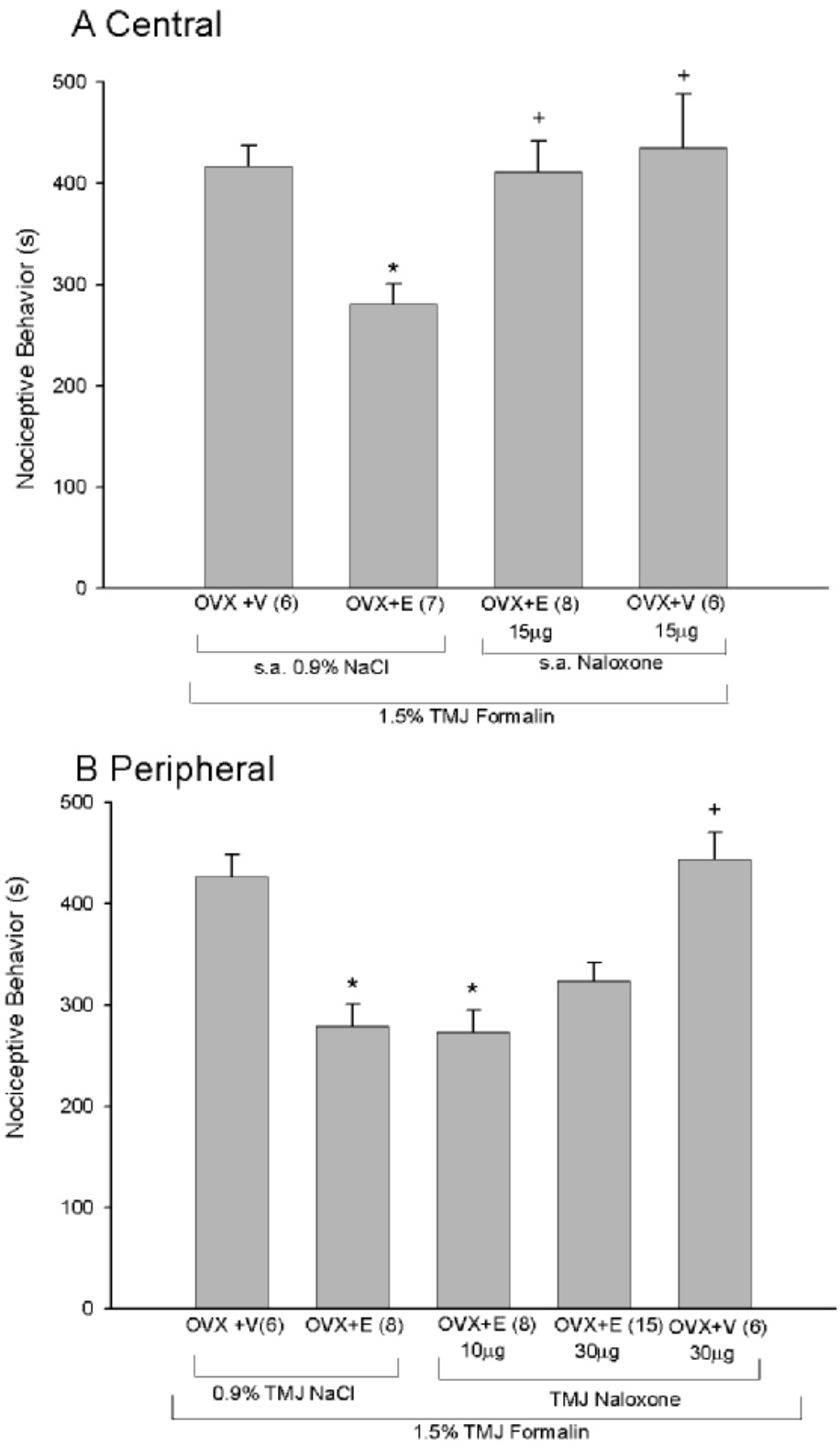
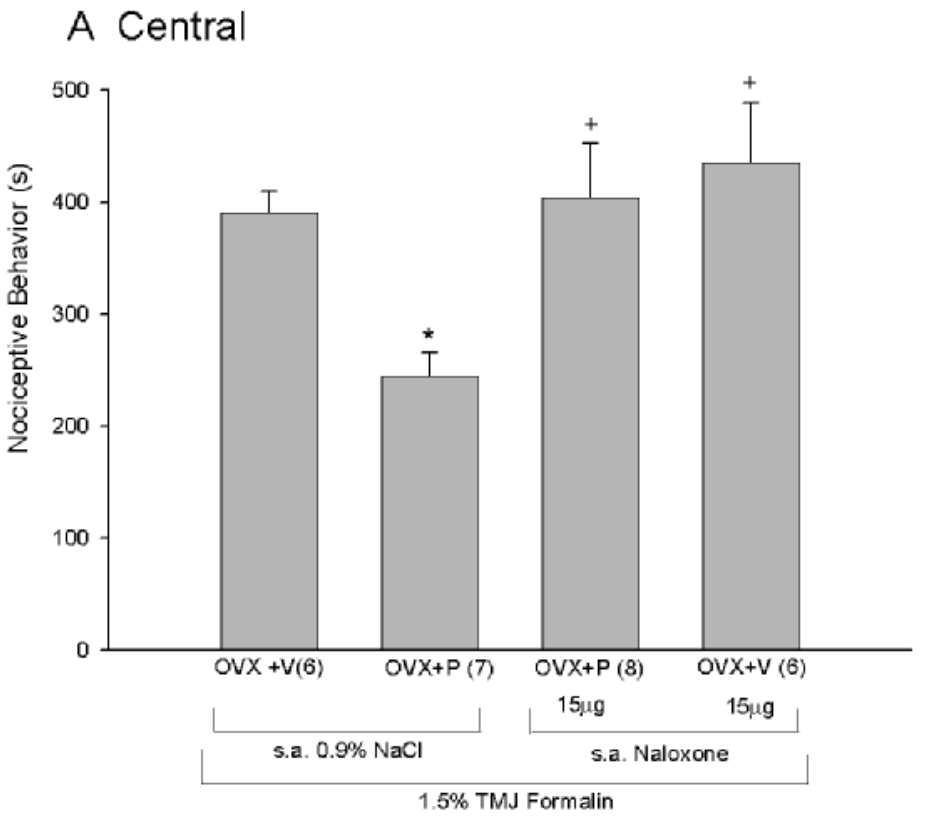
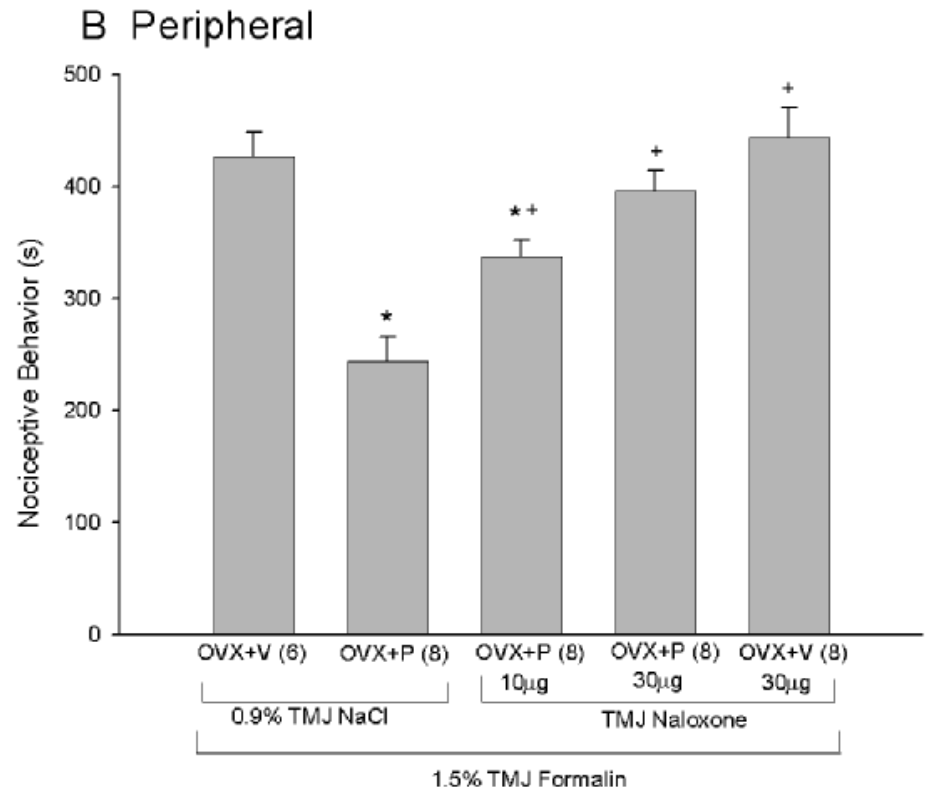

A Central
Nociceptive Behavior (s)
500
400
300
200
100
0
*
+
+
OVX +V (6)
OVX+E (7)
OVX+E (8)
15µg
OVX+V (6)
15µg
s.a. 0.9% NaCl
s.a. Naloxone
1.5% TMJ Formalin
B Peripheral
Nociceptive Behavior (s)
500
400
300
200
100
0
*
*
+
OVX +V(6)
OVX+E (8)
OVX+E (8)
10µg
OVX+E (15)
30µg
OVX+V (6)
30µg
0.9% TMJ NaCl
TMJ Naloxone
1.5% TMJ Formalin

Figure 2


A Central
Nociceptive Behavior (s)
500
400
300
200
100
0
OVX +V(6)
OVX+P (7)
OVX+P (8)
OVX+V (6)
15μg
15μg
s.a. 0.9% NaCl
s.a. Naloxone
1.5% TMJ Formalin


B Peripheral
Nociceptive Behavior (s)
500
400
300
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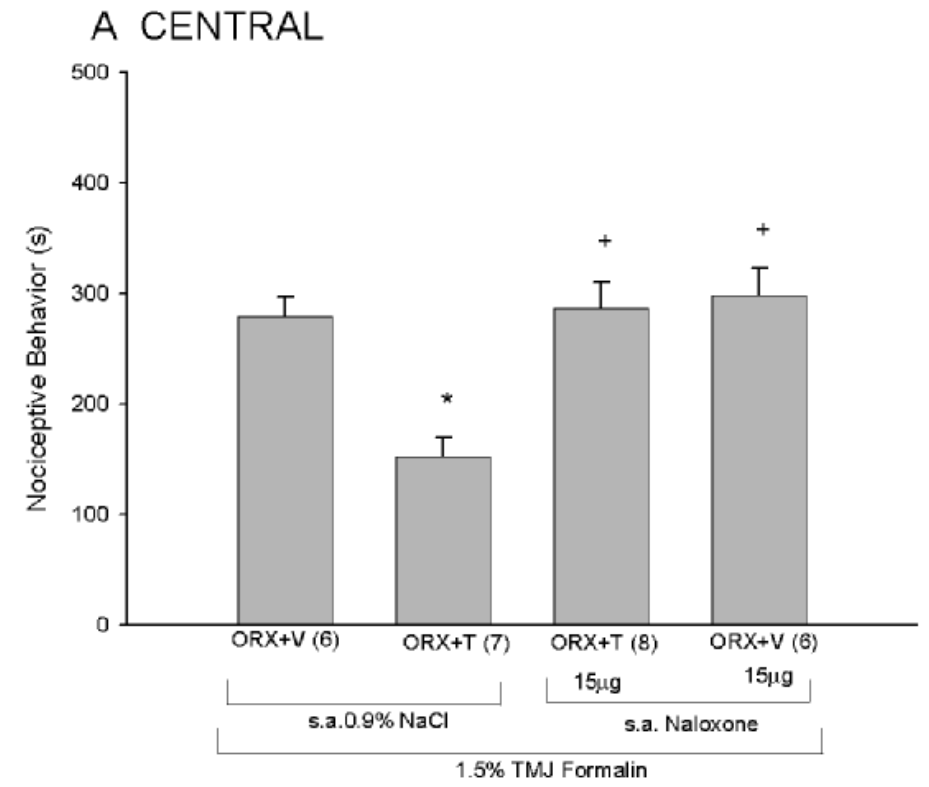
100
0
OVX+V (6)
OVX+P (8)
OVX+P (8)
OVX+P (8)
OVX+V (8)
10μg
30μg
30μg
0.9% TMJ NaCl
TMJ Naloxone
1.5% TMJ Formalin

Figure 3


A CENTRAL
Nociceptive Behavior (s)
500
400
300
200
100
0
*
+
+
ORX+V (6)
ORX+T (7)
ORX+T (8)
ORX+V (6)
15µg
15µg
s.a.0.9% NaCl
s.a. Naloxone
1.5% TMJ Formalin

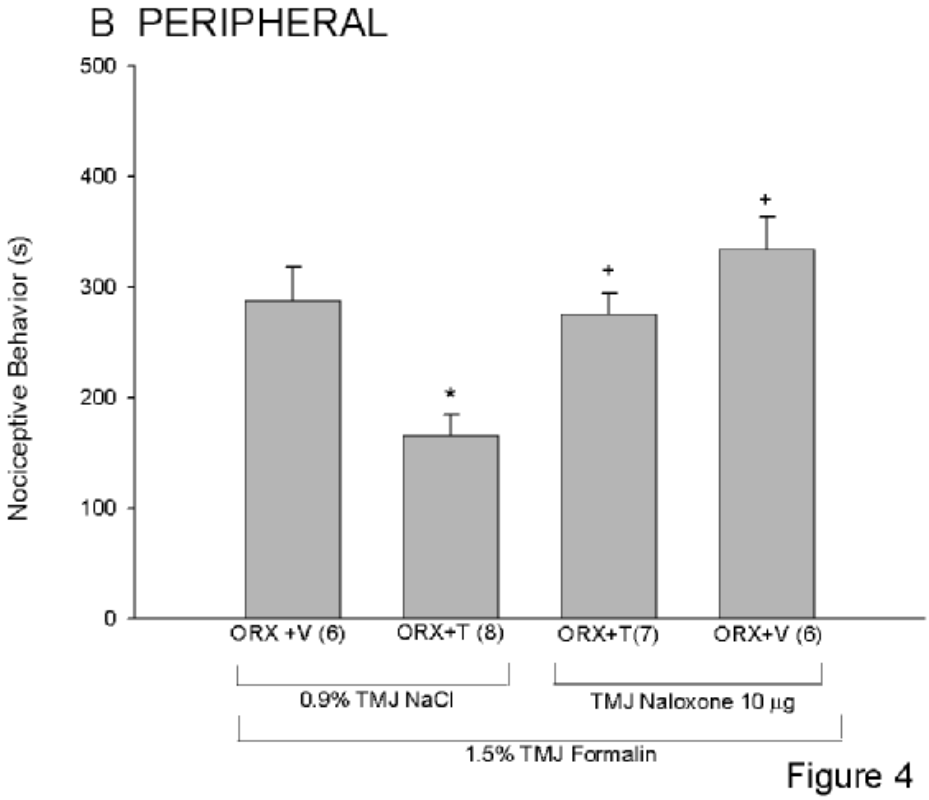

B PERIPHERAL
Nociceptive Behavior (s)
500
400
300
200
100
0
*
+
+
ORX +V (6)
ORX+T (8)
ORX+T(7)
ORX+V (6)
0.9% TMJ NaCl
TMJ Naloxone 10 µg
1.5% TMJ Formalin

Figure 4

Capítulo 5

Peripheral estradiol induces temporomandibular joint antinociception in rats through the NO/cGMP signaling pathway

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Original Article

Keywords: Estradiol ; Temporomandibular joint pain; Nitric Oxide; cyclic CMP; Formalin; membrane estrogen receptors.

Abstract

Recently, we have reported that systemic estradiol decreases formalin-induced temporomandibular joint nociception in female rats. However, the mechanisms underlying the antinociceptive effect of estradiol are presently unknown. In this study, we used the TMJ formalin model in rats to investigate whether the antinociceptive effect of estradiol is mediated by a peripheral non-genomic mechanism, and if so, whether this mechanism is mediated by the activation of the NO-cGMP signaling pathway and of opioid receptors. Co-administration of estradiol with formalin significantly reduced formalin-induced temporomandibular joint nociception in ovariectomized and diestrus females but not in males. The antinociceptive effect of estradiol was mimicked by estradiol conjugated with bovine serum albumin, which does not diffuse through the plasma membrane, and was blocked by the peripherally restricted estrogen receptor antagonist ICI 182-780. Co-administration of the nitric oxide synthase Nitro-L-arginine or of the guanilato cyclase 1H-(1,2,4)-oxadiasolo (4,2-a) quinoxalin-1-one inhibitor blocked the antinociceptive effect of estradiol and E-BSA, while the opioid receptor antagonist naloxone had no effect. These findings suggest that estradiol decreases TMJ nociception in female rats through a peripheral non-genomic activation of the nitric oxide – cyclic guanosine monophosfate signaling pathway.

Introduction

The majority of chronic pain conditions (Unruh 1996) such as temporomandibular dysfunctions (Dworkin *et al.* 1990) are more prevalent and severe in women than in men, suggesting a role of gonadal hormones in pain modulation. In fact, TMJ pain in women (LeResche *et al.* 2003) and formalin-induced TMJ nociception in female rats (Clemente *et al.* 2004) are lower during high estradiol levels of the reproductive cycle. These findings suggest that estradiol decreases TMJ pain and are further supported by other animal (Gaumond *et al.* 2002; Ceccarelli *et al.* 2003; Pajot *et al.* 2003; Kuba *et al.* 2006; Mannino *et al.* 2007) and human (Smith *et al.* 2006) studies showing an antinociceptive effect of estradiol. Although not yet tested, the antinociceptive effect of estradiol might result from a peripheral non-genomic mechanism. This idea is supported by "in vitro" studies showing that estradiol inhibits calcium channel currents in neurons of the dorsal root ganglia via a non-genomic mechanism mediated by membrane estrogen receptors (Lee et al. 2002; Chaban et al. 2003). The activation of these receptors activates the oxide nitric - cyclic guanosine mono-phosphate (cGMP) signaling pathway (NO-cGMP), as demonstrated in endothelial cells (Stefano *et al.* 2000). The activation of this pathway in primary nociceptive afferents has been associated with the antinociceptive effect of anti-inflammatory drugs (Deciga-Campos and Lopez-Munoz 2004; Ventura-Martinez *et al.* 2004) and opioids (Durate *et al.* 1990; Pol 2007). Furthermore, peripheral opioid mechanisms mediate the antinociceptive effect induced by estradiol and progesterone in the TMJ of pregnant rats (Arthuri *et al.* 2005). Therefore, in this study we used the TMJ formalin model in rats to investigate whether the antinociceptive effect of estradiol is mediated by a peripheral non-genomic mechanism, and if so, whether this mechanism is mediated by the activation of the NO-cGMP signaling pathway and of opioid receptors.

Material and methods

Animals

This study was carried out in 200- 300g ovariectomized (OVX) and diestrus female and male Wistar rats. We used OVX females because the depletion of systemic estradiol possibly facilitates the assessment of peripheral estrogen-mediated effect. We also used normally cycling diestrus females (low physiological estradiol level) to control for a possible change in estrogen receptor expression following ovariectomy (Pajot *et al.* 2003). We also included male rats in this study to evaluate if the peripheral administration of estradiol induces a sex specific antinociceptive effect, as it does the systemic administration. All animal experimental procedures and protocols were approved by the Committee on Animal Research of the University of Campinas and are in accordance with IASP guidelines for the study of pain in animals (Zimmermann 1983). The animals were maintained on a temperature-controlled room (± 23ºC) and were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle (lights on at 06:00 A.M.) with food and water available ad libitum.

Drugs

Formalin was prepared from commercially available stock formalin (an aqueous solution of 37% of formaldehyde) further diluted in 0.9% NaCl to a concentration of 1.5%; (Roveroni *et al.* 2001); Estradiol (17β-estradiol, 0.4μg, 1.2μg; (Ceccarelli *et al.* 2004) and 3.6μg) was dissolved in propileneglycol; Estradiol coupled to bovine serum albumin (E-BSA, 1.2 μg of estradiol plus BSA) was dissolved in 0.9% NaCl; the selective estrogen receptor antagonist ICI 182-780 (0.16μg, 1μg and 6μg; (Ceccarelli *et al.* 2004) was dissolved in dimethyl sulfoxide (DMSO); the NO synthase inhibitor Nitro-L-arginine (L-NNA 22μg; (Toda *et al.* 1993), was dissolved in 0.9% NaCl; the guanilato cyclase inhibitor 1H-(1,2,4)-oxadiasolo (4,2-a) quinoxalin-1-one (ODQ 0.8 and 8μg; (Cunha *et al.* 1999), was dissolved in DMSO; the opioid receptor antagonist Naloxone (10μg; (Eisenberg *et al.* 1996) and 30μg), was dissolved in 0.9% NaCl. Formalin; estradiol; E-BSA; naloxone and

L-NNA were purchased from Sigma-Aldrich St. Louis, MO, USA; ODQ and ICI182-780 were purchased from Tocris Bioscience, St. Louis, MO, USA.

Steroid hormones conjugated with bovine serum albumin have been extensively used to assess their non-genomic effects (Kelly and Levin 2001). However, it was suggested that E-BSA has biological activity not observed with estradiol (Stevis *et al.* 1999). For this reason, the experiments were performed using both 17β-estradiol and E-BSA. The dose-response curves of all drugs were performed in OVX females and the most effective dose was selected for further experiments.

Estrous phase determination

Estrous phase was determined by daily microscope examination of vaginal smears between 7 and 8 a.m. The initial phase of diestrus (first 4 hours) was identified by the predominance (>70%) of leukocytes (Butcher *et al.* 1974) in rats with at least two consecutive regular 4-5 day cycles and was confirmed before and immediately after each experiment, to ensure that the rats remained in diestrus. This phase was chosen because it is characterized by low physiological levels of estradiol (Butcher *et al.* 1974).

Gonadectomy

Ovariectomy (45 days old females; (Gordon and Soliman 1994) was performed through bilateral upper flank incisions. The ovarian bundles were tied off with 4-O silk sutures and the ovaries removed. The fascia and the skin were closed with 4-O silk sutures (Waynforth and Flecknell 1992a). Sham operated animals underwent a surgical procedure similar to that of OVX animals, except that the ovaries were not removed. The procedures were carried out under anesthesia induced by an intramuscular injection of a mixture of ketamine (55mg/Kg) and xylazine (5.5 mg/Kg). A subcutaneous injection of ketoprofen (5 mg/kg) was used for post-operative analgesia (Roughan and Flecknell 2000). OVX and sham-operated rats were used in experiments when they were three months of age. The efficacy of ovariectomy was confirmed by the absence of estrous cycle determined by observation of vaginal smears during ten days.

TMJ Injections

The animals were briefly anesthetized by inhalation of halothane to allow the TMJ injection; each animal regained consciousness approximately 30 seconds after discontinuing the anesthetic. The TMJ injection was performed with a 30-gauge needle introduced into the TMJ at the moment of injection. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50µl) (Roveroni *et al.* 2001). The volume injection was 15µl per drug. At the conclusion of the behavior test, each animal was anesthetized by an intraperitoneal injection of a mixture of urethane (1g/kg) and α-chloralose (50mg/kg). The Evans blue dye (5 mg/kg) was systemically injected and 15 minutes later the animals were submitted to cardiac perfusion with normal saline. Since this dye binds to plasma protein, the correct site of injection was indicated by the observation that the plasma extravasation induced by the TMJ injection of formalin was restricted to the TMJ region (Haas *et al.* 1992).

Testing procedure for TMJ pain

Behavior test was performed during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at ± 23ºC (Rosland 1991). The nociceptive response was assessed by an observer blinded to the experimental manipulation. Before the experiments, each animal was manipulated for 7 days in the test room (handled for approximately one minute) to be habituated to the experimental manipulation. On the day of the experiment, each animal was individually placed in a test chamber (30 x 30 x 30 cm mirrored-wood chamber with a glass at the front side) for a 15 min habituation period to minimize stress. After the TMJ injection, the animal was returned to the test chamber for counting nociceptive responses. The nociceptive behavior characterized by rubbing the orofacial region and flinching the head was counted in blocks of 5 minutes for 45 minutes. For each block of 5 min, the behavior characterized by rubbing the orofacial region was quantified by the amount of time that the animal exhibited it and the behavior characterized by flinching the head was quantified by its occurrence. Considering that the head flinching behavior follows an uniform pattern of 1 second in duration, each flinching was counted as

1 second as previously described (Roveroni *et al.* 2001). Rats did not have access to food or water during the test and each animal was used once.

Statistical analysis

The area under the curve (AUC) was calculated for each treatment group by summing the behaviors recorded in each block of 5 min during the entire duration of the experiment. To determine if there were significant differences ($p < 0.05$) between the treatment groups, one-way ANOVA using AUC as the dependent variable was performed. If there was a significant between-subjects main effect of treatment group, post-hoc contrasts, using the Tukey method test were performed to determine the basis of the significant difference. Data are expressed in figures as means ± S.E.M.

Results

Co-administration of estradiol or estradiol conjugated with bovine serum albumin (E-BSA) with formalin into the rat's TMJ significantly decreased formalin-induced TMJ nociception in OVX (Fig. 1 A) and diestrus (Fig. 1 B) females, but did not affect TMJ nociception in males, even at a dose three times higher than that used in females (Fig. 1 C). Formalin-induced TMJ nociception was similar (t- test, $p > 0.05$) in intact (422.5 $\pm$ 15.5) and sham-operated diestrus females (415.8 $\pm$ 17.6).

To discard that the antinociceptive effect induced by the TMJ injection of estradiol could derivate from a non-specific action, we evaluated the involvement of estrogen receptors in estradiol-induced antinociception by assessing the effect of the estrogen receptor antagonist ICI 182-780. Co-administration of ICI 182-780 blocked the antinociceptive effect of estradiol and of E-BSA in OVX (Fig. 2A and B, respectively) and of estradiol in diestrus females (Fig. 2C).

Co-administration of the NO synthase L-NNA or of the guanilato cyclase ODQ inhibitor blocked the antinociceptive effect of estradiol and E-BSA in OVX (Fig. 3A and B and Fig. 4 A and B, respectively) and of estradiol in diestrus (Fig. 3C and 4C, respectively) females.

The opioid receptor antagonist naloxone did not affect estradiol-induced TMJ antinociception in OVX (Fig. 5 A) and diestrus (Fig. 5 B) females.

Discussion

This study demonstrated that estradiol decreases TMJ nociception in female rats through a peripheral non-genomic mechanism mediated by the activation of the NO-cGMP signaling pathway but not of opioid receptors. The evidences are that the administration of estradiol or E-BSA into the TMJ of female rats significantly decreased formalin-induced TMJ nociception. This antinociceptive effect was blocked by the estrogen receptor antagonist ICI 182-780 and by the NO synthase and the guanilato cyclase inhibitors, L-NNA and ODQ, respectively, but not by the opioid receptor antagonist naloxone. Both ICI 182-780 (Clark *et al.* 2003) and E-BSA do not cross the blood-brain barrier, confirming the peripheral action of estradiol. The higher dose of ICI 182-780 required to block estradiol induced-antinociception in OVX than in diestrus females might result from an increased estrogen receptor expression in trigeminal primary afferent neurons after ovariectomy, as previously demonstrated in neurons of the trigeminal subnucleus caudalis (Pajot *et al.* 2003). The finding that the membrane impermeable compound E-BSA (Kelly and Levin 2001) mimicked the antinociceptive effect induced by estradiol suggests that membrane estrogen receptors mediate estradiol-induced TMJ antinociception. In fact, the antinociceptive effect observed in the current study is incompatible with the classic genomic effects of estradiol that take hours to days to occur (McEwen 2001). In males, in contrast to females, formalin-induced TMJ nociception was not affected by estradiol, confirming our previous findings that the antinociceptive effect of estradiol is sex-specific (Fischer *et al.* 2007). The mechanisms underlying the sex-specificity of estradiol-induced TMJ antinociception are unknown, however, organizational effects of estradiol during female development (Jost 1983) may contribute to that.

Our finding that estradiol induces TMJ antinociception through a non-genomic peripheral activation of the NO-cGMP signaling pathway is supported by the finding that estradiol increases the activity of the NO synthase (Chen *et al.* 1999; Simoncini *et al.* 2000) and guanilato cyclase (Fiorelli *et al.* 1996) by non-genomic mechanisms. The increased activity of these enzymes results in an increased level of cGMP, which has been associated with peripheral antinociception (Durate *et al.* 1990; Qian *et al.* 1996; Almeida and Duarte

2007). Although it has been demonstrated that the peripheral analgesic effect of opioids is mediated by the activation of NO-cGMP signaling pathway (Granados-Soto *et al.* 1997; Pol 2007) and that estradiol modulates the opioid system through non-genomic mechanisms (Lagrange *et al.* 1995; Brown *et al.* 2007) the findings of the current study suggest that the peripheral non-genomic antinociceptive effect induced by estradiol in the TMJ is not mediated by opioid mechanisms.

The peripheral activation of the NO-cGMP pathway by estradiol may contributes to the decreased TMJ pain induced by high physiological estradiol level in the TMJ of female rats (Clemente *et al.* 2004) and women (LeResche *et al.* 2003). However, in physiological conditions, other mechanisms are probably involved in estradiol-induced antinociception, since estrogen receptors are widely distributed in areas of the central nervous system involved in pain transmission and modulation (McEwen 2001).

In summary, this study shows that estradiol decreases TMJ nociception in female rats through a peripheral activation of the NO-cGMP signaling pathway. These findings suggest that the NO-cGMP signaling pathway may be a valuable molecular target for the development of drugs, such as estrogen receptor ligands devoid of classic estrogenic activity and of side effects related to anti-inflammatory and opioids, which also activate this pathway to induce peripheral antinociception.

Acknowledgements

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References

Almeida RT, Duarte ID. Nitric oxide/cGMP pathway mediates orofacial antinociception induced by electroacupuncture at the St36 acupoint. Brain Res 2007.

Arthuri MT, Gameiro GH, Tambeli CH, de Arruda Veiga MC. Peripheral effect of a kappa opioid receptor antagonist on nociception evoked by formalin injected in TMJ of pregnant rats. Life Sci 2005;76(10):1177-1188.

Brown CH, Brunton PJ, Russell JA. Rapid Estradiol-17beta Modulation of Opioid Actions on the Electrical and Secretory Activity of Rat Oxytocin Neurons In vivo. Neurochem Res 2007.

Chaban VV, Mayer EA, Ennes HS, Micevych PE. Estradiol inhibits atp-induced intracellular calcium concentration increase in dorsal root ganglia neurons. Neuroscience 2003;118(4):941-948.

Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW. Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. J Clin Invest 1999;103(3):401-406.

Clark AS, Guarraci FA, Megroz AB, Porter DM, Henderson LP. The display of sexual behaviors by female rats administered ICI 182,780. Horm Behav 2003;43(4):454-464.

Clemente JT, Parada CA, Veiga MC, Gear RW, Tambeli CH. Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint. Neurosci Lett 2004;372(3):250-255.

Cunha FQ, Teixeira MM, Ferreira SH. Pharmacological modulation of secondary mediator systems--cyclic AMP and cyclic GMP--on inflammatory hyperalgesia. Br J Pharmacol 1999;127(3):671-678.

Durate ID, Lorenzetti BB, Ferreira SH. Peripheral analgesia and activation of the nitric oxide-cyclic GMP pathway. Eur J Pharmacol 1990;186(2-3):289-293.

Dworkin SF, Huggins KH, LeResche L, Von Korff M, Howard J, Truelove E, Sommers E. Epidemiology of signs and symptoms in temporomandibular disorders: clinical signs in cases and controls. J Am Dent Assoc 1990;120(3):273-281.

Eisenberg E, Vos BP, Strassman AM. The peripheral antinociceptive effect of morphine in a rat model of facial pain. Neuroscience 1996;72(2):519-525.

Evrard HC, Balthazart J. Rapid regulation of pain by estrogens synthesized in spinal dorsal horn neurons. J Neurosci 2004;24(33):7225-7229.

Fiorelli G, Gori F, Frediani U, Franceschelli F, Tanini A, Tosti-Guerra C, Benvenuti S, Gennari L, Becherini L, Brandi ML. Membrane binding sites and non-genomic effects of estrogen in cultured human pre-osteoclastic cells. J Steroid Biochem Mol Biol 1996;59(2):233-240.

Fischer L, Clemente JT, Tambeli CH. The protective role of testosterone in the development of temporomandibular joint pain. J Pain 2007;8(5):437-442.

Fischer L, Torres-Chávez KE, Clemente-Napimoga JT, Jorge D, Arsati F, Arruda Veiga MCF, Tambeli CH. The effect of sex and ovarian hormones on temporomandibular joint nociception in rats J pain *in press*.

Gaumond I, Arsenault P, Marchand S. The role of sex hormones on formalin-induced nociceptive responses. Brain Res 2002;958(1):139-145.

Gordon FT, Soliman MR. Diurnal variation in the acute effects of estradiol and progesterone on beta-endorphin and Met-enkephalin levels in specific brain regions of ovariectomized rats. Pharmacology 1994;49(3):192-198.

Granados-Soto V, Rufino MO, Gomes Lopes LD, Ferreira SH. Evidence for the involvement of the nitric oxide-cGMP pathway in the antinociception of morphine in the formalin test. Eur J Pharmacol 1997;340(2-3):177-180.

Haas DA, Nakanishi O, MacMillan RE, Jordan RC, Hu JW. Development of an orofacial model of acute inflammation in the rat. Arch Oral Biol 1992;37(5):417-422.

Jost A. Genetic and hormonal factors in sex differentiation of the brain. Psychoneuroendocrinology 1983;8(2):183-193.

Keith JC, Jr., Albert LM, Leathurby Y, Follettie M, Wang L, Borges-Marcucci L, Chadwick CC, Steffan RJ, Harnish DC. The utility of pathway selective estrogen receptor ligands that inhibit nuclear factor-kappa B transcriptional activity in models of rheumatoid arthritis. Arthritis Res Ther 2005;7(3):R427-438.

Kelly MJ, Levin ER. Rapid actions of plasma membrane estrogen receptors. Trends Endocrinol Metab 2001;12(4):152-156.

Kuba T, Wu HB, Nazarian A, Festa ED, Barr GA, Jenab S, Inturrisi CE, Quinones-Jenab V. Estradiol and progesterone differentially regulate formalin-induced nociception in ovariectomized female rats. Hormones and behavior 2006;49(4):441-449.

Lagrange AH, Ronnekleiv OK, Kelly MJ. Estradiol-17 beta and mu-opioid peptides rapidly hyperpolarize GnRH neurons: a cellular mechanism of negative feedback? Endocrinology 1995;136(5):2341-2344.

Lantin-Hermoso RL, Rosenfeld CR, Yuhanna IS, German Z, Chen Z, Shaul PW. Estrogen acutely stimulates nitric oxide synthase activity in fetal pulmonary artery endothelium. Am J Physiol 1997;273(1 Pt 1):L119-126.

Lee DY, Chai YG, Lee EB, Kim KW, Nah SY, Oh TH, Rhim H. 17Beta-estradiol inhibits high-voltage-activated calcium channel currents in rat sensory neurons via a non-genomic mechanism. Life Sci 2002;70(17):2047-2059.

LeResche L, Mancl L, Sherman JJ, Gandara B, Dworkin SF. Changes in temporomandibular pain and other symptoms across the menstrual cycle. Pain 2003;106(3):253-261.

Ma B, Rong W, Dunn PM, Burnstock G. 17beta-estradiol attenuates alpha, beta-meATP-induced currents in rat dorsal root ganglion neurons. Life Sci 2005;76(22):2547-2558.

Mannino CA, South SM, Quinones-Jenab V, Inturrisi CE. Estradiol replacement in ovariectomized rats is antihyperalgesic in the formalin test. J Pain 2007;8(4):334-342.

Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. Braz J Biol 2002;62(4A):609-614.

McEwen BS. Invited review: Estrogens effects on the brain: multiple sites and molecular mechanisms. J Appl Physiol 2001;91(6):2785-2801.

Pajot J, Ressot C, Ngom I, Woda A. Gonadectomy induces site-specific differences in nociception in rats. Pain 2003;104(1-2):367-373.

Pol O. The involvement of the nitric oxide in the effects and expression of opioid receptors during peripheral inflammation. Curr Med Chem 2007;14(18):1945-1955.

Qian Y, Chao DS, Santillano DR, Cornwell TL, Nairn AC, Greengard P, Lincoln TM, Bredt DS. cGMP-dependent protein kinase in dorsal root ganglion: relationship with nitric oxide synthase and nociceptive neurons. J Neurosci 1996;16(10):3130-3138.

Rosland JH. The formalin test in mice: the influence of ambient temperature. Pain 1991;45(2):211-216.

Roughan JV, Flecknell PA. Effects of surgery and analgesic administration on spontaneous behaviour in singly housed rats. Res Vet Sci 2000;69(3):283-288.

Roveroni RC, Parada CA, Cecilia M, Veiga FA, Tambeli CH. Development of a behavioral model of TMJ pain in rats: the TMJ formalin test. Pain 2001;94(2):185-191.

Sachs D, Cunha FQ, Ferreira SH. Peripheral analgesic blockade of hypernociception: activation of arginine/NO/cGMP/protein kinase G/ATP-sensitive K+ channel pathway. Proc Natl Acad Sci U S A 2004;101(10):3680-3685.

Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 2000;407(6803):538-541.

Simoncini T, Mannella P, Fornari L, Caruso A, Varone G, Genazzani AR. Genomic and non-genomic effects of estrogens on endothelial cells. Steroids 2004;69(8-9):537-542.

Smith YR, Stohler CS, Nichols TE, Bueller JA, Koeppe RA, Zubieta JK. Pronociceptive and antinociceptive effects of estradiol through endogenous opioid neurotransmission in women. J Neurosci 2006;26(21):5777-5785.

Stevis PE, Deecher DC, Suhadolnik L, Mallis LM, Frail DE. Differential effects of estradiol and estradiol-BSA conjugates. Endocrinology 1999;140(11):5455-5458.

Unruh AM. Gender variations in clinical pain experience. Pain 1996;65(2-3):123-167.

Vandenput L, Swinnen JV, Van Herck E, Verstuyf A, Boonen S, Bouillon R, Vanderschueren D. The estrogen receptor ligand ICI 182,780 does not impair the bone-sparing effects of testosterone in the young orchidectomized rat model. Calcif Tissue Int 2002;70(3):170-175.

Waynforth H, Flecknell P. Experimental and surgical technique in the rat

Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16(2):109-110.

Figure Legends

Figure 1- Effect of intra-articular administration of estradiol on formalin-induced TMJ nociception.

A- 17β-estradiol or E-BSA significantly reduced formalin-induced TMJ nociception in OVX females. The symbol "*" indicates a nociceptive response significantly greater than that induced by the TMJ injection of 0.9% NaCl. The symbol "+" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol) (Tukey test, $p < 0.05$). In this and subsequent figures, data are plotted as mean ± s.e.m., group sample sizes are shown in parentheses; see Methods for additional details regarding data presentation and analysis. Abbreviations: OVX = Ovariectomized; E-BSA = Estradiol coupled to bovine serum albumin.

B- 17β-estradiol or E-BSA significantly reduced formalin-induced TMJ nociception in diestrus females. The symbol "*" indicates a nociceptive response significantly greater than that induced by the TMJ injection of 0.9% NaCl. The symbol "+" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol) (Tukey test, $p < 0.05$).

C- 17β-estradiol did not affect formalin-induced TMJ nociception in males. The symbol "*" indicates a nociceptive response significantly greater than that induced by the TMJ injection of 0.9% NaCl (Tukey test, $p < 0.05$).

Figure 2- Effect of intra-articular administration of the estrogen receptor antagonist ICI 182-780 on the antinociceptive effect induced by estradiol in the TMJ.

A- ICI 182-780 blocked the antinociceptive effect induced by estradiol in OVX females. The symbol "*" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol). The symbol "+" indicates a nociceptive response significantly greater than that induced by the TMJ injection of formalin plus estradiol plus DMSO (dimethylsulfoxide, ICI182-780 vehicle) (Tukey test, $p < 0.05$).

B- ICI 182-780 blocked the antinociceptive effect induced by E-BSA in OVX females. The symbol "*" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol). The symbol "+" indicates a nociceptive response significantly greater than that induced by TMJ injection of formalin plus E-BSA plus DMSO (dimethylsulfoxide, ICI182-780 vehicle), (Tukey test, $p< 0.05$).

C- ICI 182-780 blocked the antinociceptive effect induced by estradiol in diestrus females. The symbol "*" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol). The symbol "+" indicates a nociceptive response significantly greater than that induced by the TMJ injection of formalin plus estradiol plus DMSO (dimethylsulfoxide, ICI182-780 vehicle) (Tukey test, $p< 0.05$).

Figure 3- Effect of intra-articular administration of the NO synthase inhibitor L-NNA on the antinociceptive effect induced by estradiol in the TMJ.

A- L-NNA blocked the antinociceptive effect induced by estradiol in OVX females. The symbol "*" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol). The symbol "+" indicates a nociceptive response significantly greater than that induced by the TMJ injection of formalin plus estradiol (Tukey test, $p< 0.05$).

B- L-NNA blocked the antinociceptive effect induced by E-BSA in OVX females. The symbol "*" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol). The symbol "+" indicates a nociceptive response significantly greater than that induced by the TMJ injection of formalin plus E-BSA (Tukey test, $p< 0.05$).

C- L-NNA blocked the antinociceptive effect induced by estradiol in diestrus females. The symbol "*" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol). The symbol "+"

indicates a nociceptive response significantly greater than that induced by the TMJ injection of formalin plus estradiol (Tukey test, $p< 0.05$).

Figure 4- Effect of intra-articular administration of the Guanilato cyclase inhibitor ODQ on the antinociceptive effect induced by estradiol in the TMJ.

A- ODQ blocked the antinociceptive effect induced by estradiol in OVX females. The symbol "*" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol). The symbol "+" indicates a nociceptive response significantly greater than that induced by the TMJ injection of formalin plus estradiol plus DMSO (dimethylsulfoxide, ODQ vehicle) (Tukey test, $p< 0.05$).

B- ODQ blocked the antinociceptive effect induced by E-BSA in OVX females. The symbol "*" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol). The symbol "+" indicates a nociceptive response significantly greater than that induced by the TMJ injection of formalin plus E-BSA plus DMSO (dimethylsulfoxide, ODQ vehicle) (Tukey test, $p< 0.05$).

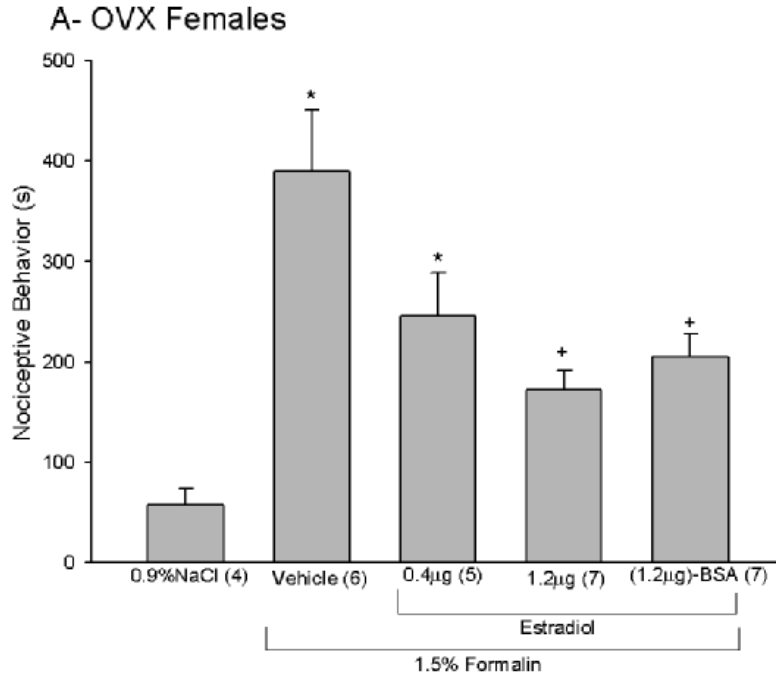
C- ODQ blocked the antinociceptive effect induced by estradiol in diestrus females. The symbol "*" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol). The symbol "+" indicates a nociceptive response significantly greater than that induced by the TMJ injection of formalin plus estradiol plus DMSO (dimethylsulfoxide, ODQ vehicle) (Tukey test, $p< 0.05$).

Figure 5- Effect of intra-articular administration of the opioid receptor antagonist naloxone on the antinociceptive effect induced by estradiol in the TMJ.

A- Naloxone did not affect the antinociceptive effect induced by estradiol in OVX females. The symbol "*" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol) (Tukey test, $p< 0.05$).

B- Naloxone did not affect the antinociceptive effect induced by estradiol in diestrus females. The symbol "*" indicates a nociceptive response significantly lower than that induced by the TMJ injection of formalin plus vehicle (propyleneglycol) (Tukey test, $p < 0.05$).

Figures



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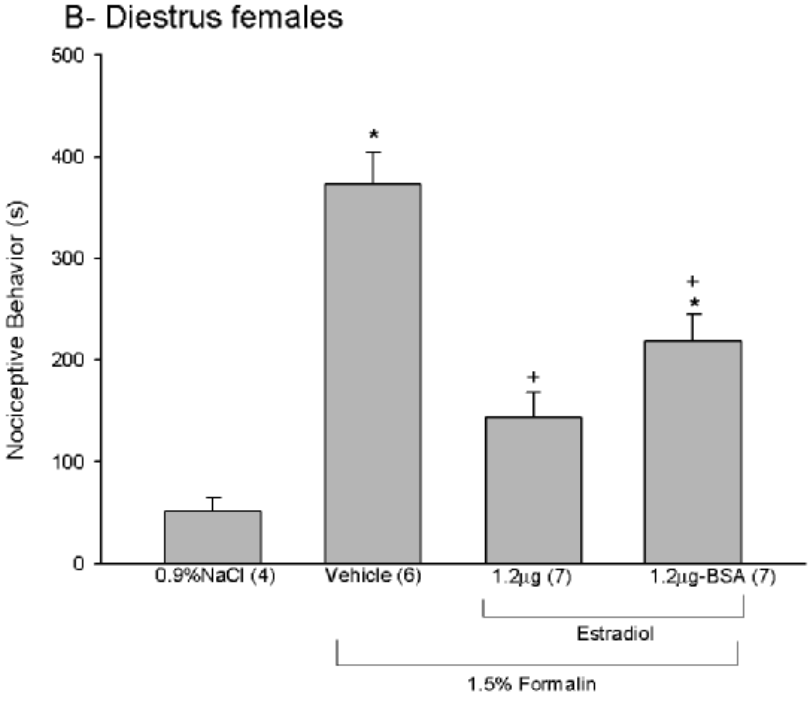

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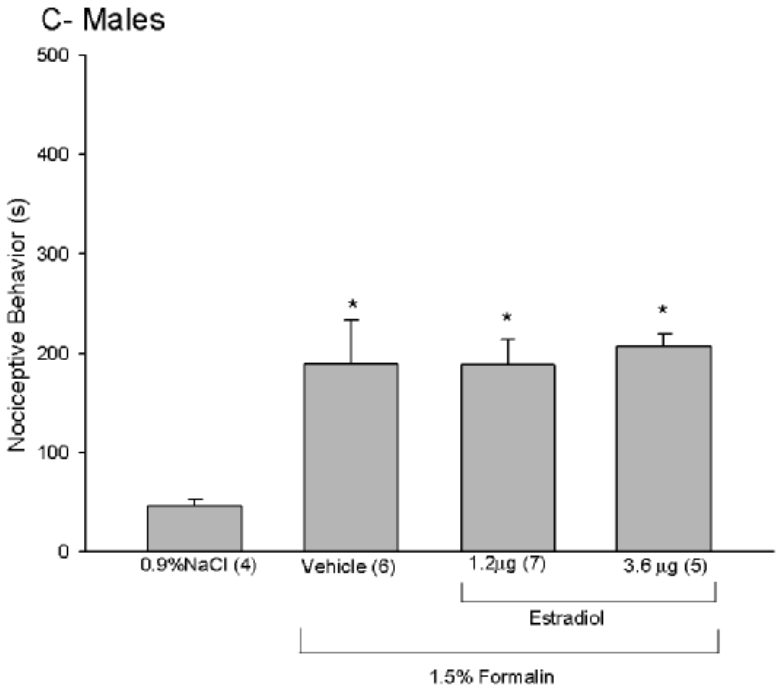

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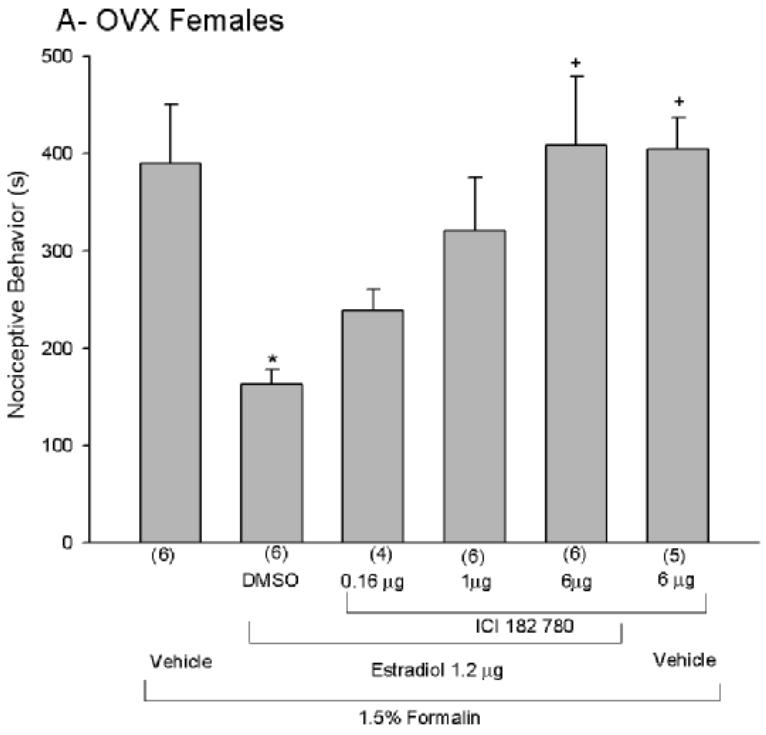

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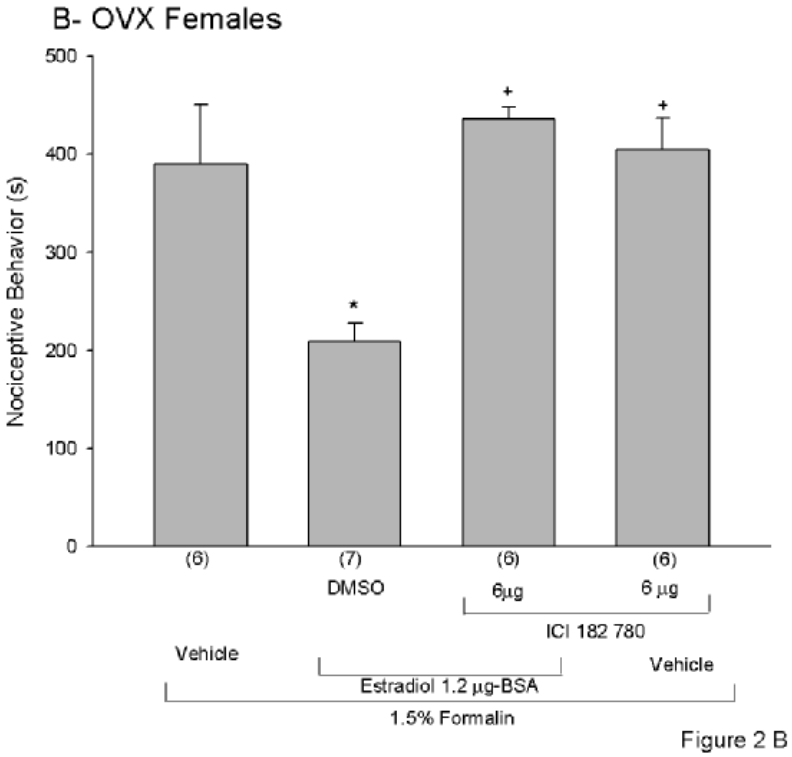

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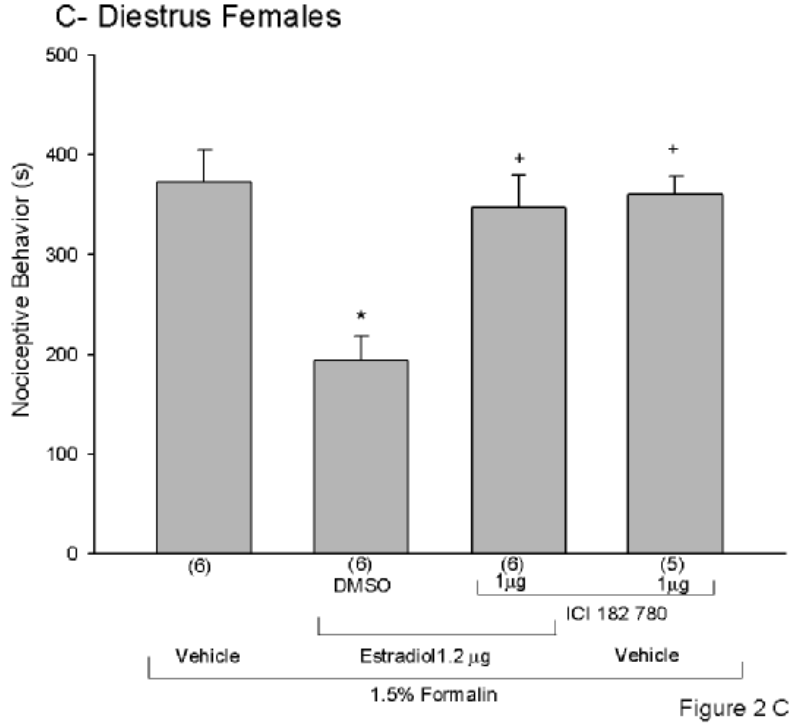

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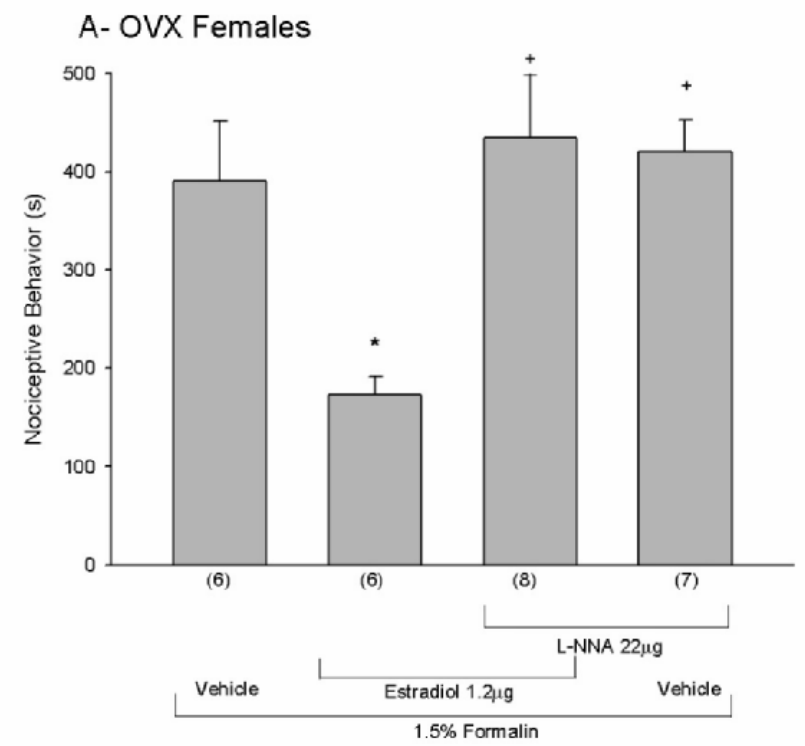

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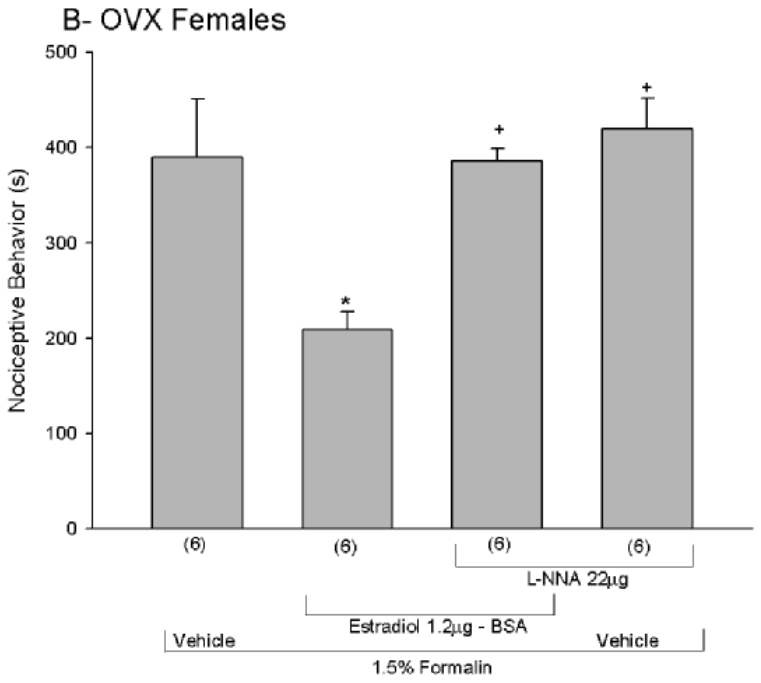

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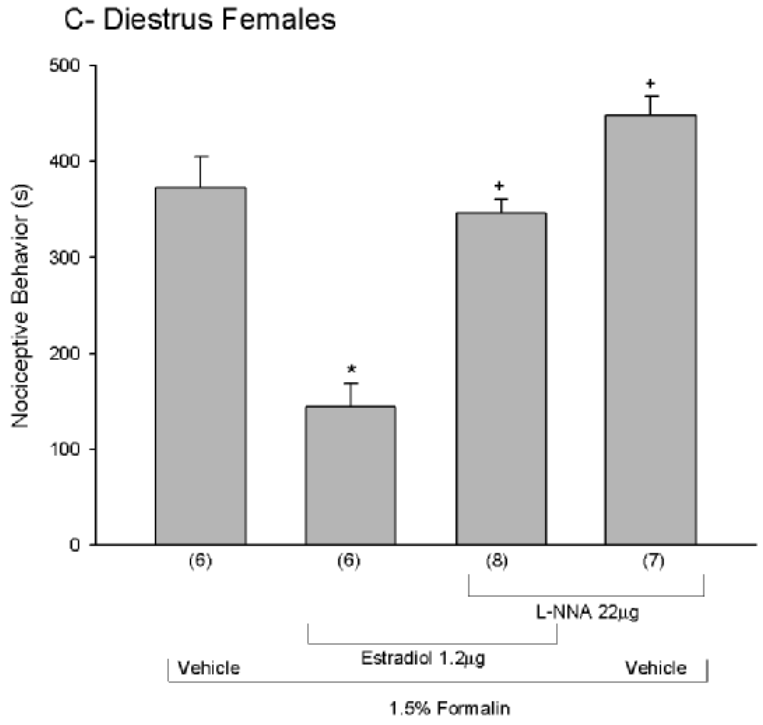

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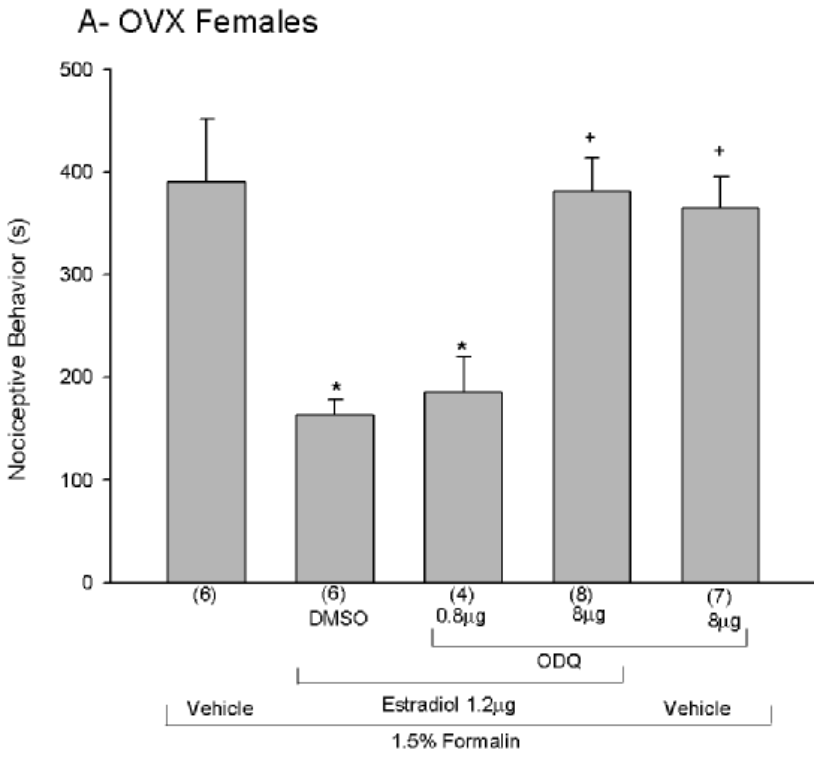

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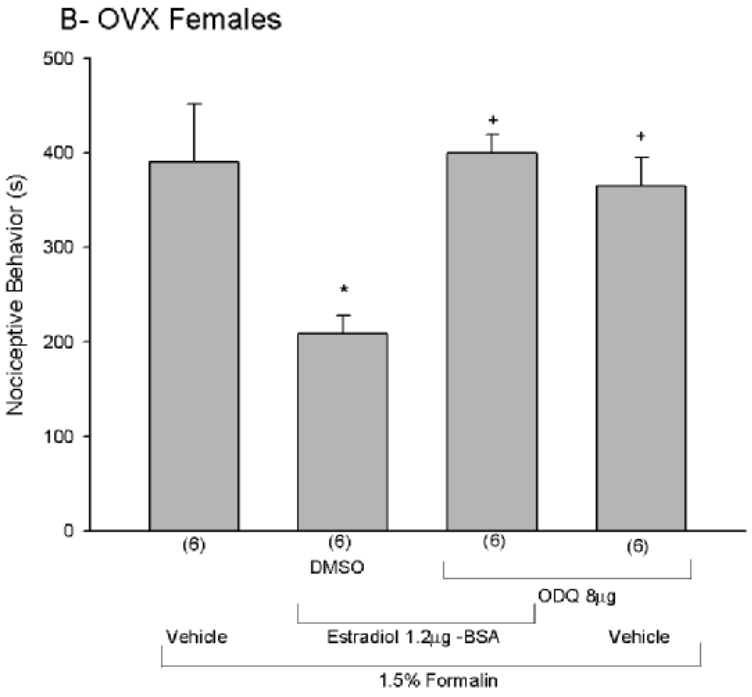

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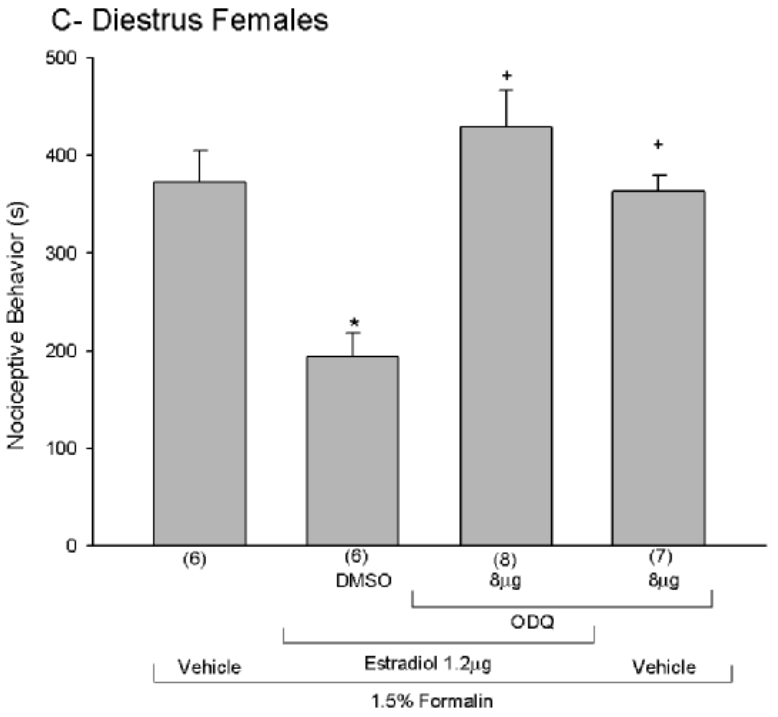

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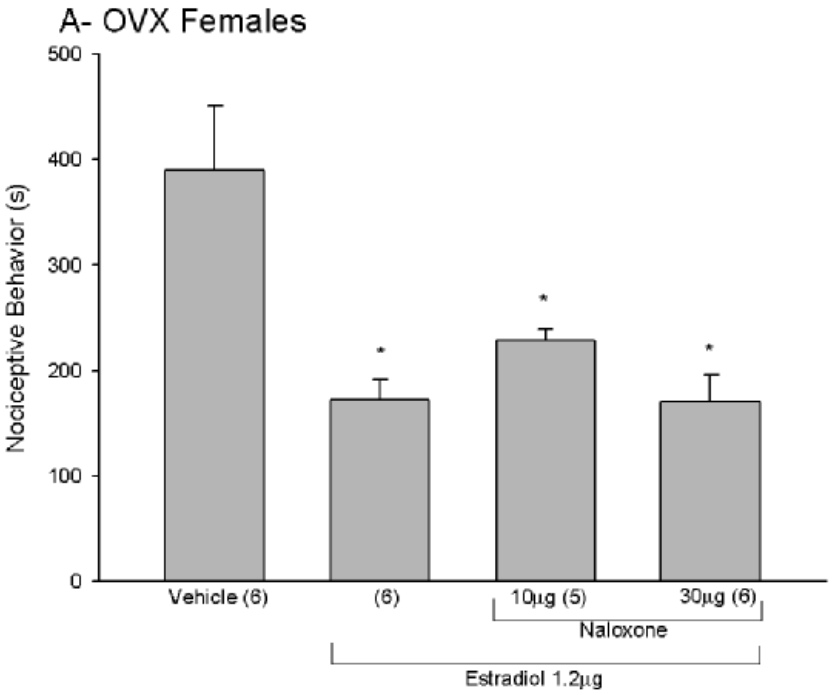

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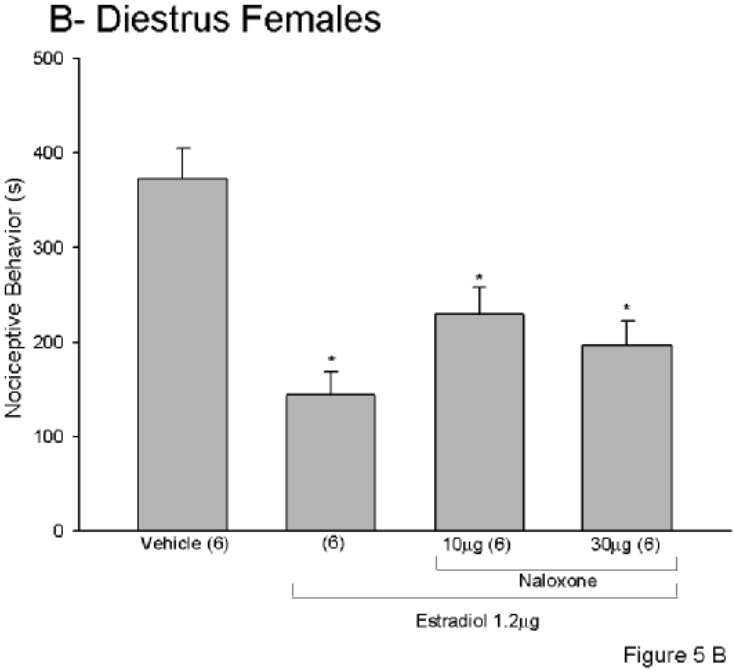

Figure 5 B

DISCUSSÃO

Os resultados desse estudo demonstram que os níveis fisiológicos de testosterona diminuem o risco de ratos desenvolverem nocicepção da ATM e os de estrógeno diminuem a nocicepção da ATM em ratas. Além disso, a nocicepção da ATM também é diminuída pela administração de estrógeno e progesterona em ratas gonadectomizadas e de testosterona em ratos gonadectomizados. O efeito antinociceptivo induzido pelo estrógeno e pela progesterona em fêmeas e pela testosterona em machos é mediado por mecanismos opióides centrais, enquanto mecanismos opióides periféricos também medeiam o efeito da progesterona e da testosterona, mas não do estrógeno. Consistente com a ausência de envolvimento do sistema opióide periférico no efeito antinociceptivo do estrógeno, demonstramos que administração de estrógeno na ATM reduz a nocicepção através de um mecanismo periférico não genômico, mediado pela ativação da via do óxido nítrico-GMPc, mas não pela ativação do sistema opióide.

Os resultados apresentados nos dois primeiros capítulos desse estudo replicam e ajudam a explicar três importantes características clínicas da modulação da dor da ATM pelos hormônios sexuais. Primeiro, a menor prevalência das condições dolorosas da ATM no sexo masculino (Carlsson and LeResche 1995) poderia ser explicada por um efeito protetor da testosterona que diminui o risco de desenvolvimento dor na ATM. Essa idéia é suportada pelos dados experimentais que demonstraram que a administração de formalina na ATM em uma concentração (0,5%) que não induziu nocicepção em machos intactos, induziu em machos gonadectomizados e em fêmeas intactas (Cap 1, Fig 2, pg 24). Segundo, a maior severidade das condições dolorosas da ATM no sexo feminino (Carlsson and LeResche 1995) poderia ser explicada pelo fato de que nas mulheres, ao contrário do que ocorre nos homens, os níveis hormonais não são constantes. Períodos de altos níveis hormonais são seguidos por quedas bruscas desses níveis e durante o período de baixos níveis hormonais a dor da ATM poderia ser exacerbada. Essa idéia é suportada pelos dados experimentais que demonstraram que a resposta nociceptiva induzida pela injeção de formalina na ATM de machos é significativamente menor que àquela induzida na ATM de fêmeas em diestro, fase do ciclo estral com baixos níveis de estrógeno, mas semelhante

àquela induzida na ATM de fêmeas em proestro, fase do ciclo estral com altos níveis de estrógeno (Cap 2, Fig 2, pg 52). Terceiro, o aumento da dor da ATM em mulheres durante os períodos de baixos níveis séricos de estrógeno (LeResche *et al.* 2003) poderia ser explicado pela interrupção do efeito antinociceptivo induzido pelo estrógeno. Essa idéia é suportada pelos dados experimentais que demonstraram que a nocicepção induzida pela injeção de formalina na ATM de fêmeas na fase diestro do ciclo estral, é maior que aquela induzida em fêmeas na fase proestro (Cap 2, Fig 2, pg 52)

Esses resultados, juntamente com aqueles que demonstraram que a administração de estrógeno e progesterona em fêmeas gonadectomizadas diminui a nocicepção induzida pela injeção de formalina na ATM (Cap 2, Fig 5, pg 55) contrastam não só com a idéia clássica de que a maior prevalência e severidade das condições dolorosas da ATM em mulheres é resultado de um efeito pró-nociceptivo dos hormônios ovarianos (LeResche 1997; Warren and Fried 2001; Craft *et al.* 2004; Cairns 2007), mas também com estudos que sugeriram um efeito pró-nociceptivo do estrógeno na nocicepção experimental da ATM em ratos (Bereiter 2001; Cairns *et al.* 2002; Okamoto *et al.* 2003; Flake *et al.* 2005). A idéia de que um efeito pró-nociceptivo dos hormônios ovarianos seria responsável pela maior prevalência e severidade das condições dolorosas da ATM no sexo feminino provavelmente resulta de estudos que demonstraram que a dor da ATM é mais intensa em mulheres (Pullinger *et al.* 1988; Magnusson *et al.* 2000; Wolf *et al.* 2001; Etoz and Ataoglu 2007). No entanto, é importante salientar que na maioria desses estudos (Pullinger *et al.* 1988; Magnusson *et al.* 2000; Wolf *et al.* 2001; Etoz and Ataoglu 2007) a fase do ciclo menstrual das mulheres não é levada em consideração. Como o período em que os níveis hormonais estão baixos é maior que quando estão elevados, a chance de analisar mulheres durante o período de baixo nível hormonal é maior, o que poderia levar a observação de que a dor da ATM é sempre maior no sexo feminino. Essa observação, juntamente com dados epidemiológicos, de que as DTMs são duas vezes mais comuns em mulheres, pode levar a conclusão de que são os hormônios ovarianos aumentam a sensibilidade dolorosa e conseqüentemente são os responsáveis pela maior prevalência das DTMs no sexo feminino. Por outro lado, a discrepância entre o presente estudo e aqueles que apontam para um efeito pró-nociceptivo do estrógeno na nocicepção experimental da

ATM em ratos (Bereiter 2001; Cairns *et al.* 2002; Okamoto *et al.* 2003; Flake *et al.* 2005) poderia ser explicada por diferenças nas metodologias experimentais. Conforme discutido no capítulo 2 (pg 40), uma possível explicação é o estado de consciência do animal, uma vez que todos esses estudos utilizaram animais anestesiados e que já foi demonstrado que a anestesia geral altera as propriedades eletrofisiológicas de neurônios em áreas envolvidas na modulação e transmissão da informação nociceptiva (Heym *et al.* 1984; Collins and Ren 1987; Oliveras *et al.* 1991; McGaraughty *et al.* 1995; Montagne-Clavel *et al.* 1995; Shaw *et al.* 2001).

É importante salientar que em nenhum dos estudos que avaliou o efeito do estrógeno na nocicepção experimental da ATM em ratos (Bereiter 2001; Cairns *et al.* 2002; Okamoto *et al.* 2003; Flake *et al.* 2005) o agente nociceptivo utilizado foi a formalina. Por esse motivo, decidimos avaliar o efeito dos hormônios ovarianos utilizando também o glutamato, que é o agente nociceptivo utilizado no estudo que demonstrou que o estrógeno aumenta a resposta eletromiográfica dos músculos mastigatórios induzida pela injeção de glutamato na ATM de ratas (Cairns *et al.* 2002). A influência do sexo e dos hormônios ovarianos na nocicepção induzida pela injeção de glutamato ou de formalina na ATM foi exatamente a mesma, o que demonstra que os resultados obtidos com o teste da formalina na ATM não são estritamente relacionados a nocicepção induzida por esse agente nociceptivo. O fato de termos obtidos resultados semelhantes utilizando dois agentes nociceptivos diferentes e de que esses resultados replicam estudos clínicos sugere que o modelo comportamental de nocicepção da ATM pode ser útil e confiável para estudar os mecanismos envolvidos no efeito antinociceptivo dos hormônios sexuais na ATM de ratos. Um procedimento experimental muito útil para o estudo desses mecanismos é a administração de drogas no líquido cefalorraquidiano da região de complexo sensorial trigeminal (Flores *et al.* 2001). Nesse contexto, a técnica que permite a injeção direta de drogas nessa região, sem a necessidade de procedimentos cirúrgicos (capítulo 3, pg 57), contribui especialmente com os estudos que utilizam a análise do comportamento nociceptivo para investigar os mecanismos envolvidos no efeito antinociceptivo dos hormônios sexuais. Evidentemente, essa é uma técnica útil para todos os experimentos,

relacionados ou não com nocicepção e com hormônios sexuais, em que faz necessária a administração de drogas na região do complexo sensorial trigeminal.

Os hormônios sexuais desempenham um papel complexo, envolvendo quase todas as estruturas do corpo (Aloisi and Bonifazi 2006). Seus receptores estão amplamente distribuídos no sistema nervoso, tanto central (Simerly *et al.* 1990; Amandusson *et al.* 1996; Voisin *et al.* 1997; Alves *et al.* 1998; VanderHorst *et al.* 1998; Kastrup *et al.* 1999; McEwen 2001; Francis *et al.* 2002) quanto periférico (Keast and Gleeson 1998; Koenig *et al.* 2000; Chaban and Micevych 2005), em células inflamatórias (King *et al.* 1996; Bebo *et al.* 1999; Phiel *et al.* 2005) e nos tecidos da região da ATM (Aufdemorte *et al.* 1986; Abubaker *et al.* 1993; Yamada *et al.* 2003). Portanto, esses hormônios podem afetar a nocicepção da ATM através da ativação de diferentes mecanismos em diferentes regiões envolvidas na transmissão e modulação da informação nociceptiva. Nesse estudo, demonstramos que o aumento da atividade opióide endógena, no sistema nervoso central, medeia o efeito antinociceptivo induzido pelo estrógeno e progesterona na ATM de fêmeas e pela testosterona na ATM de machos (Cap 4, figs 1-4, pgs 94-97). Embora vários estudos tenham demonstrado que os hormônios sexuais modulam o sistema opióide endógeno em áreas envolvidas na transmissão da informação nociceptiva (Dawson-Basoa and Gintzler 1993; Quinones-Jenab *et al.* 1997; Amandusson *et al.* 1999; Chang *et al.* 2000; Flores *et al.* 2003; Foradori *et al.* 2005; Smith *et al.* 2006), poucos estudos avaliaram a participação direta do sistema opióide no efeito antinociceptivo desses hormônios. Um estudo recente, realizado em mulheres, demonstrou que altos níveis circulantes de estrógeno estão relacionados à diminuição da dor induzida experimentalmente na região orofacial e à maior ativação do sistema opióide no sistema nervoso central (Smith *et al.* 2006). Além de mecanismos opióides centrais, mecanismos opióides periféricos também medeiam o efeito antinociceptivo da progesterona e da testosterona, mas não do estrógeno (Cap 4, figs 1-4, pgs 94-97). De fato, dados apresentados no capítulo 5 demonstram que a administração de estrógeno na ATM diminui a nocicepção por meio de um mecanismo periférico não genômico (Cap 5, figs 1 e 2, pgs 116-18) mediado pela ativação da via do óxido nítrico-GMPc (Cap 5, figs 3 e 4, pgs 119-21), mas não do sistema opióide periférico (Cap 5, fig 5, pgs 122). Esse resultado é consistente com estudos que demonstram que a ativação

periférica da via do óxido nítrico-GMPc induz antinocicepção e que o estrógeno ativa as enzimas dessa via (Fiorelli *et al.* 1996; Chen *et al.* 1999; Simoncini *et al.* 2000) e reduz a atividade da fibra nociceptiva primária (Lee *et al.* 2002; Chaban *et al.* 2003; Ma *et al.* 2005) por meio de mecanismos não genômicos.

Esse estudo dá um passo inicial para o entendimento dos mecanismos envolvidos na modulação da nocicepção da ATM pelos hormônios sexuais. No entanto, estudos adicionais são necessários tanto para compreender a via envolvida na ativação desses mecanismos, ou seja, desde a ativação do receptor hormonal até a redução efetiva da nocicepção, quanto para avaliar o envolvimento de outros possíveis mecanismos. Com relação aos mecanismos descritos nesse estudo, por exemplo, é necessário determinar o local onde os hormônios sexuais ativam do sistema opióide, no sistema nervoso central. Conforme discutido no capitulo 4 (pg 85), o subnúcleo caudal, que é o correspondente trigeminal do corno dorsal da medula espinhal, pode estar envolvido, principalmente porque a naloxona é injetada próximo a essa região. No entanto, como a naloxona pode se difundir no líquido cefalorraquidiano, não se pode descartar o envolvimento de outras regiões. Uma possibilidade bem aceitável seria de que os hormônios sexuais ativassem algum sistema de modulação descendente (Gebhart 2004). Independente do local onde essa ativação está ocorrendo, para determinar a via neuronal envolvida é importante determinar os subtipos de receptores opióides envolvidos e a sua localização, pré ou pós-sináptica. No sistema nervoso periférico, a progesterona e a testosterona poderiam modular o sistema opióide principalmente por meio de dois mecanismos. Conforme sugerido no capítulo 4 (pg 85), uma possibilidade é de que esses hormônios aumentem a transcrição dos genes que codificam os receptores opióides nas células do gânglio trigeminal. Nesse caso, a expressão desses receptores estaria aumentada tanto no terminal central, quanto no terminal periférico da fibra nociceptiva aferente primária. Esse mecanismo explicaria porque a injeção de naloxona induziu o mesmo efeito quando administrada no sistema nervoso central e na ATM. No entanto, não se pode descartar a possibilidade de que esses hormônios aumentem a liberação de opióides endógenos pelas células inflamatórias que migraram para a região da ATM após a injuria tecidual. Com relação ao efeito antinociceptivo periférico não genômico do estrógeno também ainda há muito para se estudar. Inicialmente, seria

interessante comprovar que o estrógeno aumenta a ativação, mas não a expressão, das enzimas da via do óxido-nítrico. Também é necessário determinar os mecanismos bioquímicos e moleculares envolvidos tanto na ativação dessas enzimas pelo estrógeno, possivelmente pela ativação de vias intracelulares de proteína quinases (Chen et al. 1999; Simoncini et al. 2000), quanto na diminuição da transmissão nociceptiva aferente pelo GMPc, possivelmente pela da ativação de uma proteína quinase G (Ropero *et al.* 1999) e da abertura de canais de potássio (Sachs *et al.* 2004; Ortiz *et al.* 2006).

Embora os hormônios sexuais sejam apenas um dos vários fatores que modulam a dor, os mecanismos envolvidos no efeito antinociceptivo desses hormônios são de grande interesse terapêutico. O tratamento das DTMs (Carlsson 1999; Shankland 2004), assim como da maioria das condições de dor crônica (Schnitzer 2006) é marcado por um alto índice de insucessos. Apesar de nosso conhecimento sobre os mecanismos nociceptivos ter evoluído muito nos últimos anos, os fármacos utilizados para o controle da dor hoje pertencem a mesma classe de fármacos utilizados há décadas. São basicamente antiinflamatórios e analgésicos opióides que induzem inúmeros efeitos colaterais que se intensificam com o uso crônico. O aumento da eficácia desses fármacos e principalmente, o desenvolvimento de novas classes de fármacos mais efetivos e que apresentem menos efeitos colaterais aumentaria os índices de sucesso nos tratamentos e a qualidade de vida do paciente que sofre de dor crônica. O estudo dos mecanismos pelos quais os hormônios sexuais reduzem a dor contribuirá tanto para o desenvolvimento de novas classes de fármacos quanto para aprimorar aquelas já existentes. Por exemplo, os resultados desse estudo sugerem que a maior sensibilidade dolorosa em mulheres durante os períodos em que os níveis de estrógeno estão baixos poderia ser mediada por uma diminuição da atividade opióide central (capítulo 4), e/ou por uma diminuição da ativação periférica da via do óxido nítrico-GMPc (capítulo 5). Portanto, uma alternativa terapêutica viável e eficaz para o controle da dor, especialmente no sexo feminino poderia ser o desenvolvimento de agonistas opióides específicos, que compensem os efeitos induzidos pela diminuição dos níveis de estrógeno no sistema nervoso central, ou de antiinflamatórios e opióides que compensem uma possível diminuição da ativação da via do óxido nítrico (importante salientar que essa via medeia os efeitos antinociceptivos periféricos tanto de

antiinflamatórios (Deciga-Campos and Lopez-Munoz 2004; Ventura-Martinez *et al.* 2004) quanto de opióides (Durate *et al.* 1990; Pol 2007)). Mais interessante ainda é a possibilidade de desenvolver moduladores de receptores de estrógeno que não induzam efeitos estrogênicos clássicos, mas que mimetizem ou potencializem as ações do estrógeno sobre os mecanismos nociceptivos, por exemplo, sobre o sistema opióide ou sobre a via do óxido nítrico-GMPc. O desenvolvimento desse tipo de fármaco para o controle da dor se encontra em fase de experimentação animal (Keith *et al.* 2005).

CONCLUSÕES

Os resultados apresentados nesse estudo em ratos sugerem que:

(1) Os níveis fisiológicos de testosterona exercem um efeito protetor em machos, diminuindo o risco de desenvolvimento da dor na ATM. Os níveis suprafisiológicos de testosterona em machos reduzem a dor da ATM já instalada.

(2) O alto nível fisiológico de estrógeno durante a fase proestro do ciclo estral e a adminstração de estrógeno e progesterona em fêmeas ovariectomizadas diminui a nocicepção induzida pela injeção de formalina ou de glutamato na ATM.

(3) A técnica de injeção direta de drogas no espaço subaracnóide da região de complexo sensorial trigeminal facilitará o estudo dos mecanismos nociceptivos trigeminais.

(4) Mecanismos opióides no sistema nervoso central medeiam o efeito antinociceptivo do estrógeno, da progesterona e da testosterona, enquanto mecanismos opióides periféricos medeiam o efeito antinociceptivo da progesterona e da testosterona.

(5) A co-administração de estrógeno com formalina na ATM de fêmeas reduz a nocicepção induzida pela formalina através de um mecanismo não genômico periférico envolvendo a ativação da via do óxido nítrico-GMP cíclico.

REFERÊNCIAS BIBLIOGRÁFICAS

Abubaker AO, Raslan WF, Sotereanos GC. Estrogen and progesterone receptors in temporomandibular joint discs of symptomatic and asymptomatic persons: a preliminary study. J Oral Maxillofac Surg. 1993;51(10):1096-1100.

Aigouy L, Fondras JC, Pajot J, Schoeffler P, Woda A. Intrathecal midazolam versus intrathecal morphine in orofacial nociception: an experimental study in rats. Neurosci Lett. 1992;139(1):97-99.

Ali BH, Sharif SI, Elkadi A. Sex differences and the effect of gonadectomy on morphine-induced antinociception and dependence in rats and mice. Clin Exp Pharmacol Physiol. 1995;22(5):342-344.

Almeida RT, Duarte ID. Nitric oxide/cGMP pathway mediates orofacial antinociception induced by electroacupuncture at the St36 acupoint. Brain Res. 2007.

Aloisi AM. Gonadal hormones and sex differences in pain reactivity. Clin J Pain. 2003;19(3):168-174.

Aloisi AM, Bonifazi M. Sex hormones, central nervous system and pain. Horm Behav. 2006;50(1):1-7.

Aloisi AM, Ceccarelli I, Fiorenzani P. Gonadectomy affects hormonal and behavioral responses to repetitive nociceptive stimulation in male rats. Ann N Y Acad Sci. 2003;1007:232-237.

Aloisi AM, Ceccarelli I, Fiorenzani P, De Padova AM, Massafra C. Testosterone affects formalin-induced responses differently in male and female rats. Neurosci Lett. 2004;361(1-3):262-264.

Alves SE, Weiland NG, Hayashi S, McEwen BS. Immunocytochemical localization of nuclear estrogen receptors and progestin receptors within the rat dorsal raphe nucleus. J Comp Neurol. 1998;391(3):322-334.

Amandusson A, Hallbeck M, Hallbeck AL, Hermanson O, Blomqvist A. Estrogen-induced alterations of spinal cord enkephalin gene expression. Pain. 1999;83(2):243-248.

Amandusson A, Hermanson O, Blomqvist A. Colocalization of oestrogen receptor immunoreactivity and preproenkephalin mRNA expression to neurons in the superficial laminae of the spinal and medullary dorsal horn of rats. Eur J Neurosci. 1996;8(11):2440-2445.

Anderson LC, Rao RD. Interleukin-6 and nerve growth factor levels in peripheral nerve and brainstem after trigeminal nerve injury in the rat. Arch Oral Biol. 2001;46(7):633-640.

Arthuri MT, Gameiro GH, Tambeli CH, de Arruda Veiga MC. Peripheral effect of a kappa opioid receptor antagonist on nociception evoked by formalin injected in TMJ of pregnant rats. Life Sci. 2005;76(10):1177-1188.

Ashkenazi A, Silberstein SD. Hormone-related headache: pathophysiology and treatment. CNS Drugs. 2006;20(2):125-141.

Aufdemorte TB, Van Sickels JE, Dolwick MF, Sheridan PJ, Holt GR, Aragon SB, *et al.* Estrogen receptors in the temporomandibular joint of the baboon (Papio cynocephalus): an autoradiographic study. Oral Surg Oral Med Oral Pathol. 1986;61(4):307-314.

Basaria S, Wahlstrom JT, Dobs AS. Clinical review 138: Anabolic-androgenic steroid therapy in the treatment of chronic diseases. J Clin Endocrinol Metab. 2001;86(11):5108-5117.

Bebo BF, Jr., Schuster JC, Vandenbark AA, Offner H. Androgens alter the cytokine profile and reduce encephalitogenicity of myelin-reactive T cells. J Immunol. 1999;162(1):35-40.

Beirith A, Santos AR, Calixto JB. Mechanisms underlying the nociception and paw oedema caused by injection of glutamate into the mouse paw. Brain Res. 2002;924(2):219-228.

Benoliel R, Eliav E, Tal M. Strain-dependent modification of neuropathic pain behaviour in the rat hindpaw by a priming painful trigeminal nerve injury. Pain. 2002a;97(3):203-212.

Benoliel R, Wilensky A, Tal M, Eliav E. Application of a pro-inflammatory agent to the orbital portion of the rat infraorbital nerve induces changes indicative of ongoing trigeminal pain. Pain. 2002b;99(3):567-578.

Bereiter DA. Sex differences in brainstem neural activation after injury to the TMJ region. Cells Tissues Organs. 2001;169(3):226-237.

Berg KA, Zardeneta G, Hargreaves KM, Clarke WP, Milam SB. Integrins regulate opioid receptor signaling in trigeminal ganglion neurons. Neuroscience. 2007;144(3):889-897.

Bernardi F, Pluchino N, Pieri M, Begliuomini S, Lenzi E, Puccetti S*, et al.* Progesterone and medroxyprogesterone acetate effects on central and peripheral allopregnanolone and beta-endorphin levels. Neuroendocrinology. 2006;83(5-6):348-359.

Brown CH, Brunton PJ, Russell JA. Rapid Estradiol-17beta Modulation of Opioid Actions on the Electrical and Secretory Activity of Rat Oxytocin Neurons In vivo. Neurochem Res. 2007.

Brown JT. Anabolic steroids: what should the emergency physician know? Emerg Med Clin North Am. 2005;23(3):815-826, ix-x.

Butcher RL, Collins WE, Fugo NW. Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat. Endocrinology. 1974;94(6):1704-1708.

Cairns BE. The influence of gender and sex steroids on craniofacial nociception. Headache. 2007;47(2):319-324.

Cairns BE, Hu JW, Arendt-Nielsen L, Sessle BJ, Svensson P. Sex-related differences in human pain and rat afferent discharge evoked by injection of glutamate into the masseter muscle. J Neurophysiol. 2001a;86(2):782-791.

Cairns BE, Sessle BJ, Hu JW. Evidence that excitatory amino acid receptors within the temporomandibular joint region are involved in the reflex activation of the jaw muscles. J Neurosci. 1998;18(19):8056-8064.

Cairns BE, Sessle BJ, Hu JW. Temporomandibular-evoked jaw muscle reflex: role of brain stem NMDA and non-NMDA receptors. Neuroreport. 2001b;12(9):1875-1878.

Cairns BE, Sim Y, Bereiter DA, Sessle BJ, Hu JW. Influence of sex on reflex jaw muscle activity evoked from the rat temporomandibular joint. Brain Res. 2002;957(2):338-344.

Campos M, Morais Pde L, Pupo AS. Effects of castration and of testosterone replacement on alpha(1)-adrenoceptor subtypes in the rat vas deferens. Eur J Pharmacol. 2003;471(2):149-155.

Carlsson G, LeResche L. Epidemiology of temporomandibular disorders. Seattle, WA 1995.

Carlsson GE. Epidemiological studies of signs and symptoms of temporomandibular joint-pain-dysfunction. A literature review. Aust Prosthodont Soc Bull. 1984;14:7-12.

Carlsson GE. Epidemiology and treatment need for temporomandibular disorders. J Orofac Pain. 1999;13(4):232-237.

Caulin-Glaser T, Garcia-Cardena G, Sarrel P, Sessa WC, Bender JR. 17 beta-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca2+ mobilization. Circ Res. 1997;81(5):885-892.

Ceccarelli I, Fiorenzani P, Grasso G, Lariviere WR, Massafra C, Massai L*, et al.* Estrogen and mu-opioid receptor antagonists counteract the 17 beta-estradiol-induced licking increase and interferon-gamma reduction occurring during the formalin test in male rats. Pain. 2004;111(1-2):181-190.

Ceccarelli I, Fiorenzani P, Massafra C, Aloisi AM. Long-term ovariectomy changes formalin-induced licking in female rats: the role of estrogens. Reprod Biol Endocrinol. 2003;1(1):24.

Chaban VV, Mayer EA, Ennes HS, Micevych PE. Estradiol inhibits atp-induced intracellular calcium concentration increase in dorsal root ganglia neurons. Neuroscience. 2003;118(4):941-948.

Chaban VV, Micevych PE. Estrogen receptor-alpha mediates estradiol attenuation of ATP-induced Ca2+ signaling in mouse dorsal root ganglion neurons. J Neurosci Res. 2005;81(1):31-37.

Chang PC, Aicher SA, Drake CT. Kappa opioid receptors in rat spinal cord vary across the estrous cycle. Brain Res. 2000;861(1):168-172.

Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW. Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. J Clin Invest. 1999;103(3):401-406.

Chidiac JJ, Rifai K, Hawwa NN, Massaad CA, Jurjus AR, Jabbur SJ*, et al.* Nociceptive behaviour induced by dental application of irritants to rat incisors: a new model for tooth inflammatory pain. Eur J Pain. 2002;6(1):55-67.

Christensen D, Gautron M, Guilbaud G, Kayser V. Effect of gabapentin and lamotrigine on mechanical allodynia-like behaviour in a rat model of trigeminal neuropathic pain. Pain. 2001;93(2):147-153.

Cicero TJ, Nock B, Meyer ER. Gender-related differences in the antinociceptive properties of morphine. J Pharmacol Exp Ther. 1996;279(2):767-773.

Clark AS, Guarraci FA, Megroz AB, Porter DM, Henderson LP. The display of sexual behaviors by female rats administered ICI 182,780. Horm Behav. 2003;43(4):454-464.

Clavelou P, Dallel R, Orliaguet T, Woda A, Raboisson P. The orofacial formalin test in rats: effects of different formalin concentrations. Pain. 1995;62(3):295-301.

Clemente JT, Parada CA, Veiga MC, Gear RW, Tambeli CH. Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint. Neurosci Lett. 2004;372(3):250-255.

Collins JG, Ren K. WDR response profiles of spinal dorsal horn neurons may be unmasked by barbiturate anesthesia. Pain. 1987;28(3):369-378.

Coombs DW, Colburn RW, McCarthy LE, DeLeo JA, Hoopes PJ, Twitchell BB. Neurotoxicology of chronic infusion of the ganglioside GM1 in the ewe: phase I. intrathecal administration. Anesth Analg. 1993;77(3):507-515.

Craft RM, Mogil JS, Aloisi AM. Sex differences in pain and analgesia: the role of gonadal hormones. Eur J Pain. 2004;8(5):397-411.

Cunha FQ, Teixeira MM, Ferreira SH. Pharmacological modulation of secondary mediator systems--cyclic AMP and cyclic GMP--on inflammatory hyperalgesia. Br J Pharmacol. 1999;127(3):671-678.

Danzebrink RM, Green SA, Gebhart GF. Spinal mu and delta, but not kappa, opioid-receptor agonists attenuate responses to noxious colorectal distension in the rat. Pain. 1995;63(1):39-47.

Dawson-Basoa MB, Gintzler AR. 17-Beta-estradiol and progesterone modulate an intrinsic opioid analgesic system. Brain Res. 1993;601(1-2):241-245.

Deciga-Campos M, Lopez-Munoz FJ. Participation of the L-arginine-nitric oxide-cyclic GMP-ATP-sensitive K+ channel cascade in the antinociceptive effect of rofecoxib. Eur J Pharmacol. 2004;484(2-3):193-199.

DeLeo JA, Rutkowski MD. Gender differences in rat neuropathic pain sensitivity is dependent on strain. Neurosci Lett. 2000;282(3):197-199.

Denucci DJ, Dionne RA, Dubner R. Identifying a neurobiologic basis for drug therapy in TMDs. J Am Dent Assoc. 1996;127(5):581-593.

Dib B. Intrathecal chronic catheterization in the rat. Pharmacol Biochem Behav. 1984;20(1):45-48.

Durate ID, Lorenzetti BB, Ferreira SH. Peripheral analgesia and activation of the nitric oxide-cyclic GMP pathway. Eur J Pharmacol. 1990;186(2-3):289-293.

Dworkin SF, Huggins KH, LeResche L, Von Korff M, Howard J, Truelove E*, et al.* Epidemiology of signs and symptoms in temporomandibular disorders: clinical signs in cases and controls. J Am Dent Assoc. 1990;120(3):273-281.

Dworkin SF, LeResche L. Research diagnostic criteria for temporomandibular disorders: review, criteria, examinations and specifications, critique. J Craniomandib Disord. 1992;6(4):301-355.

Eisenberg E, Vos BP, Strassman AM. The peripheral antinociceptive effect of morphine in a rat model of facial pain. Neuroscience. 1996;72(2):519-525.

Etoz OA, Ataoglu H. Evaluation of pain perception in patients with temporomandibular disorders. J Oral Maxillofac Surg. 2007;65(12):2475-2478.

Evans NA. Current concepts in anabolic-androgenic steroids. Am J Sports Med. 2004;32(2):534-542.

Fillingim RB, Edwards RR, Powell T. The relationship of sex and clinical pain to experimental pain responses. Pain. 1999;83(3):419-425.

Fiorelli G, Gori F, Frediani U, Franceschelli F, Tanini A, Tosti-Guerra C*, et al.* Membrane binding sites and non-genomic effects of estrogen in cultured human pre-osteoclastic cells. J Steroid Biochem Mol Biol. 1996;59(2):233-240.

Fischer L, Clemente JT, Tambeli CH. The protective role of testosterone in the development of temporomandibular joint pain. J Pain. 2007;8(5):437-442.

Fischer L, Parada CA, Tambeli CH. A novel method for subarachnoid drug delivery in the medullary region of rats. J Neurosci Methods. 2005;148(2):108-112.

Fischer L, Torres-Chávez KE, Clemente-Napimoga JT, Jorge D, Arsati F, Arruda Veiga MCF*, et al.* The effect of sex and ovarian hormones on temporomandibular joint nociception in rats J pain. *in press*.

Flake NM, Bonebreak DB, Gold MS. Estrogen and inflammation increase the excitability of rat temporomandibular joint afferent neurons. J Neurophysiol. 2005;93(3):1585-1597.

Flores CA, Shughrue P, Petersen SL, Mokha SS. Sex-related differences in the distribution of opioid receptor-like 1 receptor mRNA and colocalization with estrogen receptor mRNA in neurons of the spinal trigeminal nucleus caudalis in the rat. Neuroscience. 2003;118(3):769-778.

Flores CA, Wang XM, Zhang KM, Mokha SS. Orphanin FQ produces gender-specific modulation of trigeminal nociception: behavioral and electrophysiological observations. Neuroscience. 2001;105(2):489-498.

Foradori CD, Goodman RL, Adams VL, Valent M, Lehman MN. Progesterone increases dynorphin a concentrations in cerebrospinal fluid and preprodynorphin messenger ribonucleic Acid levels in a subset of dynorphin neurons in the sheep. Endocrinology. 2005;146(4):1835-1842.

Forest MG. Role of androgens in fetal and pubertal development. Horm Res. 1983;18(1-3):69-83.

Francis K, Meddle SL, Bishop VR, Russell JA. Progesterone receptor expression in the pregnant and parturient rat hypothalamus and brainstem. Brain Res. 2002;927(1):18-26.

Frot M, Feine JS, Bushnell MC. Sex differences in pain perception and anxiety. A psychophysical study with topical capsaicin. Pain. 2004;108(3):230-236.

Gameiro GH, Arthuri MT, Tambeli CH, de Arruda Veiga MC. Effects of ethanol on deep pain evoked by formalin injected in TMJ of rat. Life Sci. 2003;73(26):3351-3361.

Gaumond I, Arsenault P, Marchand S. The role of sex hormones on formalin-induced nociceptive responses. Brain Res. 2002;958(1):139-145.

Gaumond I, Arsenault P, Marchand S. Specificity of female and male sex hormones on excitatory and inhibitory phases of formalin-induced nociceptive responses. Brain Res. 2005;1052(1):105-111.

Gebhart GF. Descending modulation of pain. Neurosci Biobehav Rev. 2004;27(8):729-737.

Gonzalez-Darder JM, Gomez-Cardenas E, Gil-Salu JL. [Microsurgical catheterization of the intrathecal space in the rat for chronic infusion of drugs]. Rev Esp Anestesiol Reanim. 1989;36(3):153-156.

Gordon FT, Soliman MR. Diurnal variation in the acute effects of estradiol and progesterone on beta-endorphin and Met-enkephalin levels in specific brain regions of ovariectomized rats. Pharmacology. 1994;49(3):192-198.

Gordon FT, Soliman MR. The effects of estradiol and progesterone on pain sensitivity and brain opioid receptors in ovariectomized rats. Horm Behav. 1996;30(3):244-250.

Grabow TS, Dougherty PM. Cervicomedullary intrathecal injection of morphine produces antinociception in the orofacial formalin test in the rat. Anesthesiology. 2001;95(6):1427-1434.

Granados-Soto V, Rufino MO, Gomes Lopes LD, Ferreira SH. Evidence for the involvement of the nitric oxide-cGMP pathway in the antinociception of morphine in the formalin test. Eur J Pharmacol. 1997;340(2-3):177-180.

Gurun MS, Leinbach R, Moore L, Lee CS, Owen MD, Eisenach JC. Studies on the safety of glucose and paraben-containing neostigmine for intrathecal administration. Anesth Analg. 1997;85(2):317-323.

Haas DA, Nakanishi O, MacMillan RE, Jordan RC, Hu JW. Development of an orofacial model of acute inflammation in the rat. Arch Oral Biol. 1992;37(5):417-422.

Hammer RP, Jr., Bridges RS. Preoptic area opioids and opiate receptors increase during pregnancy and decrease during lactation. Brain Res. 1987;420(1):48-56.

Harris JA, Chang PC, Drake CT. Kappa opioid receptors in rat spinal cord: sex-linked distribution differences. Neuroscience. 2004;124(4):879-890.

Hartwig AC, Mathias SI, Law AS, Gebhart GF. Characterization and opioid modulation of inflammatory temporomandibular joint pain in the rat. J Oral Maxillofac Surg. 2003;61(11):1302-1309.

Hatch AM, Wiberg GS, Zawidzka Z, Cann M, Airth JM, Grice HC. Isolation syndrome in the rat. Toxicol Appl Pharmacol. 1965;7(5):737-745.

He J, Evans CO, Hoffman SW, Oyesiku NM, Stein DG. Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury. Exp Neurol. 2004;189(2):404-412.

Heil SH. Activational and organizational actions of gonadal hormones and the sex-specific effects of prolactin on food intake by rats. Dev Psychobiol. 1999;35(1):61-67.

Heintjes E, Berger MY, Bierma-Zeinstra SM, Bernsen RM, Verhaar JA, Koes BW. Pharmacotherapy for patellofemoral pain syndrome. Cochrane Database Syst Rev. 2004(3):CD003470.

Herradon G, Ezquerra L, Nguyen T, Wang C, Siso A, Franklin B*, et al.* Changes in BDNF gene expression correlate with rat strain differences in neuropathic pain. Neurosci Lett. 2007;420(3):273-276.

Heym J, Steinfels GF, Jacobs BL. Chloral hydrate anesthesia alters the responsiveness of central serotonergic neurons in the cat. Brain Res. 1984;291(1):63-72.

Hu JW, Yu XM, Vernon H, Sessle BJ. Excitatory effects on neck and jaw muscle activity of inflammatory irritant applied to cervical paraspinal tissues. Pain. 1993;55(2):243-250.

Imbe H, Iwata K, Zhou QQ, Zou S, Dubner R, Ren K. Orofacial deep and cutaneous tissue inflammation and trigeminal neuronal activation. Implications for persistent temporomandibular pain. Cells Tissues Organs. 2001;169(3):238-247.

Isaacs WA, Effiong CE, Ayeni O. Steroid treatment in the prevention of painful episodes in sickle-cell disease. Lancet. 1972;1(7750):570-571.

Jasmin L, Ohara PT. Long-term intrathecal catheterization in the rat. J Neurosci Methods. 2001;110(1-2):81-89.

Johansson P, Ray A, Zhou Q, Huang W, Karlsson K, Nyberg F. Anabolic androgenic steroids increase beta-endorphin levels in the ventral tegmental area in the male rat brain. Neurosci Res. 1997;27(2):185-189.

Jost A. Genetic and hormonal factors in sex differentiation of the brain. Psychoneuroendocrinology. 1983;8(2):183-193.

Kastrup Y, Hallbeck M, Amandusson A, Hirata S, Hermanson O, Blomqvist A. Progesterone receptor expression in the brainstem of the female rat. Neurosci Lett. 1999;275(2):85-88.

Keast JR, Gleeson RJ. Androgen receptor immunoreactivity is present in primary sensory neurons of male rats. Neuroreport. 1998;9(18):4137-4140.

Keith JC, Jr., Albert LM, Leathurby Y, Follettie M, Wang L, Borges-Marcucci L*, et al.* The utility of pathway selective estrogen receptor ligands that inhibit nuclear factor-kappa B transcriptional activity in models of rheumatoid arthritis. Arthritis Res Ther. 2005;7(3):R427-438.

Kelly MJ, Lagrange AH, Wagner EJ, Ronnekleiv OK. Rapid effects of estrogen to modulate G protein-coupled receptors via activation of protein kinase A and protein kinase C pathways. Steroids. 1999;64(1-2):64-75.

Kelly MJ, Levin ER. Rapid actions of plasma membrane estrogen receptors. Trends Endocrinol Metab. 2001;12(4):152-156.

King A, Gardner L, Loke YW. Evaluation of oestrogen and progesterone receptor expression in uterine mucosal lymphocytes. Hum Reprod. 1996;11(5):1079-1082.

Koenig HL, Gong WH, Pelissier P. Role of progesterone in peripheral nerve repair. Rev Reprod. 2000;5(3):189-199.

Kuba T, Wu HB, Nazarian A, Festa ED, Barr GA, Jenab S*, et al.* Estradiol and progesterone differentially regulate formalin-induced nociception in ovariectomized female rats. Horm Behav. 2006;49(4):441-449.

Kuroiwa T, Bonnekoh P, Hossmann KA. Therapeutic window of halothane anesthesia for reversal of delayed neuronal injury in gerbils: relationship to postischemic motor hyperactivity. Brain Res. 1991;563(1-2):33-38.

Lagrange AH, Ronnekleiv OK, Kelly MJ. Estradiol-17 beta and mu-opioid peptides rapidly hyperpolarize GnRH neurons: a cellular mechanism of negative feedback? Endocrinology. 1995;136(5):2341-2344.

Lantin-Hermoso RL, Rosenfeld CR, Yuhanna IS, German Z, Chen Z, Shaul PW. Estrogen acutely stimulates nitric oxide synthase activity in fetal pulmonary artery endothelium. Am J Physiol. 1997;273(1 Pt 1):L119-126.

Lee DY, Chai YG, Lee EB, Kim KW, Nah SY, Oh TH, *et al.* 17Beta-estradiol inhibits high-voltage-activated calcium channel currents in rat sensory neurons via a non-genomic mechanism. Life Sci. 2002;70(17):2047-2059.

LeResche L. Epidemiology of temporomandibular disorders: implications for the investigation of etiologic factors. Crit Rev Oral Biol Med. 1997;8(3):291-305.

LeResche L, Mancl L, Sherman JJ, Gandara B, Dworkin SF. Changes in temporomandibular pain and other symptoms across the menstrual cycle. Pain. 2003;106(3):253-261.

LeResche L, Saunders K, Von Korff MR, Barlow W, Dworkin SF. Use of exogenous hormones and risk of temporomandibular disorder pain. Pain. 1997;69(1-2):153-160.

LeResche L, Sherman JJ, Huggins K, Saunders K, Mancl LA, Lentz G, *et al.* Musculoskeletal orofacial pain and other signs and symptoms of temporomandibular disorders during pregnancy: a prospective study. J Orofac Pain. 2005;19(3):193-201.

Lipton JA, Ship JA, Larach-Robinson D. Estimated prevalence and distribution of reported orofacial pain in the United States. J Am Dent Assoc. 1993;124(10):115-121.

Lombardi G, Zarrilli S, Colao A, Paesano L, Di Somma C, Rossi F, *et al.* Estrogens and health in males. Mol Cell Endocrinol. 2001;178(1-2):51-55.

LoPachin RM, Rudy TA, Yaksh TL. An improved method for chronic catheterization of the rat spinal subarachnoid space. Physiol Behav. 1981;27(3):559-561.

Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, *et al.* Guanylyl cyclases and signaling by cyclic GMP. Pharmacol Rev. 2000;52(3):375-414.

Ma B, Rong W, Dunn PM, Burnstock G. 17beta-estradiol attenuates alpha, beta-meATP-induced currents in rat dorsal root ganglion neurons. Life Sci. 2005;76(22):2547-2558.

Magnusson T, Egermark I, Carlsson GE. A longitudinal epidemiologic study of signs and symptoms of temporomandibular disorders from 15 to 35 years of age. J Orofac Pain. 2000;14(4):310-319.

Mannino CA, South SM, Quinones-Jenab V, Inturrisi CE. Estradiol replacement in ovariectomized rats is antihyperalgesic in the formalin test. J Pain. 2007;8(4):334-342.

Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. Braz J Biol. 2002;62(4A):609-614.

Martin H, Kocher L, Chery-Croze S. Chronic lumbar intrathecal catheterization in the rat with reduced-length spinal compression. Physiol Behav. 1984;33(1):159-161.

McEwen BS. Invited review: Estrogens effects on the brain: multiple sites and molecular mechanisms. J Appl Physiol. 2001;91(6):2785-2801.

McGaraughty S, Reinis S, Tsoukatos J. A correlogram analysis of the activity in the rostral ventromedial medulla of awake rats and in rats anesthetized with ketamine or pentobarbital following the administration of morphine. Exp Brain Res. 1995;106(2):283-290.

Mermelstein PG, Becker JB, Surmeier DJ. Estradiol reduces calcium currents in rat neostriatal neurons via a membrane receptor. J Neurosci. 1996;16(2):595-604.

Montagne-Clavel J, Oliveras JL, Martin G. Single-unit recordings at dorsal raphe nucleus in the awake-anesthetized rat: spontaneous activity and responses to cutaneous innocuous and noxious stimulations. Pain. 1995;60(3):303-310.

Morales AJ, Nolan JJ, Nelson JC, Yen SS. Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age. J Clin Endocrinol Metab. 1994;78(6):1360-1367.

Moss RL, Gu Q, Wong M. Estrogen: nontranscriptional signaling pathway. Recent Prog Horm Res. 1997;52:33-68; discussion 68-39.

Nunez S, Lee JS, Zhang Y, Bai G, Ro JY. Role of peripheral mu-opioid receptors in inflammatory orofacial muscle pain. Neuroscience. 2007;146(3):1346-1354.

O'Connor TC, Abram SE. Halothane enhances suppression of spinal sensitization by intrathecal morphine in the rat formalin test. Anesthesiology. 1994;81(5):1277-1283.

Ogawa A, Ren K, Tsuboi Y, Morimoto T, Sato T, Iwata K. A new model of experimental parotitis in rats and its implication for trigeminal nociception. Exp Brain Res. 2003;152(3):307-316.

Okamoto K, Hirata H, Takeshita S, Bereiter DA. Response properties of TMJ units in superficial laminae at the spinomedullary junction of female rats vary over the estrous cycle. J Neurophysiol. 2003;89(3):1467-1477.

Oliveras JL, Montagne-Clavel J, Martin G. Drastic changes of ventromedial medulla neuronal properties induced by barbiturate anesthesia. I. Comparison of the single-unit types in the same awake and pentobarbital-treated rats. Brain Res. 1991;563(1-2):241-250.

Ortiz MI, Medina-Tato DA, Sarmiento-Heredia D, Palma-Martinez J, Granados-Soto V. Possible activation of the NO-cyclic GMP-protein kinase G-K+ channels pathway by gabapentin on the formalin test. Pharmacol Biochem Behav. 2006;83(3):420-427.

Owen MD, Unal CB, Callahan MF, Trivedi K, York C, Millington WR. Glycyl-glutamine inhibits the respiratory depression, but not the antinociception, produced by morphine. Am J Physiol Regul Integr Comp Physiol. 2000;279(5):R1944-1948.

Pajot J, Ressot C, Ngom I, Woda A. Gonadectomy induces site-specific differences in nociception in rats. Pain. 2003;104(1-2):367-373.

Petersen SL, LaFlamme KD. Progesterone increases levels of mu-opioid receptor mRNA in the preoptic area and arcuate nucleus of ovariectomized, estradiol-treated female rats. Brain Res Mol Brain Res. 1997;52(1):32-37.

Phiel KL, Henderson RA, Adelman SJ, Elloso MM. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. Immunol Lett. 2005;97(1):107-113.

Pol O. The involvement of the nitric oxide in the effects and expression of opioid receptors during peripheral inflammation. Curr Med Chem. 2007;14(18):1945-1955.

Pullinger AG, Seligman DA, Solberg WK. Temporomandibular disorders. Part I: Functional status, dentomorphologic features, and sex differences in a nonpatient population. J Prosthet Dent. 1988;59(2):228-235.

Puri V, Cui L, Liverman CS, Roby KF, Klein RM, Welch KM, *et al.* Ovarian steroids regulate neuropeptides in the trigeminal ganglion. Neuropeptides. 2005;39(4):409-417.

Qian Y, Chao DS, Santillano DR, Cornwell TL, Nairn AC, Greengard P, *et al.* cGMP-dependent protein kinase in dorsal root ganglion: relationship with nitric oxide synthase and nociceptive neurons. J Neurosci. 1996;16(10):3130-3138.

Quinones-Jenab V, Jenab S, Ogawa S, Inturrisi C, Pfaff DW. Estrogen regulation of mu-opioid receptor mRNA in the forebrain of female rats. Brain Res Mol Brain Res. 1997;47(1-2):134-138.

Riley JL, 3rd, Gilbert GH. Orofacial pain symptoms: an interaction between age and sex. Pain. 2001;90(3):245-256.

Riley JL, 3rd, Robinson ME, Wise EA, Myers CD, Fillingim RB. Sex differences in the perception of noxious experimental stimuli: a meta-analysis. Pain. 1998;74(2-3):181-187.

Ropero AB, Fuentes E, Rovira JM, Ripoll C, Soria B, Nadal A. Non-genomic actions of 17beta-oestradiol in mouse pancreatic beta-cells are mediated by a cGMP-dependent protein kinase. J Physiol. 1999;521 Pt 2:397-407.

Rosland JH. The formalin test in mice: the influence of ambient temperature. Pain. 1991;45(2):211-216.

Roughan JV, Flecknell PA. Effects of surgery and analgesic administration on spontaneous behaviour in singly housed rats. Res Vet Sci. 2000;69(3):283-288.

Roveroni RC, Parada CA, Cecilia M, Veiga FA, Tambeli CH. Development of a behavioral model of TMJ pain in rats: the TMJ formalin test. Pain. 2001;94(2):185-191.

Sachs D, Cunha FQ, Ferreira SH. Peripheral analgesic blockade of hypernociception: activation of arginine/NO/cGMP/protein kinase G/ATP-sensitive K+ channel pathway. Proc Natl Acad Sci U S A. 2004;101(10):3680-3685.

Schnitzer TJ. Update on guidelines for the treatment of chronic musculoskeletal pain. Clin Rheumatol. 2006;25 Suppl 1:S22-29.

Sessle BJ. Acute and chronic craniofacial pain: brainstem mechanisms of nociceptive transmission and neuroplasticity, and their clinical correlates. Crit Rev Oral Biol Med. 2000;11(1):57-91.

Shankland WE, 2nd. Temporomandibular disorders: standard treatment options. Gen Dent. 2004;52(4):349-355; quiz 356, 365-346.

Shaw FZ, Chen RF, Yen CT. Dynamic changes of touch- and laser heat-evoked field potentials of primary somatosensory cortex in awake and pentobarbital-anesthetized rats. Brain Res. 2001;911(2):105-115.

Simerly RB, Chang C, Muramatsu M, Swanson LW. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J Comp Neurol. 1990;294(1):76-95.

Simon P, Malatray J, Boissier JR. Antagonism by amantadine of prochlorpemazine-induced catalepsy. J Pharm Pharmacol. 1970;22(7):546-547.

Simoncini T, Genazzani AR. Non-genomic actions of sex steroid hormones. Eur J Endocrinol. 2003;148(3):281-292.

Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature. 2000;407(6803):538-541.

Simoncini T, Mannella P, Fornari L, Caruso A, Varone G, Genazzani AR. Genomic and non-genomic effects of estrogens on endothelial cells. Steroids. 2004;69(8-9):537-542.

Smith YR, Stohler CS, Nichols TE, Bueller JA, Koeppe RA, Zubieta JK. Pronociceptive and antinociceptive effects of estradiol through endogenous opioid neurotransmission in women. J Neurosci. 2006;26(21):5777-5785.

Stefano GB, Prevot V, Beauvillain JC, Cadet P, Fimiani C, Welters I, *et al.* Cell-surface estrogen receptors mediate calcium-dependent nitric oxide release in human endothelia. Circulation. 2000;101(13):1594-1597.

Stein C, Schafer M, Machelska H. Attacking pain at its source: new perspectives on opioids. Nat Med. 2003;9(8):1003-1008.

Stevis PE, Deecher DC, Suhadolnik L, Mallis LM, Frail DE. Differential effects of estradiol and estradiol-BSA conjugates. Endocrinology. 1999;140(11):5455-5458.

Stoffel EC, Ulibarri CM, Folk JE, Rice KC, Craft RM. Gonadal hormone modulation of mu, kappa, and delta opioid antinociception in male and female rats. J Pain. 2005;6(4):261-274.

Storkson RV, Kjorsvik A, Tjolsen A, Hole K. Lumbar catheterization of the spinal subarachnoid space in the rat. J Neurosci Methods. 1996;65(2):167-172.

Tambeli CH, Seo K, Sessle BJ, Hu JW. Central mu opioid receptor mechanisms modulate mustard oil-evoked jaw muscle activity. Brain Res. 2001;913(1):90-94.

Tjolsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. Pain. 1992;51(1):5-17.

Toda N, Ayajiki K, Okamura T. Neural mechanism underlying basilar arterial constriction by intracisternal L-NNA in anesthetized dogs. The American journal of physiology. 1993;265(1 Pt 2):H103-107.

Unruh AM. Gender variations in clinical pain experience. Pain. 1996;65(2-3):123-167.

VanderHorst VG, Schasfoort FC, Meijer E, van Leeuwen FW, Holstege G. Estrogen receptor-alpha-immunoreactive neurons in the periaqueductal gray of the adult ovariectomized female cat. Neurosci Lett. 1998;240(1):13-16.

Vendruscolo LF, Pamplona FA, Takahashi RN. Strain and sex differences in the expression of nociceptive behavior and stress-induced analgesia in rats. Brain Res. 2004;1030(2):277-283.

Ventura-Martinez R, Deciga-Campos M, Diaz-Reval MI, Gonzalez-Trujano ME, Lopez-Munoz FJ. Peripheral involvement of the nitric oxide-cGMP pathway in the indomethacin-induced antinociception in rat. Eur J Pharmacol. 2004;503(1-3):43-48.

Vincler M, Maixner W, Vierck CJ, Light AR. Estrous cycle modulation of nociceptive behaviors elicited by electrical stimulation and formalin. Pharmacol Biochem Behav. 2001;69(3-4):315-324.

Voisin DL, Simonian SX, Herbison AE. Identification of estrogen receptor-containing neurons projecting to the rat supraoptic nucleus. Neuroscience. 1997;78(1):215-228.

Vos BP, Strassman AM, Maciewicz RJ. Behavioral evidence of trigeminal neuropathic pain following chronic constriction injury to the rat's infraorbital nerve. J Neurosci. 1994;14(5 Pt 1):2708-2723.

Wang XM, Zhang ZJ, Bains R, Mokha SS. Effect of antisense knock-down of alpha(2a)- and alpha(2c)-adrenoceptors on the antinociceptive action of clonidine on trigeminal nociception in the rat. Pain. 2002;98(1-2):27-35.

Warren MP, Fried JL. Temporomandibular disorders and hormones in women. Cells Tissues Organs. 2001;169(3):187-192.

Waynforth H, Flecknell P. Experimental and surgical technique in the rat

Waynforth H, Flecknell P. Experimental and surgical technique in the rat. Academic, London. 1992b.

Wolf E, Petersson K, Petersson A, Nilner M. Long-lasting orofacial pain--a study of 109 consecutive patients referred to a pain group. Swed Dent J. 2001;25(3):129-136.

Wu SZ, Weng XZ. Therapeutic effects of an androgenic preparation on myocardial ischemia and cardiac function in 62 elderly male coronary heart disease patients. Chin Med J (Engl). 1993;106(6):415-418.

Yaksh TL, Grafe MR, Malkmus S, Rathbun ML, Eisenach JC. Studies on the safety of chronically administered intrathecal neostigmine methylsulfate in rats and dogs. Anesthesiology. 1995;82(2):412-427.

Yaksh TL, Rudy TA. Chronic catheterization of the spinal subarachnoid space. Physiol Behav. 1976;17(6):1031-1036.

Yamada K, Nozawa-Inoue K, Kawano Y, Kohno S, Amizuka N, Iwanaga T*, et al.* Expression of estrogen receptor alpha (ER alpha) in the rat temporomandibular joint. Anat Rec A Discov Mol Cell Evol Biol. 2003;274(2):934-941.

Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain. 1983;16(2):109-110.

Zubieta JK, Smith YR, Bueller JA, Xu Y, Kilbourn MR, Jewett DM*, et al.* mu-opioid receptor-mediated antinociceptive responses differ in men and women. J Neurosci. 2002;22(12):5100-5107.