JULIANA TRINDADE CLEMENTE NAPIMOGA

ESTUDO DOS MECANISMOS ENVOLVIDOS NO DIMORFISMO SEXUAL DA ANALGESIA MEDIADA POR RECEPTORES OPIÓIDES CAPA NA ARTICULAÇÃO TEMPOROMANDIBULAR.

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

Orientadora: Prof.ª Dr.ª Cláudia Herrera Tambeli

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"O que prevemos raramente ocorre, o que menos esperamos geralmente acontece".

(Benjamin Disraeli)

RESUMO:

Recentemente foi demonstrado que a ativação de receptores capa opióides localizados na ATM de ratos reduz o comportamento nociceptivo induzido pela injeção se formalina na ATM de ratos, especialmente nas fêmeas na fase diestro do ciclo estral. Sendo a fase diestro aquela que representa baixos níveis hormonais, estes resultados indicam que os hormônios gonadais diminuem a antinocicepção mediada pelos receptores capa na ATM. O objetivo deste trabalho foi investigar o possível mecanismo pelo qual os hormônios gonadais poderiam diminuir a antinocicepção mediada pelos receptores capa opióides através das seguintes hipóteses: (a) Os hormônios gonadais diminuem a antinocicepção mediada pelos receptores capa na ATM por diminuírem a expressão de receptores capa opióides no gânglio trigeminal; (b) O efeito periférico antinociceptivo dos agonistas dos receptores capa opióides é mediado pela ativação da via L-Arginina/NO/GMPc em machos e fêmeas; (c) Os hormônios gonadais diminuem a antinocicepção mediada pelos receptores capa na ATM por diminuírem a ativação da via L-Arginina/NO/GMPc. A análise pela técnica Western blot demonstrou que a expressão protéica dos receptores capa opióides é maior em fêmeas do que em machos, sugerindo que a testosterona induz acentuada diminuição na expressão dos receptores capa opióides. Nas fêmeas, a expressão dos receptores capa opióides foi significativamente maior nas fêmeas em diestro em relação às fêmeas em proestro, sugerindo que os hormônios gonadais femininos também diminuem a expressão dos receptores capa opióides. A co-administração do inibidor da NO-sintase, L-NMMA, ou do inibidor da guanilil ciclase sensível ao NO, ODQ, com o agonista do receptor capa opióide U50,488 bloqueou a antinocicepção mediada pelos receptores capa na ATM de machos e fêmeas, sugerindo que a antinocicepção induzida pelos receptores capa opióides é mediada pela ativação da via L-Arginina/NO/GMPc em ambos os sexos. No entanto, a co-administração de baixas doses de L-NMMA e ODQ com U50,488 significativamente diminuiu a antinocicepção mediada por receptores capa apenas nas fêmeas em diestro. Estes resultados indicam que a antinocicepção mediada pelos receptores capa opióides depende da ativação da via L-Arginina/NO/GMPc em machos e fêmeas. No entanto, o dimorfismo sexual na antinocicepção mediada pelos receptores capa opióides na ATM se deve, pelo menos em parte, pela diminuição da expressão dos receptores capa opióides no gânglio trigeminal pelos hormônios gonadais, especialmente a testosterona. Apesar do

envolvimento da via L-Arginin/NO/GMPc na antinocicepção mediada pelo receptor capa na ATM em ambos os sexos, os hormônios gonadais não diminuem a atividade desta via diminuindo o efeito antinociceptivo mediado por receptores capa opióides na ATM.

Palavras-chave: via L-Arginina/NO/GMPc, receptores capa opióides, articulação temporomandibular, difenças sexuais, ciclo estral, nocicepção.

ABSTRACT:

We have previously demonstrated that activation of kappa opioid receptors located in the TMJ of rats suppresses formalin-induced TMJ nociception behavior especially in females of the diestrus phase of the estrous cycle. Since diestrus is a phase of low gonadal hormonal serum level, these findings indicate that gonadal hormones decrease kappa-mediated TMJ antinociception. The aim of this study was to investigate some of the mechanisms by which gonadal hormones might decrease kappa-mediated antinociception by testing the following hypothesis: (a) Gonadal hormones decrease kappa-mediated TMJ antinociception through a down regulation in the expression of kappa oipoid receptors in the trigeminal ganglia; (b) The peripheral antinociceptive effect of kappa opioid receptor agonists is mediate by the activation of the L-Arginine/NO/cGMP pathway in both males and females; (c) Gonadal hormones decrease kappa-mediated TMJ antinociception by diminishing the activity of the L-Arginine/NO/cGMP. Western blot analysis demonstrated that protein expression of KORs was significantly higher in females than in males, suggesting that testosterone induces a strong down-regulation in KOR expression. In females, KOR expression was significantly higher in those in diestrus than in proestrus suggesting that female gonadal hormones also down-regulate KOR expression in the trigeminal ganglia. Co-application of the NOS inhibitor L-NMMA or of the NO-sensitive guanylyl cyclase inhibitor ODQ with the kappa opioid receptor agonist U50,488 blocked kappa-mediated TMJ antinociception in males and females suggesting that antinociception induced by activation of peripheral kappa opioid receptors is mediated by the L-arginine/NO/cGMP pathway in both sexes. However, co-application of lower doses of L-NMMA and ODQ with U50,488 significantly diminished kappa -mediated TMJ antinociception only in diestrus females. These results indicate that kappa-mediated TMJ antinociception depends on activation of the L-Arginine/NO/cGMP pathway in both males and females. However, the sexual dimorphism in kappa-mediated TMJ antinociception is mediated, at least in part, by the down regulation in the expression of kappa-oipoid receptors in the trigeminal ganglia induced by gonadal hormones, especially testosterone. Despite the involvement of the L-Arginine/NO/cGMP pathway in kappa-mediated TMJ antinociception in both sexes, gonadal hormones do not diminish the activity of this pathway to decrease kappa-mediated TMJ antinociception.

Keywords: L-Arginine/NO/cGMP pathway, kappa opioid receptor, temporomandibular joint, sex differences, estrous cycle, nociception.

SUMÁRIO

1. INTRODUÇÃO:

Este trabalho aborda o estudo dos mecanismos envolvidos no dimorfismo sexual da analgesia mediada por receptores capa opióides na articulação temporomandibular. Nosso interesse pelo assunto, inciou-se através de estudos realizados em humanos e em animais experimentais, que demonstraram que o sexo feminino apresenta uma maior sensibilidade aos efeitos analgésicos dos agonistas de receptores capa opióides. Por exemplo, em humanos a administração sistêmica de agonistas seletivos para os receptores capa opióides (Gear *et al.,* 1996a; Gear *et al*., 1996b) produz maior analgesia pós-operatória no sexo feminino com a dose eficaz menor do que a preconizada (Gear *et al.,* 2003; Gear *et al*., 1999). Estes resultados têm sido demonstrados também em animais experimentais, de forma que a analgesia mediada pela administração sistêmica de agonistas dos receptores capa opióides é significativamente maior em fêmeas quando comparadas com machos, nos modelos nociceptivos de "tail flick" e da placa quente (Binder *et al.,* 2000; Tershner *et al*., 2000).

Esses resultados sugerem que os agonistas de receptores capa opióides, podem apresentar maiores benefícios terapêuticos no tratamento de condições dolorosas que acometem mais o sexo feminino. Dentre essas condições dolorosas, está à dor proveniente da articulação temporomandibular (ATM). Dados da literatura demonstram que a maioria dos pacientes que apresentam dor associada às disfunções temporomandibulares (DTMs) é do sexo feminino (Johansson *et al.,* 2003; Krogstad *et al.,* 1992; Von Korff *et al.,* 1988) e que a prevalência de DTMs em mulheres, especialmente durante o período reprodutivo, é 1,5 a 2 vezes maior que em homens (LeResche *et al*., 1997; Warren & Fried, 2001).

Apesar dos opióides estarem entre as drogas utilizadas no tratamento das condições dolorosas da ATM, seus efeitos colaterais, tais como tontura, náusea, vômito, desenvolvimento de tolerância e dependência, limitam seu uso (Denucci *et al*., 1996). Nesse contexto, nos interessamos pelo estudo dos efeitos periféricos dos agonistas dos receptores opióides capa na dor da ATM. Apesar da expressão de RNAm de receptores opióides capa ter sido previamente detectada no gânglio trigeminal (Schafer *et al*., 1994; Xie *et al*., 1999), não se sabia até então se existiam receptores capa opióides funcionais na ATM. No entanto, dados obtidos recentemente em nosso laboratório, através da

utilização do teste da formalina na ATM (Gameiro *et al*., 2003; Roveroni *et al.,* 2001) demonstraram que a co-administração de doses de efeito local do agonista do receptor capa opióide, U50,488, com o agente nociceptivo formalina na ATM de ratos, reduz significativamente a resposta comportamental nociceptiva induzida pela formalina. Estes resultados sugerem a existência de receptores opióides capa *funcionais* na região da ATM (Clemente *et al.,* 2004) e evidenciam o potencial terapêutico de agonistas periféricos dos receptores capa opióides no tratamento da dor da ATM.

Neste mesmo trabalho (Clemente *et al*., 2004) também foi observado que, para a mesma intensidade de dor induzida pela administração de formalina na região da ATM de ratos, as fêmeas, especialmente na fase diestro do ciclo estral (baixa taxa hormonal), são mais sensíveis ao efeito antinociceptivo periférico do agonista do receptor capa opióide (Figura 1). Vale a pena ressaltar que a administração do agonista do receptor opióide capa U-50,488 na ATM contralateral (média ± E.P.M.; 210,0 ± 4,1) não reduziu as respostas nociceptivas induzidas pela formalina (227,3 ± 13,3) descartando um possível efeito sistêmico.

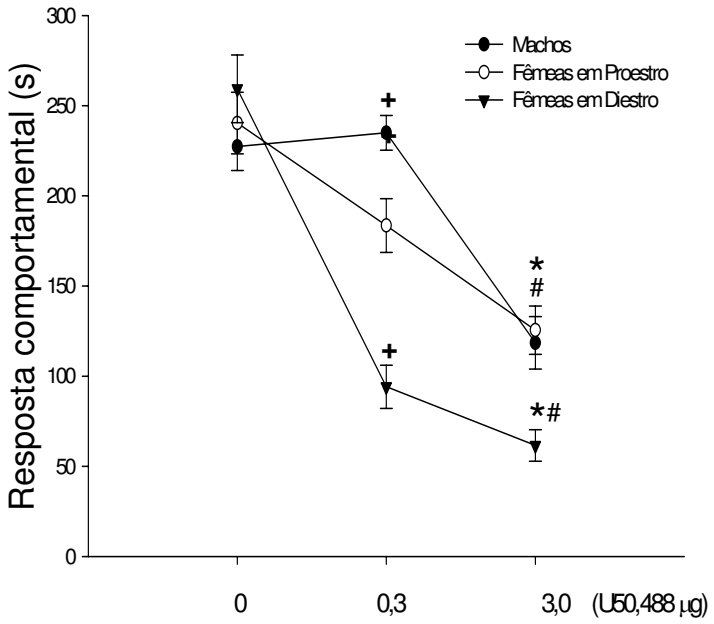

Figura 1. Dimorfismo sexual na analgesia mediada pelo agonista do receptor capa opióide (U50,488 0,3 e 3μg) na ATM. A co-administração do U50,488 com formalina na região da ATM de ratos reduziu a resposta nociceptiva induzida pela formalina em machos e fêmeas. O U50,488 foi mais eficaz em reduzir a resposta nociceptiva em fêmeas, especialmente em fêmeas em diestro. Os dados apresentados correspondem à média ± E.P.M. Símbolos iguais indicam diferença estatística entre os grupos: Machos, Fêmeas em proestro e Fêmeas em diestro ($p<0,05$, teste Tukey). Fonte: Clemente et al., 2004.

Para que possamos expandir nosso conhecimento a respeito desse dimorfismo sexual e entender porque as fêmeas são mais sensíveis à analgesia mediada pela ativação de receptores opióides capa na ATM, nos propusemos na presente pesquisa estudar os mecanismos que poderiam explicar tal fenômeno.

Relata-se que o efeito analgésico periférico dos agonistas do receptor opióide capa é mediado pela ativação da via intracelular L-arginina/NO/GMPc em machos (Amarante & Duarte, 2002; Nozaki-Taguchi & Yamamoto, 1998). Considerando estudos que demonstraram que os hormônios sexuais podem modular a ativação da via L-Arginina/NO/GMPc (Yallampalli *et al.,* 1994; Panzica *et al*., 2006), assim como modular a expressão de receptores opióides em diferentes regiões do sistema nervoso (Weiland *et al*., 1990, Shen *et al.,* 1995; Maggi *et al.,* 1999; Chang *et al*., 2000; Schafer *et al.,* 1994) , o objetivo deste trabalho foi investigar o possível mecanismo pelo qual os hormônios gonadais poderiam diminuir a antinocicepção mediada pelos receptores capa opióides através das seguintes hipóteses: (a) Os hormônios gonadais diminuem a antinocicepção mediada pelos receptores capa na ATM por diminuírem a expressão dos receptores capa opióides no gânglio trigeminal; (b) O efeito periférico antinociceptivo dos agonistas dos receptores capa opióides é mediado pela ativação da via L-Arginine/NO/GMPc em machos e fêmeas; Os hormônios gonadais diminuem a antinocicepção mediada pelos receptores capa na ATM por diminuírem a ativação da via L-Arginine/NO/GMPc.

Uma vez que, apesar da alta incidência das condições dolorosas da ATM o número de insucessos no tratamento das mesmas ainda é bastante elevado (Irving *et al*., 1999), consideramos que o trabalho proposto é de relevância clínica e farmacológica, pois além de avançar no conhecimento dos mecanismos moleculares envolvidos no dimorfismo sexual da analgesia periférica mediada pelo agonista do receptor opióide capa, o trabalho contribui para o desenvolvimento de uma nova opção terapêutica no tratamento da dor da ATM. Além disso, os resultados obtidos nestes experimentos ajudam a determinar um tratamento diferenciado das condições dolorosas da ATM entre os sexos.

2. CAPÍTULO :

O presente artigo será submetido ao periódico “Pain”.

Gonadal hormones decrease kappa-mediated TMJ antinociception through a down regulation in the expression of kappa opioid receptors in the trigeminal ganglia

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Abstract

We have previously demonstrated that activation of kappa opioid receptors located in the TMJ of rats suppresses formalin-induced TMJ nociception behavior especially in females of the diestrus phase of the estrous cycle. Since diestrus is a phase of low gonadal hormonal serum level, these findings indicate that gonadal hormones decrease kappa-mediated TMJ antinociception. The aim of this study was to investigate some of the mechanisms by which gonadal hormones might decrease kappa-mediated antinociception by testing the following hypothesis: (a) Gonadal hormones decrease kappa-mediated TMJ antinociception through a down regulation in the expression of kappa oipoid receptors in the trigeminal ganglia; (b) The peripheral antinociceptive effect of kappa opioid receptor agonists is mediate by the activation of the L-Arginine/NO/cGMP pathway in both males and females; (c) Gonadal hormones decrease kappa-mediated TMJ antinociception by diminishing the activity of the L-Arginine/NO/cGMP. Western blot analysis demonstrated that protein expression of KORs was significantly higher in females than in males, suggesting that testosterone induces a strong down-regulation in KOR expression. In females, KOR expression was significantly higher in those in diestrus than in proestrus suggesting that female gonadal hormones also down-regulate KOR expression in the trigeminal ganglia. Co-application of the NOS inhibitor L-NMMA or of the NO-sensitive guanylyl cyclase inhibitor ODQ with the kappa opioid receptor agonist U50,488 blocked kappa-mediated TMJ antinociception in males and females suggesting that antinociception induced by activation of peripheral kappa opioid receptors is mediated by the L-arginine/NO/cGMP pathway in both sexes. However, co-application of lower doses of L-NMMA and ODQ with U50,488 significantly diminished kappa -mediated TMJ antinociception only in diestrus females. These results indicate that kappa-mediated TMJ antinociception depends on activation of the L-Arginine/NO/cGMP pathway in both males and females. However, the sexual dimorphism in kappa-mediated TMJ antinociception is mediated, at least in part, by the down regulation in the expression of kappa-oipoid receptors in the trigeminal ganglia induced by gonadal hormones, especially testosterone. Despite the involvement of the L-Arginine/NO/cGMP pathway in kappa-mediated TMJ antinociception in both sexes, gonadal hormones do not diminish the activity of this pathway to decrease kappa-mediated TMJ antinociception.

Keywords: L-Arginine/NO/cGMP pathway, kappa opioid receptor, temporomandibular joint, sex differences, estrous cycle, nociception.

Introduction

Temporomandibular disorder (TMD) is a common clinical condition involving pain in the temporomandibular joint (TMJ) region and/or associated muscles with a greater prevalence in women of reproductive age than in men (LeResche et al. 1997; Warren and Fried 2001).

Data from animals (Binder et al. 2000; Tershner et al. 2000) and humans (Gear et al. 1996a; Gear et al. 1996b) studies indicate that kappa opioid agonists produce significantly greater analgesia in females than males highlighting the potential clinical relevance of kappa-mediated analgesia in females. Recently, we have demonstrated that activation of kappa opioid receptors located in the TMJ of rats suppresses formalin-induced TMJ nociception, especially in females of the diestrus phase of the estrous cycle. Since diestrus is a phase of low gonadal hormonal serum level (Butcher et al., 1974) these findings suggest that gonadal hormones decrease kappa-mediated TMJ antinociception. The mechanisms underlying this effect of gonadal hormones on kappa-mediated TMJ antinociception are presently unknown. However, both the expression of kappa oipoid receptors (Kalyuzhny et al., 1995; Ji RR et al., 1995; Rau KK et al., 2005) and the activation of the L-Arginine/NO/cGMP by kappa opioid agonists (Nozaki-Taguchi and Yamamoto 1998; Amarante and Duarte 2002) can affect kappa-mediated antinociception.

It has been previously demonstrated that sex hormones regulate the expression of opioid receptors in different regions of the nervous system. For example, female reproductive hormones regulate the expression of opioid receptors in the hypothalamus (Weiland et al., 1990, Shen et al., 1995), in neuroblastoma cells (Maggi et al., 1999) and the expression of kappa opioid receptors in the spinal cord (Chang et al., 2000). Although kappa-opioid receptor mRNA is expressed in the trigeminal ganglia (Schafer et al., 1994) it is not known whether the expression of kappa-opioid receptors is regulated by sex hormones.

It is well known that the peripheral antinociceptive effect of kappa opioid receptor agonists is mediated by the activation of the intracellular L-arginine/NO/cGMP pathway in males (Nozaki-Taguchi and Yamamoto, 1998; Amarante and Duarte, 2002). Also well known is that estrogen down-regulates the L-Arginine/NO/cGMP pathway (Yallampalli et al., 1994). Recently, it has been demonstrated that the expression of neuronal nitric oxide synthase can be up or down-regulated by testosterone in males, and

by estrogen in females (Panzica et al., 2006). Therefore, if the L-Arginine/NO/cGMP pathway is also involved in the peripheral antinociceptive effect of kappa opioid receptor agonists in females, gonadal hormones might decrease kappa-mediated antinociception by diminishing the activation of the L-Arginine/NO/cGMP pathway.

The aim of this study was to investigate some of the mechanisms by which gonadal hormones might decrease kappa-mediated antinociception by testing the following hypothesis:

(a) Gonadal hormones decrease kappa-mediated TMJ antinociception through a down regulation in the expression of kappa oipoid receptors in the trigeminal ganglia;

(b) The peripheral antinociceptive effect of kappa opioid receptor agonists is mediate by the activation of the L-Arginine/NO/cGMP pathway in both males and females;

(c) Gonadal hormones decrease kappa-mediated TMJ antinociception by diminishing the activity of the L-Arginine/NO/cGMP.

Methods

Drugs and doses

Formalin solutions were prepared from commercially (Sigma, USA) available stock formalin (an aqueous solution of 37 % of formaldehyde) further diluted in 0.9 % NaCl (saline) to concentrations of 1 and 1.5 %. The inhibitor of NO sintase L-NMMA (N^G-Monomethyl-L-arginine acetate 50, 150 or 450 μg, Ferreira et al. 1991) and the NO-sensitive guanylyl cyclase inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one 8, 32 or 80 μg, Cunha et al. 1999) were obtained from Tocris Cookson (USA); the kappa opioid receptor agonist U50,488 (0.3 or 3 μg, Clemente et al., 2004) was obtained from Sigma (USA). L-NMMA and U50,488 were dissolved in saline; ODQ was dissolved in dimethyl sulfoxide (DMSO).

Animals

Three-month-old male and female albino Wistar rats were used, and the experiments were conducted in accordance to the IASP guidelines on using laboratory animals (Zimmermann, 1983). All animal experimental procedures and protocols were approved by the Committee on Animal Research of the University of Campinas. The animal suffering and number of animals per group were kept at a minimum. The animals 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*. They were maintained on a temperature-controlled room test (± 23ºC) for a 1-hour habituation period previously to the test.

Estrous phase determination

Estrous phase was determined by daily microscope examination of vaginal smears taken by gentle lavage, between 7 and 8 A.M. Proestrus phase and the initial phase of diestrus (first 4 h) were identified by the predominance (> 70 %) of nucleated epithelial cells and leukocytes, respectively (Bereiter et al., 2001) in rats with at least two consecutive regular 4-day cycles (Smith et al., 1975). These phases were chosen because they represent phases of high and low ovarian hormonal level, respectively (Butcher et al., 1974).

TMJ Injections

Animals were briefly anesthetized by inhalation of halothane to allow the TMJ injection, which was performed with a 30-gauge needle introduced into the left TMJ at the moment of injection. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 μl). Injection volumes were 15 μl in all cases. Each animal regained consciousness approximately 30 s after discontinuing the anesthetic.

Testing procedure for TMJ pain

Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23º C (Rosland et al., 1991). Each animal was manipulated for 7 days to be habituated to the experimental manipulation. After this period, the animal was placed in a test chamber (30x30x30 cm mirrored-wood chamber with a glass at the front side) for a 15 min habituation period to minimize stress. Each animal immediately recovered from the anesthesia after TMJ injection and was returned to the test chamber for counting nociceptive responses during the followed 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 proportional to the proportion of time that the behavior occupies. Since head flinches followed a uniform pattern of 1 s of duration, each flinch was expressed as 1 s (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, animals were anesthetized by an intraperitoneal injection of a mixture of urethane (1 g/kg) and α-chloralose (50 mg/kg) (Hu et al., 1990) and Evans blue dye (1 %, 5 mg/Kg), was then intravenously administered in order to visualize formalin-induced plasma extravasation upon post-mortem examination of injected TMJs (Haas et al., 1992). This procedure also allowed confirmation that the plasma extravasation induced by TMJ injection at the correct site was restricted to the immediate TMJ region.

Western Blot analysis

Five animals in each group were used for immunoblot study. Trigeminal ganglion tissues were homogenized by sonication in cold RIPA buffer (1 % Igepal CA-630, 0,5 % sodium deoxycholate, 0,1 % SDS, 1 mM PMSF, 10 mg/ml aprotinin, 1 mM sodium orthovanadate in PBS buffer, pH 7.4). The samples were centrifuged at 10,000 g for 10 minutes at 4ºC. The supernatant was removed and centrifuged again. After centrifugation, the protein concentration was determined in the supernatants by using the Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, USA). Aliquots containing 40 μg total protein were boiled in loading Laemmli buffer (BioRad, USA), thereafter; each aliquot was loaded onto a 10 % polyacrylamide gel. After electrophoresis separation, proteins were transferred to a nitrocellulose membrane (Bio-Rad). Membrane was blocked in TBST (20 mM Tris-HCL, 150 mM NaCl, and 0.1 % Tween 20) containing 5 % non-fat dry milk overnight at 4ºC, followed by incubation with KOR-1 (N-19) goat polyclonal IgG (1:500; Santa Cruz Biotechnology) for 3 h at room temperature, rinsed six times with TBST, and then incubated for 1h in anti-goat IgG peroxidase conjugate (1:1000, Sigma). Membrane was visualized using ECL solution (Pierce) for 60 s, then exposed to x-ray film (Kodak) in a dark room. Films were scanned into Image Quant 5.2 for analysis. Banding specificity was determined by omission of primary antibody from the Western blot protocol. To compensate for any differences in the amount of loaded protein, the intensity of the kappa opioid receptor band was divided by the intensity of α-tubulin (Sigma, USA) band for each sample.

Experimantal design

To test the hypothesis that sex hormones decrease kappa-mediated antinociception through a down regulation in the expression of kappa opioid receptors in the trigeminal ganglia, western blot analyses was used to evaluate the expression of kappa opioid receptors in the trigeminal ganglia of males, proestrus and diestrus females.

To test the hypothesis that sex hormones decrease kappa-mediated antinociception by diminishing the activation of the L-arginine/NO/cGMP pathway, the NOS inhibitor L-NMMA (50, 150 or 450 μg) or the NO-sensitive guanylyl cyclase inhibitor, ODQ (8, 32 or 80 μg) was co-applied with the same volume of equi-nociceptive

concentrations of formalin (1 % to diestrus females and 1.5 % to males and proestrus females; Clemente et al., 2004) plus equi-antinociceptive doses of the kappa opioid receptor agonist U50,488 (0.3 μg to diestrus females and 3 μg to proestus females and males; Clemente et al., 2004). To confirm the local action of L-NMMA and ODQ, the highest concentration of each of them was applied into the contralateral TMJ to that injected with formalin plus U50,488.

Statistical analysis

To determine if there were significant differences ($p < 0.05$) between treatment groups, the t-test or one-way ANOVA was performed as apropriated. 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 are presented in figures as means ± S.E.M.

Results

Kappa opioid receptor expression

Protein expression of kappa opioid receptor (KOR) was significantly higher in females than in males, especially in females at the diestrus phase of the estrous cycle (Fig. 1).

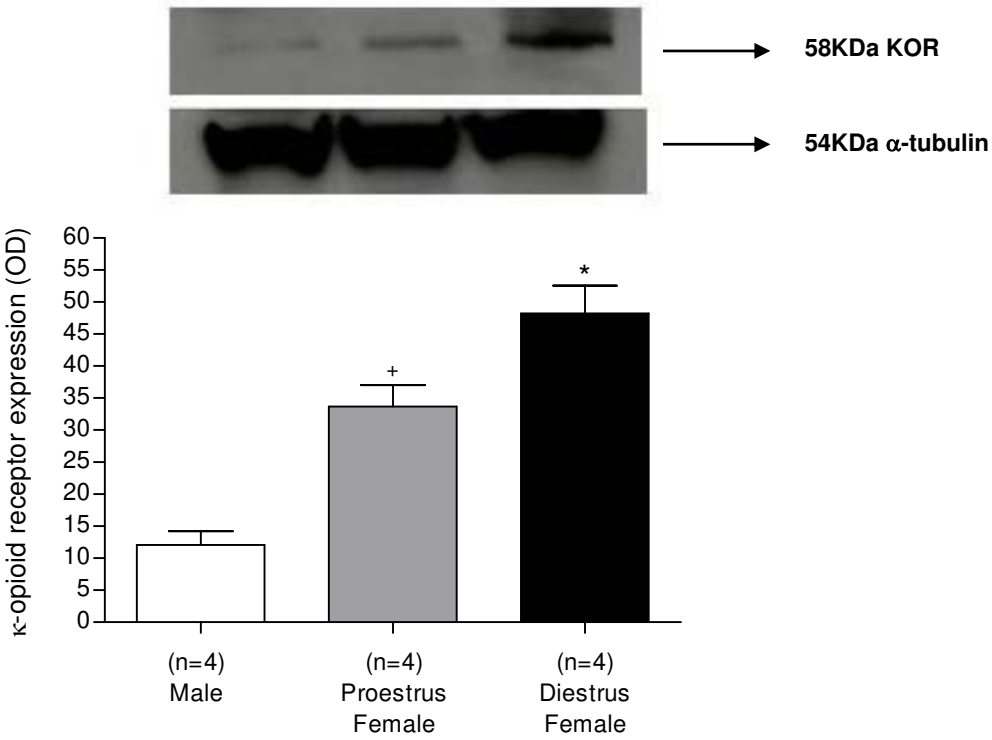

Figure 1: Kappa opioid receptor expression in the trigeminal ganglion of males and females - The symbol (*) indicates that the KOR expression was significantly higher in diestrus females than in males and proestrus females. The symbol (+) indicates that the KOR expression was significantly higher in proestrus females than in males. Immunoblot for KOR expression with the corresponding blot for α-tubulin is shown.

Inhibition of nitric oxide

Co-application of the NOS inhibitor L-NMMA (450 μg) with equi-antinociceptive doses of the kappa opioid receptor agonist U50,488 blocked kappa-mediated antinociception in the TMJ of males, proestrus females and diestrus females (Fig. 2). At lower concentrations (50 and 150 μg), L-NMMA significantly reduced the antinociceptive effect of U50,488 on TMJ formalin-induced nociceptive response only in diestrus females (Fig. 2C). To discard the possibility that the antinociceptive effect of the U50,488 induced by lower doses of L-NMMA in diestrus females was due to the use of a lower concentration of U50,488 (0.3 μg), the same concentration of U50,488 (3 μg) that was used in males and proestrus females was also co-applied with L-NMMA in diestrus females. Co application of L-NMMA (50 and 150 μg) also significantly reduced the antinociceptive effect of 3 μg of U50,488 on TMJ formalin-induced nociceptive response in diestrus females. These findings indicate that the antinociception induced by kappa opioid receptor activation in the TMJ is mediated by local release of NO in males and females.

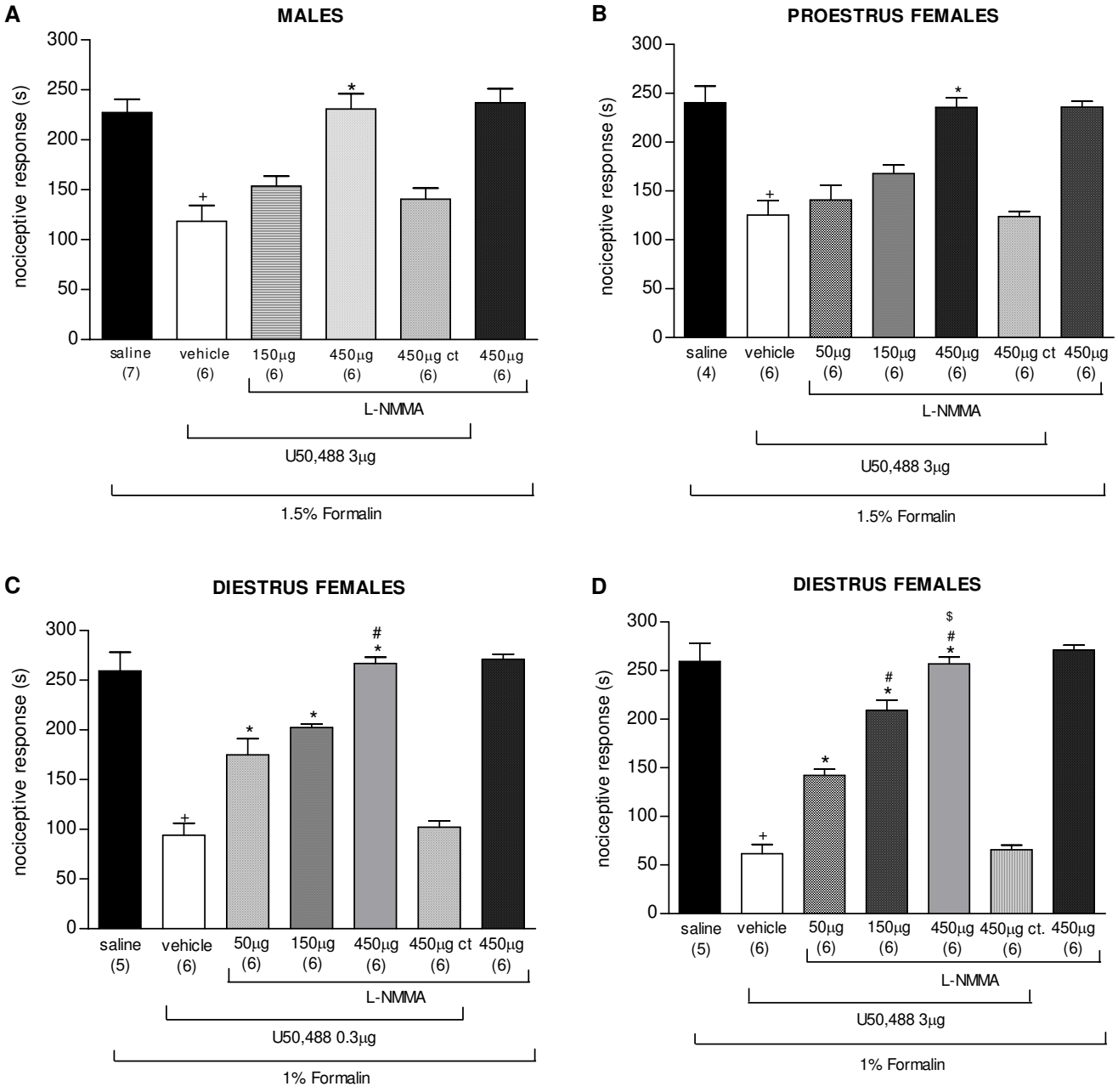

Figure 2: Effect of locally administered NOS inhibitor (L-NMMA) on the antinociception induced by the kappa opioid receptor agonist (U50,488) in the TMJ.

Figure 2A: ***Males*** - Co-application of L-NMMA (450 but not 150 µg) significantly diminished the antinociceptive effect of U50,488 (3 µg) on 1.5 % formalin-induced nociceptive response; second bar from right: application of L-NMMA (450 µg) into contralateral (ct) TMJ did not affect ($p>0.05$, t test) the antinociceptive effect of U50,488 (3 µg, second bar from left); first bar from right: co-application of L-NMMA (450 µg) did not affect ($p>0.05$, t test) 1.5 %

formalin-induced nociceptive response (first bar from left). The symbol (+) indicates a response significantly lower than that of saline + 1.5 % formalin ($p<0.05$, t test). The symbol (*) indicates a response significantly higher than that of U50,488 (3 μg) + 1.5 % formalin (second bar from left) and L-NMMA (150 μg) + U50,488 (3 μg) + 1.5 % formalin (third bar from left; $p<0.05$, ANOVA, tukey test).

Figure 2B: ***Proestrus females*** - Co-application of L-NMMA (450 but not 50 or 150 μg) significantly diminished the antinociceptive effect of U50,488 (3 μg) on 1.5 % formalin-induced nociceptive response; second bar from right: application of L-NMMA (450 μg) into contralateral (ct) TMJ did not affect ($p>0.05$, t test) the antinociceptive effect of U50,488 (3 μg, second bar from left); first bar from right: co-application of L-NMMA (450 μg) did not affect ($p>0.05$, t test) 1.5 % formalin-induced nociceptive response (first bar from left). The symbol (+) indicates a response significantly lower than that of saline + 1.5 % formalin ($p<0.05$, t test). The symbol (*) indicates a response significantly higher than that of U50,488 (3 μg) + 1.5 % formalin (second bar from left); L-NMMA (150 μg) + U50,488 (3 μg) + 1.5 % formalin (third bar from left) and L-NMMA (450 μg) + U50,488 (3 μg) + 1.5 % formalin (fourth bar from left; $p<0.05$, ANOVA, tukey test).

Figure 2C: ***Diestrus females*** – Co-application of L-NMMA (50, 150 or 450 μg) significantly diminished the antinociceptive effect of U50,488 (0.3 μg) on 1 % formalin-induced nociceptive response in a dose dependent manner; second bar from right: application of L-NMMA (450 μg) into contralateral (ct) TMJ did not affect ($p>0.05$, t test) the antinociceptive effect of U50,488 (0.3 μg; second bar from left); first bar from right: co-application of L-NMMA (450 μg) did not affect ($p>0.05$, t test) 1 % formalin-induced nociceptive response (first bar form left). The symbol (+) indicates a response significantly lower than that of saline + 1.5 % formalin ($p<0.05$, t test). The symbol (*) indicates a response significantly higher than that of U50,488 (0.3 μg) + 1.5 % formalin (second bar from left; $p<0.05$, ANOVA, tukey test). The symbol (#) indicates a response significantly higher than that of L-NMMA (50 μg) + U50,488 (0.3 μg) + 1 % formalin (third bar from left) and L-NMMA (150 μg) + U50,488 (0.3 μg) + 1 % formalin (fourth bar from left) ($p<0.05$, ANOVA, tukey test).

Figure 2D: ***Diestrus females*** - Co-application of L-NMMA (50, 150 or 450 μg) significantly diminished the antinociceptive effect of U50,488 (3 μg) on 1 % formalin-induced nociceptive response in a dose dependent manner; second bar from right: application of L-NMMA (450 μg) into contralateral (ct) TMJ did not affect ($p>0.05$, t test) the antinociceptive effect of U50,488 (3 μg, second bar from left); first bar from right: co-application of L-NMMA (450 μg) did not affect ($p>0.05$, t test) 1 % formalin-induced nociceptive response (first from left). The symbol (+) indicates a response significantly lower than that of saline + 1 % formalin ($p<0.05$, t test). The symbol (*) indicates a response significantly higher than that of U50,488 (3 μg) + 1 % formalin (second bar from left; $p<0.05$, ANOVA, tukey test). The symbol (#) indicates a response significantly higher than that of L-NMMA (50 μg) + U50,488 (3 μg) + 1 % formalin (third bar from left; $p<0.05$, ANOVA, tukey test). The symbol ($) indicates a response significantly higher than that of L-NMMA (150 μg) + U50,488 (3 μg) + 1 % formalin (fourth bar from left;$p<0.05$, ANOVA, tukey test).

Inhibition of guanylyl cyclase

Co-application of the NO-sensitive guanylyl cyclase inhibitor ODQ (80 μg) blocked the antinociceptive effect of U50,488 on TMJ formalin-induced nociceptive response in males, proestrus females and diestrus females (Fig. 3). At lower concentrations (0.8 and 8 μg) ODQ, significantly reduced the antinociceptive effect of U50,488 on TMJ formalin-induced nociceptive response only in females. The concentration of 0.8 μg was effective only in females at the diestrus phase of the estrous cycle (Fig. 3). These findings indicate that the antinociception induced by kappa opioid receptor activation in the TMJ is mediated by local release of cGMP in males and females.

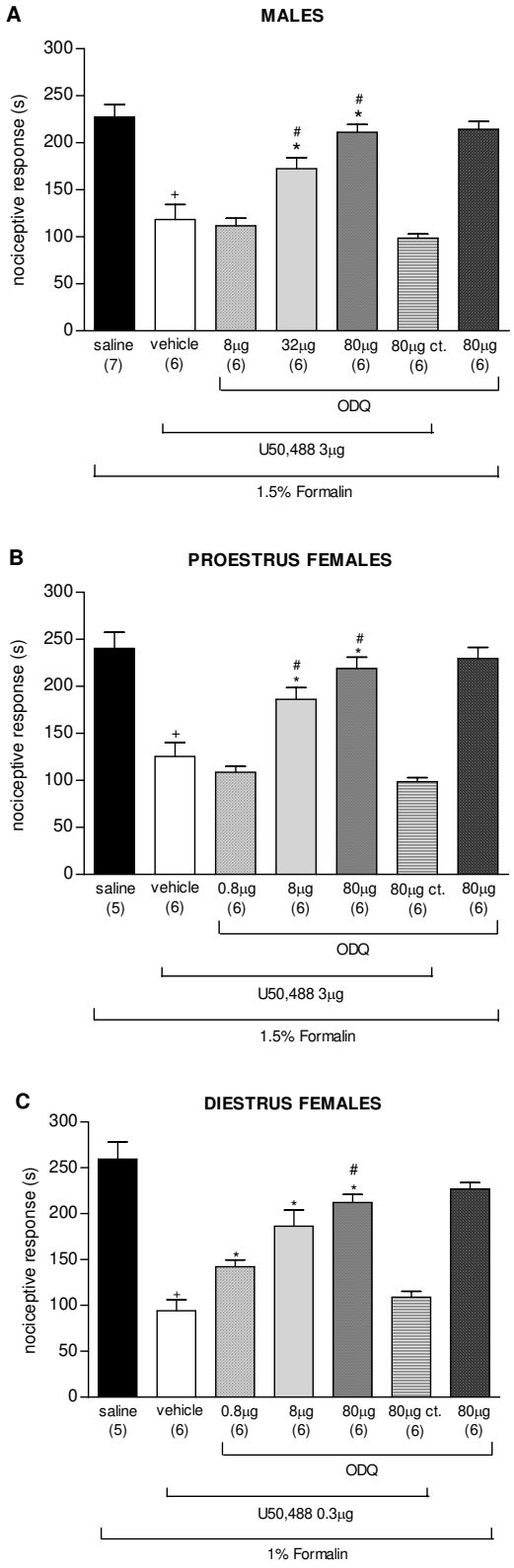
A
MALES
nociceptive response (s)
300
250
200
150
100
50
0
saline (7)
vehicle (6)
8μg (6)
32μg (6)
80μg (6)
80μg ct. (6)
80μg (6)
ODQ
U50,488 3μg
1.5% Formalin
B
PROESTRUS FEMALES
nociceptive response (s)
saline (5)
vehicle (6)
0.8μg (6)
8μg (6)
80μg (6)
80μg ct. (6)
80μg (6)
ODQ
U50,488 3μg
1.5% Formalin
C
DIESTRUS FEMALES
nociceptive response (s)
saline (5)
vehicle (6)
0.8μg (6)
8μg (6)
80μg (6)
80μg ct. (6)
80μg (6)
ODQ
U50,488 0.3μg
1% Formalin

Figure 3: Effect of locally administered NO-sensitive guanylyl cyclase inhibitor (ODQ) on the antinociception induced by kappa opioid receptor agonist (U50,488) in the TMJ.

Figure 3A: ***Males*** - Co-application of ODQ (32, 80 but not 8 μg) significantly diminished the antinociceptive effect of U50,488 (3 μg) on 1.5 % formalin-induced nociceptive response; second bar from right: application of ODQ (80 μg) into contralateral (ct) TMJ did not affect ($p>0.05$, t test) the antinociceptive effect of U50,488 (3 μg; second bar from left); first bar from right: co-application of ODQ (80 μg) did not affect ($p>0.05$, t test) 1.5 % formalin-induced nociceptive response (first bar from left). The symbol (+) indicates a response significantly lower than that of saline + 1.5 % formalin (first bar from left; $p<0.05$, t test). The symbol (*) indicates a response significantly higher than that of U50,488 (3 μg) + 1.5 % formalin (second bar from left;$p<0.05$, ANOVA, tukey test). The symbol (#) indicates a response significantly higher than that of ODQ (8 μg) + U50,488 (3 μg) + 1.5 % formalin (third bar from left;$p<0.05$, ANOVA, tukey test).

Figure 3B: ***Proestrus females*** - Co-application of ODQ (8, 80 but not 0.8 μg) significantly diminished the antinociceptive effect of U50,488 (3 μg) on 1.5 % formalin-induced nociceptive response; second bar from right: application of L-NMMA (80 μg) into contralateral (ct) TMJ did not affect ($p>0.05$, t test) the antinociceptive effect of U50,488 (3 μg; second bar from left); first bar from right: co-application of ODQ (80 μg) did not affect ($p>0.05$, t test) 1.5 % formalin-induced nociceptive response (first bar from left). The symbol (+) indicates a response significantly lower than that of saline + 1.5 % formalin (first bar from left; $p<0.05$, t test). The symbol (*) indicates a response significantly higher than that of U50,488 (3 μg) + 1.5 % formalin (second bar from left; $p<0.05$, ANOVA, tukey test). The symbol (#) indicates a response significantly higher than that of ODQ (0.8 μg) + U50,488 (3 μg) + 1.5 % formalin (third bar from left; $p<0.05$, ANOVA, tukey test).

Figure 3C: Diestrus females – Co-application of ODQ (0.8, 8 or 80 μg) significantly diminished the antinociceptive effect of U50,488 (0.3 μg) on 1 % formalin-induced nociceptive response in a dose dependent manner; second bar from right: application of ODQ (80 μg) into contralateral (ct) TMJ did not affect ($p>0.05$, t test) the antinociceptive effect of U50,488 (0.3 μg; second bar from left); first bar from right: co-application of (80 μg) did not affect ($p>0.05$, t test) 1 % formalin-induced nociceptive response (first bar from left). The symbol (+) indicates a response significantly lower than that of saline + 1 % formalin (first bar from left; $p<0.05$, t test). The symbol (*) indicates a response significantly higher than that of U50,488 (0.3 μg) + 1 % formalin (second bar from left; $p<0.05$, ANOVA, tukey test). The symbol (#) indicates a response significantly higher than that of ODQ (0.8 μg) + U50,488 (0.3 μg) + 1 % formalin (third bar from left; $p<0.05$, ANOVA, tukey test).

Discussion

The present study was undertaken to identify some of the mechanisms by which gonadal hormones might decrease kappa-mediated TMJ antinociception. Since both the expression of kappa opioid receptors (Kalyuzhny et al., 1995; Ji RR et al., 1995; Rau KK et al., 2005) and the activation of the L-Arginine/NO/cGMP by kappa opioid agonists (Nozaki-Taguchi and Yamamoto 1998; Amarante and Duarte 2002) can affect kappa-mediated antinociception, we tested the hypothesis that gonadal hormones decrease kappa-mediated TMJ antinociception through a down regulation in the expression of kappa-opioid receptors in the trigeminal ganglia and/or through a decrease in the activity of the L-Arginine/NO/cGMP pathway that mediates the antinociceptive effect of peripherally acting kappa opioid agonists.

First, we provided molecular evidence to suggest that endogenous gonadal hormones exert a pivotal role in controlling the expression of kappa opioid receptors (KORs) in the trigeminal ganglia of rats. Western blot analysis indicates that protein expression of KORs was significantly higher in females than in males, suggesting that testosterone induces a strong down-regulation in KOR expression. In females, KOR expression was significantly higher in those in diestrus than in proestrus. Given that diestrus phase has lower ovarian hormonal levels (Butcher et al., 1974) than the proestrus one, this finding suggesting that female gonadal hormones also down-regulation KOR expression in the trigeminal ganglia. This is in agreement with the findings that gonadal hormones decrease the expression of opioid receptors in hypothalamic neurons (Weiland et al., 1990) and in neuroblastoma cells (Maggi et al., 1999).

Gonadal hormones may act on cells of the trigeminal ganglia and regulate the expression of kappa-opioid receptors on the nociceptive primary afferent fibers of the TMJ. Although no previous studies appear to have examined the presence of progesterone or testosterone receptors within the trigeminal ganglia, both estrogen receptors (Puri et al., 2005a,b) and kappa-opioid receptors (current study) are expressed in this ganglia.

The biochemical mechanisms underlying the effect of gonadal hormones on the expression of KORs in the trigeminal ganglia are unknown, but could potentially involve a decrease in the expression of functional KORs, that is, KORS that are expressed on the plasma membrane. In this case, gonadal hormones would diminish the incorporation of cytoplasmatic KORs into the plasma membrane of the nociceptive primary

afferent fibers of the TMJ. Another possibility is that gonadal hormones decrease kappa opioid mRNA synthesis and/or the rate of kappa opioid mRNA degradation in these fibers, which is as important as the rate of synthesis in regulating the steady-state concentration of the mRNA. This latter suggestion is in agreement with the demonstration that sex hormones affect the concentration of receptor proteins by regulating gene expression transcriptionally, that is the mRNA synthesis or posttranscriptionally by altering mRNA stability, which is the rate of mRNA degradation (Ing, 2005).

Second, we provided pharmacological evidence to confirm that antinociception induced by activation of peripheral kappa opioid receptors is mediated by the L-arginine/NO/cGMP pathway in males (Nozaki-Taguchi and Yamamoto, 1998; Amarante and Duarte 2002) and to suggest that this kappa-opioid effect is also mediated by the L-arginine/NO/cGMP pathway in females. Co-application of the NOS inhibitor L-NMMA or of the NO-sensitive guanylyl cyclase inhibitor ODQ with the kappa opioid receptor agonist U50,488 blocked kappa-mediated antinociception in the TMJ of males and females. The mechanism by which activation of the L-arginine/NO/cGMP pathway by kappa opioid receptors results in analgesia is presently unknown. However, it could potentially involve the opening of ATP-dependent K^+ channels that, in turn, would result in K^+ outward currents thereby counteracting the lowering of the nociceptor threshold. (Rodrigues and Duarte, 2000; Soares et al., 2000).

Despite our findings that peripheral antinociceptive effect of the kappa opioid receptor agonist U50,488 is mediated by the activation of the L-Arginine/NO/cGMP pathway in both males and females, gonadal hormones do not appear to decrease kappa-mediated TMJ antinociception by diminishing the activity of this pathway. A surprising finding was that co-application of lower doses of L-NMMA and ODQ with U50,488 significantly diminished kappa -mediated TMJ antinociception only in diestrus females, that is, only in females with low ovarian hormone levels. Given that L-NMMA and ODQ were co-applied with equi-antinociceptive doses of U50,488, that is, 0.3 μg to diestrus females and 3 μg to proestus females and males (Clemente et al., 2004), the reduction in the antinociceptive effect of U50,488 induced by lower doses of L-NMMA and ODQ in diestrus females might be due to the use of a lower dose of U50,488 (0.3 μg) in these animals. However, our finding that co-application of lower doses of L-NMMA (50 and 150 μg) with the same dose of U50,488 (3 μg) that was used in males and proestrus females also

significantly reduced the antinociceptive effect of U50,488 on TMJ formalin-induced nociception only in diestrus females argues against this possibility. Therefore, at higher gonadal hormone levels such as those seen in males and proestrus females, a higher dose of L-NMMA and ODQ is necessary to significantly diminish kappa -mediated TMJ antinociception. These results were interpreted to indicate that different from our hypothesis, gonadal hormones may in fact activate the L-Arginine/NO/cGMP pathway that mediates the antinociceptive effect of peripherally applied U50,488 during formalin-induced TMJ nociception. Although the mechanism underlying this effect of sex hormones on the L-Arginine/NO/cGMP pathway is unknown, NO is synthesized from the amino acid L-arginine by a family of enzymes, the nitric oxide synthases (NOS) (Moncada, 1993), that can be modulated by sex hormones (Yallampalli et al., 1994; Chu et al., 2004; Panzica et al., 2006). The family of NOS enzymes include two constitutive Ca^{2+}/calmodulin-dependent isoforms (cNOS), namely neural NOS (nNOS) and endothelial NOS (eNOS) and one Ca^{2+}/calmodulin-independent isoform, inducible NOS (iNOS) (Xia and Krukoff, 2004). In neural cells, estrogen induces NO production through activation of cNOS, rather than iNOS, (Xia and Krukoff, 2004). Thus, our findings are compatible with the suggestion that gonadal hormones up-regulate the NOS expression, which in turn, would increase the activity of the L-Arginine/NO/cGMP pathway that mediates the antinociceptive effect of peripherally applied U50,488. The type of NOS that would be regulated by gonadal hormones in the TMJ region is unknown. Also unknown is the mechanism by which gonadal hormones would up-regulate the NOS expression. However, genomic and/or nongenomic actions of sex hormones on neuronal cells have been proposed (Prevot et al., 1999; Rachman et al., 1998; Wong et al., 2001; Scordalakes et al., 2002).

Importantly, the finding that the same dose of L-NMMA and ODQ was necessary to block the antinociceptive effect of peripherally applied U50,488 during nociception induced by formalin injection into the TMJ region of males, proestrus and diestrus females suggests that the stimulating effect of gonadal hormones on the activity of the L-Arginine/NO/cGMP pathway may be small. On the other hand, it may be necessary for the expression of kappa-mediated TMJ antinociception in the presence of high levels of gonadal hormones as those seen in males and proestrus females. The simplest explanation for this suggestion is that the high levels of these hormones down-regulate the expression of kappa-opioid receptors in the trigeminal ganglia which, in turn, decreases peripheral kappa-mediated TMJ antinociception.

Thus, while a small activation of the L-Arginine/NO/cGMP pathway by gonadal hormones can explain the expression of kappa-mediated TMJ antinociception in males and proestrus females (high levels of gonadal hormones) a strong down-regulation in the expression of kappa-opioid receptors in the trigeminal ganglia induced by these hormones, especially by testosterone, explains why kappa-mediated TMJ antinociception is significantly greater in females, especially those in diestrus (low levels of gonadal hormones).

In summary, we provide evidence that kappa-mediated TMJ antinociception depends on activation of the L-Arginine/NO/cGMP pathway in both males and females. However, the sexual dimorphism in kappa-mediated TMJ antinociception is mediated, at least in part, by the down regulation in the expression of kappa-opioid receptors in the trigeminal ganglia induced by gonadal hormones, especially testosterone.

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References

Amarante, L.H. and Duarte, I.D. The kappa-opioid agonist (+/-)-bremazocine elicits peripheral antinociception by activation of the L-arginine/nitric oxide/cyclic GMP pathway. Eur J Pharmacol 2002;454:19-23.

Bereiter, DA. Sex differences in brainstem neural activation after injury to the TMJ region. Cells Tissues Organs 2001;169:226-37.

Binder, W., Carmody, J. and Walker, J. Effect of gender on anti-inflammatory and analgesic actions of two kappa-opioids, J Pharmacol Exp Ther 2000;292:303-9.

Butcher, R.L., Collins, W.E. and Fugo, N.W. Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat. Endocrinology 1974;94:1704-8.

Chang PC, Aicher SA, Drake CT. Kappa opioid receptors in rat spinal cord vary across the estrous cycle. Brain Res 2000;861:168-72.

Chu HP, Sarkar G, Etgen AM. Estradiol and progesterone modulate the nitric oxide/cyclic gmp pathway in the hypothalamus of female rats and in GT1-1 cells. Endocrine 2004;24:177-84.

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;372:250-5.

Cunha FQ, Teixeira MM, Ferreira SH. Pharmacological modulation of secondary mediator systems--cyclic AMP and cyclic GMP--on inflammatory hyperalgesia. Br J Pharmacol 1999;127:671-8.

Ferreira, S.H., Duarte, I.D. and Lorenzetti, B.B. The molecular mechanism of action of peripheral morphine analgesia: stimulation of the cGMP system via nitric oxide release. Eur J Pharmacol 1991;201:121-2.

Gear, R.W., Gordon, N.C., Heller, P.H., Paul, S., Miaskowski, C. and Levine, J.D. Gender difference in analgesic response to the kappa-opioid pentazocine. Neurosci Lett 1996a;205:207-9.

Gear, R.W., Miaskowski, C., Gordon, N.C., Paul, S.M., Heller, P.H. and Levine, J.D. Kappa-opioids produce significantly greater analgesia in women than in men. Nat Med 1996b;2:1248-50.

Haas, D.A., Nakanishi, O., MacMillan, R.E., Jordan, R.C. and Hu, J.W. Development of an orofacial model of acute inflammation in the rat. Arch Oral Biol 1992;37:417-22.

Hu, J.W. Response properties of nociceptive and non-nociceptive neurons in the rat's trigeminal subnucleus caudalis (medullary dorsal horn) related to cutaneous and deep craniofacial afferent stimulation and modulation by diffuse noxious inhibitory controls. Pain 1990;41:331-45.

Ing NH. Steroid hormones regulate gene expression posttranscriptionally by altering the stabilities of messenger RNAs. Biol Reprod 2005;72:1290-6.

Ji RR, Zhang Q, Law PY, Low HH, Elde R, Hokfelt T. Expression of mu-, delta-, and kappa-opioid receptor-like immunoreactivities in rat dorsal root ganglia after carrageenan-induced inflammation. J Neurosci. 1995;15:8156-66.

Kalyuzhny A, Lee NM, Elde R. An opioid binding protein is specifically down-regulated by chronic morphine treatment in dorsal root and trigeminal ganglia. Neuroscience 1995;66:943-9

LeResche, L., Saunders, K., Von Korff, M.R., Barlow, W. and Dworkin, S.F.Use of exogenous hormones and risk of temporomandibular disorder pain. Pain 1997;69:153-60.

Maggi R, Ma ZQ, Pimpinelli F, Maggi A, Martini L. Decrease of the number of opioid receptors and of the responsiveness to morphineduring neuronal differentiation induced by 17beta-estradiol in estrogen receptor-transfected neuroblastoma cells (SK-ER3). Neuroendocrinology 1999;69:54-62.

Moncada S., Higgs A. The L-arginine-nitric oxide pathway. J. Med 1993;329:2002-2012.

Nozaki-Taguchi, N. and Yamamoto, T. Involvement of nitric oxide in peripheral antinociception mediated by kappa- and delta-opioid receptors. Anesth Analg 1998;87:388-93.

Panzica GC, Viglietti-Panzica C, Sica M, Gotti S., Martini M, Pinos H, Carrillo B, Collado P. Effects of gonadal hormones on central nitric oxide producing systems. Neuroscience 2006;138:987-95.

Prevot V, Croix D, Rialas CM, Poulain P, Fricchione GL, Stefano GB, Beauvillain JC. Estradiol coupling to endothelial nitric oxide stimulates gonadotropin-releasing hormone release from rat median eminence via a membrane receptor. Endocrinology 1999;140:652-9.

Puri V, Chandrala S, Puri S, Daniel CG, Klein RM, Berman NE. Ghrelin is expressed in trigeminal neurons of female mice in phase with the estrous cycle. Neuropeptides 2006;40:35-46.

Puri V, Cui L, Liverman CS, Roby KF, Klein RM, Welch KM, Berman NE. Ovarian steroids regulate neuropeptides in the trigeminal ganglion. Neuropeptides 2005;39:409-17.

Rachman IM, Unnerstal JR, Pfaff EW, Cohen RS. Regulation of neuronal nitric oxide synthase mRNA in lordosis-relevant neurons of the ventromedial hypothalamus following short-term estrogen treatment. Brain Res Mol Brain Res 1998;59:105-8.

Rau KK, Caudle RM, Cooper BY, Johnson RD. Diverse immunocytochemical expression of opioid receptors in electrophysiologically defined cells of rat dorsal root ganglia. J Chem Neuroanat 2005;29:255-64.

Rodrigues ARA, Duarte IDG. The peripheral antinociceptive e.ect induced by morphine is associated with ATP-sensitive K+ channels. British Journal of Pharmacology 2000;129:110- 114.

Rosland, J.H. The formalin test in mice: the influence of ambient temperature. Pain 1991;45:211-6.

Roveroni, R.C., Parada, C.A., Cecilia, M., Veiga, F.A. and Tambeli, C.H. Development of a behavioral model of TMJ pain in rats: the TMJ formalin test. Pain 2001;94:185-91.

Schafer MK, Bette M, Romeo H, Schwaeble W, Weihe E. Localization of kappa-opioid receptor mRNA in neuronal subpopulations of rat sensory ganglia and spinal cord. Neurosci Lett 1994;167:137-40.

Scordalakes EM, Shetty SJ, Rissman EF. Roles of estrogen receptor alpha and androgen receptor in the regulation of neuronal nitric oxide synthase. J Comp Neurol 2002;453:336-44.

Shen PJ, Smith AI, Evans RG, Clarke IJ. Effects of ovarian steroids on hypothalamic opioid receptor subtypes in ovariectomized ewes: regional changes in density and affinity. J Endocrinol 1995;145:559-67.

Smith, M.S., Freeman, M.E. and Neill, J.D. The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 1975;96:219-26.

Soares AC, Leite R, Tatsuo MA, Duarte ID. Activation of ATP-sensitive K(+) channels: mechanism of peripheral antinociceptive action of the nitric oxide donor, sodium nitroprusside. Eur J Pharmacol. 2000;400:67-71.

Tershner, S.A., Mitchell, J.M. and Fields, H.L. Brainstem pain modulating circuitry is sexually dimorphic with respect to mu and kappa opioid receptor function. Pain 2000; 85:153-9.

Warren, M.P. and Fried, J.L.Temporomandibular disorders and hormones in women. Cells Tissues Organs 2001;169:187-92.

Weiland NG, Wise PM. Estrogen and progesterone regulate opiate receptor densities in multiple brain regions. Endocrinology. 1990;126:804-8.

Wong A, Dukic-Stefanovic S, Gasic-Milenkovic J, Schinzel R, Wiesinger H, Riederer P, Munch G. Anti-inflammatory antioxidants attenuate the expression of inducible nitric oxide synthase mediated by advanced glycation endproducts in murine microglia. Eur J Neurosci 2001;14:1961-7.

Xia Y, Krukoff TL. Estrogen induces nitric oxide production via activation of constitutive nitric oxide synthases in human neuroblastoma cells. Endocrinology 2004;145:4550-7.

Yallampalli C, Byam-Smith M, Nelson SO, Garfield RE. Steroid hormones modulate the production of nitric oxide and cGMP in the rat uterus. Endocrinology 1994;134:1971-4.

Zimmermann, M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16:109-10.

3. CONCLUSÃO:

Este trabalho sugere que a antinocicepção mediada pelos receptores capa opióides na ATM depende da ativação da via L-Arginina/NO/GMPc tanto em machos como em fêmeas. No entanto, o dimorfismo sexual na antinocicepção mediada pelos receptores capa opióides se deve, pelo menos em parte, pela diminuição da expressão dos receptores capa opióides no gânglio trigeminal induzido pelos hormônios gonadais, especialmente a testosterona.

REFERÊNCIAS BIBLIOGRÁFICAS (*):

Denucci, D.J., Dionne, R.A. and Dubner, R., Identifying a neurobiologic basis for drug therapy in TMDs, J Am Dent Assoc, 127 (1996) 581-93.

Gameiro, G.H., Arthuri, M.T., Tambeli, C.H. and de Arruda Veiga, M.C., Effects of ethanol on deep pain evoked by formalin injected in TMJ of rat, Life Sci, 73 (2003) 3351-61.

Gear, R.W., Gordon, N.C., Miaskowski, C., Paul, S.M., Heller, P.H. and Levine, J.D., Sexual dimorphism in very low dose nalbuphine postoperative analgesia, Neurosci Lett, 339 (2003) 1-4.

Gear, R.W., Miaskowski, C., Gordon, N.C., Paul, S.M., Heller, P.H. and Levine, J.D., The kappa opioid nalbuphine produces gender- and dose-dependent analgesia and antianalgesia in patients with postoperative pain, Pain, 83 (1999) 339-45.

Johansson, A., Unell, L., Carlsson, G.E., Soderfeldt, B. and Halling, A., Gender difference in symptoms related to temporomandibular disorders in a population of 50-year-old subjects, J Orofac Pain, 17 (2003) 29-35.

Krogstad, B.S., Dahl, B.L., Eckersberg, T. and Ogaard, B., Sex differences in signs and symptoms from masticatory and other muscles in 19-year-old individuals, J Oral Rehabil, 19 (1992) 435-40.

Von Korff, M., Dworkin, S.F., Le Resche, L. and Kruger, A., An epidemiologic comparison of pain complaints, Pain, 32 (1988) 173-83.

Xie, G.X., Meuser, T., Pietruck, C., Sharma, M. and Palmer, P.P., Presence of opioid receptor-like (ORL1) receptor mRNA splice variants in peripheral sensory and sympathetic neuronal ganglia, Life Sci, 64 (1999) 2029-37.

(*) De acordo com a norma UNICAMP/FOP, baseadas na norma do Internacional Committe of Medical Journal Editors – Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline

ANEXO 1

Ficha utilizada para quantificar as respostas comportamentais, induzidas pela administração de formalina na região da ATM de ratos, duranto o período de observação de 45 minutos.

DATA: HORÁRIO:

GRUPO:

PESO (g):

	0-3	3-6	6-9	9-12	12-15	15-18	18-21	21-24	24-27	27-30	30-33	33-36	36-39	39-42	42-45
CO															
LC															

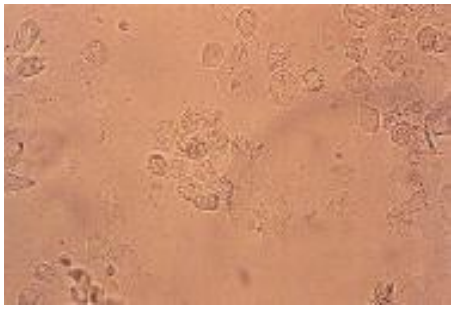
CO: comportamento de coçar a região orofacial (segundos)

LC: comportamento de levantar rapidamente a cabeça (número de vezes)

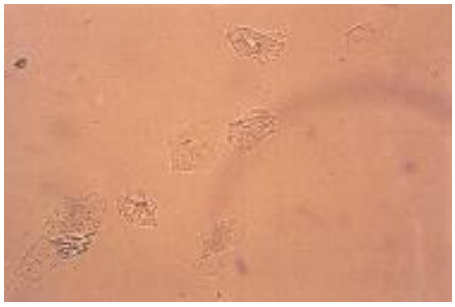
OBSERVAÇÕES:

ANEXO 2

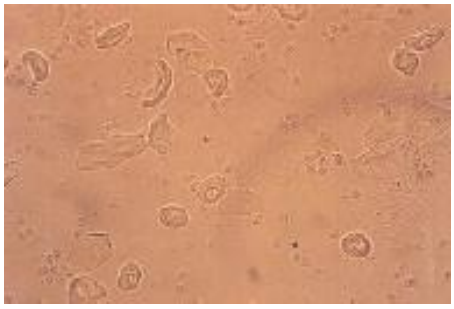
DETERMINAÇÃO DAS FASES DO CICLO ESTRAL:



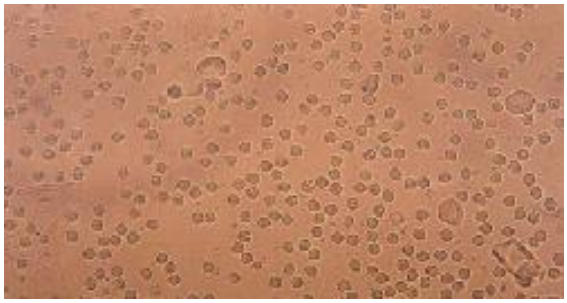
PROESTRO – predomínio de células epiteliais



ESTRO – predomínios de células queratinizadas



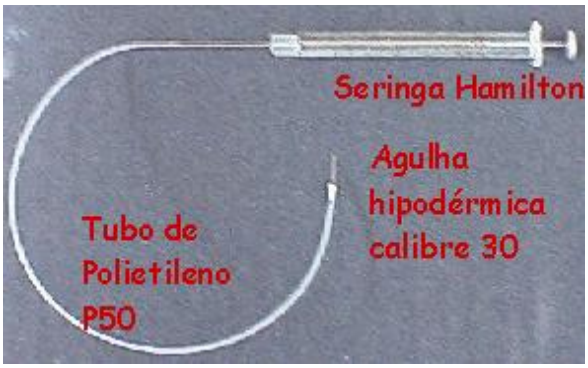
METAESTRO – proporção semelhante dos três tipos celulares



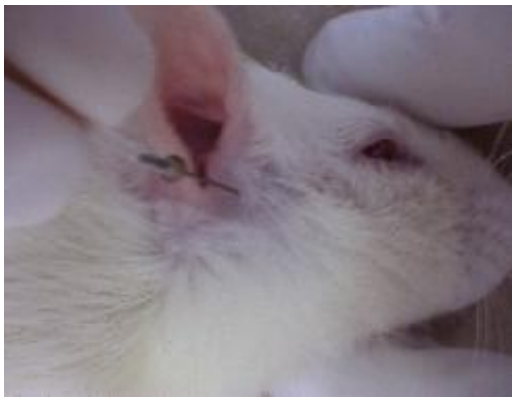
DIESTRO – predomínio de leucócitos

Imagens obtidas através de microscópio óptico, num aumento de 10 vezes.

ANEXO 3

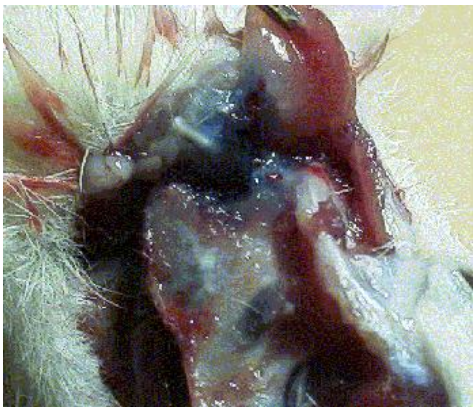


Agulha hipodérmica calibre 30 conectada a uma seringa Hamilton de 50μl, através de uma cânula de polietileno P50, utilizada para administração de drogas na região da ATM do animal.



Administração da droga na ATM do animal. Posicionamento da agulha em relação à cabeça do animal.

CONTADOR DE CÉLULAS – utilizado para quantificar o número de vezes que o animal levanta reflexamente a cabeça.
CRONÔMETRO – utilizado para quantificar o tempo, expresso em segundos, que o animal coça a região orofacial.



Identificação visual do sítio de injeção, de acordo com a aparência do corante Azul de Evans extravasado.

ANEXO 4

Universidade Estadual de Campinas
Instituto de Biologia

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

C E R T I F I C A D O

Certificamos que o Protocolo nº **672-1**, sobre "**MECANISMOS DO DIMORFISMO SEXUAL NA ANALGESIA INDUZIDA POR RECEPTORES OPIÓDES CAPA NA ATM**" sob a responsabilidade de **Profa. Dra. Claudia Herrera Tambeli / Juliana Trindade Clemente** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 06 de Maio de 2004.

C E R T I F I C A T E

We certify that the protocol nº **672-1**, entitled "**MECHANISMS FOR SEXUAL DIMORPHISM IN THE ANALGESIC RESPONSE TO ACTIVATION OF KAPPA OPIOD RECEPTORS IN THE TMJ**", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on May 6, 2004.

Campinas, 06 de Maio de 2004.

Profa. Dra. Liana Verinaud
Presidente - CEEA/IB/UNICAMP

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Secretária - CEEA/IB/UNICAMP

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