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ENVOLVIMENTO DOS RECEPTORES TRPV1, CENTRAIS E PERIFÉRICOS, NA HIPERALGESIA DA ATM INDUZIDA PELA RETIRADA DO ETANOL

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

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PIRACICABA
2008

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FACULDADE DE ODONTOLOGIA DE PIRACICABA
Bibliotecário: Marilene Girello – CRB-8ª. / 6159

Ur8e

Urtado, Marília Bertoldo.
Envolvimento dos receptores TRPV1, centrais e periféricos, na hiperalgesia da ATM induzida pela retirada do etanol. / Marília Bertoldo Urtado. -- Piracicaba, SP : [s.n.], 2008.

Orientador: Maria Cecília Ferraz de Arruda Veiga.
Dissertação (Mestrado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Dor. 2. Articulação temporomandibular. I. Veiga, Maria Cecília Ferraz de Arruda. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

(mg/fop)

Título em Inglês: Involvement of peripheral and central TRPV1 in TMJ hyperalgesia induced by ethanol withdrawal
Palavras-chave em Inglês (Keywords): 1. Pain. 2. Temporomandibular joint
Área de Concentração: Fisiologia Oral
Titulação: Mestre em Odontologia
Banca Examinadora: Maria Cecília Ferraz de Arruda Veiga, Cínthia Pereira Machado Tabchoury, Sílvia Cristina Crepaldi Alves
Data da Defesa: 22-02-2008
Programa de Pós-Graduação em Odontologia

UNICAMP

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

A Comissão Julgadora dos trabalhos de Defesa de Dissertação de MESTRADO, em sessão pública realizada em 22 de Fevereiro de 2008, considerou o candidato MARÍLIA BERTOLDC URTADO aprovado.

PROFa. DRa. MARIA CECILIA FERRAZ ARRUDA VEIGA

PROFa. DRa. SILVIA CRISTINA CREPALDI ALVES

PROFa. DRa. CINTHIA PEREIRA MACHADO TABCHOURY

Dedico esse trabalho à minha mãe Zeza, pelo amor que me sustentou em todos os momentos, ao meu pai Carlos, ao meu irmão Christiano pela dedicação, amizade e atenção dispensada e ao meu filho Nícollas por me ensinar que tudo é possível.

AGRADECIMENTOS

Agradeço primeiramente a Deus por possibilitar-me mais essa conquista.

Agradeço minha orientadora Maria Cecília Ferraz de Arruda Veiga pela excelência profissional, amizade e dedicação sempre indicando a direção a ser tomada em todos os momentos.

Sou grata especialmente a Ângela Scarparo Caldo-Teixeira e a Caroline Morini Calil pela contribuição essencial para o inicio dessa jornada.

Ao amigo Gustavo Hauber Gameiro pela presença constante.

Agradeço a amiga Luana Fischer pela contribuição em vários momentos importantes nessa minha trajetória.

Aos meus amigos Maria Claudia Gonçalves de Oliveira, Juliana Trindade Clemente Napimoga, Mariana Trevisani Arthuri Franco, Rosemary Ferreira, Ana Paula Botelho, Karla Elena Tores Chávez , Priscila Tiemi Kawashita, Vander José das Neves, que, de uma forma ou de outra, contribuíram com sua amizade e com sugestões efetivas para a realização deste trabalho, gostaria de expressar minha profunda gratidão

Aos professores Christiano Bertoldo Urtado, Adriana Pelegrini da Silva, Claudia Herreira Tambeli, Maria Cristina Volpato, Cínthia Pereira Machado Tabchoury, Ana Paula Tanno, Tatiana de Souza da Cunha, Silvia Crepaldi Alvez, que participaram ativamente dessa fase da minha vida contribuindo para a conclusão desse trabalho.

Ao senhor Carlos Alberto Feliciano, agradeço pela ajuda no dia-a-dia do laboratório.

Às secretárias Eliete Rigueto, Maria Elisa dos Santos, Eliana Melo Franco, Érica A. Pinho Sinhoreti e Raquel Q. Marcondes César Sacchi.

Agradeço à FOP-Unicamp e, em especial, o departamento de ciências fisiológicas por me darem oportunidade e todas as condições necessárias para que esse trabalho fosse realizado.

Ao CNPq, pelo auxílio financeiro durante a realização desse trabalho.

E finalmente agradeço com muito carinho meus familiares:

Meus pais Carlos e Zeza, pelo contínuo apoio em todos estes anos, ensinando-me, principalmente, a importância da construção dos meus próprios valores.

Ao meu irmão Christiano sempre pronto, sempre atento, sempre dedicado, agradeço a presença constante.

André, pelo apoio e compreensão pelas minhas horas de ausência.

Meu filho, Nícollas, o meu orgulho e a minha alegria diária.

Meus tios José Primo e Deliza pelas vibrações positivas que me sustentaram nos momentos difíceis.

Minha tia Célia pelo apoio na redação desse trabalho.

Minha Prima Sílvia e meu afilhado Felipe.

E minha querida avó Negrinha por ter sido para mim um exemplo de amor, e força ao longo da sua vida e durante os últimos dias que esteve entre nós.

"A razão cardeal de toda a superioridade humana é sem dúvida a vontade. O poder nasce do querer. Sempre que o homem aplicar a perseverante energia de sua alma a um fim, ele vencerá obstáculos e se não atingir o alvo, pelo menos fará coisas admiráveis."

José de Alencar

RESUMO

O aumento da sensibilidade nociceptiva (hiperalgesia) situa-se entre uma das complicações observadas durante o período de abstinência alcoólica. Entretanto, os mecanismos neurofisiológicos responsáveis por esse fenômeno não são bem conhecidos. Alguns estudos sugerem que o etanol pode potencializar a resposta do receptor Vanilloid tipo 1 (TRPV1) provocada por estímulos nociceptivos. Diante disso, o objetivo deste trabalho foi investigar o envolvimento dos receptores TRPV1, periféricos e centrais, na hiperalgesia induzida pelo teste da formalina na articulação temporomandibular (ATM) em ratos submetidos à ingestão crônica e à retirada do etanol. A retirada do etanol foi testada 12 horas após suspensão da ingestão crônica (etanol 6,5 %, por 10 dias). Neste grupo, foi observado um aumento das respostas nociceptivas ao teste da formalina na ATM. O envolvimento dos receptores TRPV1 periféricos nesta hiperalgesia foi avaliado com um antagonista TRPV1, a capsazepina (CPZ), administrada na ATM 30 minutos antes do teste da formalina. A CPZ reduziu significativamente a hiperalgesia induzida pela retirada do etanol, porém este efeito anti-hiperalgésico não foi observado nos animais que não beberam etanol. Quando administrada centralmente (injeção subaracnóide), o efeito anti-hiperalgésico da CPZ também foi observado nos animais submetidos à retirada do etanol. Estes resultados sugerem que a sensibilização periférica e central dos receptores TRPV1 contribui para a hiperalgesia da ATM induzida pela retirada do etanol.

Palavras-chave: Etanol, Articulação temporomandibular, Dor, TRPV1, Capsazepina, Formalina.

ABSTRACT

The increase in nociceptive sensitivity (hyperalgesia) is one of the complications observed during the alcohol abstinence period. However, the neurophysiological mechanisms responsible for this phenomenon are not well known. Some studies suggest that ethanol can potentiate the response of vanilloid receptor type 1 (TRPV1) to nocive stimuli. Thus, the aim of this study was investigate the involvement of peripheral and central TRPV1 receptors on hyperalgesia induced by the TMJ formalin test, in rats submitted to chronic ingestion and ethanol withdrawal. Ethanol withdrawal was tested 12 h after the suspension of chronic ingestion (ethanol 6.5 %, for 10 days). In this group, increased nociceptive responses were observed in the TMJ formalin test. The involvement of peripheral TRPV1 receptors in this hyperalgesia was evaluated by an TRPV1 antagonist, the capsazepine (CPZ), administrated on TMJ 30 min before the formalin test. CPZ significantly reduced the hyperalgesia induced by ethanol withdrawal, but this anti-hyperalgesic effect was not observed in animals that did not drink ethanol. When centrally administrated (subarachnoid injection), the anti-hyperalgesic effect of CPZ was also observed in animals submitted to ethanol withdrawal. These results suggest that peripheral and central sensitization of TRPV1 receptors contribute to the TMJ hyperalgesia induced by ethanol withdrawal.

Keywords: Ethanol, Pain, TRPV1, Temporomandibular joint, Capsazepine, Formalin.

SUMÁRIO

I. INTRODUÇÃO

Atualmente a dor não pode mais ser considerada simplesmente uma sensação que reflete os aspectos sensório-discriminativos de um estímulo nociceptivo, tais como: qualidade, intensidade, localização e duração (Dubner, 1978). Hoje, a dor é conceituada como uma experiência multidimensional que pode ser modificada por influências cognitivas, emocionais e motivacionais, relacionadas à vida das pessoas e às suas experiências pregressas de dor (Sessle, 1986). A dor, portanto, não está ligada somente a um fenômeno sensorial envolvendo eventos neurais periféricos provocados por uma lesão física identificável, mas com a interação desses eventos com o sistema nervoso central.

As terminações nervosas livres encontradas nos tecidos craniofaciais como pele, mucosa oral, ATM, polpa dental, periósteo, periodonto e músculos, suprem a base periférica para a dor. Muitas terminações nervosas livres atuam como nociceptores que são órgãos sensoriais especializados na detecção de estímulos nociceptivos. A ativação desses nociceptores pode resultar na excitação da fibra nervosa aferente com a qual eles estão associados e na transmissão de informações sensoriais e discriminativas sobre as características espacial e temporal de um estímulo nociceptivo, ao sistema nervoso central (Sessle, 2000). As fibras envolvidas nesse processo são do tipo A-delta (mielinizadas) e fibras-C (não mielinizadas). As primeiras transmitem a sensação de dor rápida e bem localizada enquanto as segundas

transmitem a sensação dolorosa de queimação, difusa e pobremente localizada (Sessle, 1986).

Ao ocorrer lesão tecidual, como por exemplo na ATM, uma série de respostas são desencadeadas pelo organismo por intermédio da liberação ordenada de diversas classes de mediadores inflamatórios (histamina, bradicinina, substância P, prostaglandinas, serotonina, entre outras), esses mediadores podem ativar ou sensibilizar os nociceptores. A ativação do nociceptor pode levar à despolarização da membrana com condução do sinal até o Sistema Nervoso Central (SNC). Na sensibilização nociceptores que normalmente são insensíveis a estímulos nociceptivos, nociceptores silenciosos, se tornam ativos, o que leva a ocorrência de quadros clínicos de hiperalgesia e alodinia (Coderre *et al*., 1993). Esses mediadores serão capazes, então, de promover uma série de alterações neuroplásticas periféricas e centrais (Dray, 1995; Swift *et al*., 1998) e essas alterações neuroplásticas podem sofrer influência do etanol.

Embora a literatura especializada sobre as ações do etanol no organismo esteja repleta de controvérsias, em relação à dor a maioria dos autores concorda que o etanol pode provocar alterações nos sistemas nociceptivos (Jorgensen *et al*., 198; Pohorecky & Shah, 1987). O etanol foi amplamente utilizado para o alívio da dor nos tempos antigos, embora os seus mecanismos de ação não sejam ainda bem compreendidos. Ele afeta vária funções do sistema nervoso central, podendo provocar analgesia, sedação, hipnose, distúrbio motor, distúrbio de memória, confusão, neurodegeneração e/ou dependência (Deitrich et al., 1989; Fadda & Rossetti, 1998). Estudos

sugerem que a administração crônica de etanol 6,5% por 10 dias causa antinocicepção do segundo ao sexto dia; tolerância do oitavo ao décimo dia e hiperalgesia de 6 a 12 horas após sua retirada (Gatch, 1999; Gatch & Lal, 1999, Gameiro et al., 2003). Os mecanismos pelos quais o etanol produz analgesia ainda não estão claros; de fato, vários mecanismos podem estar envolvidos. Tem sido demonstrado que o etanol possui propriedades antagônicas sobre receptores excitatórios N-Methil-D-Aspartato (NMDA) (Lovinger et al., 1989) e propriedades agonistas sobre receptores inibitórios Ácido Gama Amino Butírico (GABA) (Mihic, 1999) o que leva a supor uma possível inibição da transmissão e percepção da sensação dolorosa pelo etanol. Porém, a ingestão crônica e a retirada do etanol desencadeiam respostas neuroadaptativas compensatórias o que pode levar a quadros de tolerância e hiperalgesia, respectivamente. Muitos estudos relacionam esse estado de tolerância a um aumento na atividade (up- regulation) dos receptores NMDA e a diminuição da atividade (down-regulation) dos receptores GABA, e a hiperalgesia, observada na síndrome de abstinência, à um realce nos mecanismos de adaptação.

A síndrome da abstinência é um importante componente do alcoolismo e inclui hipersensibilidade diante de estímulos nocivos e não nocivos (West & Gossop, 1994; Gossop *et al*., 2002). Os mecanismos que envolvem o desconforto físico induzidos pela retirada do etanol não são bem entendidos. Existem algumas evidências de que a hiperalgesia pode ser induzida pela suspensão do etanol administrado cronicamente (Gatch, 1999, 2002; Gatch & Lal, 1999; Gatch & Selvig, 2002; Gameiro *et al*., 2003), mas a

base neurológica para o aumento da sensibilidade nociceptiva induzida pela retirada do etanol não é bem entendida.

Trevesani *et al.*,(2002) demonstraram que o etanol estimula a liberação de citocinas e subseqüente extravasamento plasmático pela ativação do receptor TRPV1 (transient receptor potencial vanilloid) e sugerem também que o etanol potencializa a resposta do TRPV1 para capsaicina, alterações de pH e calor nocivo (34 a 42°C).

O TRPV1 foi confirmado como um canal iônico não seletivo, depois de ser clonado em ratos (Caterina *et al.*, 1997) e em humanos (Hayes *et al.*, 2000; McIntyre *et al.*, 2001). Estudos "*in vitro"* têm demonstrado que o TRPV1 pode ser ativado por calor nocivo (Caterina *et al.*, 1997), baixo pH (Tomiaga *et al.*,1998), substâncias endógenas pró inflamatórias (Szallasi *&* Blumberg, 1999), produtos da lipoxigenase (Caterina & Julius, 2001) e também por uma variedade de compostos derivados de plantas, incluindo a capsaicina, um componente 'ardente' das pimentas vermelhas. Esse receptor é encontrado principalmente em fibras nociceptivas periféricas e centrais do corno dorsal da medula espinhal e gânglio trigeminal (Guo *et al.*, 1999; Carlton & Coggeshall, 2001; Valtschanoff *et al.*, 2001; Pei *et al.*, 2007).

Uma vez sensibilizado, o receptor TPRV1 possibilita o influxo de íons, predominantemente íons cálcio (Marsh *et al.*, 1987; Wood *et al.,* 1988), e esse influxo é propagado através da fibra e pode ser traduzido como dor pelo sistema nervoso central (Holzer, 1991). De fato, esse não é o único mecanismo pelo qual a informação nociceptiva é transmitida do neurônio de primeira para o de segunda ordem, no corno dorsal da medula espinhal. Provavelmente

neurotransmissores tais como glutamato, ATP, e uma variedade de outros neuropeptídeos estão envolvidos nesse processo (Holzer, 1991; Lundberg, 1996).

A capsazepina, um antagonista TRPV1, tem sido usada para reverter a hiperalgesia em modelos de dor crônica e inflamatória (Walker *et al.,* 2003). Kwak *et al.* (1998) demonstraram que a administração de capsazepina, reduz a expressão de neuropeptídeos inflamatórios induzida pela capsaicina, agonista TRPV1, e pela formalina e, ainda, que a redução dos neuropeptídeos inflamatórios induzida pela formalina é menor. Em adição, Caterina *et al.,* (2000) sugerem que a sensibilização do TRPV1 não é essencial para a resposta nociceptiva à formalina.

Uma ampla extensão de dores crônicas somáticas, visceral e urogenital, são associadas com o aumento da expressão de TRPV1 (Chan *et al*., 2003; Matthews *et al*., 2004; Kanai *et al.,* 2005). Em relação à região orofacial, Pei *et al*. (2007) demonstraram que o óleo de turpentine, um irritante inflamatório, injetado na região facial de ratos induziu hiperalgesia frente a estímulos como calor e frio nocivos, com o aumento da expressão de TRPV1 em neurônios do gânglio trigeminal, fibras nervosas periféricas e projeções centrais do subnúcleo caudal. O mecanismo que regula a expressão gênica do TRPV1 envolvida em condições patológicas não é conhecido, mas várias substâncias endógenas e exógenas podem exercer um importante papel nesse processo (Cortright & Szallasi, 2004). Acredita-se que substâncias tais como bradicininas (Chuang *et al*., 2001), nicotina (Liu *et al*., 2004) e o etanol (Trevisani *et al*., 2002) estejam envolvidas na sensibilização e/ou ativação direta do TRPV1.

Considerando a relação existente entre o etanol e processos dolorosos e também a capacidade do etanol em alterar a percepção e resposta à dor, estudos sobre os mecanismos das alterações nociceptivas induzidas pelo etanol e o envolvimento do TRPV1 nas dores relacionadas com tecidos profundos tornam-se relevantes para a pesquisa sobre a etiologia das desordens temporomandibulares.

Com base no exposto, o presente trabalho tem como objetivo verificar o envolvimento dos receptores TRPV1 periféricos e centrais, na hiperalgesia induzida pelo uso crônico e retirada do etanol em animais.

Capítulo 1

Involvement of peripheral TRPV1 in TMJ hyperalgesia induced by ethanol withdrawal

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Abstract

Ethanol withdrawal increases nociception after the injection of formalin into the rat's temporomandibular joint (TMJ). Little is known about the neurological basis for hyperalgesia induced by ethanol withdrawal, but it has been reported that ethanol can potentiate the response of transient receptor potential vanilloid receptor-1 (TRPV1) in superficial tissues. The present study was designed to test the hypothesis that peripheral TRPV1 could be involved on nociceptive behavioral responses induced by the injection of formalin into the TMJ region of rats exposed to chronic ethanol administration and ethanol withdrawal. Behavioral hyperalgesia was verified 12 hr after ethanol withdrawal in rats that

dorsal root ganglia as well as TRPV1-expressing cells responded to ethanol in a concentration dependent and capsazepine-sensitive fashion (Trevisani et al., 2002). Ethanol potentiated the response of TRPV1 to capsaicin, protons and heat and lowered the threshold for heat activation of TRPV1 from 42°C to 34°C (Trevisani et al., 2002). This provides a likely mechanistic explanation for the ethanol sensory responses that occur at body temperature and for the sensitivity of inflamed tissues to ethanol, such as those that might be found in asthma, esophagitis, neuralgia or wounds (Trevisani et al., 2002; Trevisani et al., 2004). Capsaicin or formalin induced Fos expression was reduced in both cases by pretreatment with capsazepine, but to a much lesser extent for formalin (Kwak et al., 1998).

To date, there are no studies about the peripheral effect of ethanol on hyperalgesia induced by ethanol withdrawal, associated with pain response evoked by deep nociceptive stimuli. Considering that the nociceptive behavioral responses elicited by the injection of formalin into the TMJ represent a valid and reliable model of deep orofacial pain (Roveroni et al., 2001), the aim of this study was to evaluate the involvement of peripheral TRPV1 on pain responses induced by the TMJ formalin test in rats exposed to chronic ethanol administration and ethanol withdrawal.

Methods

Subjects and housing

Male Wistar rats (obtained from Centro Multi – institucional de Bioterismo-Cemib, Unicamp, Campinas, Brazil), weighing 200 – 300 g, were housed

drank an ethanol solution (6.5%) for 10 days. In another group submitted to the same ethanol regimen, the selective vanilloid receptor antagonist capsazepine (300, 600 or 1200 µg/ 25 µl) or an equal volume of vehicle were injected into the TMJ regions 30 min before the TMJ formalin test. The local injections of capsazepine reduced the increased nociceptive responses induced by ethanol withdrawal. The effect of capsazepine on rats that did not drink ethanol was not significant. These results indicate that the peripheral TRPV1 can contribute to the hyperalgesia induced by ethanol withdrawal on deep pain conditions.

Keywords: Ethanol; TRPV1; Pain; Temporomandibular joint

Introduction

Ethanol-induced analgesia has been reported in both humans (Cutter and O'Farrel, 1987; Wolff et al., 1942; Woodrow and Eltherington, 1988) and animals (Bass et al., 1978; Brick et al., 1976; Jorgensen et al., 1985; Pohorecky and Shah, 1987; Yirmiya and Taylor, 1989). Research has shown that chronic exposure to ethanol (6.5% w/v) in liquid diet for 10 days resulted in antinociception from day 2 to day 6, development of tolerance on days 8-10, and marked hyperalgesia 6-12 h after withdrawal of ethanol (Gatch, 1999; Gatch and Lal, 1999). The syndrome of withdrawal is an important component of alcoholism, and includes hypersensitivity to both noxious and normally innocuous stimuli (hyperalgesia and allodynia) (West and Gossop, 1994; Gossop et al., 2002). Although the anxiogenic effects of ethanol withdrawal

have been well studied (Gatch et al., 2000; Becker, 2000), the mechanisms underlying the physical discomfort induced by ethanol withdrawal are not understood.

There are some evidences that behavioral hyperalgesia can be induced after the suspension of chronic ethanol administration (Gatch, 1999, 2002; Gatch and Lal, 1999; Gatch and Selvig, 2002; Gameiro et al., 2003), but the neurological basis for hyperalgesia induced by ethanol withdrawal is not fully explained. Trevisani et al. (2002) reported that ethanol mediated the modulation of vanilloid receptor type 1 (TRPV1) function. Ethanol (0.1–3%) caused the potentiation of TRPV1 activity produced by vanilloids, anandamide, protons or heat. The TRPV1 is a pivotal molecular integrator of noxious stimuli that is expressed on somatic and autonomic primary afferent neurons, and has been confirmed as a ligand-gated ion channel after its cloning from rat (Caterina et al.,1997) and human (Hayes et al., 2000; McIntyre et al., 2001). The TRPV1 is responsible for burning sensation elicited by capsaicin (Trevisani et al., 2002), the pungent component of hot peppers, which causes immediate burning pain or nociceptive reflexes (LaMotte et al., 1991).

Capsaicin analogues, such as resiniferatoxin, a potent TRPV1 agonist, and capsazepine, a specific competitive TRPV1 antagonist, have been discovered (Bevan et al., 1992) and the role of ethanol in nociception has been predicted. Previous research has proved that ethanol activates primary sensory neurons, resulting in neuropeptide release or plasma extravasation in the esophagus, spinal cord or skin (Trevisani et al., 2002). Sensory neurons from trigeminal or

individually with free access to food and drink solutions (water or ethanol). Body weight was determined daily, and the amount of fluid consumption per cage was recorded. They were maintained in a temperature-controlled room (23 ± 1°C) with a 12/12 light/dark cycle (lights on at 7:00 AM) for at least 1 week prior to the experiments. The study was conducted in accordance with the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

Ethanol administration: drinking

Rats (N=6/group) were given either an ethanol solution, as their sole drinking solution, or tap water for 10 days. The ethanol drinking solution was mixed to a 6.5% concentration using 99.5% ethanol and tap water (Bell et al., 1998; Gameiro et al., 2003). Pretreatment was carried out in the animals' home cages.

Ethanol withdrawal

To assess the effects of ethanol withdrawal, rats were submitted to the TMJ formalin test 12 hr after removal of the ethanol solutions (given for 10 days). The 12 hr time point was chosen because a large degree of nociception was seen at that time point in early experiments and to provide data directly comparable with other ethanol-withdrawal research (Lal et al., 1991; Wallis et al., 1995, Gameiro et al., 2003).

Testing procedure for TMJ pain

The design of this study follows the design used by Roveroni et al., 2001. Testing sessions took place between 08:00 and 13:00 hr in a quiet room

maintained at 23 ± 1ºC. Each animal was first placed in a test chamber (30 X 30 X 30 cm mirrored- wood chamber with glass at the front side) for a 30 min habituation period to minimize stress (Abbot et al., 1986). After the period of adaptation, the animal was removed from the test chamber and lightly anesthetized by inhalation of halothane to allow the TMJ injection.

Rats received 50 µl injection of diluted formalin (1.5%) into the left TMJ region. The injections were performed via a 30-gauge needle introduced into the TMJ capsule. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 µl) previously filled with formalin 1.5%.

Following the injection of formalin into TMJ region, the rat was returned to the test chamber and nociceptive behavioral responses characterized by rubbing the orofacial region (amount of time-seconds) and flinching the head (number of head flinches) were quantified for 45 min (15 blocks of 3 min). Considering that the flinching of the head behavior followed a uniform pattern of a 1s in duration, each flinching was expressed as 1s. The combination (sum) of both behaviors provides a better measure of pain intensity than any single behavior (Roveroni et al., 2001). An investigator who was blind to the rat's group assignment made the observation of the behaviors.

After the conclusion of each experiment, Evans blue dye (0.1% 5mg/Kg) was injected systemically in order to confirm the TMJ injection side at post-mortem, as previously described (Hass et al., 1992) by the visual examination of formalin-induced plasma extravasation of Evans blue dye bond to plasma protein.

***Testing the involvement of peripheral* TRPV1**

The capsazepine, a TRPV1 antagonist that has been shown to competitively inhibit capsaicin-mediated responses (Bevan et al., 1992; Urban and Dray, 1991; Lou and Ludberg, 1992; Fox et al., 1995) was used to evaluate the involvement of peripheral TRPV1 on nociception induced by the TMJ formalin test.

Rats received a 25 µl injection of diluted capsazepine (300 µg, 600 µg, 1200 µg) or vehicle (saline containing 50% dimethylsulphoxide – Kwak et al., 1998) 30 min before (Walker et al., 2003) a 50 µl injection of diluted formalin (1.5%) into the left TMJ region. The injections were performed via a 30-gauge needle introduced into the TMJ capsule. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 µl) previously filled with capsazepine.

Statistical analysis

The sum of rubbing and flinching responses exhibited by each animal was computed. The data were analyzed by One Way ANOVA followed by Tukey or Student-Newman-Keuls post-hoc tests, as appropriate. A p value of <0.05 was considered statistically significant. All statistical analyses were performed using SIGMA STAT version 3.0 for Windows.

Results

The injection of formalin (FOR) into the TMJ induced a significant degree of hyperalgesia in rats when the nociceptive behaviors were evaluated 12 hr after removal of the ethanol solutions (6.5%-given for 10 days). The increase of

nociceptive behavioral responses was highly statistically significant ($F_{6.38}$ = 22.91, p< 0.001, One way ANOVA + Tukey) when the FOR withdrawal group was compared with the FOR water group and the FOR ethanol group (Fig. 1).

Effets of capsazepine

The local injection of capsazepine in all doses tested induced a significant reduction of hyperalgesia in rats exposed to ethanol withdrawal as describes above. The decrease of nociceptive behavioral responses was statistically significant when the FOR group and vehicle + FOR group were compared with the capsazepine 300, 600 and 1200 µg groups ($F_{6.38}$ = 22.91, p < 0.001, One way ANOVA + Tukey, Fig. 1).

The effect of the highest dose of ipsilateral capsazepine (1200 µg) on animals that drank only water was not statistically significant (p=0.886, One Way ANOVA + Student-Newman-Keuls, Fig. 2). Also, the contralateral administration of capsazepine 1200 µg did not reduce the hyperalgesia observed in rats submitted to ethanol withdrawal (p=0.729, One Way ANOVA + Student-Newman-Keuls, Fig. 2).

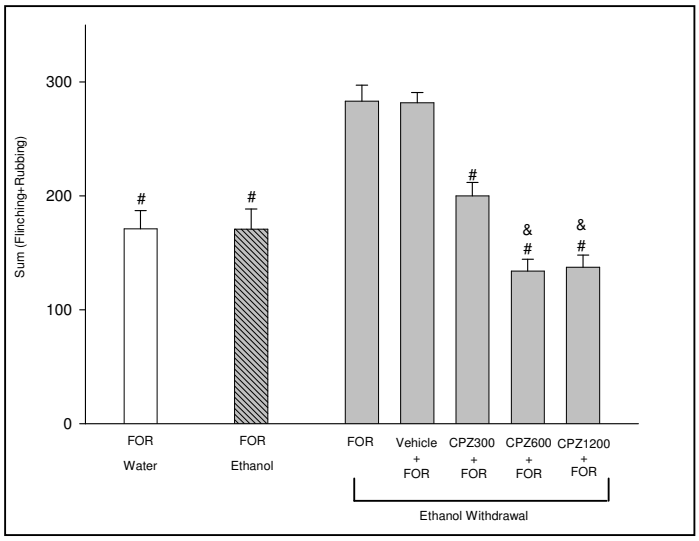

FIG 1. Sum of nociceptive responses to formalin TMJ injection in the following groups: rats that drank water (column1); rats that drank ethanol (column 2); rats exposed to ethanol withdrawal treated with formalin alone (colunm 3), vehicle + formalin (column 4), capsazepine 300 µg + formalin (column 5), capsazepine 600 µg + formalin (column 6) and capsazepine 1200 µg + formalin (column 7). $^{\#}$indicates significant differences in relation to formalin and in relation to vehicle + formalin (in ethanol withdrawal groups). $^{\&}$indicates significant differences in relation to CPZ 300µg + formalin (in ethanol withdrawal groups). FOR: formalin; CPZ: capsazepine; vehicle: dimethylsulphoxide 50%.

Discussion

The present study evaluated the peripheral effect of ethanol on hyperalgesia induced by ethanol withdrawal in rats submitted to the TMJ formalin test. The results suggest that hyperalgesia evoked by ethanol withdrawal could be mediated, at least in part, by peripheral mechanisms through the increased responses of peripheral TRPV1 within the TMJ. The fact that the local administration of capsazepine, a TRPV1 antagonist, reverted the hyperalgesia

caused by ethanol withdrawal in animals submitted to the TMJ formalin test support this idea. The results indicate that ethanol could mediate a hyper-responsiveness of TRPV1 that might play an important role in the development of some symptoms of ethanol withdrawal, especially in the modulation of nociceptive effects related to ethanol suspension.

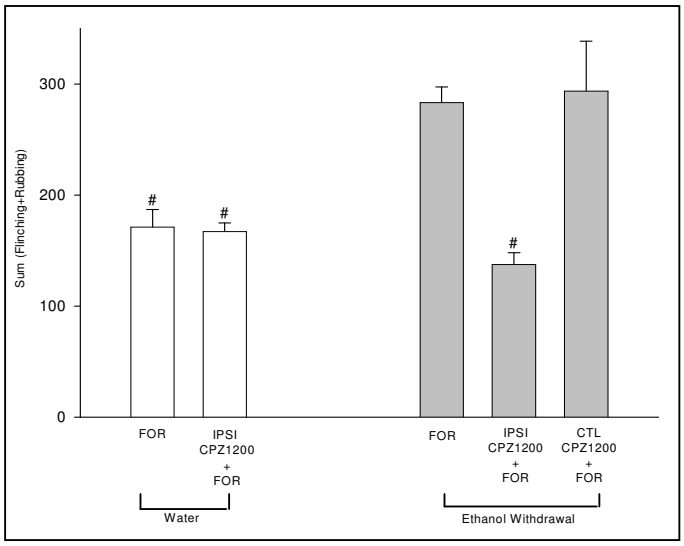

FIG 2. Sum of nociceptive responses to formalin TMJ injection in rats that drank water treated with formalin alone (column1) or with ipsilateral capsazepine 1200µg + formalin (column2). In the ethanol withdrawal groups, rats were treated with formalin alone (column3), ipsilateral capsazepine 1200µg + formalin (column4) and contralateral capsazepine 1200 µg + formalin (column5). #indicates significant differences in relation to formalin and in relation to contralateral capsazepine 1200µg + formalin (in ethanol withdrawal groups). FOR: formalin; IPSI CPZ: capsazepine ipsilateral; CTL CPZ: capsazepine contralateral.

These results are in agreement with a previous study reporting that ethanol stimulated the release of tachykinins, and subsequent plasma extravasation via TRPV1 activation. In trigeminal ganglion neurons, ethanol caused a

concentration-dependent increase in cytosolic calcium ion concentration, which was inhibited by capsazepine (Trevisani et al., 2002). Vanilloid-operated nonspecific cation channel opens the channel pore and leads to cation, predominantly calcium influx (Marsh et al., 1987; Wood et al., 1988). It is propagated along the entire length of the vanilloid-sensitive neuron and may be perceived as itch or pain the in the central nervous system (Holzer, 1991). It is not exactly known how the painful information is transmitted from the central terminals of the vanilloid-sensitive neurons to second-order neurons of the dorsal horn. The possible neurotransmitters involved in this process are glutamate, ATP, and a variety of other neuropeptides (Holzer, 1991; Lundberg, 1996).

Although little is known about the neurological basis for hyperalgesia induced by ethanol withdrawal (Gatch, 1999, 2002; Gatch and Lal, 1999; Gatch and Selvig, 2002), some forms of hyperalgesia are due in part to central sensitization in spinal nociceptive pathways (Bridges et al., 2001), including hyperalgesia associated with opioid withdrawal (Mao and Mayer, 2001). Withdrawal hyper-responsiveness is an apparent increase in excitability dependent on ethanol exposure and appearing during ethanol removal. It is not clear whether the increase in excitability develops during the presence of ethanol and it is only revealed when the depressant effects are removed, or whether it develops as a consequence of the disappearance of ethanol. Moreover, few experimental studies (Caterina et al., 1997; Hayes et al., 2000; Trevisani et al., 2002) have examined the peripheral effect of ethanol in the development of some symptoms of ethanol withdrawal.

The TRPV1 channels have been implicated in pain detection, making them prime therapeutic marks for target-oriented novel analgesic molecules. An added complication is the finding that bradykinin (and maybe also other alogogenic- proinflammatory mediators) recruit normally vanilloid-insensitive sensory neurons to respond to capsaicin and/or low pH (Stucky et al., 1998). As a consequence, inflamed tissue may contain an increase number of nociceptors that could contribute to hyperalgesia via spatial summation on spinal neurons. Kwak et al. (1998), assessing cells exhibiting Fos immunohistochemistry and the paw-withdrawal test in rats, showed that intradermal injection of capsazepine significantly reduced the number of cells exhibiting Fos-like immunoreactivity and significantly increased the paw-withdrawal latency. Moreover, capsaicin or formalin induced Fos expression was reduced in both cases by pretreatment of capsazepine, but to a much lesser extent for formalin (Kwak et al., 1998). Santos and Calixto, 1997 showed that capsazepine prevented the early phase of formalin-induced mechanical hyperalgesia when administered intradermally in mice and Kwak et al., 1998 showed that capsazepine inhibited carageenan-induced inflammatory hyperalgesia in the rat paw (Kwak et al., 1998). In contrast to these findings, our results showed that capsazepine alone did not reduce the nociceptive behaviors evoked by injection of formalin into the TMJ. It is important to point out that deep pain conditions differ from the one evoked by superficial nociceptive stimuli (Sessle and Hu, 1990; Sessle, 2002). Therefore, the absence of capsazepine-induced analgesia in our model may be related to the different site of formalin injection.

Nociceptive transmission and modulation are different even when distinct superficial nociceptive assays are used (Fang and Proudfit, 1998).

In conclusion, the results of this study showed a peripheral effect of ethanol withdrawal on nociception evoked by deep tissue injury. This conclusion was based in the fact that the local injection (ipsilateral) of capsazepine into the TMJ reduced the increased nociceptive responses induced by ethanol withdrawal, while the contralateral injection of the highest dose of capsazepine had no effect on TMJ hyperalgesia. These findings indicate that TRPV1 receptors are present in the TMJ and they could be sensitized by ethanol administration, since the TMJ hyperalgesia was observed even after the suspension of the ethanol regimen. Further research is now necessary to investigate if alcoholism could represent a contributing factor to TMJ pain in the clinical setting.

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Capítulo 2

Central TRPV1 receptors modulate the TMJ hyperalgesia induced by ethanol withdrawal

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Abstract

Ethanol withdrawal can lead to TMJ hyperalgesia in the formalin test due to peripheral changes in TRPV1 receptors. However, the role of central TRPV1 in this model should also be investigated. It is known that central TRPV1 receptors play a differential role in the effect of TRPV1 antagonists in various types of pain. The present study evaluated the central effect of the selective vanilloid receptor antagonist capsazepine (CPZ) on nociceptive behavioral responses induced by the injection of formalin into the TMJ region of rats

exposed to chronic ethanol administration and ethanol withdrawal. Behavioral hyperalgesia was observed 12 hr after ethanol withdrawal in rats that drank an ethanol solution (6.5%) for 10 days. To test the involvement of central TRPV1 receptors in this phenomenon, a method for direct subarachnoid drug delivery to the medullary dorsal horn region of rats, without introducing a catheter, was used. This method provides a reliable evaluation of nociceptive responses that require normal neck muscle activity to occur, such as those measured in this study. The subarachnoid injections of CPZ (92 µg/ 10 µl) or an equal volume of vehicle were performed 30 min before the TMJ formalin test in rats exposed to ethanol withdrawal as described above. The subarachnoid injection of CPZ, but not vehicle, reduced the increased nociceptive responses induced by ethanol withdrawal. In conclusion, these findings support the hypothesis that central TRPV1 receptor sensitization plays a significant role in the TMJ hyperalgesia induced by ethanol withdrawal.

Keywords: Ethanol; TRPV1; formalin; temporomandibular joint; hyperalgesia

Introduction

The transient receptor potential vanilloid 1 (TRPV1) receptor, previously known as the vanilloid receptor 1 (VR1), is a non-selective cation channel activated by a range of noxious stimuli, including heat, low pH, capsaicin and some endogenous substances known to be associated with tissue inflammation, particularly lipoxygenase products (Caterina and Julius, 2001). TRPV1 receptors are mainly expressed by nociceptive neurons in peripheral

and central processes of dorsal root and trigeminal ganglia (TG) (Guo et al., 1999; Carlton and Coggeshall, 2001; Valtschanoff et al., 2001, Pei et al., 2007).

In the brain, TRPV1 receptors have been identified in various regions known for their role in pain transmission or modulation (Mezey et al., 2000; Szabo et al., 2002; Roberts et al., 2004). However, there is increasing evidence that TRPV1 receptor is expressed in various cell types throughout the body, including bladder epithelial cells (Inoue et al., 2002), keratinocytes (Southall et al., 2003), epithelial cells lining human airways (Agopyan et al., 2003), dental pulp (Kim et al., 2006) and peripheral blood mononuclear cells (Saunders et al., 2007), as well as in skin (Miyamoto et al., 2005) and synovial fibroblasts (Engler et al., 2007). The wide tissue distribution of TRPV1 receptors expresses a previously unsuspected level of complexity in TRPV1 regulation in both physiological and pathological conditions.

A wide range of somatic, visceral and urogenital chronic pain conditions are associated with increased peripheral expression of TRPV1 (Chan et al., 2003; Matthews et al., 2004; and Kanai et al., 2005). Regarding the orofacial region, Pei et al. (2007) showed that the inflammatory irritant turpentine oil injected into rat facial area induced hyperalgesia to noxious heat and cold stimuli, with an increased TRPV1 expression in the neurons of the TG, peripheral nerve fibers in the vibrissal pad, and central projection processes in the trigeminal sensory nuclei caudalis. The mechanisms that regulate TRPV1 gene expression under pathological conditions are unknown, but various important endogenous as well as exogenous substances might play a role in this process (Cortright and Arpad Szallasi, 2004). Sensitization (and/or direct activation) by kinases and

desensitization by phosphatases are believed to involveTRPV1 in the mechanism of action of important substances such as bradykinin (Chuang et al., 2001), nicotine (Liu et al., 2004) and ethanol (Trevisani et al., 2002).

Trevisani et al. (2002) reported that ethanol potentiate the response of TRPV1 to capsaicin, protons and heat, lowering the threshold for heat activation of TRPV1 from 42ºC to 34ºC. Recently, a research conducted in our laboratory showed the involvement of peripheral TRPV1 on pain responses induced by the TMJ formalin test, in rats previously exposed to chronic ethanol administration and ethanol withdrawal (Urtado et al., 2007). The results of this study indicated that TRPV1 could be sensitized by ethanol administration, since the local injection (ipsilateral) of capsazepine into the TMJ reduced the increased nociceptive responses induced by ethanol withdrawal, while the contralateral injection of the highest dose of capsazepine had no effect on TMJ formalin-induced hyperalgesia (Urtado et al., 2007). Although this previous study provided support to the peripheral involvement of TRPV1 on TMJ hyperalgesia induced by ethanol withdrawal, the possible role of central TRVP1 in this model should also be investigated

On this background, the aim of this study was to evaluate the involvement of central TRPV1 on pain responses induced by the TMJ formalin test in rats exposed to chronic ethanol administration and ethanol withdrawal.

Methods

Subjects and housing

Male Wistar rats (obtained from Centro Multi – institucional de

Bioterismo-Cemib, Unicamp, Campinas, Brazil) weighing 200 – 300g were housed individually with free access to food and drink solutions (water or ethanol). Body weight was determined daily, and the amount of fluid consumption per cage was recorded. They were maintained in a temperature-controlled room (23 ± 1°C) with a 12/12light/dark cycle (lights on at 7:00 AM) for at least 1 week prior to the experiments. The study was conducted in accordance with the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

Ethanol administration: drinking

Rats (N=6/group) were given either an ethanol solution, as their sole drinking solution, or tap water for 10 days. The ethanol drinking solution was mixed to a 6.5% concentration using 99.5% ethanol and tap water (Gameiro et al., 2003). Pretreatment was carried out in the animals' home cages.

Ethanol withdrawal

12 hr after removal of the ethanol solutions (given for 10 days), rats were submitted to the TMJ formalin test. The 12 hr time point was chosen because a large degree of nociception was seen at that time point in early experiments and to provide data directly comparable with other ethanol-withdrawal research (Gatch and Lal, 1999; Gatch and Selvig, 2002; Gameiro et al., 2003).

Testing procedure for TMJ pain

The design of this study is described elsewhere (Roveroni et al., 2001). Briefly, rats received 50 µl injection of diluted formalin (1.5%) into the left TMJ region; nociceptive behavioral responses characterized by rubbing the orofacial region (amount of time-seconds) and flinching the head (number of head flinches)

were quantified for 45 min (15 blocks of 3 min). Considering that the flinching of the head behavior followed a uniform pattern of a 1s in duration, each flinching was expressed as 1s. The combination (sum) of both behaviors provides a better measure of pain intensity than any single behavior (Roveroni et al., 2001). An investigator who was blind to the rat's group assignment made the observation of the behaviors. After the conclusion of each experiment, Evans blue dye (0.1% 5 mg/Kg) was injected systemically in order to confirm the TMJ injection side at post-mortem, as previously described (Hass et al., 1992).

Testing the involvement of central TRPV1

The capsazepine, a TRPV1 antagonist that has been shown to competitively inhibit capsaicin-mediated responses (Fox et al., 1995), was used to evaluate the involvement of central TRPV1 on nociception induced by the TMJ formalin test. 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 50 µl Hamilton syringe by a polyethylene cannula was first inserted below the occipital bone up to 4 mm, and slightly inclined in a cranial direction. The needle was advanced more than 2 mm to perforate the atlanto-occipital membrane and reach the medullary subarachnoid space (Fig. 1). This technique allowed direct drug delivery in the cerebrospinal fluid in the surroundings of trigeminal subnucleus caudalis. Total injection volume in the experiment was 10 µl. All injections were performed at rate of 1µl/s (Fischer et al., 2005). Rats received a 10 µl subarachnoid injection of diluted capsazepine (92 µg – Bach et al., 1995) or vehicle (saline containing

50% dimethylsulphoxide) 30 min before (Walker et al., 2003) the TMJ formalin test.

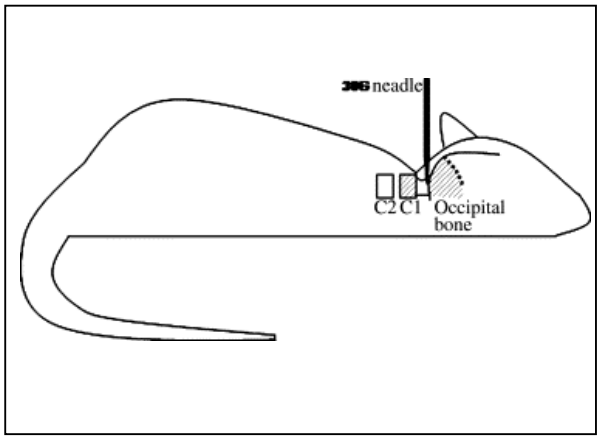

Fig.1 The approach used for the subarachnoid drug injection. The needle was inserted below the occipital bone up to 4 mm, and slightly inclined in a cranial direction. The needle was advanced more than 2 mm to perforate the atlanto-occipital membrane and reach the medullary subarachnoid space.

Statistical analysis

The sum of rubbing and flinching responses exhibited by each animal was computed. The data were analyzed by One Way ANOVA followed by Student-Newman-Keuls post-hoc test. A p value of <0.05 was considered statistically significant. All statistical analyses were performed using SIGMA STAT version 3.0 for Windows.

Results

Effects of ethanol withdrawal and capsazepine administered centrally

The injection of formalin (FOR) into the TMJ induced a significant degree of hyperalgesia in rats when the nociceptive behaviors were evaluated 12 hr after removal of the ethanol solutions. The increase of nociceptive responses was statistically significant ($F_{3.21} = 10.73$, $p< 0.001$, One way ANOVA + Student-Newman-Keuls) when the ethanol withdrawal group was compared to its control (water group). The subarachnoid injection of capsazepine (CPZ) 92µg/10µl

induced a significant reduction of hyperalgesia in rats exposed to ethanol withdrawal as described above. The decrease of nociceptive behavioral responses was statistically significant when the CPZ + FOR group was compared to vehicle + FOR and FOR groups ($F_{3.21}$= 10.73, $p < 0.001$, One way ANOVA +, Student-Newman-Keuls) (Fig. 2).

Discussion

The development of novel TRPV1 antagonists has highlighted research investigating neurological basis for hyperalgesia associated with TRPV1 receptors. The present study showed that blockade of centrally located TRPV1 receptors reduced the TMJ hyperalgesia induced by ethanol withdrawal. Increased nociceptive behavioral responses during ethanol withdrawal have already been reported in animal studies using both superficial (Gatch and Lal 1999, Gatch and Selvig, 2002) and deep nociceptive stimuli (Gameiro et al., 2003). Research on the relationship between hyperalgesia and ethanol withdrawal so far has not led to unequivocal conclusions and underlying molecular mechanisms are not fully understood.

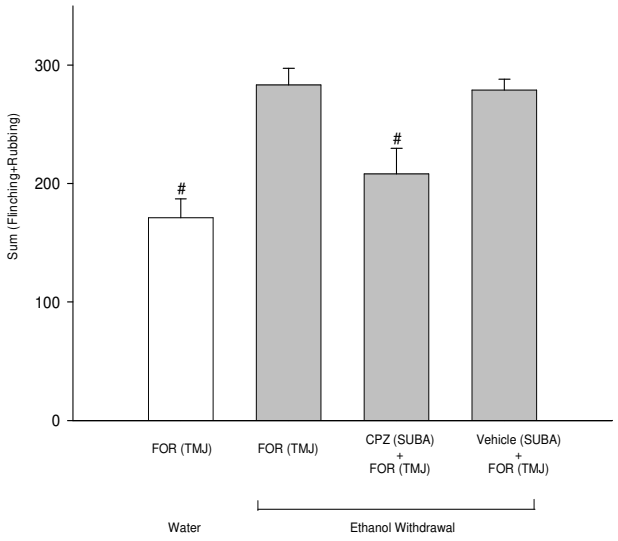

Fig. 2. Sum of nociceptive responses to formalin TMJ injection in the following groups: rats that drank water (column1); rats exposed to ethanol withdrawal treated with formalin alone (column 2), capsazepine 92µg + formalin (column 3), vehicle + formalin (column 4), #indicates significant differences in relation to formalin and in relation to vehicle + formalin (in ethanol withdrawal groups). FOR: formalin; CPZ: capsazepine; vehicle: dimethylsulphoxide 50%; TMJ: temporomandibular joint injection; SUBA: Subarachnoid medullary injection

A growing number of studies suggest that TRPV1 receptors are involved in pain and sensitization associated with tissue injury and inflammation (Walker et al., 2003; Cui et al., 2006; Chizh et al., Pei et al., 2007). However, we have only limited knowledge about the role of these receptors in hyperalgesia states induced by ethanol withdrawal.

In an experiment conducted in our laboratory, rats exposed to ethanol withdrawal exhibited increased nociceptive responses in the TMJ formalin test, and this hyperalgesia was reduced by the previous local application of CPZ into the TMJ region (Urtado el al., 2007). These findings provided the first experimental evidence that peripheral TRPV1 receptors might be involved in hyperalgesia induced by ethanol withdrawal. However, considering that ethanol

affects many functions of the central nervous system (CNS) and that formalin-induced responses are modulated by a central sensitization process (Mcnamara et al., 2007), the role of central TRPV1 receptors in TMJ hyperalgesia induced by ethanol withdrawal should not be discarded.

In the present study, the subarachnoid injection of CPZ (92 µg/10µL), but not vehicle, was able to prevent the TMJ hyperalgesia induced by ethanol withdrawal (Fig. 2). These data, together with our previous results (Urtado et al., 2007), demonstrate that both the peripheral and central TRPV1 receptors play important roles in the TMJ hyperalgesia induced by ethanol withdrawal. In fact, these findings were not surprising, since various studies have shown that TRPV1 receptors in both the periphery and the CNS play a role in inflammatory pain conditions. Receptor expression studies have demonstrated that TRPV1 receptors are upregulated in both the periphery and the CNS during persistent inflammation (Carlton and Coggeshall, 2001; Ji et al., 2002; Luo et al., 2004; Pei et al., 2007). Moreover, endogenous TRPV1 agonists and modulators such as protons, anandamide and products of the arachidonic acid metabolism can be released or up-regulated by inflammation and tissue damage (Nagy et al., 2004, Jia et al., 2005 and Krause et al., 2005). Therefore, TRPV1 represents an important target for developing novel analgesics (Doherty et al., 2007). Actually, there is clinical evidence that a potent, selective and orally bioavailable TRPV1 antagonist (SB-705498) may alleviate pain and hyperalgesia associated with inflammation and tissue injury (Chizh et al., 2007).

It should be noted that animal models of nociception will be very useful to test the efficacy profile and potential side-effects of novel TRPV1 antagonists. It is

also important to point out that pain models differ in their susceptibility to modulation by drugs (Cui 2006). For example, CPZ and ruthenium red (a cation-selective antagonist coupled to vanilloid receptor of capsaicin), administered by supraspinal site, were inactive in inhibiting formalin-induced nociception, but prevented, in a graded manner, capsaicin-induced algesic response, suggesting the participation of distinct mechanisms in the nociception induced by formalin and capsaicin (Santos and Calixto, 1997). Therefore, the mechanisms involved in a specific pain model should be understood in order to better evaluate the effect of new drugs on some specific situation. In the present study, using a deep nociception assay, we demonstrate that TRPV1 receptors in the CNS play an important role in TMJ hyperalgesia induced by ethanol withdrawal. Taken together, our previous (Urtado et al., 2007) and present data suggest that both central and peripheral TRPV1 receptors represent important targets for developing novel analgesics to treat deep orofacial pain conditions, like temporomandibular disorders (TMD). Information on the extent of ethanol-induced changes in nociceptive systems can also be of clinical value, especially in individuals who regularly drink alcohol and suffer from some kind of chronic pain conditions, including TMD. If chronic ethanol administration or ethanol dependence alters nociceptive thresholds, then the effects of pain medications on heavy drinkers may be

significantly altered, potentially leading to over- or under-use or to unpleasant or dangerous side effects.

Acknowledgments

The authors thank Maria Imaculada Montebelo for statistical analyses. This work was supported by CNPq, Brazil.

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IV - CONCLUSÃO

Os resultados do presente trabalho, sugerem que:

- Os receptores TRPV1 centrais e periféricos não participam das respostas comportamentais de dor provocadas pelo teste da formalina na ATM.

- A sensibilização periférica e central dos receptores TRPV1 contribui para a hiperalgesia da ATM induzida pela retirada do etanol

V – REFERÊNCIAS BIBLIOGRÁFICAS*

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* De acordo com a norma da UNICAMP/FOP, baseada no modelo Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

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APÊNDICE

FIGURAS

FIGURA 1 – Local de punção para a administração de formalina e capsazepina.

FIGURA 3 – Câmara de observação (30 cm x 30 cm x 30 cm) utilizada para análise comportamental no teste da formalina

FIGURA 4 - Cronômetro e contador de células utilizados para a quantificação dos comportamentos nociceptivos.

TABELAS REFERENTES AOS VALORES INDIVIDUAIS DA AMOSTRA

Tabela 1 - Valores individuais referentes à soma dos comportamentos nociceptivos [coçar (CO) + levantar rapidamente a cabeça (LC)] desencadeados pela injeção da formalina na ATM após administração crônica (10 dias) de etanol ou água (controle).

SOMA DOS COMPORTAMENTOS (CO + LC)		
Animal	**Grupo água (controle)**	**Grupo etanol (10 dias)**
1	145,410	147,56
2	131,900	156,70
3	208,800	227,73
4	188,090	110,18
5	132,850	211,46
6	219,400	170,67
Média ± EPM	**171 ± 16**	**170 ± 17**

Tabela 2 - Valores individuais referentes à soma dos comportamentos nociceptivos [coçar (CO) + levantar rapidamente a cabeça (LC)] desencadeados pela injeção da formalina na ATM após tratamento crônico e retirada do etanol e administração de veículo ou capsazepina (300, 600 e 1200 µg) na ATM

SOMA DOS COMPORTAMENTOS (CO + LC)						
Animal	**Grupo Retirada**	**Veículo (IPSI)**	**CPZ300 (IPSI)**	**CPZ600 (IPSI)**	**CPZ1200 (IPSI)**	**CPZ1200 (CTL)**
1	287,21	268,01	248,33	108,84	160,31	167,53
2	338,91	280,00	167,84	124,66	136,02	204,00
3	243,33	315,05	181,87	126,81	137,32	365,30
4	289,41	287,21	208,54	132,75	151,14	388,98
5	250,28	250,28	216,78	183,69	184,00	342,10
6	290,11	290,11	159,86	127,20	82,93	
7			215,87		115,19	
8					132,81	
Média ± EPM	**283 ± 13**	**281 ± 8**	**199 ± 11**	**133 ± 10**	**137 ± 10**	**243 ± 45**

Tabela 3 - Valores individuais referentes à soma dos comportamentos nociceptivos [coçar (CO) + levantar rapidamente a cabeça (LC)] desencadeados pela injeção da formalina na ATM e após administração da capsazepina (1200µg) ipsilateral.

SOMA DOS COMPORTAMENTOS (CO + LC)		
Animal	**Grupo água (controle)**	**CPZ1200 (IPSI)**
1	145,410	170,08
2	131,900	173,30
3	208,800	173,21
4	188,090	130,05
5	132,850	169,01
6	219,400	186,16
Média ± EPM	**171 ± 16**	**166 ± 7**

Tabela 4 - Valores individuais referentes à soma dos comportamentos nociceptivos [coçar (CO) + levantar rapidamente a cabeça (LC)] desencadeados pela injeção da formalina na ATM e após administração da capsazepina (92µg) subaracnóide (SUBA).

SOMA DOS COMPORTAMENTOS (CO + LC)		
Animal	**Grupo água (controle)**	**CPZ 92 (SUBA)**
1	145,410	277,98
2	131,900	200,76
3	208,800	230,51
4	188,090	153,42
5	132,850	272,14
6	219,400	167,57
Média ± EPM	**171 ± 16**	**217 ± 21**

Tabela 5 - Valores individuais referentes à soma dos comportamentos nociceptivos [coçar (CO) + levantar rapidamente a cabeça (LC)] desencadeados pela injeção da formalina na ATM e após administração da capsazepina (92µg) subaracnóide (SUBA) ou veículo, em animais submetidos ao tratamento crônico e retirada do etanol.

SOMA DOS COMPORTAMENTOS (CO +LC)			
Animal	**Grupo Retirada**	**CPZ 92 (SUBA)**	**Veículo (SUBA)**
1	287,21	121,00	258,380
2	338,91	165,69	285,380
3	243,33	250,20	261,100
4	289,41	255,95	294,900
5	250,28	197,77	313,000
6	290,11	282,76	259,930
7		182,94	
Média ± EPM	**283 ± 13**	**208 ± 21**	**278 ± 9**

ANEXO

Certificado do comitê de ética

Universidade Estadual de Campinas
Instituto de Biologia

UNICAMP

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 **936-1**, sobre **"ENVOLVIMENTO DO RECEPTOR VANILÓIDE (VR1) NA NOCICEPÇÃO INDUZIDA PELA FORMALINA INJETADA NA ATM DE RATOS SUBMETIDOS A ADMINISTRAÇÃO CRÔNICA DE ETANOL"** sob a responsabilidade de **Profa. Dra. Maria Cecilia Ferraz de Arruda Veiga/Marília Bertoldo Urtado** 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 12 de dezembro de 2005.

C E R T I F I C A T E

We certify that the protocol nº **936-1**, entitled **"ENVOLVIMENT OF VANILLOID 1 RECEPTOR (VR1) ON NOCICEPTION EVOKED BY FORMALIN INJETED IN TMJ OF RATS SUBMETED TO CHRONIC CONSUNPTION OF ETHANOL"**, 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 December 12, 2005.

Campinas, 12 de dezembro de 2005.

Profa. Dra. Ana Maria A. Guaraldo
Presidente - CEEA/IB/UNICAMP

Fátima Alonso
Secretária - CEEA/IB/UNICAMP

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Confirmação de Aceite do Artigo para publicação (Capítulo 1)

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Journal title: Life Sciences
Corresponding author: Prof. Maria Cecília Ferraz de Arruda Veiga
First author: Dr. Marilia B Urtado
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