
JOSIANE MIRANDA

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# “EFEITO DA CRONIFICAÇÃO DA DOR E DA DOR CRÔNICA EM UM MECANISMO DE MODULAÇÃO ENDÓGENA DE DOR EM RATOS”

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UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Orientadora: Profa. Dra. Cláudia Herrera Tambeli

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Dissertação de mestrado apresentada ao Programa de pós-graduação em Odontologia da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para obtenção do título de Mestra em Odontologia, na área de Fisiologia Oral.

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Profa. Dra. DIONÉIA ARALDI

**RESUMO**

Neste estudo, foi testada a hipótese de que a transição da hiperalgesia aguda para a persistente e a hiperalgesia persistente reduzem a atividade e induzem alterações plásticas num circuito de analgesia endógena, o controle nociceptivo ascendente (CNA). Este circuito é de grande importância para mediar uma forma de analgesia endógena, conhecida como analgesia induzida por capsaicina, e é dependente de receptores μ-opióide no núcleo accumbens. Portanto, nós também investigamos se a transição da hiperalgesia mecânica aguda para a persistente e a hiperalgesia mecânica persistente altera a participação dos receptores μ-opióide intra-accumbens na analgesia induzida por capsaicina. No modelo animal de cronificação da dor utilizado, 14 dias consecutivos de injeções intraplantares de $PGE_2$ na pata traseira de ratos (referido como o período de indução da hiperalgesia persistente), induz um estado permanente de sensibilização dos nociceptores (referido como o período de manutenção da hiperalgesia persistente), que se mantém por até 30 dias após a interrupção do tratamento com $PGE_2$. A hipersensibilidade dos nociceptores foi medida pela diminuição do intervalo de tempo para o animal responder a uma leve estimulação mecânica na pata traseira. Para avaliar a expressão dos receptores μ-opióide no núcleo accumbens, foi utilizado o método de Western Blotting. Foi encontrada uma redução significativa na duração da analgesia induzida por capsaicina nos dias 7 e 14 do período de indução e nos dias 1, 7, 14 e 21 do período de manutenção da hiperalgesia mecânica persistente. A administração intra-accumbens do antagonista seletivo de receptor μ-opióide $Cys^2$,$Tyr^3$,$Orn^5$,$Pen^7$amide (CTOP), 10 minutos antes da injeção subcutânea de capsaicina na pata dianteira dos ratos, bloqueou a analgesia induzida por capsaicina. No entanto, não ocorreram alterações significativas na expressão dos receptores μ-opióide. Tomados em conjunto, estes resultados indicam que a transição da hiperalgesia aguda para a persistente e a hiperalgesia persistente reduzem a duração da analgesia induzida por capsaicina, sem afetar sua dependência de mecanismos mediados por receptores μ-opióide no núcleo accumbens. A atenuação da analgesia endógena durante a cronificação da dor e dor crônica sugerem que os circuitos endógenos de controle da dor desempenham um importante papel no desenvolvimento e manutenção da dor crônica.

**Palavras-chave:** cronificação da dor, dor crônica, analgesia endógena, núcleo accumbens, receptores μ-opióide.

**ABSTRACT**

In this study, we tested the hypothesis that the transition from acute to persistent hyperalgesia and persistent hyperalgesia reduces the activity and induces plastic changes in an endogenous analgesia circuit, the ascending nociceptive control (ANC). An important mechanism mediating this form of endogenous analgesia, referred as capsaicin-induced analgesia, is its dependence on nucleus accumbens μ-opioid receptor mechanisms. Therefore, we also investigated whether the transition from acute to persistent mechanical hyperalgesia and persistent mechanical hyperalgesia alters the requirement for nucleus accumbens μ-opioid receptor mechanisms in capsaicin-induced analgesia. We used an animal model of pain chronification in which daily intraplantar $PGE_2$ injection into the rat's hind paw for 14 days, referred as the induction period of persistent hyperalgesia, induces a long lasting state of nociceptior sensitization referred as the maintenance period of persistent hyperalgesia, that lasts for at least 30 days following the cessation of the $PGE_2$ treatment. The nociceptor hypersensitivity was measured by the shortening of the time interval for the animal to respond to a mechanical mild stimulation of the hind paw. Western blot analysis were used to evaluate the expression of μ-opioid receptors in nucleus accumbens. We found a significant reduction in the duration of capsaicin-induced analgesia at day 7 and 14th of the induction period and at days 1, 7, 14 and 21th of the maintenance period of persistent mechanical hyperalgesia. Intra-accumbens administration of the μ-receptor selective antagonist $Cys^2$,$Tyr^3$,$Orn^5$,$Pen^7$amide (CTOP) 10 min before the subcutaneous injection of capsaicin into the rat's fore paw blocked capsaicin-induced analgesia. However, no significant changes occurred in the expression of μ-opioid receptors. Taken together, these findings indicate that the transition from acute to persistent hyperalgesia and persistent hyperalgesia reduces the duration of capsaicin-induced analgesia, without affecting its dependence on nucleus accumbens μ-opioid receptor mechanisms. The attenuation of endogenous analgesia during pain chronification and chronic pain suggests that endogenous pain circuits play an important role in the development and maintenance of chronic pain.

**Key words:** pain chronification, chronic pain, endogenous analgesia, nucleus accumbens, μ-opiod receptors.

**SUMÁRIO**

## DEDICATÓRIA

Dedico esse trabalho,

***À Deus***

Por ter me dado forças e coragem para prosseguir mesmo diante de situações difíceis e ter me abençoado, fazendo com que eu fosse alcançando grandes vitórias.

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**EPÍGRAFE**

*"Nenhuma grande vitória é possível sem que tenha sido precedida de pequenas vitórias sobre nós mesmos."*

*L. M. Leonov*

## INTRODUÇÃO

A IASP (do inglês, *International Association for the Study of Pain*, 2011), define a dor como sendo uma experiência sensorial e emocional desagradável associada a um dano tecidual real ou potencial dos tecidos, ou descrita em termos de tal dano. Ao contrário da dor aguda ou transitória que tem um caráter protetor bem definido, a dor crônica tem um caráter deletério para o organismo. É um problema de saúde pública que causa sofrimento para o indivíduo em termos de dificuldade no tratamento e gera implicações econômicas significativas (Curkovic, 2007). Em geral, as dores crônicas de origem inflamatória são precedidas de episódio inflamatório agudo e acredita-se que a inflamação aguda de longa duração pode induzir mudanças plásticas nos nociceptores contribuindo para a persistência do quadro doloroso, independentemente da resolução do processo inflamatório. Uma das dificuldades de se entender o processo de cronificação da dor se dá pela falta de modelos experimentais que mimetizem com bom grau de predição as condições clínicas observadas em humanos. A grande maioria dos modelos experimentais de hiperalgesia avalia as respostas a estímulos nocivos agudos ou transitórios.

Em 1990, Ferreira *et al*. desenvolveram um modelo animal de hiperalgesia mecânica persistente no qual a sensibilização da pata traseira do rato dura por mais de 30 dias, após o término de 14 dias de tratamento com administrações locais de prostaglandina $E_2$ ($PGE_2$) no tecido subcutâneo intraplantar (i.pl.). No período de injeções ocorre hipernocicepção aguda e, de alguma maneira, a $PGE_2$, por exemplo, induz mudanças nos nociceptores que garantem subseqüentemente à fase de hipernocicepção aguda o desenvolvimento da hipernocicepção persistente. Neste modelo, a fibra aferente primária parece adquirir memória, de modo que após a interrupção da hipernocicepção pela injeção de dipirona, esta se restabelece rapidamente com baixas doses de prostaglandina, sugerindo que neste modelo ocorrem mudanças nos nociceptores favorecendo uma espécie de "memória nociceptiva" – quadro que caracteriza dores crônicas. A sensibilização persistente do nociceptor após a interrupção do tratamento com $PGE_2$ sugere que ocorrem alterações plásticas no sistema nervoso central que estimulam o processo de cronificação da dor e a dor crônica.

Alguns sistemas de modulação endógena de dor que são ativados por estimulação nociceptiva são importantes na atenuação da hiperalgesia. Analgesia semelhante à desencadeada por uma dose elevada de morfina (10mg/kg) pode ser induzida por estimulação nociceptiva. Por exemplo, injeção subdérmica de capsaicina na pata traseira de ratos anestesiados atenua a dor aguda ao atenuar o reflexo trigeminal de abertura bucal (Gear *et al*., 1999; Schmidt *et al*., 2002a; Tambeli *et al*., 2009). A antinocicepção mediada pela estimulação nociceptiva periférica ocorre através de uma via de modulação de dor onde um estímulo nociceptivo de grande intensidade atenua a dor em uma região corporal distante do local estimulado (heterosegmental). Em animais acordados, através da utilização de outro modelo nociceptivo agudo, ou seja, o do reflexo de retirada da pata em resposta a aplicação de um estímulo mecânico na mesma, foi demonstrado que a injeção subdérmica de capsaicina na pata dianteira de ratos induz antinocicepção heterosegmental, como indicado pelo aumento do limiar para retirada da pata traseira de um estímulo mecânico (Gear *et al*., 1999). Essa analgesia induzida pela estimulação nociceptiva é mediada pela ativação de um sistema de modulação de dor ascendente denominado de **controle nociceptivo ascendente** (ANC, do inglês Ascending Nociceptive Control), que se origina na medula espinhal e ascende da mesma para o Núcleo Accumbens (Gear & Levine, 1995; Gear *et al*., 1999). Portanto, o Núcleo Accumbens, conhecido por ser um importante componente do sistema de recompensa dopaminérgico mesolímbico e implicado no abuso de drogas, também exerce um papel importante na modulação da dor. Por exemplo, a administração prévia de antagonistas opióides no Núcleo Accumbens (Gear *et al*., 1999; Schmidt *et al*., 2002b), mas não em regiões que conhecidamente participam do controle antinociceptivo descendente, como núcleo magno da rafe e a substância cinzenta periaquedutal (Schmidt *et al*., 2002b) bloqueia a analgesia induzida pela estimulação nociceptiva.

Há evidências que sob condições fisiológicas basais, existe uma atividade tônica excitatória que ascende da medula espinhal e inibe a antinocicepção mediada pelo núcleo accumbens (Tambeli *et al*., 2002). Foi observado, em modelo de dor aguda, o do reflexo nociceptivo de abertura bucal, que a inibição dessa atividade neural espinhal excitatória via

administração intratecal (i.t.) do agonista do receptor μ-opióide, DAMGO, do anestésico local lidocaína (Gear & Levine, 1995), de bloqueadores de canais de cálcio (Tambeli *et al*., 2002) ou transecção cirúrgica da medula espinhal (Gear & Levine, 1995), ou ainda pelo bloqueio de receptores de aminoácidos excitatórios através da administração intratecal de antagonistas do receptor de AMPA ou de mGluR1 (Tambeli *et al*., 2002), induz antinocicepção heterosegmental, ou seja atenua profundamente a dor aguda (reflexo de abertura bucal). A estimulação nociceptiva periférica, tal como a induzida pela administração subcutânea de capsaicina, ativa um mecanismo espinhal inibitório que bloqueia essa atividade tônica ascendente excitatória, o que por sua vez desinibe um mecanismo opiodérgico no Núcleo Accumbens para produzir antinocicepção (Tambeli, 2003a; Tambeli *et al*., 2003b). No Núcleo Accumbens, vários mecanismos são importantes para a analgesia induzida por estimulação nociceptiva (Schmidt *et al*., 2001, 2002a, 2002b, 2003), porém a dependência de receptores μ-opióides têm sido uma das mais estudadas. Por exemplo, a aplicação do antagonista seletivo de receptor μ-opióide CTOP bloqueia a analgesia induzida por estimulação nociceptiva numa variedade de condições fisiológicas, incluindo animais intactos (Schmidt *et. al.*, 2002b) e submetidos à retirada precipitada de morfina (Schmidt *et. al.*, 2003). Já os mecanismos espinhais ativados pela estimulação nociceptiva periférica incluem mecanismos excitatórios mediados por receptores de NMDA e $mGluR_5$, mas não por receptores $mGluR_1$ (Tambeli et al., 2003b) e inibitórios mediados por receptores μ-opioid, $GABA_B$, e $GABA_A$ (Tambeli et al., 2003a). Para confirmar a participação dos mecanismos espinhais acima citados na antinocicepção mediada por estimulação nociceptiva, foi verificado que a administração intratecal de antagonistas de receptores inibitórios como receptores opióides μ, k, $GABA_A$ e $GABA_B$ (Tambeli et al., 2003a) bloqueia a antinocicepção heterosegmental induzida pela estimulação nociceptiva periférica. Embora os antagonistas de NMDA sejam conhecidos em geral por produzir antinocicepção, a administração intratecal do antagonista de receptor de NMDA, LY235959, também bloqueia a antinocicepção heterosegmental induzida pela estimulação nociceptiva periférica (Tambeli et al., 2003b).

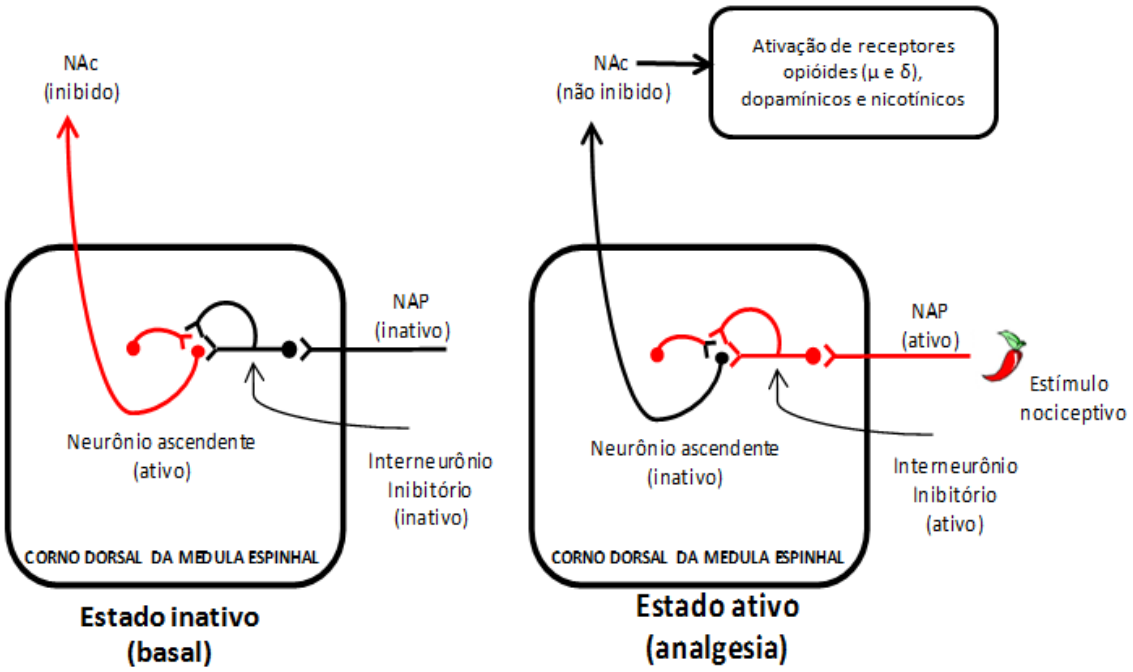


**Figura 1: Diagrama esquemático do circuito espinhal envolvido no controle nociceptivo ascendente.** Sob condições fisiológicas basais, na ausência de uma estimulação nociceptiva, o neurônio tonicamente ativo (em vermelho) projeta-se supraespinhalmente para inibir a antinocicepção mediada pelo núcleo accumbens. Note a falta de atividade no nociceptor aferente primário ("NAP") e do interneurônio inibitório (ambos em preto). A atividade no neurônio ascendente resulta de uma atividade glutamatérgica através da ativação pós-sináptica de receptores AMPA/cainato e $mGluR_1$ (Tambeli *et al*., 2002). No entanto, através de uma estimulação nociceptiva periférica, tal como a induzida pela administração de capsaicina, ativa-se o nociceptor aferente primário e o interneurônio inibitório (agora ambos em vermelho). O interneurônio inibitório é ativado via receptores NMDA e $mGluR_5$ de glutamato que é liberado a partir dos nociceptores aferentes primários em resposta a aplicação de um estímulo nociceptivo. A ativação do interneurônio inibitório libera GABA e opióides endógenos que via ativação de receptores $GABA_{(A\ e\ B)}$ e μ e κ-opióides atenuam a atividade tônica ascendente excitatória resultando em antinocicepção heterossegmental mediada pela liberação de opióides endógenos no núcleo accumbens (Tambeli *et al*., 2003b), o que caracteriza o processo de ativação do controle nociceptivo ascendente.

Apesar da ativação do controle nociceptivo ascendente através da estimulação nociceptiva induzir antinocicepção heterosegmental em modelos de dor aguda, não se sabe

se a ativação dessa via também modula a indução e manutenção da dor crônica. Utilizando o modelo experimental de hiperalgesia descrito acima (Ferreira et al., 1990; Sachs et al., 2002) demonstramos recentemente, em observações não publicadas do nosso laboratório, que o Núcleo Accumbens possui um papel facilitatório na manutenção da hiperalgesia persistente de origem inflamatória.

Nesse contexto, o objetivo deste trabalho foi testar a hipótese de que a transição da hiperalgesia aguda para a persistente e a hiperalgesia persistente reduzem a atividade e induzem alterações plásticas no controle nociceptivo ascendente (CNA). Como esse circuito é de grande importância para mediar uma forma de analgesia endógena, conhecida como analgesia induzida por capsaicina, e é dependente de receptores μ-opióide no núcleo accumbens foi investigado também se a transição da hiperalgesia mecânica aguda para a persistente e a hiperalgesia mecânica persistente alteram a participação dos receptores μ-opióide intra-accumbens na analgesia induzida por capsaicina.

**CAPÍTULO**

O presente artigo será submetido ao periódico "Neuroscience".

# EFFECT OF PAIN CHRONIFICATION AND CHRONIC PAIN ON AN ENDOGENOUS PAIN MODULATION CIRCUIT IN RATS

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Original Article

**Key words:** pain chronification, chronic pain, endogenous analgesia, nucleus accumbens, μ-opiod receptors.

**Abstract**

In this study, we tested the hypothesis that pain chronification and chronic pain reduce the activity and induce plastic changes in an endogenous analgesia circuit, the ascending nociceptive control. An important mechanism mediating this form of endogenous analgesia, referred as capsaicin-induced analgesia, is its dependence on nucleus accumbens μ-opioid receptor mechanisms. Therefore, we also investigated whether pain chronification and chronic pain alter the requirement for nucleus accumbens μ-opioid receptor mechanisms in capsaicin-induced analgesia. We used an animal model of pain chronification in which daily intraplantar $PGE_2$ injection into the rat's hind paw for 14 days, referred as the induction period of persistent hyperalgesia, induces a long lasting state of nociceptor sensitization referred as the maintenance period of persistent hyperalgesia, that lasts for at least 30 days following the cessation of the $PGE_2$ treatment. The nociceptor hypersensitivity was measured by the shortening of the time interval for the animal to respond to a mechanical mild stimulation of the hind paw. Western blot analyses were used to evaluate the expression of μ-opioid receptors in nucleus accumbens. We found a significant reduction in the duration of capsaicin-induced analgesia on days 7 and 14 of the induction period and on days 1, 7, 14 and 21 of the maintenance period of persistent mechanical hyperalgesia. Intra-accumbens administration of the μ-receptor selective antagonist $Cys^2$,$Tyr^3$,$Orn^5$,$Pen^7$amide (CTOP) 10 min before the subcutaneous injection of capsaicin into the rat's fore paw blocked capsaicin-induced analgesia. However, no significant changes occurred in the expression of μ-opioid receptors in the nucleus accumbens. Taken together, these findings indicate that the pain chronification and the chronic pain reduce the duration of capsaicin-induced analgesia, without affecting its dependence on nucleus accumbens μ-opioid receptor mechanisms. The attenuation of endogenous analgesia during the pain chronification and the chronic pain suggests that endogenous pain circuits play an important role in the development and maintenance of chronic pain.

**Key words:** pain chronification, chronic pain, endogenous analgesia, nucleus accumbens, μ-opioid receptors.

## 1- Introduction

Chronic pain syndromes cause enormous morbidity, social cost and damaging effect on quality of life (Porreca *et al*., 2002; Curkovic, 2007; Bushnell, 2013). They result from the transition from acute to chronic pain, but the mechanisms involved in pain chronification are not very well understood. This may be due, at least in part, to the lack of experimental models to study pain chronification and chronic pain with a good degree of human therapeutic predictability.

There are several causes of chronic pain but certainly many of them result from a previous inflammatory episode and are accompanied by hyperalgesia. Persistent hyperalgesia is generated by frequent periods of sensitization of the pain receptor and lasts several weeks (Woolf, 1983; Woolf, 2011).

The animal model of pain chronification developed by Ferreira *et al*. (1990) induces a state of sensitization of the nociceptors that lasts for at least 30 days following the cessation of 14 successive daily intraplantar injections of $PGE_2$. In this model, the 14 days period of successive daily intraplantar injections of $PGE_2$ corresponds to the induction period of persistent mechanical hyperalgesia and allows to study the mechanisms involved in pain chronification, while the 30 days period following the cessation of the $PGE_2$ injections corresponds to the maintenance period of persistent mechanical hyperalgesia and allow to study the mechanisms involved in chronic pain.

The persistence of nociceptor sensitization in the absence of any peripheral stimulus suggests that plastic changes in nervous system lead to pain chronification. However, whether plastic and functional changes occur in endogenous control circuits during pain chronification is not known.

In this study, we used the pain chronification model developed by Ferreira *et al* (1990) to test the hypothesis that the pain chronification and the chronic pain reduce the

activity and induce plastic changes in an endogenous analgesia circuit, the ascending nociceptive control.

This endogenous analgesia circuit is physiologically activated by a peripheral noxious stimulus such as a subcutaneous capsaicin injection at a site remote from the nociceptive testing and induces heterosegmental analgesia equivalent in magnitude to a high dose of morphine for more than an hour (Gear *et al*., 1999). This form of endogenous analgesia, referred as capsaicin-induced analgesia, is mediated by multiple mechanisms in nucleus accumbens, (Schmidt *et al*., 2001, 2002a, 2002b, 2003), but its dependence on nucleus accumbens μ-opioid receptor mechanisms is one of the most studied (Gear *et al*., 1999; Schmidt *et al*., 2002a, 2003). Therefore, we also investigated whether the pain chronification and the chronic pain alter the requirement for nucleus accumbens μ-opioid receptor mechanisms in capsaicin-induced analgesia.

## 2- Materials and Methods

### 2.1- Animals

Male albino Wistar rats (200-300 g) were obtained from the Multidisciplinary Center for Biological Research (CEMIB) - University of Campinas. The animals were housed in plastic cages with soft bedding (five rats/cage) on a 12:12 light cycle (lights on at 6:00 A.M.) with food and water available *ad libitum*. The animals were maintained in a temperature-controlled room (±23°C) and handled for at least one week prior to the experiments (Rosland, 1991). The Committee on Animal Research of the University of Campinas approved the experimental protocols (protocol number 1952-1), which conformed to the IASP guidelines for the study of pain in animals (Zimmermann, 1983). Effort was made to limit the number of animals used (168) and their discomfort.

### 2.2- Experimental Design

A guide-cannula was bilaterally stereotaxically implanted in the nucleus accumbens one week before the initiation of the $PGE_2$ injection into the rat's hind paw used

to induce persistent hyperalgesia. The μ-opioid receptor antagonist CTOP (1.0ng/0.25μl) or its vehicle was administrated into nucleus accumbens 10 minutes before the induction of acute peripheral noxious stimulation by capsaicin injection or before its vehicle injection into the fore paw on day 1, 7 or 14 after initiating the $PGE_2$ injection into the rat's hind paw (induction period) or on day 1, 7, 14, or 21 after discontinuing the $PGE_2$ injection (maintenance period of the persistent hyperalgesia model). The nociceptive threshold of the animals was recorded immediately, 15, 30, 45 and 60 min after the subcutaneous injection of capsaicin or its vehicle. The locomotor activity was evaluated in the "rota-rod" equipment immediately after the nociceptive test to exclude the possibility that the effect of intra-accumbal treatments on the intensity of the nociceptive response was due to altered motor activity (Figure 1).

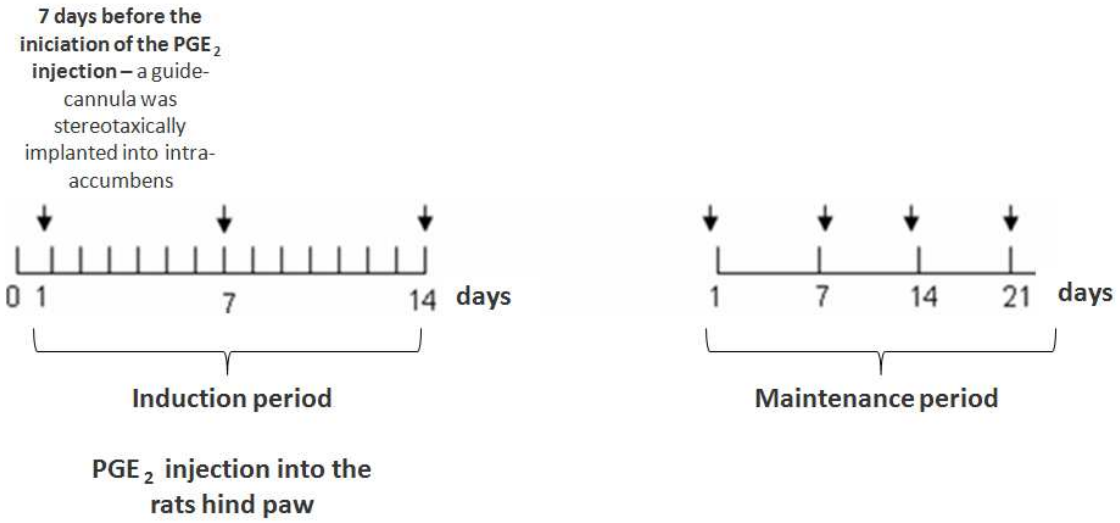



**Figure 1. Experimental Design.** On the days indicated by the arrows, CTOP or its vehicle was administrated into nucleus accumbens 10 minutes before the administration of capsaicin or its vehicle into the fore paw. The nociceptive threshold was recorded immediately, 15, 30, 45 and 60 min. after fore paw administration.

## 2.3- Persistent mechanical hyperalgesia model

$PGE_2$-induced persistent mechanical hyperalgesia was induced as previously described (Ferreira *et al*., 1990). Briefly, persistent mechanical hyperalgesia was induced by daily subcutaneously injection of $PGE_2$ (100 ng/50 µl/paw) into the dorsal surface of the rat's hind paw over 14 days. In order to avoid a local release of $PGE_2$ as a result of successive injections, all animals were treated with indomethacin (2 mg/kg) by intraperitoneal route 30 min before the $PGE_2$ injection. After the discontinuation of the 14 successive daily injections of $PGE_2$, the hyperalgesia persists for approximately 30 days. Therefore, there are two well-defined periods in this persistent hyperalgesia model, the induction and the maintenance period. The induction period was defined as the 14 days period of daily subcutaneous injection of $PGE_2$ into the rat's hind paw, and the maintenance period the 21 days period after discontinuing the daily $PGE_2$ injections. The intensity of hyperalgesia was evaluated by the mechanical nociceptive threshold measured immediately before the subcutaneous $PGE_2$ injection into the rat hind paw on days 1, 7 and 14 of the induction period of the persistent hyperalgesia model. After the discontinuation of the $PGE_2$ treatment (maintenance period) the hyperalgesia was evaluated on days 1, 7, 14 and 21.

## 2.4- Nociceptive testing

Mechanical nociceptive threshold was quantified using the Randall-Selitto nociceptive paw-withdrawal test (Randall & Selitto, 1957) in which a force that increases linearly over time is applied to the dorsum of the rat hind paw (Taiwo *et al*., 1989) (Ugo Basile Algesymeter, Stoelting). The test was performed by placing one of the paws of the animal in a compressing device that consists of two items: a flat part, which rests on the dorsal part of the animal's paw, and a conical part which exerts pressure on the plantar surface paw of the animal. Upon testing, a pedal that is a constituent part of the device is triggered by the experimenter and transmits a pressure of constant intensity that falls on the conical part of the appliance by pressing the paw of the animal. This pressure is measured by moving a cursor that slides on a linear scale and provides values in grams at the time the

animal present a hyperalgesic response, i.e. the moment at which the animal shows the paw withdrawal reflex. The nociceptive threshold was defined as the average of three measurements performed at a 5 min. interval. A decrease in mechanical paw withdrawal threshold was indicative of hyperalgesia. The nociceptive threshold of the animals was recorded immediately, 15, 30, 45 and 60 min after the subcutaneous injection of capsaicin or its vehicle.

The change of the mechanical nociceptive threshold was calculated by subtracting the average of three values obtained after the administration of capsaicin or its vehicle into the rat's fore paw of from the average of the three values obtained on the first day of experiments prior any treatment (baseline). The increase in the change of the mechanical nociceptive threshold was indicative of increased intensity of hyperalgesia.

Testing sessions took place during the light phase (between 09:00 A.M. and 5:00 P.M.) in a quiet room maintained at 23ºC (Rosland, 1991).

**2.5- Rota-Rod**

Motor function was measured as previously described (Tsuda et al., 1996) using the rotarod Ugo Basile after the bilateral injection of CTOP or its vehicle into the nucleus accumbens. Animals were assessed for their ability to maintain balance on a rotating bar at a constant speed of 18 rpm. The time from when the animal mounted the rod to when it fell from the rod was recorded. The animals were trained for 2 days before testing, which includes 3 training sessions each day. The off-stated time was 200 seconds. The procedure was made according to the model.

**2.6- Drugs and doses**

The following drugs were administered. Prostaglandin $E_2$, 100ng/50 µl/hind paw (Ferreira *et al*., 1990); the cyclooxygenase (COX) inhibitor indomethacin, 2.0 mg/Kg (Honore *et al*., 1995); E-capsaicin (capsaicin), 125µg/50 µl/fore paw (Gear *et al*., 1999) and the µ-opioid receptor antagonist CTOP ($Cys^2$,$Tyr^3$, $Orn^5$,$Pen^7$amide, 1.0 µg) which was administered into the nucleus accumbens (Schmidt *et al*., 2002b).

The stock solution of $PGE_2$ (1 µg/µL) was prepared in 10% ethanol, and additional dilutions were made in physiological saline (0.9% NaCl) to yield a final ethanol concentration less than 1%. E-capsaicin (capsaicin) was dissolved in Tween 80 (50%) and ethanol (50%) to an initial concentration of 50µg/µL and diluted in 0.9% saline to a concentration of 2.5 µg/µL. CTOP was dissolved in phosphate buffered saline (PBS).

All drugs and reagents were obtained from Sigma-Aldrich, SP, Brazil.

**2.7- Subcutaneous Injections**

Drugs or their vehicle were subcutaneously injected in the dorsum of the rat paw. $PGE_2$ was injected in the hind paw and capsaicin in the fore paw by tenting the skin and puncturing it with a 30-gauge needle prior to injecting the test agent, as previously described (Oliveira *et al*., 2007). The needle was connected to a catheter of polyethylene and also to a Hamilton syringe (50 µL). The animals were briefly restrained and the total volume administered in the paw was 50µL.

**2.8- Nucleus accumbens drug administration**

The rats were anesthetized with an intraperitoneal injection of xylazine chloride (10 mg/kg) and ketamine hydrochloride (90 mg/kg) and a bilateral 23 gauge stainless steel guide cannula was stereotaxically positioned and held in place with orthodontic resin. Each rat was allowed to recover for at least 7 days prior testing. Intra-accumbal injections were performed on the testing day in the awake rat via the insertion of a 30-gauge stainless steel injection cannula, which extended 2 mm beyond the guide cannula. The injection cannula was connected to a 2-µl syringe (Hamilton, Reno, NV, USA). The stereotaxic coordinates for nucleus accumbens core injections were as follows: 1.3 mm rostral, 7.2 mm ventral, and 1.8 mm from the bregma bilaterally (Paxinos & Watson, 1986). The injection volumes in all experiments were 0.25 µl, and the injections were performed over a period of 2 minutes. The cannula was left in place for an additional 30 seconds. The administration sites were

verified by histological examination (100-µm sections stained with cresyl violet acetate) and plotted on coronal maps (Fig. 5) adapted from the atlas of Paxinos and Watson (1986).

**2.9- Western blotting analysis**

After experimental procedures (induction period - days 1 and 14; maintenance period - days 7 and 14) rats were anesthetized and decapitated. The nucleus accumbens was dissected, frozen in liquid nitrogen and stored at -80ºC up for further use. Total proteins from each sample were extracted using the TRIzol® reagent protocol (Invitrogen, USA) and solubilized in a buffer containing 50mM phosphate buffer, pH 7.4, 1mM EDTA, 1% SDS, 0,5% beta-mercaptoethanol and 1% protease inhibitor mixture (Sigma P8340). Total proteins were separated by SDS-PAGE on 12% polyacrylamide gels and electrophoretically transferred to the nitrocellulose membranes (BioRad). Membranes were stained with Ponceau S and blocked for 1h with phosphate buffered saline 0.1% Tween 20 (PBS-Tween) containing 5% non-fat dry milk. After blockade membranes were incubated with primary antibody anti-mu opioid receptor (Rabbit polyclonal antibody, AB10275, ABCAM, Cambridge, UK) diluted 1:1000 in PBS-Tween containing 3% bovine serum albumin (12 h, 4 °C). Membranes were washed with PBS-Tween and incubated for 2 h with secondary antibody conjugated with peroxidase (1:10.000; Goat anti-Rabbit IgG, Zymed Laboratories, CA, USA). Immunoreactive bands were detected by using chemiluminescence kit SuperSignal West Pico (Pierce), photodocumented (Gbox iChemi XR, GeneSnap Software, Syngene) and quantified (GeneTools Software, Syngene). Results were calculated as the ratio between the optical density (OD) of mu opioid receptor band and the OD corresponding to all protein bands stained with Ponceau S (Vieira *et al*., 2009; Romero-Calvo *et al*., 2010). This procedure allowed the use of OD value of all protein bands as an internal control.

### 2.10- Data analysis

For figures 2, 4, 5 and 6, a two-way repeated measures ANOVA with one between subjects factor (i.e. treatment) and one within subjects factor (i.e. time) was used to determine if there were significant differences in nociceptive response among the groups. For figures 2 and 7 a one-way analysis of variance (ANOVA) was used to determine if there were significant differences between the groups. The Tukey post hoc test was employed to determine the basis of significant differences between the groups. Results were expressed as mean ± standard error of mean (SEM) of six animals per group. Data are presented in figures as means ± SEM. The level for statistical significance was $p \leq 0.05$.

## 3- Results

### 3.1.- Capsaicin-induced analgesia

Subcutaneous injection of capsaicin (125 µg) but not of its vehicle into the rat's fore paw significantly increased ($p<0.05$, Tukey Test) paw withdrawal threshold in the hind paw of naive rats immediately after and 15, 30, 45 and 60 minutes after its injection. In these experiments, the injection of 0.9% NaCl into the rat's hind paw was performed approximately 3 hours before the subcutaneous injection of capsaicin (125 µg) or of its vehicle into the rat's fore paw. These findings indicate that the intense nociceptive stimulation induced by the capsaicin injection induces antinociception in rats.

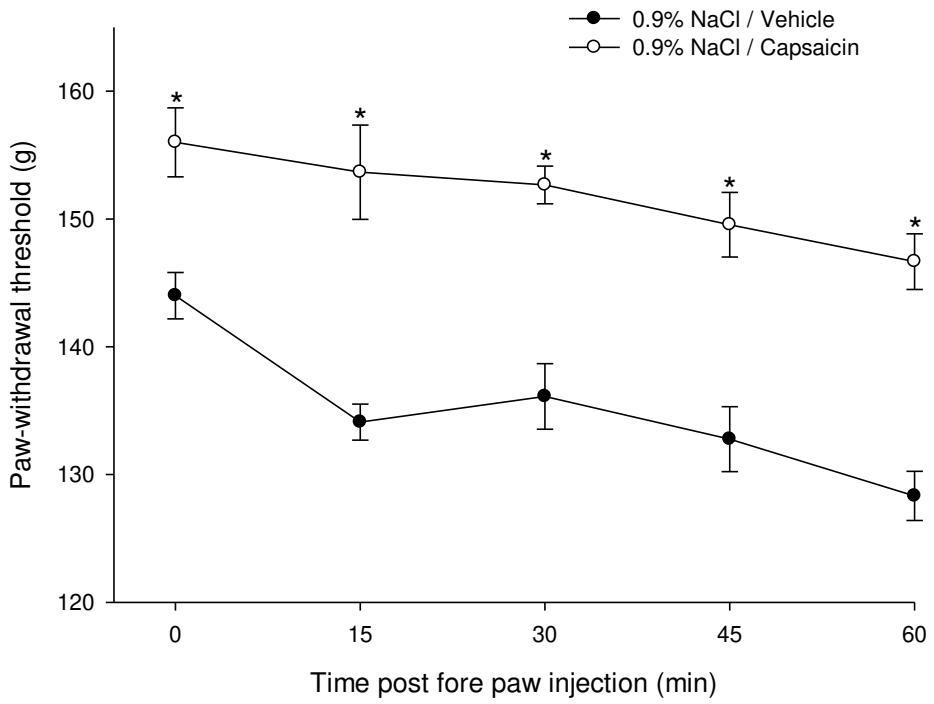



**Figure 2. Capsaicin-induced analgesia.**

The symbol "*" indicates that the subcutaneous injection of capsaicin (125 µg) or of its vehicle into the rat's fore paw significantly increased the hind paw withdrawal threshold. (Two-way repeated measures ANOVA, post hoc Tukey test, $p \leq 0.05$). PWT, Paw-withdrawal threshold. In this and in figures 2 to 5 data are plotted as mean ± s.e.m, number of rats per group was six in all cases.

## 3.2- Persistent hyperalgesia

Daily subcutaneous injections of $PGE_2$ (100ng/50µl/hind paw) for 14 days significantly reduced ($p<0.05$) the mechanical nociceptive threshold measured before $PGE_2$ injection on days 7 and 14 of the induction period and on days 1, 7, 14 and 21 of the maintenance period of persistent hyperalgesia, indicating the development of persistent hyperalgesia.

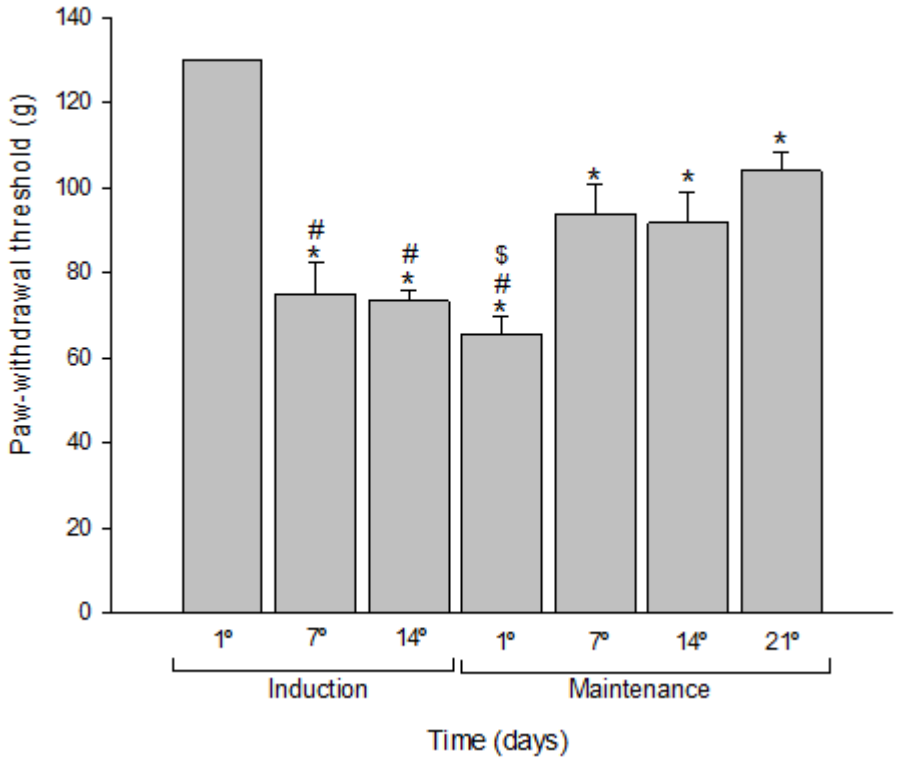


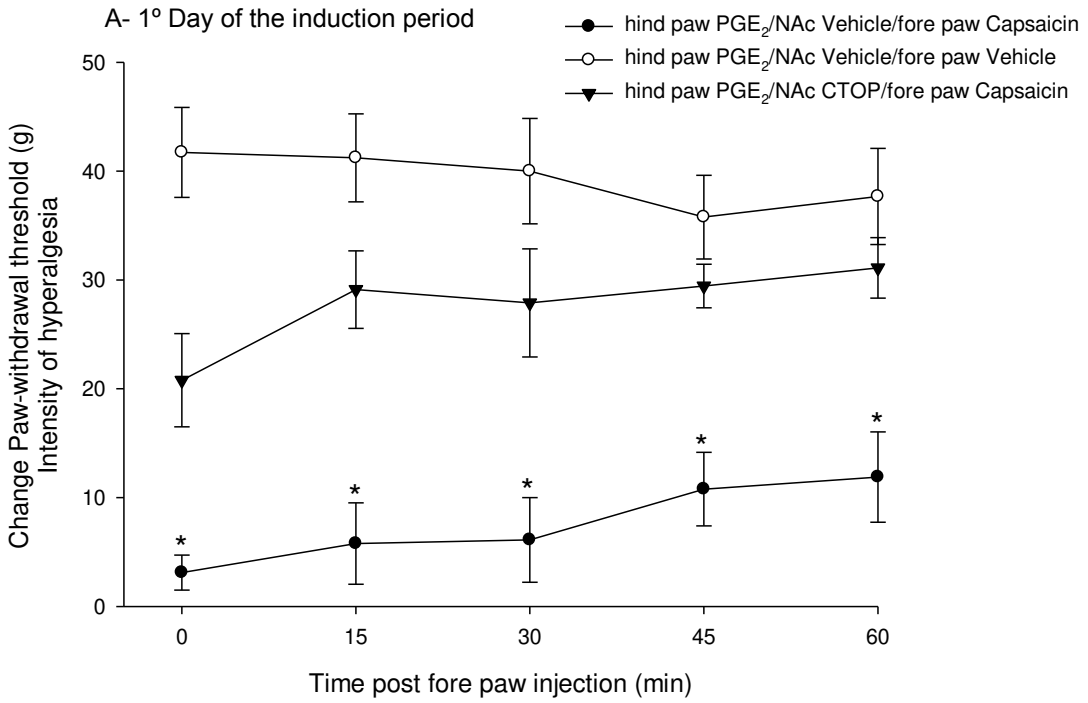

**Figure 3. Development and maintenance of the persistent hyperalgesia.**

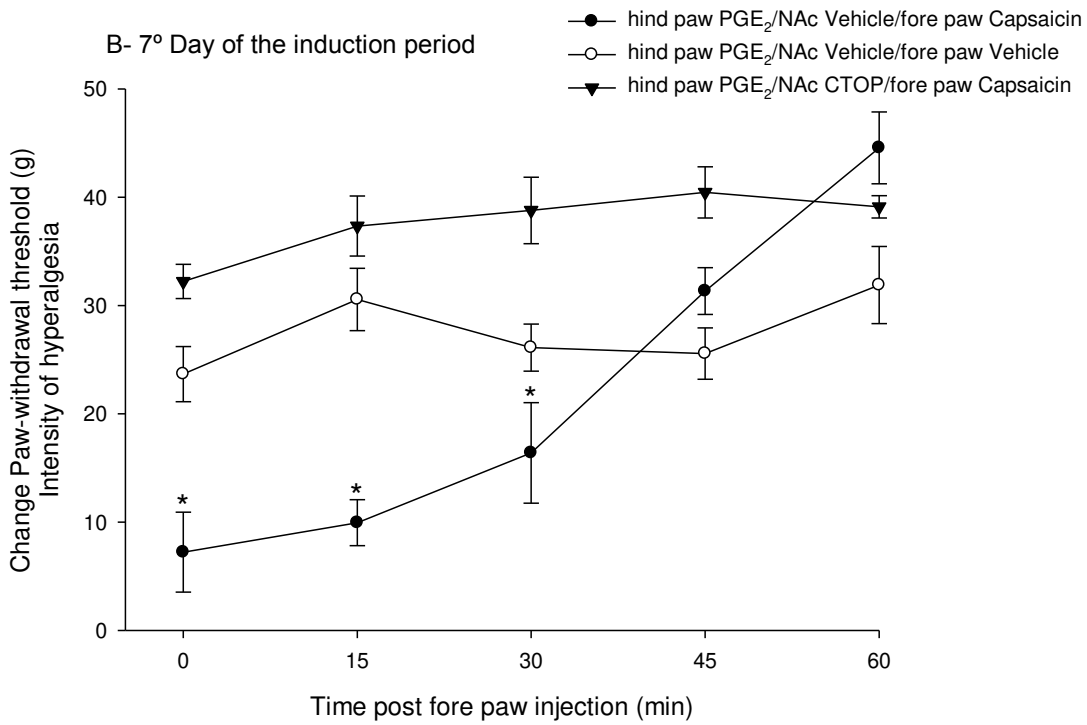
The temporal evolution of the basal mechanical threshold in the induction and maintenance period of the pain chronification model shows the development of persistent hyperalgesia. The symbol "*" indicates a response significantly lower than that of group day 1 of the induction period (first bar) of the persistent hyperalgesia. The symbol "#" indicates a response significantly lower than that of the group day 21 of the maintenance period of the persistent hyperalgesia. The symbol "$" indicates a response significantly lower than that of the groups day 7 and 14 of the maintenance period of the persistent hyperalgesia (One-way Analysis of Variance, post hoc test Tukey, $p<0.05$).

## 3.3- Effect of the persistent hyperalgesia from its induction to its maintenance on capsaicin-induced analgesia

***Induction period***

The subcutaneous injection of capsaicin (125 µg) into the rat's fore paw significantly ($p<0.05$) decreased the intensity of $PGE_2$-induced hyperalgesia for 60 min on day 1 of the induction period of the persistent hyperalgesia (Figure 4A). However, on days 7 (Figure 4B) and 14 (Figure 4C) of the induction period of the persistent hyperalgesia, the duration of capsaicin-induced analgesia was reduced to only 30 min. Capsaicin-induced analgesia was prevented by the intra-accumbal injection of the µ-opioid receptor antagonist CTOP (1.0µg) but not of its vehicle 10 min prior to the subcutaneous injection of capsaicin into the rat's fore paw at the periods of time evaluated. In these experiments, the subcutaneous injection of $PGE_2$ into the rat's hind paw was performed approximately 3 hours before the intra-accumbal injection. Rat locomotor activity measured immediately after the last measurement of paw withdrawal threshold was not significantly affected by these treatments ($p>0.05$) indicating that intra-accumbal treatments did not alter motor activity (data not shown). These results indicate that the pain chronification reduced the duration of capsaicin-induced analgesia.




B- 7º Day of the induction period
hind paw PGE2/NAc Vehicle/fore paw Capsaicin
hind paw PGE2/NAc Vehicle/fore paw Vehicle
hind paw PGE2/NAc CTOP/fore paw Capsaicin
Change Paw-withdrawal threshold (g)
Intensity of hyperalgesia
50
40
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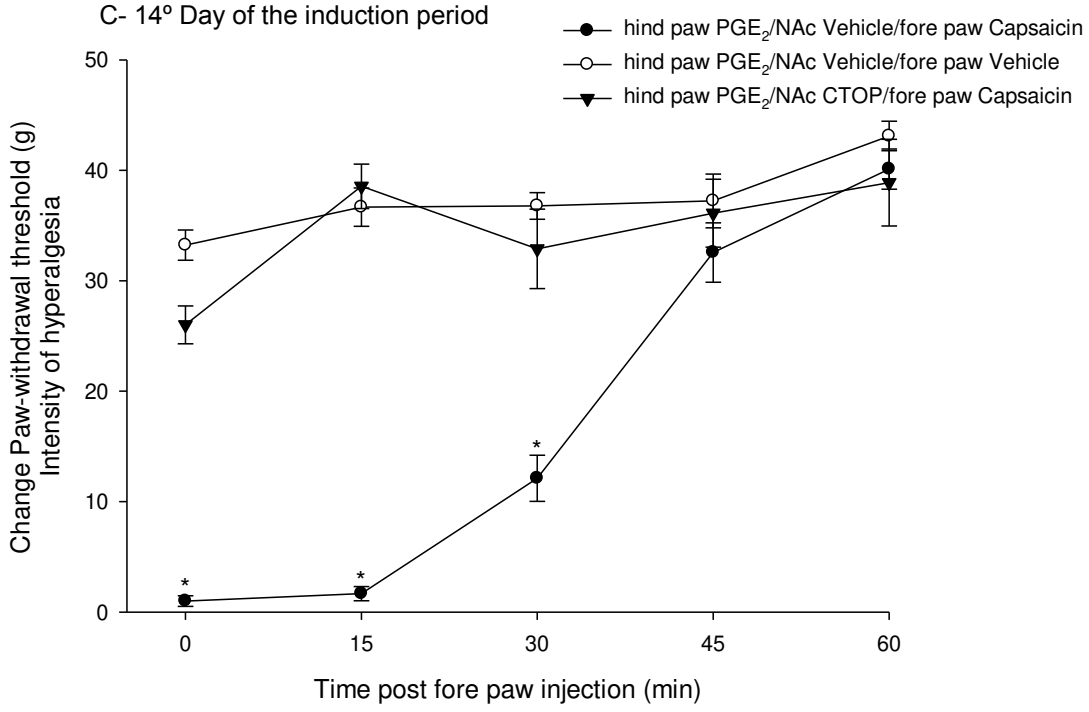
15
30
45
60
Time post fore paw injection (min)


C- 14º Day of the induction period
hind paw PGE2/NAc Vehicle/fore paw Capsaicin
hind paw PGE2/NAc Vehicle/fore paw Vehicle
hind paw PGE2/NAc CTOP/fore paw Capsaicin
Change Paw-withdrawal threshold (g)
Intensity of hyperalgesia
50
40
30
20
10
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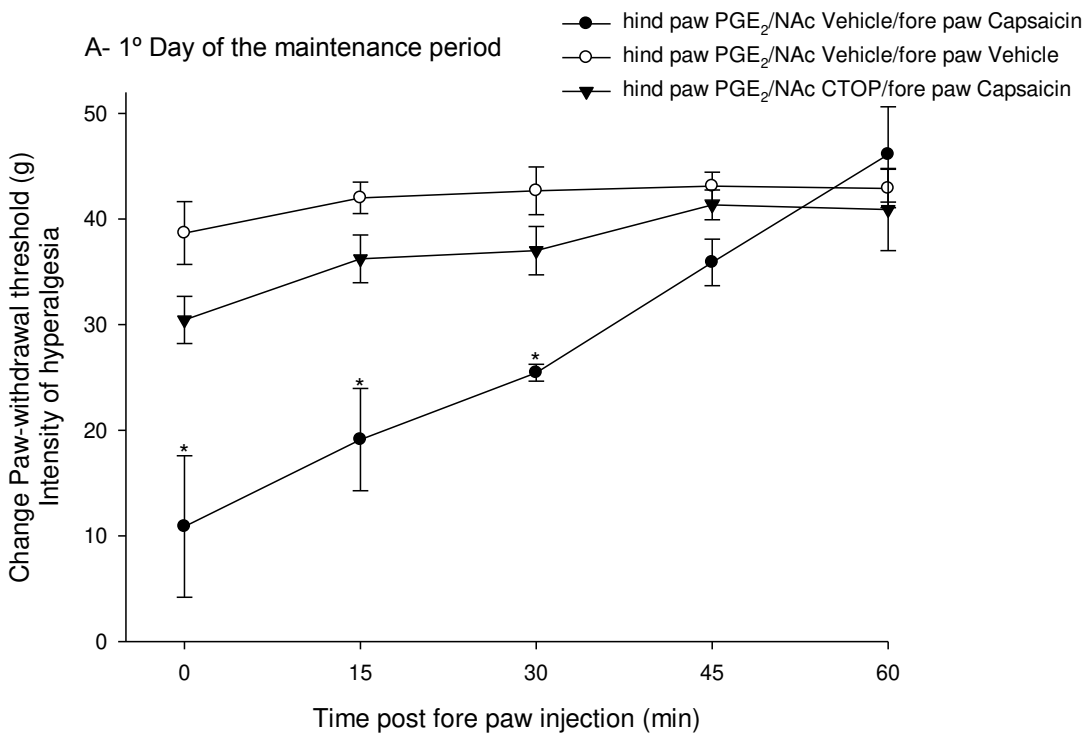
0
15
30
45
60
Time post fore paw injection (min)

**Figure 4. Effect of pain chronification on capsaicin-induced analgesia.** On day 1 (A) capsaicin-induced analgesia lasted for 60 min (Two-way repeated measures ANOVA, post hoc test Tukey, $p<0.05$). The symbol "*" indicates that capsaicin significantly reduced $PGE_2$-induced hyperalgesia for 60 min, an effect that was blocked by prior intra-accumbal administration of CTOP. On days 7 (B) and 14 (C) of the induction period of the persistent hyperalgesia model, the duration of capsaicin-induced analgesia was reduced from 60 to 30 min (Two-way repeated measures ANOVA, post hoc test Tukey, $p<0.05$). The symbol "*" indicates that capsaicin significantly reduced $PGE_2$-induced hyperalgesia for 30 min, an effect that was blocked by prior intra-accumbal administration of CTOP. Abbreviation: NAc=nucleus accumbens.

### *Maintenance period*

The subcutaneous injection of capsaicin (125 µg) into the rat's fore paw significantly ($p<0.05$) decreased the intensity of $PGE_2$-induced hyperalgesia for 30 min after its injection on days 1, 7, 14 (Figure 5A, B and C, respectively) and for 45 min on day 21 (Figure 5D) of the maintenance period of persistent hyperalgesia. The capsaicin-induced analgesia was prevented by intra-accumbal administration of the µ-opioid receptor antagonist CTOP (1.0µg) but not of its vehicle 10 min prior to the subcutaneous injection of capsaicin into the rat's fore paw ($p<0.05$) at the periods of time evaluated. In these experiments, each animal received a $PGE_2$ injection into the hind paw for 14 consecutive days for the installation of persistent hyperalgesia as described by Ferreira et al., 1990, and after discontinuation of the $PGE_2$ injection, on days 1, 7, 14 and 21 of the maintenance period of persistent hyperalgesia, the experiments were performed. Rat locomotor activity measured immediately after the last measurement of the paw withdrawal threshold was not significantly affected by these treatments ($p<0.05$) indicating that intra-accumbal treatments did not alter motor activity (data not shown). These results indicate that chronic pain reduced the duration of capsaicin-induced analgesia.

All injections of CTOP or of its vehicle were within nucleus accumbens as shown in Figure 6.


A- 1º Day of the maintenance period
hind paw PGE2/NAc Vehicle/fore paw Capsaicin
hind paw PGE2/NAc Vehicle/fore paw Vehicle
hind paw PGE2/NAc CTOP/fore paw Capsaicin
Change Paw-withdrawal threshold (g)
Intensity of hyperalgesia
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10
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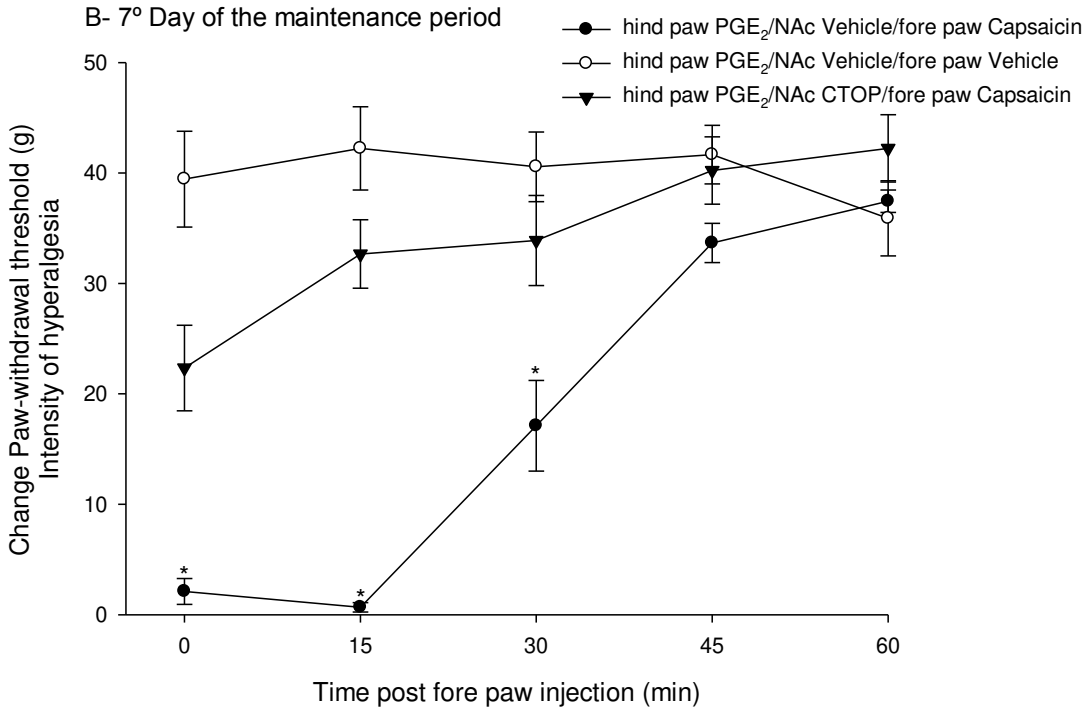
50
0
15
30
45
60
Time post fore paw injection (min)


B- 7º Day of the maintenance period
hind paw PGE2/NAc Vehicle/fore paw Capsaicin
hind paw PGE2/NAc Vehicle/fore paw Vehicle
hind paw PGE2/NAc CTOP/fore paw Capsaicin
Change Paw-withdrawal threshold (g)
Intensity of hyperalgesia
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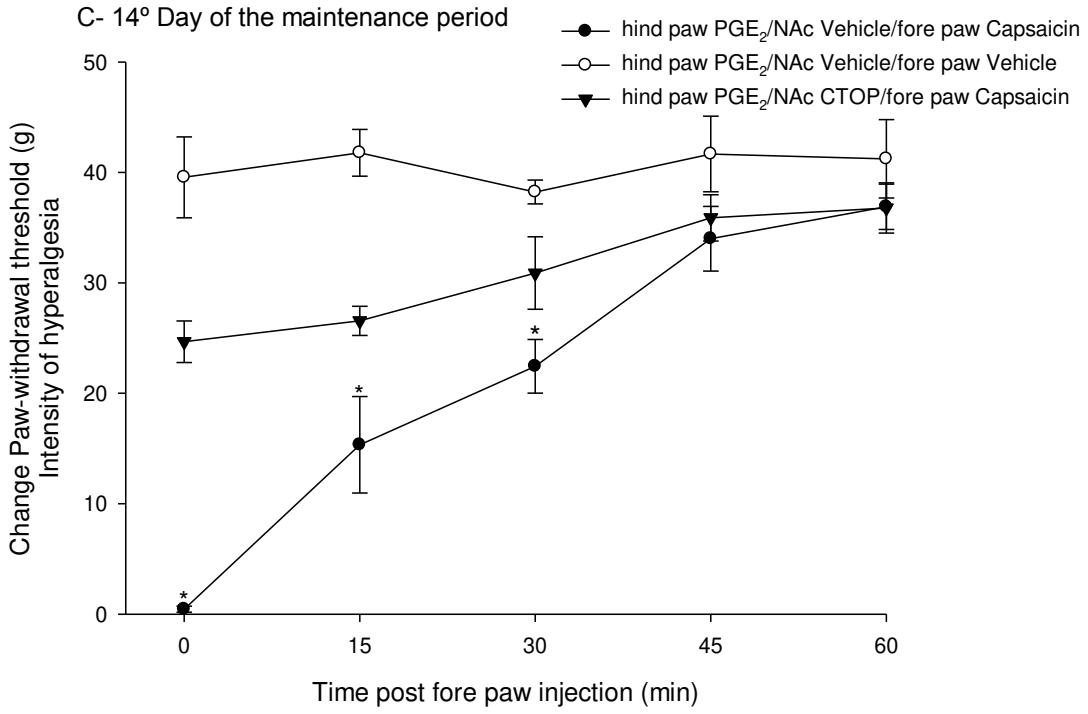
Time post fore paw injection (min)


C- 14º Day of the maintenance period
hind paw PGE2/NAc Vehicle/fore paw Capsaicin
hind paw PGE2/NAc Vehicle/fore paw Vehicle
hind paw PGE2/NAc CTOP/fore paw Capsaicin
Change Paw-withdrawal threshold (g)
Intensity of hyperalgesia
50
40
30
20
10
0
0
15
30
45
60
Time post fore paw injection (min)

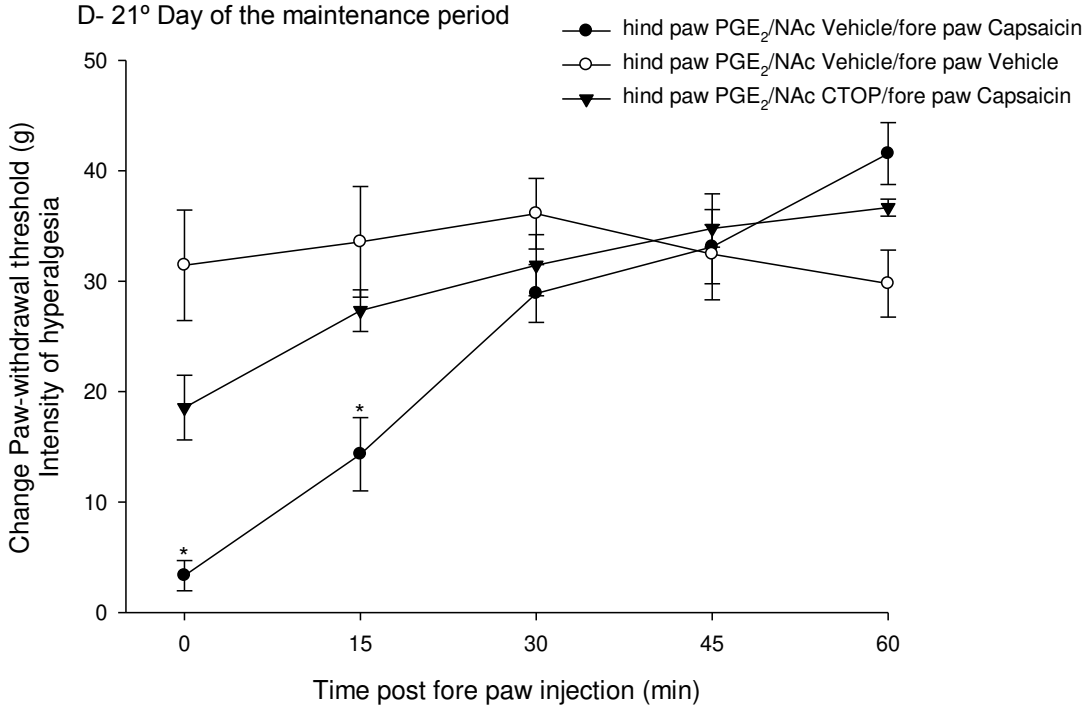

D- 21º Day of the maintenance period
hind paw PGE2/NAc Vehicle/fore paw Capsaicin
hind paw PGE2/NAc Vehicle/fore paw Vehicle
hind paw PGE2/NAc CTOP/fore paw Capsaicin
Change Paw-withdrawal threshold (g)
Intensity of hyperalgesia
50
40
30
20
10
0
0
15
30
45
60
Time post fore paw injection (min)

**Figure 5. Effect of chronic pain on capsaicin-induced analgesia.**

On days 1 (A), 7 (B) and 14 (C) capsaicin-induced analgesia lasted for 30 min (Two-way repeated measures ANOVA, post hoc test Tukey, $p<0.05$). The symbol "*" indicates that capsaicin significantly reduced $PGE_2$-induced hyperalgesia for 30 min, an effect that was blocked by prior intra-accumbal administration of CTOP. On day 21 (D) of the maintenance period of the persistent hyperalgesia model, the duration of capsaicin-induced analgesia was reduced from 30 to 15 min (Two-way repeated measures ANOVA, post hoc test Tukey, $p<0.05$). The symbol "*" indicates that capsaicin significantly reduced $PGE_2$-induced hyperalgesia for 45 min, an effect that was blocked by prior intra-accumbal administration of CTOP. Abbreviation: NAc=nucleus accumbens.

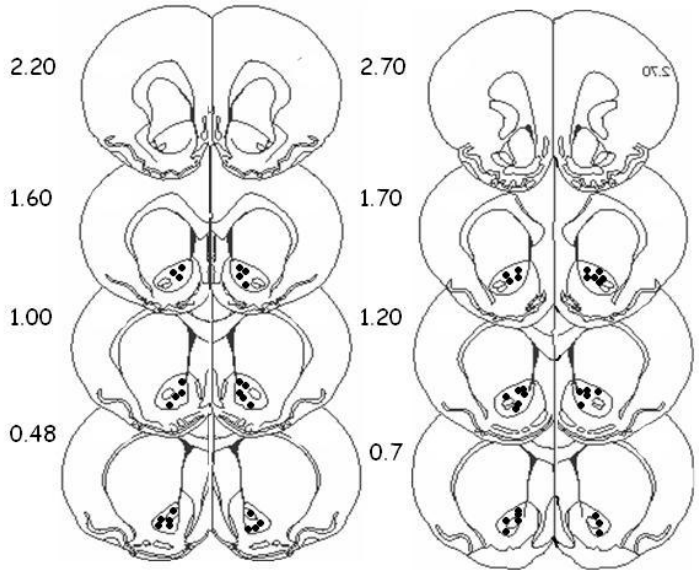



**Figure 6. Location of the injection sites in the nucleus accumbens.** All injection sites were within the nucleus accumbens. Coronal sections were taken from a brain atlas (Paxinos & Watson, 1986) to demonstrate the areas of the injection sites (filled circles). The numbers on the left refer to the distance in mm, rostral to bregma.

## 3.4- Western blotting analysis

Persistent hyperalgesia from its induction to its maintenance period did not affect the expression of μ-opioid receptors in nucleus accumbens.

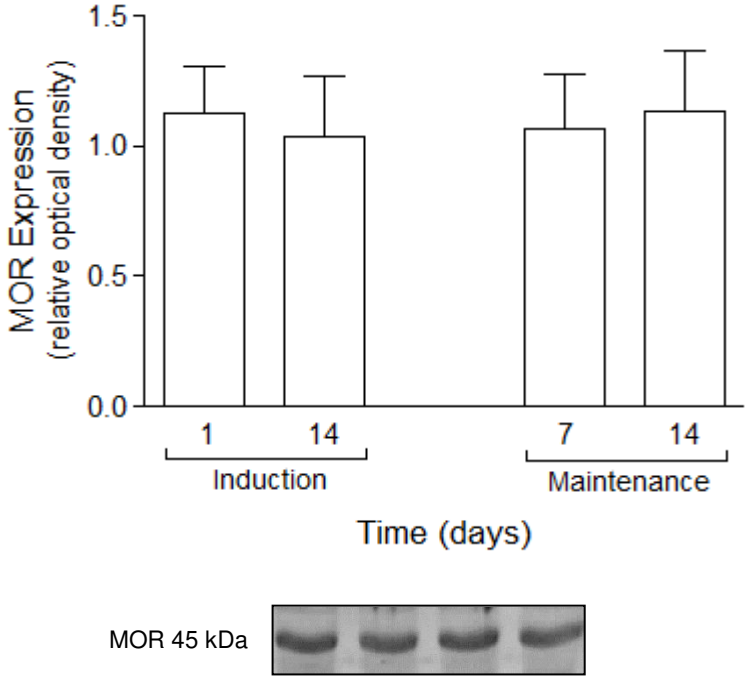



**Figure 7.** Western blot revealed no significant difference in μ-opioid receptor (MOR) expression in the nucleus accumbens of rats during the induction (days 1 and 14) and maintenance period (days 7 and 14) of the persistent hyperalgesia. Graph represents the ratio of MOR bands optical density. Data are presented as mean ± S.E.M. (n=4 per group). One-way ANOVA followed by Tukey test (p>0,05). Representative MOR bands of each group are displayed under the graph.

## 4- Discussion

In this study, by using a model of pain chronification we showed that the pain chronification and the chronic pain reduce the activity of an endogenous analgesia circuit, the ascending nociceptive control. Specifically, at different stages of the pain chronification process such as at the $7^{th}$ and $14^{th}$ day of the induction period of the persistent hyperalgesia and at the $1^{rst}$ , $7^{th}$, $14^{th}$ and $21^{th}$ days of the maintenance period of the persistent hyperalgesia (after the cessation of the 14 successive daily subcutaneous injections of $PGE_2$ into the rat's hind paw) analgesia was markedly shortened. These findings are consistent with recent evidences that in other chronic pain models (i.e., alcoholic neuropathy, late-phase oxaliplatin neuropathy, and chronic unpredictable stress) the chronic pain reduces the

activity of the ascending nociceptive control (Ferrari et al., 2010). However, our findings are the first to show attenuation in the activity of an endogenous analgesia circuit during the process of pain chronification indicating that the pain chronification can produce functional changes in endogenous analgesia circuits that may contribute to the maintenance of the chronic pain.

Another novelty of this study is that the pain chronification and the chronic pain do not affect the requirement for nucleus accumbens opioidergic neurotransmission in capsaicin-induced analgesia. This is supported by our findings that intra-accumbal administration of CTOP blocked capsaicin-induced analgesia in both the induction and maintenance period of the persistent hyperalgesia. Although multiple supra spinal mechanisms contribute to capsaicin-induced analgesia, the activation of μ-opioid receptors in nucleus accumbens (Gear *et al*., 1999; Schmidt *et al*., 2002a, 2003) is one of the most studied. The finding that μ-opioid receptors in nucleus accumbens still mediate capsaicin-induced analgesia during the pain chronification and the chronic pain indicate that a supraspinal μ-opioid receptor mechanism may be effective in the management of chronic pain.

The pain chronification and the chronic pain may have shortened capsaicin-induced analgesia by several mechanisms. One is a change in the expression and/or in the functional activity of intra-accumbens μ-opioid receptors. For example, it was demonstrated that the antinociceptive effect of the μ-opioid receptor agonist DAMGO, is significantly reduced in the locus coeruleus under persistent inflammatory pain due to a decrease in the expression of μ-opioid receptors in the locus coeruleus (Jongeling *et al*., 2009). However, our finding that the persistent hyperalgesia from its induction to its maintenance period did not affect the expression of μ-opioid receptors in nucleus accumbens do not support this hypothesis. Alternatively, the pain chronification and the chronic pain may have shortened capsaicin-induced analgesia by shortening the endogenous opioid release duration in nucleus accumbens, but future experiments will test this hypothesis.

Another mechanism by which the pain chronification and the chronic pain may have shortened capsaicin-induced analgesia is the increase of the nucleus accumbens pro-nociceptive efferent activity. It has been recently proposed that the output of nucleus

accumbens is pronociceptive (Gear and Levine, 2011). For example, intra-accumbens injection of quaternary lidocaine (QX-314) attenuates the jaw-opening reflex while intra-accumbens injection of the excitatory amino acid agonist kainate enhances this reflex response. The activation of endogenous opioids in nucleus accumbens produces antinociception by inhibiting this pro-nociceptive efferent activity (Gear and Levine, 2011). Importantly, enhancing nucleus accumbens efferent activity facilitates nociception, raising the possibility that such modulation of activity in nucleus accumbens could contribute to acute or chronic pain syndromes.

Finally, the pain chronification and the chronic pain may have shortened capsaicin-induced analgesia by other mechanisms implicated in this form of analgesia in nucleus accumbens, spinal cord and/or rostral ventral medulla. In nucleus accumbens, in addition to μ-opioid receptors other receptors such as δ-opioid receptors (Schmidt *et al*., 2002a), nicotinic receptors (Schmidt *et al*., 2001; Gear & Levine, 2009) and dopamine receptors (Gear *et al*., 1999) contribute to capsaicin-induced antinociception. In the spinal cord, it has been previously proposed that ongoing glutamate-driven activity tonically suppress an antinociceptive mechanism in nucleus accumbens, and that inhibition of this spinal activity disinhibits accumbens-mediated antinociception (Tambeli *et al*., 2002). Furthermore, it was demonstrated that capsaicin-induced analgesia is mediated by spinal excitation through the activation of excitatory receptors such as AMPA/KAINATE glutamate receptors and mGluR 1 receptors (Tambeli, 2003a) and spinal inhibition through the activation of μ- and κ-opioid receptors and GABA receptors (Tambeli *et al*., 2003b). Based on these findings, it was proposed that capsaicin-induced analgesia is mediated by the release of opioids and GABA in the spinal cord, which, in turn, inhibits tonic pro-nociceptive spinal activity to produce heterosegmental antinociception mediated in nucleus accumbens. Finally, in rostral ventral medulla, nicotinic acetylcholine receptors are also involved in capsaicin-induced analgesia (Gear and Levine, 2009). Therefore, important questions that remain to be investigated are whether the pain chronification and the chronic pain affect these mechanisms to attenuate capsaicin-induced analgesia duration and whether they affect the endogenous analgesia induced by the activation of others endogenous pain modulation circuits such as the diffuse noxious inhibitory control (DNIC) (Le Bars *et al*., 1992,

Villanueva & Le Bars, 1995) and descending pain modulation circuits (Porreca, et al., 2002, Vanegas and Schaible, 2004).

It has been previously demonstrated that the ascending nociceptive control contributes to acupuncture analgesia. Therefore our findings that the pain chronification and the chronic pain attenuate the activity of this endogenous analgesia circuit suggest that they can degrade the efficacy of acupuncture and help to explain the variable efficacy and the short-lasting benefits (Wang et al., 2008) of acupuncture in some chronic pain conditions.

In summary, we provide evidence that the pain chronification and the chronic pain induce a dysfunction in the ascending nociceptive control without affecting an important mechanism mediating this form of endogenous analgesia, that is the requirement for nucleus accumbens opioidergic neurotransmission. The attenuation of a form of endogenous analgesia during the pain chronification and the chronic pain suggests that endogenous pain circuits play an important role in the development and maintenance of chronic pain. Therefore, the study of the mechanisms by which the endogenous analgesia is attenuated during pain chronification and chronic pain is an important challenge in research to improve clinical treatment of chronic pain and to decrease the risk of chronic pain development.

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## CONCLUSÃO

Este estudo demonstrou que durante os períodos de indução e manutenção da hiperalgesia persistente ocorre uma diminuição na duração da analgesia pela ativação de um circuito de modulação endógena de dor denominado controle nociceptivo ascendente, de maneira que a analgesia induzida pela ativação desse mecanismo de modulação de dor teve sua duração reduzida a partir do 7º dia do período de indução da hiperalgesia persistente. Outro achado importante foi que a analgesia induzida pela ativação do controle nociceptivo ascendente continua dependendo dos receptores μ-opióide intra-accumbens, uma vez que antagonistas destes receptores bloquearam o efeito analgésico induzido pela ativação do controle nociceptivo ascendente.

Uma vez que a cronificação da dor é um dos maiores desafios em pesquisa na área e os tratamentos utilizados apresentam sucesso terapêutico limitado, este estudo demonstrou que os circuitos de modulação endógena de dor tem sua eficácia diminuída durante a transição da dor aguda para a persistente, sugerindo que os mesmos desempenham um importante papel no processo de cronificação da dor. O estudo de qual mecanismo é alterado durante a cronificação da dor pode possibilitar um aumento na duração da analgesia mediada pelos mecanismos de controle de dor endógenos e aumentar a eficácia dos efeitos analgésicos das terapias contra-irritação utilizadas atualmente para a dor crônica, como a acupuntura e a capsaicina que induzem analgesia através da ativação do controle nociceptivo ascendente, além de diminuir o risco de desenvolvimento da dor crônica.

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## ANEXO 1

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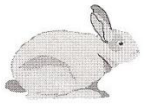

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**C E R T I F I C A D O**

Certificamos que o Protocolo nº **1952-1**, sobre **"Papel do controle nociceptivo ascendente na hiperalgesia persistente em ratos"**, sob a responsabilidade de **Profa. Dra. Claudia Herrera Tambeli / Josiane Miranda**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **05 de outubro de 2009**.

**C E R T I F I C A T E**

We certify that the protocol nº **1952-1**, entitled **"The role of ascending nociceptive control in persistent hyperalgesia in rats"**, 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 **October 5, 2009**.

Campinas, 05 de outubro de 2009.

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