SIMONE MONALIZA SILVA LAMANA

“EFFECT OF PERSISTENT HYPERALGESIA ON THE DEFENSIVE BEHAVIOR OF RATS AND IN THE ABILITY OF AN ENDOGENOUS ANALGESIA CIRCUIT TO MODULATE IT”

“EFEITO DA HIPERALGESIA PERSISTENTE NO COMPORTAMENTO DEFENSIVO DE RATOS E NA CAPACIDADE DE UM CIRCUITO DE ANALGESIA ENDÓGENO EM MODULÁ-LO”

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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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ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA DISSERTAÇÃO
DEFENDIDA PELA ALUNA SIMONE MONALIZA SILVA LAMANA
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Dedico esse trabalho,

*À **Deus***

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"É melhor tentar e falhar,

que preocupar-se e ver a vida passar.

É melhor tentar, ainda que em vão,

que sentar-se fazendo nada até o final.

Eu prefiro na chuva caminhar,

que em dias tristes em casa me esconder.

Prefiro ser feliz, embora louco,

que em conformidade viver"

(Martin Luther King)

RESUMO

A resposta de imobilidade dorsal é um comportamento defensivo inato, caracterizado por um estado temporário de profunda e reversível inibição motora, induzido por algumas formas de restrição física. Quando um animal está em uma situação de perigo ele apresenta comportamentos defensivos para tentar assegurar sua sobrevivência. No entanto, não se sabe se a hiperalgesia inflamatória persistente (HIP) poderia afetar o comportamento defensivo de imobilidade dorsal (CDID) em ratos. Recentemente, foi demonstrado por nosso grupo de pesquisa que a dor aguda periférica, por meio da ativação de um circuito neural endógeno de modulação de dor conhecido por controle nociceptivo ascendente (ANC), potencializa a duração CDID em ratos por um mecanismo opióide-dependente no núcleo accumbens (NAc). No entanto, também não se sabe se a HIP afeta esse processo. Nesse contexto, o objetivo do presente estudo foi investigar se a HIP afeta a duração do CDID e a modulação que a dor aguda periférica intensa, via ativação do ANC, exerce neste comportamento defensivo. A HIP foi induzida por um modelo experimental no qual a hiperalgesia dura por aproximadamente 30 dias após o término da administração diária de prostaglandina E_2 no tecido subcutâneo da pata traseira de ratos por 14 dias. O CDID foi induzido através da suspensão do rato adulto pela pele da nuca. Para investigar se a HIP afeta o CDID, este foi avaliado nos dias 1, 7 e 14 de administração de prostaglandina E_2 (período de indução da HIP) ou seu veículo no tecido subcutâneo da pata traseira de ratos e nos dias 1, 7, 14 e 21 após a interrupção do tratamento com a prostaglandina E_2 (período de manutenção da HIP). Para investigar se a HIP afeta a modulação que a dor aguda periférica intensa, via ativação do ANC, exerce sobre o CDID, a capsaicina foi administrada subcutaneamente na pata dianteira para induzir estimulação nociceptiva periférica e ativar o ANC nos dias 1, 7, e 14 de administração de PGE_2 e nos dias 1, 7, 14 e 21 após a interrupção do tratamento com a PGE_2. Dez minutos antes da administração de capsaicina o antagonista do receptor opióide μ, CTOP ou seu veículo, foi administrado no NAc e imediatamente após a administração da capsaicina, foi avaliado o CDID e a atividade locomotora dos ratos no equipamento Rota-rod. Os resultados demonstram, pela primeira vez, que a HIP reduz a duração do CDID em ratos em ambos os períodos de indução e manutenção da HIP. Além disso, demonstram que a estimulação nociceptiva periférica intensa, via ativação do ANC, aumenta a duração do

CDID em ratos durante os períodos de indução e manutenção da HIP, efeito que é prevenido pela administração prévia do antagonista de receptor opióide μ, CTOP (Cys^2, Tyr^3, Orn^5, Pen^7amide) intra-NAc. Esses resultados sugerem que a HIP reduz a duração do CDID, mas não afeta a capacidade de mecanismos endógenos de modulação de dor, como o ANC, em facilitar comportamentos defensivos.

Palavras-chave: comportamento defensivo, comportamento de imobilidade dorsal, hiperalgesia inflamatória persistente, núcleos accumbens, estimulação nociceptiva periférica, controle nociceptivo ascendente.

ABSTRACT

The dorsal immobility response is an inborn defensive behavior characterized by a temporary state of profound and reversible motor inhibition induced by some forms of physical restraint. When the animal is in a dangerous situation it needs to engage in defensive responses without interference from the motivational conflicts to engage in recuperative behaviors. However, it is unclear whether the persistent inflammatory hyperalgesia (PIH) could affect the defensive behavior of dorsal immobility (DBDI) in rats. Recently, our research group has demonstrated that acute peripheral pain through activation of endogenous neural circuitry of pain modulation known for ascending nociceptive control (ANC), enhances the duration of the DBDI in rats through a mechanism opioid-dependent on the nucleus accumbens (NAc). Moreover, the PIH could be the conflict source. Based on this, the proposal of the present study was to investigate whether PIH affects the DBDI and the modulation that intense acute peripheral pain, via ANC activaction, exerts on this defensive behavior. The PIH was induced by using an experimental model in which hyperalgesia lasting for about 30 days after the daily administration of prostaglandin E_2 (PGE_2) for 14 days in the subcutaneous tissue of the rats' hind paw. The DBDI was induced by suspending the adult rat the skin of the nape. Investigating whether PIH affects the DBDI, immobility dorsal behavior was evaluated on days 1, 7, and 14, prostaglandin E_2 (induction period of PIH) or its vehicle in the subcutaneous tissue of the rats' hind paw and on days 1, 7, 14, and 21 after cessation of treatment with prostaglandin E_2 (maintenance period of PIH). Investigating whether PIH affects modulation of the intense peripheral acute pain, via activation of ANC, carries on the DBDI, capsaicin was administered subcutaneously in the fore paw to induce peripheral nociceptive stimulation and activate the ANC on days 1, 7, and 14 prostaglandin E_2 and on days 1, 7, 14, and 21 after discontinuation of PGE_2. Ten minutes before administration of the capsaicin μ-opioid receptor antagonist, CTOP, or its vehicle was administered in the NAc and immediately after administration of capsaicin, the dorsal immobility response was evaluated, and the locomotor activity of the animals in the equipment Rota-rod. The results demonstrate for the first time that PIH decreases the duration of DBDI in rats in both induction and maintenance periods of PIH. They also show that intense peripheral nociceptive stimulation via activation ANC increases the duration of dorsal immobility response in rats during periods of

induction and maintenance of PIH, and that this effect is prevented by the prior intra-accumbal administration of the μ-opioid receptor antagonist, CTOP (Cys^2, Tyr^3, Orn^5, Pen^{7}amide). These results suggest that PIH affects the DBDI, by reducing its duration, but does not affect the ability of endogenous pain modulation mechanisms, such as the ANC, to facilitate defensive behaviors.

Key words: defensive behavior, dorsal immobility behavior, persistent inflammatory hyperalgesia, nucleus accumbens, peripheral nociceptive stimulation, ascending nociceptive control.

SUMÁRIO

INTRODUÇÃO

A dor é definida pela Associação Internacional para o Estudo da Dor (IASP) (do inglês, *International Association for the Study of Pain*) como sendo "uma experiência emocional e sensorial desagradável associada com uma lesão tecidual real ou potencial ou descrita em termos de tal lesão". É uma experiência complexa e que não envolve apenas a transdução de estímulo nocivo ambiental, mas também o processamento cognitivo e emocional pelo encéfalo (Chapman *et al*., 1999; Julius *et al*., 2001; Almeida *et al*., 2004). O fenômeno doloroso, portanto, possui dois componentes: um componente sensorial-discriminativo, que permite identificar e localizar o estímulo doloroso; e outro que envolve a aprendizagem, memória, e que pode estar associado a mecanismos de recompensa, e a comportamentos defensivos, como de fuga/luta, sendo denominado de componente aversivo-cognitivo-motivacional (Melzack, 1975; Almeida *et al*., 2004).

A dor pode ser classificada em aguda ou crônica. A dor aguda pode ser causada por um estímulo nociceptivo ocorrido devido a uma lesão, por uma doença ou funcionamento anormal de um músculo ou víscera (Russo & Brose, 1998), sendo caracterizada por uma resposta defensiva protetora, pois alerta o indivíduo para uma lesão iminente ou real dos tecidos, induzindo respostas reflexas e comportamentais coordenadas com o objetivo de evitar ferimentos ou a progressão de uma lesão já ocorrida (Woolf *et al*., 1999). Desta forma é fundamental para a sobrevivência, pois induz no indivíduo reações de defesa, fuga ou remoção do agente causal. No entanto, quando a dor passa a se repetir ou sustentar-se por período prolongado, persistindo por semanas, meses ou até anos é classificada como dor crônica (Russo & Brose, 1998).

Na natureza os animais apresentam reações de defesa em resposta a uma variedade de situações de perigo que podem comprometer sua integridade física ou até mesmo sua sobrevivência, como o confronto com predadores e animais da mesma espécie em competições, em situações de dor e estímulos ambientais aversivos. Diante dessas situações a estratégia defensiva adotada depende de fatores como as características do ambiente e sua familiaridade, a proximidade do estímulo, as experiências anteriores em situações semelhantes, a posição do indivíduo na hierarquia social do seu grupo. Geralmente os animais utilizam uma de quatro estratégias defensivas comportamentais básicas, conhecidas como fuga, imobilidade, ataque

defensivo e submissão (Adams, 1979 e Marks, 1994 apud Zangrossi; Graeff, 2004; Blanchard & Blanchard, 1988).

O comportamento de imobilidade é uma resposta inata desencadeada em muitas espécies de vertebrados e invertebrados por diferentes estímulos sensoriais (Klemm, 1971; Webster *et al*., 1981), sendo também conhecida como congelamento, hipnose animal ou fingir-se de morto (Ratner, 1967; Thompson *et al*., 1981). É caracterizado pelo estado reversível de uma profunda inibição motora e relativa perda da capacidade de resposta ao ambiente (Vieira *et al*., 2011; Volchan *et al*., 2011) que ocorre durante o confronto presa-predador. O comportamento de imobilidade geralmente é desencadeado em situações que geram medo intenso (Zamudio *et al*., 2005; Michelan *et al*., 2006; Vieira *et al*., 2011), com o objetivo de proteger o animal de ataques por predadores (Klemm, 1971; Thompson *et al*., 1981; Gallup & Rager 1996; Monassi *et al*., 1999), pois a falta do movimento frequentemente interrompe o ataque promovendo uma oportunidade de fuga (Sargeant *et al*., 1975; Thompson *et al*., 1981), uma vez que, o debater da presa parece ser necessário para que o predador continue o ataque (Thompson *et al*., 1981). Eventos traumáticos que envolvem ameaça à vida e são acompanhados de medo intenso também têm o poder de evocar a imobilidade em humanos (Heidt *et al*., 2005; Rocha-Rego *et al*., 2009; Volchan *et al*., 2011), por exemplo, a violência urbana ou sexual.

A imobilidade dorsal pode ser facilmente desencadeada no laboratório suspendendo o rato adulto pela pele da nuca e elevando-o no ar (Smith & Meyer, 1985), na tentativa de simular a restrição física e o medo intenso que ocorre durante o ataque do predador (Leite-Panissi *et al*., 2001). Nessa condição, o animal exibe imediatamente uma postura imóvel estereotipada, que persiste até o mesmo apresentar comportamentos de fuga (Smith & Meyer, 1985; Pellis *et al*, 1990; Meyer *et al*., 1993). Este tipo de manobra enfatiza as sensações táteis e proprioceptivas que são importantes para a indução deste tipo de comportamento (Vieira *et al*., 2011).

A modulação da dor pelo sistema nervoso central consiste na inibição ou facilitação da excitabilidade de neurônios do corno dorsal espinhal. Estímulos ambientais fisiologicamente relevantes tais como os estímulos nociceptivos, em particular estímulos nociceptivos intensos, como a injeção subcutânea de capsaicina ou

a imersão da pata do rato em água quente, produzem profunda antinocicepção heterossegmental como demonstrado previamente por Gear *et al.*(1999).

Dados da literatura indicam a existência de um potente sistema de modulação da dor capaz de produzir antinocicepção equivalente à induzida por 10 mg/Kg de morfina com duração de aproximadamente 3 horas (Gear *et al*., 1999; Schmidt *et al.,* 2002; Tambeli *et al*., 2003a; Tambeli *et al.,* 2003b; Tambeli *et al*., 2009). Esse sistema de modulação de dor denominado de **controle nociceptivo ascendente,** origina-se na medula espinhal e ascende da mesma para o núcleo accumbens (Gear & Levine, 1995). O controle nociceptivo ascendente medeia uma forma heterossegmental de analgesia induzida pela dor conhecida como antinocicepção induzida por um estímulo nociceptivo (Gear *et al*., 1999; Tambeli *et al.*, 2003b, 2009), que inclui mecanismos específicos na medula espinhal (Tambeli et al., 2003b, 2009), colinérgicos no bulbo rostroventral (RVM) (Gear & Levine, 2009) e opióides endógenos no núcleo accumbens (Schmidt et al., 2001, 2003) o maior componente do estriado ventral. Há evidências que sob condições fisiológicas basais, na ausência de uma estimulação nociceptiva, há uma atividade tônica excitatória mediada pelo glutamato que ascende da medula espinhal e inibe a antinocicepção mediada pelo núcleo accumbens (Tambeli *et al*., 2009). 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 heterossegmental, ou seja atenua profundamente a dor aguda (reflexo de abertura bucal). Foi demonstrado também que a estimulação nociceptiva periférica, tal como a induzida pela administração subcutânea de capsaicina, ativa um mecanismo espinhal inibitório (Tambeli *et al*., 2003b), o qual, por sua vez, inibe a atividade neural tônica excitatória (Gear & Levine, 1995) na medula espinhal (Tambeli *et al*., 2002), o que por sua vez desinibe mecanismos opiodérgico/dopaminérgico no núcleo accumbens resultando em antinocicepção heterossegmental (Tambeli *et al*., 2009). Os mecanismos espinhais ativados pela

estimulação nociceptiva periférica incluem mecanismos excitatórios mediados por receptores de NMDA e $mGluR_5$, mas não por receptores $mGluR_1$, e inibitórios mediados por receptores (μ e κ-opióide, $GABA_B$ e $GABA_A$) (Tambeli *et al.*, 2003b).

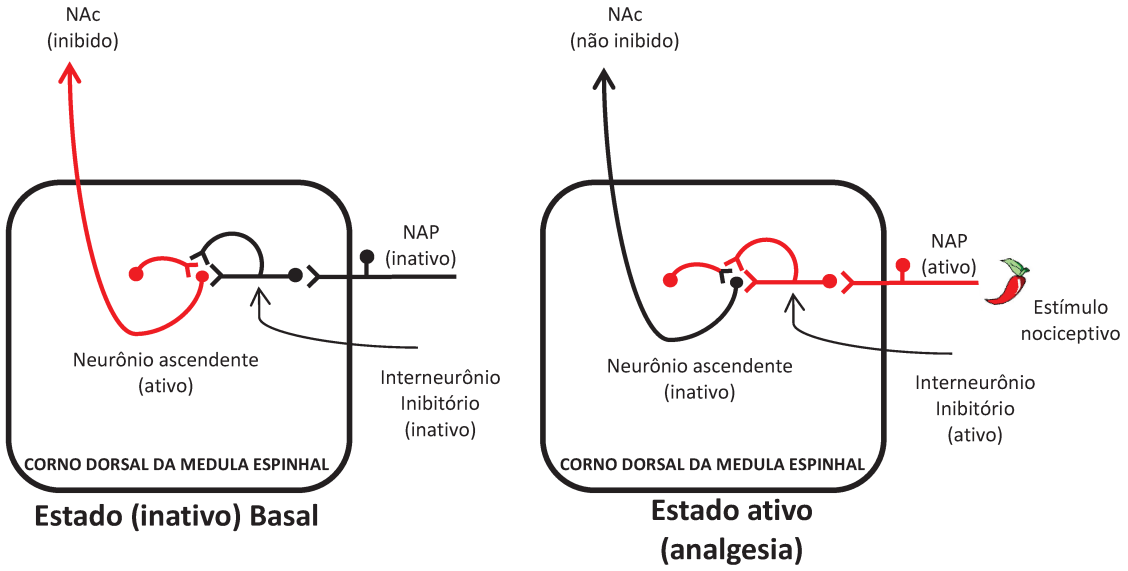

Figura 1: Diagrama esquemático do circuito espinhal envolvido no controle nociceptivo ascendente. Sob condições fisiológicas basais, na ausência de uma estimulação nociceptiva, o neurônio tonicamente ativo (em vermelho) projeta-se supraespinhalmente para inibir a antinocicepção mediada pelo núcleo accumbens. 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/kainate 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.

A analgesia induzida pela ativação do controle nociceptivo ascendente se assemelha ao Controle Inibitório Difuso (DNIC, do inglês, *Diffuse Noxious Inhibitory Controls*), pois ambos são dependentes do estímulo nociceptivo. No entanto, ao contrário do controle nociceptivo ascendente, o DNIC não depende do núcleo accumbens (Le Bars & Villanueva, 1988; Villanueva *et al.*, 1988). Além disso, a analgesia induzida pela ativação do DNIC tem duração menor que 5 (cinco) minutos, na

maioria dos estudos, e é dependente da presença do estímulo nocivo (Le Bars *et al*., 1979), enquanto aquela induzida pelo controle nociceptivo ascendente dura em torno de 2.5 h, e persiste mesmo após bloqueio neural periférico.

Tem sido proposto que quando a segurança do organismo está ameaçada acionam-se respostas comportamentais defensivas, tais como a imobilidade dorsal, ativando mecanismos analgésicos endógenos responsáveis pela redução das respostas nociceptivas (Leite-Panissi *et al*., 2001; Rhudy & Meagher, 2000) de modo a prevenir comportamentos recuperativos gerados pela lesão tecidual (Fanselow, 1984). Por outro lado, a ativação de sistemas de modulação da dor endógenos, facilita comportamentos defensivos. Por exemplo, a estimulação nociceptiva periférica, via ativação do controle nociceptivo ascendente, aumenta a duração do comportamento de imobilidade dorsal em ratos por um mecanismo opióide-dependente no núcleo accumbens (Tambeli *et al*., 2012) Além do núcleo accumbens, outras estruturas envolvidas na modulação da dor, como a substância cinzenta periaquedutal (PAG) *(*Monassi *et al*., 1997), região parabraquial (Menescal-de-Oliveira & Hoffmann, 1993) e amígdala (Ramos *et al*., 1999) também modulam o comportamento defensivo (Harris, 1996) com o objetivo de aumentar as chances de sobrevivência em situações de perigo.

A dor crônica, ao contrário da dor aguda, não tem função de defesa nem de alerta para a preservação da vida e é mediada por mecanismos de adaptação que induzem à incapacidade e a repercussões biopsicossociais desfavoráveis (Teixeira, 2006). Apesar de estar associada a uma diminuição da capacidade de se defender contra uma situação perigosa, não há evidência experimental na literatura de que a dor crônica afeta comportamentos defensivos.

Tem sido recentemente demonstrado que a dor crônica pode reduzir a capacidade de modulação da dor dos circuitos de analgesia endógena, tais como o controle nociceptivo ascendente, através da diminuição da duração do efeito antinociceptivo desencadeado pela ativação do mesmo (Ferrari *et al*, 2010; Miranda *et al*., 2011). No entanto, não é conhecido se isso afeta a capacidade desse circuito em modular comportamentos defensivos.

CAPÍTULO

O presente foi submetido ao periódico "Behavioural Brain Research".

PERSISTENT INFLAMMATORY HYPERALGESIA AFFECTS A DEFENSIVE BEHAVIOR, BUT NOT THE ABILITY OF AN ENDOGENOUS ANALGESIA CIRCUIT TO MODULATE IT

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Original Article

Key words: defensive behavior, dorsal immobility behavior, persistent inflammatory hyperalgesia, nucleus accumbens, peripheral noxious stimulation, ascending nociceptive control.

Abstract

Noxious stimulus-induced antinociception potentiates the duration of the defensive behavior of dorsal immobility in rats via an opioid-dependent mechanism in the nucleus accumbens. The dorsal immobility response is an inborn defensive behavior characterized by a temporary state of profound and reversible motor inhibition elicited by some forms of physical restraint. When the animal is in a dangerous situation it needs to engage in defensive responses without interference from the motivational conflicts to engage in recuperative behaviors. The persistent inflammatory pain could be the conflict source. Based on this, the proposal of the present study was to investigate whether persistent inflammatory hyperalgesia affects the duration of the dorsal immobility behavior and the modulation that noxious stimulus-induced antinociception exerts on defensive behavior. The results of this study demonstrate, for the first time, that chronic pain disrupts defensive behaviors by showing that persistent inflammatory hyperalgesia significantly decreases the duration of the dorsal immobility response in rats. However, chronic pain does not affect the ability of noxious stimulus-induced antinociception to facilitate defensive behaviors via an opioid-dependent mechanism in the nucleus accumbens. These findings suggest that chronic pain states may decrease the ability to engage in defensive behaviors, but not the ability of acute noxious stimulus-induced antinociception to facilitate these behaviors.

Key words: defensive behavior, dorsal immobility behavior, persistent inflammatory hyperalgesia, nucleus accumbens, peripheral noxious stimulation, ascending nociceptive control.

1- Introduction

Acute pain is a type of pain that can be described as a warning that an injury has occurred and a change in behavior is warranted. Defensive behaviors may be among the helpful behavioral changes in response to acute pain. They are a set of responses to threat stimuli and situations with the adaptive function of reducing harm to the threatened organism. The immobility response is an innate response of profound inactivity and relative lack of responsiveness to the environment [1] that occurs during

prey-predator confrontations. The immobility response may increase the chances of survival because the predator requires the prey to struggle for the attack to continue [2]. However, within a context of noxious stimulation, a concomitant decrease in pain perception could be decisive to prevent conflicting motivations to engage in recuperative behaviors in response to acute pain instead of an appropriate defensive response. We have previously characterized the interaction among acute pain, endogenous analgesia and defensive behaviors by showing that intense acute pain can activates endogenous pain modulation mechanisms that serve to control defensive responses in animals [3]. For example, noxious stimulus-induced antinociception (NSIA) induced by peripheral administration of capsaicin is mediated by a pain modulation system that ascend from the spinal cord to the nucleus accumbens in the ventral striatum [4]. NSIA potentiates the duration of the dorsal immobility response in rats via an opioid mechanism in the nucleus accumbens [3].

When acute pain persists beyond a reasonable time for tissue damage to heal, it becomes chronic [5]. Unlike acute, chronic pain has neither defense nor warning function for life preservation. Although it is associated to an impaired ability to defend itself against a dangerous situation, there is no experimental evidence in the literature that chronic pain affects defensive behaviors. It has been recently demonstrated that chronic pain can reduce the duration of NSIA [6,7], but whether it affects the ability of NSIA to modulate defensive behaviors is not known.

In this study, we asked whether chronic inflammatory pain affects defensive responses in rats and the ability of NSIA to modulate them. To answer this question, we used a model of persistent inflammatory hyperalgesia that lasts for at least 30 days following the cessation of 14 successive daily subcutaneous injections of prostaglandin E_2 (PGE_2) into the rat hind paw [8] which has been found to be representative of human chronic inflammatory pain [9], and the dorsal immobility response in rats as a model of defensive responses.

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. The Committee on Animal Research of the University of Campinas approved the experimental protocols, which conformed to the IASP guidelines for the study of pain in animals [10]. 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 hind paw used to induce persistent inflammatory 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 7 or 14 after initiating the PGE_2 injection into the rat hind paw (induction period) or on day 1, 7, 14, or 21 after discontinuing the PGE_2 injection (maintenance period of the persistent inflammatory hyperalgesia model). The dorsal immobility response was recorded immediately after the subcutaneous injection of capsaicin or its vehicle. The locomotor activity of animals was evaluated in the "rota-rod" equipment immediately after the dorsal immobility test to exclude the possibility that the effect of intra-accumbal treatments on the duration of dorsal immobility response was due to altered motor activity.

2.3- Persistent inflammatory hyperalgesia model.

PGE_2-induced persistent inflammatory hyperalgesia was induced as previously described [8]. Briefly, persistent hyperalgesia was induced by daily subcutaneously injection of PGE_2 (100 ng/50 μl/paw) in the dorsal surface of the rat hind paw over 14 days. In order to avoid a local release of PGE_2 caused by 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 inflammatory hyperalgesia persists for aproximately 30 days. Therefore, there are two well-defined periods in this persistent inflammatory 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 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 inflammatory hyperalgesia model. After the discontinuation of the PGE2 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 [11] in which a force that increases linearly over time is applied to the dorsum of the rat hind paw [12] (Ugo Basile Algesymeter, Stoelting). Mechanical nociceptive threshold was defined as the force in grams at which the rat withdrew its paw and as the mean of three readings performed immediately before the subcutaneous PGE_2 injection in the rat hind paw. A decrease in mechanical nociceptive threshold was indicative of hyperalgesia

Testing sessions took place during light phase (between 09:00 A.M. and 5:00 P.M.) in a quiet room maintained at 23ºC [13].

2.5- Dorsal immobility recording

The rats were gently held by the skin of the napes of their necks between the experimenter's thumb and index finger, lifted off of the ground and suspended in the air. The dorsal immobility response (DIR) duration was measured (in seconds) using a chronometer from the time of suspension until the rat performed escape-like movements [14,15]. The DIR duration was measured before any experimental intervention and immediately after the subcutaneous injection of capsaicin into the rat fore paw on days

1, 7 and 14 of the induction period and on days 1, 7, 14 and 21 of the maintenance period of the persistent inflammatory hyperalgesia model.

2.6- Rota-Rod

Motor function was measured 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 [16] model.

2.7- Drugs and doses

The following drugs were administered. Prostaglandin E_2, 100ng/50 µl/hind paw [8]; the cyclooxygenase (COX) inhibitor indomethacin, 2.0 mg/Kg [17]; E-capsaicin (capsaicin, 125µg/50 µl/fore paw) [4] and the µ-opioid receptor antagonist CTOP (Cys^2,Tyr^3, Orn^5,Pen^7amide) [18] which was administered into the nucleus accumbens (1.0 µg).

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.

2.8- 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 [19]. 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.

2.9- 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 [20]. 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 [20].

2.10- Data analysis

Data in Figure 1 is presented as absolute DIR duration. However, because DIR duration differed between groups during the induction and maintenance period of the persistent inflammatory hyperalgesia model, DIR duration data are presented as change in DIR duration in seconds to normalize the differences in Figures 2, 3 and 4. Change in DIR duration was calculated for days 7 and 14 of the induction period and for days 1, 7, 14 and 21 of the maintenance period of the persistent inflammatory hyperalgesia model by subtracting the DIR duration measured before any experimental

intervention performed on that day from DIR duration measured immediately after the capsaicin injection into the rat fore paw. A one-way analysis of variance (ANOVA) determined the significant ($p < 0.05$) differences in the responses between the groups. Student-Newman-Keuls post hoc tests determined the basis of the significant differences. Data are presented in figures as means ± SEM.

3- Results

3.1- Dorsal immobility behavior in the presence of persistent inflammatory 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 the persistent inflammatory hyperalgesia model, confirming the development of persistent inflammatory hyperalgesia (Figure 1A). Persistent inflammatory hyperalgesia significantly reduced the duration of dorsal immobility behavior (Figure 1B), indicating impairment of this defensive behavior.

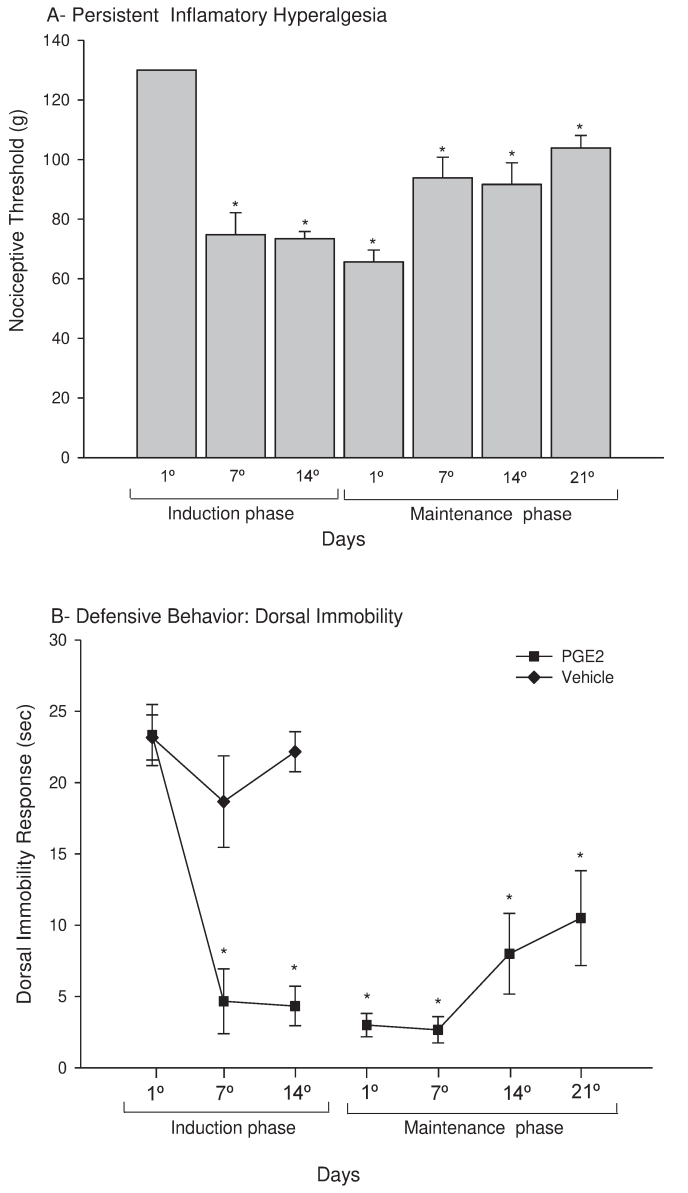

Figure 1

Figure 1. Effect of the progression of inflammatory hyperalgesia on the duration of the dorsal immobility behavior.

A- The temporal evolution of the basal mechanical threshold in the induction and maintenance period of the persistent inflammatory hyperalgesia model shows the development of persistent inflammatory hyperalgesia.

B- Persistent inflammatory hyperalgesia significantly reduced the duration of dorsal immobility behavior. The symbol "*" indicates a response significantly lower than that of group day 1 of the induction period of persistent inflammatory hyperalgesia (One-way Analysis of Variance, Student-Newman-Keuls Method, $p<0.05$). Data in this and subsequent figures are plotted as mean ± s.e.m, there are six animals per group.

3.2- Dorsal immobility behavior modulation by acute pain in the *absence* of persistent inflammatory hyperalgesia

Peripheral acute noxious stimulus induced by a subcutaneous injection of capsaicin (125 µg) but not of its vehicle into the rat fore paw significantly increased ($p<0.05$) the duration of the dorsal immobility behavior (Figure 2). This effect was prevented by intra-accumbal administration of the µ-opioid receptor antagonist CTOP (1.0µg) but not its vehicle 10 min prior to the injection of capsaicin, confirming that peripheral intense acute pain modulates the defensive behavior.

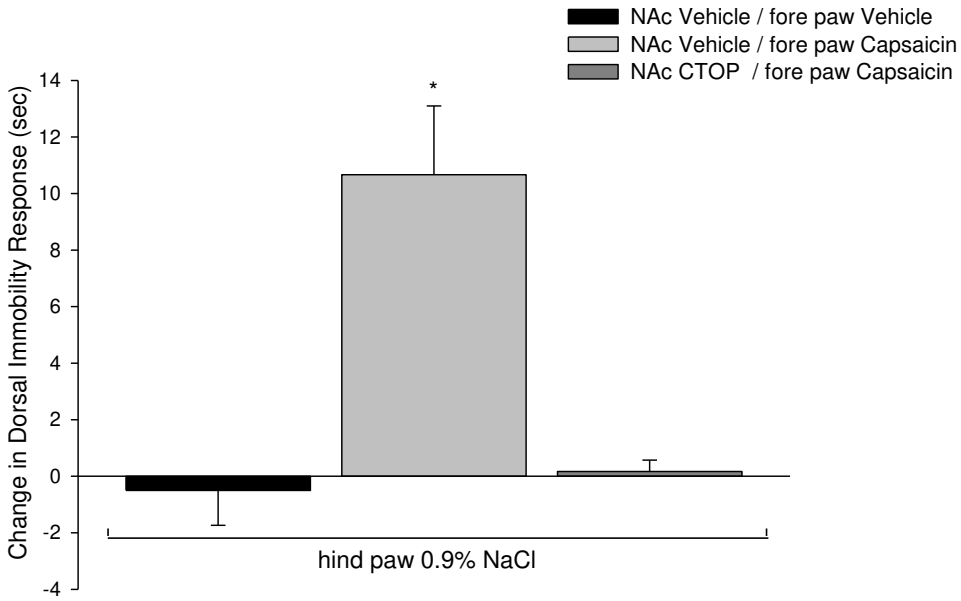

Figure 2

Figure 2. Effect of peripheral noxious stimulation on dorsal immobility behavior.
The symbol "*" indicates that the subcutaneous administration of capsaicin (125 µg) into the rat fore paw significantly increased the duration of the dorsal immobility behavior (One-way Analysis of Variance, Student-Newman-Keuls Method, $p<0.05$), an effect that was prevented by previous intra-accumbal administration of CTOP.

3.3- Dorsal immobility behavior modulation by acute pain in the *presence* of persistent inflammatory hyperalgesia

Induction period

Intense acute pain induced by a subcutaneous injection of capsaicin (125 µg) into the rat fore paw significantly increased ($p<0.05$) the duration of the dorsal immobility behavior on days 7 (Figure 3A) and 14 (Figure 3B) of the induction period of persistent inflammatory hyperalgesia. This effect was prevented by the intra-accumbal administration of the µ-opioid receptor antagonist CTOP (1.0µg) but not by its vehicle 10 min prior to the subcutaneous injection of capsaicin into the rat fore paw ($p<0.05$). In these experiments, the subcutaneous injection of PGE_2 into the rat hind paw was performed approximately 3 hours before the intra-accumbal injection. Rat locomotor activity measured immediately after the DIR recordings was not significantly affected by these treatments ($p>0.05$) indicating that intra-accumbal treatments do not alter motor activity. These results indicate that the induction of persistent inflammatory hyperalgesia does not affect the ability of NSIA to modulate the dorsal immobility behavior.

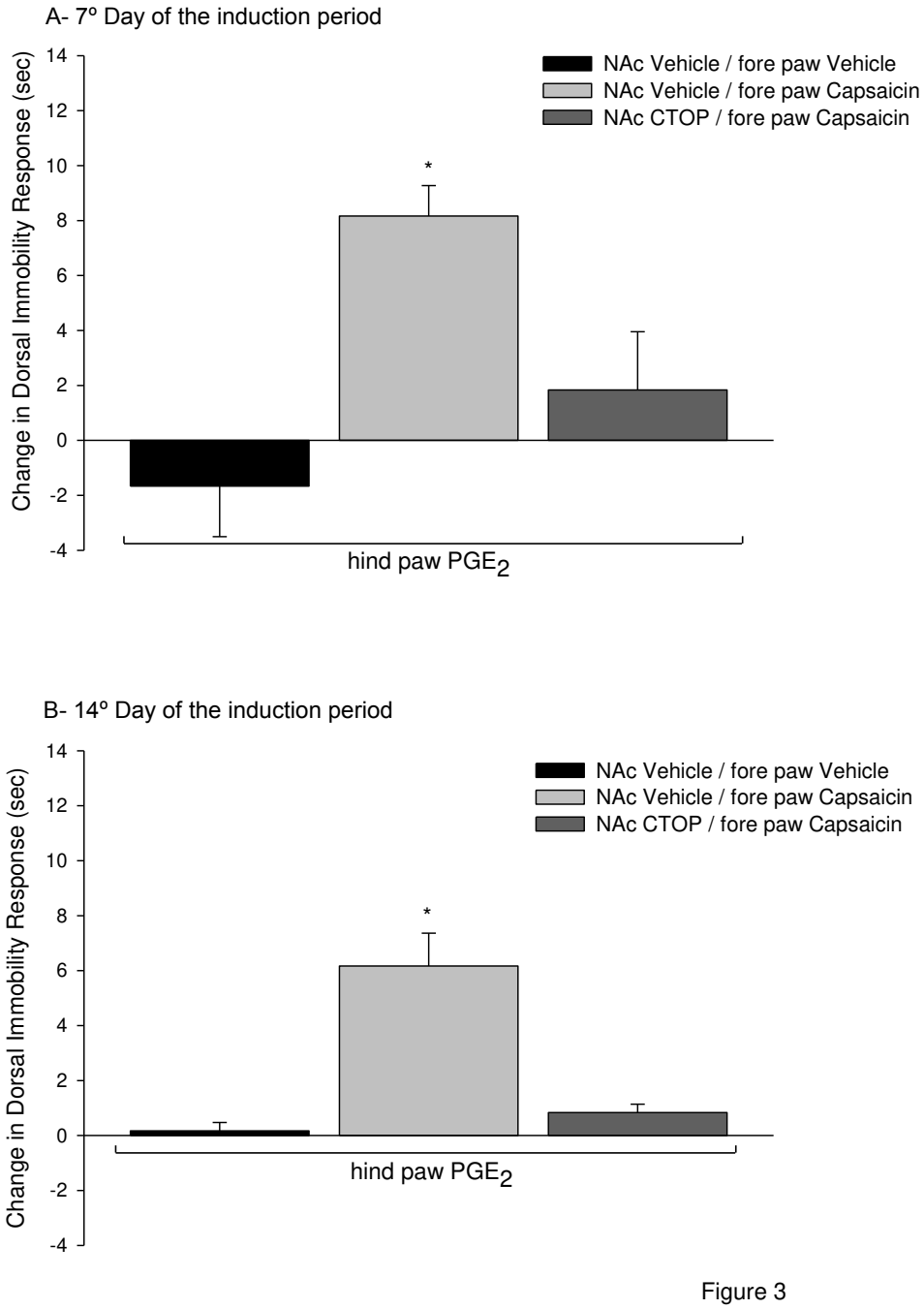

Figure 3

Figure 3. Effect of peripheral noxious stimulation on the dorsal immobility duration during the induction period of persistent inflammatory hyperalgesia.

On days 7 (A) and 14 (B) of the induction period of the persistent inflammatory hyperalgesia model, intense acute pain induced by the capsaicin injection (125 µg) into the rat fore paw significantly increased (One-way Analysis of Variance, Student-Newman-Keuls Method, $p<0.05$) the duration of the dorsal immobility as indicated by the symbol "*", an effect that was blocked by prior intra-accumbal administration of CTOP.

Maintenance period

Intense acute pain induced by a subcutaneous injection of capsaicin (125 µg) into the rat fore paw significantly increased ($p<0.05$) the duration of the dorsal immobility behavior on days 1, 7, 14 and 21 (Figure 4A, B, C, and D, respectively) of the maintenance period of persistent inflammatory hyperalgesia. This effect was prevented by the intra-accumbal administration of the µ-opioid receptor antagonist CTOP (1.0µg) but not by its vehicle 10 min prior to the subcutaneous injection of capsaicin into the rat fore paw ($p<0.05$) in all periods evaluated. Rat locomotor activity measured immediately after the DIR recordings was not significantly affected by these treatments ($p<0.05$) indicating that intra-accumbal treatments do not alter motor activity. These results indicate that persistent inflammatory hyperalgesia does not affect the ability of NSIA to modulate the dorsal immobility behavior. All injections of CTOP or its vehicle were within nucleus accumbens as shown in Figure 5.

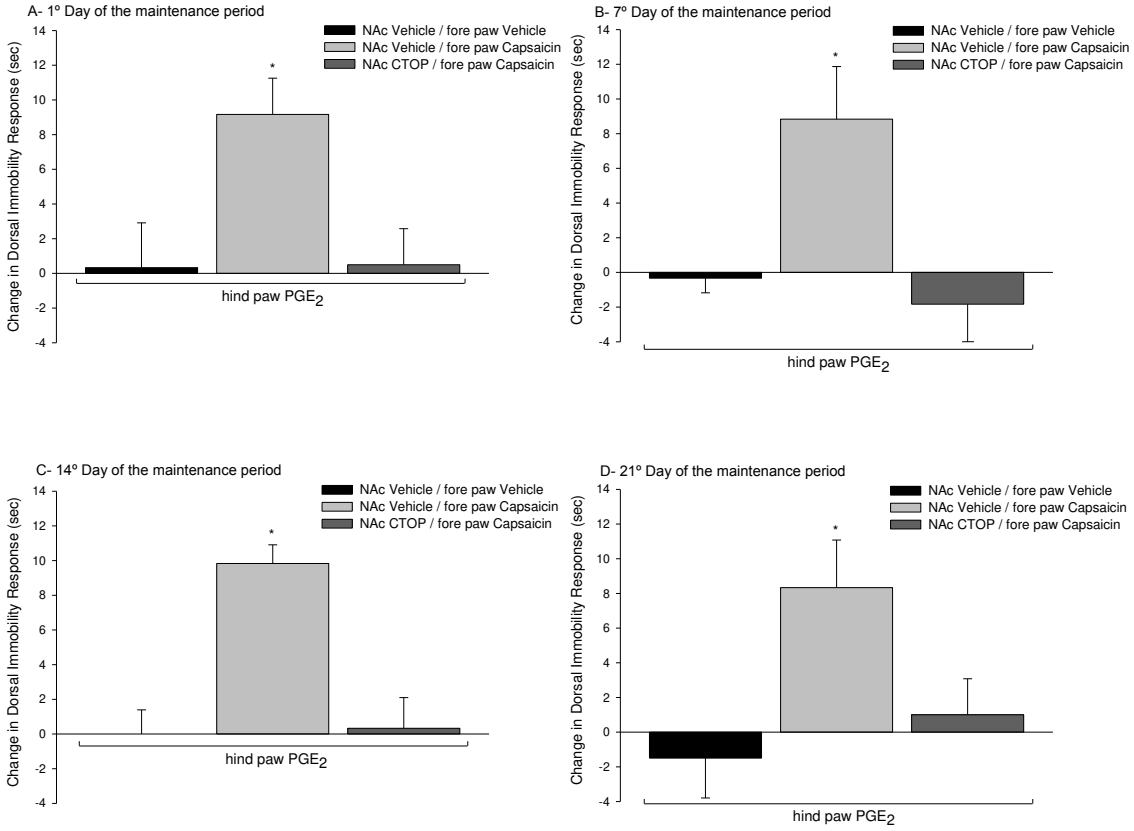

Figure 4

Figure 4. Effect of peripheral noxious stimulation on the dorsal immobility duration during the maitenance period of persistent inflammatory hyperalgesia.

On all days evaluated (A, B, C and D), intense acute pain induced by the capsaicin injection (125 µg) into the rat fore paw significantly increased (One-way Analysis of Variance, Student-Newman-Keuls Method, $p<0.05$) the duration of the dorsal immobility as indicated by the symbols "*", an effect that was prevented by prior intra-accumbal administration of CTOP.

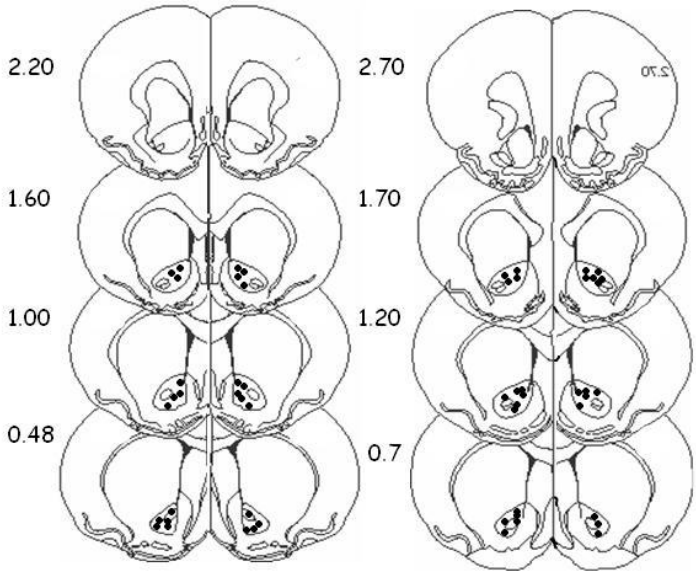

Figure 5. Location of the injection sites in the nucleus accumbens.

All injection sites were within the nucleus accumbens. Coronal sections were taken from a brain atlas [20] to demonstrate the areas of the injection sites (filled circles). The numbers on the left refer to the distance in mm, rostral to bregma.

4- Discussion

This study presented the first experimental evidence that chronic pain disrupts defensive behaviors (Figure 6, #1) by showing that persistent inflammatory hyperalgesia significantly decreases the duration of dorsal immobility response in rats. However, chronic pain does not affect the ability of NSIA to facilitate defensive behaviors via opioid mechanisms in nucleus accumbens. This was evidenced by findings showing that peripheral acute pain induced by a fore paw injection of capsaicin [21,4,18,22,6] significantly increased the duration of the dorsal immobility response in rats with persistent inflammatory hyperalgesia, via an opioid dependent mechanism in nucleus accumbens.

Chronic pain is characterized by plastic changes in the nervous system that predispose to impaired mood, cognitive functions and recuperative behaviors [5]. In this study, we have used a model of persistent inflammatory hyperalgesia characterized by a state of nociceptor sensitization that lasts for aproximately 30 days following the cessation of 14 successive daily subcutaneous injections of PGE_2 into the rat hind paw (Figure 1A). The persistence of nociceptor sensitization in the absence of any peripheral

stimulus suggest that plastic changes in nervous system leaded to chronification of a pain state which is thought to be correlated with human chronic inflammatory pain [9]. Seven days after the initiation of daily subcutaneous injections of PGE_2, the duration of dorsal immobility response was significantly decreased. This decrease persisted through the induction and maintenance period of the persistent inflammatory hyperalgesia model (Figure 1B). A slight and not significant increase in dorsal immobility response duration occurred on days 14 and 21 of the maintenance period (e.g. 14 and 21 days after discontinuing the daily subcutaneous injections of PGE_2 into the rat hind paw), which suggest a tendency to return to the basal condition. The immobility response is the last antipredator resort, employed when flight or fight responses are ineffective in an attempt to increase the chances of prey survival by decreasing the likelihood that the predator will continue its attack [2,23]. In humans, this response was reported to be induced in the context of life threat and to be accompanied by intense fear [24,25,26]. Therefore, the present findings demonstrate that a pre-existing pain condition, particularly chronic inflammatory pain, affects the ability of that subject to engage in defensive behaviors important to survival during life threatening situations (Figure 6, #1). In addition to immobility behavior, other defensive and adaptive behaviors used to pursue biologically relevant goals could be affected in chronic pain states. Although further studies are needed to address this issue, the inability to engage in self-protective behaviors could contribute to the difficulties in chronic pain treatment, notoriously the most difficult pain problem to manage. The mechanisms underlying the disruption of defensive behaviors in the presence of chronic pain are presently unknown. However, they probably lie on the plastic changes in central nervous system associated with pain chronification.

In contrast to chronic pain, acute pain has a warning function, being by itself a defense mechanism to avoid injury or to hinder the progression of an already existing injury. Therefore, the facilitation of defensive behaviors in response to acute noxious stimulation could be of great value to increases the chances of survival in situation of life threat accompanied by acute pain. In fact, as we have previously demonstrated [3] and here (Figure 2), acute pain facilitates defensive behaviors, since the subcutaneous injection of capsaicin significantly increases the duration of dorsal immobility response. The link between acute pain and facilitation of defensive behaviors appears to be the

antinociception induced by peripheral noxious stimulation (Figure 6, #3 and 4). The biologically goal of NSIA in pain and defensive behavior modulation is evident because analgesia may facilitate defensive behaviors increasing the chances of survival in life threatening situations. We have previously characterized the role of NSIA in defensive behaviors [3] and here we confirmed our previous data by showing that intra-accumbal administration of a μ-opioid receptor antagonist prevents the potentiated effect of subcutaneous capsaicin injection on dorsal immobility response (Figure 2). Subcutaneous capsaicin injection and intra-accumbal μ-opioid receptor antagonists administration have been classically used to induce and to block NSIA, respectively, as previously demonstrated [4,18,27,21,28,29]. Therefore, we used these procedures to investigate the ability of NSIA in modulating defensive behaviors during chronic pain states.

Although chronic pain disrupts defensive behaviors (Figure 6, #1), it does not hinder the ability of NSIA to facilitate them. This was demonstrate by findings showing that the injection of capsaicin into the fore paw of animals with persistent hyperalgesia significantly increased the duration of dorsal immobility response, an effect that was prevented by prior intra-accumbal administration of the selective μ-opioid receptor antagonist CTOP. This effect was observed with similar magnitude during the induction (Figure 3) and the maintenance (Figure 4) period of the persistent hyperalgesic state. These findings demonstrate that the ability of acute noxious stimuli to recruit opioid dependent neuronal mechanisms in nucleus accumbens, (Figure 6, #3) to facilitate defensive behaviors (Figure 6, #4) is preserved, even in the presence of a chronic pain condition (Figure 6, #2) that normally impairs defensive behaviors. The preservation of such mechanism could have the biological purpose of increasing the chances of survival of an animal previously wounded or sick and submitted to a novel threatening situation.

In addition to NSIA, other endogenous pain modulation systems facilitate defensive behaviors (Figure 6, #4). In fact, several evidences in the literature have demonstrated a reciprocal interaction between defensive behaviors and endogenous pain controls (Figure 6, #4 and 5). Fear (Figure 6, #6) and activation of defensive systems (Figure 6, #5) activate endogenous analgesic mechanisms to inhibit pain when an organism safety is threatened [30]. The adaptive function of pain inhibition is to prevent

conflicting motivations to engage in recuperative behaviors in response to pain instead of an appropriate defensive response [31,32,33,34,35,3]. On the other hand, activation of endogenous pain modulation systems, by themselves (in the absence of fear or life threat), facilitates defensive behaviors [3,23]. This reciprocal interaction has an anatomical support, since defensive responses depends on central nervous system regions also involved in endogenous analgesia, such as periaqueductal Gray Matter (PAG) [36], rostral ventromedial medulla (RVM) [37,] and nucleus accumbens [14]. Therefore, neuronal circuits in these regions may be mobilized simultaneously by the same *environmental stimulus* in a situation of confrontation, resulting in analgesia to allow defensive behaviors. Our present and previous [3] findings demonstrated that NSIA facilitates the immobility response. The biological value of this mechanism is expected to be so high that it is preserved even in the presence of chronic pain, a condition that, by itself, disrupts defensive behaviors.

Although it is possible that NSIA represents an undiscovered element of the PAG–RVM pain modulation system, that system is mediated by endogenous opioids in both PAG and RVM, whereas NSIA is not. In addition, NSIA is mediated by cholinergic mechanisms in RVM [22] and evidence argues against PAG participation in RVM cholinergic mechanisms [38]. The analgesia mediated by NSIA resembles that produced by diffuse noxious inhibitory controls (DNIC) since both are dependent on noxious stimulation. However, NSIA lasts much more, persists long after a local anesthetic blockade at the site of the initiating noxious stimulus and depends on supraspinal mechanisms [4,29]. Therefore, a functional connection between NSIA and other pain modulation system is improbable, suggesting that NSIA is a novel identified mechanism to link noxious stimulation to analgesia and facilitation of defensive behaviors.

In summary, the present findings demonstrated that a persistent hyperalgesic state disrupts the defensive behavior of dorsal immobility (Figure 6, #1) in rats but does not affect the ability of NSIA (Figure 6, #2 and #3) to facilitate it (Figure 6, #4). These findings suggest that a chronic pain state is associated to a decreased ability to engage in defensive behaviors, but a preserved ability to respond to acute noxious stimulation by activating endogenous analgesic mechanisms to facilitate these behaviors. The preservation of such mechanism may contribute to increase the chances of survival of

an animal previously wounded and submitted to a novel threatening situation. There is a complex interaction between endogenous pain control and defensive systems (Figure 6), in that the activation of defensive systems activates endogenous analgesic mechanisms (Figure 6, #5) and the activation of endogenous analgesic mechanisms facilitates defensive behaviors (Figure 6, #4). This reciprocal interaction ensures a decrease in pain perception during life threatening situations, preventing conflicting motivations to engage in recuperative behaviors in response to pain instead of an appropriate defensive response.

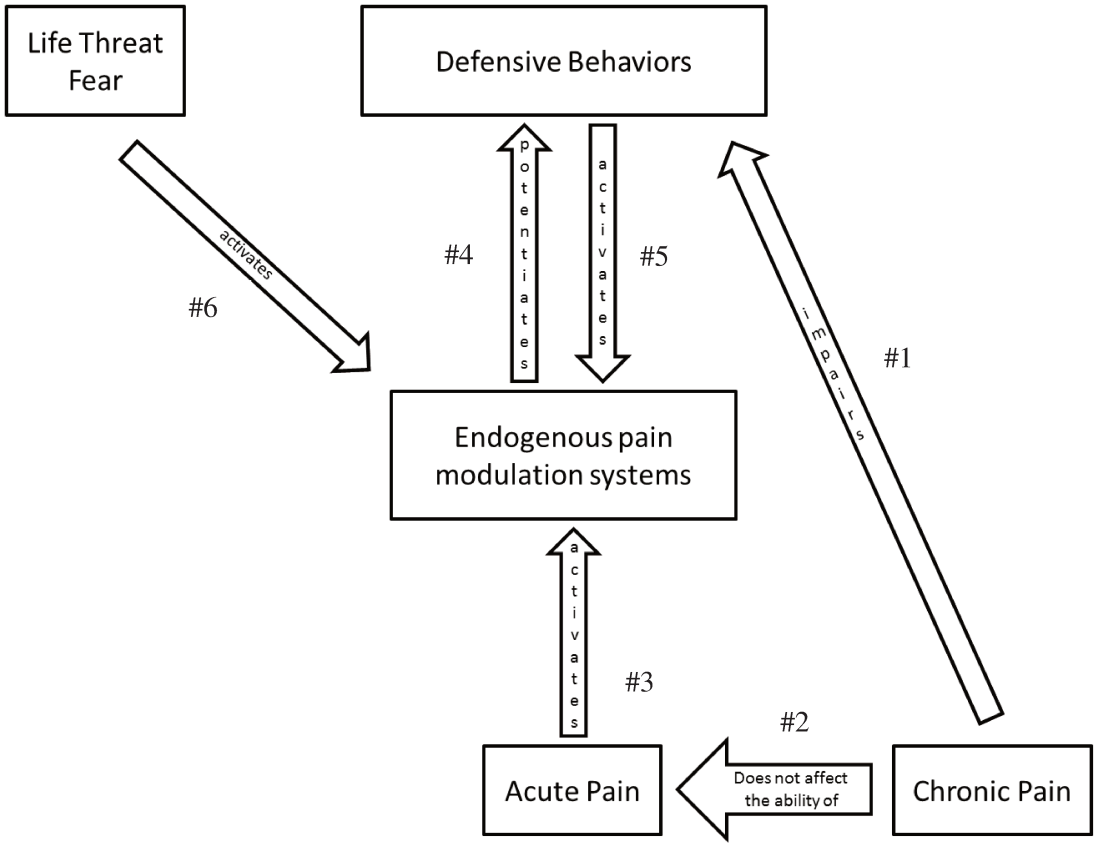

Figure 6. Proposed model of interaction between chronic pain, endogenous pain modulation systems and defensive behaviors.

Chronic pain impairs defensive behaviors (#1), but does not affect the ability of acute pain (#2), via endogenous pain modulation systems such as ANC to potentiate (#4) defensive behaviors such as the dorsal immobility behavior. Defensive behaviors (#5), and life threat and fear (#6) can also activate endogenous pain modulation systems.

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5- References

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CONCLUSÃO

O presente trabalho demonstrou que a hiperalgesia inflamatória persistente reduz a duração do comportamento defensivo de imobilidade dorsal em ratos, mas preserva a capacidade do estímulo nociceptivo agudo em facilitar este tipo de comportamento através da ativação de mecanismos analgésicos endógenos. Portanto, pode-se sugerir que nos estados de dor crônica a capacidade de expressão de comportamentos defensivos pode ser diminuída, mas a capacidade de responder a estímulos dolorosos agudos e, consequentemente, ativar mecanismos endógenos de modulação de dor para facilitar comportamentos defensivos é preservada. A preservação de tais mecanismos pode contribuir para aumentar as chances de sobrevivência do animal quando submetido a uma situação que ameaça sua vida.

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ANEXO 1

Certificado de aprovação pela Comissão de Ética na Experimentação Animal (CEEA) – UNICAMP.

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Comissão de Ética na Experimentação Animal
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CERTIFICADO

Certificamos que o Protocolo nº **1953-1**, sobre **"Efeito da hiperalgesia persistente sobre o comportamento defensivo de ratos: participação do controle nociceptivo ascendente"**, sob a responsabilidade de **Profa. Dra. Claudia Herrera Tambeli / Simone Monaliza Silva Lamana**, 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**.

CERTIFICATE

We certify that the protocol nº **1953-1**, entitled **"Effect of persistent hyperalgesia on the defensive behavior of rats: participation of ascending nociceptive control"**, 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.

Prof. Dr. Stephen Hyslop
Vice-Presidente

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ANEXO 2

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