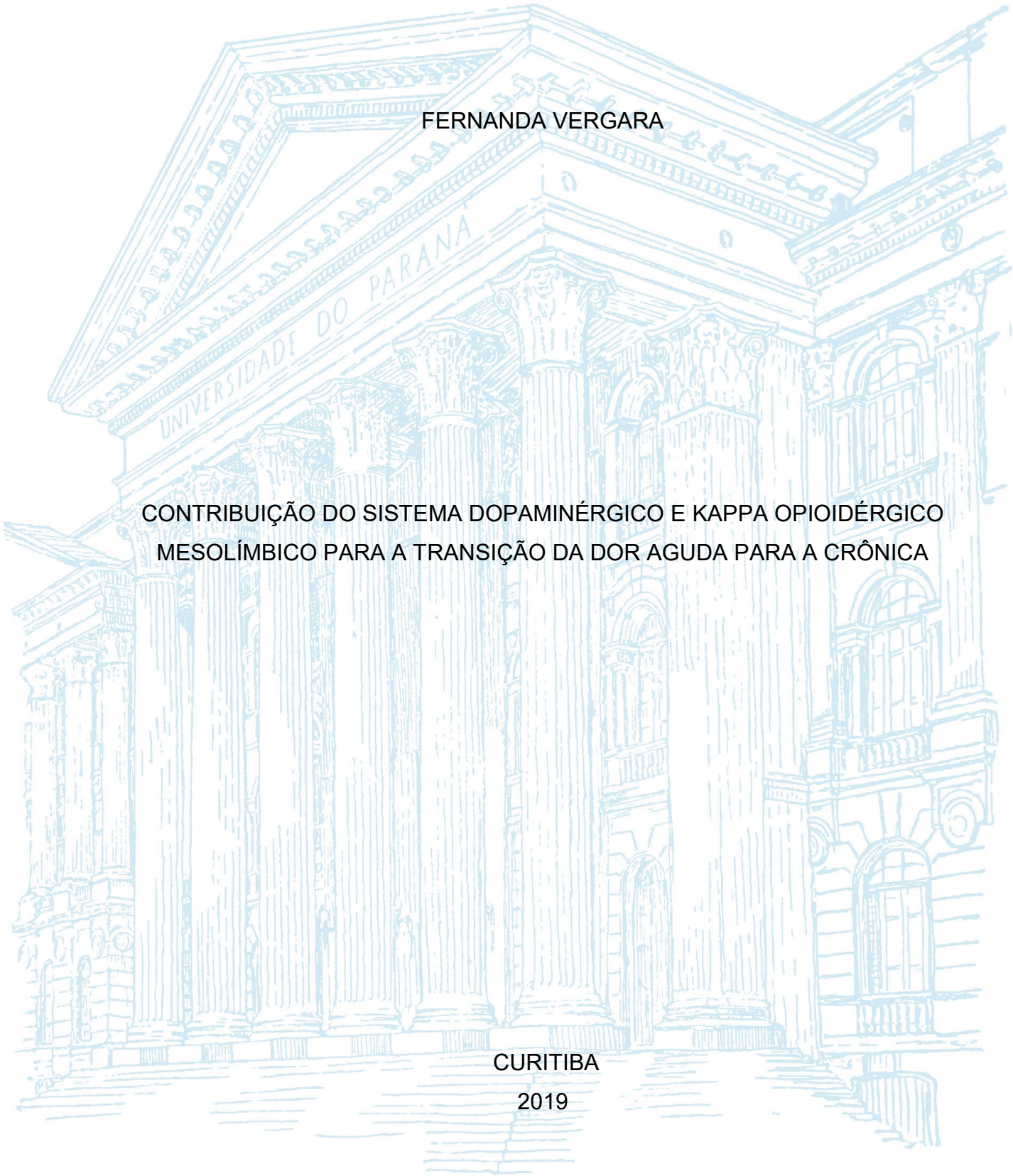
UNIVERSIDADE FEDERAL DO PARANÁ

FERNANDA VERGARA



CONTRIBUIÇÃO DO SISTEMA DOPAMINÉRGICO E KAPPA OPIOIDÉRGICO MESOLÍMBICO PARA A TRANSIÇÃO DA DOR AGUDA PARA A CRÔNICA

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Dissertação apresentada ao Programa de Pós-Graduação em Fisiologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Fisiologia.

Orientadora: Profa. Dra. Luana Fischer

Coorientadora: Profa. Dra. Juliana Jeremias Chichorro

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FISIOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **FERNANDA VERGARA**, intitulada: **"CONTRIBUIÇÃO DO SISTEMA DOPAMINÉRGICO E KAPPA OPIOIDÉRGICO MESOLÍMBICO PARA A TRANSIÇÃO DA DOR AGUDA PARA A CRÔNICA."**, sob orientação da Profa. Dra. LUANA FISCHER, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de Mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 23 de Agosto de 2019.

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A menos que modifiquemos à nossa maneira de pensar, não seremos capazes de resolver os problemas causados pela forma como nos acostumamos a ver o mundo (Albert Einstein)

RESUMO

A maioria das condições clínicas de dor crônica se desenvolve após uma lesão inicial, que leva à dor persistente que se cronifica. A dor crônica está relacionada ao afeto emocional negativo, ansiedade e depressão. O sistema mesolímbico tem sido implicado na patogênese de vários distúrbios neuropsiquiátricos e, recentemente, da dor crônica. Sua atividade dopaminérgica é influenciada por diversos mecanismos, sendo o sistema kappa opioide associado à diminuição da dopamina. O aumento da atividade mesolímbica kappa opioide e diminuição da atividade da dopamina está relacionado aos componentes afetivo-motivacionais da dor crônica, mas seu papel no processo de cronificação da dor não está estabelecido. O objetivo deste estudo foi avaliar sua atividade dopaminérgica da área tegmental ventral e o sistema kappa opioide no núcleo accumbens contribuem para o processo de cronificação da dor. Para isso, usamos um modelo de dor crônica no qual 14 injeções subcutâneas diárias de prostaglandina E2 (PGE_2) na pata traseira de ratos induz um estado crônico de hiperalgesia que dura por pelo menos 30 dias após o término das injeções. A lesão das células dopaminérgicas da área tegmental ventral, através da administração da toxina 6-hidroxidopamina impediu o desenvolvimento da hiperalgesia crônica. Esse achado indica que a transição de dor aguda para a dor crônica requer atividade de dopaminérgica mesolímbica. No entanto, à medida que a dor se torna crônica, os níveis de dopamina no nucleus accumbens diminuem e uma correlação positiva entre baixos níveis de dopamina e diminuição do limiar nociceptivo pode ser detectada. O bloqueio dos receptores kappa opioide no nucleus accumbens tanto preveniu quanto reverteu o desenvolvimento do estado hiperalgésico crônico. Complementarmente, a ativação farmacológica desses receptores possibilitou o desenvolvimento do estado hiperalgésico crônico em metade do tempo habitual. Esses achados indicam que o sistema opioide kappa mesolímbico promove a transição da dor aguda para a dor crônica. É importante ressaltar que nenhuma dessas intervenções afetou as respostas nociceptivas agudas. Este estudo sugere que os sistemas dopaminérgicos e kappa opioide mesolimbico são alvos potenciais para o desenvolvimento de estratégias terapêuticas que visem interromper ou reverter o processo de cronificação da dor.

Palavras-chave: dor crônica; dopamina; núcleo accumbens; área tegmental ventral; sistema mesolímbico; receptor kappa opioide; dinorfina,

ABSTRACT

It has been demonstrated that a decrease in dopaminergic activity and an increase in kappa opioid activity in the mesolimbic system underlie the negative affective states, pro-depressive and anti-reward behaviors related to chronic pain. However, we do not know whether these neuroplastic changes and their behavioral outcomes are a consequence of chronic pain or, in fact, contribute to its development. In this study, we we asked whether the mesolimbic dopamine and kappa opioid systems contribute to the transition from acute to chronic pain. With this purpose, we used a chronic pain model in which 14 daily subcutaneous injection of prostaglandin E2 (PGE_2) in the rat's hind paw induces a chronic hyperalgesic state that persists for at least 30 days after the discontinuation of the injections. The lesion of the dopaminergic cells of the ventral tegmental area, by locally injecting 6-hydroxydopamine, prevented the development of the chronic hyperalgesic state. This finding idicates that the transitioin from acute to chronic pain requires mesolimbic dopamine activity. However, as pain becomes chronic, the dopamine levels in the nucleus accumbens decrease with a positive correlation between low dopamine levels and decreased nociceptive threshold. The blockade of the kappa opioid receptors in the nucleus accumbens both prevented and reversed development of the chronic hyperalgesic state. Complementarily, the pharmacological activation of the kappa opioid receptors in the nucleus accumbens made it possible the development of the chronic hyperalgesic state in half the usual time. These findings indicate that the mesolimbic kappa opioid system drives the transitioin from acute to chronic pain. Importantly, none of these interventions affected acute nociceptive responses. This study suggests that mesolimbic dopamine and kappa opioid systems are potential targets for the development of therapeutic strategies to stop or reverse the pain chronification process.

Keywords: chronic pain; dopamine; nucleus accumbens; ventral tegmental area; mesolimbic system; kappa opioid receptor; dynorphin.

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LISTA DE ABREVIATURAS OU SIGLAS

AMPA - alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico

AMY - Amigdala

cAMP - adenosina 3',5'-monofosfato cíclico

CREB – proteína de ligação ao elemento de resposta cAMP, do inglês “cAMP-response-element-binding protein”

DA – dopamina

DHPG- (S) -3,5-Di-hidroxifenilglicina, do inglês “ (S) -3,5-Dihydroxyphenylglycine”

DOPAC - Ácido 3,4-diidroxifenilacético, do inglês “3,4-dihydroxyphenylacetic acid”

HIAA- ácido 5-hidroxindolacético, do inglês 5-Hydroxyindoleacetic acid

HVA – ácido homovanílico, do inglês “homovanillic acid”

KO – nocaute, do inglês “knock-out”

MSNs - neurônios espinhosos médios, do inglês “medium spiny neurons”

NAc - Nucleus accumbens

NAcSh- Nucleus accumbens shell

NMDA - N-metil-D-aspartato

nor-BNI - norbinaltorfimina

PFC – Córtex pré-frontal, do inglês “Pre Frontal Cortex”

PGE_2 – Prostaglandina E 2

SHAM - simulado

SNL - lesão de ligadura de nervo, do inglês “spinal nerve ligation”

VTA – Área tegmental ventral, do inglês “Ventral Tegmental Area”

µg – micrograma

µ- letra grega “mi”

δ- letra grega “delta”

κ- letra grega “kappa”

6-OHDA - 6-hidroxidopamina

SUMÁRIO

1. INTRODUÇÃO

A dor aguda é essencial à sobrevivência, pois ela chama a atenção e guia o comportamento do indivíduo com a intenção de proteger o organismo(Porreca e Navratilova, 2017). Quando a dor persiste, ela se torna crônica (McCormick e Law, 2016) e perde seu caráter protetor, tornando-se patológica. A dor crônica é um problema médico de ordem mundial (Tsang *et al.*, 2008). Ela afeta negativamente a qualidade de vida dos indivíduos e está associada a enormes custos socioeconômicos (Gaskin e Richard, 2012).

Os mecanismos envolvidos na transição da dor aguda para a dor crônica ainda são amplamente desconhecidos. Com o entendimento dos mecanismos de plasticidade sináptica envolvidos no aprendizado e memória na década de 1970 (Bliss e Lomo, 1973), a visão de que todas as sinapses são plásticas começa a emergir em outras áreas das neurociências, inclusive no estudo da dor (Woolf, 1983). A partir da década de 1980, os estudos começam a identificar os mecanismos envolvidos no que foi chamado de processo de sensibilização central (Latremoliere e Woolf, 2009; Woolf e Salter, 2000). Esse processo se inicia com a intensa ativação das fibras nociceptivas, levando a modificações bioquímicas nas sinapses das vias centrais da dor que culminam em um estado anormal de responsividade do sistema nociceptivo (Latremoliere e Woolf, 2009; Woolf e Salter, 2000).

Recentemente, com o avanço das técnicas de neuroimagem ficou claro que a dor crônica também está associada a modificações morfológicas (como diminuição da densidade da massa cinzenta) e funcionais (como alterações na conectividade entre regiões encefálicas) (Apkarian *et al.*, 2004; Baliki *et al.*, 2014; Bushnell, Ceko e Low, 2013; Fritz *et al.*, 2016; Kucyi *et al.*, 2014; Moayedi *et al.*, 2011, 2012; Pleger *et al.*, 2014; Tracey e Bushnell, 2009). Interessantemente, essas alterações envolvem majoritariamente os circuitos cerebrais responsáveis pelos estados emocionais e motivacionais (Baliki *et al.*, 2006; Baliki e Apkarian, 2016; Bushnell, Ceko e Low, 2013; Navratilova *et al.*, 2016; Oluigbo, Salma e Rezai, 2012; Ossipov, 2012). Sendo assim, o alvo do tratamento nessas situações deve ser o sistema nervoso central e não a periferia (Latremoliere e Woolf, 2009; Woolf, 2011)

A dor é aversiva, e assim como outros estados aversivos, sinaliza o desvio da homeostase. Portanto, recruta respostas comportamentais motivadas pela busca do alívio da dor e, consequentemente, da restauração do equilíbrio homeostático (Craig,

2003; Navratilova *et al.*, 2016; Porreca e Navratilova, 2017). Dessa forma, o alívio da dor, ao cessar um estado aversivo, é gratificante, e está associado a ativação do sistema de recompensa (Bair *et al.*, 2003; Baliki, Marwan N *et al.*, 2010; Becker, Gandhi e Schweinhardt, 2012; Craig, 2003; Kato, Ide e Minami, 2016; Leknes e Tracey, 2008; Mccutcheon *et al.*, 2012; Navratilova *et al.*, 2016; Navratilova, Edita *et al.*, 2012). O processamento neural da recompensa no encéfalo ocorre no sistema mesolímbico a partir de projeções dopaminérgicas da área tegmental ventral (VTA) para o núcleo accumbens (NAc), amígdala (AMY), córtex pré-frontal (PFC) e outras regiões encefálicas (Elman e Borsook, 2016; Gardner, 2011; Kelley e Berridge, 2002). Sua função biológica é aprendizagem para evitar situações aversivas e buscar recompensas (Mayer *et al.*, 2018; Salgado e Kaplitt, 2015), algo amplamente dependente da sinalização dopaminérgica no NAc. Por exemplo, o aumento do nível de dopamina no NAc é um potente elemento sinalizador a eventos que levam a recompensa, como o alívio da dor (Kato, Ide e Minami, 2016; Martins e Tavares, 2017; Navratilova *et al.*, 2016; Navratilova, Edita *et al.*, 2012).

Recentemente ficou claro que a liberação de dopamina no NAc também pode estar também associada a respostas aversivas, como por exemplo a dor (Brooks e Berns, 2013; Budygin, Park e Bass, 2012; Lammel *et al.*, 2011). No entanto, evidências indicam que o aumento da atividade dopaminérgica no NAc diminui a dor aguda (Dias *et al.*, 2015; Gear, Aley e Levine, 1999; Siahposht-Khachaki *et al.*, 2017). Portanto, a liberação de dopamina no NAc em resposta a dor aguda parece ser um mecanismo endogenamente recrutado para modular a própria dor (Gear e Levine, 1995; Siahposht-Khachaki *et al.*, 2017).

Diferente da dor aguda, a dor crônica está associada a hipofunção tanto dos sistemas de modulação da dor (Austin *et al.*, 2010) quanto do sistema dopaminérgico mesolimbico (Melis, Spiga e Diana, 2005; Self, 2004; Volkow, Fowler e Wang, 2003) e o NAc parece ter um papel central no processo de cronificação da dor. Em humanos, foi demonstrado que durante a cronificação da dor ocorre um aumento da conectividade entre o NAc e o córtex pré-frontal e que esse aumento não persiste quando a dor crônica está estabelecida (Baliki *et al.*, 2012; Baliki, Marwan N. *et al.*, 2010; Vachon-Presseau *et al.*, 2016). Em animais, o aumento da atividade dopaminérgica no NAc contribui, enquanto seu bloqueio dificulta o processo de cronificação da dor (Dias *et al.*, 2015). Portanto, a dopamina no NAc aumenta na fase

aguda da dor, mas sua atividade persistente parece contribuir para o desenvolvimento da fase crônica.

O aumento da liberação de DA no NAc está relacionado a uma ativação de fatores de transcrição, incluindo CREB (cAMP-response-element-binding protein), levando a transcrição de dinorfina, um kappa opióde endógeno (Ji *et al.*, 2003; Muschamp e Carlezon, 2013). A dinorfina, então, através de um feedback negativo, diminui a liberação de dopamina (Muschamp e Carlezon, 2013; Niikura *et al.*, 2010). Estudos recentes mostraram que a liberação de dinorfina por um estímulo doloroso contribui para o estado de afeto negativo da dor (Liu *et al.*, 2019; Massaly *et al.*, 2019), mas a sua contribuição para o processo de cronificação da dor não foi estabelecido.

Diante disso, o objetivo desse trabalho foi verificar se a neurotransmissão dopaminérgica em paralelo com a ativação do sistema kappa opioide no NAc conduz o processo de cronificação da dor, que culmina com uma diminuição nos níveis de dopamina quando o estado de dor crônica é estabelecido.

2. REVISÃO DE LITERATURA

2.1 DOR

A nocicepção é definida como a estimulação das fibras nervosas que transmitem informações sobre danos ou potenciais danos aos tecidos ao cérebro, seja por estímulos térmicos, mecânicos ou químicos nocivos (Basbaum *et al.*, 2009; Seth e Gray, 2016). A experiência dolorosa, em si, resulta da transdução, transmissão e modulação da informação nociceptiva pelas vias encefálicas, onde há uma integração com o componente emocional (Seth e Gray, 2016). Dor, de acordo com a Associação InterNAcional para Estudos da dor (em inglês – InterNAcional Association for Study of Pain, IASP), é uma experiência sensorial e emocional desagradável associada a um dano real ou potencial, ou retratados em termos de tais danos (Merskey e Bogduk, 1994). Portanto, a dor, em si, resulta da transdução, transmissão e modulação da informação nociceptiva pelas vias encefálicas, onde há uma integração com o componente emocional (Seth e Gray, 2016) (Figura A).

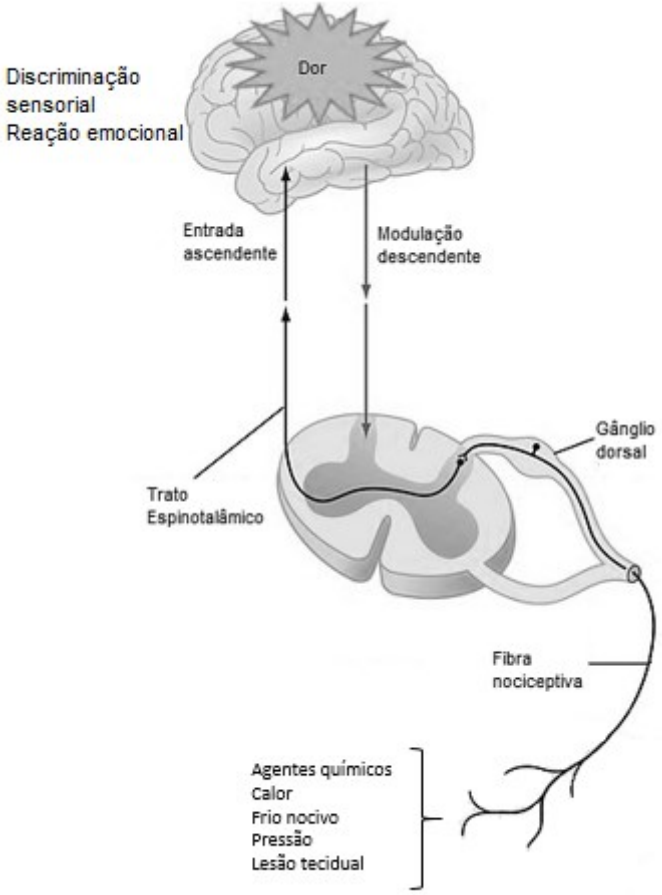

Figura A. Visão esquemática da via nociceptiva. Adaptado de https://www.ceces.ca/lessons/mechanism-of-action-therapeutic-action-and-side-effects/

A dor pode ser classificada quanto à sua duração, neste sentido a dor pode ser aguda ou crônica.

Em geral, a dor aguda é consequência de uma lesão ou doença, portanto conforme o processo de cicatrização evolui, ocorre a diminuição da intensidade da dor. Normalmente dura menos de três meses, com previsão baseada na causa, local e tipo de lesão (McCormick e Law, 2016; Seth e Gray, 2016). Entretanto, sob condições persistentes ou patológicas, este sistema pode se alterar e se tornar sensibilizado, transformado a dor aguda protetiva em dor persistente, patológica (Davis e Moayedi, 2013; Ji *et al.*, 2003; Zeilhofer, 2005).

A dor crônica pode ser definida como dor que persiste além do processo de cura, sendo caracterizada por dor espontânea (percepção da dor na ausência de estímulos físicos), bem como respostas exageradas a estímulos físicos (hiperalgesia e alodinia) (Apkarian, 2008).

2.2 DOR CRÔNICA E SISTEMA MESOLÍMBICO

Os mecanismos envolvidos na transição da dor aguda para a dor crônica ainda não foram totalmente elucidados. Voscopoulos e Lema afirmam que a mudança da dor aguda para a crônica parece transcorrer em passos patológicos e histopatológicos discretos (Voscopoulos e Lema, 2010). Um dos possíveis mecanismos envolvidos nessa transição seria uma ativação intensa de uma fibra nociceptiva levando a uma modificação bioquímica, alterações sinápticas, uma modulação por mediadores endógenos e/ou uma sensibilização central nas vias encefálicas da dor (Kuner, 2010; Latremoliere e Woolf, 2009; Woolf e Salter, 2000).

A sensibilização periférica acontece por meio de dano tecidual, infecção, inflamação, neuropatia, entre outros, fazendo com que as terminações nociceptivas fiquem hipersensibilizadas (Tal, 1999; Zeilhofer, 2008). Essa constante ativação periférica acaba causando modificações como hipertrofia e brotamento de terminações nervosas, aumento da atividade espontânea e excitabilidade da fibra, aumento de transmissão glutamatérgica, causando um aumento de receptores AMPA e NMDA no corno dorsal da medula (Kuner, 2010; Peirs e P. Seal, 2016; Rocha et al., 2007) e que levaria à sensibilização central.

Os neurônios dopaminérgicos do sistema mesocorticolímbico, que participam do processamento de recompensa e reforço no encéfalo, projetam-se da área tegmental ventral (VTA) para o núcleo accumbens (NAc), amígdala (AMY), córtex pré-frontal (CPF) e outras regiões encefálicas (Elman e Borsook, 2016; Gardner, 2011; Kelley e Berridge, 2002). O aumento do nível de dopamina no NAc é um elemento comum de recompensa assim como no uso de drogas de abuso (Chiara, Di e Imperato, 1988; Gardner, 2011; Kelley e Berridge, 2002; Kringelbach e Berridge, 2016)

Pacientes com dores crônicas frequentemente apresentam distúrbios neurológicos, incluindo ansiedade e depressão (Asmundson e Katz, 2009; Bair *et al.*, 2003; Elman, Borsook e Volkow, 2013; Gureje, 2008; Marbach e Lund, 1981). Estudos de ressonância magnética funcional mostram que modificações morfológicas e fisiológicas (como diminuição da densidade da massa cinzenta e conectividade entre regiões encefálicas) são encontradas nesses pacientes, geralmente acompanhadas de outras doenças neuropsiquiátricas (Apkarian *et al.*, 2004; Asmundson e Katz, 2009; Baliki *et al.*, 2014; Bushnell, Ceko e Low, 2013; Davis e Moayedi, 2013; Elman, Borsook e Volkow, 2013; Fritz *et al.*, 2016; Kucyi *et al.*, 2014; Moayedi *et al.*, 2011, 2012; Pleger *et al.*, 2014; Simons, Elman e Borsook, 2014; Tracey e Bushnell, 2009).

Essas alterações ocorrem principalmente no sistema mesolímbico, composto de neurônios que se projetam reciprocamente da área tegmentar ventral (VTA) do mesencéfalo para o núcleo accumbens (NAc), amígdala (AMY), córtex pré-frontal (CPF) e outras regiões encefálicas (Elman e Borsook, 2016; Gardner, 2011; Kelley e Berridge, 2002). Esse sistema está envolvido na aprendizagem ao qual situações que levam ao desvio ou restauração da homeostase ajudam o organismo a evitar situações aversivas e a encontrar recompensas (Bauch, Rausch e Bunzeck, 2014; Porreca e Navratilova, 2017), sendo que neurônios dopaminérgicos emergentes da VTA liberam dopamina no NAc durante reforços, como alimentação, interação social ou drogas de abuso.

ADICÃO DE DROGAS

Os neurônios dopaminérgicos do sistema mesocorticolímbico participam do processamento de recompensa e reforço no encéfalo, como comportamentos apetitivos instruídos por sugestões condicionadas (Ambroggi *et al.*, 2008; Baliki, Marwan N *et al.*, 2010; Carlezon e Thomas, 2009). O NAc é responsável pela codificação de erros de predição para estímulos apetitivos (Baliki, Marwan N. *et al.*, 2010; Fields *et al.*, 2007; Schultz, 1998) e aversivos (Becerra *et al.*, 2001; Brooks e Berns, 2013; Budygin, Park e Bass, 2012) gerando comportamento motivado. Os sinais de predição favoráveis e de recompensa envolvem diferentes respostas de dopamina no NAc (Bromberg-martin, Matsumoto e Hikosaka, 2010; Kringelbach e Berridge, 2016; Porreca e Navratilova, 2017).

O aumento do nível de dopamina (DA) no NAc é um elemento comum de recompensa assim como no uso de drogas de abuso (Chiara, Di e Imperato, 1988; Gardner, 2011; Kelley e Berridge, 2002; Kringelbach e Berridge, 2016).

Acredita-se que em alguns indivíduos, o uso repetitivo da droga de abuso leve a sensibilização do sistema dopaminérgico e à um consequente incentivo excessivo ao ato de utilizar a droga. Com o uso crônico da droga, modificações neuroplásticas alteram o nível basal de humor, que se torna progressivamente mais negativo. Portanto, o desenvolvimento da adição ocorre com a progressão de um estado de reforço positivo (com a recompensa associada ao uso agudo da droga) para um estado de reforço negativo (quando seu uso crônico tem o objetivo de aliviar o humor negativo) (Koob, 2015; Koob *et al.*, 2014; Koob e Moal, 2008; Kringelbach e Berridge, 2016) .

A base mecanicista para todo o processo parece depender, inicialmente, da liberação persistente de dopamina no NAc em resposta ao uso repetitivo da droga (Borsook *et al.*, 2016). Durante o uso crônico de drogas de abuso, os receptores de dopamina presentes no NAc, na presença de excesso de dopamina causada pelo uso prolongado da droga, estimulam uma cascata de eventos intracelulares, aumentam cAMP e levam a produção de dinorfina, um agonista kappa opioide, que suprime a via de recompensa (Margolis *et al.*, 2003; Narita *et al.*, 2005; Nestler, 2004). A hipoatividade do sistema mesolímbico que resulta desse processo contribui para a depressão do humor (Chartoff *et al.*, 2012; Graziane *et al.*, 2012; Koob, 2015; Kringelbach e Berridge, 2016; Mantsch *et al.*, 2014). A dinorfina tem sido apontada como um dos fatores para o estado emocional negativo (ansiedade, disforia, respostas aversivas ao estresse) durante a fase de retirada das drogas (Bali, Randhawa e Jaggi, 2015; Bruchas, Land e Chavkin, 2010; Koob *et al.*, 2014; Koob e Moal, 2008; Shippenberg, Zapata e Chefer, 2007).

Estudos apontam que o antagonista de receptor kappa opioide nor-BNI (norbinaltorfimina), no núcleo accumbens, bloqueia o estado emocional negativo em modelos animais na fase de retirada de drogas (Chartoff *et al.*, 2012; Graziane *et al.*, 2012; Margolis *et al.*, 2003; Narita *et al.*, 2005; Nealey *et al.*, 2011; Schlosburg *et al.*, 2013; Zan *et al.*, 2015).

2.3 DOPAMINA E DINORFINA NA DOR CRÔNICA

A neurotransmissão de opiáceos dentro da rede de núcleos subcorticais (por exemplo, NAc, VTA, pálido ventral e hipotálamo) e do tronco cerebral (por exemplo, PAG) é outro componente de recompensa (Leknes e Tracey, 2008). A ativação de receptores opioides facilitam (μ e δ) ou suprimem (κ) a liberação de dopamina enquanto mediam efeitos prazerosos e aversivos (por exemplo, disforia, irritabilidade e dor física e emocional), respectivamente (Tejeda e Bonci, 2019; Wise e Koob, 2014).

A dinorfina é agonista de receptores kappa opioide (KOR) e está presente nas projeções para a VTA de várias estruturas fortemente implicadas em motivação e reforço, incluindo NAc, amígdala e hipotálamo (Fallon, Leslie e Cone, 1985; Meredith, 1999; Muschamp e Carlezon, 2013). Os receptores de dopamina do tipo D1 presentes no NAc, ao serem estimulados por dopamina ou agonistas, levam a produção de dinorfina (Muschamp e Carlezon, 2013; Ren *et al.*, 2016). Essa elevação na produção

de dinorfina, provoca um mecanismo de feedback no sistema mesolímbico levando a diminuição de DA liberada pela VTA (Carlezon e Thomas, 2009; Koob *et al.*, 2014; Margolis *et al.*, 2003). A dinorfina tem sido apontada como um dos fatores para o estado emocional negativo durante a fase de retirada das drogas, estados aversivos e perda motivacional em animais com dor (Koob *et al.*, 2014; Liu *et al.*, 2019; Massaly *et al.*, 2019)

A relação da dinorfina e dor vem sendo estudada há algum tempo. Esse agonista kappa opioide endógeno foi identificado inicialmente como um possível agente antinociceptivo endógeno, dependendo da circunstância (Beyer, Schäfer e Stein, 1997; Herman e Goldstein, 1985; Spampinato e Candeletti, 1985; Tiseo, Geller e Adler, 1988), entretanto inúmeros estudos indicam que a expressão aumentada da dinorfina é pró-nociceptiva (Gardell *et al.*, 2001; Kajander *et al.*, 1990; Mika, Obara e Przewlocka, 2011). Por exemplo, inflamação crônica e lesão de nervo periférico, que são acompanhadas por manifestações de dor anormal, incluindo dor espontânea, alodinia e hiperalgesia, também estão associados a um elevado índice de dinorfina (Gardell *et al.*, 2001; Vanderah *et al.*, 2001). E, um estudo com camundongos selvagens e knock-out (KO) para o gene pró-dinorfina demonstrou que os camundongos KO apresentavam hiperalgesia mais branda que os selvagens em testes nociceptivos e de dor aguda (formalina) e em grupos com lesão de igadura de nervo (SNL) (Gardell *et al.*, 2001). Os camundongos KO apresentavam menos hiperalgesia térmica e mecânica do que os selvagens. Quando foram testados os níveis dinorfinérgicos, os selvagens SHAM e SNL, no dia 2 apresentavam mesmo nível de dinorfina espinhal. Já ao 10º dia, os SNL apresentavam um aumento significativo de dinorfina espinhal (Gardell *et al.*, 2001).

Recentemente ficou claro que a liberação de DA no NAc também pode estar associada a respostas aversivas, como por exemplo a dor (Brooks e Berns, 2013; Budygin, Park e Bass, 2012; Lammel *et al.*, 2011). No entanto, evidências indicam que o aumento da atividade dopaminérgica no NAc diminui a dor aguda (Gear; Aley; Levine, 1999; Dias et al., 2015; Pourreza; Ezzatpanah; Haghparast, 2017). Portanto, a liberação de dopamina no NAc em resposta a dor aguda parece ser um mecanismo endogenamente recrutado para modular a própria dor (Gear e Levine, 1995; Siahposht-Khachaki *et al.*, 2017). Diferente da dor aguda, a dor crônica está associada a hipofunção tanto dos sistemas de modulação da dor (Austin *et al.*, 2010) quanto do sistema dopaminérgico mesolimbico (Melis, Spiga e Diana, 2005; Self, 2004; Volkow,

Fowler e Wang, 2003) e além disso, o NAc parece ter um papel central no processo de cronificação da dor. Em humanos, foi demonstrado que durante a cronificação da dor ocorre um aumento da conectividade entre o NAc e o córtex pré-frontal e que esse aumento não persiste quando a dor crônica está estabelecida (Vachon-Presseau *et al.*, 2016). Em animais, o aumento da atividade dopaminérgica no NAc contribui, enquanto seu bloqueio dificulta o processo de cronificação da dor(Dias *et al.*, 2015; Watanabe *et al.*, 2018). Portanto, assim como acontece durante o desenvolvimento da adição, a DA no NAc aumenta na fase aguda da dor, mas sua atividade persistente parece contribuir para o desenvolvimento da fase crônica.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Testar a hipótese de que a neurotransmissão dopaminérgica paralela à ativação do sistema kappa-opioide no NAc conduz o processo de cronificação da dor, que culmina com uma diminuição nos níveis de dopamina quando o estado de dor crônica é estabelecido.

3.2 OBJETIVOS ESPECÍFICOS

1- Determinar se a lesão das células dopaminérgicas da VTA impede a cronificação da dor;
2- Determinar se o bloqueio farmacológico dos receptores kappa opioide no NAc impede a cronificação da dor;
3- Determinar se a ativação farmacológica dos receptores kappa opioide no NAc facilita a cronificação da dor;
4- Determinar se os níveis de dopamina e seus metabólitos, no núcleo accumbens, variam nas fases aguda, durante a cronificação e fase crônica da hiperalgesia perisistente;

4 ARTIGO CIENTÍFICO

Contribution of mesolimbic dopamine and kappa opioid systems to the transition from acute to chronic pain

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Original Article

Introduction

Chronic pain is a worldwide public health problem with an overall prevalence of 30% (Gaskin e Richard, 2012; Tsang *et al.*, 2008). In addition to a huge socioeconomic burden (Gaskin e Richard, 2012), chronic pain is associated with a dramatic decrease in the quality of life (Elman, Borsook e Volkow, 2013). As pain progresses from acute to chronic ('pain chronification'), the negative emotional affection of pain is worsened by the development of several neuropsychiatric comorbidities such as depression, anxiety, and decision-making abnormalities (Doan, Manders e Wang, 2015; Elman, Borsook e Volkow, 2013). The understanding of the mechanisms underlying the transition from acute to chronic pain is essential to the development of therapeutic strategies to stop or reverse the pain chronification process.

The mesolimbic system, consisting mainly of dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) has been implicated in the pathogenesis of several neuropsychiatric disorders (Doan, Manders e Wang, 2015; Elman, Borsook e Volkow, 2013), including recent evidence for chronic pain (Dias *et al.*, 2015; Liu *et al.*, 2019; Massaly *et al.*, 2019; Vachon-Presseau *et al.*, 2016; Watanabe *et al.*, 2018). For example, a greater functional connectivity between NAc and prefrontal cortex appears to predict the transition from acute to chronic pain in humans (Vachon-Presseau *et al.*, 2016) and the increase in dopaminergic neurotransmission into the NAc facilitates the pain chronification process in rats (Dias *et al.*, 2015). However, evidence obtained from human (Martikainen *et al.*, 2015; Wood *et al.*, 2007) and animal (Ren *et al.*, 2016; Watanabe *et al.*, 2018) studies demonstrated that chronic pain is characterized by a mesolimbic hypodopaminergic tone. The dopaminergic activity in the mesolimbic system is influenced by several mechanisms, being the kappa opioid system a key factor associated with its decrease (Margolis e Karkhanis, 2019). Interestingly, recent evidence demonstrated that chronic pain increases endogenous kappa opioid activity in the NAc (Liu *et al.*, 2019; Massaly *et al.*, 2019). There is now ample evidence that this decrease in dopaminergic activity and increase in kappa opioid activity in the mesolimbic system underlie the negative affective states, pro-depressive and anti-reward behaviors related to chronic pain (Austin *et al.*, 2010; Chang *et al.*, 2014; Dias *et al.*, 2015; Liu *et al.*, 2019; Massaly *et al.*, 2019; Ren *et al.*, 2016; Watanabe *et al.*, 2018).

In recent years, we have seen a shift in pain science, if it used to be mainly focused on the sensory aspect of pain, now it is increasingly focused on the affective and motivational components of pain. This is an important change, because pain is much more than a sensory experience and we have to understand it in all its complexity. However, while the contribution of decreased dopamine and increased kappa opioid mesolimbic activities to the affective-motivational components of chronic pain is well established, their role in the transition from acute to chronic pain has received little attention. For example, we do not know whether these neuroplastic changes and their behavioral outcomes are a consequence of chronic pain or, in fact, contribute to its development. This is of great importance because for the most part, the affective and motivational aspects of chronic pain are closely related to its sensory aspect and preventing the transition from acute to chronic pain could prevent the whole clinical picture. Therefore, in this study we asked whether the dopaminergic activity of VTA and the kappa opioid system of the NAc underlie the transition from acute to chronic pain.

Materials and Methods

Animals

The experiments were performed in male Wistar rats (290–320 g). The animals were randomly housed in groups of four or five in polypropylene cages lined with shavings, with *ad libitum* access to rat chow and water. They were maintained in a room with controlled 12:12-h light/dark cycle and temperature (22°C ± 2). All animal experimental procedures and protocols were approved by the local Committee on Animal Research of the Federal University of Parana (approval ID #1156) and followed the guidelines of the Ethics Standards of the International Association for the Study of Pain in animals (Zimmermann, 1983).

Drugs

Prostaglandin E 2 (100ng/30µL) (Ferreira, Lorenzetti e de Campos, 1990), nor-BNI (nor-Binaltorphimine dihydrochloride) (1.25µg/0.30µL per side)(Kelley, Bless e Swanson, 1996), U50,488 (trans-(1S,2S)-U-50488 hydrochloride hydrate) (1.6µg/0.30µL per side)(Cortez *et al.*, 2010), 6-OHDA (6-hydroxydopamine) (3µL/0.25µL per side)(Zhou *et al.*, 2015) were purchased from Sigma, St Louis, MO, USA and were dissolved in 0.9 % NaCl, except 6-OHDA that was dissolved in 0.9% NaCl containing 0.2% ascorbic acid.

6-OHDA was microinfused once in the VTA, five days prior the experiments; nor-BNI, the highly selective and slow-onset kappa opioid receptor antagonist, has been shown to have long-lasting effects (up to 21 days) after administration (Jones e Holtzman, 1992) and therefore it was microinjected into the NAc twice either during the early or during the late induction phase; U50,488 was daily microinjected into the NAc during 7 days in the induction phase.

Stereotaxic Surgery and drug infusion

The rats were anesthetized with intraperitoneal xylazine (10 mg/kg) and ketamine (60 mg/kg) and placed in a stereotaxic apparatus. The skull was exposed and a small hole was made to introduce a 26-gauge guide cannula into NAc Shell (NAcSh), bilaterally. The coordinates from bregma were: +1.8 mm from the AP, L +/−0.9 mm, and DV -5.2 mm, 2 mm above the site.

The cannula was fixed into place with orthodontic resin (L.D. Caulk Co., Milford, DE, USA), and a small screw was fixed in the skull to ensure its immobility. After surgery, the animals received dipyrone (50 mg/kg) and gentamicin (0.5 mg/kg), and experiments were carried out 5-7 days later.

During experiments, all drugs were injected bilaterally in the NAcSh at two levels into the NAc shell layer, because of its narrow structure: 0.15 µl in DV – 6.7mm and 0.15µl in DV – 7.7mm.

After experiments, the animals were transcardially perfused and the injection sites were verified histologically. Evans blue dye was injected through the guide cannula (1%, 0.3 µl) and postmortem 50 µm coronal sections of the brain (Gear e Levine, 1995) were performed to determine the location of the dye (Paxinos and Watson, 2007). Only animals where the dye is restricted to the NAc shell were included in the figures and data analysis.

6-OHDA lesion

Selective dopaminergic lesions were performed bilaterally in the VTA by injecting 6-OHDA (3 µg/0.25 µL) through an electronic infusion pump, during the surgical procedure. Sham operations followed the same procedure, but vehicle (0.25 µL) was injected instead of 6-OHDA. The coordinates were: AP -4.8 mm, L+/- 1mm, DV -8.3 mm. After surgery, the skin was sutured and the animals received dipyrone (50 mg/kg) and gentamicin (0.5 mg/kg). Experiments were carried out 5-7 days later in order to allow animal´s recovery. Lesion location and extension were histologically assessed.

Prostaglandin E_2-induced acute increase in nociceptive response

The hind paw injection of PGE_2 was performed using a 26-gauge needle connected to a Hamilton syringe. PGE_2 (100 ng) or vehicle was subcutaneously administered in a volume of 30 µL into the dorsal surface of the rat´s hindpaw. The mechanical nociceptive threshold (see below) was evaluated before injection (baseline) and 3 h and 24 h later (Ferreira, Lorenzetti e Campos, de, 1990).

Prostaglandin E_2-induced chronic increase in nociceptive response

In this chronic pain model, 14 daily subcutaneous injections of PGE_2 (100 ng/30 µl/paw) into the rat's hind paw induced a chronic decrease in the

mechanical nociceptive threshold, defined as hyperalgesia, that persists for at least 30 days after the discontinuation of the injections (Ferreira, Lorenzetti e de Campos, 1990).

There are two well-defined phases in this model: the induction phase and the maintenance phase. The induction phase is defined as the 14-day period of daily PGE_2 injections; while the maintenance phase is defined as the long-lasting period after the discontinuation of the PGE_2 injections.

During the induction phase it is possible to study the mechanisms underlying pain chronification. In this phase, the experimental manipulations that difficult pain chronification will prevent the maintenance of chronic hyperalgesia after the discontinuation of the PGE_2 injections. Complementarily, experimental manipulations that facilitate pain chronification lead to the maintenance of chronic hyperalgesia after only 7 daily PGE_2 injections (Dias *et al.*, 2015).

The mechanical nociceptive threshold (see below) was evaluated on days -1 (baseline) and at different time points (see figures) during the induction and maintenance phases. Importantly, the mechanical nociceptive threshold during the induction phase was evaluated always before the daily PGE_2 injection (and before microinjection, when it was the case), therefore the effect observed in graphics is the chronic cumulative effect of previous injections.

Mechanical Nociceptive threshold test

The nociceptive behavioral test was performed during the light phase (between 7 a.m. and 12 a.m.), in a quiet room maintained at 22 °C. Before the experiments, each animal was manipulated for 7 days to be habituated to the experimental manipulation.

The Randall–Selitto test (Randall e Selitto, 1957) was used to assess the nociceptive mechanical paw withdrawal threshold, as a measure of the nociceptive response. In this test, a continuous pressure is applied to the dorsal surface of the rat's left hind paw until the animal withdrew the paw. The nociceptive mechanical threshold was defined as the force in grams at which the rat withdrew its paw. The value was obtained from the mean of three readings made at intervals of 2 min. Data were expressed as mean ± SEM of the mechanical paw withdrawal threshold (g) at each time point, except bar charts, expressed as mean + SEM.

Open-Field Test

The open-field test was used to provide an overall indication of locomotor activity, discarding the possibility that any experimental manipulation could affect animals' motor behavior. The open-field arena consists of a circular area (90 cm of diameter), divided into 7-12 squares, limited by a 50-cm-high wall. The number of crossed squares was quantified for 5 minutes and used as a measure of the exploratory behavior. The test was performed in the last day of microinjections (into the NAc) or in the last day of PGE_2 injections in the VTA lesion groups.

Histological sample preparation

The rats were anesthetized by an intraperitoneal injection of xylazine (10mg/kg) and ketamine (60 mg/kg) and transcardially perfused with saline followed by 4 % paraformaldehyde in 0.1-phosphate buffer, pH 7.4. Brains were removed and immersed in paraformaldehyde at 4 °C for two weeks, in 30 % sucrose solution for another week and stored at −80°C until sectioning. Four sections (30 µm), between bregma +1.92 and +1.56 mm (NAcSh) and another four sections (30 µm), between bregma -4.68mm and -4.92 mm (VTA), were sliced per animal.

Tyrosine Hydroxylase Immunohistochemistry

The expression of tyrosine hydroxylase (TH), the enzyme responsible for dopamine synthesis, was assessed in the VTA, in order to determine lesion intensity and extension, and in the NAcSh, in order to determine the effect of VTA lesion in the NAc dopaminergic terminals. Free-floating sections of the NAc shell and VTA were rinsed in 0.1M phosphate-buffered saline (PBS) and treated with 0.5% H_2O_2 in 0.1M PBS for 30 min to suppress endogenous peroxidase activity. Then incubated with primary mouse anti-TH antibody, diluted in phosphate-buffered saline containing 0.3% Triton X-100 (1:3000; cat # AB152 Chemicon, CA, USA) overnight at 10°C. Biotin-conjugated secondary antibody incubation (1:200; cat # S-1000 Vector Laboratories, USA), was performed for 2h at room temperature. After several washes in phosphate-buffered saline, the antibody complex was localized using the ABC system (Vectastain ABC Elite kit cat # PK6101, Vector Laboratories, USA) followed by 3,3'-diaminobenzidine reaction with nickel enhancement. The sections were then mounted onto gelatin-coated slides and cover slipped after dehydration in ascending concentrations of ethanol-xylene solutions.

The slices were digitized with a microscope scanner (Axio Imager Z2, Carl Zeiss, Jena, DE) coupled to an imaging system (Metasystems, Altlussheim, DE). Quantification of TH immunoreactive immunoreactive cells was performed automatically by optical density using ImageJ 1.37c (Public Domain) image analysis software.

High performance liquid chromatography (HPLC)

For determining DA, DOPAC (3,4-dihydro-xyphenylacetic acid), HVA (homovallinic acid), NA (noradrenaline), DHPG (3,5-dihydroxyphenylglycine), 5-HT (5-hidroxitriptamine) and HIAA (5-hydroxyindoleacetic acid) contents in brain, the rats were killed by decapitation at different days of the PGE_2 treatment. The brains were immediately removed, and the NAc freshly dissected, frozen in liquid nitrogen, and stored at −80◦C. The concentration of DA, DOPAC, HVA, NA, DHPG, 5-HT and HIAA was assayed by reverse-phase HPLC with electrochemical detection (ED).

The system consisted of a Synergi Fusion-RPC-18 reverse-phase column (150 × 4.6 mm i.d., 4 µm particle size) fitted with a 4 × 3.0 mm precolumn (SecurityGuard Cartridges Fusion-RP), an electrochemical detector (ESA Coulochem III Electrochemical Detector) equipped with a guard cell (ESA 5020), with the electrode set at 350 mV and a dual electrode analytical cell (ESA 5011A); and a LC-20AT pump (Shimadzu) equipped with a manual Rheodyne 7725 injector with a 20 µl loop. The column was maintained inside a temperature-controlled oven (25◦C, Shimadzu). The cell had two chambers in series: each chamber included a porous graphite colorimetric electrode, a double counter electrode, and a double reference electrode. Oxidizing potentials were set at 100 mV for the first electrode and at 450 mV for the second electrode. DA, DOPAC, HVA, NA, DHPG, 5-HT and HIAA were detected at the second electrode. The tissue samples were homogenized with an ultrasonic cell disrupter (Sonics) in 0.1 M perchloric acid containing sodium metabisulfite 0.02% and internal standard. After centrifugation at 10000 × g for 30 minutes at 4◦C, 20 µl of the supernatant was injected into the chromatograph. The mobile phase used at a flow rate of 1 ml/min had the following composition: 20 g citric acid monohydrate (Merck), 200 mg octane-1-sulfonic acid sodium salt (Merck), 40 mg ethylenediaminetetraacetic acid (EDTA) (Sigma), and 900 ml HPLC-grade water. The pH of the buffer running solution was adjusted to 4.0 then filtered through a 0.45 µm filter. Methanol (Merck) was added to give a final composition of 10% methanol (v/v). The peak areas of the

external standards were used to quantify the sample peaks. The data was expressed as ng/g of tissue and the turnover of dopamine (DA), was expressed accordingly to the equations (DOPAC + HVA)/DA, based on previous works (Moreira *et al.*, 2012).

Statistical Analysis

Data from mechanical nociceptive threshold were analyzed by repeated measures analysis of variance (ANOVA) with one within-subjects factor (time) and one between-subjects factor (treatment intra-NAc and paw injection). Data from open field were analyzed by two-way ANOVA with PGE_2 or saline and drug or vehicle as factors. Data from the levels of monoamines and their metabolites were analyzed by t-test or by one-way ANOVA. All post hoc contrasts, when appropriate, were performed using Tukey's test. The level for statistical significance was $P \leq 0.05$. The correlation between dopamine levels and nociceptive threshold was determine by the Pearson's correlation test. STATISTICA® software (StatSoft, Tulsa, OK, USA) was used to perform data analysis. SigmaPlot® software (Systat Software, San Jose, CA, USA) was used to perform graphical representation and analysis. Data are plotted in figures as mean ± SEM, except bar charts, expressed as mean + SEM.

Results

Prostaglandin E_2-induced acute and chronic increases in nociceptive response

The injection of PGE_2 (100 ng) into the hindpaw induced an acute increase in nociceptive response, characterized by a significant decrease in the mechanical nociceptive threshold (hyperalgesia) 3h after PGE_2 injection. The nociceptive response returned to baseline 24 hours later (Figure 1 A, repeated-measures ANOVA - $F_{treatment\ x\ time}(3, 7) = 144.25$, $p<0.001$, followed by Tukey's post hoc test, $p < 0.05$).

Daily intraplantar injections of PGE_2 (100 ng) for 14 days (induction phase) induced a chronic increase in nociceptive response, characterized by a significant decrease in the mechanical nociceptive threshold 24 h after the last injection (the nociceptive tests were performed before the daily injections). Such hyperalgesic response persisted for at least 16 days (maintenance phase) after the injections were discontinued (Figure 1 B, repeated-measures ANOVA - $F_{treatment}(1, 9) = 1023.7$, $p=0.000001$; $F_{time}(14, 126) = 40.813$, $p<0.001$; $F_{treatment\ x\ time}(14, 126) = 63.480$, $p=0.00001$, followed by Tukey's post hoc test, $p < 0.05$).

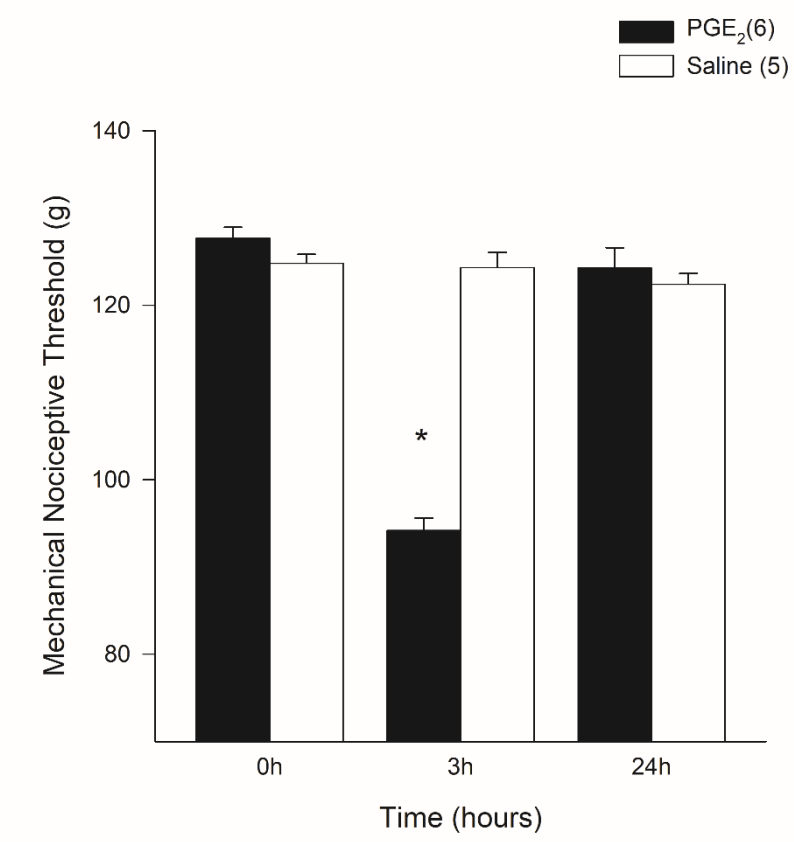


Figure 1. A- PGE_2-induced acute increase in nociceptive response. Three hours after PGE_2 injection in the left hindpaw, mechanical nociceptive threshold was significantly decreased and it returned to its baseline value after 24h. Repeated measures ANOVA, with one within-subjects factor (time) and one between-subjects factors (treatment). The symbol "*" indicates a mechanical nociceptive threshold significantly lower than that of the control group at the same time point (Tukey's post-hoc test, $p < 0.0002$). In this and in subsequent bar charts data are expressed as mean + S.E.M for. Number in parenthesis indicates the number of animals in each group.

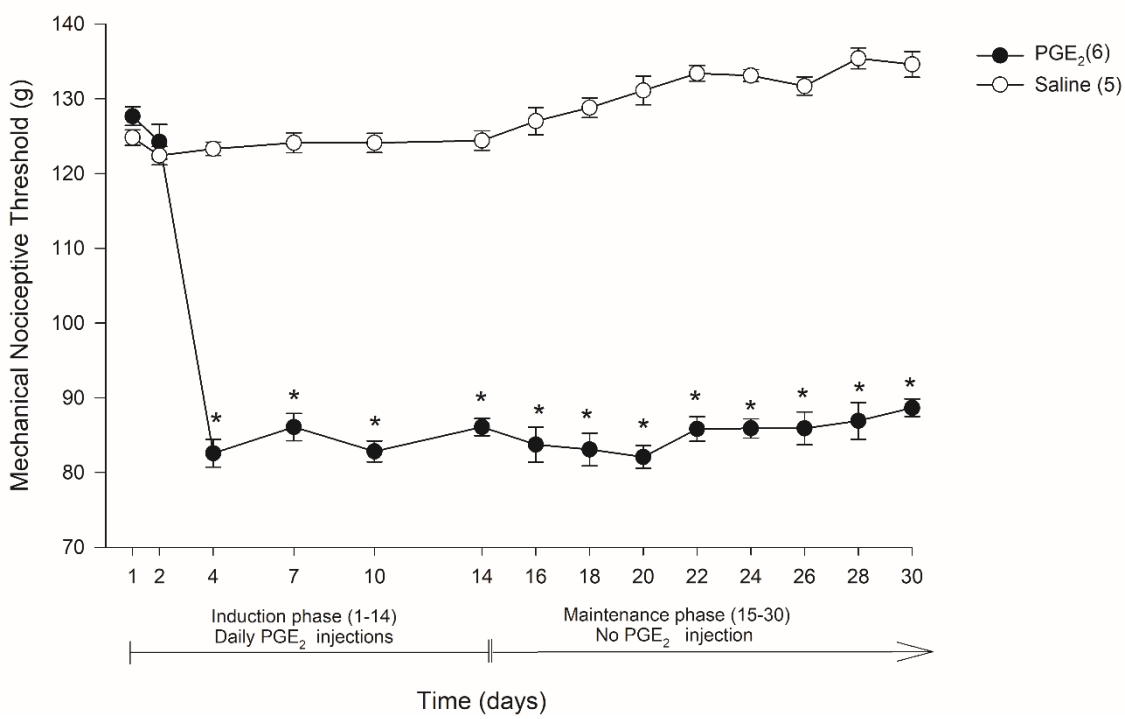


Figure 1. B- PGE_2-induced chronic increase in nociceptive response. Daily intraplantar injections of prostaglandin E_2, for 14 days, induced a chronic hyperalgesic state that lasted for at least 16 days after the injections were discontinued. Repeated measures ANOVA, with one within-subjects factor (time) and one between-subjects factors (treatment). The symbol "*" indicates a mechanical nociceptive threshold significantly lower than that of the control group at the same time point (Tukey's post-hoc test, $p < 0.0002$). In this and in subsequent figures showing chronic nociceptive responses, the nociceptive tests were performed before the daily PGE_2 injection during the induction phase. In this and in subsequent figures data are expressed as mean ± S.E.M Number in parenthesis indicates the number of animals in each group.

The dopaminergic lesion of the Ventral Tegmental Area prevented the chronic, but not the acute prostaglandin E_2-induced increase in nociceptive response

The dopaminergic lesion of the VTA by bilateral microinjection of 6-OHDA (3µg/0.25µL) had no effect either on basal nociceptive threshold or on PGE_2-induced acute hyperalgesia (Figure 2.A, repeated-measures ANOVA - $F_{\text{treatment x time}}(4, 50) = 173.24$, $p=0.00001$).

In contrast, the dopaminergic lesion of the VTA prevented the development of the chronic hyperalgesic state. This effect is seen early in the induction phase, when the nociceptive threshold was only slightly reduced. When the PGE_2 injections were discontinued (maintenance phase) the nociceptive response return to the baseline

values (Figure 2.B, repeated-measures ANOVA, $F_{treatment}(2, 25) = 973.45$, $p=0.00001$; $F_{time}(10, 250) = 150.07$, $p<0.001$; $F_{treatment \times time}(20, 250) = 101.70$, $p=0.00001$, followed by Tukey's post hoc test, $p < 0.05$).

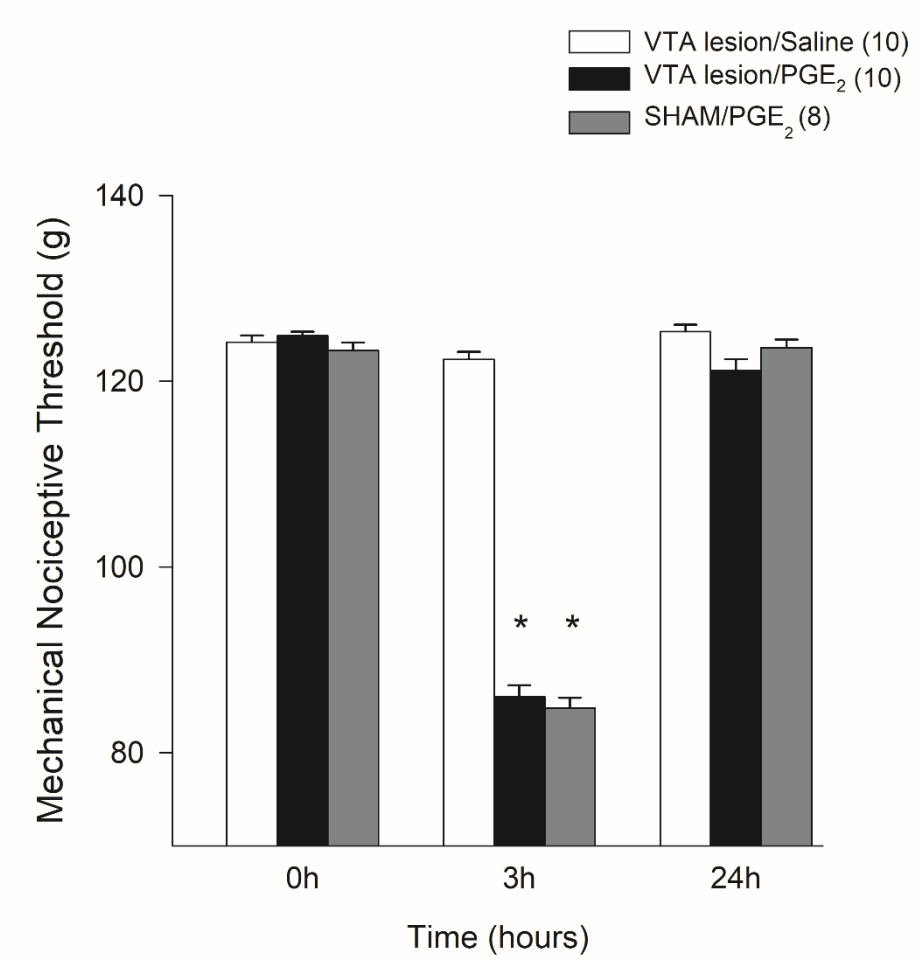


Figure 2. A- The effect of the dopaminergic lesion of the VTA in PGE_2-induced acute increase in nociceptive response. The administration of 3µg of OHDA into the VTA 5 days before experiments did not affect either the basal nociceptive threshold or the acute hyperalgesia induced by PGE_2. Repeated measures ANOVA, with one within-subjects factor (time) and one between-subjects factors (treatment). The symbol "*" indicates a mechanical nociceptive threshold significantly lower than that of the saline group at the same time point (Tukey's post-hoc test, $p < 0.0001$). Number in parenthesis indicates the number of animals in each group.

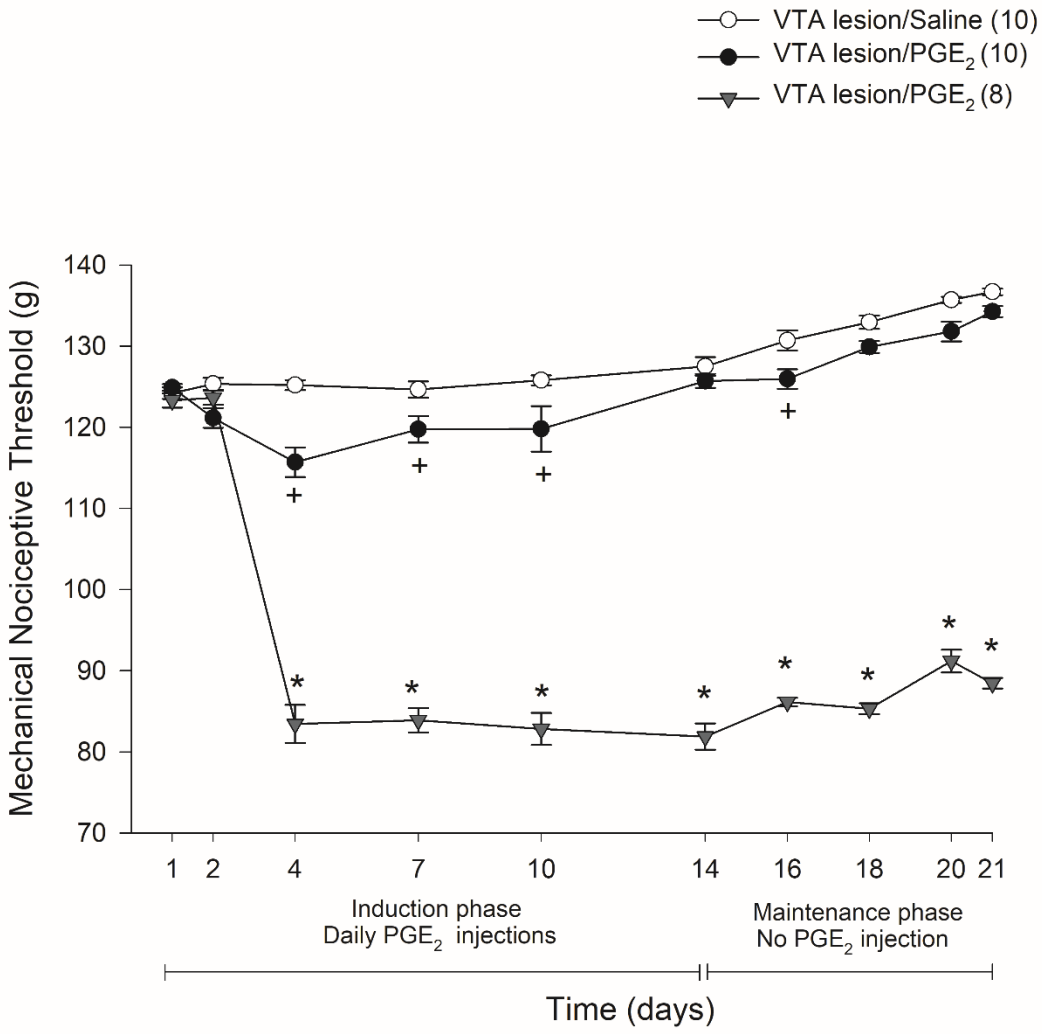


Figure 2. B- The effect of the dopaminergic lesion of the VTA in PGE_2-induced chronic increase in nociceptive response. The administration of 3µg of OHDA into the VTA 5 days before experiments prevented the development of the chronic hyperalgesia. Repeated measures ANOVA, with one within-subjects factor (time) and one between-subjects factors (treatment). The symbol "*" indicates a mechanical nociceptive threshold significantly lower than that of the other groups at the same time point; the symbol "+" indicates a mechanical nociceptive threshold significantly lower than that of the saline group within the same time point (Tukey's post-hoc test, $p < 0.0001$). Number in parenthesis indicates the number of animals in each group.

The dopaminergic lesion of the VTA significantly decreased the number of neurons expressing tyrosine hydroxylase (TH+ neurons), both in the NAc (Figure 2 C, ANOVA, $F(2,22) = 4.253$, $p < 0.029$) and in the VTA (Figure 2 D, One Way ANOVA, $F(2,21) = 12.450$, $p<0.001$). Since TH is expressed in dopaminergic neurons, these data indicate a reduction in the number of such neurons in the VTA and in dopaminergic terminals in the NAc.

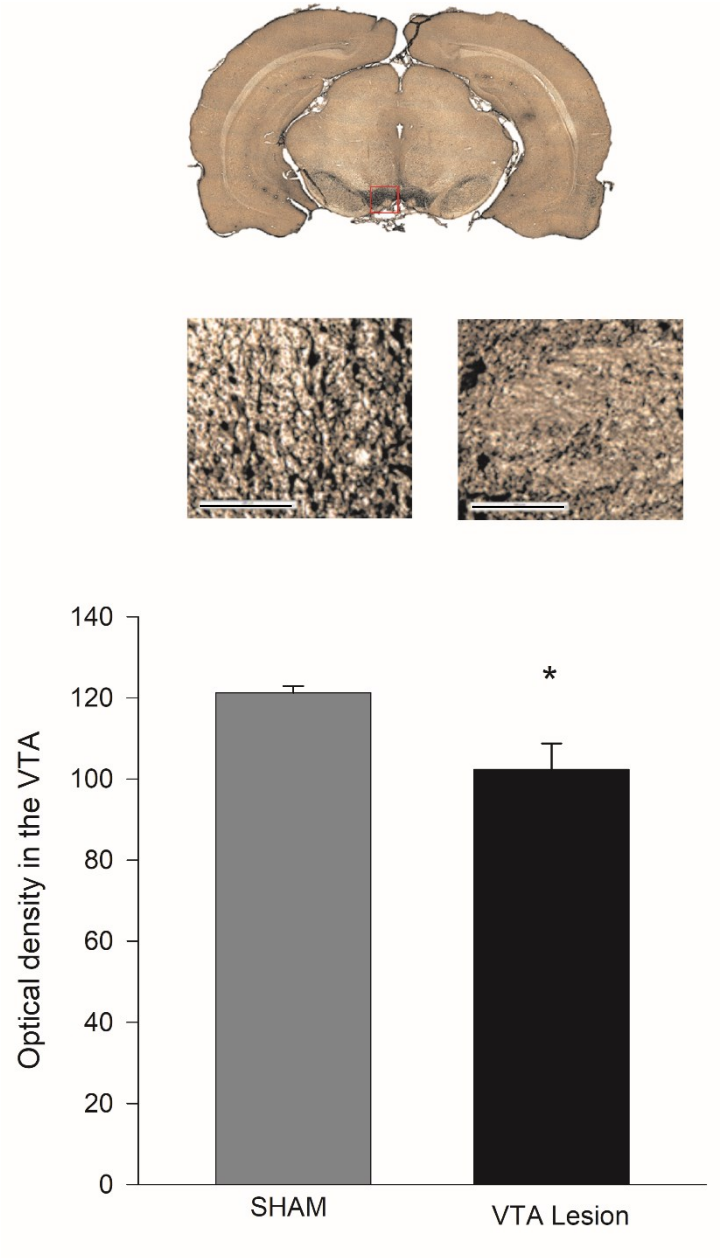


Figure 2. C- Tyrosine hydroxylase expression within the VTA. Top: Photomicrograph of representative sections for each group of TH immunoreative density in lesion and sham groups. Red square represents VTA localization. The black line in the pictures represents 100 µm. Bottom: quantification of the optical density of TH, the symbol "*" indicates a significantly lower optical density than that of the sham group, t test, $p<0.005$.

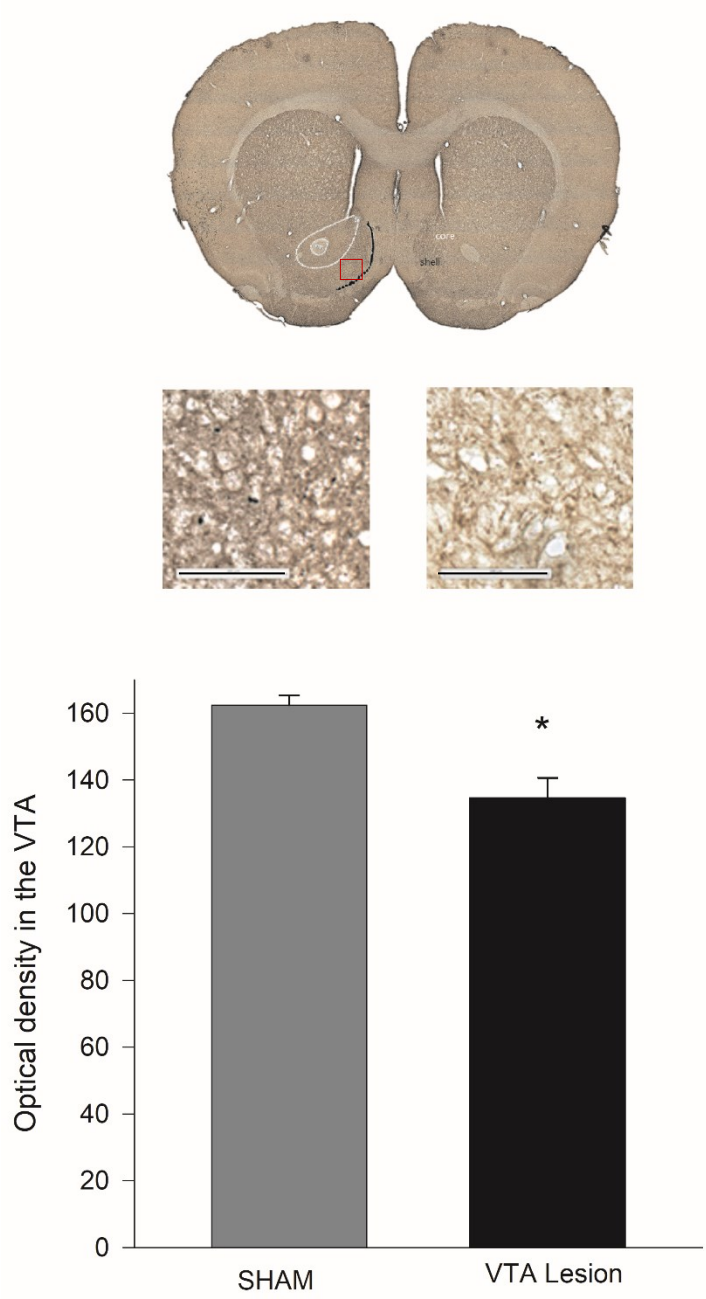


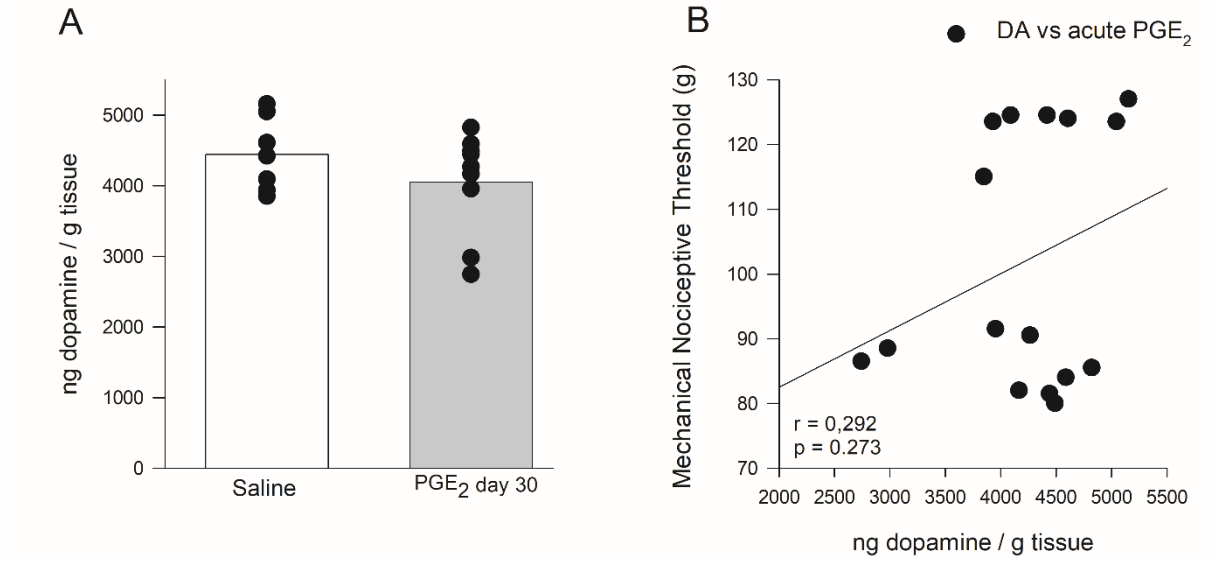
Figure 2. D- Tyrosine hydroxylase expression within the NAc Shell. Top: Photomicrograph of representative sections for each group of TH immunoreative density in lesion and sham groups. Red square represents NAcSh localization. The black line in the pictures represents 100 μm. Bottom: quantification of the optical density of TH, the symbol "*" indicates a significantly lower optical density than that of the sham group, t test, $p<0.005$.

NAc dopamine levels progressively decrease as the increased nociceptive response becomes chronic.

The dopamine levels in the NAc were not affected by a single PGE_2 injection (Figure 3 A, t-test, $t(14) = 1.211$, $p= 0.246$) and there is no correlation between dopamine levels and the acute decrease in nociceptive threshold (Figure 3 B,

Pearson's correlation analysis). However, PGE_2 injection significantly increased the levels of DOPAC, the deaminated metabolite of dopamine, but not that of HVA into the NAc (Table 1). Nonetheless, the dopamine DA turnover, as indicated by the (DOPAC+HVA)/DA ratio was significantly increased 3 h after PGE_2 injection (Table 1).

The dopamine levels significantly decreased after 14 daily injections of PGE_2 (Figure 3 C Tukey post hoc test, F (2,22) = 3.815, p= 0.038) and there is a positive correlation between low dopamine levels and decreased nociceptive threshold during days 7 and 14 of the induction phase of the chronic hyperalgesic state (Figure 3 D, Pearson's correlation analysis). The dopamine levels remained significantly decreased 16 days after the discontinuation of the 14 daily injections of PGE_2, on day 30 of the experiment (Figure 3 E, t-test, t (14) = 3,2, p= 0.006). There is a positive correlation between low dopamine levels and decreased nociceptive threshold on day 30 of the maintenance phase of the chronic hyperalgesic state (Figure 3 F, Pearson's correlation analysis). While the NAc levels of the dopamine metabolites DOPAC and HVA did not significantly change in either the induction or maintenance phases (Table 1), the dopamine DA turnover was significantly increased in both phases (Table 1). The NAc levels of noradrenaline, serotonin and their main metabolites, DHPG and HIAA, respectively, did not significantly change in either the acute, the induction or the maintenance phases, except for a decrease in DHPG levels during the induction phase (Table 1).



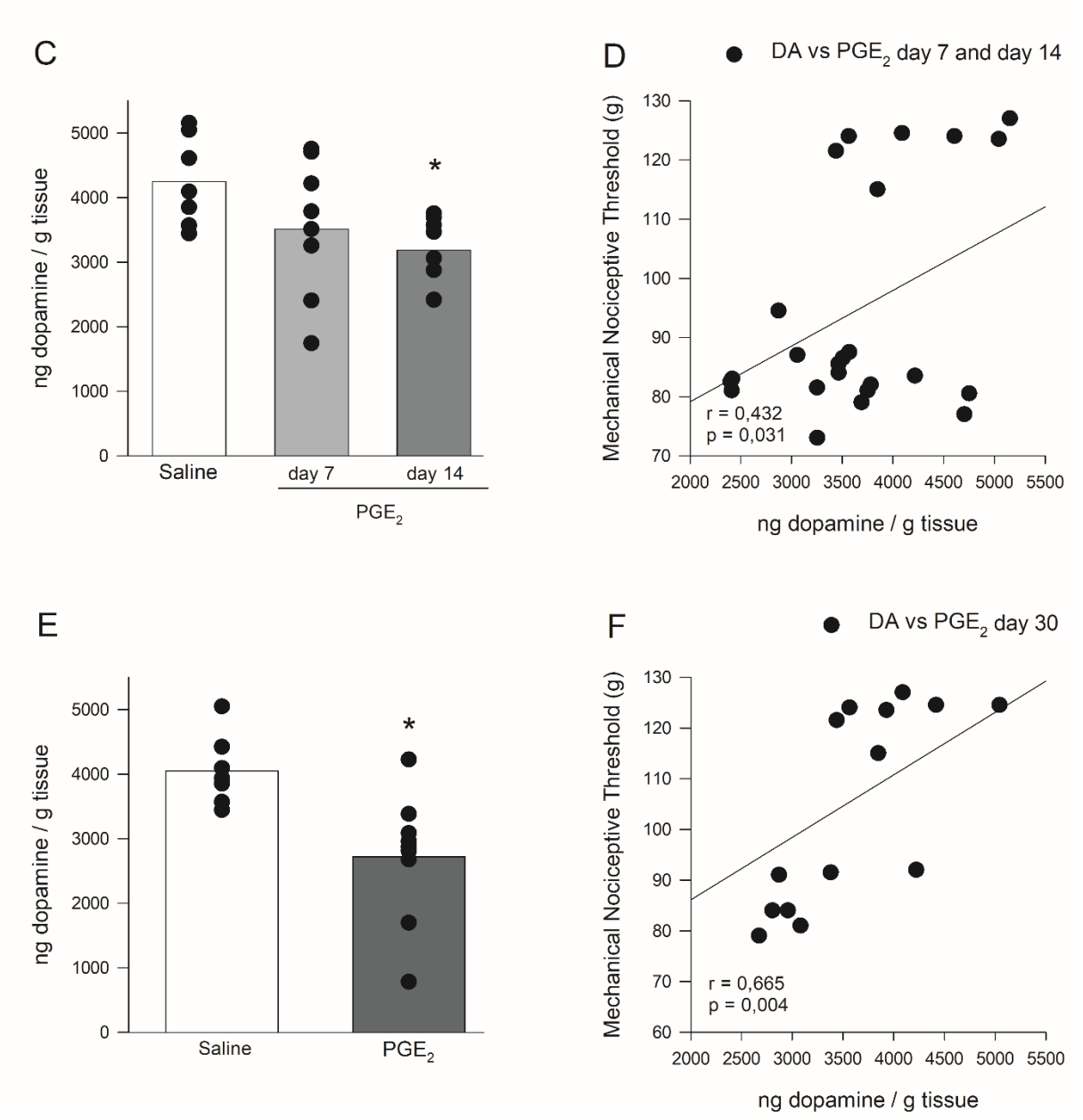


Figure 3. Dopamine levels during the chronification process and their correlation with the nociceptive response. A- A single PGE_2 injection did not affect dopamine levels in the NAc 3 h later; **B**- dopamine levels did not correlate with the nociceptive threshold 3 h later PGE_2 injection; **C**- 14 daily PGE_2 injections did significantly decreased dopamine levels in the NAc; **D**- the decreased dopamine levels positively correlate with the decreased nociceptive threshold; **E**- at day 30 (16 days after the discontinuation of daily PGE_2 injections) dopamine levels in the NAc remained significantly decreased; **F**- at day 30 the decreased dopamine levels positively correlate with the decreased nociceptive threshold. The symbol "*" indicates dopamine levels significantly lower than that of the saline groups, $p<0.005$.

CONCENTRATIONS OF MONOAMINES AND METABOLITES IN THE NAc			
Monoamine/ metabolite	Group	Mean±SEM	P
DOPAC	Saline	521.68 ± 39.03	*0.01
	PGE_2 *	742.84 ± 57.7	
	Saline	567.19 ± 24.93	0.937
	PGE_2 day 7	592.36 ± 59.97	
	PGE_2 day 14	585.96 ± 46.24	
	Saline	584.25 ± 39.96	0.368
	PGE_2 day 30	481.25 ± 71.32	
HVA	Saline	182.34 ± 10.55	0.723
	PGE_2	188.78 ± 13.33	
	Saline	177.24 ± 12.1	0.4
	PGE_2 day 7	161.94 ± 15.21	
	PGE_2 day 14	151.96 ± 9.29	
	Saline	168.62 ± 7.11	0.089
	PGE_2 day 30	137.96 ± 13.63	
DOPAC+HVA / DOPAMINE	Saline	0.15 ± 0.011	*<0.001
	PGE_2	0.22 ± 0.009	
	Saline *	0.18 ± 0.01	* 0.023
	PGE_2 day 7	0.21 ± 0.006	
	PGE_2 day 14 *	0.23 ± 0.009	
	Saline	0.18 ± 0.014	* 0.025
	PGE_2 day 30	0.24 ± 0.029	
Noradrenaline	Saline	103.83 ± 34.47	0.873
	PGE_2	94.54 ± 27.06	
	Saline	72.56 ± 7.37	0.409
	PGE_2 day 7	169.12 ± 46.25	
	PGE_2 day 14	174.72 ± 43.8	
	Saline	95.15 ± 35.25	0.29
	PGE_2 day 30	194.97 ± 61.67	
DHPG	Saline	46.89 ± 17.7	0.341
	PGE_2	27.31 ± 0.87	
	Saline	45.5 ± 17.93	*0.012
	PGE_2 day 7	18.79 ± 1.54	
	PGE_2 day 14	23.15 ± 3.95	
	Saline	44.24 ± 18.13	0.072
	PGE_2 day 30	21.26 ± 1.57	
Serotonin	Saline	949.11 ± 66.07	0.098
	PGE_2	763.46 ± 76.5	
	Saline	915.13 ± 70.7	0.573
	PGE_2 day 7	815.41 ± 73.61	
	PGE_2 day 14	894.28 ± 63.56	
	Saline	893.72 ± 80.45	0.456
	PGE_2 day 30	807.87 ± 76.17	
HIAA	Saline	404.33 ± 28.26	0.811
	PGE_2	394.98 ± 25.56	
	Saline	393.01 ± 27.9	0.467
	PGE_2 day 7	418.71 ± 38.55	
	PGE_2 day 14	441.31 ± 23.28	
	Saline	399.28 ± 25.1	0.449
	PGE_2 day 30	442.21 ± 44.25	

Table 1. Monoamines and their metabolites concentrations in the NAc (mean ± S.E.M.) The symbol "*" indicates a concentration significantly lower than that of the saline groups. DOPAC-dihydroxyphenylacetic acid; DHPG-Dihydroxyphenylglycine; HIAA- Hydroxyindoleacetic acid; HVA - homovanillic acid.

The blockade of kappa opioid receptors into the nucleus accumbens did not change the acute, but prevented and reversed the chronic prostaglandin E2-induced increase in nociceptive response

The microinjection of the long-acting kappa opioid receptor antagonist, nor-BNI, into the NAc had no effect either on basal nociceptive threshold or on PGE_2-induced acute hyperalgesia (Figure 3. A, repeated-measures ANOVA - $F_{treatment}$ (1, 20) = 0.77635, p=0.38872; $F_{treatment\ x\ time}$ (3, 60) =100.33, p<0.001; $F_{paw\ injection\ x\ treatment\ x\ time}$ (3, 60) = 0.80813, p=0.49432, followed by Tukey's post hoc test, $p < 0.05$).

The microinjection of nor-BNI into the NAc early in the induction phase prevented the development of the chronic hyperalgesic state (Figure 3 B, repeated-measures ANOVA - $F_{treatment}$ (1, 20) = 67,773, p<0.001; $F_{paw\ injection\ x\ time}$ (11, 220) =32.767, p<0.001; $F_{paw\ injection\ x\ treatment\ x\ time}$ (11, 220) =13.980, p<0.001, followed by Tukey's post hoc test, $p < 0.05$).

Importantly, the microinjection of nor-BNI into the NAc late in the induction phase interrupted the chronification process, preventing the development of the chronic hyperalgesia. Only two days after the discontinuation of PGE_2 injections, the group that received nor-BNI returned to the basal nociceptive threshold (Figure 3.C, repeated-measures ANOVA - $F_{treatment}$ (1, 23) =86.598, p=0.000001; $F_{paw\ injection\ x}$ F_{time} (10, 230) = 35,270, p<0.001; $F_{paw\ injection\ x\ treatment\ x\ time}$ (10, 230)=17.060, p<0.001, followed by Tukey's post hoc test, $p < 0.05$).

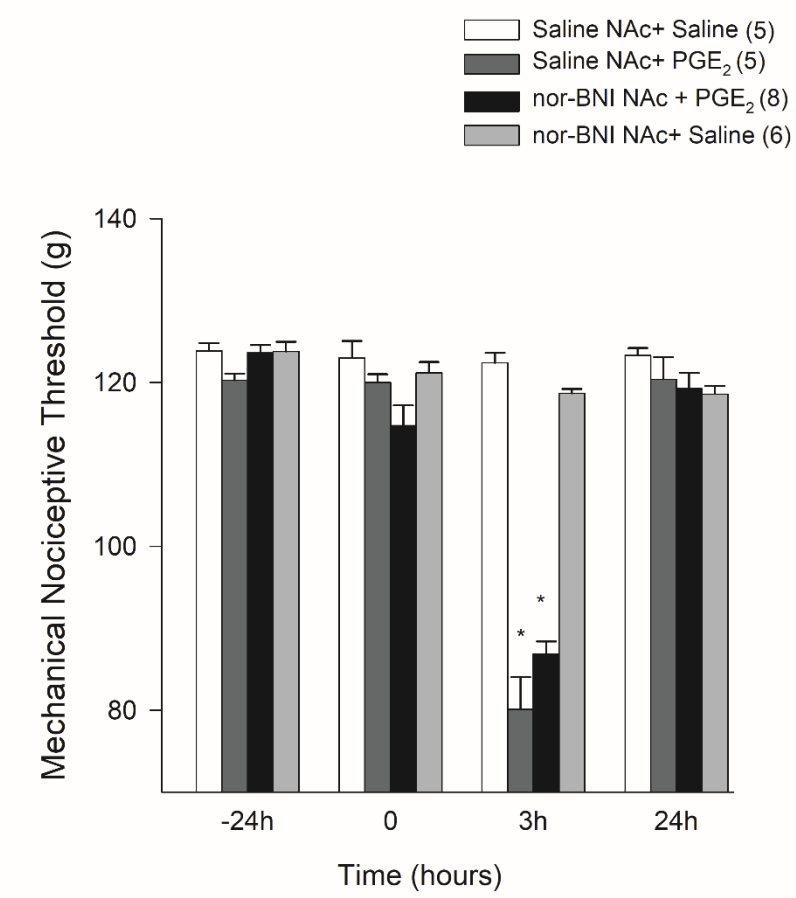


Figure 4. A- The effect of kappa opioid receptor blockade on PGE_2 induced acute increase in nociceptive response. The microinjection of the slow-onset and long-acting kappa opioid receptor antagonist nor-BNI into the NAc 24h before the paw injection did not affect either the basal nociceptive threshold (saline into the hindpaw) or the acute hyperalgesia induced by PGE_2. Repeated measures ANOVA, with one within-subjects factor (time) and one between-subjects factors (treatment intra-NAc and paw injection). The symbol "*" indicates a mechanical nociceptive threshold significantly lower than that of the saline groups at the same time point (Tukey's post-hoc test, $p < 0.0001$). Number in parenthesis indicates the number of animals in each group.

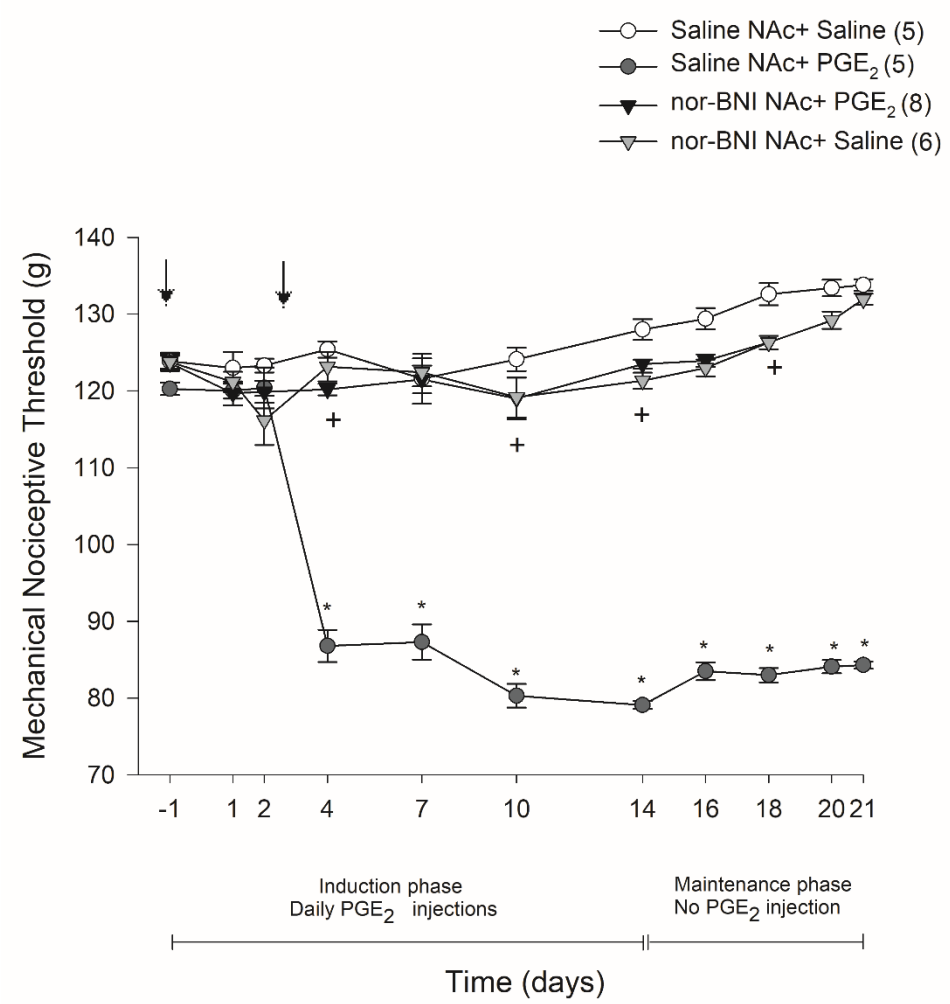


Figure 4. B- The effect of early kappa opioid receptor blockade on PGE_2 induced chronic increase in nociceptive response. The microinjection of the long-acting kappa opioid receptor antagonist nor-BNI into the NAc at the days -1 and 3 of the induction phase (arrows) prevented the development of the chronic hyperalgesia. Repeated measures ANOVA, with one within-subjects factor (time) and one between-subjects factors (treatment intra-NAc and paw injection). The symbol "*" indicates a mechanical nociceptive threshold significantly lower than that of the other groups at the same time point; the symbol "+" indicates a mechanical nociceptive threshold significantly lower than that of the saline group at the same time point (Tukey's post-hoc test, $p < 0.0001$). Number in parenthesis indicates the number of animals in each group.

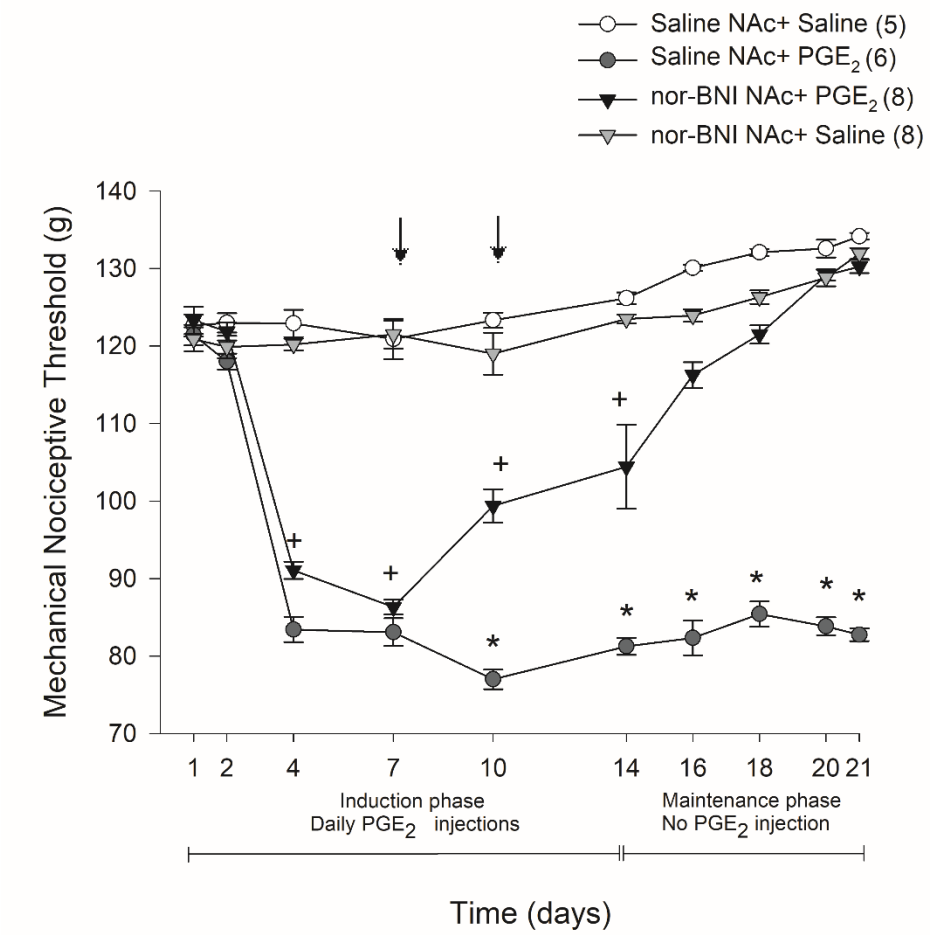


Figure 4. C- The effect of late kappa opioid receptor blockade on PGE_2 induced chronic nociceptive response. The microinjection of the long-acting KAPPA OPIOID RECEPTOR antagonist nor-BNI into the NAc at days 7 and 14 of the induction phase (arrows) stopped the development of the chronic hyperalgesia. The symbols "*" and "+" indicate a mechanical nociceptive threshold significantly different from that of all the other groups at the same time point (Tukey's post-hoc test, $p < 0.0001$). Number in parenthesis indicates the number of animals in each group.

The pharmacological activation of kappa opioid receptors did not change acute, but facilitated chronic prostaglandin E2-induced increase in nociceptive response

The microinjection of the kappa opioid receptor agonist, U50,488, into the NAc had no effect either on basal nociceptive threshold or on PGE_2-induced acute hyperalgesia (Figure 4 A repeated-measures ANOVA - $F_{treatment}$ (1, 27) = 6.2298, p=0.01897; $F_{paw\ injection\ x\ time}$ (1, 27) =129.03, p<0.001; $F_{paw\ injection\ x\ treatment\ x\ time}$ (2, 54) = 0.04400, p=0.95699, followed by Tukey's post hoc test, $p < 0.05$).

In order to investigated whether the pharmacological kappa opioid receptor activation in the NAc facilitates pain chronification, we performed 7 daily injections of PGE_2 into the hindpaw of animals receiving 7 daily microinjections of U50,488 or saline into the NAc. As expected, the mechanical nociceptive threshold rapidly and progressively returned to the baseline values in animals that received saline into de NAc. On the other hand, in animals that received the 7 daily microinjections of U50,488 into the NAc, hyperalgesia persisted long after the discontinuation of the 7 daily PGE_2 injection into the hindpaw (Figure 4 B repeated-measures ANOVA - $F_{treatment}$ (1, 27) = 217.14, $p<0.001$; $F_{paw\ injection\ x\ time}$ (10, 270) = 58.287, $p=0.00001$; $F_{paw\ injection\ x\ treatment\ x\ time}$ (10, 270) = 27.855, $p<0.001$, followed by Tukey's post hoc test, $p < 0.05$).

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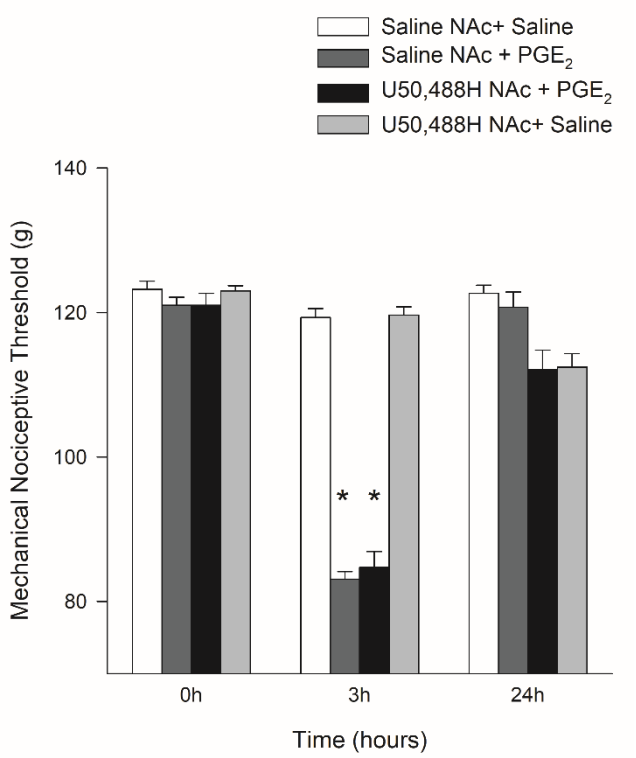


Figure 5. A- The effect of kappa opioid receptor activation on PGE_2 induced acute nociceptive response. The microinjection of the kappa opioid receptor agonist U50,488 into the NAc did not affect either the basal nociceptive threshold (saline into the hindpaw) or the acute hyperalgesia induced by PGE2. Repeated measures ANOVA, with one within-subjects factor (time) and one between-subjects factors (treatment intra-NAc and paw injection). The symbol "*" indicates a mechanical nociceptive threshold significantly lower than that of the saline groups at the same time point (Tukey's post-hoc test, $p < 0.0001$). Number in parenthesis indicates the number of animals in each group.

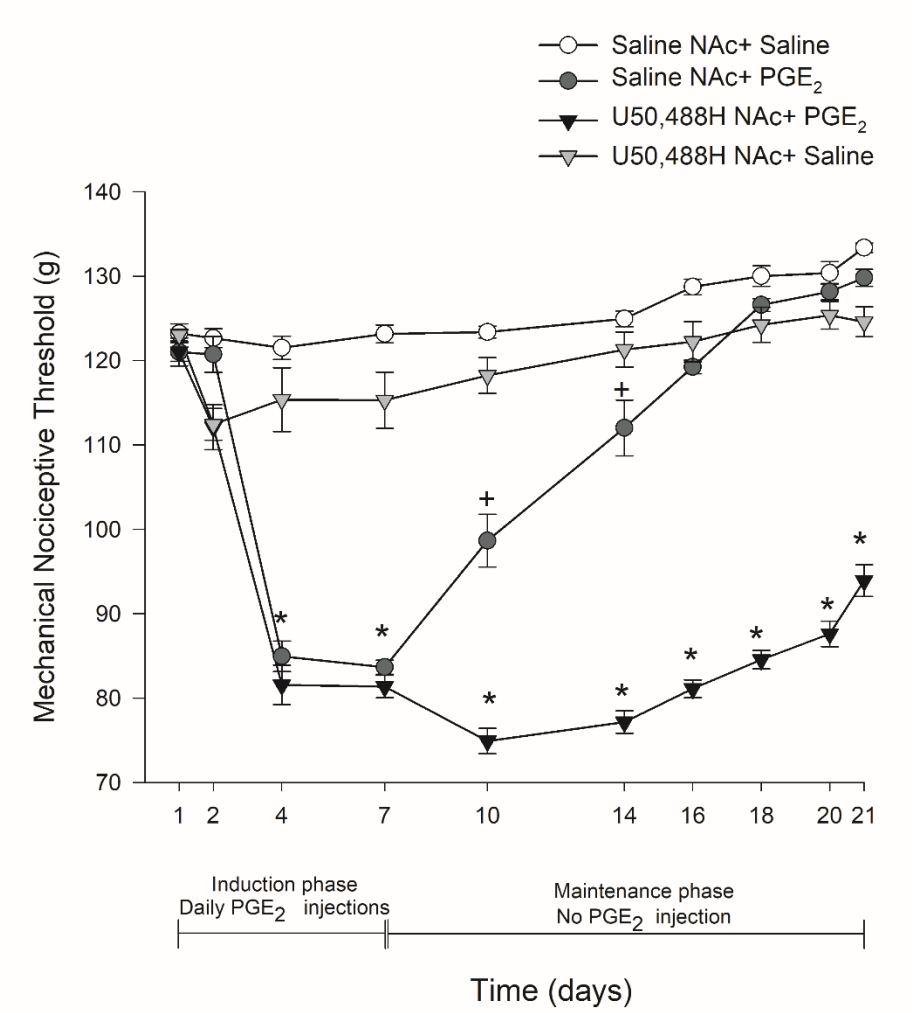


Figure 5. B- The effect of pharmacological kappa opioid receptor activation on PGE_2-induced chronic increase in nociceptive response. In this experiment, only 7 daily PGE_2 injections were performed and they were enough to brought about the chronic hyperalgesic state in animals that received 7 daily microinjections of U50,488 into the NAc. In contrast, the nociceptive threshold rapidly returned to the baseline values in animals that received saline into the NAc. Repeated measures ANOVA, with one within-subjects factor (time) and one between-subjects factors (treatment intra-NAc and paw injection). The symbols "*"and "+" indicate a mechanical nociceptive threshold significantly different from that of all the other groups (Tukey's post-hoc test, $p < 0.0001$). Number in parenthesis indicates the number of animals in each group.

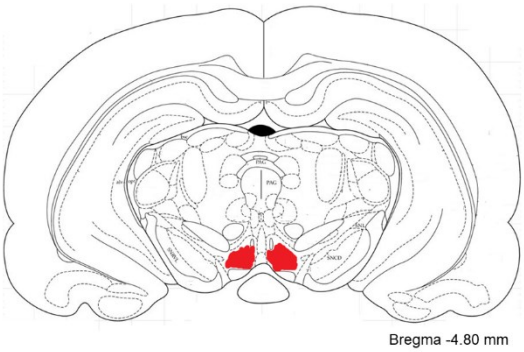
Spontaneous Locomotor Activity

None of the experimental interventions affected the animals' locomotion in an open field arena (Table 2).

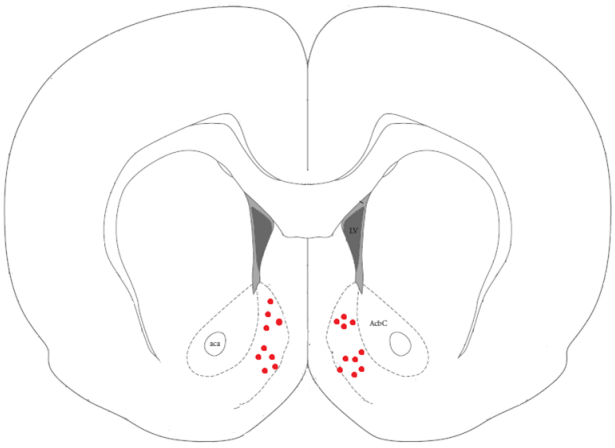
SPONTANEOUS LOCOMOTOR ACTIVITY					
Injection site	Group	Mean	SEM	F	P
NAc	Vehicle+vehicle (14 days)	54.2	1.71	0.09	0.766
	Vehicle+PGE2 (14 days)	59.0	3.94		
	nor+BNI (-1/3 day) +vehicle(14 days)	55.0	1.88		
	nor+BNI (-1/3 day)+PGE2 (14 days)	56.1	1.36		
	Vehicle+vehicle (14 days)	53.6	3.40	0.875	0.359
	Vehicle+PGE2 (14 days)	55.2	4.41		
	nor+BNI (7/10 day) +vehicle(14 days)	60.6	2.56		
	nor+BNI (7/10 day)+PGE2 (14 days)	56.9	1.03		
	Vehicle+vehicle (7 days)	52.3	2.50	0.373	0.546
	Vehicle+PGE2 (7 days)	55.6	2.40		
	U50,488 (7 days) +vehicle(7 days)	57.6	3.38		
	U50,488 (7 days) +PGE2(7 days)	57.6	3.91		
VTA	SHAM+PGE2 (14 days)	59.3	2.03	0.488	0.619
	Lesion+PGE2(14 days)	61.9	2.17		
	Lesion+vehicle(14 days)	59.9	1.68		
N/A	Vehicle (14 days)	59.4	1.17	0.424	0.833
	PGE2 (14 days)	59.5	2.19		

Table 2. Effect (mean ± S.E.M.) of experimental manipulations on locomotor activity in the open field test. None of the treatments significantly affected locomotion ($p > 0.05$, one-way ANOVA). N/A = not applicable; vehicle = 0.9% NaCl.

The anatomical reconstruction of the injection sites into the VTA and NAc shell sites is shown in supplementary material (Supplementary Figure S1 and S2).



Supplementary Figure S1. Diagrammatic representation of 6-OHDA lesion on cross-section from the atlas of Paxinos and Watson (2007). Red draw represents the local and extension of 6-OHDA lesion in the VTA.



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Supplementary Figure S2. Diagrammatic representation of injection sites in the NAc on cross-section from the atlas of Paxinos and Watson (2007). All injection sites were within the NAc shell. Red circles represent the local of drug infusion.

Discussion

The main findings of this study demonstrate that the transition from acute to chronic pain (1) requires mesolimbic dopaminergic activity; but (2) it is associate with a decrease in mesolimbic dopamine levels; and while (3) it is both prevented and reversed by the blockade of kappa opioid receptors within the NAc; (3) it is facilitated by the pharmacological activation of such receptors. Taken together, these findings demonstrate that the mesolimbic dopamine and kappa opioid systems are essential for pain to become chronic.

Most of clinical chronic pain conditions develop after an initial injury, which leads to persistent pain that becomes chronic. If we could understand the neuroplastic mechanisms that drive the transition from acute to chronic pain, we could develop strategies to stop or reverse them, preventing chronic pain development. In this aspect, the animal model we have used in this study may be of great translational relevance, since the chronic pain evolves in two well-defined phases. The induction phase (daily injection-period), when the pain chronification process develops and the maintenance phase, when chronic pain is already established lasting for at least 30 days (Figure 1). Therefore, the neuroplastic changes necessary for the transition from acute to chronic pain take place during the induction phase and strategies that prevent this transition are of interest as potential targets for the development of therapeutic approaches to prevent pain chronification. This study provided evidence to support two potential targets within the mesolimbic system.

The mesolimbic dopaminergic system has been implicated in the pathogenesis of different neuropsychiatric disorders, such as depression and drug addiction (Doan, Manders e Wang, 2015; Elman e Borsook, 2016; Elman, Borsook e Volkow, 2013). Recently its role in chronic pain conditions has reached the spotlight. However, a consideration of the utmost importance is whether the reported changes in mesolimbic dopaminergic activity are just consequence of chronic pain development or whether they actually contribute to the chronification process. This issue has not been directly addressed by most studies that are focused on the contribution of mesolimbic system to the negative affect, decreased motivation and anhedonia associated with chronic pain (Liu *et al.*, 2019; Massaly *et al.*, 2019; Schwartz *et al.*, 2014; Watanabe *et al.*, 2018). To our knowledge, this was the first study to demonstrate that the mesolimbic dopaminergic system is necessary for acute pain to become chronic. The evidence is that the lesion of mesolimbic dopaminergic cells, by injecting 6-OHDA into the VTA,

prevented chronic pain development without affecting the acute nociceptive response (Figure 2). These findings extend a previous study showing that the pharmacological blockade of NAc dopamine receptors impairs pain chronification (Dias et al., 2015). Together, these complementary data set suggest that the dopaminergic mesolimbic system may be a driver of the pain chronification process. This idea fits well with a human longitudinal study showing that a greater structural and functional corticolimbic connectivity predicts the transition from acute to chronic pain in humans (Vachon-Presseau et al., 2016).

Although mesolimbic dopamine is essential for the transition from acute to chronic pain, our findings show that its levels decrease as pain becomes chronic. Initially, NAc dopamine levels were neither affected by an acute nociceptive stimulus, nor they correlate with the intensity of nociceptive response (Figure 3 A and B). However, the increase in DOPAC + HVA/DA levels suggest an enhancement in dopaminergic turnover, that might be related to an increase in dopaminergic neurons activity during the acute pain (Nissbrandt e Carlsson, 1987; Wickham *et al.*, 2013). Although in this line, the literature is not conclusive because acute pain has been shown to increase (Budygin, Park e Bass, 2012); decrease (Leitl *et al.*, 2013) or not change (Park *et al.*, 2015) NAc dopamine levels. However, as the pain chronification process evolves, data become more conclusive. The decrease in NAc dopamine levels reaches statistical significance on day 14, when the chronification process ends, falling further on day 30 when chronic pain is well-established (Figure 3C and E). Interestingly, the lower is the dopamine level, the higher is the nociceptive response during both the induction and maintenance phases, as evidenced by the positive correlation between low dopamine levels and decreased nociceptive threshold (Figure 3D and F). In addition, the increased DA turnover during chronic pain, as indicated by the (DOPAC+HVA)/DA ratio (Table 1), might suggest an attempt to compensate the DA deficit by the dopaminergic neurons, a mechanism of plasticity also observed in animals models of dopamine deficiency (Moreira *et al.*, 2012). The impact of pain on the mesolimbic dopaminergic system contrast with almost no changes in the other monoamine systems. We did not detect any change in serotonin, noradrenaline and their metabolites, except that DHPG is significantly decreased during the induction phase and a decreasing tendency can be detected in the maintenance phase. These findings may reflect a decreased NE reuptake or metabolism, which is associated with pain facilitation (Martins *et al.*, 2013). Our findings of decreased NAc dopamine levels

in animals with chronic pain are consistent with evidence from both the animal and human literature showing that chronic pain leads to a hypodopaminergic state. For example, decreased dopamine levels and D2 receptors have been demonstrated both in chronic pain patients (Hagelberg et al., 2003; Loggia et al., 2014; Martikainen et al., 2015; Wood, Patrick B et al., 2007) and in animal models of chronic pain (Chang et al., 2014; Ren et al., 2016; Sagheddu et al., 2015). There are now multiple lines of evidence suggesting that this impairment of mesolimbic dopamine activity underlie the anhedonia related to depression-like behavior and decreased motivation common with chronic pain. For example, while the hedonic value of food rewards appears not to be significantly affected by chronic pain, it decreases the drive to obtain these rewards (Massaly et al., 2019; Schwartz et al., 2014). Although pain relief is associated with dopamine release into the NAc (Kato, Ide e Minami, 2016; Navratilova *et al.*, 2016; Navratilova, E. *et al.*, 2012), this release is no longer observed during late phases of chronic pain (Kato, Ide e Minami, 2016). These observations fit well with a clinical study showing that chronic pain patients have reduced hedonic response to rewards, which correlates with lower nucleus accumbens volume (Elvemo et al., 2015).

This consistent literature showing that the hypodopaminergic tone in the mesolimbic system underlies the negative affective dimensions of pain, has supported the view that strategies to restore dopamine signaling may represent a promising approach to manage chronic pain (Taylor *et al.*, 2016; Watanabe *et al.*, 2018). We cannot refute this idea, but this study sheds light on a new perspective because it has shown that mesolimbic dopaminergic activity is indeed essential for acute pain to become chronic. Based on our data, we believe that persistent pain continuously recruits mesolimbic dopaminergic neurotransmission, but over time, neuroplastic changes appear that decrease dopamine activity. Although the dopaminergic activity is initially essential to the neuroplastic changes, its later decrease drives in parallel the pain chronification process and its affective sequelae. A question that naturally arises is what mechanism is responsible for decreasing dopamine activity in chronic pain. Recent evidence points to the dynorphin/kappa opioid receptor system as a possible candidate.

The kappa opioid system has received attention in the last years in light of its role in several neuropsychiatric disorders associated with negative affective states, such as depression and drug addiction (Tejeda e Bonci, 2019). The activation of the kappa opioid system decreases mesolimbic dopamine activity and drives aversion,

pro-depressive behavior and anti-reward effects (Tejeda e Bonci, 2019). More recently, it was demonstrated that chronic pain is related to the increase in the kappa opioid system activity within the mesolimbic circuitry and that this underlies the negative affective component of pain (Liu *et al.*, 2019; Massaly *et al.*, 2019). However, no prior study has shown the role of the kappa opioid system in the pain chronification process. The present study demonstrated that the endogenous activation of the NAc kappa opioid receptors is necessary for acute pain to become chronic. The evidence is that the intra NAc administration of the kappa opioid receptor antagonist, nor-BNI, both prevented and reversed the pain chronification process (Figures 4 B and 4 C). Complementarily, the pharmacological activation of the NAc kappa opioid receptors facilitates the transition from acute to chronic pain. The evidence is that the intra NAc administration of the kappa opioid receptor agonist, U50,488 made it possible for the pain to become chronic in half the usual time (Figure 5 B). Interestingly, neither the kappa opioid receptor antagonist nor the agonist affected acute nociceptive responses (Figures 4 A and 5 A), which suggests that the mesolimbic kappa opioid system may only be recruited in the presence of chronic, but not transient pain states. This last observation is in agreement with a previous study showing that the mesolimbic kappa opioid system mediates chronic but not acute pain-induced aversion (Liu *et al.*, 2019).

While acute pain has a primary protective role that is critical for survival, chronic pain is pathological and devoid of biological significance. The transition from a protective/natural (acute pain) to a pathological (chronic pain) state underlies the development of most neuropsychiatric disorders, such as stress and anxiety disorders, depression and drug addiction. For example, drug addiction develops when the natural behavior of seeking reward is misrepresented, driving a compulsive behavior that, at this stage, aims to alleviate a pathological negative affective state. It is well-established that the increase in dopamine levels in NAc during the initial use of the drug drives its repetitive use. Over time, the resultant dopaminergic activity leads to allostatic changes, among them, the activation of the kappa opioid system, which in turn, reduces dopaminergic activity through a negative feedback mechanism. The addiction process is complete when a hypodopaminergic state is established, inducing the negative affective state that drives the compulsive use. It has been suggested that there are similarities between this process and the pain chronification process (Apkarian et al., 2013; Elman e Borsook, 2016) and the present study provide a direct experimental evidence to support this idea. This is because our data suggest that the

mesolimbic dopaminergic neurotransmission in parallel with the activation of the kappa opioid system into the NAc drive the pain chronification process, which culminates with a decrease in mesolimbic dopamine levels when the chronic pain state is established.

Summarizing, in this study we provide evidence that acute pain does not become chronic if mesolimbic dopamine or kappa opioid activities is blocked; in a complementary way, its chronification is facilitated if the mesolimbic kappa opioid activity is stimulated. Interestingly, none of the interventions that either prevented or facilitated pain chronification affected acute nociceptive responses, suggesting that the mesolimbic dopamine and kappa opioid systems may enable selective control of pathological pain, without interfering with physiological pain. Despite this evidence, the role of other neurotransmitters system in the transition from acute to chronic pain cannot be ignored. Similarly, neuroplastic changes outside of the mesolimbic system may also contribute to the pain chronification process. Nonetheless, our results support that the mesolimbic dopamine and kappa opioid systems are potential targets for the development of future therapeutic strategies to prevent acute pain from developing into chronic pain.

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4. DISCUSSÃO

Os principais achados deste estudo demonstram que a transição da dor aguda para a crônica requer atividade dopaminérgica mesolímbica, que a cronificação é tanto prevenida quanto revertida pelo bloqueio dos receptores opioides kappa ou facilitada pela ativação farmacológica desses receptores dentro do núcleo accumbens shell.

A dor quando aguda, demanda atenção e tem a capacidade de controlar o comportamento do indivíduo (Porreca e Navratilova, 2017), conforme ela vai cronificando, exige envolvimento contínuo dos circuitos motivacionais e emocionais do cérebro, levando à um processo de constante busca por alívio, sendo capaz de suprimir outras emoções, incluindo recompensas naturais, podendo levar à anedonia, ansiedade, depressão e outras doenças psiquiátricas, diminuindo drasticamente a qualidade de vida do indivíduo (Elman, Borsook e Volkow, 2013; Elvemo *et al.*, 2015; Simons, Elman e Borsook, 2014).

Na maioria dos casos clínicos de dor crônica, ela se desenvolve após uma lesão inicial, geralmente concomitante à inflamação, que leva à dor persistente que se torna crônica. Da mesma forma, o modelo utilizado é divido em fases (indução e manutenção), mimetizando a transição da dor aguda para a crônica. (Ferreira, Lorenzetti e Campos, de, 1990). Mostramos que ao longo de 14 dias de injeção de PGE_2 na pata, os animais permaneceram em um estado de hiperalgesia persistente que perdura mais de 15 dias após as injeções terem cessado (Figura 1). Essa divisão, em fases, nos permitiu estudar mecanismos subjacentes à cronificação da dor, antes dela se tornar crônica.

Alterações encontradas em pacientes com dor crônica envolvem principalmente os circuitos cerebrais responsáveis pelos estados emocionais e motivacionais, em especial, o sistema mesolímbico (Apkarian, Baliki e Farmer, 2013; Becerra *et al.*, 2013; Elvemo *et al.*, 2015; Vachon-Presseau *et al.*, 2016). o que resulta de uma possível neuroplasticidade do sistema mesolímbico na presença constante da dor, pois a dor persistente dispõe uma demanda alta do sistema motivacional (Apkarian, 2012), podendo resultar em adaptações ao longo do tempo, que levam à cronificação.

Modelos animais também comprovam a contribuição do sistema mesolímbico para o afeto negativo, diminuição da motivação e anedonia associada à dor crônica

(Bilbao *et al.*, 2018; Massaly *et al.*, 2019; Narita *et al.*, 2005). A diminuição da atividade das células dopaminérgicas da VTA é encontrada nos modelos de dor neuropática e câncer e sua ativação reverte a alodinia termal (Watanabe *et al.*, 2018) e é consistente com a diminuição da sinalização dopaminérgica no NAc e perda da inibição do receptor dopaminérgico D2 em neurônios da via para promover a alodinia no modelo neuropático (Chang *et al.*, 2014; Ren *et al.*, 2016).

Em nosso estudo, demonstramos que uma lesão na VTA, núcleo responsável pela transmissão dopaminérgica mesolímbica, previne a transição da dor aguda para a dor crônica. Embora não tenha afetado a resposta nociceptiva aguda à PGE_2 (Figura 2 A), a lesão de células dopaminérgicas da VTA impede o desenvolvimento da dor crônica (Figura 2 B), o que demonstra que é necessário a atividade da dopamina mesolímbica para a dor aguda se tornar crônica. Esses achados estendem um estudo anterior mostrando que o bloqueio farmacológico dos receptores de dopamina NAc prejudica a cronificação da dor (Dias *et al.*, 2015). Juntos, esses dados complementares sugerem que uma neuroplasticidade do sistema mesolímbico dopaminérgico pode ser um fator determinante do processo de cronificação da dor.

Classicamente, a liberação de dopamina no NAc está relacionada à recompensa (Lammel, Lim e Malenka, 2014), mas nos últimos anos, ela também está associada a respostas aversivas como a própria dor (Brooks e Berns, 2013; Mccutcheon *et al.*, 2012). Durante a presença de um estímulo doloroso, ocorre liberação de DA (Budygin, Park e Bass, 2012). Em torno de 90–95% dos neurônios do NAc, são células de projeção, chamados de neurônios espinhosos médios (MSNs), que são divididos em duas vias: via direta e via indireta. A via direta é formada por neurônios de projeção direta GABAérgica (dSPNs), cuja atividade é aumentada pela dopamina (DA), atuando nos receptores pós-sinápticos D1 de DA (D1-MSNs), inibindo diretamente o mesencéfalo dopaminérgico ventral e desinibindo o tálamo, promovendo comportamentos associados à recompensa e ao afeto positivo. Já a via indireta, sustentada por neurônios de projeção indireta GABAérgicos (iSPNs), cuja atividade é suprimida por DA ao atuar em receptores pós-sinápticos D2 de DA (D2-MSNs), inibindo o pálido ventral, que inibe o mesencéfalo ventral, resultando na inibição do tálamo promovendo comportamentos associados à eventos aversivos e afeto negativo (Gerfen e Surmeier, 2011; Kupchik e Kalivas, 2017; Ren *et al.*, 2016).

Os mecanismos de transmissão dopaminérgica podem acontecer através de liberações fásicas ou tônicas. A liberação tônica resulta em baixos níveis

de DA que levam principalmente a ativação de receptores D2, pois estes apresentam maior afinidade pela DA (Danjo *et al.*, 2014; Jarcho *et al.*, 2012). Já a liberação fásica resulta em altos níveis de DA, levando a excitação tanto de D1 quanto de D2 (Danjo *et al.*, 2014; Jarcho *et al.*, 2012). Tanto a recompensa quanto a aversão liberam DA no NAc (Yang *et al.*, 2018). Estímulos nociceptivos excitam a VTA, liberando a DA de forma fásica nos MSNs do NAc (Brischoux *et al.*, 2009; Jong, de *et al.*, 2019). Os terminais dopaminérgicos provindos da VTA medial são excitados e liberam DA no NAcSh medial por estímulos aversivos (Jong, de *et al.*, 2019). A estimulação aguda de receptores D2 no NAc inibiu dor inflamatória em modelos animais (Dias *et al.*, 2015; Taylor, Joshi e Uppal, 2003).

Através de técnica de HPLC, demonstramos que ao longo de diferentes dias da hiperalgesia persistente (Figura 3), a concentração de DA no NAc se altera. Ocorre sua diminuição conforme a dor vai se tornando persistente, como já foi demonstrado em estudos anteriores em modelos neuropáticos (Chang *et al.*, 2014; Kato, Ide e Minami, 2016; Ren *et al.*, 2016) e em pacientes humanos (Martikainen *et al.*, 2015; Wood, Patrick B *et al.*, 2007). Curiosamente, quanto menor o nível de dopamina, maior é a resposta nociceptiva durante as fases de indução e manutenção, como evidenciado pela correlação positiva entre baixos níveis de dopamina e diminuição do limiar nociceptivo (Figura 3 D e F). Além disso, tanto a dor aguda quanto a crónica claramente aumentam o turnover da dopamina, conforme indicado pela relação (DOPAC + HVA) / DA (Tabela 1), demonstrando um aumento do metabolismo dopaminérgico de modo compensatório, como uma tentativa de aumentar a síntese de DA. O impacto da dor no sistema dopaminérgico mesolímbico contrasta quase sem alterações nos outros sistemas monoaminérgicos.

A permanente liberação de DA, leva a estimulação ininterrupta de neurônios D1-MSNs, provocando a liberação de dinorfina, um agonista kappa opioide endógeno, no NAc (Muschamp e Carlezon, 2013). A dor também induz um aumento de dinorfina e essa liberação de dinorfina está relacionada ao estado afetivo negativo (Liu *et al.*, 2019; Massaly *et al.*, 2019). Finalmente o aumento da dinorfina reduz a DA através de um feedback negativo (Muschamp e Carlezon, 2013).

A dinorfina e os receptores kappa opioide são conhecidos por serem importantes reguladores do circuito NAc e a ativação de KOR inibe a transmissão de glutamato e dopamina no NAc (Muschamp e Carlezon, 2013). A dor neuropática em animais aumenta a expressão e ativação de KOR, e subsequentemente a diminuição

da liberação de dopamina em regiões do cérebro importantes, como NAc e AMY, para recompensa e afeto (Liu *et al.*, 2016). Os neurônios do NAcSh são responsáveis para liberação de dinorfina na presença de dor (Massaly *et al.*, 2019), e ela, a dinorfina, é responsável pelo afeto negativo e perda motivacional causados pela dor (Liu *et al.*, 2019; Massaly *et al.*, 2019). Antagonistas kappa opioide apresentam melhora desses sintomas em animais, sendo um possível alvo terapêutico em pacientes humanos (Liu *et al.*, 2019; Massaly *et al.*, 2019). Nosso estudo evidencia o papel do sistema kappa opioidérgico no NAcSh como mecanismo paralelo e auxiliar à cronificação da dor. Em relação a dor aguda, é importante notar que nem o antagonista irreversível nor-BNI ou agonista U50,488 afetaram o efeito da PGE_2 (Figuras 4 A e 5 A). Em relação ao processo de cronificação, quando os efeitos do kappa opioide são bloqueados por nor-BNI, a dor persistente é tanto prevenida (Figura 4 B) quanto revertida (Figura 4 C). Já, quando o agonista kappa U50,488 é injetado no NAcSh durante 7 dias, juntamente com 7 dias de PGE_2, os animais apresentam hiperalgesia persistente (Figura 5 B), sendo que 7 dias de PGE_2 não são suficientes para levar a hiperalgesia persistente.

É importante considerar a possibilidade de que outros centros de neurônios contendo dinorfina, como o pálido ventral e o hipotálamo lateral também são recrutados durante as condições de dor (Baldo *et al.*, 2003; Peyron *et al.*, 1998). A ativação do sistema kappa localizado fora do NAc pode ser necessária para conduzir adaptações induzidas pela dor em afeto negativo ou estresse, como a ativação de kappa opioide na amígdala (Nation *et al.*, 2018; Navratilova *et al.*, 2018), assim como a liberação de outros neuropeptídios que podem estar envolvidos no mecanismo alostático através do qual a dor crônica afeta o microcircuito do NAc.

Outro ponto importante a ser considerado é que uma pequena porção dos neurônios MSNs do NAc co-expressam receptores D1 e D2, formando um receptor heterômero D1/D2 (Hasbi *et al.*, 2009; Perreault *et al.*, 2010). A ativação desses heterômeros D1/D2 desencadeia a sinalização intracelular de cálcio, levando a produção de fator neurotrófico derivado do cérebro (BNDF), geralmente envolvido na plasticidade sináptica (Hasbi *et al.*, 2009; Verma *et al.*, 2010). É relevante que esses heterômeros D1/D2 também estão localizados em neurônios produtores de encefalinas e dinorfinas (Perreault *et al.*, 2010). Perreault *et al,* demonstraram que a sensibilidade heterométrica de D1/D2 e a sua atividade funcional foram positivamente reguladas no estriado de ratos quando expostos a tratamento crônico de anfetamina,

podendo contribuir para psicopatologias de abuso de drogas, esquizofrenia ou outras desordens envolvendo transmissão de dopamina (Perreault *et al.*, 2010). Portanto, esses heterômeros poderiam atuar durante a cronificação da dor, levando ao aumento da liberação de dinorfina e neuroplasticidade que torna a dor crônica.

Em resumo, esse estudo constatou a importância da dopamina durante o processo de transição da dor aguda para a dor crônica no microcircuito VTA- NAcshell. Diferentemente de outros estudos, que focavam a relevância da dinorfina e ativação de KOR no processo de afeto negativo causado pela dor (Liu *et al.*, 2019; Massaly *et al.*, 2019), esse trabalho demonstrou a importância da ativação de KOR para tornar a dor crônica, abrindo novos horizontes para futuros tratamentos.

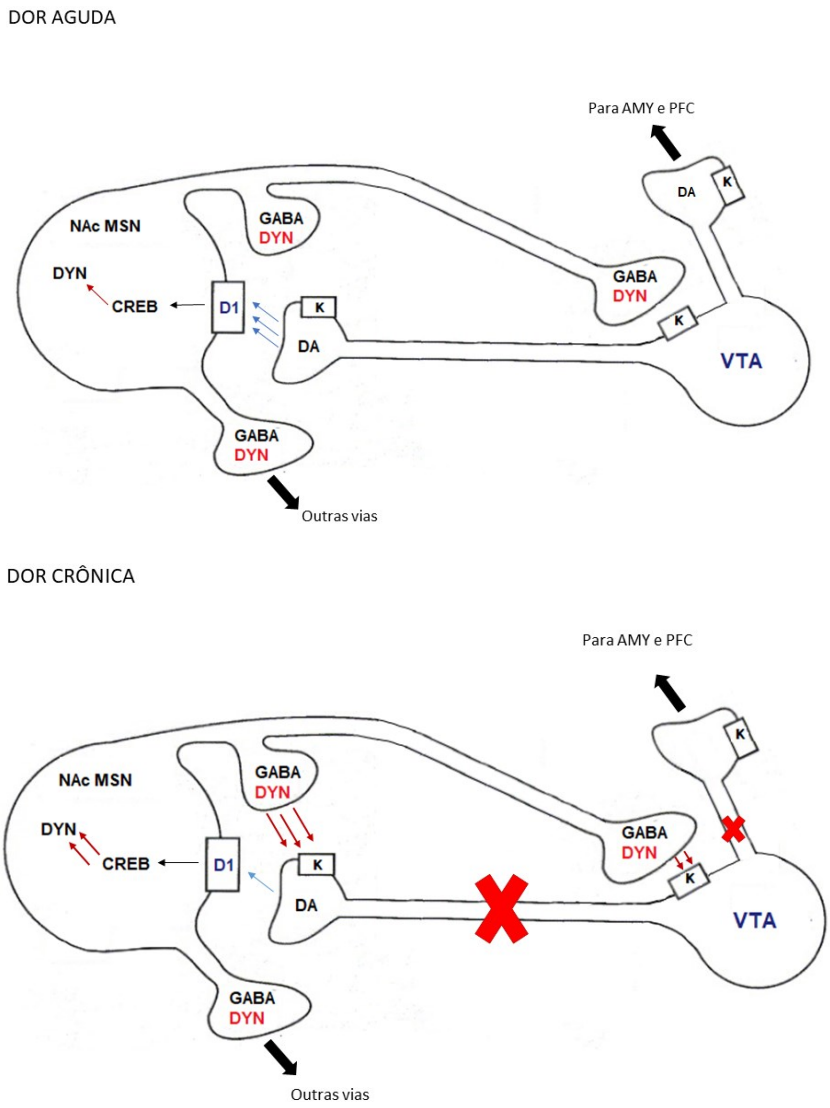

Figura A. Resumo do papel da dinorfina na cronificação da dor. Na presença de dor aguda, a dinorfina endógena é liberada, auxiliando no estado afetivo negativo. Já na dor crônica, ocorrem

mudanças neuroplásticas, há um alto tônus de dinorfina sendo liberado no NAc e na VTA, diminuindo a liberação de dopamina.

5. CONCLUSÃO

Neste estudo proporcionamos evidências de que a dor aguda não se torna crônica se as atividades mesolímbicas de dopamina ou kappa opioides estiverem bloqueadas e sua cronificação é facilitada se a atividade kappa opioide mesolímbica for estimulada. Surpreendentemente, nenhuma das intervenções que preveniram ou facilitaram a cronificação da dor afetou as respostas nociceptivas agudas, sugerindo que os sistemas mesolímbico de dopamina e opioides kappa podem permitir o controle seletivo da dor patológica, sem interferir na dor fisiológica. Apesar dessa evidência, o papel de outros sistemas de neurotransmissores na transição da dor aguda para a crônica não pode ser ignorado. Da mesma forma, alterações neuroplásticas fora do sistema mesolímbico também podem contribuir para o processo de cronificação da dor. Apesar disso, nossos resultados confirmam que os sistemas mesolímbico de dopamina e kappa opioides são potenciais alvos para o desenvolvimento de futuras estratégias terapêuticas para evitar que a dor aguda evolua para dor crônica.

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ANEXO

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Comissão de Ética no Uso de Animais
(CEUA)

Nº 1156

CERTIFICADO

A Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR), instituída pela Resolução Nº 86/11 do Conselho de Ensino Pesquisa e Extensão (CEPE), de 22 de dezembro de 2011, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado estão de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais para a experimentação animal.

STATEMENT

The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), established by the Resolution Nº 86/11 of the Teaching Research and Extension Council (CEPE) on December 22nd 2011, **CERTIFIES** that the procedures using animals in the research project specified below are in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation.

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TÍTULO: Mecanismos mesolímbicos envolvidos na cronificação da dor.

TITLE: Mesolimbic mechanisms underlying pain chronification.

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DEPARTAMENTO/DEPARTMENT: Fisiologia

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