

**UNIVERSIDADE FEDERAL DE SANTA CATARINA
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Daniel Fernandes Martins

**ANÁLISE DOS MECANISMOS NEUROFISIOLÓGICOS
ENVOLVIDOS NO EFEITO DA TERAPIA MANUAL
(MOBILIZAÇÃO ARTICULAR) NA DOR AGUDA E CRÔNICA**

Florianópolis
2013

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Tese submetida ao Programa de Pós-Graduação em Neurociências, da Universidade Federal de Santa Catarina, para a obtenção do Grau de Doutor em Neurociências.

Orientador: Prof. Adair Roberto Soares dos Santos, Dr.

Co-orientador: Vinícius de Maria Gadotti, Dr.

Florianópolis
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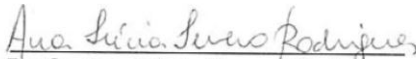
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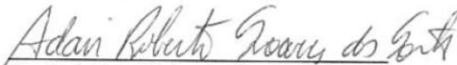
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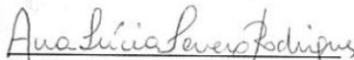
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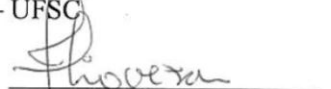
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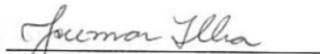

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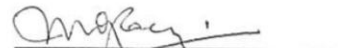
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RESUMO

Evidências sugerem que as técnicas de terapia manual são efetivas no tratamento de problemas músculo-esqueléticos incluindo dor lombar, síndrome do túnel do carpo, osteoartrite de joelho e quadril. No entanto, apesar dos seus resultados positivos e da sua eficácia, os mecanismos neurofisiológicos destas técnicas foram pouco estudados. Assim, o objetivo deste estudo foi de verificar o efeito da mobilização articular do tornozelo (MAT) na dor aguda e crônica de origem pós-operatória e neuropática, respectivamente, além de investigar os mecanismos de ação envolvidos neste efeito. Para tal, foram utilizados camundongos Swiss machos (25-35g) submetidos ao modelo de dor pós-operatória através da incisão plantar (IP) e ao tratamento com a MAT por 3 ou 9 minutos. A avaliação da dor (nociceção) foi realizada através da mensuração da hiperalgesia mecânica, utilizando-se o filamento de von Frey (0,4 g) pelo método de frequência de resposta, antes e após a incisão e 30 minutos após a MAT, diariamente. Além disto, os animais também receberam tratamento diário com MAT durante 9 minutos por 6 dias. No estudo do mecanismo de ação, as análises foram realizadas 24 horas após a IP e o envolvimento dos sistemas opioidérgico, adenosinérgico, monoaminérgico e endocanabinoidérgico no efeito da MAT foram investigados. Para avaliar a influência da MAT sobre a dor neuropática, utilizou-se o modelo do esmagamento do nervo isquiático em ratos Wistar machos (250-280 g), sendo que os animais receberam 15 tratamentos (9 minutos de MAT) em dias alternados. A hiperalgesia mecânica foi avaliada antes e ao longo do tratamento por 5 semanas. Trinta e cinco dias após a lesão, a medula espinal (L4-L5) foi coletada

para determinação da imunoreatividade de CD11b/c (microglia) e de GFAP (astrócito). Os resultados demonstram que 9 minutos de MAT reduz a hiperalgesia mecânica induzida pela IP, efeito mediado pelos sistemas opioidérgico, adenosinérgico, monoaminérgico e endocanabinoidérgico. Além disso, a MAT produz efeito antihiperalgésico e neuroregenerativo na lesão do nervo isquiático, ocorrendo estes efeitos em paralelo com a redução da imunoreatividade para microglia e astrócito na medula espinal. Assim, conclui-se que: i) a MAT apresenta grande potencial terapêutico na redução da dor pós-operatória e neuropática, bem como em acelerar a regeneração nervosa periférica; ii) os sistemas opioidérgico, adenosinérgico, monoaminérgico e endocanabinoidérgico estão envolvidos no efeito antihiperalgésico da MAT e; iii) a redução da ativação de células microgлияis e astrocítárias na medula espinal contribui para o efeito antihiperalgésico da MAT. Neste sentido, estes dados fornecem à literatura substanciais subsídios neurofisiológicos do efeito terapêutico da MAT para o direcionamento de futuros ensaios clínicos.

Palavras-chave: Fisioterapia. Terapia manual. Reabilitação. Dor aguda. Dor neuropática.

ABSTRACT

Manual therapy techniques are effective in the treatment of musculoskeletal problems including low back pain, carpal tunnel syndrome, osteoarthritis of the knee and hip. However, despite their positive results and its effectiveness, the neurophysiological mechanisms of these techniques are not thoroughly known. This study investigates the effect of ankle joint mobilization (AJM) in acute and chronic pain of postoperative and neuropathic origin, respectively, and the possible mechanisms involved in this effect. To this end, male Swiss mice (25-35g) were subjected to a plantar incision procedure (PI), a model of postoperative pain, and treated with AJM for 3 or 9 minutes. Pain levels (nociception) were assessed by measuring mechanical hyperalgesia to a von Frey filament (0.4g) by the method of frequency response before and after PI and 30 minutes after daily AJM. In addition, the animals also received daily 9 minutes sessions with AJM for 6 days. For the investigation of the mechanisms of action, the tests were performed 24 hours after PI and opioid, adenosinergic, monoaminergic and endocannabinoid systems were analyzed at different sites (central, peripheral and local). In order to evaluate the influence of AJM on neuropathic pain the model of sciatic nerve crush was used in male Wistar rats (250-280 g). These animals received 15 treatments (9 minutes AJM) on alternate days. The mechanical hyperalgesia was assessed before and during treatment for 5 weeks. Thirty-five days after injury, the spinal cord (L4-L5) was collected for determination of immunoreactivity to CD11b / c (microglia) and GFAP (astrocytes). The results show that 9 minutes AJM reduces the mechanical hyperalgesia

induced by PI, an effect that is mediated by opioid, adenosinergic, monoaminergic and endocannabinoid systems. Finally, we showed that AJM produces antihyperalgesic and regenerative effects, and these effects occur in parallel to the reduction in immunoreactivity to GFAP CD11b/c in the spinal cord. In conclusion, we demonstrated that: i) AJM has great therapeutic potential in reducing postoperative and neuropathic pain as well as accelerates peripheral nerve regeneration; ii) opioid, adenosinergic, monoaminergic and endocannabinoid systems are involved in the antihyperalgesic effect of AJM and iii) reducing the activation of microglial cells and astrocytes in the spinal cord contributes to the antihyperalgesic effect of AJM. Thus, these data provide substantial input to the neurophysiological literature of the therapeutic effect of AJM that will help to guide future clinical trials.

Keys words: Physiotherapy. Manual Therapy. Rehabilitation. Acute pain. Neuropathic pain.

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LISTA DE ABREVIATURAS E SIGLAS

- ACP - analgesia controlada pelo paciente
- AD - adenosina desaminase
- AEA - anandamida
- AQ - adenosina quinase
- AMPA - α -amino-3-hidroxi-5-metil-4-isoxazolepropionato
- ATP - trifosfato de adenosina
- AVDs - atividades de vida diárias
- CB - canabinóides
- CCL2 - CC *ligand 2*
- CGRP - peptídeo relacionado ao gene da calcitonina
- CR3 - receptor para o complemento-3
- CX3CL1 - CX3C *ligand 1*
- DAG - diacil glycerol
- DOR - receptor opióide do tipo delta
- DRG - glânglio da raiz dorsal
- ERK - quinase regulada por sinais extracelulares
- FAAH - amido hidrolase de ácidos graxos
- GABA - ácido gama-aminobutírico
- GFAP - proteína glial fibrilar acídica
- GLU - glutamato
- GRPCs - receptores acoplados a proteína G
- PENK - proencephalina
- PGs – prostaglandinas
- POMC - proopiomelanocortina
- IL-1 β - interleucina - 1 beta

IL-6 - interleucina - 6

i.p. - intraperitoneal

i.pl. - intraplantar

i.t. - intratecal

IASP - Associação Internacional para o Estudo da Dor

JNK - quinase terminal C-jun-N

KOR - receptor opióide do tipo kappa

MA - Mobilização articular

MAT - Mobilização articular do tornozelo

MAGL - mono acil glicerol lipase

MAPK - quinases ativadas por mitógenos

MHC - complexo principal de histocompatibilidade

MOR - receptor opióide do tipo mu

NO - óxido nítrico

PAG - substância cinzenta periaquedutal

PK - proteína quinase

RsCBs - receptores canabinóides

RVM - bulbo ventral rostral

SNC - sistema nervoso central

SNP - sistema nervoso periférico

SP - substância P

TNF- α - fator de necrose tumoral - alfa

TTM - técnicas de terapia manual

VAS - escala de dor análoga visual

2-AG - 2 araquidonil glicerol

Δ^9 -THC - Δ^9 -tetrahydrocannabinol

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1 APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos, os quais encontram-se no item **ARTIGOS CIENTÍFICOS**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos e representam a íntegra deste estudo.

Os itens, **DISCUSSÃO E CONCLUSÕES**, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre os artigos científicos contidos neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente à citações que aparecem nos itens **INTRODUÇÃO** e **DISCUSSÃO** desta tese.

2 DESENVOLVIMENTO

2.1 PROCESSAMENTO DA DOR

Tem sido descrito que a dor, diferentemente da audição, visão, propriocepção, ou olfação, não é um sentido primário, mas ao invés disso é mais do que uma experiência emocional. Muitos especialistas envolvidos no tratamento da dor a consideram como uma percepção complexa evocada por um estímulo nocivo. Talvez seja a mais complexa entre as modalidades perceptuais do nosso corpo. Por exemplo, num quadro de lesão nervosa, onde a dor é persistente, o estímulo que evoca dor pode mudar, nestas condições estímulos inócuos prontamente evocam a percepção da dor (alodinia) (BASBAUM; BUSHNELL, 2009).

No entanto, mesmo esta percepção incomum (alodinia) não descreve claramente as características que fazem da dor uma das mais complexas percepções. A Associação Internacional para o Estudo da Dor (IASP) define a dor como "Uma experiência sensorial e emocional desagradável associada a dano tecidual real ou potencial, ou descrita em termos de tal dano" (LOESER; TREEDE, 2008). Em outras palavras, apesar de existir uma base anatômica e fisiológica específica para a detecção e transmissão das mensagens que são interpretadas como dolorosas, o que torna a experiência de dor tão especial é que há sempre uma qualidade emocional à esta experiência (BASBAUM, BUSHNELL, 2009). A dor, no sentido fisiológico, envolve um sistema sensorial que transmite uma importante informação adaptativa sobre o ambiente para o organismo. Este tipo de sinalização é conhecida como nocicepção. A

sinalização da dor geralmente inicia pela ativação de fibras do tipo δ e C (Figura 1). Neurônios aferentes nociceptivos do tipo C podem ser divididos em dois principais grupos neuroquímicos: aqueles que contêm neuropeptídeos, como a substância P (LAWSON; CREPPS; PERL, 1997), e aqueles que não contêm (SNIDER; MCMAHON, 1998). Estes dois grupos possuem distintas zonas de terminação dentro da lâmina superficial. Fibras não-peptidérgicas do tipo C estão principalmente associadas com a pele, que fazem parte da inervação da epiderme (TAYLOR; PELESHOK; RIBEIRO-DA-SILVA, 2009), enquanto as fibras peptidérgicas inervam vários outros tecidos, bem como regiões mais profundas da pele (BENNETT et al., 1996; PERRY; LAWSON, 1998; PLENDERLEITH; SNOW, 1993).

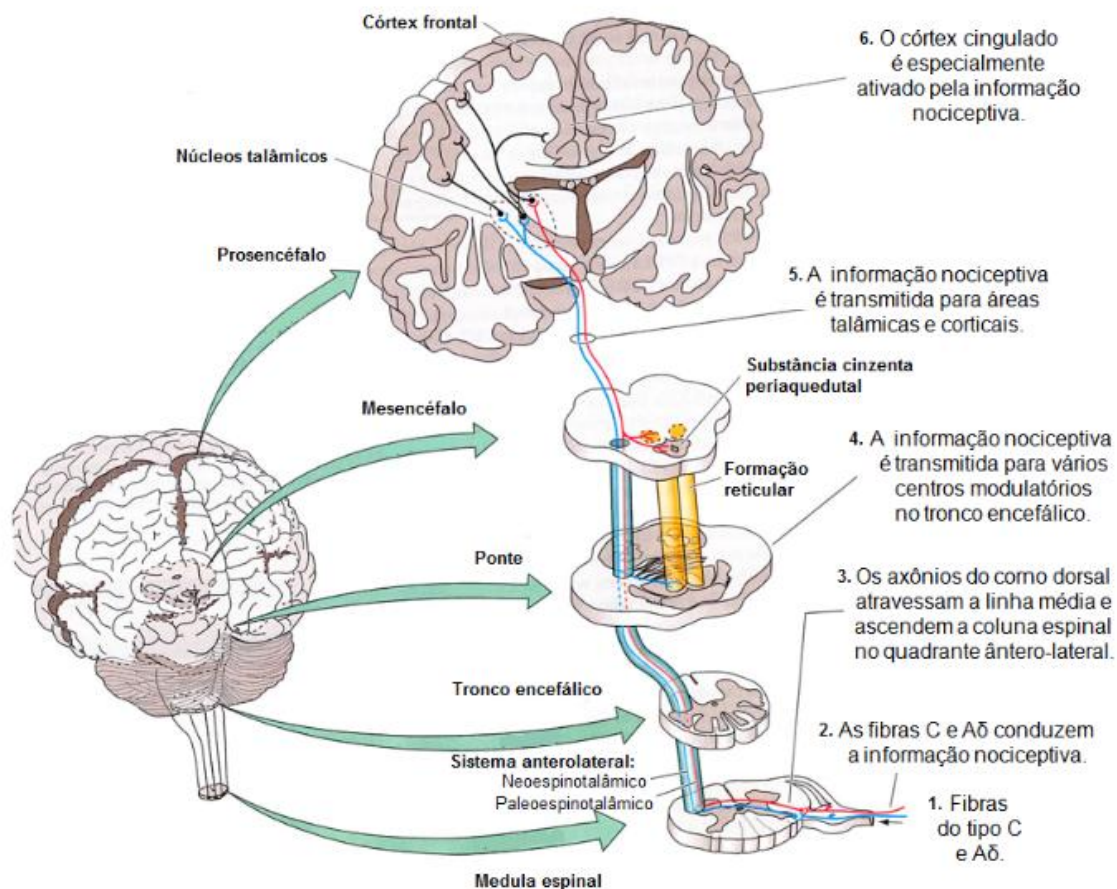
As fibras aferentes primárias do tipo $A\delta$ e C entram na medula espinal via divisão lateral da zona de entrada da raiz posterior. As fibras penetram no corno posterior e se bifurcam em ramos ascendentes e descendentes. Alguns colaterais terminam em interneurônios da substância cinzenta medular. Estas conexões participam de circuitos que medeiam reflexos espinhais tais como o reflexo flexor de retirada. Os neurônios do corno posterior da medula espinal exibem numerosos receptores de membrana. Estes incluem receptores da neurocinina 1 (NK1, receptor para substância P), receptores para o ácido gama-aminobutírico (GABA), serotonina, peptídeos opióides, glicina e receptor do tipo α -amino-3-hidroxi-5-metil-4-isoxazolepropionato (AMPA) para o glutamato. As propriedades dos neurônios do corno posterior da medula espinal refletem o tipo de informação da fibra aferente primária recebida. Estes neurônios são classificados como de baixo limiar (não-nociceptivos), nociceptivos-específicos (nociceptivos),

de ampla variação dinâmica (não-nociceptivos e nociceptivos), ou profundo com base em suas respostas a diferentes modalidades de estímulos. Em geral, o alvo central das fibras aferentes primárias nociceptivas incluem as lâminas I, II e V do corno posterior. Fibras aferentes do tipo A δ têm como alvo as lâminas I e V (PURVES et al., 2010).

O sistema ântero-lateral (SAL) é um feixe composto que inclui fibras espinotalâmicas, espinomesencefálicas, espinoreticulares, espinobulbares e espino-hipotalâmicas. As fibras espinotalâmicas projetam-se da medula espinal para o núcleo postero-lateral ventral (NPLV), o grupo nuclear posterior e os núcleos intralaminares (núcleos central lateral e parafasciculares centromedianos) do tálamo. Colaterais para a formação reticular emergem de alguns destes axônios. Os axônios espinomesencefálicos se projetam para a substância cinzenta periaquedutal (PAG) e para o tectum; as últimas fibras são espinotectais. Embora as fibras espinoreticulares se projetem para a formação reticular do bulbo, ponte e mesencéfalo, as colaterais podem ascender para outros alvos como o tálamo (PURVES et al., 2010).

No encéfalo existem múltiplos sítios e vias que estão envolvidos na modulação descendente da dor, desde o córtex cerebral até a região caudal do bulbo (BASBAUM; BUSHNELL, 2009). Até o presente momento, a via modulatória endógena da dor mais bem caracterizada envolve um circuito que liga a PAG mesencefálica, o bulbo rostral ventral (RVM), e a medula espinal (BASBAUM; BUSHNELL, 2009).

Figura 1 - Processamento da dor.



Fonte: adaptado de Purves et al., 2010.

A figura demonstra o processamento da dor envolvendo vários componentes, incluindo a ativação nervosa sensorial, transmissão aferente à medula espinal, integração e modulação espinal, sinalização supra-espinal, e regulação descendente (passos 1-6).

2.2 DOR PÓS-OPERATÓRIA

Raros são os indivíduos que tem a sorte de viver uma vida livre de qualquer dor, mas, felizmente para a maioria, tais experiências não são graves. Para muitas pessoas, no entanto, a dor é duradoura, debilitante e devastadora e surge após procedimentos cirúrgicos, ferimentos ou início de doença. Pesquisadores estimam que apenas um em cada quatro pacientes submetidos a procedimentos cirúrgicos nos

EUA recebe tratamento adequado para a dor pós-operatória. O reconhecimento da natureza generalizada da dor pós-operatória levou ao desenvolvimento de muitas diretrizes médicas e sociais e mais notadamente de novas normas regulamentadoras (por exemplo, *Joint Commission on Accreditation of Healthcare Organizations*) para a avaliação e tratamento da dor aguda. Uma das principais ênfases dessas novas normas tem sido a avaliação de rotina da dor como o chamado quinto sinal vital (PHILLIP, 2000).

Neste sentido, apesar da introdução de novas normas, diretrizes e esforços educacionais, dados em escala mundial sugerem que o tratamento da dor pós-operatória continua sendo insatisfatório. Uma avaliação de 1.490 pacientes submetidos a procedimentos cirúrgicos na Holanda revelou que, apesar da presença de um protocolo de controle da dor aguda, 41% dos pacientes apresentaram dor moderada a severa no dia da cirurgia, com quase 15% dos pacientes sofrendo de dor moderada a severa no quarto dia pós-operatório (SOMMER et al., 2008). Em um estudo randomizado com 250 pacientes adultos norte-americanos submetidos a procedimentos cirúrgicos, mostrou-se que 80% deles tiveram dor pós-operatória e, destes, 86% tiveram dor moderada a grave, com a maioria dos pacientes relatando dor com intensidade maior do que antes da internação hospitalar (APFELBAUM et al., 2003). Mais de 40 milhões de procedimentos cirúrgicos são realizados anualmente nos Estados Unidos (CULLEN; HALL; GOLOSINSKIY, 2009), e a dor pós-operatória de intensidade moderada a severa tem sido relatada em mais da metade destes pacientes seguido à cirurgia (KEHLET; DAHL, 2003; SOMMER et al., 2008). Isso explica o gasto de uma significativa parte dos recursos do país com cuidados relacionados à saúde.

2.2.1 Patofisiologia da dor pós-operatória

Na última década, foi reconhecido que a etiologia e o tratamento da dor pós-operatória causada por cirurgia é diferente de outras condições de dores clínicas tais como: artrite reumatóide, fibromialgia ou Herpes Zoster aguda (BRENNAN; ZAHN; POGATZKI-ZAHN, 2005; MEYER et al., 2006). Até então, muitos modelos animais de dor eram utilizados para o estudo dos mecanismos patofisiológicos da dor pós-operatória, mas menos adequados para o desenvolvimento de novos alvos no tratamento de particulares condições de dores clínicas, pois os mesmos não mimetizavam tais condições. Para avançar no tratamento da dor pós-operatória, foi necessário reconhecer que muitos modelos pré-clínicos de dor, como por exemplo: inflamação específica-antigênica ou estímulo químico receptor-específico (ex. formalina ou capsaicina), não necessariamente traduzem adequadamente os mecanismos da dor incisional (HONORE et al., 2006; ZAHN; BRENNAN, 1998). Além disso, pelo fato de que efetiva analgesia pós-operatória reduz a morbidade seguida de procedimento cirúrgico, novos tratamentos devem ser continuamente investigados. Assim, quando a dor é estudada através de uma abordagem pré-clínica (básica) e clínica, surgem novas teorias, vários tratamentos e estratégias preventivas para o manejo da dor, em especial da dor pós-operatória.

Estudos farmacológicos e neurofisiológicos utilizando um modelo animal de dor pós-operatória aguda, que mimetizam esta condição dolorosa, aumentaram nosso conhecimento sobre os mecanismos de dor resultante de incisão cirúrgica e lesão tecidual associada (BRENNAN; VANDERMEULEN; GEBHART, 1996;

POGATZKI-ZAHN; ZAHN; BRENNAN, 2007). Estes estudos sugerem que os mecanismos da dor pós-operatória são diferentes dos estados de dor inflamatória e neuropática, bem como dos modelos químicos citados anteriormente (formalina ou capsaicina). Tem sido demonstrado que a hiperalgesia na região da incisão é mediada pela sensibilização de fibras aferentes primárias do tipo A δ e do tipo C e pela ativação de nociceptores mecânicos silenciosos de fibras aferentes primárias do tipo A δ após a incisão (POGATZKI; GEBHART; BRENNAN, 2002). Outro estudo demonstrou uma importante contribuição dos receptores ionotrópicos para aminoácido excitatório AMPA e cainato na dor, hiperalgesia e sensibilização central induzidas pela incisão (ZAHN; UMALI; BRENNAN, 1998). O aumento nas concentrações de lactato e a redução do pH foram observados na pele e músculo lesionados após a incisão, sugerindo que um mecanismo de dor isquêmica poderia contribuir para dor pós-operatória (KIM et al., 2007). Sensibilização neuronal central provavelmente contribui para a dor pós-operatória e hiperalgesia. Estudos neurofisiológicos em animais têm mostrado um aumento na prevalência e da taxa de atividade espontânea de neurônios do corno posterior da medula espinal após incisão da pele e músculos profundos (BRENNAN, 2011). No entanto, o papel preciso da sensibilização central no desenvolvimento da dor pós-operatória persistente ainda não é totalmente compreendido (LAVAND'HOMME, 2011; BRENNAN; KEHLET, 2005).

Após um procedimento cirúrgico envolvendo a incisão da pele e de estruturas profundas, ocorre dor durante o repouso e em resposta à estímulos como pressão e toque, os quais geralmente não são dolorosos (RICHMOND; BROMLEY; WOOLF, 1993; MOINICHE et al., 1997).

Estas respostas exageradas podem ser mensuradas usando pressão, por exemplo: algometria de pressão após histerectomia (MOINICHE et al., 1997) e toracotomia (KAVANAGH et al., 1994); ou toque, por exemplo: dor induzida pelo filamento de von Frey após nefrectomia (STUBHAUG et al., 1997). Com o intuito de compreender os mecanismos da dor causada por incisões Kawamata et al. (2002) realizaram uma pequena incisão na face anterior do antebraço (área frequentemente utilizada para testes sensoriais em humanos) de voluntários sadios, e observaram que a dor ao repouso desapareceu 2 horas após a incisão experimental. No entanto, a resposta a estímulos mecânicos foi evidenciada por 7 dias. Os mesmos autores ainda encontraram a presença de áreas hiperalgésicas, causadas pela incisão em regiões não lesionadas, o que foi similar a pacientes no pós-operatório. Assim, pela análise de aspectos básicos fundamentais da dor pós-operatória, pode ser possível desenvolver uma ligação entre modelos clínicos e pré-clínicos de dor pós-operatória.

Recentes estudos sobre resultados cirúrgicos têm focado em melhorar a recuperação pós-operatória, determinada pelo tempo de estadia no hospital, morbidade, mortalidade, tempo para o retorno às atividades de vida diária (AVDs) e a satisfação do paciente. Muitas dessas medidas têm melhorado, atualmente, pelo aumento da atenção prestada à muitos fatores envolvidos na qualidade de realização da cirurgia e aos cuidados com o paciente no período pós-operatório (WU; RAJA, 2011).

Tradicionalmente, a analgesia pós-operatória seguida por alguns procedimentos cirúrgicos como, por exemplo, uma artroplastia total de uma articulação, tem focado na analgesia controlada pelo paciente

(ACP), analgesia epidural ou narcóticos parenterais. No entanto, estas modalidades frequentemente resultam no pobre controle da dor e na insatisfação do paciente (ETHGEN et al., 2004). O controle inadequado da dor conseqüentemente pode gerar complicações e prejuízos psicológicos (SINATRA; TORRES; BUSTOS 2002). Além disso, o uso da ACP com altas doses de opióides resulta em sedação, constipação, confusão mental, retenção urinária, náusea, vômito, prurido, além de induzir tolerância. Embora, as infusões epidurais possam fornecer uma analgesia superior, elas estão associadas com hipotensão, retenção urinária, bloqueio motor que limita a mobilização, e o potencial para hematomas espinais secundários à anti-coagulação (CHOI et al., 2003).

Nos últimos anos, o conceito de cirurgia de via rápida baseada em evidência tem incluído a mobilização precoce do paciente como um fator importante para aumentar a recuperação e reduzir complicações, tais como: trombose venosa profunda, hipotrofia muscular, pneumonia, ansiedade, depressão e dor crônica (KEHLET; WILMORE, 2002). Além disso, recentes estudos têm demonstrados que para evitar doses altas de medicamentos, efeitos adversos e potencial toxicidade associada com dependência de um agente ou técnica, esquemas analgésicos multidisciplinares têm sido defendidos. Geralmente, um programa de recuperação multidisciplinar consiste em três componentes principais: (1) mobilização precoce, (2) completa analgesia perioperatória, e (3) nutrição oral precoce. Com objetivo de acelerar a reabilitação do paciente e reduzir o tempo das hospitalizações (DAVY; SHARP; LYNCH, 2003).

2.3 DOR NEUROPÁTICA

A dor neuropática é a condição mais debilitante entre as diferentes condições de dor crônica, podendo ser intensa, ininterrupta e muitas vezes resistente a todos os tratamentos atualmente disponíveis (JENSEN et al., 2001). Ela pode ser causada por lesões traumáticas, inflamatórias, ou metabólicas do sistema nervoso central (SNC) ou periférico (SNP). Além disso, a dor neuropática ocorre na ausência de qualquer estímulo, devido à redução do limiar de ativação e estimulação dos nociceptores. A dor torna-se autônoma e perde sua função adaptativa associada com a recuperação tecidual. A dor neuropática é relativamente comum, ocorrendo em cerca de 1% da população (JENSEN et al., 2001) e é definida pela IASP como "dor originada como consequência direta de uma lesão ou doença que afeta o sistema somatossensorial" (LOESER; TREEDE, 2008). Esta lesão no tecido neural produz alterações da função neuronal que são percebidas pelo paciente como os sinais e sintomas de dor neuropática. Ao exame clínico, tanto sintomas negativos quanto positivos podem ser evidenciados. Sintomas positivos incluem dor, parestesia, disestesia, hiperalgesia e alodinia. Sinais e sintomas negativos envolvem déficits sensoriais (hipoestesia e hipoalgesia), fraqueza e mudanças nos reflexos. Clinicamente, os pacientes podem se queixar de dor espontânea (na ausência de estímulos), sensações lancinante do tipo choque elétrico ou queimações intermitentes e/ou por hipersensibilidade dolorosa evocada em resposta à estímulos (na presença de estímulos), sendo estes considerados hiperalgesia e alodinia (JENSEN et al., 2001).

Após uma lesão nervosa periférica, a parte distal,

transeccionada, da fibra neuronal desconectada do corpo neuronal sofre degeneração Walleriana, assim, deixando os órgãos periféricos denervados. Em paralelo, uma série de alterações moleculares e celulares conhecidas como reação retrógrada e cromatólise ocorrem no corpo dos neurônios seccionados (ALLODI; UDINA; NAVARRO, 2012). A degeneração Walleriana cria um microambiente distal ao sítio da lesão que favorece o recrescimento axonal, enquanto que a reação retrógrada conduz à alterações metabólicas necessárias para a regeneração e alongamento axonal. O significado funcional da regeneração axonal é a substituição do segmento distal do axônio perdido durante a degeneração, permitindo a reinervação dos órgãos-alvo e da restituição das suas correspondentes funções (ALLODI; UDINA; NAVARRO, 2012). Através desta sequência de eventos, os axônios lesionados do sistema nervoso periférico são capazes de regenerar e reinervar os órgãos-alvo. Após uma axoniotmese, onde as bainhas de tecidos conjuntivos do nervo (endoneuro, perineuro e epineuro) são preservadas e somente os axônios são lesionados, a recuperação funcional é geralmente boa. No entanto, após neurotmese (corte transversal do nervo), quando os tubos endoneurais perdem a continuidade, os axônios são frequentemente mal direcionados e geralmente reinervam órgãos-alvo incorretos, mesmo quando realizado reparo cirúrgico (BODINE-FOWLER et al., 1997; MOLANDER; ALDSKOGIUS, 1992; VALERO-CABRE; NAVARRO, 2002).

Uma lesão traumática de nervos expõe as proteínas nervosas periféricas P0 e P2, que ativam o sistema imunológico, iniciando uma resposta auto-imune semelhante à provocada por patógenos (WATKINS; MAIER, 2002). A lesão do nervo também leva à

degeneração Walleriana caracterizada por desmielinização e remielinização seguida por denervação e reinervação (STOLL; JANDER; MYERS, 2002). Neste processo, vários tipos celulares são ativados e recrutados para o sítio da lesão, incluindo mastócitos, macrófagos, fibroblastos, neutrófilos e células de Schwann, estas células liberam trifosfato de adenosina (ATP), citocinas pró-inflamatórias, prostaglandinas (PGs), e o fator de crescimento do nervo (NGF), que contribuem para hipersensibilidade na periferia (MARCHAND; PERRETTI; MCMAHON, 2005).

2.3.1 Dor neuropática e células gliais

A ativação de nociceptores periféricos resulta também em plasticidade do SNC. Esta plasticidade modifica o funcionamento da via nociceptiva, aumentando e prolongando as respostas à subseqüentes estímulos periféricos. Estas mudanças ocorrem na medula espinal, bem como no encéfalo, e são referidas como sensibilização central (CAO; ZHANG, 2008). Nas últimas décadas, tornou-se evidente o papel das células gliais espinais, especialmente, microglias e astrócitos no desenvolvimento e manutenção da sensibilização central nos estados de dor crônica (MARCHAND; PERRETTI; MCMAHON, 2005).

A glia, refere-se a um diverso conjunto de tipos celulares especializados encontrados tanto no SNP (células de Schwann, glia satélite, glia perineural), quanto no SNC (astrócitos, oligodendrócitos, microglia e células da glia perivascular) (KRIEGSTEIN; ALVAREZ-BUYLLA, 2009). As células da glia constituem 70% da população total de células no encéfalo e medula espinal. Elas podem ser subdivididas em duas categorias principais: microglia, que compreende 5% a 10% da

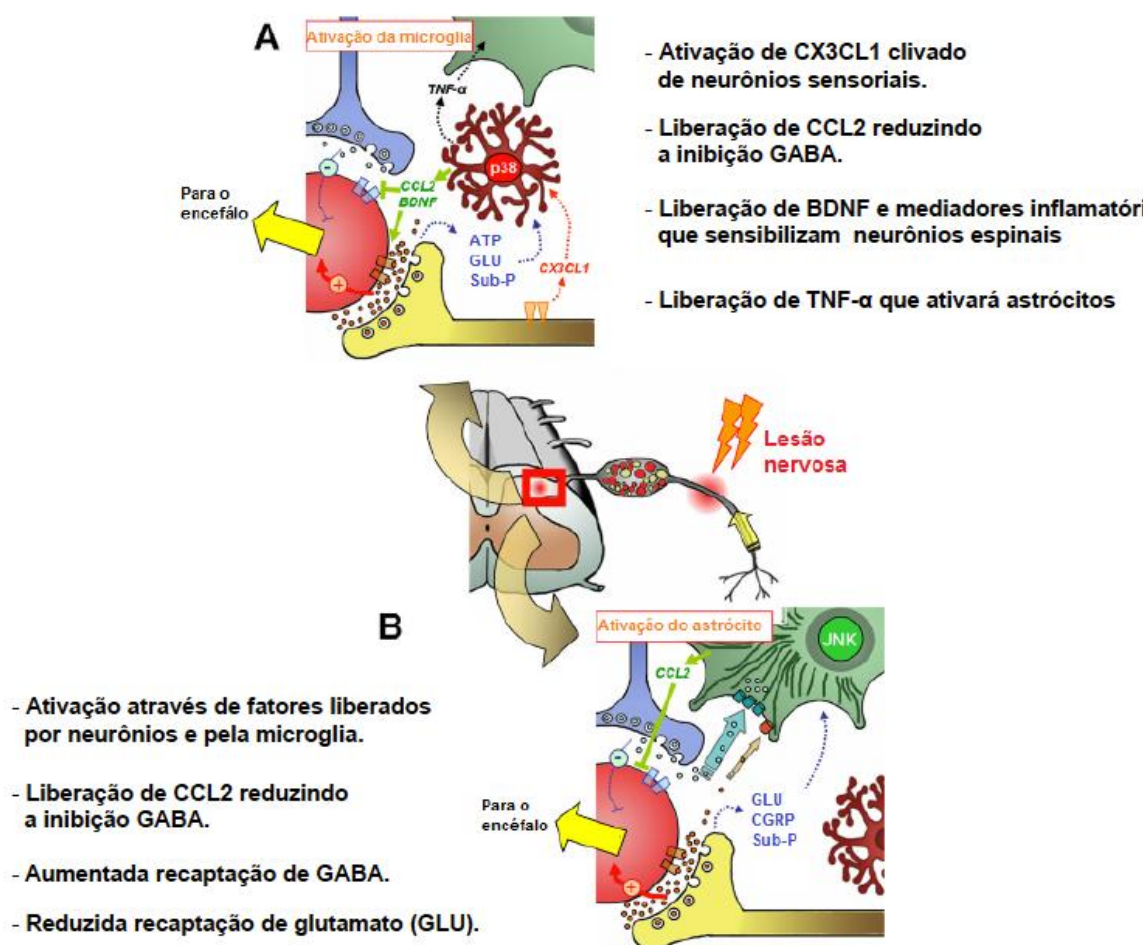
população glial, e macroglia, que incluem os astrócitos e os oligodendrócitos (MOALEM; TRACEY, 2006) (Figura 2).

As células da glia não conduzem impulsos nervosos, no entanto, fornecem suporte estrutural para o encéfalo (Figura 2), auxiliando no reparo, desenvolvimento e manutenção do sistema nervoso, fornecimento de nutrientes e substratos neuronais, funções metabólicas para os neurônios, destruindo e removendo neurônios lesionados ou mortos contribuindo na patofisiologia de doenças neurodegenerativas e, finalmente, na regulação do microambiente neuronal. As células microgliais podem proteger ou prejudicar os neurônios dependendo de onde e como elas são ativadas (JHA; JEON; SUK, 2012).

As células microgliais totalmente ativadas são prejudiciais para os neurônios, mas, em outros estágios na sequência do estado reativo elas podem melhorar a sobrevivência neuronal, liberando fatores neurotróficos ou removendo o excesso de glutamato do espaço extracelular (AAMODT, 2007). As células da glia, incluindo oligodendrócitos, astrócitos e microglia desempenham papéis importantes na dor neuropática. Tendo em vista o pouco conhecimento do envolvimento dos oligodendrócitos na dor, este trabalho concentrou-se principalmente no envolvimento da microglia e astrócito na dor neuropática.

apresentada à proteínas ou estímulos específicos promove uma resposta imunológica (Figura 3). Evidências sugerem um papel importante da resposta imune no desenvolvimento da dor neuropática, em particular através da atividade da microglia na medula espinal (CAO; ZHANG, 2008). A ativação de células gliais tem sido observada em vários modelos animais de dor crônica, incluindo lesão do nervo espinal (RAGHAVENDRA; TANGA; DELEO, 2003), inflamação periférica (BAO et al., 2001), lesão tecidual periférica (OBATA et al., 2006), inflamação de nervos periféricos (MILLIGAN et al., 2003), lesão medular (HAINS; WAXMAN, 2006), e câncer ósseo (ZHANG et al., 2005). A microglia quando ativada contribui para os estados de hipersensibilidade à dor, através da liberação de citocinas pró-inflamatórias e de moléculas efetoras que estimulam ainda mais as células microglias vizinhas aumentando a produção de mais mediadores pró-inflamatórios no corno posterior da medula espinal. Este ciclo de *feedback* positivo pode significativamente agravar o desenvolvimento de síndromes neuropáticas (HAINS; WAXMAN, 2006). Uma vez que uma resposta imune é iniciada, vários agentes são liberados pela microglia, tais como proteases, óxido nítrico (NO), cininas, e outras moléculas imuno-moduladoras, as quais medeiam os danos secundários e são conhecidas por estarem envolvidas no desenvolvimento da dor neuropática (Figura 3). A ativação microglial também é caracterizada pelo aumento da expressão de moléculas como o complexo principal de histocompatibilidade (MHC), o receptor para o complemento-3 (CR3), as integrinas β 2 (CD11b e CD11c) e uma variedade de receptores para citocinas, quimiocinas e outras substâncias liberadas no sistema nervoso central (CAO; ZHANG, 2008).

Figura 3 - Eventos microgliais e astrocitários na medula espinal na dor neuropática.



Fonte: Adaptado de Gosselin, 2010.

A figura mostra o envolvimento de células microgliais e astrocárias ativadas e as suas conseqüentes liberações de mediadores pró-inflamatórios, contribuindo assim para a dor neuropática.

*GFAP: proteína glial fibrilar ácida; CX3CL1: *CX3C ligand 1*. GABA: ácido gama-aminobutírico; GLU: glutamato; SP: substância P; CGRP: peptídeo relacionado ao gene da calcitonina; JNK: quinase terminal C-jun-N.

2.3.1.2 Astrócito

As células da glia (principalmente astrócito e microglia) atraíram a atenção de pesquisadores da dor no início dos anos 90. Em seu pioneiro trabalho Garrison et al. (1991) demonstraram, pela primeira

vez, que uma lesão neuropática periférica pode levar à ativação de astrócitos na medula espinal. Usando a técnica de imunohistoquímica, avaliando a expressão de GFAP (proteína glial fibrilar acídica), eles mostraram que: 1) a constrição do nervo isquiático que induz dor neuropática, também promove ativação astrocitária, e 2) drogas que bloqueiam a dor neuropática também bloqueiam a ativação dos astrócitos. Desde então, a ativação glial (ambos, astrócito e microglia) tem sido observada na região lombar da medula espinal em vários modelos animais de lesão nervosa periférica (COLBURN et al., 1997; GARRISON et al., 1991; SWEITZER et al., 2001; WINKELSTEIN et al., 2001; ALDSKOGIUS et al., 2011).

Os astrócitos apresentam processos longos e numerosos e em formato estrelar. Normalmente estão ativos e desempenham diferentes funções tais como nutrição, suporte da função neuronal através da manutenção da concentração local de íons, estocagem de glicogênio, limpeza de resíduos neuronais e recaptação de neurotransmissores (NAIR; FREDERICK; MILLER, 2008). Tornam-se ativados em resposta ao mesmo estímulo capaz de ativar a microglia ou por produtos liberados pela microglia ativada (DONG; BENVENISTE, 2001). Quando ativados apresentam corpo celular com processos espessos, aumentada expressão de GFAP, e aumento na produção e liberação de uma variedade de substâncias pró-inflamatórias (WATKINS; MAIER, 2003).

Astrócitos e células microgliais expressam vários receptores para neurotransmissores e são ativados por neurotransmissores e neuromoduladores clássicos, assim, podem receber e responder a sinais durante a transmissão sináptica. Os astrócitos são mais conhecidos por

seu papel ativo durante a transmissão sináptica, e ambos, microglia e astrócito estão em contato físico com os neurônios, assim como com outros astrócitos e células microgliais, realizando funções modulatórias (POCOCK; KETTENMANN, 2007; HAYDON, 2001). Quando os neurônios liberam ATP, glutamato e SP, estes mediadores excitatórios agem como fontes de estímulos para astrócitos e microglia. Por sua vez, astrócitos e células microgliais liberam glutamato e/ou ATP, levando a uma maior ativação neuronal e glial. Em tais condições, ambas, subsequente resposta glial a neurotransmissores clássicos e interações neurônio-glia são alteradas (MILLIGAN; WATKINS, 2009). Em condições neuropáticas, a glia ativada libera as mesmas substâncias que são liberadas nas condições imunogênicas, tais como interleucina - 1 beta (IL-1 β), Fator de necrose tumoral - alfa (TNF- α) e interleucina - 6 (IL-6), na indução e manutenção da dor neuropática em modelos animais (WATKINS; MILLIGAN; MAIER, 2001). Além disso, toda a sinalização neuroexcitatória e neuromodulatória que a glia libera (como, glutamato, NO, ATP e SP) ou receptores expressos na superfície de células gliais são elementos fundamentais para o estado de dor crônica de origem neuropática.

Na dor crônica de origem neuropática, sob a influência de fatores neuronais (tais como: CX3CL1, ATP ou neuromediadores), a microglia sofre alterações no seu fenótipo, incluindo a ativação da via quinases ativadas por mitógenos (MAPK) p38. Os fatores liberados pela microglia (citocinas) reforçam a nocicepção, reduzindo a potência da inibição GABAérgica, sensibilizando neurônios espinais e ativando os astrócitos (Figura 3A). A ativação dos astrócitos é caracterizada por uma ativação da quinase terminal C-jun-N (JNK) e ocorre,

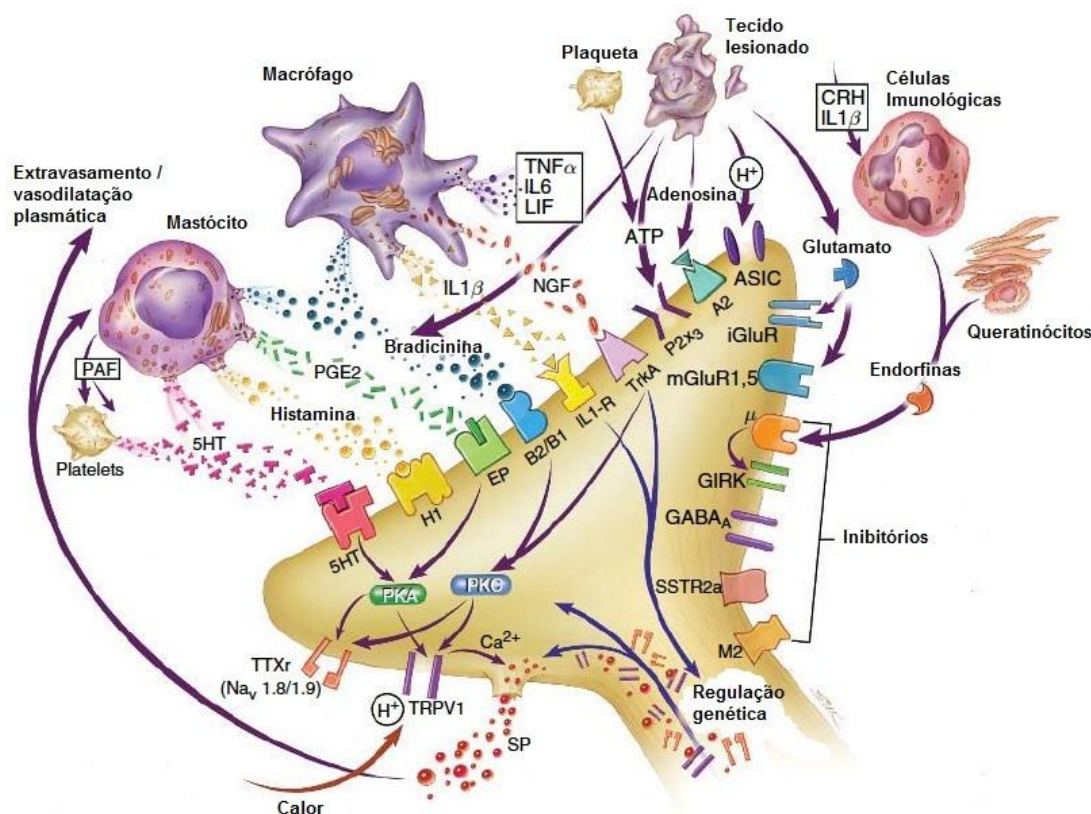
consequentemente, pela liberação neuronal de altas quantidades de neuromediadores e/ou produção microglial de citocinas tais como TNF- α . Como resultado da reação astrocitária, uma redução na capacidade de eliminação de glutamato associada com um aumento na absorção de GABA, reforça também a sinalização da dor. Além disso, astrócitos reativos liberam citocinas, tais como CCL2, ajudando na perda da inibição GABAérgica (Figura 3B).

Interessantemente, foi demonstrado que nas lesões do SNC ocorre uma ativação precoce de células microglias seguida por uma ativação tardia de células astrocítárias (COLBURN et al., 1997). Além disso, várias evidências fortemente indicam que a microglia pode ser responsável por iniciar a hipersensibilidade dolorosa induzida por lesão nervosa periférica, e os astrócitos podem estar envolvidos na manutenção da mesma (PARKITNA et al., 2006; ZHUANG et al., 2005).

2.4 SISTEMAS ENDÓGENOS DE CONTROLE DA DOR

A modulação endógena da dor pode ser definida como as adaptações do corpo a informações nociceptivas momentâneas, bem como a longo prazo. Esta definição se aproxima muito da definição de homeostase proposta por William Cannon em 1900, “como a capacidade de um sistema vivo em regular o seu ambiente interno mantendo uma condição constante estável” (CANNON, 1929). A modulação da dor pode ocorrer em todos os níveis do sistema nervoso, tais como periférico (Figura 4), espinal (Figura 5) ou supra-espinal (Figura 1) e é dependente do contexto da lesão, bem como de fatores endógenos.

Figura 4 - Forças facilitatórias e inibitórias da nocicepção na periferia.



Fonte: adaptado de Ringkamp e Meyer, 2009.

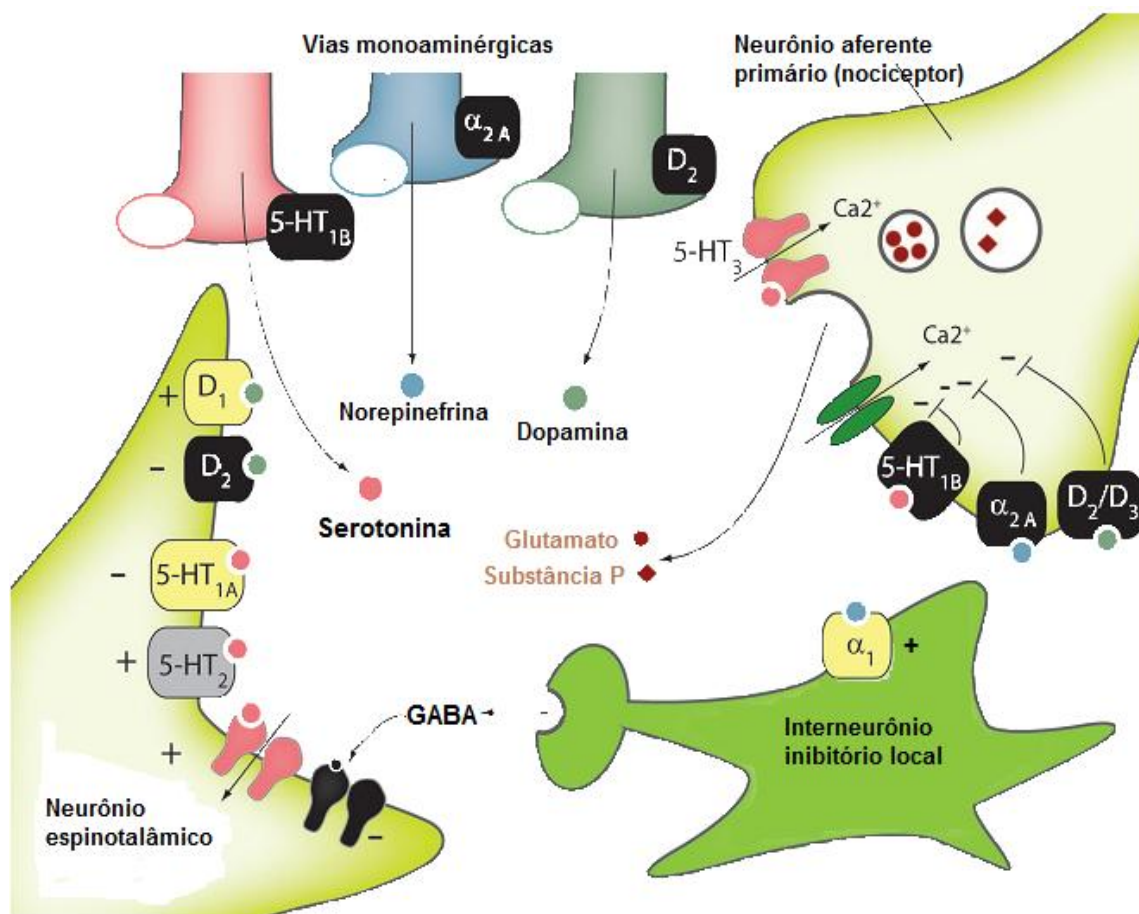
A figura ilustra um terminal neuronal periférico com a expressão de receptores membranares pró- e anti-nociceptivos que são ativados por substâncias liberadas por vários tipos celulares após uma lesão tecidual.

*PAF: Fator de ativação plaquetária; PGE₂: prostaglandina E₂; IL-1β: interleucina - 1 beta; TNF-α: Fator de necrose tumoral - alfa; IL-6: interleucina - 6; 5HT: receptor para serotonina; PK: proteína quinase; TRPV1: receptor vanilóide de potencial transiente do tipo 1; Na_v 1.8/1.9 TTRr: canal de cálcio voltagem dependente 1.8/1.9 tetratoxina resistente; SP: substância P; SSRT2a: receptores para somatostatina periférico do tipo 2a; M2: receptor muscarínico do tipo 2; GIRK: canais de potássio retificadores de influxo; mGlu1,5: receptores glutamatérgicos metabotrópicos do tipo 1,5. iGluR: receptores glutamatérgicos ionotrópicos; ASIC: canal iônico sensível ao ácido; A2: P2X₃: receptor purinérgico do tipo 2X3; TrKA: receptor tirosina quinase A; IL1-R: receptor para IL1; B2/B1: receptor para bradicinina; EP: receptor para prostaglandinas; H1: receptor para histamina do tipo 1.

Estudos sugerem que a resposta individual à dor é dependente do balanço dinâmico entre a facilitação e a inibição da mesma, o que explica, em parte, as diferentes respostas dos indivíduos ao mesmo

estímulo nocivo (EDWARDS, 2005). Este balanço entre a facilitação e a inibição da dor é controlado por uma rede modulatória neuronal originada em regiões do tronco encefálico e encefálicas intimamente ligadas à medula espinal (Figura 5) (BENARROCH, 2008). Os efeitos do sistema modulatório descendente sobre o processo nociceptivo no corno posterior da medula espinal é complexo (BENARROCH, 2008). Por exemplo: noradrenalina, serotonina e dopamina podem exercer papéis duais na nocicepção, dependendo do tipo de receptor envolvido, do sítio de ação no corno posterior e/ou do *cross talk* entre a sinalização neuroquímica local e as vias descendentes, incluindo adenosina, opióides endógenos e NO (Figura 5) (MILLAN, 2002; PERTOVAARA, 2006).

Figura 5 - Forças facilitatórias e inibitórias da nocicepção na medula espinal.



Fonte: adaptado de Benarroch, 2008.

A figura demonstra o processamento nociceptivo espinal e as forças inibitórias da dor através do controle descendente (vias monoaminérgicas) e interneurônios locais inibitórios.

*5-HT: receptor para serotonina; D: receptor para dopamina; α : receptor adrenérgico.

2.4.1 Sistema opioidérgico

Os peptídeos opióides são agonistas endógenos para receptores opióides. Três famílias de peptídeos opióides estão bem caracterizadas nos sistemas nervoso e neuroendócrino. Cada família deriva de uma das três proteínas precursoras: proopiomelanocortina (POMC),

proencefalina (PENK), e prodinorfina. O processamento apropriado produz os respectivos peptídeos opióides: as endorfinas, encefalinas e dinorfinas. Estes peptídeos exibem diferentes afinidade e selectividade para os três receptores opióides, μ (endorfinas, encefalinas), δ (encefalinas, endorfinas) e κ (dinorfinas). Além disso, foram identificados em 1997 a endomorfina-1 e a endomorfina-2, dois peptídeos opióides adicionais que são seletivos para receptores μ . No entanto, seus precursores ainda não foram identificados (AKIL et al., 1998).

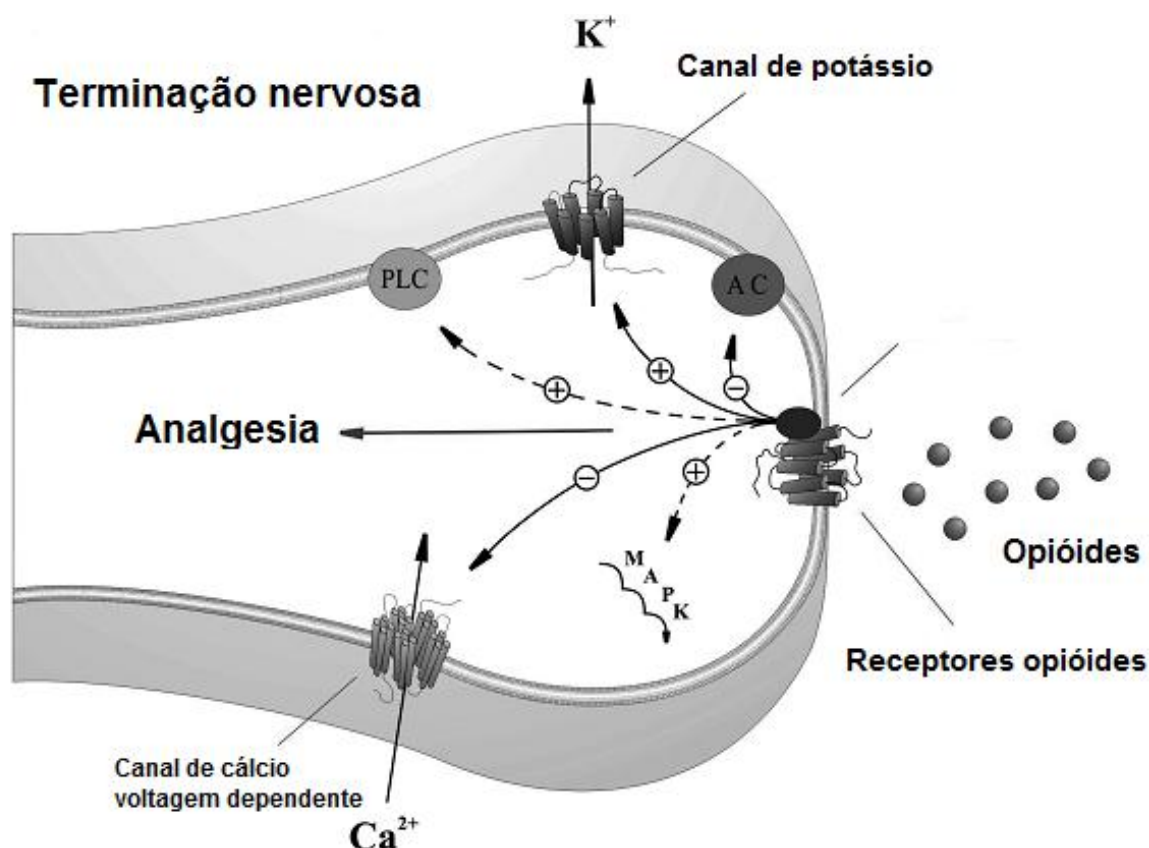
Os opióides, cujo o protótipo é a morfina, estão entre os medicamentos mais eficazes no alívio da dor. Eles atuam em sítios periféricos, espinais, e supra-espinais através de uma variedade de receptores para opióides (μ , κ e δ). Estes receptores são considerados alvos para o sistema opioidérgico endógeno que foi extensivamente revisado (YAKSH, 1997). A ativação dos receptores opióides causam muitos efeitos secundários no SNC, incluindo sedação, disforia, depressão respiratória e constipação. Assim, há uma tentativa vigorosa de explorar as ações antinociceptivas periféricas dos opióides, como um meio de se evitar efeitos colaterais no SNC. Neste sentido, sabe-se que os neurônios aferentes primários expressam e transportam os receptores opióides para seus terminais centrais e periféricos. Nos terminais centrais, os opióides reduzem a liberação do neurotransmissor das fibras aferentes primárias nociceptivas, bloqueando assim a transmissão sináptica, enquanto que, na periferia a ativação dos receptores opióides hiperpolariza diretamente os neurônios sensoriais e atenua a sensibilização neuronal ou a hiperexcitabilidade induzida por inflamação ou lesão (HURLEY; HAMMOND, 2000).

2.4.1.1 Receptores opioidérgicos

Os primeiros estudos de ligação e bioensaios definiram três principais tipos de receptores opióides (OR) no sistema nervoso central: mu (MOR), delta-(DOR) e kappa-(KOR) (KIEFFER et al., 1992). A clonagem molecular resultou na identificação de três genes de receptores opióides e permitiu o estudo dos tipos individuais de receptores opióides em relação aos seus perfis farmacológicos, mecanismos bioquímicos intracelulares, distribuição anatômica e regulação de expressão. Os receptores opióides pertencem à família de receptores acoplados a proteína G (GPCRs) de sete domínios transmembranares e apresentam 50-70% de homologia entre os seus genes (EVANS et al., 1992; KIEFFER et al., 1992). Os receptores opióides são expressos por neurônios periféricos e centrais, por células neuroendócrinas (hipófise e glândulas supra-renais), imunológicas e ectodérmicas (ZÖLLNER; STEIN, 2007). Além disto, os receptores opióides são expressos no gânglio da raiz dorsal (DRG) em neurônios de pequeno, médio e grande diâmetro (BUZAS; COX, 1997; CHEN; DYMSHITZ; VASKO, 1997; GENDRON et al., 2006); e são transportados para os terminais dos nervos periféricos, sendo acoplados a proteínas Gi/o que inibem a ciclase de adenilil e modulam canais iônicos (ZÖLLNER et al., 2003). A diminuição das correntes de Ca^{2+} parece ser um mecanismo importante para a inibição das funções neuronais sensoriais (AKINS; MCCLESKEY, 1993). Recentemente, foi observado que canais de K^+ retificadores acoplados à proteína G e MOR estão colocalizados em terminações nervosas sensoriais na epiderme (Figura 6) (KHODOROVA et al., 2003). A ativação de receptores

opióides também suprime a modulação pronociceptiva de canais iônicos de Na⁺ tetrodotoxina-resistentes e correntes catiônicas não-seletivas (INGRAM; WILLIAMS, 1994), correntes mediadas pelo receptor P2X (CHIZHMAKOV et al., 2005), bem como as correntes dos canais TRPV1 mediadas pela proteína Gi/o e por reduzir os níveis de AMPc (CHIZHMAKOV et al., 2005). Como resultado, os agonistas opióides podem atenuar o aumento da excitabilidade de neurônios aferentes primários, aumento este induzido pela inflamação e liberação de neuropeptídeos pró-inflamatórios, tais como, a SP e o CGRP, a partir dos terminais nervosos periféricos e centrais (STEIN; SCHÄFER; MACHELSKA, 2003). Em particular, dentro do tecido lesionado, estes eventos levam à antinocicepção e reduzem a inflamação.

Figura 6 - Receptores opióides periféricos.



Fonte: adaptado de Kapitzke, Vetter e Cabot, 2005.

A figura ilustra a expressão de receptores opióides periféricos e sua sinalização intracelular que contribui para a analgesia.

*PL: fosfolipase; AC: ciclase de adenilil.

2.4.2 Sistema adenosinérgico

Ainda é uma questão de conjectura porque o ATP, o principal combustível celular e elemento essencial de todas as células vivas, e o seu metabólito, a adenosina, estão diretamente envolvidos na comunicação das células nervosas. A adenosina é 'onipresente', isto é, sendo produzida e liberada de aparentemente todas as células, incluindo neurônios e células neurogлияis. A adenosina é reconhecida como uma

substância muito importante na homeostasia das células do sistema nervoso, nomeada como 'um sinal de vida' (ENGLER, 1991).

Enquanto o ATP pode funcionar como um neurotransmissor em algumas áreas encefálicas (MORI et al., 2002), a adenosina não é armazenada nem liberada como um neurotransmissor clássico, uma vez que não é armazenada em vesículas sinápticas, sendo liberada do citoplasma para o espaço extracelular através de um transportador de nucleosídeo. Os transportadores de adenosina também medeiam a recaptação de adenosina, e a direção do transporte depende do gradiente de concentração de ambos os lados da membrana (GU et al., 1995).

A adenosina intracelular é fosforilada em AMP pela adenosina quinase (AQ), ou desaminada em inosina pela adenosina desaminase (AD). A AQ é uma enzima citosólica que está amplamente distribuída no encéfalo, enquanto AD é citosólica e extracelular, e se encontra em certas regiões do encéfalo e medula espinal (GEIGER; PARKINSON; KOWALUK, 1997). No interior da célula a AD exerce funções metabólicas, mas do lado de fora da célula, ela também tem efeitos extra-enzimáticos envolvendo a modulação da ligação do ligante ao receptor adenosinérgico A_1 e regulação da ativação do receptor e endocitose (SAURA et al., 1998).

Uma vez que a adenosina está no espaço extracelular, ela é removida por captação através da membrana celular por moléculas transportadoras específicas de nucleosídeos. A neuromodulação pela adenosina é exercida através da ativação dos receptores de alta afinidade (A_1 e A_{2A}), que são, provavelmente, de importância fisiológica, e de receptores de baixa afinidade (A_{2B}), os quais podem ser relevantes em condições patológicas (Figura 7). O receptor de adenosina A_3 é um

receptor de alta afinidade em seres humanos, mas têm uma baixa densidade na maioria dos tecidos. Estes quatro receptores de adenosina são também conhecidos como receptores P1, da nomenclatura P1 (seletivo a adenosina) / P2 (seletivo ao ATP) (BURNSTOCK, 1978). Eles pertencem à família dos GPCRs, e todos têm sido clonados e caracterizados a partir de várias espécies de mamíferos, incluindo seres humanos (revisar em FREDHOLM et al., 2001).

O receptor adenosinérgico A_1 é altamente expresso no córtex cerebral, cerebelo, hipocampo, e no corno posterior da medula espinal. O receptor de adenosina A_{2A} é altamente expresso em neurônios GABAérgicos estriato-palidais e do bulbo olfatório, e expresso em níveis inferiores em outras regiões do encéfalo (revisar em SEBASTIÃO; RIBEIRO, 1996). Os receptores para adenosina estão presentes também no sistema nervoso periférico autonômico e somático. Além disso, estudos mostrando ações inibitórias pré-sinápticas mediadas por receptores A_1 em terminais de neurônios motores (GINSBORG; HIRST, 1972) e ações excitatórias pré-sinápticas mediadas por receptores para adenosina A_{2A} (CORREIA-DE-SÁ; SEBASTIÃO; RIBEIRO, 1991) inspiraram vários estudos sobre as ações neuromodulatórias da adenosina no sistema nervoso central.

Embora o mesmo receptor para adenosina possa se acoplar a diferentes proteínas G, o par de receptores A_1 e A_3 geralmente estão associados à proteínas G "inibitórias" (G_i e G_o), enquanto que o par de receptores A_{2A} e A_{2B} estão associados proteínas G "estimulatórias" (G_s) (ver LINDEN, 2001). Os receptores A_{2A} estimulam a ciclase de adenilil, enquanto que os receptores A_1 podem levar à inibição desta enzima. A modificação da atividade da fosfolipase C foi descrita após a ativação de

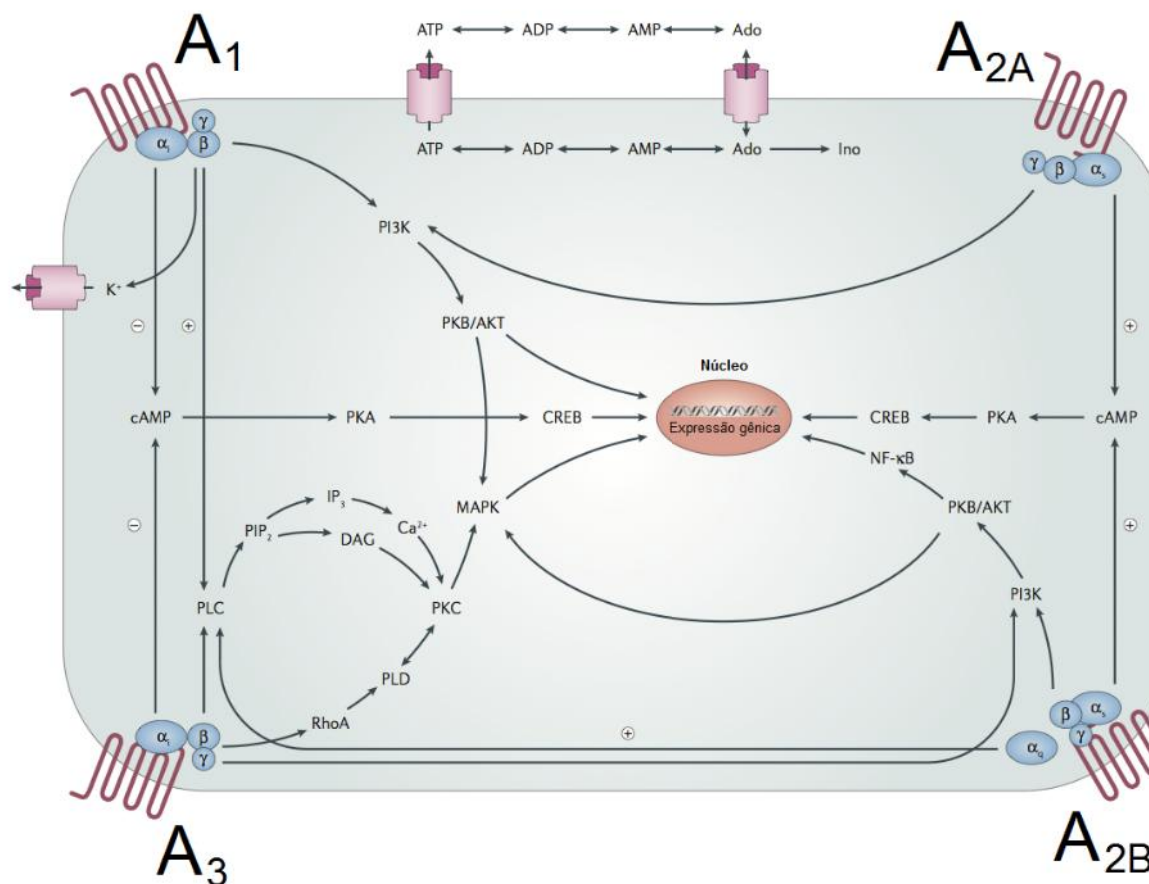
receptores A_1 (Figura 7) (CASCALHEIRA; SEBASTIÃO; RIBEIRO, 2002). Os canais iônicos, incluindo diferentes tipos de canais de K^+ e de Ca^{2+} também estão sob controle direto ou indireto (através de segundos mensageiros) de receptores para adenosina. Por exemplo, os receptores A_1 inibem as correntes de Ca^{2+} do tipo N através de uma via dependente de voltagem e sensível à toxina pertussis (PARK et al., 2001).

Na medula espinal, a ativação do receptor A_1 produz antinocicepção em modelos de nocicepção aguda, dor inflamatória e neuropática (revisar em SAWYNOK, 1998). Portanto, verificou-se um interesse crescente no desenvolvimento de drogas que, ao influenciar os níveis de adenosina extracelular, possam desempenhar ações analgésicas. Exemplos bem sucedidos foram os inibidores da quinase de adenosina cujas propriedades antinociceptivas mediadas espinalmente foram observadas há uma década (KEIL; DELANDER, 1992). Provavelmente devido às ações anti-inflamatórias da adenosina, inibidores da quinase de adenosina administrados por via oral são ainda mais eficazes em reduzir a dor inflamatória do que a dor aguda ou neuropática (JARVIS et al., 2002). Ao comparar as propriedades antinociceptivas e anti-inflamatórias dos inibidores da quinase de adenosina administrados ipsilateral e contralateral à lesão, concluiu-se que grande parte da ação anti-inflamatória é mediada localmente, enquanto que o efeito antinociceptivo é mediado sistemicamente, exercido predominantemente a nível do corno posterior da medula espinal (POON; SAWYNOK, 1999). De fato, os inibidores da quinase de adenosina são capazes de reduzir o aumento da expressão de c-fos no corno posterior da medula espinal, induzida pela injeção periférica de uma substância inflamatória (carragenina) (POON; SAWYNOK, 1999).

A modulação da dor na medula espinal mediada por receptores A_1 provavelmente está relacionada com a sua capacidade de inibir pré-sinápticamente a transmissão excitatória para neurônios da substância gelatinosa na medula espinal (LAO et al., 2001).

Na periferia, a adenosina exerce efeitos complexos sobre a sinalização da dor por suas ações em diferentes receptores e alvos celulares. Assim, ela pode inibir ou aumentar a dor por agir sobre os aferentes nociceptivos via receptores A_1 e A_{2A} , e isto resulta em diminuições e aumentos nos níveis intracelulares de AMPc, respectivamente (TAIWO; LEVINE, 1990).

Figura 7 - Receptores adenosinérgicos.



Fonte: adaptado de Jacobson e Gao 2006.

A figura demonstra os quatro tipos de receptores adenosinérgicos e suas diferentes sinalizações intracelulares.

*Ado: adenosina; Ino: inosine; CREB: proteína ligante ao elemento de resposta do AMPc; DAG: diacil glicerol; IP₃, inositol 1,4,5-trifosfato; PI3K: fosfatidil inositol 3 quinase; PIP₂: fosfatidil inositol-4,5 bifosfato; PK: proteína quinase; PL: fosfolipase; NF-κB: fator nuclear - κB; RhoA: *Ras homologous gene A*.

2.4.3 Sistema endocanabinóide

A planta *Cannabis sativa*, a fonte de *cannabis* (ou seja, maconha), é nativa da Ásia Central. Várias culturas e povos (por exemplo, a medicina ayurvédica, tibetana e tradicional chinesa) evoluíram em sua "medicina tradicional" fazendo uso desta substância (MECHOULAM, 1986). Em 1830, um médico servindo à coroa

britânica na Índia realizou uma série de experimentos de laboratório, incluindo estudos com animais e testes clínicos, para determinar a segurança e a eficácia da *Cannabis*. Ele posteriormente introduziu a *Cannabis* na Inglaterra e foi nomeado cavaleiro pela rainha Victoria (ROBSON, 2005). A maconha foi amplamente utilizada para fins medicinais, incluindo analgesia, nos Estados Unidos da América, antes de ser classificada como entorpecente e psicotrópica. Como psicotrópica, a maconha está sujeita à lei das substâncias controladas; e como entorpecente, a aprovação da maconha passou a ser utilizada com fins medicinais por diversos estados da América do Norte (FEIN, 2011). Pesquisas em animais têm indicado que os canabinóides (CB) produzem efeitos analgésicos em sítio periférico, bem como espinhal e supra-espinhal. No entanto, o uso de CB como analgésico em seres humanos é prejudicado por seus efeitos adversos, tais como: alucinações, euforia ou disforia. Seria necessário, antes disso, obter um agonista CB com eficácia analgésica, porém com efeitos adversos mínimos (FEIN, 2011).

2.4.3.1 Endocanabinóides, síntese e degradação

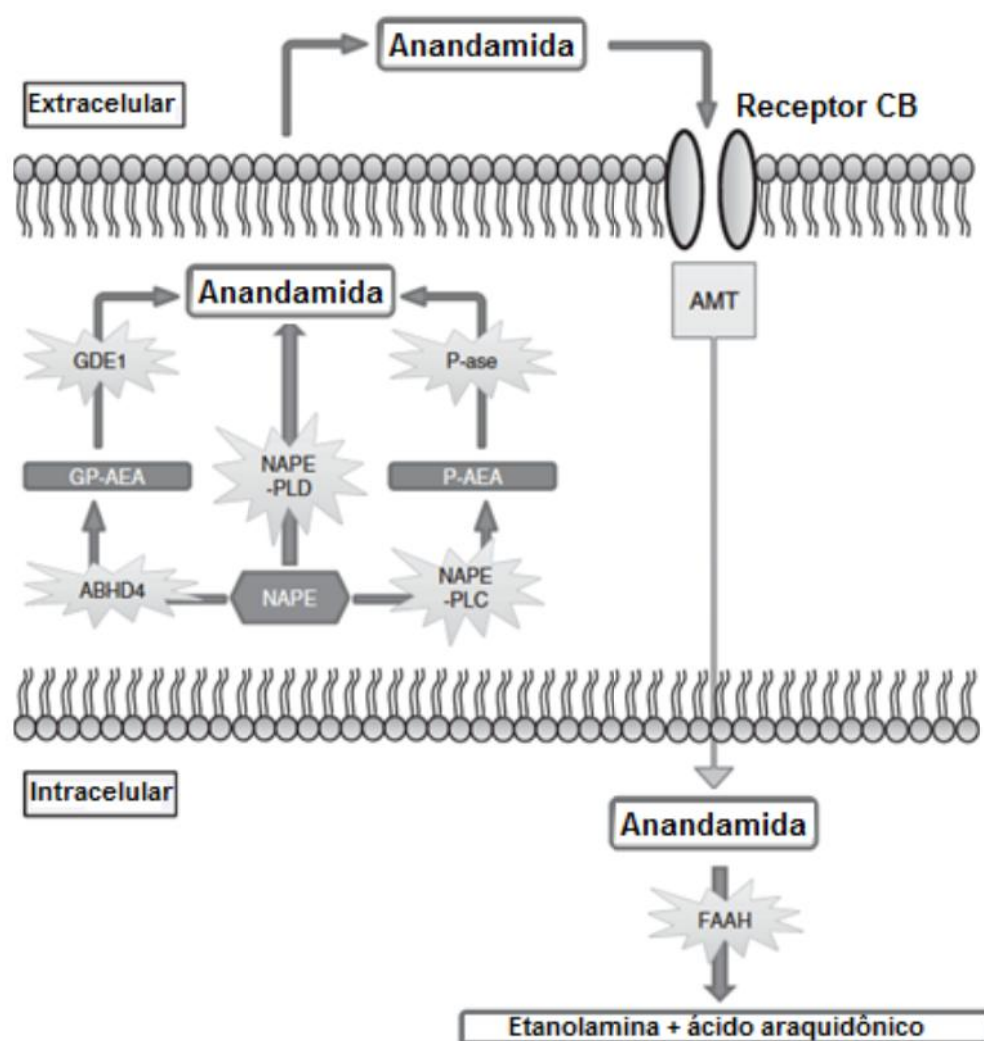
Os endocanabinóides são moléculas de sinalização lipídicas endógenas, geradas na membrana celular à partir de precursores de fosfolipídios e possuem propriedades canabinéticas porque se ligam e ativam um ou mais subtipos de receptores canabinóides (DI MARZO, 1998; PIOMELLI, 2005). O sistema de sinalização endocanabinóide foi somente recentemente foco de pesquisas médicas e considerado um potencial alvo terapêutico. Os endocanabinóides mimetizam as ações farmacológicas do princípio psicoativo da maconha, Δ^9 -

tetrahydrocannabinol (Δ^9 -THC), e estão envolvidos em diferentes funções fisiológicas e patológicas (regulação da ingestão alimentar, imunomodulação, inflamação, analgesia, câncer, comportamento aditivo, epilepsia e outras) (DI MARZO; DE PETROCELLIS, 2006; PACHER; BATKAI; KUNOS, 2006).

A descoberta da etanolamida do ácido araquidônico, anandamida (AEA) (DEVANE et al., 1992), o primeiro endocanabinóide isolado no encéfalo, foi seguida alguns anos mais tarde, pela identificação do 2 araquidonil glicerol (2-AG) (MECHOULAM et al., 1995; SUGIURA et al., 1995). Desde então, vários endocanabinóides derivados do ácido araquidônico como a oleamida (LEGGETT et al., 2004), a O-araquidonil etanolamina (virodamina) (PORTER et al., 2002), o 2-araquidonil gliceril éter (noladina) (HANUS et al., 2001) e a N-araquidonil dopamina (NADA) (HUANG et al., 2002) foram isolados. Ao contrário dos neurotransmissores clássicos, os endocanabinóides não são pré-formados e armazenados em vesículas, em vez disso, são produzidos "sobre demanda", em resposta à um estímulo externo. Uma similaridade estrutural entre os endocanabinóides é a sua natureza lipídica, sendo constituídos por uma cadeia de ácido graxo poliinsaturado derivado do ácido araquidônico e um grupamento polar, que pode ser a etanolamina no caso da AEA ou glicerol no caso do 2-AG (DI MARZO; DEUTSCH, 1998). Apesar de estruturalmente e algumas vezes funcionalmente similares, os dois endocanabinóides melhor caracterizados até hoje são produzidos por rotas biosintéticas distintas, ainda que não totalmente independentes (DI MARZO; DEUTSCH, 1998).

A AEA é formada por uma fosfolipase D (PLD) seletiva para a N-araquidonil fosfatidil etanolamina (NAPE), mas com baixa afinidade para outros fosfolipídeos de membrana, sendo conhecida como NAPE-PLD (Figura 8) (DI MARZO et al., 1994). Os níveis de AMPc e de Ca^{2+} modulam a atividade da enzima N-acil transferase, e por consequência, os níveis de substrato disponíveis para a síntese de AEA (CADAS et al., 1996). Alternativamente, a NAPE pode ser clivada pela α,β -hidrolase 4 (ABHD4) e fosfolipase C (NAPE-PLC) para produzir anandamida glicerofosfato (GP-AEA) e anandamida fosfato (P-AEA) intermediários, respectivamente. GP-AEA é então hidrolisada por glicerofosfodiesterase-1 (GDE-1) produzindo anandamida, enquanto que a P-AEA é hidrolisada pela fosfatase (P-ase) produzindo anandamida. Seguindo a sinalização através de receptores canabinóides, a anandamida sofre ativa recaptção pela célula sendo transportada intracelularmente por um transportador de membrana para anandamida (AMT). Uma vez dentro da célula, a anandamida é quebrada por uma amido hidrolase de ácidos graxos (FAAH) produzindo etanolamina e ácido araquidônico (Figura 8).

Figura 8 - Síntese e degradação da AEA.



Fonte: adaptado de Mcdougall, 2009.

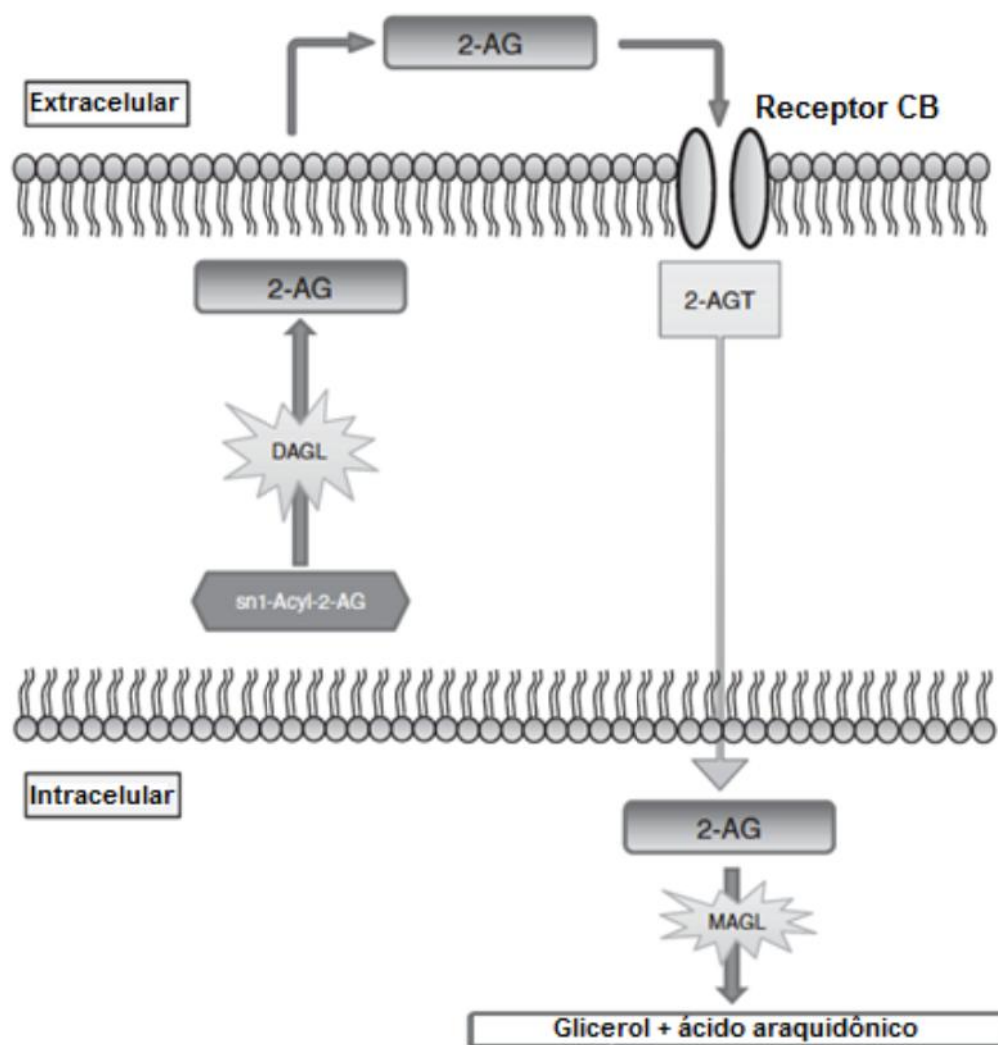
A figura apresenta uma visão geral da via biossíntese e inativação da anandamida.

*NAPE- N-araquidonil fosfatidil etanolamina; PLD: fosfolipase D; ABHD4: α,β -hidrolase 4; PLC: fosfolipase C; GP-AEA: anandamida glicerofosfato; P-AEA: anandamida fosfato; GDE-1: glicerofosfodiesterase - 1; P-ase: fosfatase; AMT: transportador de membrana para anandamida; FAAH: amido hidrolase de ácidos graxos.

O 2-AG é produzido também pela hidrólise de precursores derivados do metabolismo fosfolipídico (sn1-acil-2-araquidonil glicerol) (Figura 9). As enzimas chave são duas diacil glicerol lipases recentemente clonadas *sn*-1-seletivas (DAGL- α e DAGL- β), que

pertencem a família das serinas lipases. Estas enzimas, assim como a NAPE-PLD, são sensíveis ao cálcio, de forma consistente com a atividade do Ca^{2+} intracelular como estímulo fisiológico para a síntese dos endocanabinóides. As DAGLs estão localizadas em axônios e terminações axônicas pré-sinápticas durante o desenvolvimento, mas localizam-se pós-sinápticamente em dendritos e corpos celulares de neurônios adultos, em conformidade com o papel do 2-AG no crescimento neuronal e de um mediador retrógrado no encéfalo adulto. Seguindo a sinalização do receptor canabinóide o 2-AG é transportado para dentro da célula por um transportador de 2-AG (2-AGT) e é degradado pela mono acil glicerol lipase (MAGL) produzindo então ácido araquidônico e glicerol (Figura 9).

Figura 9 - Síntese e degradação do 2-AG.



Fonte: adaptado de Mcdougall, 2009.

A figura apresenta uma visão geral da via biossíntese e inativação do 2-AG.

*sn1-acil-2-AG: sn1-acil-2-araquidonil glicerol; DAGL: diacil glicerol lipase; 2-AG: 2-araquidonil glicerol; MAGL: mono acil glicerol lipase.

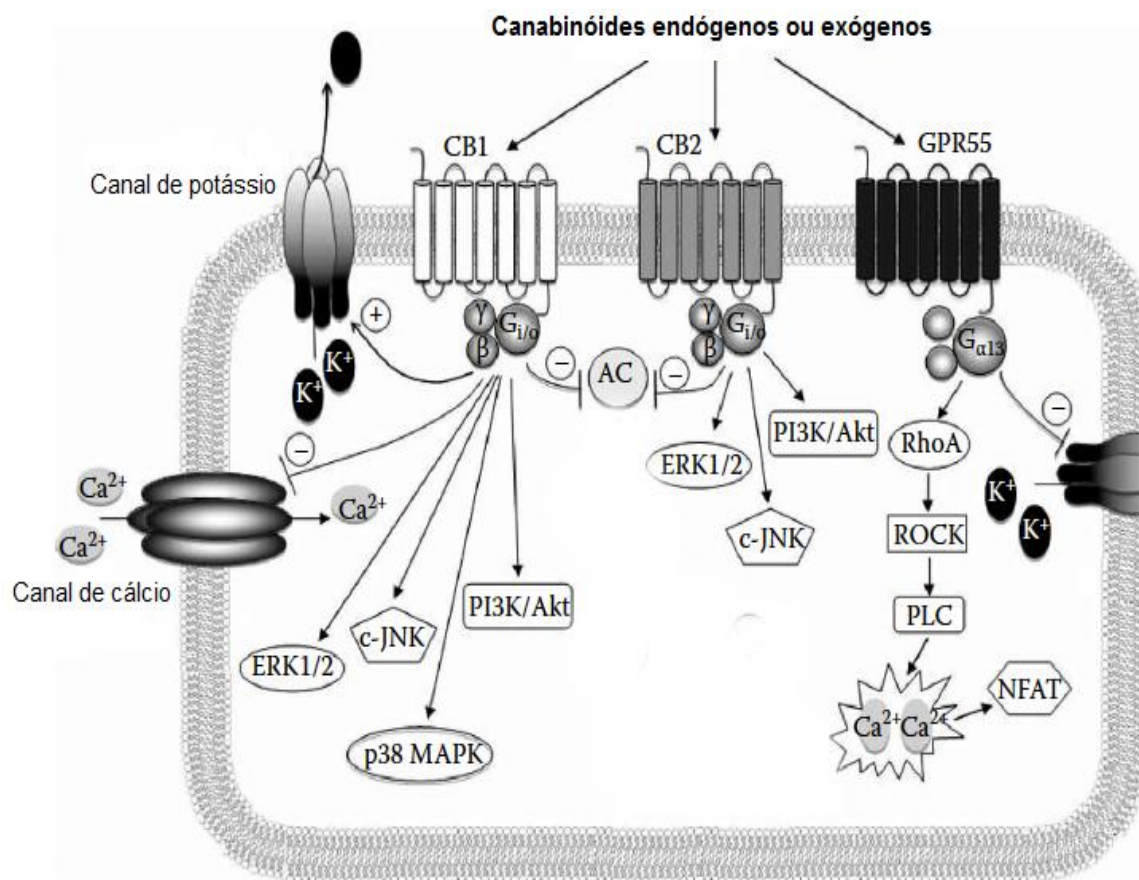
A anandamida e o 2-AG são sintetizados em resposta ao aumento de Ca^{2+} intracelular, o que pode ocorrer por uma despolarização da membrana ou através da mobilização dos estoques intracelulares de Ca^{2+} , seguido pela ativação do receptor acoplado à proteína $\text{G}_{q/11}$. A meia vida dos endocanabinóides é relativamente curta.

Uma visão geral da via biossíntese e inativação da anandamida e 2-AG estão ilustradas nas Figuras 8 e 9.

2.4.3.2 Receptores CBs

A família dos receptores canabíóides (RsCBs) no momento inclui dois receptores farmacologicamente distintos: o receptor CB₁ (RCB₁) predominantemente encontrado no encéfalo e em outros tecidos do sistema nervoso, e o RCB₂, principalmente encontrado no sistema imunológico, mas também expressado em menor densidade no encéfalo. Outra adição recente à família dos receptores para canabinóides é o GPCR, GPR55, o qual está acoplado a proteínas G_{α11-13} (Figura 10) (BEGG et al., 2005). Coerente com sua ampla distribuição, os receptores canabinóides regulam uma variedade de funções fisiológicas centrais e periféricas, incluindo o desenvolvimento neuronal, processos neuromoduladores, metabolismo energético, bem como funções cardiovasculares, respiratórias e reprodutivas. Além disso, estes receptores também modulam a proliferação, motilidade, adesão e a apoptose de células (BOSIER et al., 2010).

Figura 10 - Receptores canabinóides.



Fonte: adaptado de Spigelman, 2010.

A figura ilustra os três tipos de receptores canabinóides descritos na literatura e suas diferentes sinalizações intracelulares.

*AC: ciclase de adenilil; Akt: proteína quinase serina/treonina; ERK: quinase regulada por sinal extracelular; MAPK: proteína quinase ativada por mitógenos; c-JNK: quinase c-Jun N-terminal; NFAT: fator nuclear ativado por células T; PI3K: fosfatidil inositol 3 quinase; PKA: proteína quinase A; PLC: Fosfolipase C; RhoA: *Ras homologous gene A*; ROCK, proteína quinase associada a Rho.

Como membros da superfamília de GPCR ambos RsCB₁ e RsCB₂ exercem seus efeitos biológicos através da ativação de proteínas heterotriméricas do tipo G_{i/o} (HOWLETT et al., 2002). Como consequência deste acoplamento preferencial, a ativação destes receptores essencialmente leva à inibição da ciclase de adenilil e reduções nos níveis intracelulares de AMPc na maioria dos tecidos. Além disso, ambos RsCB₁ e RsCB₂ regulam a fosforilação e ativação de

diferentes membros da família de MAPKs, incluindo quinase regulada por sinais extracelulares-1 e -2 (ERK_{1/2}), MAPK p38 e JNK. Além disso, os RsCB₁ podem regular negativamente canais de Ca²⁺ regulados por voltagem do tipo L, N, P, e ativar canais de K⁺ do tipo A (BOSIER et al., 2010).

2.4.3.1.1 Receptor CB₁

O RCB₁, é na verdade, o GPCR mais abundante no SNC, expresso em níveis elevados no hipocampo, córtex, cerebelo e núcleos da base (HERKENHAM et al., 1990; MACKIE, 2005; MATSUDA et al., 1990; TSOU et al., 1998). A ativação de RsCB₁ leva à inibição da ciclase de adenilil (HOWLETT, 1984), bloqueio de vários canais de Ca²⁺ dependentes de voltagem (BROWN; SAFO; REGEHR, 2004), e ativação de vários canais de K⁺ (STUMPF et al., 2005). (Figura 5.1). Os RsCB₁ centrais também estão localizados em regiões envolvidas na transmissão e modulação da dor, especificamente no corno posterior da medula espinal e substância cinzenta periaquedutal (PAG) (TSOU et al., 1998).

No sistema nervoso periférico, os RsCB₁ foram encontrados no DRG de neurônios de tamanhos heterogêneos (SANUDO-PENA et al., 1999), com graus variáveis de RNAm do RCB₁ e de proteínas em diferente subtipos de neurônios sensoriais. Assim, vários grupos relataram uma predominante localização de RsCB₁ em neurônios de grande diâmetro (HOHMANN; HERKENHAM 1999b; PRICE et al., 2003), e outros demonstraram a expressão de RCB₁ principalmente em neurônios nociceptivos de pequeno diâmetro (AHLUWALIA et al.,

2002; BINZEN et al., 2006). Um fluxo axoplasmático de RsCB₁ têm sido demonstrado em axônios sensoriais periféricos, implicando em um transporte para os terminais onde se presume que os canabinóides produzem seus efeitos antinociceptivos (HOHMANN; HERKENHAM 1999a).

Estudos imunoistoquímicos também revelaram imunorreatividade para o RCB₁ em ambos, pequenos e grandes feixes de fibras nervosas não-mielinizadas e mielinizadas na pele humana (STÄNDER et al., 2005). Estes estudos demonstraram também RsCB₁ em macrófagos, mastócitos, células sebáceas, e queratinócitos (STÄNDER et al., 2005). A localização dos RsCB₁ nos terminais centrais de aferentes primários foi controversa por muitos anos, em parte, porque os estudos anteriores ultra-estruturais não conseguiram detectar RsCB₁ nestes terminais em ratos e primatas (FARQUHAR-SMITH et al., 2000; ONG; MACKIE, 1999). No entanto, após a detecção de RsCB₁ nos terminais glutamatérgicos no hipocampo e cerebelo (KATONA et al., 1999, KAWAMURA et al., 2006), um "reavaliação" dos terminais de fibras aferentes primárias do tipo Aδ e C excitatórias (glutamatérgicos) na medula espinal revelou a presença de RsCB₁ nestes terminais (NYILAS et al., 2009). Estes RsCB₁ pré-sinápticos são a provável explicação para a capacidade dos agonistas canabinóides de diminuírem a frequência das correntes excitatórias registradas pós-sinapticamente em neurônios da medula espinal, contribuindo assim para a modulação da neurotransmissão nociceptiva espinal (MORISSET et al., 2001).

2.4.3.2.2 Receptor CB_2

Do mesmo modo que o RCB_1 , o RCB_2 é capaz de modular a atividade da ciclase de adenilil e MAPK, por meio da ativação de proteínas $G_{i/o}$ (FELDER et al., 1995). No entanto, em contraste com os $RsCB_1$, a ativação de $RsCB_2$ não modula a função de canais iônicos, tal como demonstrado em células transfectadas AtT-20 com RCB_2 (FELDER et al., 1995) e oócitos de *Xenopus* transfectados com RCB_2 e com canais de K^+ (MCALLISTER et al., 1999).

Os $RsCB_2$ estão presentes principalmente no sistema imunológico, um nível de expressão muito mais elevado do que os $RsCB_1$ (GALIEGUE et al., 2005). Até recentemente, pensava-se que os $RsCB_2$ não estavam constitutivamente presentes no encéfalo, no entanto, foi demonstrada a presença de $RsCB_2$ em células da microglia encefálica (NUNES et al., 2004) e em neurônios em diversas regiões do encéfalo, incluindo o cerebelo (ASHTON et al., 2006), tronco encefálico (VAN SICKLE et al., 2005), PAG, tálamo, *striatum*, córtex, amígdala e hipocampo (GONG et al., 2006). Os RCB_2 estão presentes também na pele (IBRAHIM et al., 2005) e na medula espinal (BELTRAMO et al., 2006). A presença de $RsCB_2$ em neurônios do DRG é mais controversa, existem evidências tanto de sua presença (BELTRAMO et al., 2006) quanto de sua ausência (HOHMANN; HERKENHAM, 1999a). A base para estas diferenças não está clara, mas pode surgir como resultado de condições de culturas que influenciam a expressão do receptor ou pela baixa especificidade dos anticorpos utilizados. Foi hipotetizado que após uma lesão nervosa, os $RsCB_2$ são sintetizados no DRG de neurônios sensoriais e rapidamente transportado para os terminais nervosos *in vivo*,

(WOTHERSPOON et al., 2005) semelhante ao processo previamente demonstrado para os R_sCB_1 (HOHMANN; HERKENHAM, 1999a). Além disso, foi observada a presença de proteínas do RCB_2 em DRGs isolados de ratos com lesão do nervo isquiático; o aumento da imunorreatividade para o RCB_2 na parte proximal, mas não distal, de axônios do nervo isquiático de animais submetidos à ligadura do nervo suportam a expressão de R_sCB_2 em neurônios sensoriais (WOTHERSPOON et al., 2005).

2.8 FISIOTERAPIA NO TRATAMENTO DA DOR

2.8.1 Movimento passivo

O movimento passivo significa movimento de qualquer parte do corpo, realizado por outra pessoa ou equipamento. Pode referir-se a movimentos articulares, acessórios de seus movimentos fisiológicos. Estes últimos são aqueles que o paciente pode realizar ativamente, sem auxílio; no entanto, movimentos acessórios são os de articulações, que não podem ser realizados ativamente, mas que podem ser feitos sobre elas por outras pessoas. Qualquer um destes movimentos podem ser feitos vagarosamente ou com certa velocidade, gentil ou vigorosamente e através de pequenas ou grandes amplitudes e, assim, serem chamados de movimentos passivos (MAITLAND, 1991).

2.8.2 Mobilização articular

As técnicas de mobilização são procedimentos realizados para aumentar a amplitude de movimento e promover analgesia em uma articulação. Estes procedimentos envolvem posicionamentos específicos e manobras oscilatórias, tanto na amplitude média, como no limite disponível da amplitude articular de movimento. O movimento pode incluir tanto um deslizamento acessório ou um movimento fisiológico da articulação. A determinação da dosagem envolve ambos, alteração da posição na amplitude de movimento ou modificação do tempo de duração da mobilização. Tipicamente, a duração do tratamento pode variar de 30 segundos a vários minutos (WRIGHT; SLUKA, 2001).

A mobilização é um movimento passivo, realizado de tal maneira (particularmente em relação à velocidade do movimento) que está sempre relacionada com a habilidade do paciente em prevenir o movimento, se ele assim desejar. Há 2 tipos de mobilização: 1) movimentos passivos, realizados com o propósito de aliviar a dor e restaurar o arco de movimento livre e funcional, sendo esta modalidade ainda subdividida em a) movimentos passivos oscilatórios e b) movimentos passivos em estiramento sustentado; e 2) movimentos passivos, realizados com o propósito de manter um arco de movimento funcional, em pacientes que estão conscientes ou que tenham doença articular inflamatória, como, por exemplo, artrite reumatóide (MAITLAND, 1991). Como mencionado acima, estas técnicas podem ser realizadas em diferentes posições, assim como podem ser usados movimentos de pequena ou grande amplitude. Os tipos de movimentos usados na aplicação das técnicas empregadas na reabilitação de

pacientes, sejam para reduzir a dor ou aumentar a amplitude de movimento, geralmente são divididos em quatro graus: Grau I - Uma pequena amplitude de movimento proximal à posição inicial do arco; Grau II - Uma grande amplitude de movimento, na amplitude do arco. Ela pode ocupar qualquer parte do percurso que esteja livre de tensões ou espasmos; Grau III - Uma grande amplitude também, porém dentro de seu arco de tensão ou espasmo muscular e Grau IV - Movimento de pequena amplitude executado, de forma forçada, com espasmo muscular ou tensão.

2.8.2.1 Mecanismo de ação da mobilização articular

A aplicação de movimentos passivos nas articulações no tratamento da dor é uma prática muito antiga de terapia manual. Embora, a mobilização espinal e periférica continue sendo amplamente aplicada na prática clínica, há poucos dados experimentais fundamentando sua eficácia na redução da dor ou melhora da função. Evidências sobre a eficácia da mobilização do membro inferior são particularmente escassas, pois a maioria dos estudos de articulações periféricas utilizam o membro superior como modelo experimental (VICENZINO; COLLINS; WRIGHT, 1996; PAUNGMALI et al., 2003). Até o presente momento, poucos estudos investigaram o efeito hipoalgésico da mobilização articular dos membros inferiores, principalmente com foco sobre articulação do tornozelo (COLLINS; TEYES; VICENZINO, 2004;. YEO; WRIGHT, 2004). Embora a literatura científica tenha começado a caracterizar os efeitos da terapia manual da coluna vertebral (WRIGHT; SLUKA, 2001), existem poucos

estudos que investigam os efeitos hipalgésico de técnicas de mobilizações articulares periféricas (YEO; WRIGHT, 2004).

Tem sido habitualmente considerado que movimentos passivos manuais produzem efeitos tanto por mecanismos “neurológicos” como “mecânicos” (BIALOSKY et al., 2009). No entanto, em termos de consequências clínicas a separação é artificial. Todos os parâmetros de movimentos passivos são uma forma mecânica de estimulação de células excitáveis, tais como nervos e músculos. Por isso, foi proposto que o movimento passivo ativa vias endógenas inibitórias da dor, através da estimulação e/ou placebo (ROCHE, 2002; ZUSMAN, 1994). Este parece ser um dos mecanismos mais plausíveis para o alívio (temporário) da dor evidenciado clinicamente, e ganhou algum apoio dos estudos realizados em animais e humanos (SKYBA et al., 2003; VICENZINO; COLLINS; WRIGHT, 1996).

Neste sentido, modelos multifatoriais foram descritos tentando explicar o efeito da terapia manual no tratamento da dor (WRIGHT, 1995). Acredita-se que essas técnicas podem ter efeitos benéficos sobre os tecidos locais e que podem suprimir a dor pela ativação de mecanismos neurofisiológicos a níveis espinais ou supra-espinais. Evidências emergentes sugerem que técnicas de terapia manual (TTM) aplicadas à coluna cervical provocam alterações simultâneas na percepção da dor, no sistema nervoso autonômico e na função motora em padrões que são semelhantes à mudanças induzidas por estimulação direta da PAG (WRIGHT, 2001; VICENZINO et al., 1998). Curiosamente, os tratamentos com terapia manual parecem exercer uma influência predominante sobre a nocicepção mecânica, mais do que sobre a nocicepção térmica (VICENZINO et al., 1998) (para revisar os

efeitos neurofisiológicos da MA, ver WRIGHT, 2001; BIALOSKY et al., 2009). Estas evidências emergentes apoiam o conceito de que as TTM exercem importantes efeitos neurofisiológicos que podem contribuir para a redução da dor.

2.9 JUSTIFICATIVA

Várias terapias físicas são utilizadas no tratamento da dor aguda e crônica. Estas podem ser categorizadas como modalidades de eletroterapia (por exemplo, a estimulação elétrica nervosa transcutânea [TENS]) (KOCYIGIT et al., 2012), acupuntura (VAS et al., 2012), térmicas (por exemplo, calor e frio) (SECO et al., 2011), terapias manuais (por exemplo, manipulação) (RUBINSTEIN et al., 2012) ou mobilização articular (MOSS; SLUKA; WRIGHT, 2003), ou ainda exercícios físicos (NIJS et al., 2012). Na clínica, os fisioterapeutas usam uma combinação dessas modalidades para as necessidades de cada paciente, com base nas conclusões do exame físico.

No passado, algumas terapias foram particularmente adotadas em diferentes países ou por diferentes grupos de fisioterapeutas. No entanto, cada vez mais, fisioterapeutas estão adotando uma abordagem baseada em evidências científicas para o tratamento do paciente. Cada uma das categorias terapêuticas acima mencionadas foram objetos de estudos nas últimas décadas (WRIGHT; SLUKA, 2001). Existem ainda muitas terapias específicas ou regimes específicos de dosagens que não foram submetidos à investigação científica. Dada a variedade de terapias físicas existentes, as diferentes maneiras pelas quais elas podem ser aplicadas, bem como a falta de uma fonte de investimento em pesquisas,

pode-se levar algumas décadas para que todas as terapias físicas sejam exaustivamente investigadas.

Neste sentido, evidências sugerem que as técnicas de terapia manual (TTMs) são efetivas no tratamento de problemas músculo-esqueléticos incluindo dor lombar (LICCIARDONE et al., 2003), síndrome do túnel do carpo (AKALIN et al., 2002), osteoartrite de joelho (DEYLE et al., 2000) e quadril (MACDONALD et al., 2006). No entanto, apesar do apoio literário em sua eficácia, os mecanismos de ação das TTM não estão bem estabelecidos. Isso levou o *National Institutes of Health* (NIH) a realizar uma chamada para projetos de pesquisa especificamente para suprir esta deficiência (KHALSA et al., 2006).

A importância de identificar os mecanismos de ação das TTMs se dá pelo fato de que isso proporcionaria uma maior aceitação de tais técnicas pelos profissionais de saúde. Apesar de vários estudos na literatura apoiarem a eficácia da terapia manual (TM) em determinadas condições músculo-esqueléticas, os clínicos ainda são resistentes a encaminhar os pacientes para a TM, ou encaminham uma parcela menor do que a esperada (BISHOP; WING, 2003). A falta de um mecanismo de ação definido das TTMs limita a sua aceitabilidade. Desta forma, o conhecimento do mecanismo de ação associado aos efeitos das TTMs pode promover o uso mais adequado destas técnicas pelos profissionais da área da saúde.

2.10 OBJETIVOS

2.10.1 Objetivo geral

O objetivo geral deste trabalho foi verificar o efeito da mobilização articular do tornozelo (MAT) na dor aguda pós-operatória e na dor crônica de origem neuropática, além de investigar os mecanismos neurofisiológicos envolvidos neste efeito.

2.10.2 Objetivos específicos

i) Avaliar em camundongos submetidos a incisão plantar (dor pós-operatória):

- o efeito de diferentes tempos (regimes de tratamento) e do tratamento diário com MAT sobre a hiperalgesia mecânica;

- a participação dos sistemas endógenos: opioidérgico, adenosinérgico, endocanabinóide e monoaminérgico sobre o efeito antihiperalgésico da MAT;

ii) Avaliar em ratos submetidos ao esmagamento do nervo isquiático (dor neuropática):

- o efeito do tratamento diário com MAT na hiperalgesia mecânica e regeneração nervosa periférica;

- o papel das células gliais no efeito antihiperalgésico da MAT.

3 ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos, os quais encontram-se aqui organizados. Os **artigos 1, 2 e 4** estão dispostos da mesma forma que foram publicados nas edições das revistas científicas *PAIN MEDICINE*, *PHYSICAL THERAPY* e *PAIN* respectivamente, ou submetido, que é o caso do **Artigo 3**, o qual foi encaminhado para a revista *NEUROSCIENCE*.

3.1 Mobilização Articular do Tornozelo Diminui Hipersensibilidade pela Ativação de Receptores Opióides Periféricos em um Modelo de Dor Pós-operatória em Camundongos

Artigo 1

**ANKLE JOINT MOBILIZATION DECREASES
HYPERSENSITIVITY BY ACTIVATION OF PERIPHERAL
OPIOID RECEPTORS IN A MOUSE MODEL OF
POSTOPERATIVE PAIN**

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Nascimento FP, Gadotti VM, Santos ARS.
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TRANSLATIONAL RESEARCH SECTION

Original Research Article

Ankle Joint Mobilization Decreases Hypersensitivity by Activation of Peripheral Opioid Receptors in a Mouse Model of Postoperative Pain

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Abstract

Objective. Investigate whether ankle joint mobilization (AJM) decreases hypersensitivity in the mouse plantar incision (PI) model of postoperative pain as well as to analyze the possible mechanisms involved in this effect.

Design. Experiment 1: PI male Swiss mice (25–35 g, N = eight animals per group) were subjected to five sessions of AJM, each lasting either 9 or 3 minutes. AJM movement was applied at a grade III as defined by Maitland. Paw withdrawal frequency to mechanical stimuli was assessed before realization of PI and before and after daily AJM sessions. Mechanical hypersensitivity was also assessed following systemic (intraperitoneal [i.p.]) and local (intraplantar) injection of naloxone (a nonselective opioid receptor antagonist; 1 mg/kg, i.p.; 5 µg/paw, respectively, experiment 2); and systemic injection of fucoidin (100 µg/mouse, i.p., an inhibitor of leukocyte rolling, experiment 3) in different groups of mice.

Results. Nine but not 3 minutes of AJM reduced mechanical hypersensitivity caused by PI, an effect that was prevented by systemic and local administrations of naloxone but not by fucoidin.

Conclusions. Our results indicate that joint mobilization reduces postoperative pain by activation of the peripheral opioid pathway. However, antihypersensitivity induced by AJM is apparently not limited by the number of opioid-containing leukocytes but by opioid receptors availability in sensory neurons. A better understanding of the peripheral mechanisms of AJM could stimulate therapists to integrate joint mobilization with strategies also known to influence endogenous pain control, such as exercise, acupuncture, and

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transcutaneous electrical nerve stimulation to potentiate endogenous analgesia.

Key Words. Incisional Pain; Manual Therapy; Opioid, Passive; Rehabilitation

Introduction

It is estimated that more than 73 million surgeries are performed annually in the United States, and up to 75% of the patients experience pain after surgery [1,2]. Economic burden of treating chronic pain that develops from acute pain in a 30-year-old individual over a lifetime could be as much as \$1 million. The prevention and effective relief of acute pain may improve clinical outcomes, avoid clinical complications, save health care resources, and improve quality of life [1,2]. Joint mobilization is a treatment approach commonly used by physical therapists for the management of a variety of painful conditions [3]. Mobilization-induced analgesia has been demonstrated in a number of clinical [3,4] and preclinical studies [5,6]. Sluka and Wright [6] have reported that unilateral knee joint mobilization reduces secondary mechanical hyperalgesia induced by capsaicin injection into the ankle joint of rats.

It has also been suggested that spinal manipulation may relieve pain of spinal origin by arousing, to clinically effective levels, a pain control system that is encoded by opioid peptides (endorphin system) [7]. Ward [8] suggested that pain relief through spinal manipulation therapy (SMT) is largely an opioid-mediated placebo response. However, Zusman, Edwards and Donaghy [9] (unpublished material) hypothesized that the information produced with passive joint movement, theoretically unsuitable for relieving pain in terms of the gate control theory, may be capable of arousing the opioid system. The opioid hypothesis was preliminarily investigated by attempting to reverse the reduction in pain reported following treatment with the opioid antagonist naloxone [10]. Despite emerging evidence for manual physiotherapy in the treatment of musculoskeletal disorders, little is known about the mechanisms through which manual physiotherapy achieves its clinically beneficial effects.

In the late 1980s, studies began to show that opioids do not only activate opioid receptors in the brain and spinal cord but also on peripheral sensory neurons. Since then, a detailed model of opioid pathways outside of the central nervous system has been developed, and applications such as intra-articular morphine injection have been introduced into routine clinical practice [11,12].

Several studies indicate that a large proportion (about 50–80%) of the analgesic effects produced by systemically administered opioids can be mediated by peripheral opioid receptors [13–15]. Peripherally acting opioid agonists would be most attractive for their lack of central side effects (respiratory depression, nausea, dysphoria, addiction, and tolerance) and due to the typical adverse

effects of nonsteroidal anti-inflammatory drugs (gastrointestinal bleeding, ulcers, and thromboembolic complications) [12].

To date, few studies have reported the effects of peripheral joint mobilization techniques on pain responses. In this context, the present study was designed to investigate the effect of passive ankle joint mobilization (AJM) in a model of incision-induced postoperative pain, different from those previously described. In addition, we verified the role of peripheral opioid receptors and the mechanism underlying peripheral opioid analgesic effect, such as the release of opioid peptides from inflammatory cells, in a passive peripheral joint mobilization-induced antihypersensitivity.

Materials and Methods

Experimental Animals

All animal care and experimental procedures were carried out in accordance with the National Institutes of Health Animal Care Guidelines (NIH publications number 80-23) and were approved by the Ethics Committee of the Universidade Federal de Santa Catarina (protocol number PP00527). All experiments were conducted using male Swiss mice (25–35 g, N = eight animals per group), housed at $22 \pm 2^\circ\text{C}$ under a 12 hours light/12 hours dark cycle (lights on at 6:00 AM) and with free access to food and water. Mice were acclimated to the laboratory for at least 1 hour before testing that was carried out between 8:00 and 12:00 hours. Animals were used only once throughout the experiments. A number of experiments were conducted to test analgesic effects of AJM and its mechanisms [16].

Plantar Incision (PI) Surgery

The PI surgery was performed as previously described [17]. Briefly, mice were anesthetized with 1–2% isoflurane delivered via a nose cone. After sterile preparation of the right hind paw, a 5-mm longitudinal incision was made through skin and fascia of the plantar surface using a number 11 scalpel blade. The incision started 2 mm from the proximal edge of the heel and extended toward the toes. After wound homeostasis, the skin was apposed with an 8.0 nylon mattress suture, and the wound was covered with 10% povidone-iodine solution. Control animals underwent a sham procedure. That is, they were subjected to anesthesia but not to PI. Animals were allowed to recover in their cages, and sutures were removed on the second postoperative day.

Experiment 1: AJM Treatment

The treatment procedure involved an AJM at grade III movement as defined by Maitland [18]. The knee joint was stabilized, and the ankle joint was rhythmically flexed and extended to the end of the range of movement, according to a previously reported dosage regime [6]. The treat-

AJM Reduces Postoperative Pain

ments with AJM were carried out in animals lightly anesthetized with 1–2% isoflurane, prior to and during joint mobilization. The two treated groups were as follows: 1) three repetitions of 1-minute mobilizations with 30-second rest periods in between mobilizations; and 2) three repetitions of 3-minute mobilizations with 30-second rest periods in between mobilizations. The joint mobilization procedure described is a standard treatment technique, and the treatment intervals are according to a previously reported dosage regime [6]. For this experiment, the following groups (N = 8) were used: 1) sham, 2) incision (control); 3) incision + AJM 3 minutes, and 4) incision + AJM 9 minutes.

Control

Mice were lightly anesthetized with 1–2% isoflurane, and the ankle was maintained in a neutral position using the same hand contact and positioning as the treatment technique. Three repetitions of 3-minute hand contact with 30-second intervals were utilized. This procedure controlled for the effects of holding the limb and applying pressure to the musculature of the paw as well as for the influence of anesthesia.

Mechanical Hypersensitivity

Mechanical hypersensitivity was measured as previously described [19]. Mice were acclimated in individual clear boxes (9 × 7 × 11 cm³) on an elevated wire mesh platform to allow access to the ventral surface of the hind paws. The right hind paw was stimulated with a constant pressure of 0.4 g von Frey filaments (VFF) (Stoelting, Chicago, IL, USA). The withdrawal frequency to 10 applications was considered as representative of nociceptive behavior. The results are expressed as the percentage of withdrawal frequency. In analyses monitoring time course effect caused by AJM, animals received 9-minute or 3-minute AJM, and repeated measures were obtained. Mechanical hypersensitivity was assessed before (baseline), 24 hours after PI and at 15, 30, 45, 60, and 120 minutes after AJM. In a separate series of experiments, we evaluated mechanical hypersensitivity before (baseline), 24 hours after PI and 30 minutes after AJM at each day after PI, for 6 days.

Experiment 2: Involvement of the Opioid System

Experiment 2.1

To assess the participation of the opioid system in the antihypersensitivity produced by 9-minute AJM, mice were submitted to PI. Mechanical hypersensitivity was tested 24 hours after PI. Mice were pretreated with an intraperitoneal (i.p.) injection of saline (10 mL/kg) or naloxone (a nonselective opioid receptor antagonist, 1 mg/kg). After 20 minutes, animals received control or AJM for 9 minutes. Mechanical hypersensitivity was evaluated using a von Frey monofilament (0.4 g) 30 minutes after AJM. Control animals were subjected to 9 minutes of anesthesia and were assessed over the same time intervals. For

this experiment, the following groups (N = 8) were used: 1) incision saline (10 mL/kg, i.p.) + control; 2) incision naloxone (1 mg/kg, i.p.) + control; 3) incision saline (10 mL/kg, i.p.) + AJM 9 minutes; and 4) incision naloxone (1 mg/kg, i.p.) + AJM 9 minutes.

Another group of mice was pretreated with saline (10 mL/kg, i.p.) or naloxone (1 mg/kg, i.p.) and, after 20 minutes, received subcutaneous (s.c.) saline (10 mL/kg) or morphine (5 mg/kg). These groups were assessed 30 minutes after saline or morphine treatment. For this experiment, the following groups (N = 8) were used: 1) incision saline (10 mL/kg, i.p.) + saline (10 mL/kg, s.c.); 2) incision naloxone (1 mg/kg, i.p.) + saline (10 mL/kg, s.c.); 3) incision saline (10 mL/kg, i.p.) + morphine (5 mg/kg, s.c.); and 4) incision naloxone (1 mg/kg, i.p.) + morphine (5 mg/kg, s.c.).

Experiment 2.2

To evaluate the involvement of peripheral opioid receptors in the antihypersensitivity induced by AJM, animals received an intraplantar injection (i.pl.) of 20 µl of naloxone (5 µg/site) or saline solution (20 µl/site) in the ipsilateral (IL; incision) or contralateral (CL; nonincision) hind paw. After 15 minutes, animals received control or AJM for 9 minutes. Mechanical hypersensitivity was evaluated using the VFF 30 minutes after AJM. Control animals received 9 minutes of anesthesia and were assessed over the same time intervals. For this experiment, the following groups (N = 8) were used: 1) incision saline (20 µl/i.pl.) + control; 2) incision naloxone (5 µg/i.pl.) + control; 3) incision saline (20 µl/i.pl.) + AJM 9 minutes; and 4) incision naloxone (5 µg/i.pl.) + AJM 9 minutes.

Another group of mice was pretreated with an i.pl. of saline (20 µl/paw) or naloxone (5 µg/paw) and, after 20 minutes, received saline (10 mL/kg, s.c.) or morphine (5 mg/kg, s.c.). These groups were assessed 30 minutes after saline or morphine treatment. For this experiment, the following groups (N = 8) were used: 1) incision saline (20 µl/i.pl.) + saline (10 mL/kg, s.c.); 2) incision naloxone (5 µg/i.pl.) + saline (10 mL/kg, s.c.); 3) incision saline (20 µl/i.pl.) + morphine (5 mg/kg, s.c.); and 4) incision naloxone (5 µg/i.pl.) + morphine (5 mg/kg, s.c.).

Experiment 3: Involvement of the Recruitment of Opioid-Containing Leukocytes to Inflamed Tissue in Peripheral Opioid Analgesic Effect Induced by AJM

To examine the mechanisms underlying peripheral opioid analgesic effects such as leukocytes infiltrating to inflamed tissue, a set of animals was pretreated with saline (10 mL/kg, i.p.) or fucoidin (100 µg/mouse, i.p., an inhibitor of leukocyte rolling) 20 minutes before the 9-minute AJM. Mechanical hypersensitivity was evaluated using the VFF 30 minutes after AJM. For this experiment, the following groups (N = 8) were used: 1) incision saline (10 mL/kg, i.p.) + control; 2) incision fucoidin (100 µg/mouse,

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i.p.) + control; 3) incision saline (10 mL/kg, i.p.) + AJM 9 minutes; and 4) incision fucoidin (100 µg/mouse, i.p.) + AJM 9 minutes.

Drugs

Morphine sulfate (União Química, Brazil) and naloxone hydrochloride from Tocris Cookson Inc. (Ellisville, MO, USA), fucoidin (Sigma Chemicals Co., St. Louis, MO, USA) were dissolved in saline solution (0.9%).

Data Analysis and Statistics

The results are presented as the mean \pm standard errors of the mean for each group. The analyses were performed using a two-way repeated measures analysis of variance (ANOVA). A multi-comparison post hoc test was performed using Bonferroni test. Data were converted to the area under the curve (AUC) using one-way ANOVA followed by Student Newman-Keuls test when appropriate. Differences with a value of $P < 0.05$ were considered significant.

Results**AJM Reduces Mechanical Hypersensitivity**

To evaluate the effects of AJM on postoperative pain, we performed a PI in mice. The results presented in Figure 1A show that acute treatment with 9-minute AJM (incision + AJM 9 minutes group) reduced mechanical hypersensitivity induced by PI. Significant differences between groups (incision vs incision + AJM 9 minutes) were observed at 15 minutes ($P < 0.001$), 30 minutes ($P < 0.001$), and 45 minutes ($P < 0.001$) after AJM. However, 3 minutes of mobilization (AJM 3 minutes) had no effect on withdrawal response frequency and was not significantly different from control incision animals. AUC analysis showed that mechanical hypersensitivity in the AJM 9 minutes group was significantly lower than incision group (15–120 minutes) ($P < 0.05$; see Figure 1B). In addition, the daily treatment of animals with 9-minute AJM decreased the mechanical hypersensitivity induced by PI evaluated 30 minutes after treatment. This effect was evident until the fifth day of AJM treatment (see Figure 1C). AUC analysis shows that mechanical hypersensitivity was significantly reduced by daily treatment of animals with AJM 9 minutes (D1–D6) ($P < 0.001$, see Figure 1D).

Involvement of the Opioid System in the AJM-Induced Antihypersensitivity**Naloxone Administrated Systemically**

The results depicted in Figure 2A show that the pretreatment of mice with naloxone (1 mg/kg, i.p.) significantly prevented the antihypersensitivity caused by AJM in the von Frey test, compared with the saline plus AJM group. The results obtained in this experiment were analyzed by a two-way ANOVA. There were significant effects of AJM ($F[1,26] = 30.77$; $P < 0.001$), naloxone ($F[1,26] = 4.76$; $P < 0.038$), and AJM \times naloxone ($F[1,26] = 42.36$;

$P < 0.001$). Post hoc analyses indicated that the pretreatment of mice with systemic naloxone prevented the antihypersensitivity produced by AJM 9 minutes ($P < 0.001$). The results presented in Figure 2B show that the pretreatment of mice with naloxone (1 mg/kg, i.p.), given 20 minutes beforehand, completely reversed the antihypersensitivity effect caused by morphine (5 mg/kg, s.c., used as a positive control) ($F[1,26] = 33.11$; $P < 0.001$) compared with the saline plus morphine group.

Naloxone Administrated Locally

Figure 2C shows the effect of naloxone (5 µg/paw, i.pl.) IL₁ administered locally, on the antihypersensitivity promoted by 9 minutes AJM as evaluated by the von Frey test compared with the saline (5 µl/paw, i.pl.) plus AJM group. Two-way ANOVA revealed significant main effects of local naloxone pretreatment in the 20 minutes ($F[1,24] = 26.29$; $P < 0.001$) and AJM 9 minutes group at 30 minutes ($F[1,24] = 55.50$; $P < 0.001$) and a local naloxone pretreatment \times AJM 9 minutes interaction at 30 minutes ($F[1,24] = 32.58$; $P < 0.001$). Post hoc analyses indicated that the pretreatment of mice with local naloxone prevented ($P < 0.001$) the antihypersensitivity elicited by AJM 9 minutes (see Figure 2C). However, CL (nonincised paw) naloxone (5 µg/paw, i.pl.) administration did not prevent the antihypersensitivity elicited by AJM 9 minutes compared with the saline (5 µl/paw, i.pl., CL) plus AJM group (see Figure 2D).

Furthermore, the pretreatment of mice with local naloxone (5 µg/paw, i.pl.), given 20 minutes beforehand, produced partial but significant reversal of the antihypersensitivity caused by morphine (s.c., 5 mg/kg) ($F[1,22] = 2.74$; $P < 0.001$) compared with the saline plus morphine group (see Figure 2E).

Migration of opioid-containing leukocytes to inflamed tissue did not mediate peripheral opioid analgesic effect induced by AJM.

Finally, to examine the functional involvement of leukocytes, animals were pretreated with an injection of fucoidin. The results depicted in Figure 3 show that the treatment of mice with fucoidin (100 µg/mouse, i.p., an inhibitor of leukocyte rolling) 20 minutes before AJM did not reverse the antihypersensitivity caused by AJM compared with the saline plus AJM group.

Interestingly, the migration of opioid-containing inflammatory cells into injured tissue does not appear to be the mechanism underlying peripheral opioid analgesic effects induced by AJM.

Discussion

Joint mobilization is a treatment approach commonly used by physical therapists for the management of a variety of painful conditions [4,20]. Despite its clinical effectiveness, its peripheral mechanisms of action are not fully known [8,20]. Because opioids do not only activate

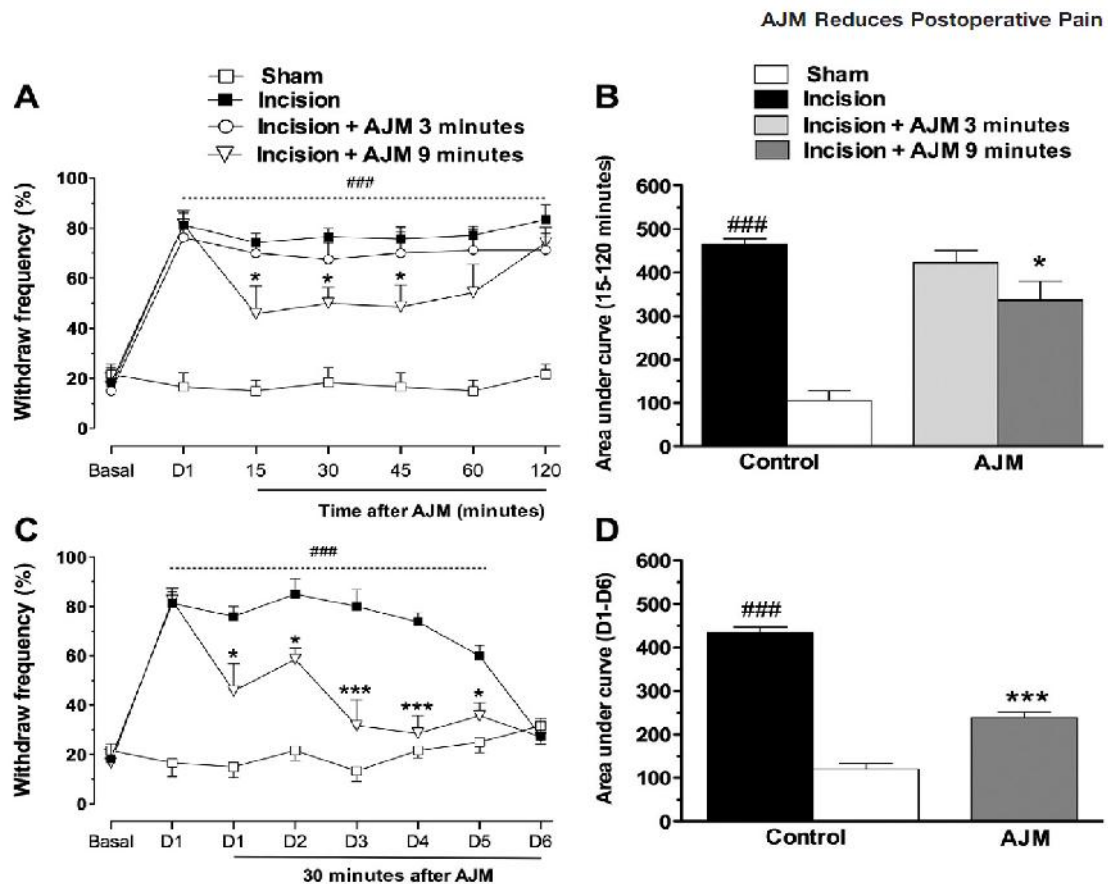


Figure 1 Ankle joint mobilization reverses mechanical hypersensitivity induced by PI. Effect of acute treatment with 9-minute or 3-minute AJM on the mechanical hypersensitivity (panel A) and analysis of the area under the curve (AUC; 15–120 minutes, panel B). Daily treatments with 9 minutes AJM (panel C) and analysis of the AUC (D1–D6, panel D). Each point represents the mean of eight animals, and vertical lines show the standard error of the mean. The symbols denote a significant difference of $*P < 0.05$ or $***P < 0.001$ when compared with control group or $***P < 0.001$ when compared with sham-operated group. D = day (s); AJM = ankle joint mobilization.

opioid receptors in the brain and spinal cord but also on peripheral sensory neurons [11], and in addition to the demonstration that the opioid system is involved exercise-induced analgesia [21]. We raised the question of whether peripheral opioid could be involved in AJM-induced analgesia. The experiments reported here investigated, through the use of a mouse model of postoperative pain, the role of peripheral opioid receptors in the antihypersensitivity induced by AJM. Furthermore, the mechanism underlying peripheral opioid analgesic effect such as the release of opioid peptides from inflammatory cells was investigated.

AJM Reduces Hypersensitivity in a Mouse Model of Postoperative Pain

Because of its relevance to postoperative pain management, the hind paw incision model has been used to study pain mechanisms and analgesic efficacy [22,23]. The model has been described for both rats and mice [17,24]. In general, both thermal and mechanical nociceptive thresholds drop immediately after the incisions are made, and these changes resolve after 4–7 days. The analgesic effects of endogenous opioids have been studied using this model, although little work has focused on the

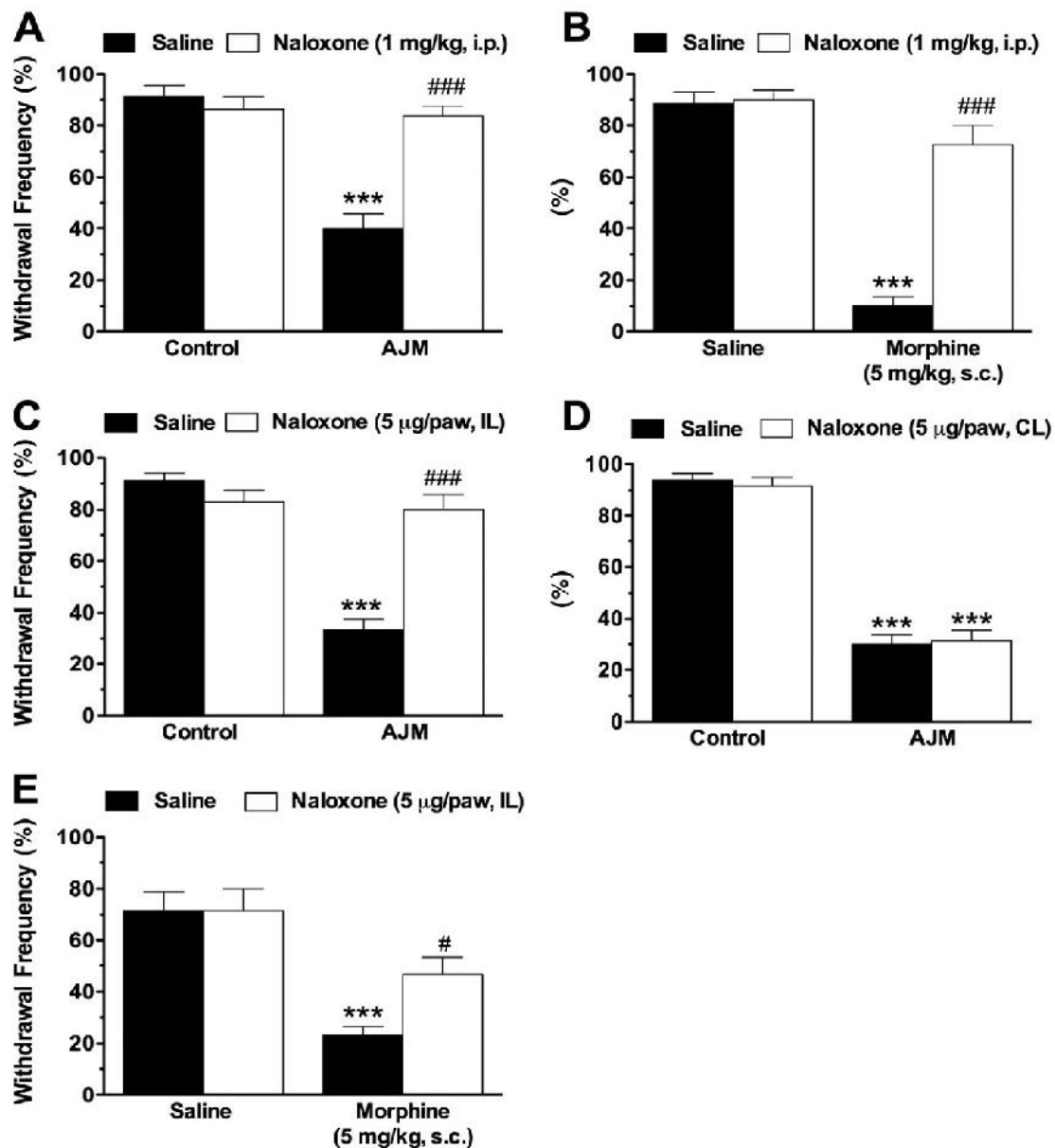


Figure 2 Peripheral opioid receptors mediate AJM-induced antihypersensitivity. Systemic pretreatment with naloxone (1 mg/kg, i.p.) panel A or 5 μ g/paw, panel C (ipsilateral) and D (contralateral) on the antihypersensitivity induced by AJM in mice. Black bars show the saline or antinociceptive effect of morphine (5 mg/kg s.c., panel). Open bars show the effects of naloxone (1 mg/kg, i.p., panel B, 5 μ g/paw, ipsilateral panel C and 5 μ g/paw, contralateral, panel D) antagonist injected at ipsilateral location 20 minutes before the morphine injection (panel E). Each point represents the mean of eight animals, and vertical lines show the standard error of the mean. The symbols denote a significant difference of *** P < 0.001 when compared with control group or * P < 0.05 and ### P < 0.001 when compared with AJM or morphine group. AJM = ankle joint mobilization; IL = ipsilateral; CL = contralateral.

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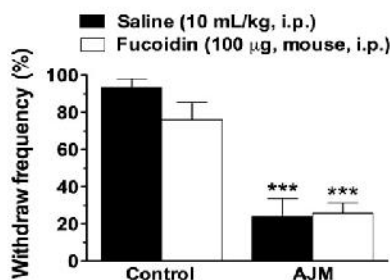


Figure 3 Systemic pretreatment with fucoidin do not reverse antihypersensitivity induced by AJM. Black bars show the control saline or AJM. Open bars show the effects of Fucoidin (100 µg/mouse, i.p.) injected intraperitoneally 20 minutes before the AJM or control. Each point represents the mean of eight animals, and vertical lines show the standard error of the mean. The symbols denote a significant difference of $***P < 0.001$ when compared with control group. AJM = ankle joint mobilization.

peripheral effects of opioids specifically [23]. The present study demonstrates that 9 but not 3 minutes of AJM effectively reduced mechanical hypersensitivity. In surgery, after tissue injury and inflammation, nociceptors are sensitized in such a way that a slight stimulation becomes painful [25]. In postoperative pain, the sensitization of A δ and C fibers occurs at the same mechanical stimuli intensity.

As shown in the present study, mechanical hypersensitivity was markedly reduced by AJM treatment for at least 5 days. Previously, it has been demonstrated that joint mobilization produces a specific hypoalgesic effect to mechanical stimulus. Our group found that AJM reduced mechanical hypersensitivity (neuropathic pain) for at least 30 days of treatment in rats [26].

Systemic Administration of Naloxone Prevents Antihypersensitivity Induced by AJM

A considerable number of mechanisms have been proposed to explain how the analgesic effects of passive joint mobilization may be mediated [4,5]. Movements might trigger segmental inhibitory mechanisms of pain [27]. Thus, recently, it has been suggested that spinal release of both serotonin and noradrenaline from descending neurons might play important modulatory effect of joint mobilization [4,5]. Sluka and Wright [6] reported that knee joint mobilization produces a significant reversal of secondary mechanical hypersensitivity induced by intra-articular injection of capsaicin in rats. Our data extend these findings by demonstrating that AJM reduces mechanical hypersensitivity in a postoperative pain model in mice, which is different from those previously described

for joint mobilization. Thus, these data favor a possible therapeutic use of joint mobilization for the relief of postoperative pain.

Much of the research on the mechanisms of action of manual therapies have focused on spinal and Mulligan's mobilization with movement techniques (application of active movement across a peripheral joint). However, the role of peripheral opioids in passive joint mobilization-induced analgesia has not been investigated. Opioids are involved in both segmental inhibition and descending inhibition [7], although the role of the opioid system in passive joint mobilization-induced analgesia is controversial in the literature. In humans, systemic blockage of opioid receptors with naloxone has no effect on the analgesia produced by manipulation [28]. However, the authors acknowledge the limitations of this study in relation to the dose of naloxone administered and the timing of the naloxone injection.

The results reported here indicate, to our knowledge for the first time, that the antihypersensitivity effect of AJM is mediated by the opioid system once the pretreatment of mice with systemic administration of naloxone was able to prevent the decrease in withdrawal response frequency elicited by AJM (see Figure 2A). Under the same conditions, naloxone significantly modified the antihypersensitivity caused by morphine in the von Frey test (see Figure 2B). This result strongly suggests that the activation of the opioid system is responsible for the antinociceptive effect of joint mobilization.

Local (Paw) Administration of Naloxone Reverses Antihypersensitivity Induced by AJM

Recently, opioid receptors have been identified on peripheral processes of sensory neurons. These findings provide new insights into the intrinsic mechanisms of pain control and suggest innovative strategies for alternative approaches to pain treatment. Another interesting result of the present study is the demonstration, for the first time, that the antihypersensitivity elicited by peripheral joint mobilization is mediated by peripheral opioid peptides release. This conclusion is derived from the fact that the pretreatment of mice with naloxone, administered locally (paw) [29], prevented the effect of AJM (see Figure 2A). Under the same conditions, naloxone significantly blocked the antihypersensitivity of morphine (see Figure 2B).

Many studies showed that an opioid could produce potent analgesia by activating opioid receptors on peripheral sensory neurons [12,30]. Opioid receptors are expressed in small-, medium- and large-diameter dorsal root ganglion (DRG) neurons [12], which are coexpressed with prototypical sensory neuropeptides such as substance P and calcitonin-gene-related peptide [30–32]. Furthermore, they are transported to the peripheral nerve terminals [33,34] and are coupled to Gi/o proteins that inhibit adenylyl cyclase and modulate ion channels [35]. The decrease of Ca $^{2+}$ currents, but not the

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modulation of K⁺ channels, appears to be a major mechanism for the inhibition of sensory neuron functions [36]. Thus, it is plausible to hypothesize that AJM could induce an increased synthesis of opioid receptors DRG neurons, leading to an enhanced analgesic efficacy of peripherally active opioids after injury and inflammation of peripheral tissues.

Systemic Administration of Fucoidin Does Not Affect Antihypersensitivity Induced by AJM

It is accepted that inflammatory mediators released from leukocytes contribute to the generation of pain [37]. However, it is less known that immune cells also produce mediators that can effectively counteract pain [37]. Pain can be effectively controlled by various endogenous mechanisms. In peripheral inflamed tissue, an interaction between immune cell-derived opioids and opioid receptors localized on sensory nerve terminals can result in strong, clinically measurable analgesia [38–40].

Opioid-containing immune cells migrate preferentially to inflamed sites, where they release β -endorphin that activates peripheral opioid receptors to inhibit pain [39]. The major source of local endogenous opioid ligands (β -endorphin, enkephalins, endomorphins, and dynorphin) are leukocytes [41]. Fucoidin (a polymer of L-fucose and L-fucose-4-sulphate) belongs to a group of sugar analogs that are therapeutically interesting because of their relative lack of antigenicity and ease of handling [42]. Fucoidin potently binds to selectins and blocks leukocyte “rolling,” leading to a reduction of leukocyte accumulation at inflammatory sites [39].

It has been demonstrated that pretreatment of rats with a selectin blocker (fucoidin) decreases the number of β -endorphin-containing immunocytes infiltrating the inflamed tissue [9]. The fact that fucoidin did not significantly change antihypersensitivity caused by AJM suggests that AJM effect does not rely upon recruitment of opioid-containing leukocytes into the injured tissue. Thus, antihypersensitivity induced by AJM is apparently not limited by the number of opioid-containing leukocytes but by opioid receptors availability in sensory neurons.

Undoubtedly, studies on the possible biochemical mechanisms involved in AJM-induced antihypersensitivity are needed. Further research is required to provide a detailed understanding of this mechanism.

Study Limitation

Our findings represent data from a small number of animals, and therefore any generalization to acute human pain response is speculative.

Clinical Implications and Conclusions

Physiotherapists often employ lower limb joint mobilization to reduce pain and increase function. However, there are few experimental data confirming its efficacy [5,6].

However, there is evidence showing that these procedures may have beneficial effects on local tissues and that they can suppress pain by activating neurophysiologic mechanisms at peripheral, spinal, or supraspinal levels [8,20]. In summary, the present results demonstrated that joint mobilization reduces hypersensitivity induced by PI surgery in mice. Moreover, the systemic and local administrations of naloxone, but not the systemic pretreatment with an inhibitor of leukocyte rolling fucoidin, were able to prevent the antihypersensitivity elicited by AJM. The recruitment of opioid-containing leukocytes into injured tissues does not appear to be the mechanism underlying the peripheral opioid analgesic effects induced by AJM.

These data, taken together, allow us to hypothesize that joint mobilization produces an opioid form of analgesia, mediated by local peripheral opioid receptors. A better understanding of the peripheral mechanisms of AJM could stimulate therapists to integrate joint mobilization with strategies also known to influence endogenous pain control such as exercise, acupuncture, and transcutaneous electrical nerve stimulation, to maximize the endogenous analgesic effect produced.

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3.2 Mobilização Articular do Tornozelo Afeta Dor Pós-operatória Através dos Receptores Centrais e Periféricos para Adenosina A₁

Artigo 2

ANKLE JOINT MOBILIZATION AFFECTS POSTOPERATIVE PAIN THROUGH PERIPHERAL AND CENTRAL ADENOSINE A₁ RECEPTORS

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Ankle Joint Mobilization Affects Postoperative Pain Through Peripheral and Central Adenosine A₁ Receptors

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Background. Physical therapists frequently use joint mobilization therapy techniques to treat people with musculoskeletal dysfunction and pain. Several studies suggest that endogenous adenosine may act in an analgesic fashion in various pain states.

Objective. The purpose of this study was to investigate the contribution of the adenosinergic system on the antihyperalgesic effect of ankle joint mobilization (AJM).

Design. This was an experimental study.

Methods. To test this hypothesis, mice (25–35 g) submitted to plantar incision surgery were used as a model of acute postoperative pain. The mice were subjected to AJM for 9 minutes. Withdrawal frequency to mechanical stimuli was assessed 24 hours after plantar incision surgery and 30 minutes after AJM, adenosine, clonidine, or morphine treatments. The adenosinergic system was assessed by systemic (intrapertoneal), central (intrathecal), and peripheral (intraplantar) administration of caffeine. The participation of the A₁ receptor was investigated using a selective adenosine A₁ receptor subtype antagonist. In addition, previous data on the involvement of the serotonergic and noradrenergic systems in the antihyperalgesic effect of AJM were confirmed.

Results. Ankle joint mobilization decreased mechanical hyperalgesia, and this effect was reversed by pretreatment of the animals with caffeine given by intraperitoneal, intraplantar, and intrathecal routes. In addition, intraplantar and intrathecal administrations of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, a selective adenosine A₁ subtype receptor antagonist) or systemic administration of yohimbine or *p*-chlorophenylalanine methyl ester hydrochloride (PCPA) blocked the antihyperalgesia induced by AJM.

Limitations. The results are limited to animal models and cannot be generalized to acute pain in humans.

Conclusions. This study demonstrated the involvement of the adenosinergic system in the antihyperalgesic effect of AJM in a rodent model of pain and provides a possible mechanism basis for AJM-induced relief of acute pain.

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Ankle Joint Mobilization Affects Postoperative Pain

Physical therapists frequently use manual physical therapy techniques to treat people with musculoskeletal dysfunction and pain. Evidence for the efficacy of lower-limb mobilization is particularly scarce, as the majority of the studies of peripheral joints have used an upper-limb model.^{1,2} Although the scientific literature has begun to characterize the effects of spinal manual physical therapy,^{3,4} there are only a few studies that have investigated the hypoalgesic effects of peripheral joint mobilization techniques. There is consequently an urgent need for further lower-limb ankle joint mobilization (AJM) studies. In the last decade, clinical and experimental studies on joint mobilization therapy have indicated the possible neurophysiological effects for analgesia induced by mobilization.⁵⁻⁷

A synopsis of current evidence for the initial mechanism of action of joint mobilization therapy indicates, in part, a neurophysiological basis.⁸ A clinical study on patients with osteoarthritis showed that knee joint mobilization induces an increased threshold for mechanical pressure, both locally and distant from the treated joint.⁷ Sluka and Wright⁹ reported that knee joint mobilization reversed mechanical hyperalgesia induced by intra-articular injection of capsaicin in the ankle joint. Martins et al¹⁰ have shown that AJM suppressed pain behavior caused by a neuropathic pain model and attenuated astroglial activation in the spinal cord of rats. In addition, Martins et al¹¹ recently demonstrated that 9 minutes of AJM, administered once a day over 5 days, reduced postoperative pain; this effect was prevented by systemic and local administrations of naloxone, but not by fucoidin.

A recent animal study showed that the analgesia produced by joint

mobilization involves serotonin and norepinephrine receptors in the spinal cord, thereby supporting a role for descending pain modulatory systems.⁵ The descending pain inhibitory pathway in rodents and humans is activated by the perception of pain in the brain. Endorphins, endocannabinoids, serotonin, norepinephrine, and adenosine play important roles in this pathway.¹² Adenosine regulates pain transmission in the spinal cord and in the periphery, and a number of agents can alter the extracellular availability of adenosine and subsequently modulate pain transmission, particularly by activation of adenosine A₁ receptors (A₁Rs).^{13,14} Of note, adenine nucleotides are frequently released following tissue stimulation and have pain-related functions in animals.¹⁴

Goldman and coauthors¹⁵ uncovered a role for peripheral adenosine receptor activation in the antinociceptive effects of acupuncture. It has been hypothesized that nonallopathic treatments of pain (eg, chiropractic manipulations, massage), modalities that involve the mechanical manipulation of joints and muscles, also might be associated with an efflux of cytosolic ATP that is sufficient to elevate extracellular adenosine.¹⁵

Based on this evidence, we asked whether AJM could increase endogenous adenosine levels and produce antihyperalgesia through signaling via A₁Rs. The present study investigated the relative contributions of central (spinal) and peripheral (paw) A₁Rs subtype on the antihyperalgesic effect of AJM. In addition, we confirmed and extended previous data from the literature by demonstrating that AJM activates the descending inhibitory pathways (serotonin and noradrenaline) in a model of postoperative pain in mice.

Materials and Method

Animals

All experiments were conducted using male Swiss mice (25–35 g), housed at 22±2°C, under a 12 hours light/12 hours dark cycle (lights on at 6:00 AM) and with free access to food and water. Animals were acclimated to the laboratory for at least 1 hour before testing and were used only once throughout the experiments. The experiments were performed after approval of the protocol (PP00622) by the Institutional Ethics Committee (blinded) and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals.

Postoperative Pain Model

The postoperative pain model was carried out according to the procedure described for rats¹⁶ and adapted for mice.^{17,11} Mice were anesthetized with 2% isoflurane via a nose cone. After antiseptic preparation of the right hind paw with 10% povidone-iodine solution, a 5-mm longitudinal incision was made with a number 11 blade through the skin and fascia of the plantar side of the foot. The incision was started 2 mm from the proximal edge of the heel and extended toward the toes. The underlying muscle was elevated with a curved forceps, leaving the muscle origin and insertion intact. The skin was apposed with a single mattress suture of 6.0 nylon.

Intrathecal Injections

Intrathecal injections were given to fully conscious mice using the method previously described by Hylden and Wilcox.¹⁸ Briefly, the animals were manually restrained, the dorsal fur of each mouse was shaved, the spinal column was arched, and a 30-gauge needle was inserted into the subarachnoid space between the L4 and L5 vertebrae. Correct intra-

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theccal positioning of the needle tip was confirmed by a characteristic tail-flick response in the animal. A 5- μ L volume containing the test agent was slowly injected with a 25- μ L Hamilton microsyringe (Hamilton, Birmingham, United Kingdom). In addition, a constant volume of 5 μ L of saline or vehicle (saline + 5% dimethyl sulfoxide [DMSO]) was injected simultaneously. Intrathecal injections were given over a period of 5 seconds.¹⁸

AJM Treatment

The AJM treatment was carried out according to the procedure described for humans and adapted for mice.¹¹ The knee joint was stabilized, and the ankle joint was rhythmically flexed and extended to the end of the range of movement, as previously described.¹¹ The mice were anesthetized with 1% to 2% isoflurane prior to and for the duration of the joint mobilization procedure via a nose cone. The treatment group received 3 applications of mobilization, each of 3 minutes' duration, and were separated by 30 seconds of rest, described here as 9 minutes of AJM. Recently, our group has demonstrated that this time frame is optimal for producing antihyperalgesia in this model.¹¹

Placebo AJM

Mice were lightly anesthetized with 2% isoflurane, and the ankle was maintained in a neutral position using the same hand contact and positioning as in the actual treatment technique.¹¹

Behavioral Measurement: Mechanical Hyperalgesia

Animals were tested for withdrawal thresholds to mechanical stimuli (von Frey filaments) applied to the plantar aspect of the hind paw.^{19,20} The mice were acclimated in individual clear boxes (9 \times 7 \times 11 cm³) on an elevated wire mesh platform to allow access to the ventral surface of

the hind paws. The right hind paw was stimulated with a constant pressure of a 0.4-g von Frey filament (Stoelting, Chicago, Illinois). The withdrawal frequency was measured as the number of times (out of 10) that the animal withdrew the paw after the application of the 0.4-g filament. The results were expressed as the percentage of withdrawal frequency.²⁰

In all experiments below, the animals were submitted to plantar incision surgery (operated), and the tests were performed before (baseline, data not shown), 30 minutes after placebo AJM or 9 minutes of AJM, agonists or antagonists + 9 minutes of AJM treatments (see experiments 1, 2, and 3). The withdrawal response before surgery procedure was 20% to 30%, as observed in our previous study.¹¹ The plantar incision procedure induced hyperalgesia, as noted by the high percentage (80%-100%) of withdrawal responses, 24 hours²¹ after surgery.

Experiment 1: Involvement of the Adenosinergic Receptors in the Antihyperalgesic Effect of AJM

Systemic administration of caffeine. The role of the adenosinergic receptors on the antihyperalgesic action of AJM was investigated by treating the mice with saline (10 mL/kg, intraperitoneal) or caffeine (a nonselective adenosine receptors antagonist, 10 mg/kg, intraperitoneal)²² 20 minutes before placebo AJM or 9 minutes of AJM. Mechanical hyperalgesia was evaluated 30 minutes after placebo AJM or 9 minutes of AJM. Placebo AJM animals were subjected to 9 minutes of anesthesia and were assessed over the same time intervals. For this experiment, the following groups (n=8) were used: (1) operated (control) + saline + placebo AJM, (2) operated + caffeine + placebo AJM, (3) operated + saline + 9 minutes of

AJM, and (4) operated + caffeine + 9 minutes of AJM.

In the positive control experiment, mice were pretreated with an intraperitoneal injection of saline (10 mL/kg) or caffeine (10 mg/kg), and after 20 minutes they received an intraperitoneal injection of saline (10 mL/kg) or adenosine (30 mg/kg, a nonselective adenosine receptor agonist).¹⁴ For this experiment, the following groups (n=8) were used: (1) operated + saline + saline, (2) operated + caffeine + saline, (3) operated + saline + adenosine, and (4) operated + caffeine + adenosine.

Peripheral (paw) administration of caffeine. In an attempt to investigate the involvement of peripheral adenosine receptors on the antihyperalgesia induced by 9 minutes of AJM, the animals received an intraplantar injection of saline (20 μ L) or caffeine (150 nmoL)²² in the right hind paw. After 15 minutes, they received placebo AJM or 9 minutes of AJM. Mechanical hyperalgesia was evaluated 30 minutes after placebo AJM or 9 minutes of AJM. In this experiment, the following groups (n=8) were used: (1) operated + saline + placebo AJM, (2) operated + caffeine + placebo AJM, (3) operated + saline + 9 minutes of AJM, and (4) operated + caffeine + 9 minutes of AJM.

In the positive control experiment, mice were pretreated with an intraplantar injection of saline (20 μ L/paw) or caffeine (150 nmol/paw). After 15 minutes, they received an intraperitoneal injection of saline (10 mL/kg) or adenosine (30 mg/kg). In this experiment, the following groups (n=8) were used: (1) operated + saline + saline, (2) operated + caffeine + saline, (3) operated + saline + adenosine, and (4) operated + caffeine + adenosine.

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Central (spinal) administration of caffeine. In another set of experiments, to evaluate the involvement of spinal adenosine receptors on the antihyperalgesia induced by 9 minutes of AJM, the animals received an intrathecal injection of saline (5 $\mu\text{L}/\text{site}$) or caffeine (150 nmol/site). After 15 minutes, the animals received placebo AJM or 9 minutes of AJM. Mechanical hyperalgesia was evaluated 30 minutes after placebo AJM or 9 minutes of AJM. In this experiment, the following groups (n=8) were used: (1) operated + saline + placebo AJM, (2) operated + caffeine + placebo AJM, (3) operated + saline + 9 minutes of AJM, and (4) operated + caffeine + 9 minutes of AJM.

In the positive control experiment, mice were pretreated with an intrathecal injection of saline (20 $\mu\text{L}/\text{site}$) or caffeine (150 nmol/site). After 15 minutes, the animals received an intraperitoneal injection of saline (10 mL/kg) or adenosine (30 mg/kg). In this experiment, the following groups (n=8) were used: (1) operated + saline + saline, (2) operated + caffeine + saline, (3) operated + saline + adenosine, and (4) operated + caffeine + adenosine.

Experiment 2: Involvement of Adenosine A₁ Subtype Receptor in the Antihyperalgesic Effect of AJM

Peripheral (paw) administration of 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX). We investigated the involvement of peripheral A₁Rs on the antihyperalgesia induced by 9 minutes of AJM. The animals received an intraplantar injection of vehicle (saline solution with 5% DMSO) or DPCPX (10 nmol, a selective adenosine A₁ receptor antagonist)²⁵ in the right hind paw. After 15 minutes, the animals received placebo AJM or 9 minutes of AJM. Mechanical hyperalgesia was evaluated 30 minutes after placebo

AJM or 9 minutes of AJM. In this experiment, the following groups (n=8) were used: (1) operated + vehicle + placebo AJM, (2) operated + DPCPX + placebo AJM, (3) operated + vehicle + 9 minutes of AJM, and (4) operated + DPCPX + 9 minutes of AJM.

In the positive control experiment, mice were pretreated with an intraplantar injection of vehicle (20 $\mu\text{L}/\text{paw}$) or DPCPX (10 nmol/paw). After 15 minutes, the animals received an intraperitoneal injection of vehicle (10 mL/kg) or adenosine (30 mg/kg). In this experiment, the following groups (n=8) were used: (1) operated + vehicle + saline, (2) operated + DPCPX + saline, (3) operated + vehicle + adenosine, and (4) operated + DPCPX + adenosine.

Central (spinal) administration of DPCPX. To determine the involvement of spinal A₁Rs on the antihyperalgesia induced by AJM, the animals received an intrathecal injection of vehicle (5 $\mu\text{L}/\text{site}$) or DPCPX (10 nmol/site).²⁴ After 15 minutes, the animals received placebo AJM or 9 minutes of AJM. Mechanical hyperalgesia was evaluated 30 minutes after placebo AJM or 9 minutes of AJM. In this experiment, the following groups (n=8) were used: (1) operated + vehicle + placebo AJM, (2) operated + DPCPX + placebo AJM, (3) operated + vehicle + 9 minutes of AJM, and (4) operated + DPCPX + 9 minutes of AJM.

In the positive control experiment, mice were pretreated with an intrathecal injection of vehicle (20 $\mu\text{L}/\text{site}$) or DPCPX (10 nmol/site). After 15 minutes, the animals received an intraperitoneal injection of saline (10 mL/kg) or adenosine (30 mg/kg). In this experiment, the following groups (n=8) were used: (1) operated + vehicle + saline, (2)

operated + DPCPX + saline, (3) operated + vehicle + adenosine, and (4) operated + DPCPX + adenosine.

Experiment 3: Involvement of Descending Monoaminergic Systems in the Antihyperalgesic Effect of AJM

Some studies indicate that the antinociception induced by adenosine depends on the spinal cord noradrenergic system.²⁵⁻²⁹ Thus, to confirm and extend previous data from the literature, we verified whether 9 minutes of AJM activates the descending inhibition pathways (serotonin and noradrenaline)⁵ to produce analgesia in the postoperative pain model. For this purpose, mice were pretreated with yohimbine (an α -2-adrenoceptor antagonist) or *p*-chlorophenylalanine methyl ester (PCPA, an inhibitor of serotonin synthesis), as described below.

Involvement of the noradrenergic pathways: systemic administration of yohimbine. To assess the possible contribution of the activation of α -2-adrenergic receptors, the mice received an intraperitoneal injection of saline (10 mL/kg) or yohimbine (0.15 mg/kg).²⁶ After 20 minutes, the animals received placebo AJM or 9 minutes of AJM. Mechanical hyperalgesia was evaluated 30 minutes after placebo AJM or 9 minutes of AJM. In this experiment, the following groups (n=8) were used: (1) operated + saline + placebo AJM, (2) operated + yohimbine + placebo AJM, (3) operated + saline + 9 minutes of AJM, and (4) operated + yohimbine + 9 minutes of AJM.

In the positive control experiment, mice were pretreated with an intraperitoneal injection of saline (10 mL/kg) or yohimbine (0.15 mg/kg). After 20 minutes, they received an intraperitoneal injection of saline (10

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mL/kg) or clonidine (0.1 mg/kg). In this experiment, the following groups (n=8) were used: (1) operated + saline + saline, (2) operated + yohimbine + saline, (3) operated + saline + clonidine, and (4) operated + yohimbine + clonidine.

Involvement of the serotonergic pathways: systemic administration of PCPA. To assess the possible contribution of endogenous serotonin on the antihyperalgesic effect of 9 minutes of AJM, the animals were pretreated with an intraperitoneal injection of saline (10 mL/kg) or PCPA (100 mg/kg, an inhibitor of serotonin synthesis) once a day, for 4 consecutive days, 3 days before and 24 hours after plantar incision surgery.²⁶ Twenty minutes after the last administration, placebo AJM or 9 minutes of AJM were performed, and after 30 minutes, mechanical hyperalgesia was assessed. In this experiment, the following groups (n=8) were used: (1) operated + saline + placebo AJM, (2) operated + PCPA + placebo AJM, (3) operated + saline + 9 minutes of AJM, and (4) operated + PCPA + 9 minutes of AJM.

In the positive control experiment, mice were pretreated with an intraperitoneal injection of saline (10 mL/kg) or PCPA (100 mg/kg). After 20 minutes, they received saline (10 mL/kg, s.c.) or morphine (5 mg/kg, s.c.). In this experiment, the following groups (n=8) were used: (1) operated + saline + saline, (2) operated + PCPA + saline, (3) operated + saline + morphine, and (4) operated + PCPA + morphine.²⁶

Drugs

The following substances were used: DMSO (solvent used to dissolve DPCPX, experiment 2), morphine hydrochloride (a nonselective opioid receptor agonist, experiment 3) (Merck, Darmstadt, Germany), caffeine (a nonselective adenosine

receptors antagonist, experiment 1), clonidine hydrochloride (a selective α -2-adrenergic receptors agonist, experiment 3), yohimbine hydrochloride (a selective α -2-adrenergic receptors antagonist, experiment 3), PCPA (experiment 3) (Sigma Chemical Co, St Louis, Missouri), adenosine (an agonist with higher affinity for adenosine A_1 and A_{2A} adenosine receptors, experiments 1 and 2), and DPCPX (experiment 2) (Tocris Bioscience, Ellisville, Missouri). The DPCPX was dissolved in saline with 5% DMSO. The final concentration of DMSO did not exceed 5% and did not cause any effect. Other substances were dissolved in saline. When drugs were delivered by intraperitoneal route, a constant volume of 10 mL/kg body weight was injected. When drugs were administered by intrathecal or intraplantar routes, a volume of 5 μ L or 20 μ L was injected, respectively. Appropriate control-treated groups also were assessed simultaneously. The doses of all substances used were chosen based on data in the literature^{14,22-24,26} or were selected from preliminary experiments conducted in our laboratory.

Data Analysis

Behavioral tests were analyzed using one-way analysis of variance following the Student Newman-Keuls test. Results were presented as the mean \pm SEM for each group. $P < .05$ was considered significant.

Role of the Funding Source

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Results

The Adenosinergic System Mediates the Antihyperalgesic Effect of AJM

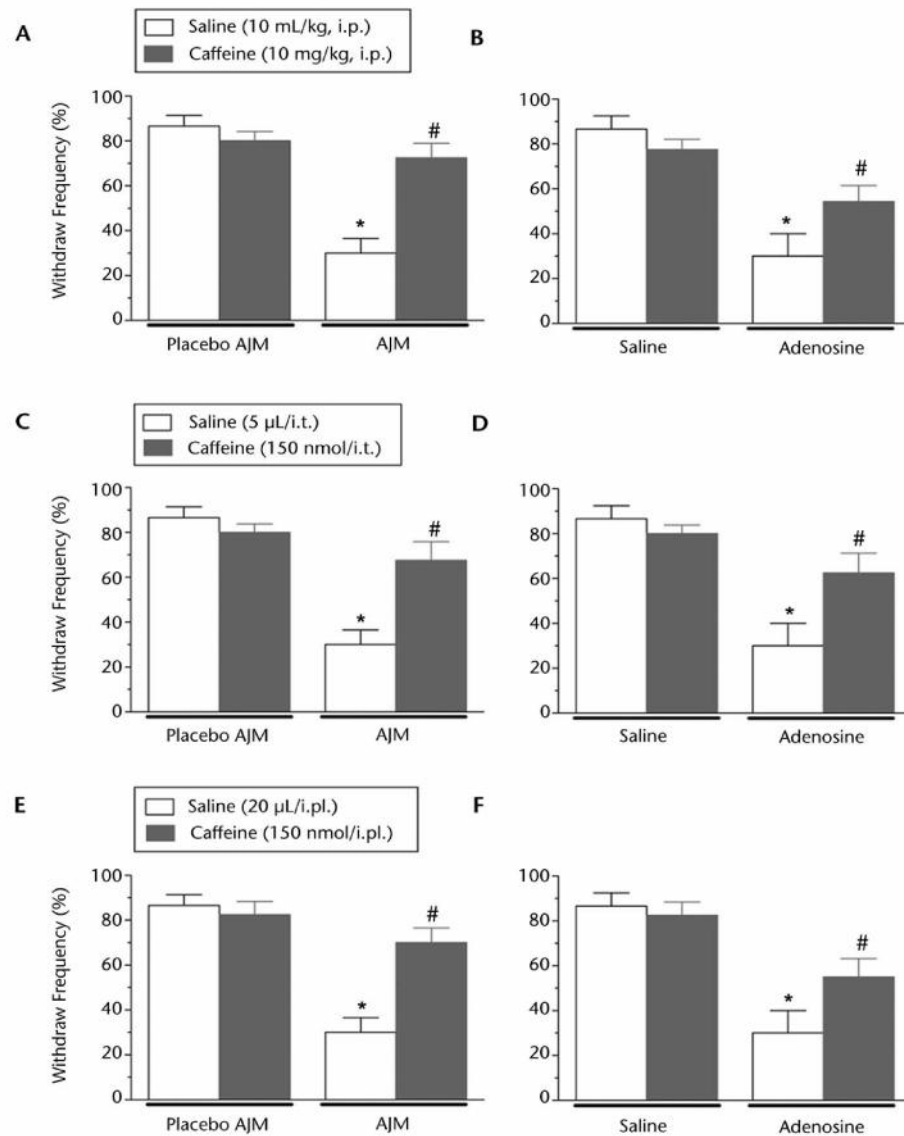
The fact that adenosine nucleotides are frequently released following tissue stimulation³⁰ and have pain-related functions in animals, and that Goldman and coauthors¹⁵ discovered that peripheral adenosine receptor activation mediates the antinociceptive effects of acupuncture, raises the question of whether the antihyperalgesic effect of AJM can be mediated via activation of adenosine receptors. To our knowledge, the adenosinergic system has not previously been implicated in mobilization-induced antihyperalgesia. Caffeine administration without 9 minutes of AJM had no effect on the withdrawal frequency, and the thresholds were similar to those of the intraperitoneal, intrathecal, and intraplantar injections of saline without 9 minutes of AJM groups (Figs. 1A, 1C, and 1E) and operated + saline + saline groups (Figs. 1B, 1D, and 1F). The pretreatment intraperitoneal, intrathecal, and intraplantar of mice with caffeine prevented the decrease in withdrawal frequency resulting from the 9 minutes of AJM ($P < .05$) or adenosine treatment ($P < .05$) (Figs. 1A, 1C and 1E). Thus, these results suggest that central and peripheral activation of A_1 Rs contributes to the antihyperalgesic effect of 9-minute AJM in the postoperative pain model.

Peripheral and Central A_1 Rs Mediate the Antihyperalgesic Effect of AJM

We determined the role of A_1 Rs on the antihyperalgesic effect of 9 minutes of AJM in the postoperative pain model. We administered the selective A_1 Rs antagonist DPCPX in 2 different sites: intrathecal and intraplantar (Figs. 2A-D).

The pretreatment with DPCPX had no effect on withdrawal frequency,

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**Figure 1.**

Intraperitoneal (panels A and B), intrathecal (panels C and D), and intraplantar (panels E and F) pretreatment with caffeine and the antihyperalgesic effect of 9 minutes of ankle joint mobilization (AJM) or adenosine (30 mg/kg, i.p.) in mice. White bars show the operated (control) + saline (10 mL/kg, i.p.) group or the antihyperalgesic effect of 9 minutes of AJM or adenosine (30 mg/kg, i.p.) treatment. Gray bars show the effects of caffeine (10 mg/kg, i.p.; 150 nmol/site, i.t.; or 150 nmol/paw, i.pl.) antagonist injected before 9 minutes of AJM or adenosine (30 mg/kg, i.p.) treatment. Each point represents the mean of 8 animals, and vertical lines show the standard error of the mean. *Significant difference ($P < .05$) compared with operated + saline + placebo AJM groups (panels A, C, and E) or operated + saline + saline groups (panels B, D, and F). #Significant difference ($P < .05$) compared with operated + saline + 9 minutes of AJM groups (panels A, C, and E) or operated + saline + adenosine groups (panels B, D, and F). Abbreviations: i.p.=intraperitoneal, i.t.=intrathecal, i.pl.=intraplantar.

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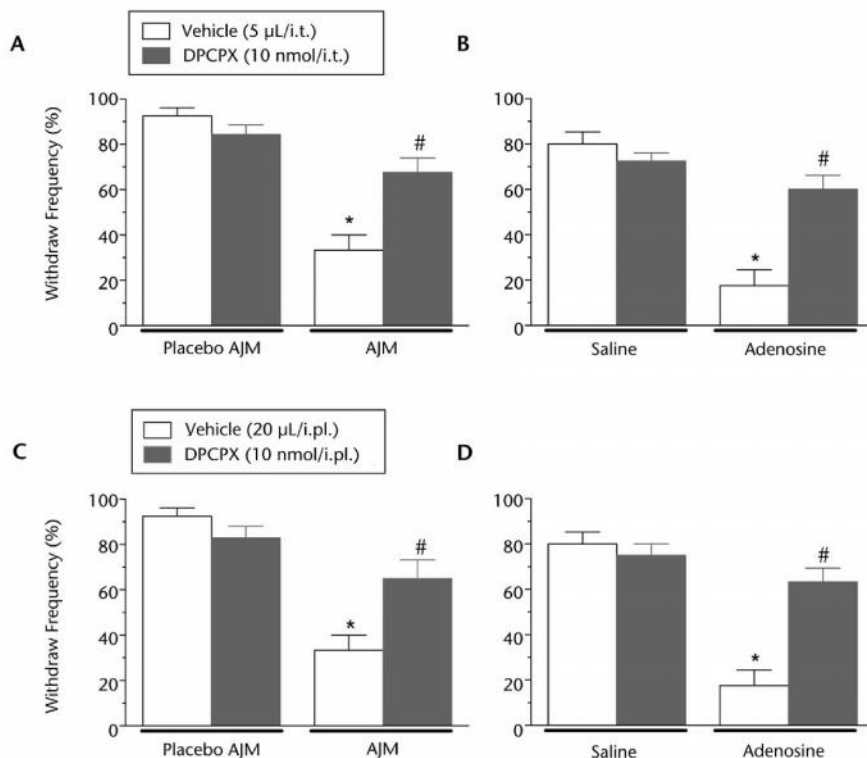


Figure 2.

Intrathecal (panels A and B) and intraplantar (panels C and D) pretreatment with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and the antihyperalgesic effect of 9 minutes of ankle joint mobilization (AJM) or adenosine (30 mg/kg, i.p.) in mice. White bars show the operated (control) + saline (10 mL/kg, i.p.) group or antihyperalgesic effect of 9 minutes of AJM or adenosine (30 mg/kg, i.p.). Gray bars show the effects of DPCPX (150 nmol/site, i.t.; 150 nmol/paw, i.pl.) antagonist injected before 9 minutes of AJM or adenosine (30 mg/kg, i.p.) treatment. Each point represents the mean of 8 animals, and vertical lines show the standard error of the mean. *Significant difference ($P < .05$) compared with operated + vehicle + placebo AJM groups (panels A and C) or operated + vehicle + saline (panels B and D) groups. #Significant difference ($P < .05$) compared with operated + vehicle + 9 minutes of AJM groups (panels A and C) or operated + vehicle + adenosine groups (panels B and D). Abbreviations: i.t.=intrathecal, i.pl.=intraplantar.

and the thresholds were similar to those of intrathecal and intraplantar injections of vehicle without AJM for 9 minutes (Figs. 2A and 2C). In addition, the withdrawal frequency values for the groups pretreated with DPCPX via intrathecal and intraplantar routes were significantly higher compared with those of the vehicle + 9 minutes of AJM group 30 minutes after treatment (Figs. 2A and 2C). Spinal and plantar administration of DPCPX, 15 minutes before AJM for 9 minutes, prevented the

decrease in the withdrawal frequency resulting from the treatment ($P < .05$) (Figs. 2A and 2C). These findings suggest that central and peripheral activation of A_1 Rs contributes to the antihyperalgesic effect of 9 minutes of AJM in the postoperative pain model.

Although the doses of caffeine and DPCPX were chosen based on previously published studies,^{24,25} we tested them against adenosine (an agonist with higher affinity for A_1

and A_{2A} receptors)¹⁴ in this model to confirm that they were adequate to block systemic, spinal, and peripheral adenosine receptors, respectively (Figs. 1B, 1D, and 1F). Administration of adenosine decreased withdrawal frequency 30 minutes after treatment (Figs. 1B, 1D, and 1F). In addition, pretreatment in different sites with caffeine (intraplantar, intrathecal, or intraplantar) or with DPCPX (intrathecal or intraplantar) completely blocked the

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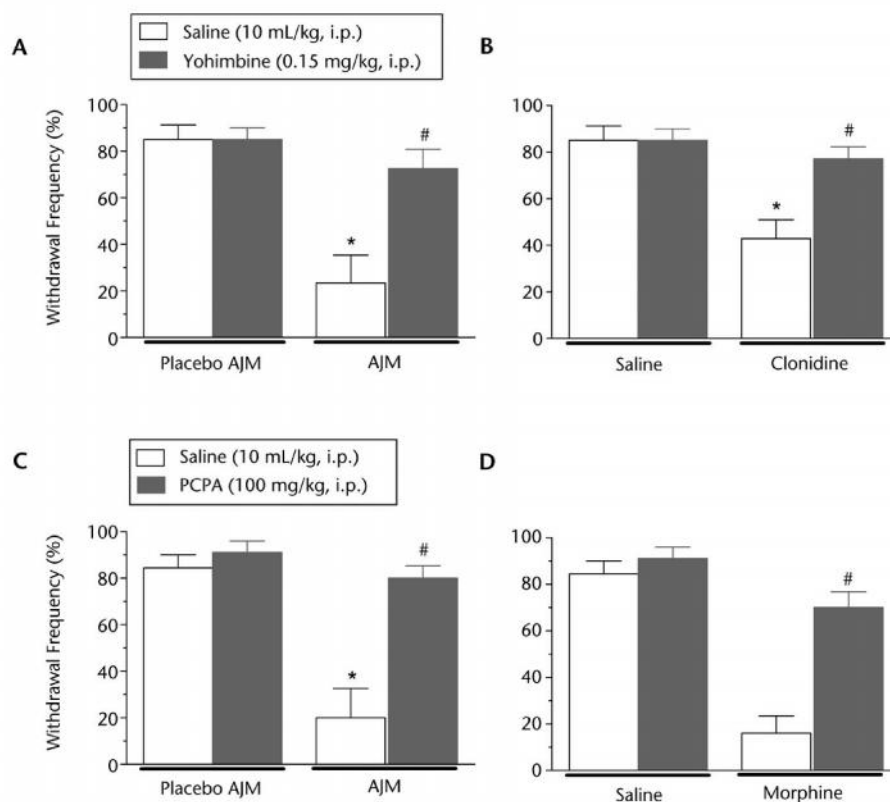


Figure 3.

Intraperitoneal pretreatment with yohimbine (0.15 mg/kg, i.p., panels A and B) or *p*-chlorophenylalanine methyl ester hydrochloride (PCPA) (100 mg/kg, i.p., panels C and D) and the antihyperalgesic effect of 9 minutes of ankle joint mobilization (AJM) (panels A and B, respectively), clonidine (0.1 mg/kg, i.p., panel B), or morphine (5 mg/kg, i.p., panel D) in mice. White bars show the operated (control) + saline (10 mL/kg, i.p.) group or the antihyperalgesic effect of 9 minutes of AJM (panels A-C), clonidine (0.1 mg/kg, i.p., panel B), or morphine (5 mg/kg, s.c., panel D). Gray bars show the effects of yohimbine (panels A and B) or PCPA (panels C and D) antagonist injected before 9 minutes of AJM, clonidine, or morphine (5 mg/kg, s.c.) treatments. Each point represents the mean of 8 animals, and vertical lines show the standard error of the mean. *Significant difference ($P < .05$) compared with operated + saline + placebo AJM group (panels A and C) or operated + saline + saline groups (panels C and D). #Significant difference ($P < .05$) compared with operated + saline + 9 minutes of AJM groups (panels A and C), operated + saline + clonidine groups (panel B), or operated + saline + morphine groups (panel D). Abbreviations: i.p.=intraperitoneal, s.c.=s.c.

effect of adenosine (Figs. 1B, 1D, 1F, 2B, and 2D, respectively).

The Descending Monoaminergic System Mediates the Antihyperalgesic Effect of AJM

Here we investigated whether the descending monoaminergic system was activated by 9 minutes of AJM. The pretreatment with yohimbine or PCPA without AJM had no effect on the withdrawal frequency, and the

thresholds were similar to those of the saline without AJM group (Fig. 3A and 3C). The withdrawal frequency values for the group pretreated with yohimbine or PCPA were significantly higher compared with the vehicle + 9 minutes of AJM group 30 minutes after treatment (Figs. 3A and 3C). The intraperitoneal administrations of yohimbine or PCPA 20 minutes before of 9 minutes of AJM prevented the decreased

in withdrawal frequency resulting from the treatment ($P < .05$, Figs. 3B and 3D). The results presented in Figures 3B and 3D showed that the pretreatment of animals with yohimbine or PCPA completely reversed the antihyperalgesia caused by clonidine ($P < .05$, Fig. 3B) or morphine ($P < .05$, Fig. 3D), used as a positive control. Thus, we confirmed an largely extended previous data from the literature⁵ by demonstrating that

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9 minutes of AJM activates serotonin and noradrenergic descending inhibitory pathways to produce analgesia.

Discussion

This study represents the first direct demonstration of the role of the adenosinergic system in supporting the antihyperalgesic effect of AJM. Our findings indicate that the activation of adenosine receptors is central to the mechanistic actions of AJM. Herein, we have reported that the antihyperalgesic effect of AJM is directly related to its ability to activate central and peripheral adenosine A_1 Rs.

Clinical Significance of the Postoperative Pain Model

Acute postoperative pain remains a significant medical problem. For those patients undergoing major surgical procedures, ongoing pain or pain at rest and pain during activities are important clinical symptoms. Pain at rest is usually moderate during the first 2 to 3 days after surgery.⁵¹ These pain scores occur even when parenteral treatments are administered. Usually, pain at rest resolves within the first week after surgery. Pain with activities, such as coughing or walking, is severe during the first 2 to 3 days. Pain with activities is moderate or severe for many days and even weeks later. Functional capability is limited during this period as well. Thus, pain can be moderate, and the patient's ability to cough or the walking distance to evoke this pain is reduced.^{52,53}

Several studies have indicated that effective postoperative analgesia reduces morbidity following surgery, thereby improving patient outcome and reducing clinical expenses. Adequate knowledge regarding treatment of postoperative pain is important to reduce the morbidity and mortality of patients after surgery.^{54,55} However, recent surveys

have demonstrated that about 50% to 70% of patients experience moderate to severe pain after surgery, indicating that despite the development of new therapies and improved analgesic techniques, postoperative pain remains undervalued and poorly treated.⁵⁵ There is now plenty of evidence that postoperative pain is based on distinct pathophysiologic and pharmacologic mechanisms compared with other pain models.^{56,57} Therefore, it is extremely important to investigate new treatments that are efficient to reduce postoperative pain.

Analgesic Effect Induced by Activation of A_1 Rs

Prior studies characterize adenosine as an important modulator of the nociceptive processes. It can act as either a pronociceptive or an antinociceptive agent, depending on the level of the nociceptive system (central or peripheral) and on the receptor subtype activated. There is a great body of evidence indicating that the activation of A_1 Rs produces antinociception in models of postoperative,³⁸ neuropathic,³⁹⁻⁴¹ and inflammatory pain.^{42,43} In addition, mice lacking A_1 Rs exhibited increased nociceptive responses and failed to show the antinociceptive effects of an A_1 R agonist, confirming the importance of A_1 Rs in antinociception.^{44,45} Recently, it has been demonstrated that spinal A_1 Rs but not A_{2A} receptors play an important role in the maintenance of non-evoked and evoked pain behaviors after an incision. In addition, A_1 R-induced spinal antinociception is mediated by interactions with pertussis toxin-sensitive G proteins.³⁸

Antihyperalgesia Induced by AJM: Interaction Between the Descending Monoaminergic System and A_1 Rs

The effects of the descending monoaminergic system on nociceptive processing in the dorsal horn are

complex.⁴⁶ Serotonin, norepinephrine, and dopamine may exert either antinociceptive or pronociceptive effects according to the type of receptor involved, site of action in the dorsal horn, and cross talk between descending and local neurochemical signals, including adenosine, endogenous opioids, and nitric oxide.^{47,48} Recent studies indicate a dependence of antinociception mediated by adenosine on the spinal cord noradrenergic system. For example, the antinociceptive effect of adenosine was blocked by coadministration of the α -2-receptor antagonist idazoxan.²⁶ Adenosine receptor agonists^{22,27} enhanced α -2-adrenergic antinociception. Finally, the destruction of spinal noradrenergic terminals with neurotoxins abolishes the antihyperalgesia effects of adenosine and adenosine modulators.²⁹

Skyba and coauthors⁵ demonstrated that descending pain inhibitory systems could be activated by mobilization, being this analgesia produced by activation of serotonin and adrenergic receptors in the spinal cord. Here, we largely extend previous data from the literature demonstrating in a postoperative pain model in mice that 9 minutes of AJM activates descending inhibitory pathways using serotonin and norepinephrine to produce analgesia. Another relevant finding of our study is that central (spinal) administration of caffeine or DPCPX before 9 minutes of AJM prevented the decrease in the withdrawal frequency resulting from the treatment. Our results suggest that central activation of adenosine A_1 Rs contributes to the antihyperalgesic effect of AJM on postoperative pain. These previous findings and the present results have led to the hypothesis that activation of A_1 Rs might be the first step by which AJM activates descending pain inhibitory systems (ie, the descending serotonergic and noradrenergic pathways

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that act upon spinal serotonin and adrenergic receptors).

Peripheral A₁Rs Mediate the Antihyperalgesic Effect of AJM

Adenine nucleotides are frequently released following tissue stimulation and have pain-related functions in animals.^{13,30} Recently, it has been demonstrated that adenosine mediates acupuncture analgesia via activation of A₁Rs.¹⁵ Vibratory stimulation applied to the skin depressed the activity of nociceptive neurons in the lower lumbar segments of cats by release of adenosine.⁴⁹ It has been hypothesized¹⁵ that other nonallopathic treatments of pain, such as chiropractic manipulations and massage modalities that involve the mechanical manipulation of joints and muscles, also might be associated with an efflux of cytosolic ATP that is sufficient to elevate extracellular adenosine.¹⁵ As in acupuncture, adenosine may accumulate during joint mobilization and dampen pain, in part, through the activation of A₁Rs on sensory afferents of ascending nerve tracks.¹⁵ Our results confirm this hypothesis, once peripheral (paw) administration of caffeine or DPCPX before 9 minutes of AJM prevented the decrease in the withdrawal frequency resulting from the treatment. These findings suggest that peripheral activation of A₁Rs contributes to the antihyperalgesic effect of AJM on postoperative pain.

Limitations

Further studies are necessary to more directly investigate the supraspinal involvement of the adenosinergic system on the antihyperalgesia induced by AJM in other pain models. Additionally, it will be relevant to assess whether other mobilization techniques that involve minimal joint movement will activate the adenosinergic system. The lack of biochemical analysis may be a limitation of this study and should be considered in future studies.

Clinical Significance

Clinical trials and meta-analyses provide increasing evidence to support the use of manual physical therapy in the management of painful spinal conditions.^{50–52} As evidence increases to support the use of manual physical therapy, it is becoming increasingly important to establish the mechanism of action of manual physical therapy techniques. The present study investigated the pharmacological properties of AJM because of the possibility that it represents the neural basis at the central and peripheral levels for the antihyperalgesic effect of AJM in humans. The implication of adenosine in the antihyperalgesia induced by AJM necessitates some new perspectives in evaluating the efficacy of AJM in clinical trials. Caffeine can block adenosine A₁Rs in modest doses and can inhibit antihyperalgesia induced by AJM.⁵⁰ It is important to note that caffeine (200 mg) has been reported to inhibit the efficacy of transcutaneous electrical nerve stimulation when given prior to the stimulation in a small experimental pain trial in humans.⁵³ Future studies should determine whether daily caffeine intake alters the effectiveness of AJM in animals and humans.

Conclusion

The results of this study demonstrated that AJM decreased injury-induced mechanical hyperalgesia, and this effect was prevented by the pretreatment with caffeine given via intraperitoneal, intrathecal, and intraplantar routes. We also showed that intrathecal and intraplantar administrations of DPCPX blocked the antihyperalgesic effect of AJM. To our knowledge, this is the first direct demonstration of the role of the adenosinergic system in mediating the antihyperalgesic effect of AJM. In addition, we confirmed and largely extended previous data by demonstrating that AJM activates the descending control of pain using

serotonin and norepinephrine to produce analgesia.

Mr Martins, Ms Mazzardo-Martins, Mr Cidral-Filho, and Dr Santos provided concept/idea/research design and writing. All authors provided data collection. Mr Martins and Ms Mazzardo-Martins provided data analysis. Dr Santos provided project management, fund procurement, and facilities/equipment.

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3.3 Ativação Periférica e Central dos Receptores para Canabinóides pela Mobilização Articular do Tornozelo Alivia Dor Pós-operatória em Camundongos

Artigo 3

PERIPHERAL AND SPINAL ACTIVATION OF CANNABINOID RECEPTORS BY JOINT MOBILIZATION ALLEVIATES POSTOPERATIVE PAIN IN MICE

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Abstract: The present study was undertaken to investigate the relative contribution of cannabinoid receptors (CBRs) subtypes and analyze cannabimimetic mechanisms involved on the inhibition of AEA and 2-AG degradation on the antihyperalgesic effect of ankle joint mobilization (AJM). Mice (25-35g) were submitted to plantar incision (PI) and 24 hours after surgery animals received the following treatments, AJM for 9 minutes, anandamide (10 mg/kg, intraperitoneal [i.p.]), WIN 55,212-2 (1.5 mg/kg, i.p.), URB937 (0.01-1 mg/kg, i.p.; a fatty acid amide hydrolase [FAAH] inhibitor) or JZL184 (0.016-16 mg/kg, i.p.; a monoacylglycerol lipase [MAGL] inhibitor). Withdrawal frequency to mechanical stimuli was assessed 24 hours after PI and at different times after treatments. Receptor specificity was investigated using selective CB1R (AM281) and CB2R (AM630) antagonists. In addition, the effect of the FAAH and MAGL inhibitors on the antihyperalgesic action of AJM was investigated. AJM, anandamide, WIN 55,212-2, URB937 and JZL184 decreased mechanical hyperalgesia induced by PI. The antihyperalgesic effect of AJM was reversed by pretreatment with AM281 given by intraperitoneal and intrathecal routes, but not intraplantarly. Additionally, intraperitoneal and intraplantar, but not intrathecal administration of AM630 blocked antihyperalgesia AJM-induced. Interestingly, mice pretreated with FAAH or MAGL inhibitor exhibited a significant prolongation of antihyperalgesic effect of AJM. This article presents data addressing the cannabinoid receptor mechanisms underlying the antihyperalgesic effects actions of joint mobilization and endocannabinoid catabolic enzyme inhibitors in the mouse postoperative pain model. Joint mobilization and these enzymes offer potential targets to treat postoperative pain.

**PERIPHERAL AND SPINAL ACTIVATION OF CANNABINOID
RECEPTORS BY JOINT MOBILIZATION ALLEVIATES
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Abbreviations

AJM, ankle joint mobilization; FAAH, fatty acid amide hydrolase;
MAGL, monoacylglycerol lipase; CBRs, cannabinoid receptors; AEA,
anandamide.

Abstract

The present study was undertaken to investigate the relative contribution of cannabinoid receptors (CBRs) subtypes and analyze cannabimimetic mechanisms involved on the inhibition of AEA and 2-AG degradation on the antihyperalgesic effect of ankle joint mobilization (AJM). Mice (25-35g) were submitted to plantar incision (PI) and 24 hours after surgery animals received the following treatments, AJM for 9 minutes, anandamide (10 mg/kg, intraperitoneal [i.p.]), WIN 55,212-2 (1.5 mg/kg, i.p.), URB937 (0.01-1 mg/kg, i.p.; a fatty acid amide hydrolase [FAAH] inhibitor) or JZL184 (0.016-16 mg/kg, i.p.; a monoacylglycerol

lipase [MAGL] inhibitor). Withdrawal frequency to mechanical stimuli was assessed 24 hours after PI and at different times after treatments. Receptor specificity was investigated using selective CB₁R (AM281) and CB₂R (AM630) antagonists. In addition, the effect of the FAAH and MAGL inhibitors on the antihyperalgesic action of AJM was investigated. AJM, anandamide, WIN 55,212-2, URB937 and JZL184 decreased mechanical hyperalgesia induced by PI. The antihyperalgesic effect of AJM was reversed by pretreatment with AM281 given by intraperitoneal and intrathecal routes, but not intraplantarly. Additionally, intraperitoneal and intraplantar, but not intrathecal administration of AM630 blocked antihyperalgesia AJM-induced. Interestingly, mice pretreated with FAAH or MAGL inhibitor exhibited a significant prolongation of antihyperalgesic effect of AJM. This article presents data addressing the cannabinoid receptor mechanisms underlying the antihyperalgesic effects actions of joint mobilization and endocannabinoid catabolic enzyme inhibitors in the mouse postoperative pain model. Joint mobilization and these enzymes offer potential targets to treat postoperative pain.

Key words: Acute pain; Cannabinoid receptors; Passive mobilization; Rehabilitation; Mice.

Introduction

Endocannabinoid system (ES) has emerged as an attractive therapeutic target for pain management in recent years (Pacher et al., 2006; Vicenzino et al., 1998). It consists of 2 known cannabinoid receptors (CBRs) subtypes CB₁R and CB₂R (Matsuda et al., 1990); a number of endogenous ligands including anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) (Mechoulam et al., 1998); a high-affinity reuptake transport system; and endocannabinoid synthesizing and metabolizing enzymes (Beltramo et al., 1997). AEA is hydrolyzed by fatty acid amide hydrolase (FAAH) (Facci et al., 1995), and 2-AG is primarily metabolized by monoacylglycerol lipase (MAGL) (Hanus et al., 1999). Whereas exogenously administered endocannabinoids are rapidly degraded by FAAH and MAGL, pharmacological inhibition of these enzymes result in elevated levels of endocannabinoids in the brain and spinal cord tissues (Facci et al., 1995; Malan et al., 2002), which might represent an alternative approach that can be used to harness the potential therapeutic effects of cannabinoids. Prevention of endocannabinoids metabolism produces behavioural analgesia in models

of acute pain (Roques et al., 2012). Targeting FAAH and/or MAGL activity, therefore, presents a promising new therapeutic strategy for the treatment of pain.

Health practitioners commonly use joint mobilization to treat musculoskeletal pain and dysfunction (Vicenzino et al., 1998). Mobilization-induced analgesia has been demonstrated in several studies in humans (Vicenzino et al., 1998; Zusman et al., 1989) and laboratory animals (Martins et al., 2011; Sbyba et al., 2003; Sluka et al., 2001). However, the mechanisms through which joint mobilization (JM) acts are not fully known. McPartland et al. (2005) had demonstrated that manual therapy (MT), including JM increases AEA blood levels in humans.

Studies have been demonstrated that patients can develop persistent or chronic pain following surgical procedures, in addition, nearly half of all surgical patients still have inadequate pain relief; therefore, it is becoming more important to understand the mechanisms involved in postoperative pain in order to better treat it (Brennan, 2011). A recent report show that endocannabinoid signaling via CB₁R and CB₂R is necessary for the resolution of paw incision-induced behavioral hypersensitivity and for the limitation of pro-inflammatory signaling in astrocytes following surgical insult (Alkaitis et al., 2010). Additionally,

we have recently reported that AJM suppressed pain behavior and attenuated astroglial activation in rats (Martins et al., 2011). Here, we used a pre-clinical model of postoperative pain described for rats (Brennan et al., 1996) and adapted for mice (Pogatzki and Raja, 2003). The model consists of a small incision made on the plantar surface of one hind paw. Paw incision in rodents induces a variety of nocifensive behaviors that is parallel the time course of postoperative pain in humans (Brennan et al., 1996).

The present study was undertaken to explore the relative contributions of central (spinal) and peripheral (paw) CBRs subtypes that mediate the antihyperalgesic effect of AJM. Additionally, we analyzed cannabimimetic mechanisms involved on the inhibition of AEA and 2-AG degradation through evaluation of effects of the JZL184 (MAGL inhibitor) and URB937 (FAAH inhibitor) on the antihyperalgesic effect of AJM.

EXPERIMENTAL PROCEDURES

2.1 Animals

All animal care and experimental procedures were carried out in accordance with the National Institutes of Health Animal Care Guidelines (NIH publications No. 80-23), and conducted following the

protocol approved by the Institutional Animal Care and Use Committee. All experiments were conducted using male Swiss mice (25-35 g), housed at $22 \pm 2^{\circ}\text{C}$ under a 12 hours light/12 hours dark cycle (lights on at 6:00 a.m.) and with free access to food and water. All manipulations were carried out between 11:00 am and 3:00 pm. The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of treatments. Control animals received the same vehicle used to dilute the compounds. When drugs were delivered by intraperitoneal (i.p.) route, a constant volume of 10 mL/kg body weight was injected. When drugs were administered by intrathecal (i.t.) or intraplantar (i.pl.) routes, volumes of 5 μL or 20 μL were injected, respectively. Appropriate vehicle-treated groups were also assessed simultaneously.

2.2 Plantar incision surgery

The postoperative pain model was carried out according to the procedure described for rats (Roques et al., 2012) and adapted for mice (Pogatzki and Raja, 2003). Briefly, mice were anesthetized with 1-2% isoflurane delivered via a nose cone. After sterile preparation of the right hind paw, a 5-mm longitudinal incision was made through skin and fascia of the plantar surface using a number 11 scalpel blade. The

incision was started 2 mm from the proximal edge of the heel and extended toward the toes. The underlying muscle was elevated with curved forceps, leaving the muscle origin and insertion intact. After wound homeostasis, the skin was apposed with an 8.0 nylon mattress suture, and the wound was covered with 10% povidone-iodine solution. Control animals underwent a sham procedure, remaining anesthetized only.

2.3 Intrathecal injections

Intrathecal (i.t.) injections were given to fully conscious mice using the method previously described by Hylden and Wilcox (1980). Briefly, the animals were manually restrained, the dorsal fur of each mouse was shaved, the spinal column was arched, and a 30-gauge needle was inserted into the subarachnoid space between the L4 and L5 vertebrae. Correct i.t. positioning of the needle tip was confirmed by a characteristic tail-flick response in the animal. A 5 μ L volume of solution containing the test agent was slowly injected with a 25 μ L Hamilton microsyringe (Hamilton, Birmingham, UK). Intrathecal injections were given over a period of 5 seconds.

2.4 Ankle joint mobilization

The knee joint was stabilized, and the ankle joint was rhythmically flexed and extended to the end of the range of movement, according to a previously reported dosage regime (Martins et al., 2011; Skyba et al., 2003). The treatment with AJM was carried out in animals lightly anesthetized with 1-2% isoflurane prior to and for the duration of joint mobilization. The treatment group received three applications of mobilization, each 3 minutes in duration separated by 30 seconds of rest. Our group has previously shown this time frame to be optimal for producing antihyperalgesia in this model. Placebo AJM mice were lightly anaesthetized with 1-2% isoflurane and the ankle was maintained in a neutral position using the same hand contact and positioning as the treatment technique (Martins et al., 2012).

2.5 Behavioral measurement: mechanical hyperalgesia

Animals were tested for withdrawal thresholds to mechanical stimuli (von Frey filaments) applied to the plantar aspect of the hindpaw (Bobinski et al., 2011; Bortalanza et al., 2002). Mice were acclimated in individual clear boxes (9 x 7 x 11 cm³) on an elevated wire mesh platform to allow access to the ventral surface of the hind paws. The right hind paw was stimulated with a constant pressure of 0.4 g von Frey filament (VFF) (Stoelting, Chicago, USA). The response frequency to

10 applications was taken as the nociceptive behavior. The results are expressed as the percentage of withdrawal frequency (Martins et al., 2012). Tests were performed before and 24 hours after plantar incision surgery and in different times after treatments (see sections 2.6.1-3; 2.7; 2.8; 2.9 and 2.10).

2.6 Investigation of the endocannabinoid signaling via CB₁R and CB₂R

1.6.1 Systemic administration of CB₁R and CB₂R antagonists

The role of cannabinoid receptors in the antihyperalgesic action of AJM was investigated by treating mice with the selective CB₁R antagonist AM281 (0.5 mg/kg, intraperitoneally [i.p.]) (Robinson et al., 2010) or the selective CB₂R antagonist AM630 (3 mg/kg, i.p.) (Rousseaux et al., 2007) 20 minutes before AJM. Each receptor antagonist exhibits more than a 100-fold difference in affinity for CB₁R and CB₂R, respectively (Lan et al., 1999; Ross et al., 1999). These treatments were performed 24 hours after plantar incision surgery. The positive controls experiments were performed using the CB₁R agonist anandamide (AEA) (10 mg/kg, i.p.) (Calignano et al., 2001) or the CB₂R agonist WIN 55,212-2 (1.5 mg/kg, i.p.) (Cano et al., 2007). Mechanical hyperalgesia was evaluated using the von Frey filament (0.4 g) 30 minutes after AJM.

Placebo AJM animals were subjected to 9 minutes of anesthesia and were assessed over the same time intervals in all experiments.

2.6.2 Spinal administration of CB₁R and CB₂R antagonists

In another set of experiments, to evaluate the involvement of spinal cannabinoid receptors in the antihyperalgesia induced by AJM, the animals received an intrathecal (i.t.) injection with 5 μ L of vehicle (10 mL/kg, site), AM281 (2 μ g/site) or AM630 (2 μ g/site) (Gu et al., 2011). After 15 minutes, the animals received AJM for 9 minutes. Mechanical hyperalgesia was evaluated using the von Frey filament 30 minutes after AJM. In parallel experiments, mice were pre-treated with an intrathecal injection of vehicle (5 μ L/site) or AM281 (2 μ g/site) and after 15 minutes, received vehicle (10 mL/kg, i.p.) or AEA (10 mL/kg, i.p.) (Calignano et al., 2001). Mechanical hyperalgesia of these groups were assessed 30 minutes after vehicle or AEA treatments. Another group of mice was pre-treated with an intrathecal injection of vehicle (5 μ L/site) or AM630 (2 μ g/site) (Gu et al., 2011) and after 15 minutes, received vehicle (10 mL/kg, i.p.) or WIN 55,212-2 (1.5 mg/kg i.p.) (Cano et al., 2007). Mechanical hyperalgesia of these groups was assessed 30 minutes after vehicle or WIN 55,212-2 treatments.

2.6.3 Peripheral administration of CB₁R and CB₂R antagonists

In an attempt to investigate the involvement of peripheral cannabinoid receptors in the antihyperalgesia induced by AJM, animals received an intraplantar injection (i.pl.) with 20 µL of vehicle, AM281 (10 µg/paw) (Khasabova et al., 2011) or AM630 (4 µg/paw) (Khasabova et al., 2011) in the right hind paw. After 15 minutes, the animals received AJM for 9 minutes. Mechanical hyperalgesia was evaluated using the von Frey filament 30 minutes after AJM.

In positive control experiments, mice were pre-treated with an intraplantar injection of vehicle (20 µL/paw) or AM281 (10 µg/paw) (Khasabova et al., 2011) and after 15 minutes, received vehicle (10 mL/kg, i.p.) or AEA (10 mg/kg, i.p. [Calignano et al., 2001]). Another groups of mice were pre-treated with an intraplantar injection of vehicle (20 µL/paw) or AM630 (4 µg/paw) (Khasabova et al., 2011) and after 15 minutes, received vehicle (10 mL/kg, i.p.) or WIN 55,212-2 (1.5 mg/kg, i.p.) (Cano et al., 2007). The mechanical hyperalgesia of these groups was assessed 30 minutes after AEA or WIN 55,212-2 or vehicle treatments.

2.7 Effect of fatty acid amide hydrolase (FAAH) inhibition

To evaluate the effect of inhibition, of the catabolic endocannabinoid enzyme, fatty acid amide hydrolase on postoperative pain, the animals received an i.p. injection of vehicle (10 mL/kg) or URB937 (0.01-1mg/kg a peripherally restricted FAAH inhibitor) (Clapper et al., 2010). Mechanical hyperalgesia was evaluated using the von Frey filament in 0.5, 1, 2, 4, 6, 8 and 24 hours after vehicle or URB937 treatments.

2.8 Effect of monoacylglycerol lipase (MAGL) inhibition

In order to determine whether inhibition of the MAGL reduces hyperalgesia-induced by plantar incision surgery, the animals received an i.p. injection of vehicle (10 mL/kg) or JZL184 (0.016-16 mg/kg, an irreversible MAGL inhibitor) (Clapper et al., 2010). Mechanical hyperalgesia was evaluated using the von Frey filament in 0.5, 1, 2, 4, 6, 8 and 24 hours after vehicle or JZL184 treatments.

2.9 Effects of combined administration of subeffective dose of FAAH inhibitor and AJM

To test the hypothesis that the antihyperalgesic effect of AJM is mediated through of inhibition of the FAAH, animals were pretreated with vehicle (10 mL/kg, i.p.) or a subeffective dose of URB937 (0.1 mg/kg, i.p.), 1.5 hour later they received AJM treatment for 9 minutes.

Mechanical hyperalgesia was evaluated using the von Frey filament in 0.5, 1, 1.5 and 2 hours after AJM treatment.

2.10 Effects of combined administration of subeffective dose of MAGL inhibitor and AJM

To test the hypothesis that the antihyperalgesic effect of AJM is mediated through of inhibition of the MAGL, animals were pretreated with vehicle (10 mL/kg, i.p.) or a subeffective dose of JZL184 (1.6 mg/kg, i.p.), 1.5 hour later they received AJM treatment for 9 minutes. Mechanical hyperalgesia was evaluated using the von Frey filament test in 0.5, 1, 1.5, 2 and 2.5 hours after AJM treatment.

2.11 Chemicals

AM281, AM630, Anandamide, WIN 55,212-2, JZL184 and URB937 were obtained from Cayman (Michigan, U.S.A.). Dimethyl sulfoxide, NaCl and ethanol were obtained from Sigma (St Louis, MO, U.S.A.). All drugs were dissolved, just before use, in DMSO and ethanol, in amount that did not exceed a final concentration of 1 and 2.5% respectively.

2.12 Data Analysis

Results are presented as the mean \pm standard errors of the mean (SEM) for each group. Behavioral testing was analyzed using both one-way analysis of variance following Student Newman-Keuls test and two-way repeated measures analysis of variance (ANOVA). A multi-comparison post hoc test was performed using Bonferroni's test. Data were converted to the area under the curve (AUC) using one-way ANOVA followed by Student Newman-Keuls test when appropriate. Differences with a value of $P < 0.05$ were considered significant.

RESULTS

3.1 Antihyperalgesic effect of joint mobilization requires activation CB₁R and CB₂R

To verify whether the antihyperalgesic effect of AJM can be mediated via activation CB₁R and CB₂R we administrated selective CB₁R antagonist, AM281 in different sites. Systemic and spinal administration of AM281, 20 and 15 minutes before mobilization of the ankle joint, respectively, prevented the decreased in percentage of withdrawal frequency resulting from the treatment. Percentage of withdrawal frequency values for the group pretreated with AM281 via i.p. or i.t. routes was significantly higher when compared to vehicle plus

mobilization group, 30 minutes after mobilization (Figs. 1A and 1C). AM281 without joint mobilization had no effect on withdrawal frequency, and thresholds were similar to intraperitoneal and intrathecal injection of vehicle without joint mobilization. However, intraplantar administration of AM281 prior to mobilization of the ankle joint had no effect on the resultant decrease in mechanical withdrawal frequency when compared to vehicle plus mobilization group (Fig. 1E).

We next determined the role of CB₂R in AJM-produced antihyperalgesic effect in postoperative pain. We administered selective CB₂R antagonist AM630 in different sites. Systemic and intraplantar administration of AM630, 20 and 15 minutes before mobilization of the ankle joint, respectively, prevented the decreased in percentage of withdrawal frequency resulting from the treatment. Percentage of withdrawal frequency values for the group pretreated with AM630 via i.p. or i.pl. routes were significantly higher when compared to vehicle plus mobilization group, 30 minutes after mobilization (Figs. 2A and 2E). AM630 without joint mobilization had no effect on the increased withdrawal frequency, and thresholds were similar to intraperitoneal and intrathecal injection of vehicle without joint mobilization. However, intrathecal administration of AM630 prior to mobilization of the ankle joint had no effect on the resultant decrease in mechanical withdrawal

frequency when compared to vehicle plus mobilization group (Fig. 2C). These findings suggest that activation of CB₁R central (spinal) and CB₂R peripheral (paw) contribute to the antihyperalgesic effect of AJM on postoperative pain.

Further, neither drugs without joint mobilization affected the increased mechanical withdrawal frequency produced by plantar incision (PI) surgery, and thresholds were similar to vehicle without joint mobilization (Figs. 1A-F and 2A-F). This suggests that the changes in withdrawal thresholds noted in treatment groups result from joint mobilization and not from administration of the receptor antagonists.

While the doses of AM281 and AM630 were chosen based on previously published studies (Gu et al., 2011; Khasabova et al., 2011; Robinson et al., 2010; Rousseaux et al., 2007) we tested them against their agonists in this model to confirm that they were adequate to block systemic, central and peripheral CB₁R and CB₂R, respectively. Administration of either anandamide or WIN 55,212-2 in vehicle groups decreased percentage of withdrawal frequency 30 minutes after intraperitoneal administration (Figs. 1B, 1D, 1F, 2B, 2D and 2F). However, pretreatment in different sites (i.p., i.t. or i.pl.) with AM281 completely blocked the effect of anandamide (Figs. 1B, 1D and 1F) and pretreatment in different sites (i.p., i.t. or i.pl.) with AM630 completely

blocked the effect of WIN 55,212-2 on mechanical withdrawal frequency (Figs. 2B, 2D, 2F). Thus, the inability of peripheral (paw) AM281 (Fig. 1E) and central (spinal) AM630 (Fig. 2C) to prevent or attenuate mobilization-induced changes in mechanical withdrawal frequency is not due to ineffective blockade of peripheral CB₁R or spinal CB₂R, respectively.

3.3 Inhibition of endocannabinoid catabolic enzymes prolongs AJM effect

The endocannabinoids anandamide and 2-arachidonoylglycerol are predominantly regulated by the respective catabolic enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL). Since that the inhibition of these enzymes elevates endocannabinoid levels and attenuates neuropathic pain (Kinsey et al. 2010) and manual therapy (MT) techniques, including JM, increases serum levels of endocannabinoids in humans (McPartland et al., 2005; McPartland, 2008a) raises the question of whether URB937 or JZL184 could increase or prolong the antihyperalgesic effect of AJM. We first characterized the effect of URB937 and JZL184 on postoperative pain. The results, presented in Fig. 3A, show that the treatment with URB937 reduced hyperalgesia induced by PI. A significant difference between

groups (PI + vehicle vs. PI + URB937 [1 mg/kg, i.p.]) for withdrawal response frequency occurred at 1 hour ($P < 0.05$), 2 hours ($P < 0.001$) and 4 hours ($P < 0.01$) after URB937 (1 mg/kg, i.p.) treatment. Area under curve (AUC) analysis showed that mechanical hyperalgesia on PI + URB937 (1 mg/kg, i.p.) group was significantly lower than PI + vehicle group ($P < 0.001$, see Fig. 3B). Fig. 4A shows that the pretreatment of mice with JZL184 decreased hyperalgesia induced by PI. A significant difference between groups (PI + vehicle vs. PI + JZL184 [16 mg/kg, i.p.]) for withdrawal response frequency occurred at 2 hours ($P < 0.001$) after JZL184 treatment. AUC analysis showed that mechanical hyperalgesia on PI + JZL184 (16 mg/kg, i.p.) group was significantly lower than PI + vehicle group ($P < 0.001$, see Fig. 4B). To test our second hypothesis animals were pretreated with a subeffective dose of endocannabinoid catabolic enzymes inhibitors and submitted to AJM. We compared the antihyperalgesic effect of AJM alone to the response of mice that received a subeffective dose of URB937 (0.1 mg/kg, i.p.) or a subeffective dose of JZL184 (1.6 mg/kg, i.p.) or vehicle plus AJM. The 9-minutes joint mobilization treatment reduced hyperalgesia for a duration of ~30 minutes in mice that received vehicle (Figs. 3C and 4C). Notably, mice mobilized pretreated with a subeffective of URB937 (0.1 mg/kg, i.p.) exhibited a significant

prolongation ($P < 0.001$) of antihyperalgesic effect caused by AJM, the hyperalgesia was suppressed for ~1.5 hour ($P < 0.001$). AUC analysis showed that mechanical hyperalgesia on PI + URB937 (0.1 mg/kg, i.p.) + AJM group was significantly lower than PI + vehicle + Placebo AJM group ($P < 0.001$, see Fig. 3D). Interestingly, mice mobilized pretreated with a subeffective dose of JLZ184 (1.6 mg/kg, i.p.) exhibited a significant prolongation ($P < 0.001$) of antihyperalgesic effect produced by AJM, the hyperalgesia was suppressed for ~2.5 hours ($P < 0.001$) (Fig. 4C). AUC analysis showed that mechanical hyperalgesia on PI + JZL184 (1.6 mg/kg, i.p.) + AJM group was significantly lower than PI + vehicle + Placebo AJM group ($P < 0.001$, see Fig. 4D).

DISCUSSION

This study represents the first direct demonstration of the role of endocannabinoid system on the antihyperalgesic effect of ankle joint mobilization (AJM). The most relevant findings of the present work are the following: i) the antihyperalgesic effect of AJM was reversed by pretreatment with AM281 given by intraperitoneal and intrathecal routes, but not intraplantarly; ii) intraperitoneal and intraplantar, but not intrathecal administration of AM630 blocked antihyperalgesia AJM-

induced; iii) mice pretreated with FAAH or MAGL inhibitor exhibited a significant prolongation of antihyperalgesic effect of AJM.

Health practitioners commonly use joint mobilization to treat musculoskeletal pain and dysfunction (Vicenzino et al., 1998). However, the mechanisms through which JM acts are not fully known. A clinical study has reported that manual therapy (MT) techniques, including JM, induces anxiolysis, eases suffering, increases sense of well-being, and even induces euphoria-psychotropic changes that can be described as cannabimimetic. The authors suggest that manipulative treatment may induce such effects by boosting endocannabinoid levels (McPartland et al., 2005). A blinded, randomized controlled trial of 31 healthy subjects measured anandamide (AEA) levels pre- and post-manipulative treatment. The manipulative treatment (MT) consisted manual therapy techniques, including JM. In subjects receiving MT, serum levels of AEA obtained after MT more than doubled the pre-MT levels. No change was seen in control subjects. In another study a smaller MT trial reported little change in AEA levels pre- and post-MT but showed significant post-MT augmentation of N-palmitoylethanolamine (Degenhardt et al., 2007).

It is now well known that, CB₁Rs are expressed in pain transmission and modulation pathways such as amygdale, cerebellum,

periaqueductal gray, rostral ventromedial medulla, spinal cord and primary afferent fibers (Agarwal et al., 2007; Hohmann et al., 2005; Svizenska et al., 2008). CB₁Rs are located pre-synaptically on axons and terminals of neurons and negatively coupled to adenylate cyclase, block voltage-dependent calcium channels, and activate potassium channels and mitogen-activated protein kinase at the cellular level, the overall effect being cellular inhibition (Hohmann et al., 2005; Ameri, 2008; Childers et al., 1992). On the other hand, in addition to peripheral tissues, several studies reported that CB₂R might also exist in the central nervous system, and exert an antinociceptive effect in some chronic pain models (Malan et al., 2002; Whiteside et al., 2005). To explore the specific contribution of CB₁R subtypes at different levels of the nociceptive pathways in mobilization-induced antihyperalgesia on postoperative pain model, the selective CB₁R antagonist AM281 was systemically or locally applied prior to AJM treatment or i.p. AEA administration. The pretreatment with the CB₁R antagonist AM281 by i.p., and i.t., but not i.pl., routes of administration prevented systemic AJM- or anandamide-induced antihyperalgesia, supporting the hypothesis that AJM-induced antihyperalgesic effect is produced via activation CB₁R centrally.

The descending pain inhibitory pathway in rodents and humans is activated by the perception of pain in the brain. Endorphins, endocannabinoids, serotonin, norepinephrine, and adenosine play important roles in this pathway (Walker and Hohmann, 2005). Spinally projecting descending serotonergic and noradrenergic pathways modulate pain transmission utilizing endogenous 5-HT and NA through activation of serotonergic and noradrenergic receptors at the spinal cord level (Millan, 2002). A large body of evidence shows the involvement of descending serotonergic and noradrenergic pathways in CB₁R-mediated antinociception. First, surgical lesion of dorsolateral funiculus, which is the main route of descending projections that runs from supraspinal sites through spinal dorsal horn, totally inhibits systemically administered WIN 55,212-2 and arachidonyl-2'-chloroethylamide (ACEA)-induced antinociceptive effects on tail-flick test (Seyrek et al., 2010). Second, CB₁R and endocannabinoid degrading enzyme FAAH were expressed on the serotonergic cells of dorsal raphe nucleus (DRN) (Mendiguren and Pineda, 2009; Bambico et al., 2007). Third, local administration of WIN 55,212-2 into the DRN elicited an acute antinociceptive effect and enhanced the firing activity of serotonergic neurons and 5-HT release through CB₁R dependent mechanism (Bambico et al., 2007; Martin et al., 1995). In another study, the

antinociception induced by WIN 55,212-2 was attenuated in spinal NA depleted rats by i.t. 6-hydroxydopamine (6-OHDA) on tail flick test (Gutierrez et al., 2003). Taken together, these results provide strong evidence that cannabinoids reinforce the descending serotonergic and noradrenergic pathways to produce acute antinociceptive effects. It has been demonstrated that joint mobilization might activate descending pain inhibitory systems, mediated supraspinally. Recent animal studies show that the analgesia produced by joint mobilization involves serotonin and norepinephrine receptors in the spinal cord, thereby supporting a role for descending pain modulatory systems (Skyba et al., 2003). These previous findings and the present results have led to the hypothesis that joint mobilization might produce antihyperalgesia via CB₁R mediated mechanism through activation of descending serotonergic and noradrenergic pathways that act upon on spinal 5-HT and NA receptors.

Emerging evidence suggests that CB₂ agonists are analgesic in a number of neuropathic, inflammatory pain and postoperative pain models (Hohmann et al., 2004; Ibrahim et al., 2003; LaBuda et al., 2005; Nackley et al., 2003). However, there is little evidence for CB₂R expression in normal spinal cord (Buckley et al., 1998). CB₂R expression is induced in the spinal cord, likely in microglial cells,

following nerve injury and the development of a neuropathic state and postoperative pain (LaBuda et al., 2005; Zhang et al., 2003). In order to provide a functional evidence of the role of CB₂R in antihyperalgesia AJM-produced, we investigated the specific contribution of CB₂R subtype at different levels of the nociceptive pathways in mobilization-induced antihyperalgesia in the postoperative pain. The selective CB₂R antagonist AM630 was systemically or locally applied prior to AJM application or i.p. WIN 55,212-2 administration. The pretreatment with the CB₂R antagonist AM630 by i.p., or i.pl., but not i.t., routes of administration prevented systemic antihyperalgesia AJM- or WIN 55,212-2-induced, suggested that antihyperalgesic effect AJM-induced might be mediated via activation peripheral CB₂R.

Keratinocytes are one type of cells that may mediate the analgesic actions of the AEA, WIN 55,212-2 and AJM treatment, which are abundantly present in the skin and express CB₂R and contain endogenous opioid peptides. It has been reported that the CB₂R-selective agonist AM1241 may result in the release of the endogenous opioid peptide β -EP, which then acts on primary afferent terminals to inhibit inflammatory pain (Ibrahim et al., 2005). However, others have shown that the antihyperalgesic effects of the CB₂R-selective agonists

are not dependent upon the release of endogenous opioids (Whiteside et al., 2005).

The endocannabinoid system alters fibroblast “focal adhesions,” by which fibroblasts link the extracellular collagen matrix to their intracellular cytoskeleton—the mechanism of fascial remodeling. In addition, it has been demonstrated that many connective tissue-related cells express CB₁R, CB₂R, and endocannabinoid-metabolizing enzymes such as fibroblasts, myofibroblasts, chondrocytes, and synoviocytes (McPartland, 2008b). Based on these studies and since ankle joint mobilization treatment stimulates all these cells, we suggested that the effect produced by AJM might be mediated via increase expression CBR_s mobilization-induced on these cells. However, other studies are necessary to further substantiate this assertion.

The fact that manual therapy (MT) techniques, including JM, increases serum levels of anandamide and N-palmitoylethanolamine in human raises the hypothesis that the JM treatment could increase the endocannabinoids (AEA or 2-AG) levels, acting by mechanisms involving the inhibition of endocannabinoids degradation. To test this hypothesis, mice were pretreated with a subeffective dose of URB937 or JZL184, 1.5 hour later they received AJM treatment for 9 minutes. Herein, we provide evidence that the antihyperalgesic effect of AJM can

be mediated by inhibition of degradation of the endocannabinoids, since subeffective dose of URB937, a fatty acid amide hydrolase inhibitor, combined with AJM prolonged for until 1.5 hour the antihyperalgesic mobilization-induced on postoperative pain model. Additionally, to confirm our hypothesis, the combination of subeffective dose the monoacylglycerol lipase inhibitor with AJM also prolonged (2.5 hours) the antihyperalgesia caused by AJM. Considering that endocannabinoid (ECB) enzyme inhibitors partially increase the AEA and 2-AG levels in tissue by inhibition of degradation of what is synthesized endogenously (Roques et al., 2012), it is possible that the prolonged effect antihyperalgesic of AJM by endocannabinoid (ECB) enzyme inhibitors is dependent on the increase of the AEA or 2-AG levels induced by AJM. Thus, we can speculate that prolonged effect observed might be the result of the increase in the ECB levels induced by both ECB enzyme inhibitors plus AJM treatment. While our approach cannot rule out the involvement of other systems, these findings strongly suggest that endocannabinoid are required for the antihyperalgesia induced by AJM. Thus, we also could suggest that ECB enzyme inhibitors can be used as an adjuvant to AJM treatment. The data from human subjects are consistent with the current findings in this animal model. Taken

together, we hypothesize that joint mobilization produces a form of analgesia mediated by endocannabinoid.

CONCLUSION

In summary, the present study suggests that activation of CB₁R central (spinal) and CB₂R peripheral (paw) appear contribute to the antihyperalgesic effect of AJM on postoperative pain model. Additionally, we suggested that AJM could exert its antihyperalgesic effect via cannabimimetic mechanisms involving inhibition of endocannabinoid degradation. Also, it would be relevant to assess via biochemical analysis if AJM increases the endocannabinoid levels. The lack of biochemical analysis may be a limitation of this study and should be considered in future studies.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

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Figures to Legends

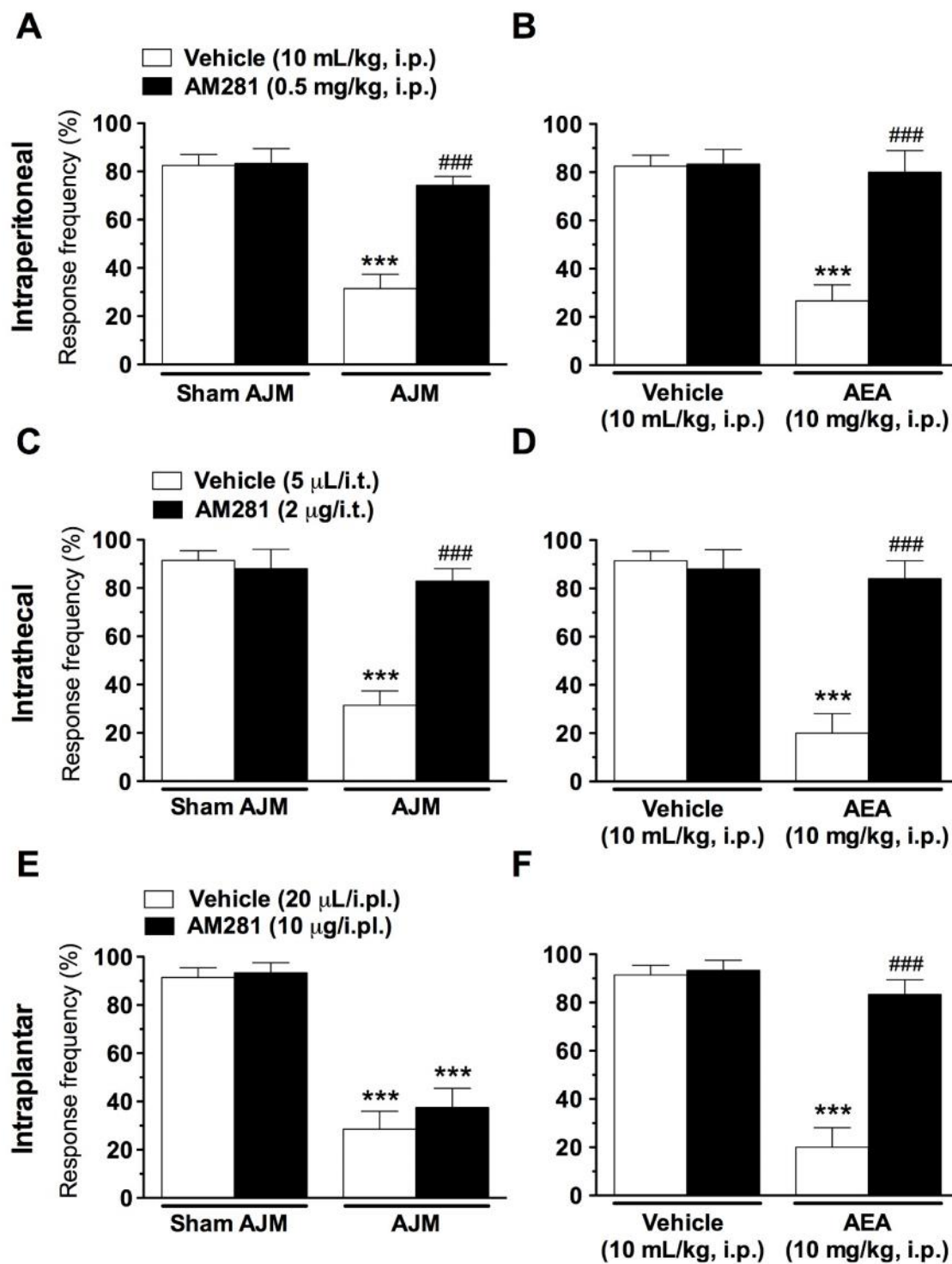


Figure 1

Figure. 1. Evidence for the involvement of CB₁R in the antihyperalgesia caused by AJM or AEA treatments on mechanical hyperalgesia induced by plantar incision (PI). Intraperitoneal (A-B), intrathecal (panel, C-D) and intraplantar (panel, E-F) pre-treatment with AM281 (CB₁R selective antagonist) on the antihyperalgesic effect of AJM and AEA (10 mg/kg, i.p.) in mice. Black bars show the effects of AM281 antagonist injected at ipsilateral location before the AJM or AEA treatments. Each point represents the mean of 8 animals and vertical lines show the S.E.M. Statistical analyses were performed by one-way ANOVA followed by Student-Newman-Keuls test. The symbols denote a significant difference of *** $P < 0.001$ when compared with Sham AJM + vehicle (control) group or ### $P < 0.001$ when compared with AJM + vehicle or AEA + vehicle group. CB₁: cannabinoid receptor 1; AJM: ankle joint mobilization; AEA: anandamide.

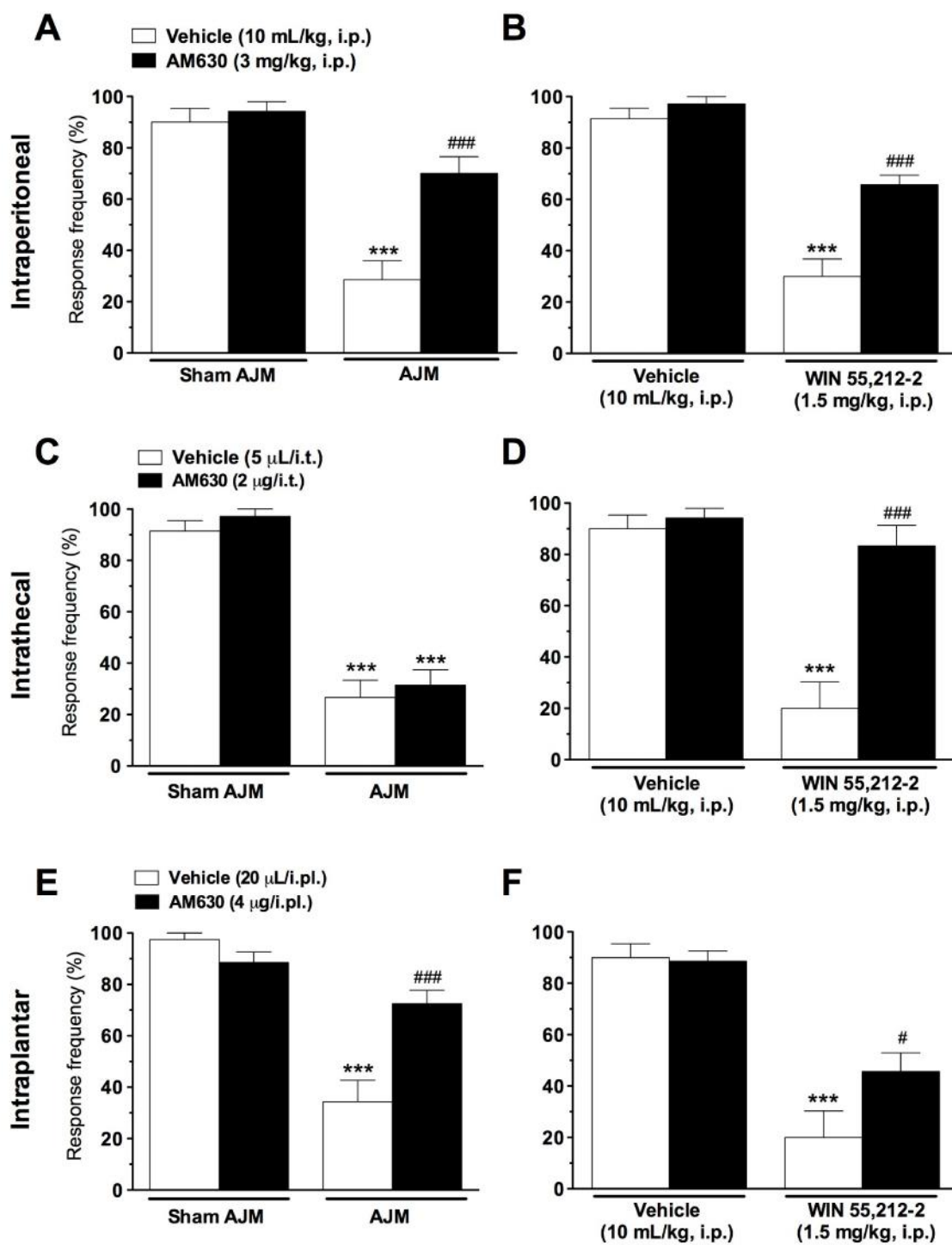


Figure 2

Figure. 2. Involvement of CB₂R in the antihyperalgesia caused by AJM or WIN 55,212-2 treatments on mechanical hyperalgesia induced by plantar incision (PI). Intratraperitoneal (panel A-B), intrathecal (panel, C-D) and intraplantar (panel, E-F) pre-treatment with AM630 (CB₂R selective antagonist) on the antihyperalgesic effect of AJM or WIN 55,212-2 (1.5 mg/kg, i.p.) in mice. Black bars show the effects of AM630 antagonist injected at ipsilateral location before the AJM or WIN 55,212-2 treatments. Each point represents the mean of 8 animals and vertical lines show the S.E.M. Statistical analyses were performed by one-way ANOVA followed by Student-Newman-Keuls test. The symbols denote a significant difference of *** $P < 0.001$ when compared with Sham AJM + vehicle (control) group or # $P < 0.05$ and ### $P < 0.001$ when compared with AJM + vehicle or WIN 55,212-2+ vehicle group. AJM: ankle joint mobilization.

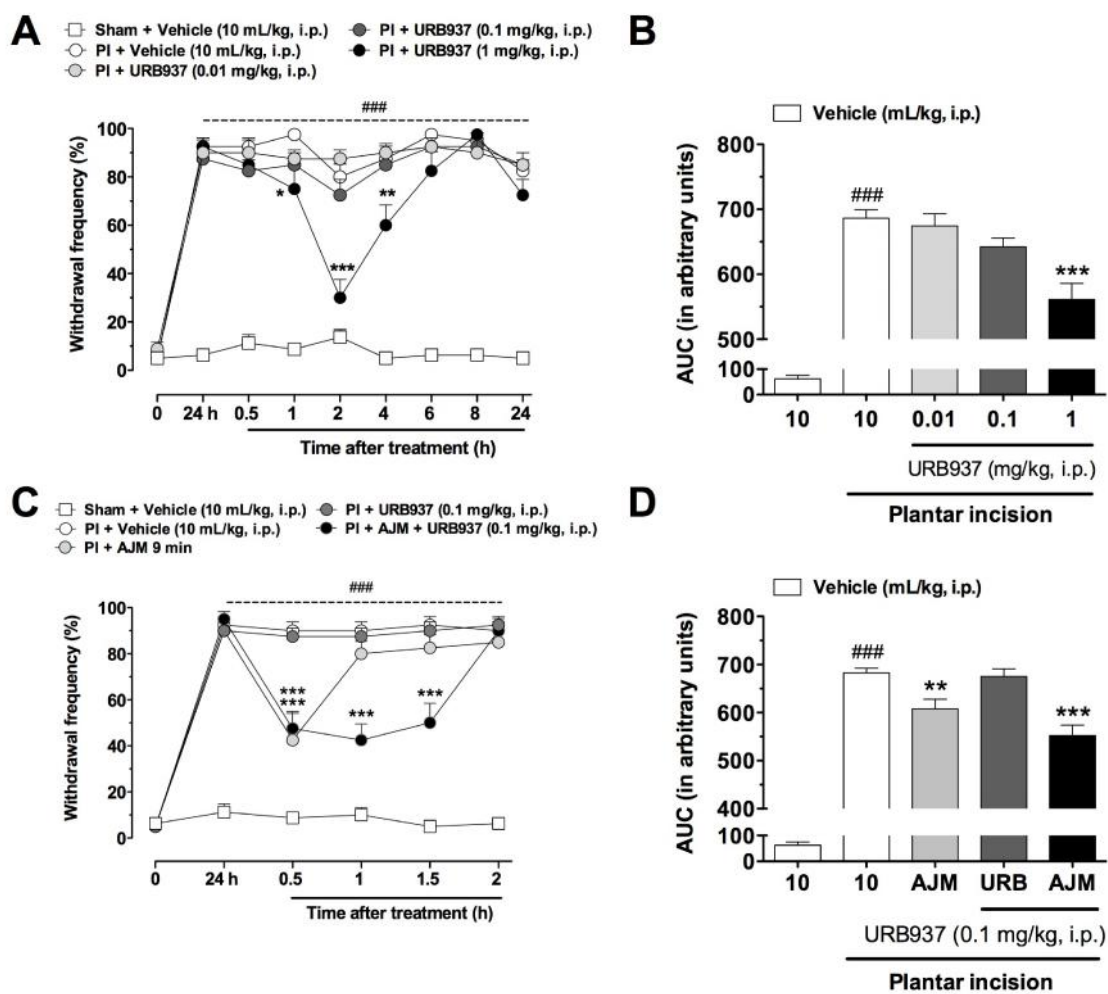


Figure 3

Figure. 3. The URB937 attenuated plantar incision-induced mechanical hyperalgesia (panel A) and prolonged the antihyperalgesia caused by AJM (panel C). Panels C and D represent the areas under the curve of panels A and B, respectively. Each point represents the mean of 8 animals and vertical lines show the S.E.M. Statistical analyses were performed by two-way ANOVA followed by Bonferroni test (panel A and C) and one-way ANOVA followed by Student Newman-Keuls test (panel B and D). The symbols denote a significant difference of $^{###}P < 0.001$ when compared with Sham + vehicle group or $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ when compared with PI + vehicle group. PI: plantar incision; AJM: ankle joint mobilization; FAAH: fatty acid amide hydrolase.

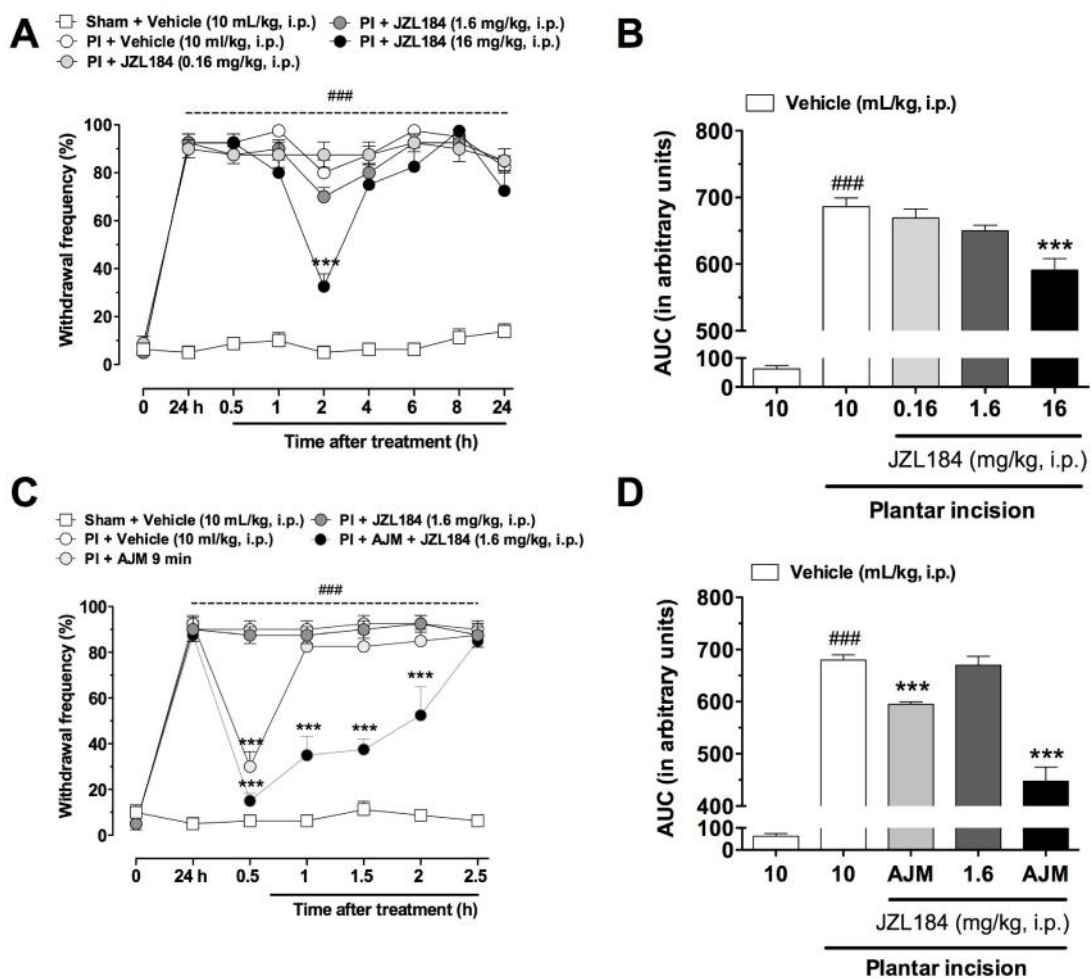


Figure 4

Figure. 4. The JZL184 reduced plantar incision-induced mechanical hyperalgesia (panel A) and prolonged the antihyperalgesia caused by AJM (panel C). Panels C and D represent the areas under the curve of panels A and B, respectively. Each point represents the mean of 8 animals and vertical lines show the S.E.M. Statistical analyses were performed by two-way ANOVA followed by Bonferroni test (panel A and C) and one-way ANOVA followed by Student Newman-Keuls test (panel B and D). The symbols denote a significant difference of $###P < 0.001$ when compared with Sham + vehicle group or $***P < 0.001$ when compared with PI + vehicle group. PI: plantar incision; AJM: ankle joint mobilization; MAGL: monoacylglycerol lipase.

3.4 Mobilização Articular do Tornozelo Reduz Dor Neuropática e Ativação Glial Induzida por Axoniotmese e melhora regeneração nervosa em ratos

Artigo 4

ANKLE JOINT MOBILIZATION REDUCES AXONOTMESIS-INDUCED NEUROPATHIC PAIN AND GLIAL ACTIVATION IN THE SPINAL CORD AND ENHANCES NERVE REGENERATION IN RATS

Martins DF, Mazzardo-Martins L, Gadotti VM, Nascimento FP, Lima DA, Speckhann B, Favretto GA, Bobinski F, Cargnin-Ferreira E, Bressan E, Dutra RC, Calixto JB, Santos ARS.

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Ankle joint mobilization reduces axonotmesis-induced neuropathic pain and glial activation in the spinal cord and enhances nerve regeneration in rats

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ABSTRACT

An important issue in physical rehabilitation is how to protect from or to reduce the effects of peripheral nerve injury. In the present study, we examined whether ankle joint mobilization (AJM) would reduce neuropathic pain and enhance motor functional recovery after nerve injury. In the axonotmesis model, AJM during 15 sessions every other day was conducted in rats. Mechanical and thermal hyperalgesia and motor performance deficit were measured for 5 weeks. After 5 weeks, we performed morphological analysis and quantified the immunoreactivity for CD11b/c and glial fibrillary acidic protein (GFAP), markers of glial activation, in the lumbar spinal cord. Mechanical and thermal hyperalgesia and motor performance deficit were found in the Crush + Anesthesia (Anes) group ($P < 0.001$), which was significantly decreased after AJM ($P < 0.001$). In the morphological analysis, the Crush + Anes group presented reduced myelin sheath thickness ($P < 0.05$), but the AJM group presented enhanced myelin sheath thickness ($P < 0.05$). Peripheral nerve injury increased the immunoreactivity for CD11b/c and GFAP in the spinal cord ($P < 0.05$), and AJM markedly reduced CD11b/c and GFAP immunoreactivity ($P < 0.01$). These results show that AJM in rats produces an antihyperalgesic effect and peripheral nerve regeneration through the inhibition of glial activation in the dorsal horn of the spinal cord. These findings suggest new approaches for physical rehabilitation to protect from or reduce the effects of nerve injury.

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1. Introduction

Peripheral nerves are often exposed to physical injuries, including nerve crush, entrapment with nerve compression, and traumatic or iatrogenic neurotmesis [9,20]. These injuries produce long-lasting neuropathic pain and hypersensitivity that is particularly prominent with mechanical and cold stimulation of the skin [21]. These effects have been shown to involve glial activation in the spinal cord [13,31]. The nerve injury affects neuronal excitability in the dorsal horn of the spinal cord partly by activation of glial cells, which release neurotrophins and proinflammatory cytokines

[13,31]. Additionally, changes in descending pain modulation contribute to neuropathic symptoms [18,19]. Joint mobilization has long been used for pain relief [23–25], and the mechanism for mobilization-related pain relief appears to involve descending inhibitory mechanisms, an event that is dependent on serotonin and norepinephrine [23].

In addition, poor muscle and nerve functional recovery is still a serious clinical problem. Nerve crushing results in muscle spindle denervation and intrafusal fiber atrophy [3]. Following peripheral nerve injury, the target muscle loses its weight and strength, the number of fibrils is reduced, and intrafascicular fibrosis occurs [17]. However, brain-derived neurotrophic factor [7], glial cell line-derived neurotrophic factor [11], neurotrophin 3 [10], and nerve growth factor [22] have been shown to be synthesized in skeletal muscle. Therefore, denervated target muscle tissue may

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be an important source for neurotrophic factors during sciatic nerve regeneration [10].

Evidence shows that physical forces, such as passive stretching or active contraction, may counteract various kinds of skeletal muscle atrophy due to, for instance, muscle immobilization, pathophysiology, or denervation. Accordingly, active muscle contraction induced by functional electric stimulation helps reduce the atrophic state of denervated muscle [26].

Based on the above evidence, we hypothesized that the ankle joint mobilization would reduce neuropathic pain and enhance motor functional recovery after peripheral nerve injury. In the current study, we investigated the effects of joint mobilization after sciatic nerve crush injury in an axonotmesis rat model, using behavioral, morphological, and biochemical tests.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (250–280 g) obtained from the Universidade Federal de Santa Catarina (Florianópolis, SC, Brazil) were used in this study. Animals were housed under a 12-hour light/12-hour dark cycle (lights on at 6 am) in a room with controlled temperature (22 ± 2 °C), and given food and water ad libitum. Animals were habituated to the laboratory conditions for at least 1 hour before testing. Experiments were performed between 8 am and 6 pm. All experiments were approved by the Ethics Committee for Animal Research of the Universidade Federal de Santa Catarina (Protocol No. PP00621) and were performed in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain on conscious animals [34].

2.2. Sciatic nerve crush injury in rats

Surgical procedures were performed under deep anesthesia induced by a premixed solution containing ketamine (80 mg/kg, intraperitoneally [i.p.]) and xylazine (5 mg/kg, i.p.). After shaving and preparing the skin with 10% povidone iodine, the right sciatic nerve was exposed through a skin incision extending from the greater trochanter to the mid thigh, distally followed by a muscle division. The sciatic nerve was exposed in the right thigh and crushed 1 cm above its trifurcation to ensure good reproducibility of the axonotmesis lesion. The sciatic nerve was crushed once for 30 seconds with 2-mm-wide forceps, as described in a previous report, with minor modifications (smooth forceps) [2]. The sham-operated group was subjected to the surgical procedures, although the sciatic nerve was not crushed.

2.3. Ankle joint mobilization

After a 24-hours recovery period, ankle joint mobilization was performed in lightly anesthetized rats. Anesthesia was induced using 2% isoflurane in 100% oxygen; anesthesia was subsequently maintained using 1% isoflurane. The rats were placed in the lateral recumbent position and subjected to manual ankle joint mobilization. Dorsal flexion and plantar flexion were realized until the end range of motion. Three treatment sessions of 3 minutes were performed with rest intervals of 30 seconds, according to a previously reported dosage regime [25]. Ankle joint mobilization was performed every other day, with 48 hours of rest between each session. Treatment comprised 15 sessions. The control group received the same duration of anesthesia but without mobilization. The following groups were used: sham-operated group (Sham group, $n = 8$); sham-operated + anesthesia group (Sha-

m + Anes group, $n = 8$); sham-operated + ankle joint mobilization group (Sham + AJM group, $n = 8$); crush-operated group (Cr group, $n = 8$); crush-operated + anesthesia group (Crush + / group, $n = 8$); crush-operated + ankle joint mobilization group (Crush + AJM group, $n = 8$).

2.4. Behavioral tests

All behavioral testing was performed blinded with respect to group assignment. Mechanical and thermal hyperalgesia was examined using the von Frey and acetone tests, respectively. Motor functional recovery was performed using the sciatic functional index and static sciatic index before surgery (0) and at 1, 3, 7, 10, 17, 21, 28, and 35 days after surgery (see Section 2.4.3.1). Assessments were made 24 hours after treatment.

2.4.1. Mechanical hyperalgesia

Mechanical thresholds of the right hind paw were assessed using von Frey filament. The withdrawal response frequency of 10 applications of 4-g von Frey filament, each 1 second in duration (VFH, Stoelting; Chicago, IL, USA), was measured as described previously [4]. This test was carried out with the rats confined in glass cylinders (20 cm diameter) and atop an elevated wire platform to allow access to the ventral surface of the hind paw. The response to mechanical stimulation (the number of paw withdrawals) was recorded and expressed as a percentage of the withdrawal response [4].

2.4.2. Cold hyperalgesia

To assess hyperalgesia to cold stimulus, the acetone drop method with minor modifications was used. Briefly, a 50- μ L droplet of acetone was applied to the ventral mid-plantar hind paw using a micropipette. Behavioral responses within the first 20 seconds were scored as follows: (0) no response; (1) one rapid hind paw flick/stamp; (2) 2 or more hind paw flicks/stamps; and (3) persistent flicking/stamping with licking of plantar hind paw. If no response was observed within the initial 20 seconds, scoring continued in the same way for another 20 seconds (if normal response at all, the response is of short latency, but hypersensitive animals display a prolonged period of response episodes) [8]. Acetone application was repeated 3 times for each hind paw, with a 1-minute interval between each application. For each rat, the sum of the 3 scores was then used for data analysis.

2.4.3. Walking track analysis using video recordings

To assess motor recovery, we used the mirror method introduced by Westergaard and Gramsbergen [33]. Briefly, animals were tested in a confined glass walkway with a length of 42 cm, width of 8.2 cm, and height of 15 cm. A mirror was placed at an angle of 45° below the apparatus, and a darkened cage was connected at the end of the corridor to attract the animals. The walking track was recorded with a video camera (Panasonic Camcorder PV-GS19; Panasonic, Secaucus, NJ, USA) while the rat walked through the glass runway. For analyzing the foot placements, 10 still frames were used, 5 of each foot. This image was loaded in a computer using a frame grabber, as described previously [6]. Evaluations of the motor functional recovery were made via digital images, which were captured and analyzed using the image analysis software Image Pro Plus software 6.0 (Media Cybernetics, Bethesda, MD, USA).

2.4.3.1. Sciatic functional index (SFI) and static sciatic index (SSI). From the footprints, the following parameters were obtained: distance from the heel to the third toe, or print length (PL); distance from the first to the fifth toe, or toe spread (TS); and distance from the second to the fourth toe, or intermediary toe spread (ITS).

both dynamic (sciatic functional index [SFI]) and static assessment (static sciatic index [SSI]), all measurements were taken from the experimental (E) and normal (N) sides. The mean distances of 3 measurements were used to calculate the following factors (dynamic and static), as described previously [5]: toe spread factor (TSF) = (ETS – NTS)/NTS; intermediate toe spread factor (ITSF) = (EITS – NITS)/NITS; print length factor (PLF) = (EPL – NPL)/NPL. SFI was calculated, as described previously [1], according to the following equation:

$$\begin{aligned} \text{SFI} &= -38.3 \left(\frac{\text{EPL} - \text{NPL}}{\text{NPL}} \right) + 109.5 \left(\frac{\text{ETS} - \text{NTS}}{\text{NTS}} \right) \\ &+ 13.3 \left(\frac{\text{EIT} - \text{NIT}}{\text{NIT}} \right) - 8.8 \\ &= (-38.3 \times \text{PLF}) + (109.5 \times \text{TSF}) + (13.3 \times \text{ITSF}) - 8.8. \end{aligned}$$

Static foot placings were obtained during at least 4 occasional rest periods. For the SSI, only the parameters TS and ITS were measured, as described previously [1]:

$$\text{SSI} = (108.44 \times \text{TSF}) + (31.85 \times \text{ITSF}) - 5.49.$$

SFI and SSI are standard methods for evaluating crush peripheral nerve injury. The SFI is a tool that evaluates the degree of functional loss (or recovery). It compares parameters from the normal and experimental footprints by a mathematical formula. SFI shows a high correlation with functional recovery [1,5]. The SSI is another way of assessing recovery of function after sciatic injury in animal models. It uses the footprints, acquired when the animal is in a position and related to gait velocity.

Motor functional assessment by means of the SFI and SSI was conducted as previously described [1,5]. A value of 0 corresponds to normal function (ie, both sides with the same functional status), and –100 corresponds to a total functional impairment of one side [1,5].

2.5. Histology

On the 35th day post injury, after functional assessment, the rats were deeply anesthetized with 15% chloral hydrate (0.5 g/kg, i.p.). The distal portion of the right sciatic nerve was excised and immediately immersed in a buffered fixative solution of zinc-formalin (1.6% zinc chloride, 4% formaldehyde, 20% calcium acetate) for 24 hours. After fixation, the samples were placed in 5% potassium dichromate for 5 days. Then they were put in running tap water overnight to wash out all the dichromate before dehydrating in graded concentrations of ethanol. All samples were embedded in paraffin wax. Sections 5- μm thick were obtained, and the slides were stained with Cason [28].

2.6. Morphological analysis

All morphological analysis was performed blinded with respect to group assignment. Once stained, the slices were observed and photographed under light microscopy. Five parameters were quantified: (1) Degeneration debris (%). To reach this parameter, we took a photo at 100 \times magnification. First, we calculated the total area of the nerve; then we recognized areas of debris, connective tissue, and myelinated fibers. The other parameters, (2) area of connective tissue (%), (3) area of myelinated fibers (%), (4) density of myelinated fibers/mm², and (5) myelin sheath thickness (μm), were measured in photographs at 1000 \times magnification. For myelin sheath thickness, we chose a representative area where we could count 10 whole myelinated axons. The histological examination was restricted to endoneuro and the myelin sheath area. Fields with folds or poorly preserved tissue components in histological sections were excluded. Digital images were acquired using a light microscope (Olympus, BX-41; Olympus America, Central Valley, PA, USA) a digital camera (3.3 Mpixel QCOLOR3C, QImaging, Surrey, BC, Canada) and image acquisition software (Qcapture

Pro 5.1, QImaging). Afterward, images were digitized (initially 1000 \times and further amplified 200 \times for analysis). The images were captured by Image Pro Plus Software 6.0 (Media Cybernetics). In each case, photomicrographs of 1600 \times 1200 pixels were obtained from noncoincident and consecutive fields.

2.7. Immunohistochemistry

We aimed to evaluate the immunoreactivity of specific markers, glial fibrillary acidic protein (GFAP) and CD11b/c, as indicators of astroglial and microglial activation, respectively. In this experiment, a naive group was added. Thirty-five days after surgery, rats were deeply anesthetized with 15% chloral hydrate (0.5 g/kg, i.p.) (Vetec Química Fina LTDA, Rio de Janeiro, Brazil) and perfused transcardially with cold phosphate-buffered saline (PBS; 0.1 M; pH 7.4; 300 mL) followed by cold buffered paraformaldehyde (4%; pH 7.4; 500 mL, Vetec Química Fina LTDA). The lumbar spinal cord segments (L4-L5) were resected and cut into 12- μm cryosections with a cryostat (8 sections/animal). The sections were washed with PBS (3 \times 5 minutes) and incubated at room temperature in PBS containing 0.1% Triton X-100 for 30 minutes. To avoid nonspecific labeling, slides were incubated at room temperature for 1 hour in PBS containing 5% normal bovine serum and 1% bovine serum albumin. Subsequently, sections were incubated overnight at 4 $^{\circ}\text{C}$ with the primary antibodies: polyclonal rabbit anti-GFAP (1:500; Dako Cytomation, Carpinteria, CA, USA) and monoclonal mouse anti-rat CD11b/c (1:200; Invitrogen, Grand Island, NY, USA). After incubation, the slides were washed with PBS (3 \times 5 minutes) and incubated at room temperature with secondary antibodies: Alexa Fluor 488 chicken anti-rabbit immunoglobulin G (1:500; Invitrogen) and Alexa Fluor 546 goat anti-mouse immunoglobulin G (1:500; Invitrogen), respectively. The antibodies were diluted in PBS containing 2% normal bovine serum. Following the incubation, the slides were washed with PBS (3 \times 5 minutes). Coverslips were mounted in buffered glycerin (pH 9) and visualized under fluorescence microscopy. To minimize variability in staining, tissues from all treatment groups were run in the same immunohistochemical session. A negative control omitting the primary antibody was performed for all experiments.

2.7.1. Glial activation analysis

To quantify positive glial cells immunoreactivity in the ipsilateral side of dorsal horn of the spinal cord, 3 sections per animal were randomly selected and analyzed by experimenters blinded as to the treatment groups. Three to five rats were included in each group. Identification of laminae areas was visualized by Hoechst staining of cell nuclei. Images were captured with an epifluorescent light microscope (Olympus, BX-41) equipped with adequate filters for Fluorescein isothiocyanate (FITC) and Alexa Fluor 546 (U-MWG2 and U-MWB2, Olympus), a digital camera (3.3 Mpixel QCOLOR3C, QImaging), and image acquisition software (Qcapture Pro 5.1, QImaging). From each sample, photomicrographs of 1600 \times 1200 pixels of resolution were obtained from each animal. The images were visualized under 10 \times magnification, captured in a predefined region of interest in the lumbar dorsal horn (L4–L5), including laminae I and II (superficial laminae). Image processing and analysis were performed with the CHPTool 5.0 program as previously described [26]. The mean fluorescence intensity occupied by the immunoreactive tissue was used as a descriptor for the magnitude of astrocytes and microglial activation in immunolabeled sections. [26]. We use a semiautomatic method for the identification of immunohistochemical (IHC) staining in digitized samples. Briefly, some typical positive stained regions on a sample image were stained and used as a reference for the construction of a distance metric. In this analysis, the global optimum is obtained by induction employing higher polynomial terms of the Mahalanobis

distance, extracting nonlinear features of the IHC pattern distributions. A nonlinear color distance metric was used for the identification of spinal cord immunohistochemical staining [26]. This method showed a high correlation to a pathologist's manual analysis [26]. Measurements were performed by an experimenter who was blind to the experimental group. The proposed approach has an accurate discrimination between stained and nonstained areas of the IHC patterns, avoiding the need of threshold color plugging commonly used on related approaches. Data are presented as the mean \pm SEM of the GFAP- and CD11b/c-immunoreactive mean fluorescence intensities in arbitrary units.

2.8. Statistical analysis

Behavioral testing was compared using 2-way analysis of variance for repeated measures, with Bonferroni multiple comparison as the post hoc test. Histological and immunohistochemical analysis were performed using one-way analysis of variance following Student–Newman–Keuls test. Results are presented as the mean \pm SEM for each group. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of ankle joint mobilization on mechanical and cold hyperalgesia in rats after sciatic nerve injury

Sciatic nerve crush produced marked and long-lasting mechanical and cold hyperalgesia, with behavioral abnormalities still evident 17 and 35 days, respectively, after the injury compared with baseline response ($P < 0.001$). Relevantly, we found that ankle joint mobilization treatment produced a significant mechanical antihyperalgesic effect on days 3 ($P < 0.001$), 7

($P < 0.001$), 10 ($P < 0.001$), and 17 ($P < 0.05$) when compared the Crush and Crush + Anes group (Fig. 1A). The results presented in Fig. 1C show that the Crush + AJM group exhibited a decrease in cold hyperalgesia at day 3 ($P < 0.01$) compared to the Crush and Crush + Anes groups. Sham, Sham + Anes, and Sham + AJM groups did not change mechanical or cold thresholds (Fig. 2B and D).

3.2. Effect of ankle joint mobilization on motor functional recovery after sciatic nerve injury

There were no signs of functional impairment on the last day of the assessment period. The Crush and Crush + Anes groups displayed an accelerated pattern during the entire assessment period as demonstrated by the SFI and SSI (Fig. 2A and C). In the first post lesion, the animals with nerve injury showed the most pronounced loss of function, but recovered gradually after this. In marked contrast, the Crush + AJM group showed a significant increase in motor performance when observed on days 7 ($P < 0.05$), 10 ($P < 0.05$), 14 ($P < 0.001$), and 17 ($P < 0.01$), measured by SFI, and on days 7 ($P < 0.05$) and 17 ($P < 0.05$), measured by SSI, compared with the Crush and Crush + Anes groups (Fig. 2A and C). Sham, Sham + Anes, and Sham + AJM groups did not change motor functional recovery (Fig. 2B and D).

3.3. Effect of ankle joint mobilization on morphological recovery of sciatic nerve in rats after sciatic nerve injury

The Crush and Crush + Anes groups showed a lower average myelin sheath thickness and percentage of myelinated fiber compared to Sham group ($P < 0.05$, Figs. 3A, B and D and 4A/B, respectively). However, there was an increase in the percentage

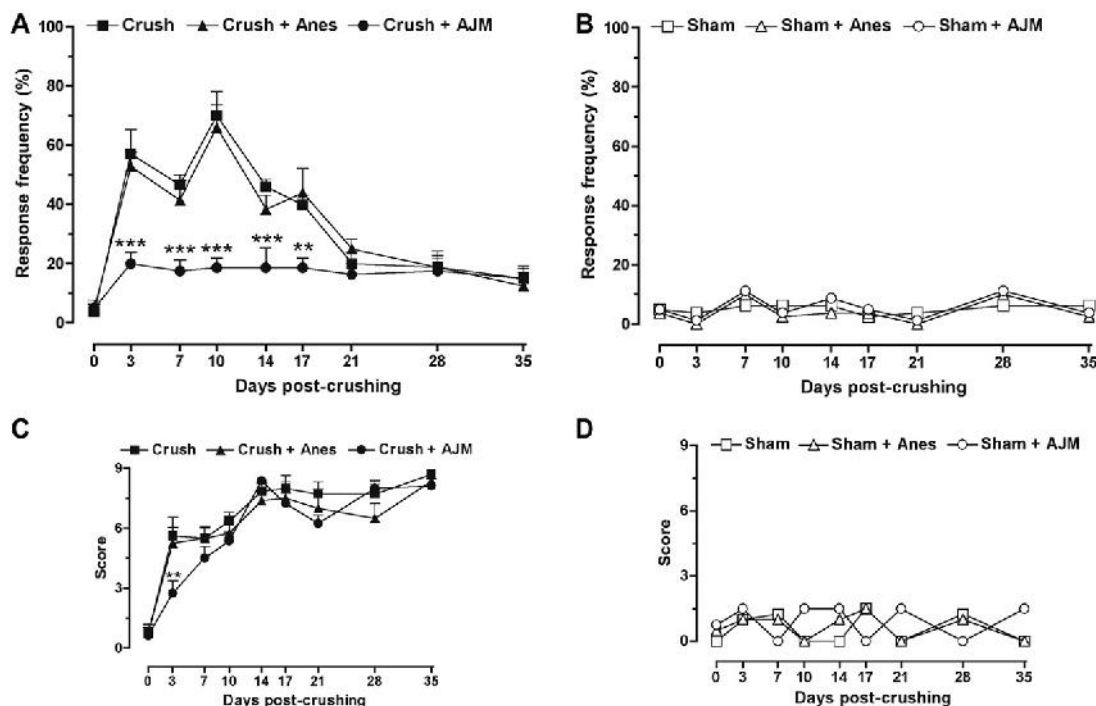


Fig. 1. Effect of ankle joint mobilization on hyperalgesia in rats after sciatic nerve injury. Mechanical hyperalgesia (A and B) and cold hyperalgesia (C and D). Values represent the mean of 8 animals; vertical lines indicate SEM. $**P < 0.01$, $***P < 0.001$. Sham, sham-operated; Sham + Anes, sham-operated + anesthesia; Sham + AJM, sham-operated + ankle joint mobilization; Crush, Crush-operated; Crush + Anes, Crush-operated + anesthesia; Crush + AJM, Crush-operated + ankle joint mobilization.

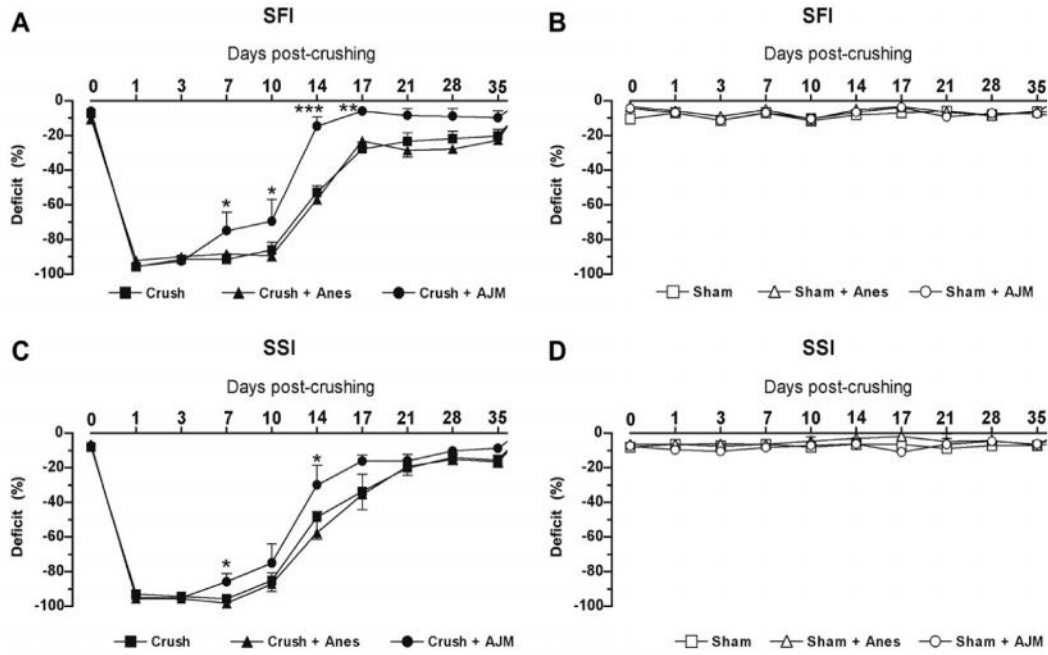


Fig. 2. Effect of ankle joint mobilization on motor functional recovery. Sciatic functional index (SFI, A and B) and static sciatic index (SSI, C and D). Values represent the mean of 8 animals; vertical lines indicate SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Sham, sham-operated; Sham + Anes, sham-operated + anesthesia; Sham + AJM, sham-operated + ankle joint mobilization; Crush, Crush-operated; Crush + Anes, Crush-operated + anesthesia; Crush + AJM, Crush-operated + ankle joint mobilization.

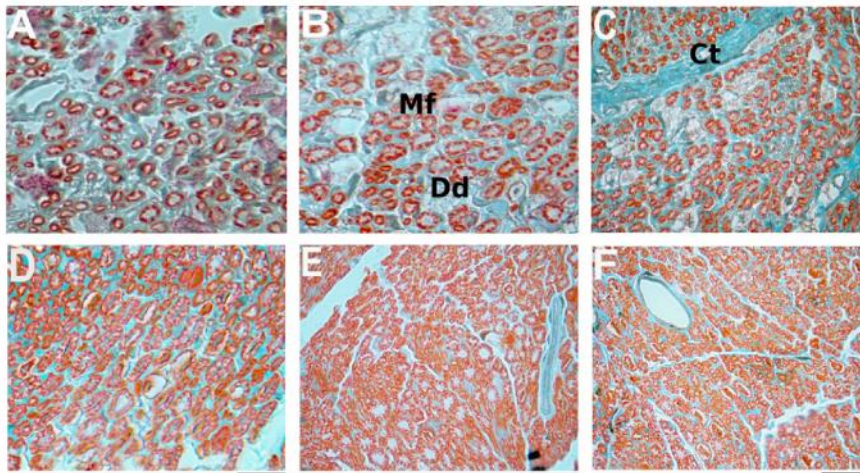


Fig. 3. Light micrographs obtained from regenerating sciatic nerves 5 weeks after nerve crush. Sections stained with Mason's trichrome. Crush and Crush + anesthesia groups (A and B), showing the predominance of small-diameter thin myelin sheath fibers, increased endoneurial connective tissue between the nerve fibers and the presence of degeneration debris; Crush + ankle joint mobilization group (C), showing the distal portion of regenerating nerves, and myelinated fibers appear similar to the Sham-operated. Sham-operated, sham-operated + anesthesia and sham-operated + ankle joint mobilization (D, E and F, respectively). Dd, degeneration debris; Mf, myelinated fiber; Ct, connective tissue. Scale bar = 5 μ m.

of endoneurial connective tissue area and the percentage of degeneration debris area when compared with the Sham group ($P < 0.05$, Figs. 3A and B and 4C and D). Only the average myelin sheath thickness of the Crush + AJM group was larger than in the Crush and Crush + Anes groups ($P < 0.05$, Figs. 3A–C and 4A). Sham, Sham + Anes, and Sham + AJM groups did not change morphological recovery (Fig. 3D, E, and F).

3.4. Effect of ankle joint mobilization on GFAP and CD11b/c immunoreactivity in the spinal cord of rats after sciatic nerve injury

Analyses of glial markers in spinal cord sections postsciatic nerve crush demonstrate glial activation. Compared to naive rats, at day 35 postsciatic nerve crush, in Crush ($P < 0.01$) and Crush + Anes ($P < 0.001$) groups, we observed an increased expression of

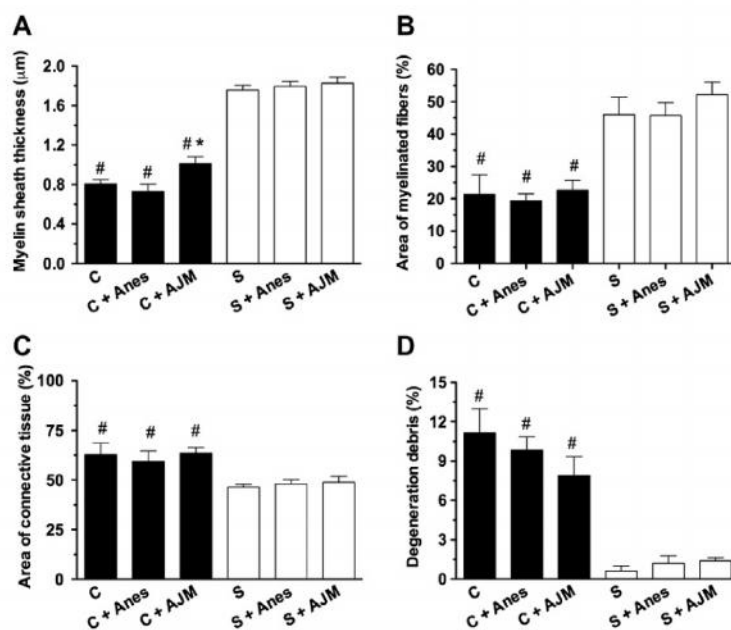


Fig. 4. Effect of ankle joint mobilization on the morphological recovery of the sciatic nerve in rats after sciatic nerve injury. Morphometric quantitative assessment of myelin sheath thickness (A), area of myelinated fibers (B), connective tissue (C), and degeneration debris (D) of regenerated sciatic nerve fibers at week 5 after axotomy. Values represent the mean of 8 animals; vertical lines indicate SEM. [#] $P < 0.05$ vs Sham; ^{*} $P < 0.05$ vs Crush-operated (determined by one-way analysis of variance followed by Student–Newman–Keuls test). S, sham-operated; S + Anes, sham-operated + anesthesia; S + AJM, sham-operated + ankle joint mobilization; C, Crush-operated; C + Anes, Crush-operated + anesthesia; C + AJM, Crush-operated + ankle joint mobilization.

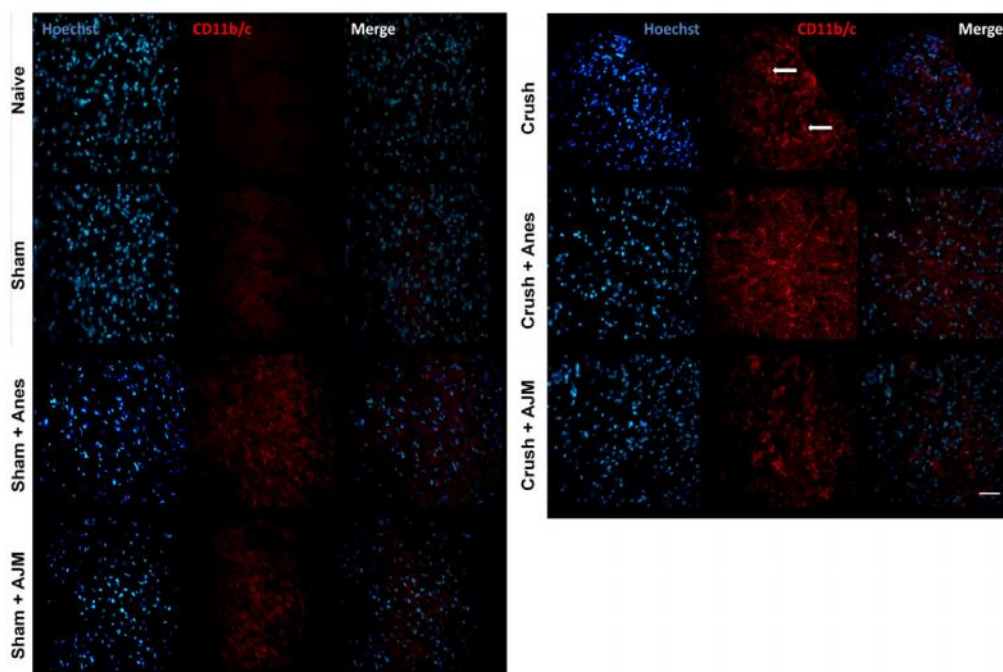


Fig. 5. Ankle joint mobilization inhibited the CD11b/c expression in the spinal cord of rats after sciatic nerve injury. The spinal lumbar cords (L4–L5) obtained at the 35th day after surgery from different experimental groups were processed for immunohistochemistry assays. Representative images of microglial activation (CD11b/c) immunoreactivity in the spinal cord from naive, sham-operated (Sham), sham + anesthesia (Sham + Anes), sham + ankle joint mobilization (Sham + AJM), crush (Crush), crush + anesthesia (Crush + Anes), crush + ankle joint mobilization (Crush + AJM) were captured. Specifically, 8 alternate 12- μm sections of dorsal horn of lumbar spinal cord (3–5 mice per group) with an individual distance of 150 μm were obtained between L4 and L5 regions. Representative sections from 2 independent experiments are shown. Scale bar corresponds to 25 μm and applies throughout.

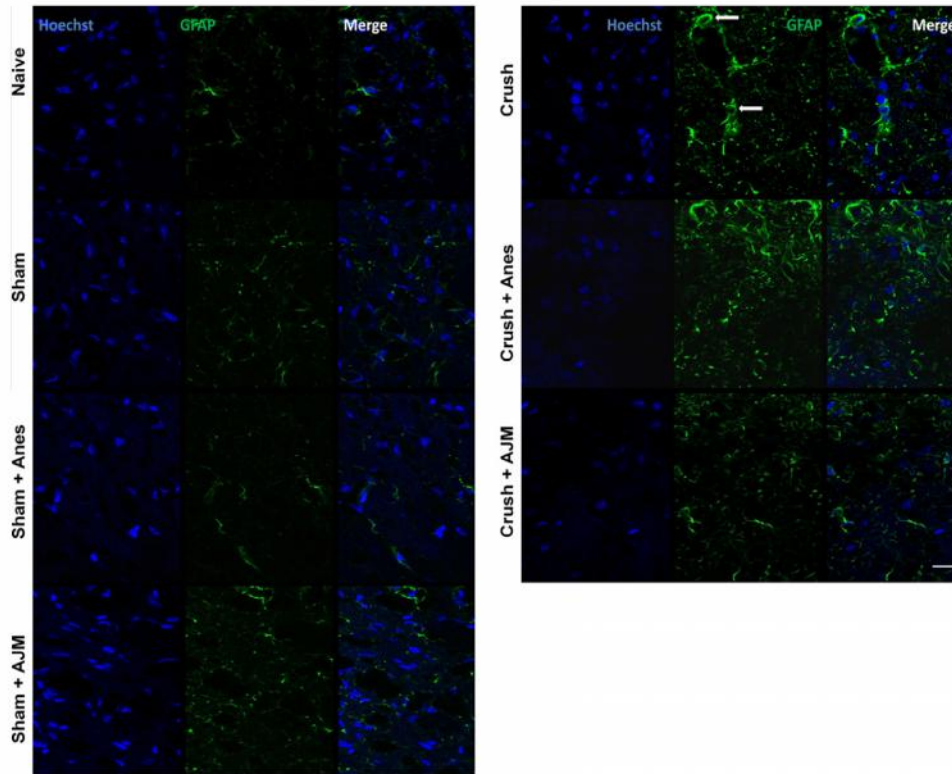


Fig. 6. Ankle joint mobilization suppressed the astrocytes activation in the spinal cord of rats after sciatic nerve injury. The spinal lumbar cords (L4–L5) obtained at the 35th day after surgery from different experimental groups was processed for immunohistochemistry assays. Representative images of astroglial protein [glial fibrillary acidic protein (GFAP)] immunoreactivity in the spinal cord from naive, sham-operated (Sham), sham + anesthesia (Sham + Anes), sham + ankle joint mobilization (Sham + AJM), crush (Crush-operated), crush + anesthesia (Crush + Anes), crush + ankle joint mobilization (Crush + AJM) were obtained. Specifically, 8 alternate 12- μ m sections of dorsal horn of lumbar spinal cord (3–5 mice per group) with an individual distance of 150 μ m were obtained between L4 and L5 regions. Representative sections from 2 independent experiments are shown. Scale bar corresponds to 25 μ m and applies throughout.

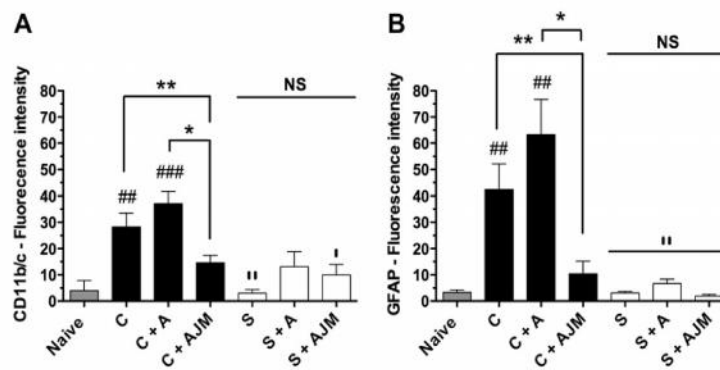


Fig. 7. Effect of ankle joint mobilization on microglial and astroglial activation markers in the dorsal spinal cord in rats after sciatic nerve injury. Graphic representation of the CD11b/c (A) and glial fibrillary acidic protein (GFAP) (B) immunostaining in the spinal cord. Thirty-five days after surgery, specifically, 8 alternate 12- μ m sections of the dorsal horn of lumbar spinal cord were obtained between L4 and L5 from naive group, naive, sham-operated (Sham), sham + anesthesia (Sham + Anes), sham + ankle joint mobilization (Sham + AJM), crush (Crush-operated), crush + anesthesia (Crush + Anes), crush + ankle joint mobilization (Crush + AJM), and were analyzed for immunoreactivity of CD11b/c (A), and GFAP (B). Data are presented as mean \pm SEM of 3–5 mice/group and are representative of 2 independent experiments. The symbols denote the levels of significance: * P < 0.05 and *** P < 0.001 vs naive and sham group; * P < 0.05 and ** P < 0.01; # P < 0.05 and ## P < 0.01 vs Crush-operated group and nonsignificant (NS) (one-way analysis of variance followed by Student–Newman–Keuls test).

microglia (representative examples in Fig. 5) and of astrocytes markers Crush ($P < 0.01$) and Crush + Anes ($P < 0.01$) (representative example in Fig. 6). In comparing Crush + Anes groups to Crush + AJM, we detected a significantly decreased expression of microglia and astrocytes markers. Quantified data showed significant differences in the expression of microglia and astrocytes markers between Sham, Crush, Crush + Anes, and Crush + AJM groups ($P < 0.05$; $P < 0.01$; Fig. 7A and B, respectively). Sham, Sham + Anes, and Sham + AJM groups did not change expression glial cells markers (Fig. 7A and B).

4. Discussion

Stretch-related injuries sustained in motor vehicle accidents account for the most common nerve trauma. Severe nerve injury has a shocking impact on patients' quality of life [21]. Typical symptoms are sensory and motor function defects that could result in complete paralysis of an affected limb or development of intractable neuropathic pain. Here, we examined the effect of ankle joint mobilization on sciatic nerve injury, focusing on motor and sensory (neuropathic pain) functional deficits induced by injury. A recent report suggests that joint mobilization reduces hyperalgesia associated with chronic muscle and joint inflammation in rats [24]. However, whether joint mobilization can suppress neuropathic pain and enhance nerve regeneration remains unknown.

The main results emerging from the present study are, to the best of our knowledge, the first evidence showing that *in vivo* sciatic nerve crush produced neuropathic pain (mechanical and cold hyperalgesia), and ankle joint mobilization caused pronounced and long-lasting analgesic effects. Furthermore, we found that nerve injury induced activation of glial cells in dorsal horn neurons. Our data also show that the mechanisms responsible for ankle joint mobilization-mediated analgesic actions are primarily associated with their ability to prevent the activation of microglial and astroglial cells in dorsal horn of spinal cord of rats after sciatic nerve crush-induced neuropathic persistent pain.

Astrocytes and microglia (collectively referred to as glia) have well-documented roles in pain facilitation: they can modulate neuronal synaptic function and neuronal excitability [14]. Altered activity of primary sensory nociceptors is relevant to the pain pathway, leading to persistent neuropathic pain. The first pain-relevant chemical signals to be identified at first-order synapses include glutamate and substance P. In addition, chronic astrocytes activation after nerve injury has been shown to involve downregulation of excitatory amino acid transporters, glutamate transporter 1 and glutamate-aspartate transporter, leading to a decrease in glutamate uptake and an increase in excitatory synaptic transmission [14].

It has been hypothesized that mobilization may activate descending pain inhibitory systems, mediated supraspinally. Recent animal studies show that the analgesia produced by joint mobilization involves serotonin and norepinephrine receptors in the spinal cord, thereby supporting a role for descending pain modulatory systems [23]. The spinal cord mechanisms by which the descending norepinephrine and serotonin systems relieve pain appear to involve the following: (1) direct hyperpolarization of gelatinous substance neurons, (2) inhibition of glutamate release from the A δ and C-afferent fibers, and (3) increase of γ -aminobutyric acid and glycine release from interneurons [30]. Thus, points 1 and 2 seem to form a common underlying mechanism that leads to decreased glutamate release in the spinal cord and therefore reduced glial activation.

As shown in the present study (Fig. 1A), mechanical hyperalgesia was reduced by ankle joint mobilization treatment markedly for at least 17 days, whereas cold hyperalgesia was reduced only during the first 3 days (Fig. 1C). Our data confirm and largely

extend previous data, which demonstrated that joint mobilization produces a specific hypoalgesic effect to mechanical but not to thermal pain stimuli [29]. Interestingly, these studies have shown that mechanical hyperalgesia uses noradrenergic neurons, whereas thermal antinociception occurs through serotonergic pathways [12]. These previous findings and the present results have led to the hypothesis that joint mobilization might selectively stimulate a specific activation of endogenous descending pain inhibitory system.

Another interesting finding demonstrated in the present study was that sciatic nerve crush caused motor performance deficit. The most pronounced loss of motor function occurred in the first day post lesion and recovered gradually after this time. Moreover, the present study is the first to demonstrate that the ankle joint mobilization accelerated nerve regeneration in rats after axonotmesis.

Available data indicate that the detrimental consequences of muscle disuse can be ameliorated by passive range of motion physiotherapy [15]. Passive mobilization or stretches may increase muscle protein synthesis, slow protein degradation in innervated muscle, stimulate reinnervation, retard denervation atrophy, increase muscle tension, and influence the process of healing in bone cartilage, tendons, ligaments, and skin [15,16]. These findings were extended and confirmed by other authors, who demonstrated that passive mobilization influences skeletal muscle and therefore functional recovery. Additionally, rats with denervated soleus muscle submitted to repetitive stretches (every 5 seconds for 15 minutes/day) for 2 weeks exhibit reduced denervation-induced muscle atrophy, as assessed by histochemical analysis (cross-sectional area of denervated soleus muscle fibers) [20]. Furthermore, Watson and coauthors demonstrated that ankle passive range of motion physiotherapy for 2.5-minutes sessions each day conducted in rats with denervation of the ankle plantar flexors improves force production in these muscles after 14 days of treatment [32].

A direct comparison of our study with others examining functional recovery is not easy, mainly because previous reports quantified the passive mobilization effect in other models. It is important to note that these studies also demonstrated that ankle joint mobilization reversed muscle atrophy and increased strength but due to the limitations of the experimental model, they did not show the influence that passive mobilization exerts on nerve regeneration. In this regard, the present study markedly demonstrates that passive joint mobilization (Crush + Anes vs Crush + -AJM) accelerates the processes of sciatic nerve regeneration, as assessed by SFI, SSI, and morphological analysis (enhanced myelin sheath thickness).

Studies have shown that forces such as passive stretching and active contraction may counteract various kinds of skeletal muscle atrophy [27]. Moreover, there is evidence that passive mechanical stimulation of the sarcolemmic membrane may reduce muscle atrophy. Stretch-activated channels (SACs) of the sarcolemma and the cytoskeleton permit a Ca^{2+} inflow that activates Ca^{2+} -dependent signaling. SACs are expressed in normal soleus muscle and SAC currents are potentiated by muscle stretching [27]. These facts suggest that the effects reported here are in agreement with the hypertrophy-inducing effects of exercise and with the effects of functional electrical stimulation in denervated humans [27]. In addition, brain-derived neurotrophic factor [10], glial cell line-derived neurotrophic factor [11], neurotrophin 3 [10], and nerve growth factor [22] are synthesized in skeletal muscle. Therefore denervated target muscle tissue may be an important source for neurotrophic factors during sciatic nerve regeneration [10]. Thus these previous findings and the present results suggest that passive joint mobilization stimulates skeletal muscle similarly to electrical stimulation or active exercise that results in release of neurotrophic factors, which enhance functional recovery nerve regeneration. Other studies should address the same question by

analyzing neurotrophic factor expression in nerve and muscle to clarify the mechanism of the passive mobilization action. Furthermore, the precise mechanism for the enhanced nerve regeneration and reduction of neuropathic pain induced by axonotmesis after prolonged periods of joint mobilization treatment needs further elucidation.

In the present study, no difference in sensory and motor functional recovery, morphological and immunohistochemical analysis was found between Crush and Sham group, regardless of whether or not it received isoflurane administration. Such results discard the possible confounded effect induced by anesthesia that could be an interaction between anesthesia and ankle joint mobilization.

In summary, the present study demonstrates that sciatic nerve crush produced mechanical and cold hyperalgesia, a motor performance deficit, and glial activation in the dorsal horn of the spinal cord. Furthermore, we found that ankle joint mobilization suppressed pain behavior and attenuated astroglial activation, as well as accelerated motor functional recovery. This experimental study on nerve regeneration suggests that joint mobilization can restore motor and nerve function to a substantial degree when initiated at the earliest possible postinjury stage. These findings could have direct therapeutic applications for preserving function after peripheral nerve injury and could provide a basis for new approaches to treating nerve injury.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgements

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4 DISCUSSÃO

A dor pós-operatória aguda permanece como um significativo problema médico. O paciente pós cirúrgico apresenta dor pós-operatória clinicamente significativa, mesmo quando é realizada a combinação da administração de drogas opióides e não-opióides ou administração de narcóticos parenterais (APFELBAUM et al., 2003). A dor ao repouso é geralmente de moderada a média e no escore da escala de dor análoga visual (VAS) é de 3 a 4 nos primeiros 3 dias após a cirurgia, sendo 10 pontos o máximo desta escala (MOINICHE et al., 1997). Geralmente, a dor no repouso persiste somente na primeira semana após a cirurgia. A dor durante as atividades tais como: andar ou tossir, é mais severa durante os primeiros 3 dias, apresentando escore entre 7 a 8 na VAS (SINGELYN et al., 1998).

A aplicação de movimentos passivos nas articulações para o tratamento da dor é uma prática muito antiga de terapia manual. Embora a mobilização vertebral e periférica continua sendo amplamente aplicada na prática clínica, há poucos dados experimentais fundamentando sua eficácia na redução da dor ou na melhora da função. Vários ensaios clínicos randomizados sobre manipulação da coluna vertebral e técnicas de MA foram documentados, permitindo a realização de revisões sistemáticas e meta-análises (AKER et al., 1996; HURWITZ et al., 1996). Os primeiros estudos sugerem efeitos benéficos da manipulação da coluna vertebral ou MA na dor, flexibilidade, e melhora da aptidão física, no entanto, esses efeitos perduram por curto período de tempo (OTTENBACHER et al., 1985). O uso da manipulação e MA no tratamento da dor cervical também foi investigado através de revisões

sistemáticas e meta-análises (AKER et al., 1996; HURWITZ et al., 1996). De acordo com uma destas revisões, Hurwitz et al. (1996) concluíram que os efeitos de ambas, tanto da manipulação quanto da MA, apresentam curta duração no tratamento da dor aguda e subaguda na região cervical. Eles também concluíram que estas técnicas de terapia manual são benéficas no tratamento de cefaléia tensional.

4.1 MAT REDUZ DOR PÓS-OPERATÓRIA

Os resultados do presente estudo corroboram com os achados clínicos supra citados, pois, verificou-se que o tratamento com a MAT nos camundongos apresenta efetividade por um curto período de tempo, muito similar ao que é encontrado nos estudos clínicos. Deste modo, enfatiza-se que o modelo de MAT experimental utilizado apresenta grande relevância, traduzindo na bancada experimental os eventos que são observados na prática clínica, validando o presente modelo.

De acordo com nosso conhecimento, o primeiro estudo pré-clínico a reportar o uso da MA no tratamento da dor foi realizado por Sluka e Wright (2001). Os pesquisadores utilizaram a MA do joelho no tratamento da dor causada por injeção intra-articular de capsaicina no tornozelo de ratos. Os autores observaram que somente as mobilizações realizadas por 9 ou 15 minutos, mas não 3 minutos, aumentam o limiar de resposta (mecânico) por até 30 minutos após o tratamento. Assim os autores concluíram que a MA do joelho (9 ou 15 minutos de duração) produz uma significativa redução da hiperalgesia mecânica. O segundo trabalho do mesmo grupo foi um pouco mais além, pois analisou os mecanismos neurofisiológicos espinais da MA. Utilizando os mesmos

modelos de dor (capsaicina intra-articular) e MA (joelho) os autores demonstraram que o bloqueio dos receptores (α)₂-adrenérgico ou 5-HT_{1/2} com metisergida ou com NAN-190, previnem o efeito antihiperalgésico da MA. Com base nestes resultados os autores sugerem que a antihiperalgesia produzida pela MA envolve mecanismos inibitórios da dor descendentes que utilizam serotonina e noradrenalina (SKYBA et al., 2003). Na publicação mais recente deste mesmo grupo sobre o tema, os autores avaliaram o efeito da MA do joelho na hiperalgesia associada com inflamação articular e muscular crônica em ratos. Em animais com inflamação crônica, a MA do joelho reduziu a hiperalgesia bilateral quando realizada na 1^o, 2^o e 4^o semanas após a indução da inflamação. No entanto, nos animais com inflamação articular, a MA do joelho diminuiu a hiperalgesia somente na 4^o semana após a indução da inflamação articular. Portanto, os autores demonstraram que a MA do joelho reduz a hiperalgesia bilateral induzida pela inflamação muscular e articular crônica (SLUKA et al., 2006).

Os estudos citados acima serviram como base para os parâmetros utilizados no presente estudo. Semelhante aos tempos adotados pelo estudo de Sluka e Wright (2001), no presente estudo observou-se que a MAT também reduziu a hiperalgesia mecânica quando realizada por 9 minutos, mas não quando realizada por 3 minutos. Além disso, estes resultados estendem os dados da literatura por demonstrar que a MAT também produz efeito antihiperalgésico no modelo de dor pós-operatória.

4.2 MECANISMOS NEUROFISIOLÓGICOS DA MAT

4.2.1 Envolvimento do sistema opioidérgico

Estudos foram realizados para avaliar uma variedade de tratamentos manuais sobre os níveis de beta-endorfinas (BE), um tipo de opióide endógeno. Assim, os trabalhos de Kaada e Torsteinbo (1989) e Vernon et al. (1986) demonstraram uma correlação positiva entre tratamentos manuais e aumento nos níveis de BE (massagem do tecido conectivo e manipulação espinal). No entanto, outros pesquisadores não encontraram tal correlação (SANDERS et al., 1990; SOUVLIS; WRIGHT, 1997). Isto pode ocorrer em relação às variáveis metodológicas, pequeno número amostral, que levaram aos resultados inconsistentes destes estudos. Assim, conclusões sólidas sobre a elevação dos níveis de BE pelos tratamentos manuais, não podem ser tiradas destes estudos e devem ser investigada adequadamente (DEGENHARDT et al., 2007). Por outro lado, foi descrito que o efeito produzido com o movimento articular passivo, é inadequadamente explicado pela teoria da comporta da dor e pode ser capaz de ativar o sistema opioidérgico (ZUSMAN; EDWARDS; DONAGHY, 1989).

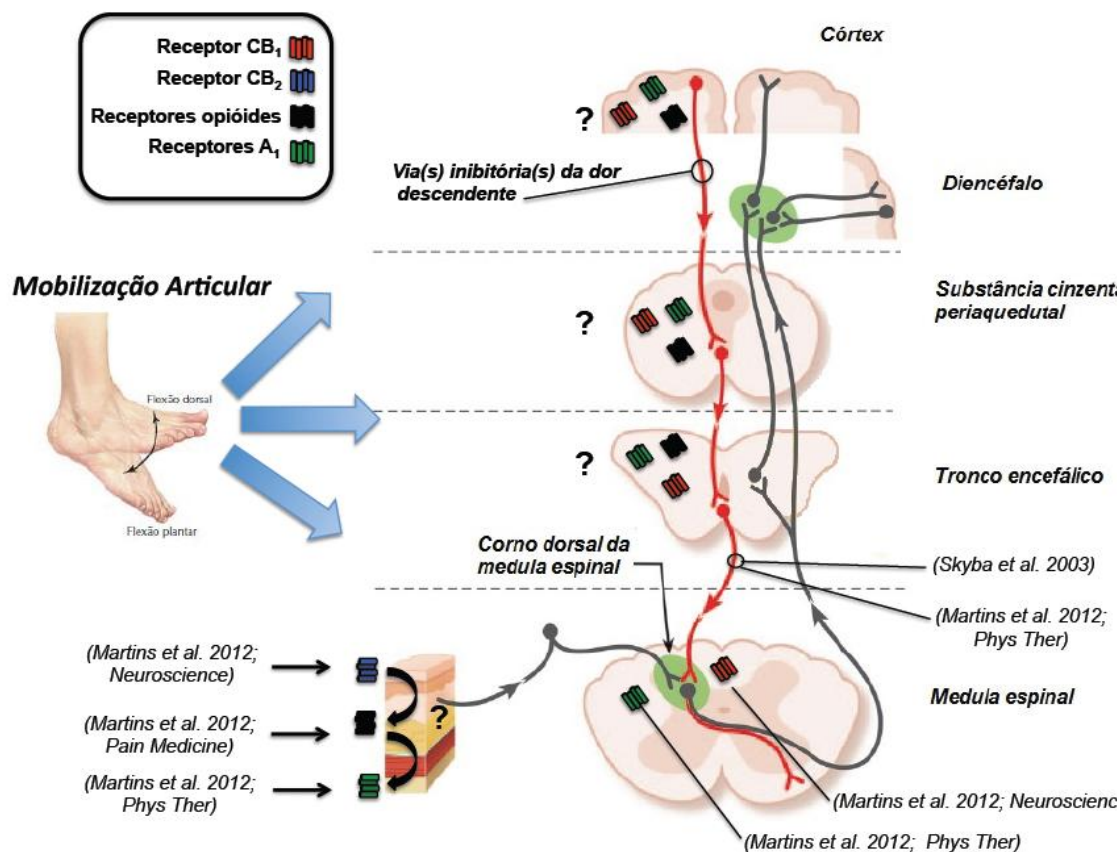
Primeiramente é preciso ter em mente que as TTMs englobam procedimentos, tais como: manipulações ou mobilizações da coluna vertebral, mobilizações do sistema nervoso ou muscular, conceito Mulligan (mobilização com movimento ativo), mobilizações das articulações periféricas (tema do presente estudo), dentre outras. A maioria das pesquisas que procuram demonstrar os efeitos neurofisiológicos da terapia manual se concentram em técnicas na

coluna vertebral, sugerindo que a analgesia induzida pela mobilização não envolve a liberação de opióides endógenos (WRIGHT, 1995). Esta afirmação surgiu de estudos em humanos que mostraram que a analgesia induzida por TTM (manipulação da coluna vertebral) não foi antagonizada pela administração de naloxona (VICENZINO et al., 2000), não produziu tolerância (SOUVLIS; WRIGHT, 1997) e ocorreu sem mudanças nos níveis plasmáticos de endorfinas (SANDERS et al., 1990). É importante ressaltar que, até o presente momento, não há estudos na literatura realizados com técnicas de mobilização articular periférica passiva, sendo todos os estudos supracitados realizados com técnicas de mobilização ou manipulação da coluna vertebral ou técnicas de mobilização com movimento, sendo assim impossível compará-los com o presente estudo.

No entanto, o trabalho de Skyba et al. (2003), mencionado anteriormente, analisou o efeito da administração de naloxona espinal para verificar a contribuição dos receptores opióides espinais na antihiperalgisia produzida pela MA do joelho. Os autores não observaram diferença no limiar mecânico destes animais sugerindo que os receptores opióides não participam deste efeito. Com base neste estudo e na literatura que apóia a hipótese opioidérgica como mecanismo neurofisiológico da TM, a primeira tentativa foi de verificar um possível papel do sistema opioidérgico no efeito antihiperalgésico da MAT através da administração sistêmica de naloxona, antagonista não seletivo dos receptores opióides. Assim, foi observado que a naloxona previne completamente o efeito antihiperalgésico da MAT em camundongos submetidos à dor pós-operatória aguda, corroborando a hipótese de que opióides endógenos podem mediar o efeito

antihiperálgico da MAT. No entanto, é importante salientar que a resposta contrastante entre os resultados demonstrados por Skyba et al. (2003) e os obtidos no presente estudo poderia ser resultante do sítio de ação. Para sanar esta dúvida, a naloxona foi administrada perifericamente (local) na pata dos animais como uma forma de verificar especificamente o papel dos receptores opióides periféricos sobre o efeito antihiperálgico da MAT. Nota-se que o efeito antihiperálgico da MAT foi completamente prevenido pela injeção de naloxona na pata. Assim, os resultados do presente estudo sugerem que o efeito observado pela administração sistêmica de naloxona é devido a sua ação periférica. A figura 11 apresenta um resumo esquemático dos mecanismos de ação neurofisiológicos da MA, e seus respectivos sítios de ação, com a compilação de trabalhos prévios e os resultados do presente estudo.

Figura 11 - Compilação dos mecanismos neurofisiológicos da MA estudados até o momento.



Fonte: Elaboração dos autores, 2013.

A figura demonstra que de acordo trabalhos prévios e os dados do presente estudo (publicados ou submetidos para publicação) a MA pode ativar os receptores CB₂ (azul), opióides (preto) e/ou A₁ (verde) periféricamente (pata) ou os receptores CB₁ e/ou A₁ centralmente (espinal). Além disso, mostra que a MA pode ativar vias inibitórias da dor descendentes (flechas vermelhas).

Com base na constatação do envolvimento dos receptores opióides periféricos no efeito antihiperálgico da MAT, a pergunta seguinte foi pautada na origem deste efeito, ou melhor, destes opióides periféricos. Examinados os alvos farmacológicos tradicionais, tentou-se elaborar uma hipótese de trabalho que fosse plausível o suficiente para ser testada. Encontrou-se na literatura que células imunes contendo opióides migram preferencialmente para locais inflamados, onde

liberam β -endorfinas que ativam receptores opióides periféricos inibindo a dor (STEIN, 1995), sendo assim, os leucócitos são importante fonte de opióides endógenos locais (MACHELSKA et al., 1998). Neste sentido, foi demonstrado que o polissacarídeo fucoidina possui a capacidade de ligar-se potently às selectinas bloqueando o rolamento dos leucócitos, levando a uma redução no acúmulo dos mesmos no sítio inflamatório (STEIN, 1995). No entanto, os resultados dos experimentos desenhados para testar esta hipótese demonstraram que a fucoidina não altera o efeito antihiperálgico da MAT, refutando a hipótese de que a MAT poderia de alguma forma aumentar os níveis locais de opióides através dos leucócitos. Desta forma, outras explicações para o efeito periférico da MAT seriam: 1) outras células inflamatórias como fontes de opióides endógenos locais; ou 2) a disponibilidade de receptores opióides nas terminações periféricas das fibras aferentes primárias. No entanto, estas hipóteses ainda precisam ser investigadas em estudos futuros.

4.2.2 Envolvimento do sistema adenosinérgico

A adenosina regula a transmissão da dor na periferia e na medula espinal, e vários agentes podem alterar a disponibilidade extracelular de adenosina e subsequentemente modular a transmissão da dor, particularmente pela ativação dos receptores adenosinérgicos do tipo A₁ (SAWYNOK, 1998; SAWYNOK; LIU, 2003).

Goldman et al. (2010) recentemente demonstraram o papel da ativação dos receptores adenosinérgicos periféricos no efeito antinociceptivo da acupuntura. Os autores hipotetizaram que tratamentos

não alopáticos da dor, tais como: quiropraxia e/ou massagem, modalidades que envolvem mobilizações das articulações ou músculos, poderiam também ter seus efeitos associados com um efluxo de ATP citosólico suficiente para elevar os níveis de adenosina extracelular. A adenosina, assim, seria outro candidato a mediador endógeno dos efeitos antihiperalgésico da MAT. Para testar a hipótese de que a adenosina poderia mediar os efeitos da MAT, inicialmente verificamos o efeito da cafeína nos três principais sítios de modulação adenosinérgica (i.p., i.t. e i.pl.), com o intuito de analisar o possível envolvimento dos receptores A_1 e A_{2A} neste efeito. Os resultados do presente estudo demonstraram que a cafeína previne o efeito antihiperalgésico da MAT em todos os sítios testados. Do ponto de vista de importância clínica, a interpretação destes resultados sugere que o consumo de cafeína deve ser evitado para não comprometer a eficácia da MAT. A partir desta informação, voltaram-se as atenções aos receptores A_1 , os quais são os subtipos de receptores com ação antinociceptiva mais bem caracterizados, tanto na periferia quanto na medula espinal (SAWYNOK; LUI, 2003). Desta forma, é importante verificar diretamente o papel específico dos receptores A_1 , tanto em sítio periférico, quanto espinais, nesta antihiperalgesia produzida pela MAT. Por isso testou-se os efeitos da administração de DPCPX na pata ou na medula espinal. Observa-se que o pré-tratamento dos animais com DPCPX previne o efeito antihiperalgésico da MAT. Seguindo esta linha de raciocínio e com estes experimentos sugere-se que a prevenção do efeito antihiperalgésico da MAT pela cafeína é resultante do bloqueio periférico e espinal dos receptores A_1 .

4.2.3 Envolvimento do sistema endocanabinóide

Tem sido demonstrado que endocanabinóides como a AEA e o 2-AG produzem ação antinociceptiva em modelos pré-clínicos de dor, incluindo dores agudas, inflamatórias e neuropáticas (DOGRUL et al., 2002 GUNDUZ et al., 2011; HOHMANN; SUPLITA, 2006). Além disso, os efeitos analgésicos dos canabinóides também foram observados em humanos saudáveis e pacientes com dores (HOSKING; ZAJICEK, 2008). No presente estudo, foi verificado também a relevância do sistema endocanabinóide no controle da dor pós-operatória, uma vez os agonistas AEA e WIN (agonistas dos RsCB₁ e RsCB₂) inibem significativamente a hiperalgesia mecânica induzida pela incisão plantar.

Recentemente foram encontradas mudanças nos níveis sanguíneos de endocanabinóides em pacientes submetidos à terapia manual (MCPARTLAND et al., 2005). Para explorar a contribuição específica dos subtipos de RsCB₁ em diferentes sítios de modulação da dor, o antagonista seletivo para o RCB₁ (AM281) foi administrado pelas vias i.p., i.t. ou i.pl. Os resultados do presente estudo suportam a hipótese de que o efeito antihiperálgico da MAT é mediado por RCB₁ e ocorre predominantemente a nível central. Assim, especula-se que a ativação do RCB₁ no SNC possa contribuir para os efeitos observados no trabalho de McPartland et al. (2005).

A demonstração de que o efeito antihiperálgico da administração sistêmica de canabinóides é significativamente diminuído seguido à lesão cirúrgica do funículo dorsolateral fornece evidências que sítios supra-espinais e vias modulatórias da dor descendentes

desempenham papéis cruciais na analgesia dependente de canabinóide (LICHTMAN; MARTIN, 1991; SEYREK et al., 2010). O principal papel da PAG na analgesia induzida por canabióides é também suportada pelo aumento na liberação de AEA e 2-AG na PAG após estímulos nocivos tais como: injeção intraplantar de formalina ou modelos de lesão nervosa periférica (FIELDS; BASBAUM; HEINRICHER, 2006). Além disso, foi observado um aumento nos níveis de AEA e 2-AG no bulbo ventrorostral (RVM) na constrição crônica do nervo isquiático de ratos sugerindo que o sistema endocanabinóide pode ser cronicamente ativado como um mecanismo adaptativo, cujo objetivo, seria se contrapor a transmissão da dor neuropática (PALAZZO et al., 2006). A PAG é uma estrutura chave da via descendente inibitória da dor, a qual estabelece um circuito principal e importante com o RVM (WILDER-SMITH, 2011). O RVM é considerado a principal estação *relay* de muitas regiões encefálicas envolvidas na modulação da dor descendente (GEBHART, 2004).

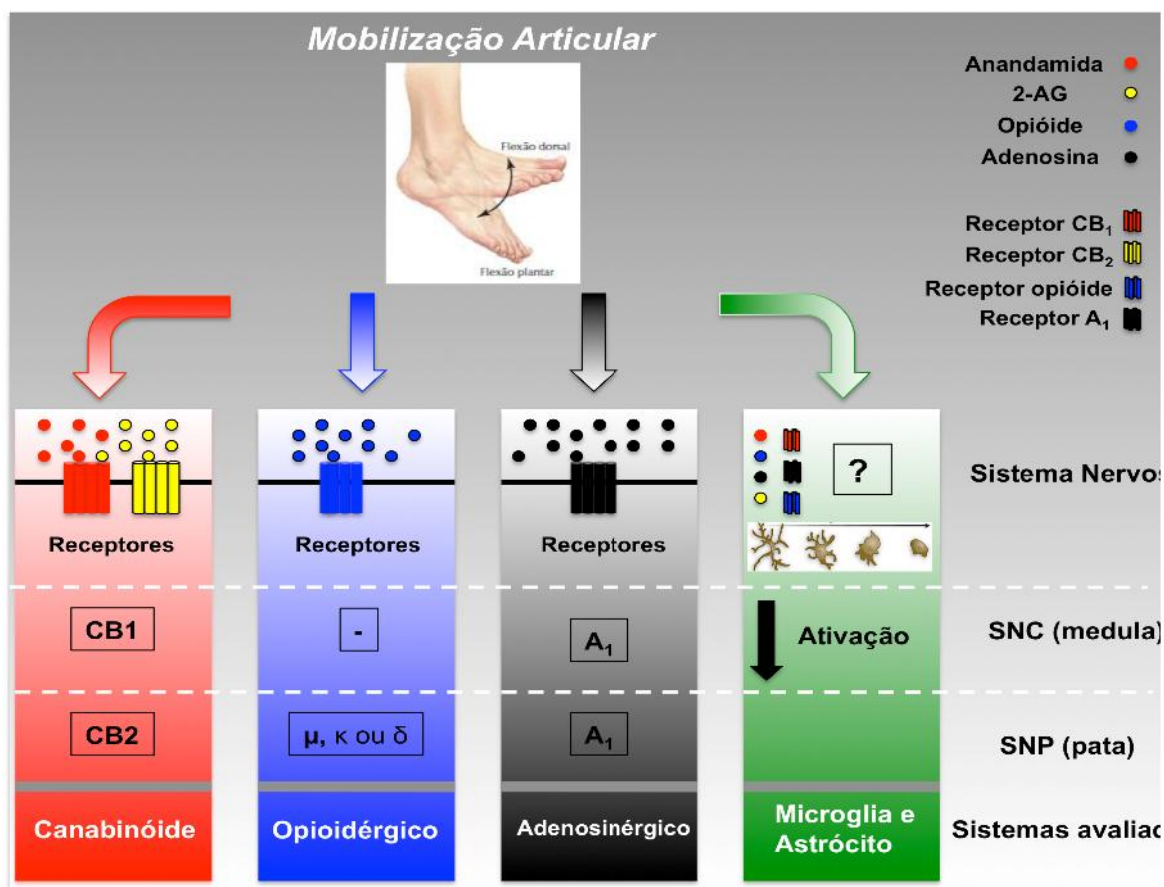
Por outro lado, LaBuda et al. (2005) demonstraram que agonistas seletivos para o RCB₂ produzem efeito antihiperalgésico no modelo da dor pós-operatória em ratos. Em 2007, Romero-Sandoval e Eisenach mostraram que ativação de RCB₂ leva à redução da hiperalgesia mecânica e que este efeito é mediado pela redução da ativação de células microgлияis localizadas na medula espinal de ratos. Com o intuito de investigar a hipótese de que o RCB₂ pudesse também mediar o efeito da MAT em diferentes sítios de modulação da dor, o seletivo antagonista para o RCB₂ (AM630) foi também administrado pelas vias i.p., i.t. ou i.pl. Os resultados destes experimentos revelam que a administração do AM630 previne o efeito antihiperalgésico da

MAT quando administrado por via i.p. ou i.pl., mas não por via i.t., suportando a hipótese de que o efeito antihiperálgico da MAT é mediado por RCB₂ e que tal mediação é predominantemente periférica. Estes achados corroboram com vários trabalhos na literatura que sugerem uma maior expressão dos RCB₂ na periferia e com seu importante papel na antinocicepção (IBRAHIM et al., 2005; ZHU et al., 2009; KHASABOVA et al., 2011).

Após a caracterização do envolvimento dos RsCBs no efeito antihiperálgico da MAT e seus respectivos sítios de ação, a próxima questão a ser respondida estava pautada nos ligantes endógenos dos RsCBs. Assim direcionaram-se as atenções aos 2 principais endocanabinóides: a AEA e o 2-AG. O endocanabinóide AEA é considerado um agonista parcial RCB₁/RCB₂, enquanto que o 2-AG é agonista total do RCB₁/RCB₂ induzindo resposta máxima (ABOOD, et al., 1997; BISOGNO, et al., 2006). Primeiramente, encontrou-se que os inibidores das enzimas FAAH e MAGL, que degradam a AEA e o 2-AG, respectivamente, apresentam significativo efeito antihiperálgico no modelo de dor pós-operatória. Assim, o passo seguinte foi utilizar uma dose subefetiva de cada um dos inibidores associadas à MAT. Encontrou-se que tanto o inibidor da FAAH quanto o inibidor da MAGL potencializam o efeito da MAT, sugerindo que a MAT provavelmente libera AEA e 2-AG e conseqüentemente ativa os RCBs.

Um esquema final dos sistemas endógenos de controle da dor que contribuem para o efeito antihiperálgico da MAT demonstrados no presente estudo é ilustrado na figura 12.

Figura 12 - Sistemas endógenos de controle da dor que contribuem para o efeito antihiperálgico da MAT.



Fonte: Elaboração dos autores, 2013.

A figura apresenta um esquema final dos sistemas endógenos de controle da dor que contribuem para o efeito antihiperálgico da MAT, verificados no presente estudo.

4.3 INTERAÇÕES ENTRE OS SISTEMAS ENDÓGENOS QUE MEDEIAM O EFEITO DA MAT

Um fenômeno que nos chamou a atenção foi o fato de que a realização da MAT ou a administração sistêmica de morfina (agonista opióide- μ), adenosina (agonista A₁ e A_{2A}), ou clonidina (agonista- α_2) produziram consistente efeito antihiperálgico. Além disso, encontrou-se que estes efeitos antihiperálgicos são completamente prevenido pela pré-administração por via i.pl. de naloxona (antagonista opióide- μ),

DPCPX (antagonista- A_1) ou por via i.p. de ioimbina (antagonista- α_2), respectivamente. Intrigantemente, a pré-administração i.pl. e i.p. de naloxona ou cafeína (antagonista A_1 e A_{2A}), i.pl. de DPCPX ou i.p. de ioimbina preveniram completamente o efeito antihiperálgico da MAT. Uma das explicações para o fato que tais antagonistas tenham prevenido completamente este efeito pode estar no trabalho de Aley e Levine (1997).

O trabalho publicado pelo grupo do professor Jon Levine propôs a existência de um complexo de três receptores envolvidos na antinociceção periférica (ALEY; LEVINE, 1997). Os autores encontraram que agonistas (opióide- μ , adrenérgico- α_2 ou adenosinérgico- A_1) podem não agir independentemente para produzir antinociceção, mas podem requerer a presença física de outros receptores para levar à antinociceção por qualquer agonista. Esta afirmação foi sugerida pelos achados de que a antinociceção induzida pela clonidina foi bloqueada não somente pela ioimbina (antagonista- α_2), mas também pelo PACPX (antagonista- A_1) e pela naloxona (antagonista opióide- μ), e que a antinociceção do DAMGO (agonista opióide- μ) e do CPA (agonista adenosinérgico- A_1) foram bloqueadas não somente pela naloxona e PACPX (antagonista- A_1), respectivamente, mas também pela ioimbina (antagonista- α_2). Para testar a hipótese de que a presença física do receptor opióide- μ é requerida não somente para antinociceção μ mas também α_2 , antisenses oligodeoxinucleotídeos para o receptor opióide- μ (AS-ODN opióide- μ) e receptor adrenérgico α_{2C} (AS-ODN adrenérgico α_{2C}) foram administrados intratecalmente para reduzir a expressão destes receptores nos neurônios aferentes primários, neste estudo de Aley e Levine

(1999). Estes experimentos demonstraram que a administração de AS-ODN opióide- μ não somente diminui a antinocicepção opióide- μ mas também α_2 ; mas não afeta a antinocicepção A_1 . Ao contrário, o AS-ODN adrenérgico α_{2C} diminuiu a antinocicepção induzida por todos as três classes de agonistas.

Assim, acredita-se que cada vez que um antagonista destes receptores é administrado a formação deste complexo de receptores fica prejudicada, refletindo diretamente na redução da antinocicepção induzida pelo mesmo. Estes dados também fornecem evidências para sugerir que a MAT pode induzir a liberação de opióides e adenosina na periferia, conforme ilustrado na figura 11.

Skyba et al. (2003) demonstraram que a MA ativa vias inibitórias da dor descendentes que ativam receptores serotoninérgicos e noradrenérgicos na medula espinal. Corroborando com estes resultados e estendendo a outro modelo pré-clínico de dor, o presente trabalho mostra que o pré-tratamento i.p. dos animais com PCPA (um inibidor da síntese de serotonina) ou com ioimbina (como já mencionado acima) também previnem o efeito antihiperálgico da MAT. Interessante é o fato que também evidencia-se no presente estudo, de que a pré-administração i.t. de cafeína ou DPCPX previnem o efeito antihiperálgico da MAT. Neste sentido, tem sido demonstrado que a serotonina (5-HT) pode liberar adenosina de sinaptossomas da medula espinal *in vitro* e *in vivo*, esta liberação é dependente de Ca^{2+} , originada de nucleotídeo (AMPc), e de neurônios sensíveis a capsaicina (SWEENEY; WHITE; SAWYNOK, 1990, SAWINOK; LIU, 2003). Além disso, esta liberação é mediada por receptor, pois é bloqueada pela administração de metilsergida (antagonista dos receptores 5-HT₁ e 5-

HT₂). Em estudos comportamentais a administração i.t. de metilxantinas (antagonista adenosinérgico) inibe a analgesia induzida pela administração i.t. de 5-HT (DELANDER; HOPKINS, 1987).

Sweeney et al. (1989) mostraram que a norepinefrina (NE) também induz a liberação de adenosina de ambas partes anterior e posterior da medula espinal em iguais proporções, e esta liberação difere da liberação induzida pela morfina ou 5-HT. Tal liberação é mediada pelo receptor α_2 e é dependente de Ca^{2+} . A proeminente liberação na medula espinal anterior e posterior indica que a NE pode liberar adenosina de múltiplos sítios, talvez refletindo ações sobre estruturas profundas dentro da medula espinal regulando atividade motora e simpática (SAWINOK; LIU, 2003). Corroborando com estes achados, o estudo de Gomes et al. (1999) mostrou que o efeito antihiperalgésico da adenosina administrada i.t. é bloqueado pelo antagonista do receptor α_2 e pela destruição dos terminais nervosos espinais noradrenérgicos. Além disso, no mesmo estudo os autores constataram que a perfusão das fatias da medula espinal com um agonista adenosinérgico aumenta de maneira dose dependente a liberação espinal de noradrenalina.

Os dados do presente estudo demonstram que a naloxona e a cafeína, ambas administradas sistêmica ou perifericamente, previnem o efeito antihiperalgésico da MAT e fortemente sugerem que este efeito pode ser observado em função da interação entre estes dois sistemas endógenos de controle da dor. Várias evidências têm demonstrado que a morfina induz a liberação endógena de adenosina em sinaptossomas obtidas da medula espinal posterior, e esta liberação origina primariamente adenosina (SWEENEY; WHITE; SAWYNOK, 1987). A liberação de adenosina induzida pela morfina é dependente de Ca^{2+} ,

principalmente pela ativação do canal de cálcio do tipo N, e ocorre através de um transportador bidirecional que está presente em neurônios sensíveis a capsaicina (SWEENEY; WHITE; SAWYNOK, 1989). Estudos comportamentais fornecem evidências corroborativas da liberação espinal de adenosina por opióides. Assim, a administração espinal de metilxantinas inibe a analgesia espinal induzida pela morfina (DELANDER; HOPKINS, 1986, SWEENEY; WHITE; SAWYNOK, 1987). A liberação de adenosina induzida por opióide pode resultar na ativação da proteína quinase C (PKC). Nas células do músculo cardíaco, a fosforilação induzida pela PKC resulta na estimulação da 5'-nucleotidase a qual leva a um aumento da formação intracelular de adenosina (KITAKAZE et al., 1995; OBATA; KUBOTA; YAMANAKA, 2001; OBATA, 2002). Alternativamente, a PKC poderia inibir a adenosina quinase (enzima responsável pela conversão da adenosina em AMP) (SINCLAIR et al., 2000), levando assim ao aumento intracelular de adenosina e ao conseqüente efluxo da mesma. Nos neurônios sensoriais, a ativação da PKC aumenta a liberação de neuropeptídeos (BARBER; VASKO, 1996), e há evidência de que a morfina possa ativar a PKC nestes neurônios (INOUE; UEDA, 2000). Uma vez que os opióides estimulam a PKC regulando a produção de adenosina, este é um plausível mecanismo da liberação de adenosina induzida por opióide.

Quando uma substância é administrada por via i.p., logo a mesma alcança o SNC, uma vez que a mesma seja uma molécula capaz de atravessar a barreira hemato-encefálica. Assim, quando a naloxona é administrada sistemicamente e previne o efeito antihiperálgico da MAT parte deste efeito se deve a interação da mesma com receptores

opioides localizados em regiões supra-espinais, sugerindo que a MAT possa liberar peptídeos opioides endógenos centralmente (Figura 11). Interessantemente, tem sido demonstrado que a administração de morfina supra-espinal por injeção intracerebroventricular resulta na liberação espinal de adenosina (SWEENEY; WHITE; SAWYNOK, 1991). Esta liberação ocorre secundariamente à liberação de 5-HT seguida da ativação de vias inibitórias da dor descendentes, originadas no tronco encefálico (SWEENEY; WHITE; SAWYNOK, 1991). Corroborando com a hipótese de que a MA pode ativar regiões supra-espinais, o estudo de Malisza et al. (2003) utilizando ressonância magnética funcional mostrou que a MA foi capaz de reduzir a ativação de áreas encefálicas relacionadas com o processamento nociceptivo tais como os córtices cingulado anterior, frontal e sensório-motor em um modelo animal de dor inflamatória em ratos.

Os canabinóides são historicamente usados em combinação com opioides no tratamento de várias condições dolorosas em humanos, devido as suas interações sinérgicas na modulação do estímulo nocivo (CICHEWICZ, 2004). Opioides e canabinóides ligam-se em diferentes receptores (MOR, DOR, KOR, RsCB₁ ou RsCB₂), os quais estão acoplados a proteína Gi/Go (WANG; VAN BOCKSTAELE; LIU-CHEN, 2008; MACKIE, 2008). A ativação de receptores opioides e canabinóides inibem a ciclase de adenilil, bloqueiam canais de Ca²⁺ dependentes de voltagem, ativam canais de K⁺ e estimulam a cascata das MAPKs (CHILDERS et al., 1992). Devido as suas localizações pré-sinápticas, o principal efeito da ativação dos receptores canabinóides e opioides é a inibição da liberação de vários neurotransmissores como

por exemplo: noradrenalina, dopamina, glutamato e GABA (SCHLICKER; KATHMANN, 2000).

Evidências acumuladas nos últimos anos sugerem a existência de interação entre os mecanismos analgésicos dos canabinóides e opióides (PAROLARO et al., 2010). Embora numerosos estudos sugerem que opióides possam também estar envolvidos na regulação da dor por canabinóides. Esta hipótese é suportada por vários estudos os quais indicam que antagonistas para receptores opióides podem bloquear a antinocicepção induzida por canabinóides (MANZANARES et al., 1999).

Os resultados do presente estudo também mostram que as administrações sistêmica ou i.t. do antagonista do RCB₁ (AM251) previnem o efeito antihiperálgico da MAT, sugerindo a participação central de RCB₁ (Figura 11). O trabalho de Wilson-Poe et al. (2012) demonstrou pela primeira vez, o alto grau de co-localização dos RsCB₁ e opióides- μ em áreas encefálicas que são criticamente envolvidas na modulação descendente da nocicepção. Os resultados do estudo de Wilson-Poe et al. (2012) são consistentes com observações prévias de que os receptores RsCB₁ e opióides- μ estão co-localizados em perfis somatodendríticos no corno posterior da medula espinal (PUGH et al., 1996; SALIO et al., 2001). O recente trabalho de Wilson-Poe et al. (2013) demonstrou que a interação canabinóide/opióide é bidirecional. Os autores observaram que a micro-injeção de morfina duas vezes ao dia, por dois dias, dentro da PAG ventrolateral, aumenta a antinocicepção induzida por agonista canabinóide (HU-210) avaliado um dia após a administração. Do mesmo modo, administrações

sistêmicas, duas vezes ao dia de THC aumenta o efeito antinociceptivo da morfina administrada um dia após a administração de THC em ratos.

No presente estudo também foi encontrado que a administração sistêmica e i.pl. do antagonista do RsCB₂ (AM630) previne o efeito antihiperálgico da MAT, sugerindo participação periférica dos RsCB₂ neste efeito. Um recente estudo de Ibrahim et al. (2004) investigou os mecanismos pelos quais agonistas seletivos para os RsCB₂ induzem a liberação de opióides endógenos. Os autores demonstraram que a ativação do RsCB₂ no queratinócito, um tipo de célula que tem sido reportado expressar RsCB₂ (CASANOVA et al., 2003) e conter peptídeos opióides endógenos (KAUSER et al., 2003) libera β -endorfinas produzindo antinocicepção periférica por agir em receptores opióides- μ nos neurônios aferentes primários. Estes mecanismos permitem a liberação local de opióides endógenos limitada ao sítio onde os receptores estão presentes, assim levando a especificidade anatômica dos efeitos opióides (IBRAHIM et al., 2005). Além disso, foi mostrado que o THC modula opióides endógenos diferencialmente em ratos normais *vs* artríticos dependendo dos níveis basais de dinorfina (COX; WELCH, 2004), sugerindo que os RsCBs podem servir como moduladores homeostáticos da liberação tônica de opióides na via nociceptiva espinal.

Outra interessante interação é entre os sistemas endocanabinóide e adenosinérgico. Na literatura encontram-se poucos estudos que analisam esta relação. No entanto, foi observado que ambos, anandamida e adenosina, interagem produzindo analgesia (TUBOLY et al., 2009). O estudo de Tuboly et al. (2009) sugere que os receptores adenosinérgicos podem mediar o efeito antihiperálgico da

anandamida, uma vez que, a pré-administração espinal de cafeína previne o efeito antihiperálgico da anadamida. Além disso, Hohmann et al. (2010) investigaram o papel da adenosina e do receptor A_1 na inibição da transmissão glutamatérgica mediada pela ativação do RCB_1 em neurônios piramidais do hipocampo em camundongos. Nos resultados do estudo os autores encontraram que a ativação de A_1 pelo aumento nos níveis de adenosina, reduz ou elimina a inibição mediada por CB_1 na liberação de glutamato, e o bloqueio dos receptores adenosinérgicos pela cafeína reverte este efeito. Assim receptores CB_1 e A_1 em neurônios piramidais do hipocampo exercem controle inibitório diminuindo a liberação de glutamato.

Outra consistente evidência desta interação foi o estudo que mostrou que o metabolismo da adenosina pela adenosina deaminase aumentou a ligação estimulada por agonista canabinóide de [35 S]-GTP γ S a proteína G ativada no hipocampo de ratos, demonstrando uma potencial base molecular para a modulação negativa dos RCB_1 pela adenosina (MOORE et al. 2000).

No entanto, não encontrou-se estudos que analisaram a relação funcional entre os $RsCB_1$ e receptores A_1 em regiões espinais ou supra-espinais responsáveis pela modulação da dor.

Considerando o conjunto de resultados deste estudo a hipótese mais provável de mecanismo de ação para explicar os efeitos da MAT pelos sistemas endógenos de controle da dor é a de que a MAT é capaz de liberar endocanabinóides ou opióides endógenos sistêmica e periféricamente que, por sua vez, após ativarem seus respectivos receptores podem diretamente inibir a atividade neuronal e promover a liberação de adenosina. Uma vez liberada, a adenosina endógena pode

mediar a antihiperálgia induzida pela MAT por ativar receptores para adenosina A₁, conseqüentemente por reduzir a atividade neuronal dos neurônios aferentes primários e de projeção, modulando o processo nociceptivo periférico e centralmente.

4.4 MAT REDUZ DOR NEUROPÁTICA ATRAVÉS DA REDUÇÃO DA ATIVIDADE DE CÉLULAS GLIAIS

Os principais mecanismos responsáveis pela dor neuropática incluem a geração de impulsos ectópicos (que podem ocorrer no local de axônios danificados ou em corpos celulares de neurônios lesionados ou neurônios vizinhos intactos), degeneração de fibras sensoriais, brotamento de fibras sensoriais para áreas que elas normalmente não inervam, brotamentos de nervos simpáticos, desinibição (atividade diminuída ou perda de neurônios inibitórios), aumento da atividade facilitatória descendente ou atividade prejudicada da transmissão inibitória descendente, ativação de células gliais e neuroinflamação. Esses eventos estão associados a alterações nos níveis de neurotransmissores, expressão e sinalização de receptores e de canais iônicos levando a sensibilização periférica e central (ou seja, reduzem o limiar de ativação e produzem respostas exageradas nos neurônios periféricos e centrais) (MACHELSKA, 2011).

Após a caracterização do efeito antihiperálgico da MAT e seu mecanismo de ação em um modelo de dor aguda. O passo seguinte, foi avaliar o efeito da MAT em um modelo de dor crônica ainda não explorado. O modelo de dor neuropática foi então escolhido por sua grande relevância clínica. No passado, a visão clássica da dor apontava

para os neurônios como o principal responsável pela iniciação e manutenção da dor neuropática. Naquela visão, o papel da glia (microglias e astrócitos) era negligenciado (WATKINS; MILLIGAN; MAIER, 2001). No entanto, nas últimas décadas várias evidências confirmaram o importante papel das células gliais localizadas no corno posterior da medula espinal na iniciação e manutenção do estado de dor crônica (MILLIGAN; WATKINS, 2009, MILLIGAN et al., 2003). Assim, utilizando um modelo de neuropatia traumática induzida pelo esmagamento do nervo isquiático de ratos, analisou-se o efeito antihiperálgico da MAT na dor crônica. Verificou-se que o esmagamento do nervo isquiático produz significativa hiperálgia mecânica a partir do 3º dia, que perdura até o 17º dia. Observa-se que a MAT reduz a hiperálgia em todos os dias avaliados evidenciando assim que a MAT tem potencial terapêutico para o tratamento da dor crônica de origem neuropática. Interessantemente, analisou-se o papel das células microgliais e astrocíticas no modelo de esmagamento do nervo esquiático. Nota-se que a lesão nervosa periférica ativa células microgliais e astrócíticas na medula espinal e que o tratamento em dias alternados com a MAT reduz significativamente esta ativação. Estes achados são os primeiros na literatura a demonstrar a efetividade da MAT na dor neuropática e a mostrar o envolvimento do sistema imunológico neste efeito, através da avaliação da atividade das células gliais.

A primeira questão do presente achado a ser discutida é: como uma lesão nervosa periférica pode ativar células gliais no corno posterior da medula espinal?

Dados sobre ativação de células gliais induzidas por esmagamento de nervos periféricos não são encontrados na literatura, no entanto, observa-se uma rápida ativação microglial e astrogliar próxima à neurônios axotomizados (transecção). É interessante lembrar que ambos, esmagamento e transecção de nervos periféricos, causam um fenômeno conhecido como degeneração Walleriana, a qual envolve uma grande participação do sistema imunológico (ROTSHENKER, 2011). Seguida à lesão do neurônio motor, os astrócitos aumentam a expressão da proteína de junção comunicante conexina-43 dentro de horas, e de GFAP em um dia. Concomitante, células microgliais proliferam e migram para o corpo celular de neurônios axotomizados. Resposta semelhante ocorre no território central (corno posterior da medula espinal) das fibras sensoriais lesionadas (ALDSKOGIUS; KOZLOVA, 1998).

Quando o nervo periférico é submetido a uma lesão de qualquer natureza, seja ela química, térmica ou traumática, como o esmagamento, isso leva a um intenso aumento da expressão de proteínas CD11b/c no corno posterior da medula espinal na área onde terminam as fibras nociceptivas, evidenciando a ativação de células microgliais (WILLIS; COGGESHALL, 1991). Assim, quando lesionadas, fibras nociceptivas periféricas liberam em seus terminais centrais aminoácidos excitatórios, SP, ATP, NO e prostaglandinas que excitam os neurônios espinais e também ativam as células gliais (MILLIGAN, et al., 2003; MILLIGAN; WATKINS, 2009). Neste sentido, a ativação microglial é induzida por neurônios responsáveis pela transmissão dolorosa. Esta comunicação neurônio-glia é mediada pela fractalcina expressa na superfície extracelular do neurônio (VERGE et al., 2004). Além disso, tem sido

demonstrado que lesões nervosas periféricas ativam várias vias de sinalização intracelular (por ex: PKA, PKC e proteína quinase dependente de cálcio/calmodulina) em neurônios sensoriais primários e secundários que então ativam MAPKs incluindo MAPK p38, ERK e JNK no corno posterior da medula espinal em neurônios, microglias e astrócitos. Esta ativação induz a produção glial de mediadores inflamatórios (TNF- α e IL1- β) que sensibilizam os neurônios do corno posterior induzindo hiperalgesia e alodinia (CHIANG; SESSLE; DOSTROVSKY, 2012). A ativação astrocitária de JNK mediada pela subunidade NR2B do receptor NMDA no corno posterior libera IL1- β que através de um mecanismo de feedback positivo aumenta e prolonga a dor neuropática (WANG, et al., 2011).

4.4.1 Prováveis mecanismos neurofisiológicos da MAT envolvidos na redução da atividade das células gliais

A segunda questão a ser discutida no presente estudo é: como a MAT pode reduzir a ativação de células gliais e conseqüentemente a dor neuropática?

Interessantemente, o estudo de Malisza et al. (2003) utilizando ressonância magnética funcional mostrou que a MA reduz a ativação de áreas medulares (corno posterior) relacionadas ao processamento nociceptivo em um modelo animal de dor inflamatória em ratos.

O presente estudo e o trabalho Malisza et al. (2003) corroboram a hipótese de que a MA é capaz de reduzir a dor por reduzir a atividade de neurônios espinais, no entanto, qual é o mecanismo neurofisiológico plausível para explicar tal fenômeno?

Uma evidência interessante a ser mencionada é a de que uma pequena incisão na superfície plantar de ratos é capaz de ativar células microgliais (e a MAPK p38) na medula espinal de ratos (WEN et al., 2009). Além disso, a ativação dual de RsCB₁/RsCB₂ é capaz de reduzir a hiperalgesia mecânica bem como, a ativação de MAPK p38 e astrócitos na medula espinal (ALKAITIS et al., 2010). Estes estudos demonstram que a ativação glial na medula espinal é um mecanismo comum entre os modelos animais estudados no presente estudo (dor pós-operatória e dor neuropática).

Com base nas evidências observadas no presente estudo de que os sistemas opioidérgico, adenosinérgico e endocanabioideérgico estão envolvidos na antihiperalgesia induzida pela MAT no modelo de dor pós-operatória, algumas hipóteses são levantadas na tentativa de explicar o efeito observado no modelo crônico de dor neuropática para o direcionamento de futuras pesquisas.

O mecanismo neurofisiológico inicialmente aceito para explicar o efeito antihiperalgésico da MA é o de que a MA ativa vias inibitórias da dor descendentes que envolvem a ativação espinal de receptores para serotonina e noradrenalina. Esta hipótese foi inicialmente levantada por Wright (2001), corroborada por Skyba et al. (2003) e confirmada pelo presente trabalho. Assim, a ativação espinal dos receptores para serotonina e noradrenalina produzem uma hiperpolarização direta dos neurônios da substância gelatinosa, reduzem a liberação de glutamato liberado pelas fibras do tipo A δ e do tipo C e aumentam a liberação de GABA e glicina dos interneurônios inibitórios (MILLAN, 2002). Reduzindo a liberação de glutamato na medula espinal e

consequentemente a ativação das células gliais (MITHILESH; SANGMIN; KYOUNGHO, 2012).

4.4.2 Pode o sistema opioidérgico ser ativado pela MAT em células gliais e em neurônios na dor neuropática e produzir antihiperalgisia?

As endomorfina são agonistas dos receptores opióides e apresentam maior afinidade e seletividade para os MOR (MIZOGUCHI et al., 2002). Elas estão distribuídas por todo o encéfalo, tronco encefálico e medula espinal (FINCHA et al., 2007). Os MOR estão presentes em astrócitos onde desempenham um papel no controle da atividade neuroinflamatória (HANSSON et al., 2008). Em condições inflamatórias, há um aumento na produção de endomorfina, uma resposta que foi mostrada ter significados funcionais no controle da dor, produzindo potente analgesia na dor inflamatória e neuropática em roedores (HORVATH; KEKESI, 2006). Além disso, das três classes de receptores opióides μ , κ e δ até agora identificados, a microglia expressa os receptores opióides μ e κ . Quando ativados, na microglia, os MOR promovem mudanças morfológicas, expressão de BDNF (DOBRENIS et al., 1995) e inibem a quimiotaxia e a migração microglial, indicando um papel antiinflamatório destes receptores na microglia (CHAO et al., 1997).

No neurônio, a inibição dos canais de Ca^{2+} parece ser o principal mecanismo de ação da ativação dos receptores opióides periféricos. Além disso, a ativação de receptores opióides também suprime os canais de Na^{+} tetrodotoxina-resistentes e as correntes

catiônicas não-seletivas (INGRAM; WILLIAMS, 1994), correntes mediadas pelo receptor P2X (CHIZHMAKOV et al., 2005), bem como pelos canais TRPV1 que envolve a inibição da ciclase de adenilil (CHIZHMAKOV et al., 2005). Assim, opióides podem atenuar o aumento da excitabilidade de neurônios aferentes primários, aumento este induzido pela liberação de neuropeptídeos pró-inflamatórios, tais como: a SP e o CGRP, a partir dos terminais nervosos periféricos e centrais (STEIN; SCHÄFER; MACHELSKA, 2003). Como no modelo da dor pós-operatória, é plausível sugerir que a MAT poderia reduzir a dor neuropática através da ativação de receptores opióides expressos em células gliais e neurônios.

4.4.3 Pode o sistema adenosinérgico ser ativado pela MAT em células gliais e em neurônios na dor neuropática e produzir antihiperalgisia?

Também acredita-se que a MAT possa reduzir a dor neuropática (hiperalgesia mecânica) e a ativação de células gliais na medula espinal através da ativação do sistema adenosinérgico. Está bem estabelecido que o ATP pode ser liberado por astrócitos e neurônios. No entanto, sob condições fisiológicas, a liberação vesicular de ATP, não neuronal, mas astrocitária, tem sido identificada como uma das principais fontes de adenosina sináptica (PASCUAL et al., 2005). Uma vez que os astrócitos contactam milhares de sinapses e coordenam redes sinápticas, (VENTURA; HARRIS, 1999), é concebível que a liberação de ATP astrocitário e sua subsequente degradação em adenosina tem uma função reguladora importante no tônus inibitório mediado pela adenosina dentro

de uma rede neuronal. Além disso, todos os quatro receptores para adenosina são encontrados nos astrócitos (BJORKKLUND et al., 2008), e foi reportado que todos estão expressos em células microgliais ou em linhagens das mesmas (DARE et al., 2007). Os receptores A_1 ajudam a proteger os astrócitos de dano ou morte celular (BJORKKLUND et al., 2008; CICCARELLI et al., 2007), particularmente através da ativação da via PI3K e fosforilação de ERK1/2. Na microglia os receptores A_1 reduzem a sua excessiva ativação. Assim acredita-se que a MAT por ativar os receptores A_1 na medula espinal, possa reduzir diretamente atividade das células gliais, inibindo a liberação de mediadores pró-inflamatórios liberados pelas mesmas e conseqüentemente a hiperatividade neuronal.

Nos neurônios, os receptores A_1 (especialmente nos terminais nervosos) são criticamente importantes na mediação do efeito inibitório sobre a atividade neuronal mediada pela adenosina, gerada a partir do ATP liberado pelo astrócito (MARTIN et al., 2007). Os receptores A_1 são altamente expressos nas terminações nervosas e sinalizam preferencialmente via proteína G_o inibindo os canais de cálcio transientes, enquanto que os mesmos receptores no DRG e dendritos regulam preferencialmente a condutância dos canais de potássio via proteínas G_1 (BOISON; CHEN; FREDHOLM, 2010).

De acordo com esta literatura e com os resultados do presente estudo, especula-se que a MAT possa diminuir diretamente a atividade neuronal e indiretamente a atividade glial através da ativação dos receptores A_1 nos neurônios e nas células gliais da medula espinal, ou mesmo produzir a inibição de ambos simultaneamente.

4.4.4 Pode o sistema endocanabinóide ser ativado pela MAT em células gliais e em neurônios na dor neuropática e produzir antihiperalgisia?

Os dados do presente estudo claramente demonstram que a ativação dos RsCB₁ espinais e periféricos pelo provável aumento dos endocanabinóides AEA e 2-AG, medeiam o efeito da MAT. Ao mesmo tempo, tem sido demonstrado que os RsCB₁/RsCB₂ são expressos na microglia e astrócito (STELLA, 2010). Os RsCB₁, uma vez ativados no astrócito reduzem a ativação da MAPK p38 levando conseqüentemente a redução da produção de mediadores inflamatórios (IL1-β) reduzindo hiperalgisia e alodinia (CHIANG; SESSLE; DOSTROVSKY, 2012). Além disso, ativação neuronal de RsCB₁ na medula espinal leva a redução dos níveis de AMPc, inibindo canais de Ca²⁺ dependentes de voltagem e ativando canais de K⁺ retificadores de influxo, reduzindo a excitabilidade neuronal e inibindo a liberação de neurotransmissores pré-sinápticos, diminuindo conseqüentemente a ativação glial (PACHER; BATKAI; KUNOS, 2006).

As questões levantadas nos itens 5.4.2, 5.4.3 e 5.4.4 apesar de estarem apoiadas na literatura, elas são apenas especulativas. Assim, para responder definitivamente estas questões são necessárias a realização de futuras pesquisas as quais investiguem o papel dos sistemas opioidérgico, adenosinérgico e endocanabinoidérgico na redução da ativação de células gliais espinais induzida pela MAT.

A dor crônica, incluindo a dor neuropática, não envolve apenas as alterações biológicas descritas acima, mas também é influenciada por fatores cognitivos (expectativa, memória), emocionais (ansiedade,

depressão) e ambientais (condicionamento, reforço). Muitos pacientes com dor crônica sofrem de depressão, ansiedade, raiva, limitada mobilidade e falta de motivação. O conceito biopsicossocial da dor crônica dá uma base para gestão interdisciplinar, que inclui terapia psicológica, terapia física e ocupacional, combinadas com o tratamento farmacológico (STEIN; KOPF, et al., 2009). Neste sentido, torna-se imprescindível o conhecimento dos mecanismos de ação não apenas de medicamentos, mas também das terapias físicas as quais serão combinadas com o tratamento farmacológico para que o clínico possa escolher o melhor tratamento para seu paciente.

4.5 MAT ACELERA A RECUPERAÇÃO FUNCIONAL MOTORA DO NERVO ISQUIÁTICO DE RATOS

Estrategicamente, o modelo animal de esmagamento do nervo isquiático foi escolhido pela possibilidade de avaliar além de dor neuropática, a recuperação funcional motora que é um dos principais objetivos da fisioterapia. Na clínica em geral, muitas estratégias são traçadas com o objetivo de reduzir a dor do paciente e aumentar a função motora após uma lesão traumática nervosa. O tratamento mais utilizado no alívio da dor neuropática é a gabapentina, no entanto, é ineficaz na regeneração nervosa (WHITLOCK et al., 2007). Ao contrário, vários fatores neurotróficos como NGF, BDNF, neurotrofina 3 (NT-3) e NT-4 induzem hiperalgesia (DENG et al., 2000). Assim, a busca por um tratamento capaz de agregar efeitos analgésicos à atividade neuroregenerativa é um desafio de muitos pesquisadores. Na fisioterapia, o exercício físico é um dos recursos mais utilizados e

estudados até o presente momento, e está entre as ferramentas mais utilizadas atualmente para promover regeneração nervosa (BOBINSKI et al., 2011; UDINA et al., 2011). Assim, foi demonstrado que a atividade muscular mantida artificialmente durante a denervação/paralisia pode melhorar a reabilitação neuromuscular (UDINA et al., 2011). A eletroestimulação do músculo denervado, exercícios passivos (MA) e treinamento da marcha são estratégias efetivas no retardo da atrofia muscular e na melhora da resposta contrátil após reinervação em modelos animais de denervação (EBERSTEIN; PACHTER, 1986; MARQUESTE et al., 2004; SOUCY; SEBURN; GARDINER, 1996). A atividade muscular induzida pela estimulação pode resultar na liberação autócrina de fatores tróficos e assim evitar os efeitos deletérios do desuso seguido de lesões nervosas periféricas ou centrais (GOLDSPINK; YANG, 2001). As fibras musculares são capazes de ajustar suas propriedades fenotípicas em resposta a estimulação nervosa ou muscular (MARQUESTE et al., 2006), ao alongamento estático (SAKAKIMA; YOSHIDA, 2003) e também ao exercício físico (BOOTH; THOMASON, 1991). A este respeito, foi sugerido que as atividades induzidas que mimetizam os movimentos fisiológicos poderiam produzir melhor preservação/recuperação das propriedades funcionais musculares do que a estimulação elétrica convencional (MARQUESTE et al., 2004; 2006).

Neste sentido, os resultados do presente estudo corroboram com os achados da literatura por demonstrar que a MAT acelera a regeneração nervosa periférica (análises funcionais e morfológicas). Um interessante aspecto que deve ser salientado, do presente estudo, é o de que pouco é conhecido sobre os possíveis efeitos isolados da

mobilização passiva, do exercício físico voluntário ou forçado, diferenças que podem afetar a interpretação dos resultados. A natação ou corrida na esteira melhora a regeneração, mas pode não necessariamente ser a sobrecarga física ou as mudanças relacionadas ao estresse o que influencia nos resultados dos experimentos com treinamento físico (VAN MEETEREN et al., 1997). Alguns estudos têm demonstrado que a mobilização passiva do músculo denervado antes da reinervação preserva a estrutura da placa motora e aumenta a reinervação (PACHTER; EBERSTEIN, 1989). Além disso, recentemente foi demonstrado que a estimulação manual (5 minutos/dia por 2 meses) das vibrissas de ratos após a transecção e reparo do nervo facial resulta na melhora da restauração dos movimentos faciais e na redução de poli-inerções (ANGELOV et al., 2007; GUNTINAS-LICHIUS et al., 2007). Em contraste aos resultados do presente estudo e da literatura, foi demonstrado que a mobilização passiva contínua do membro posterior de ratos após uma secção nervosa não influencia na regeneração, quando este tratamento é realizado somente durante os primeiros 14 dias após a lesão (KIM et al., 1998).

Intessantemente, demonstra-se no presente estudo que a MAT reduz a atividade de células microgliais e astrócitos na medula espinal (dados mencionados acima) em paralelo com a observação de que a MAT melhora a regeneração nervosa periférica. Estes resultados sugerem que a inibição destas células na medula espinal possam contribuir para regeneração nervosa periférica.

Apesar de estabelecido que existe uma resposta rápida glial imediata a lesão periférica de nervos (axoniotomia) e que tal resposta está integrada à sobrevivência neuronal, regeneração dos axônios e

restauração do contato no alvo dos neurônios lesionados (ALDSKOGIUS, 2011), o papel das células gliais na medula espinal foi pouco estudado. No entanto, uma série de experimentos foram realizados objetivando analisar os efeitos da inibição microglial na recuperação neuronal e regeneração axonal (ALDSKOGIUS; KOZLOVA, et al., 1998). Assim, a infusão intraventricular de um inibidor mitótico, citosina arabinosídeo, erradicou a população local de células microgliais nos núcleos de neurônios axoniotomizados. Os resultados deste experimento mostram que a resposta neuronal, a taxa de crescimento axonal, bem como a extensão da substituição sináptica não foram afetadas pela eliminação das células microgliais, indicando que estas células não participam deste processo (SVENSSON; ALDSKOGIUS, 1993).

No entanto, a microglia é uma potencial fonte de fatores de crescimento e pode expressar em sua superfície moléculas que promovem o remodelamento de processos neuronais. Em cultura, a microglia libera moléculas que promovem o prolongamento de neuritos (NAKAJIMA; KOHSAKA, 1993). Além disso, microglia que foram ativadas pelo fator neurotrófico ciliar (CNTF) expressam o receptor para o fator de crescimento do nervo de baixa afinidade p75 (HAGG; VARON; LOUIS, 1993). Estes achados sugerem que a microglia reativa parece não fornecer significativa influência na regeneração dos axônios dos neurônios motores. Contudo, a microglia pode contribuir para o subsequente remodelamento estrutural nas conexões dos neurônios motores lesionados. Assim, o papel geral desempenhado pelas células gliais após uma lesão nervosa periférica é ainda pouco compreendido.

Neste sentido, o presente trabalho compartilha a hipótese de que existe um papel importante das células gliais na regeneração nervosa periférica e que isso possa contribuir para os efeitos regenerativos da MAT, além disso, especula-se que este mecanismo possa ocorrer inclusive nos efeitos regenerativos induzidos pelo exercício físico. Mais estudos são necessários para corroborarem ou refutarem esta hipótese.

5 CONCLUSÕES

De acordo com os resultados apresentados no presente estudo, conclui-se que:

i) O melhor regime de tratamento com MAT para reduzir a dor pós-operatória é quando realizada por 9 minutos;

ii) O tratamento diário com a MAT apresenta grande potencial terapêutico na redução da dor pós-operatória e neuropática;

iii) O efeito antihiperálgico da MAT depende da ativação de receptores opióides periféricos;

iv) Corrobora-se os dados prévios de que a MA ativa vias inibitórias da dor descendentes que envolvem os sistemas serotoninérgico e noradrenérgico;

v) O sistema adenosinérgico contribui para o efeito antihiperálgico da MAT através da ativação de receptores adenosinérgicos A_1 periféricos (pata) e centrais (espinais);

vi) O sistema endocanabinóide está envolvido no efeito antihiperálgico da MAT através da ativação de $RsCB_1$ centrais (espinal) e $RsCB_2$ periféricos (pata) e;

vii) A redução da ativação de células microgliais e astrocitárias na medula espinal contribui para o efeito antihiperálgésico da MAT.

viii) O tratamento diário com MAT também apresenta grande potencial terapêutico na regeneração morfológica e funcional do nervo isquiático;

Assim, esta tese demonstra alguns mecanismos neurofisiológicos envolvidos no efeito terapêutico da MAT no controle de dores agudas e crônicas, como pode ser observado na Figura 12. Além disso, estes resultados fornecem substanciais subsídios à literatura para os direcionamentos de futuros estudos clínicos.

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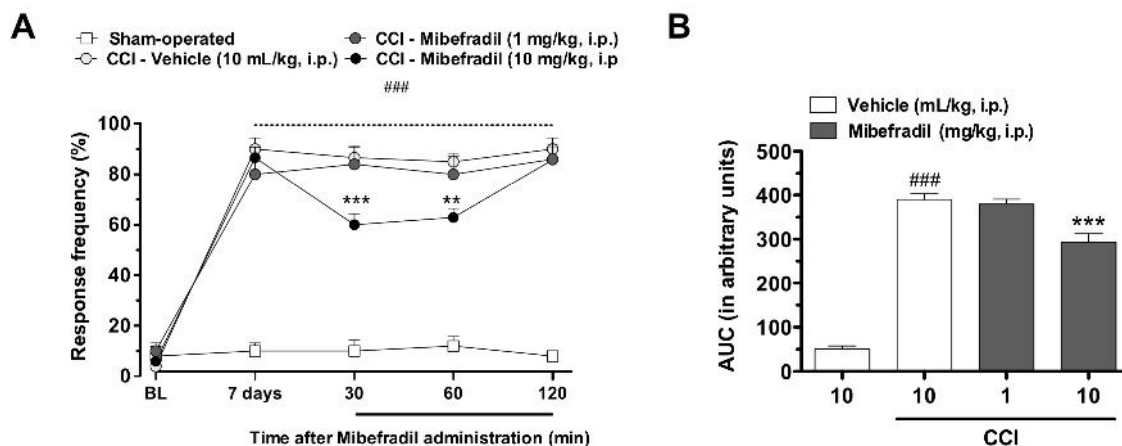
APÊNDICE

APÊNDICE - Experimentos realizados durante o período de doutorado sanduíche no exterior.

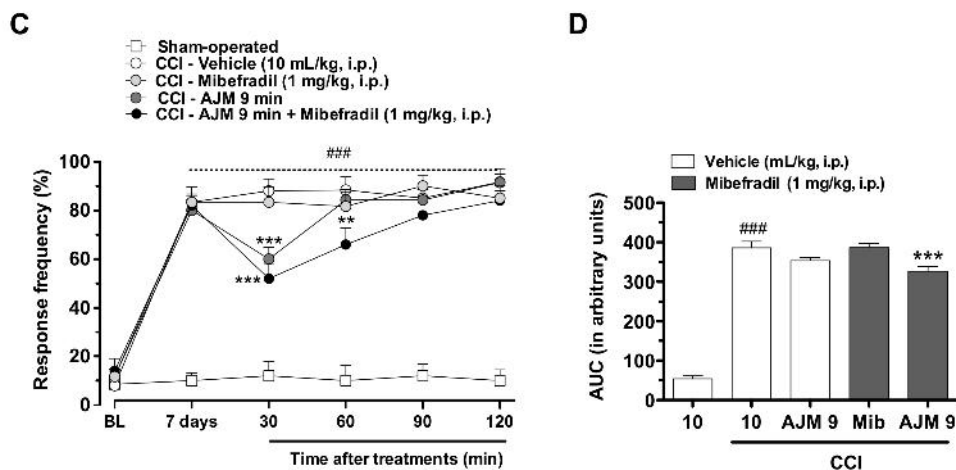
Durante o doutorado fui contemplado com uma bolsa do Programa de Doutorado Sanduíche no Exterior (PDSE) da CAPES, onde tive o privilégio de estagiar no Laboratório do Pesquisador Gerald W. Zamponi na University of Calgary, localizado no *Hotchkiss Brain Institute*, Edifício HRIC 1A25A, na cidade de Calgary, Alberta, Canadá, no período de março à julho de 2012. Neste instituto tive a oportunidade de utilizar camundongos knockout ($Ca_v 3.2$) para os canais de cálcio do tipo T, podendo assim acrescentar uma importante informação a literatura sobre os efeitos da mobilização articular do tornozelo (tema de minha tese). Além disso, trabalhei com inibidores dos receptores canabinóides CB_1 e CB_2 e inibidores das enzimas que degradam os mesmos, além de avaliar o envolvimento dos canais de cálcio no efeito analgésico dos canabinóides. Aproveitando a oportunidade, realizei cursos no *Hotchkiss Brain Institute*, os quais foram: 1) *Biological Safety - Introduction to Biosafety - Module 1 (Biosafety 1)*; 2) *Workplace Hazardous Materials Information System (WHMIS)*; 3) *animals care for mice and Rats* e 4) *Biohazards orientation*, que foram muito importantes para minha carreira como professor e pesquisador. Assim, este estágio permitiu não só a realização de experimentos com a utilização de animais geneticamente modificados, mas foi de grande valia para minha trajetória acadêmica, pois possibilitou conhecer um laboratório de pesquisa de reconhecida qualidade internacional e trabalhar com um pesquisador que é referência na área da dor. Certamente, os resultados obtidos resultarão em um artigo que possivelmente será publicado em

colaboração com o referido laboratório canadense em uma revista Internacional de referência na área da dor.

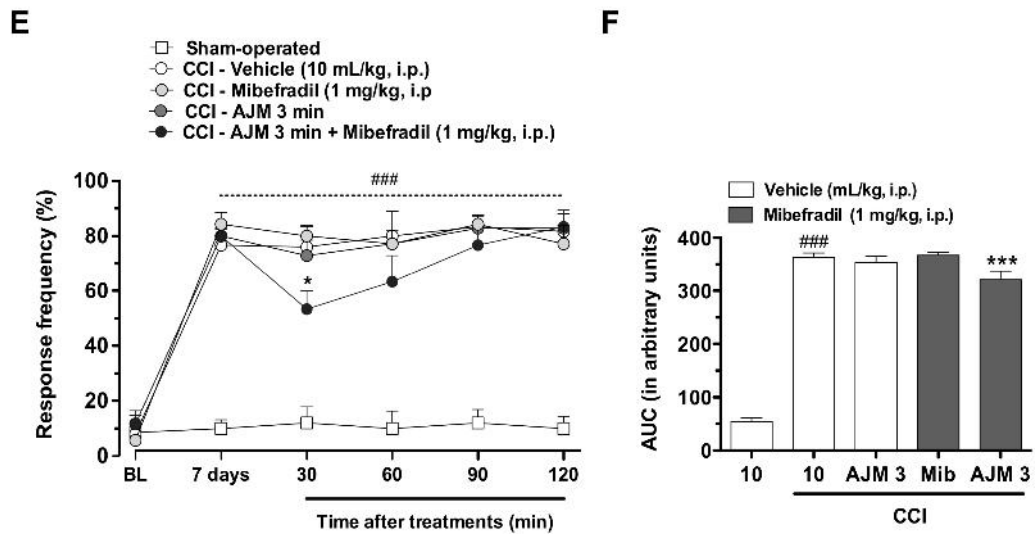
Os experimentos realizados no Canadá estão apresentados nas figuras A-I, a seguir.



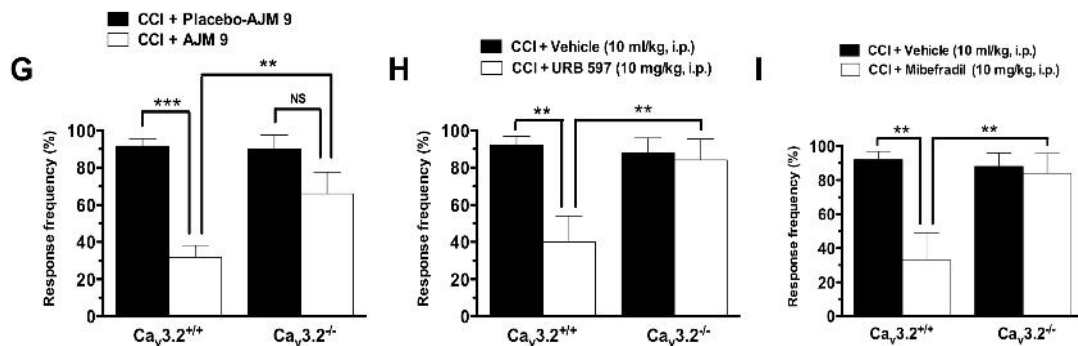
Legenda: Efeito do mibefradil sobre a hiperalgesia mecânica induzida pela constrição crônica do nervo isquiático. Em A está representado o efeito temporal (time course) do mibefradil e em B a área sob a curva (AUC). Os dados representam a média \pm E.P.M (n=8 camundongos). Os símbolos denotam os níveis de significância $**P < 0,01$ e $***P < 0,001$ quando comparados com seus respectivos controles (ANOVA de duas (A) e uma via (B), seguido do teste de Bonferroni ou Student Newman-Keuls, respectivamente).



Legenda: Efeito sinérgico entre a MAT e uma dose subefetiva de mibefradil sobre a hiperalgesia mecânica induzida pela constrição crônica do nervo isquiático. Em A está representado o efeito temporal (time course) e em B a área sob a curva (AUC). Os dados representam a média \pm E.P.M (n=8 camundongos). Os símbolos denotam os níveis de significância $**P < 0,01$ e $***P < 0,001$ quando comparados com seus respectivos controles (ANOVA de duas (A) e uma via (B), seguido do teste de Bonferroni ou Student Newman-Keuls, respectivamente).



Legenda: Efeito sinérgico entre a MAT (realizada por 3 min, um tempo que não possui efeito) e uma dose subefetiva de mibefradil sobre a hiperálgia mecânica induzida pela constrição crônica do nervo isquiático. Em A está representado o efeito temporal (time course) e em B a área sob a curva (AUC). Os dados representam a média \pm E.P.M ($n=8$ camundongos). Os símbolos denotam os níveis de significância $*P < 0,05$ e $***P < 0,001$ quando comparados com seus respectivos controles (ANOVA de duas (A) e uma via (B), seguido do teste de Bonferroni ou Student Newman-Keuls, respetivamente).



Legenda: Observação do efeito da MAT, URB 597 (inibidor da degradação da anandamida) e do mibefradil em animais selvagens e nocautes ($Cav 3.2$) sobre a hiperálgia mecânica induzida pela constrição crônica do nervo isquiático. Os dados representam a média \pm E.P.M ($n=8$ camundongos). Os símbolos denotam os níveis de significância $**P < 0,01$ e $***P < 0,001$ quando comparados com seus respectivos controles (ANOVA de uma via seguido do teste de Student Newman-Keuls).